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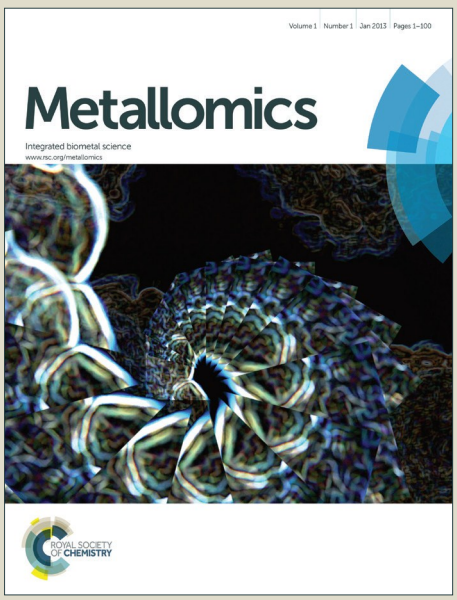

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ARTICLE

Comparative metalloproteomic approaches for the investigation proteins involved in the toxicity of inorganic and organic forms of mercury in rice (*Oryza sativa* L.) roots

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The toxicity mechanisms of rice roots under inorganic mercury (IHg) or methylmercury (MeHg) stress was investigated using metalloproteomic approaches. Rice seedlings were cultivated in nutrient solutions with IHg or MeHg for three weeks. Proteins were extracted from the roots and separated by two-dimensional electrophoresis (2-DE). Differentially expressed proteins were analyzed usingESI-MS/MS and identified by PMF. 26 and 29 protein spots were differentitally expressed in the IHg- and MeHg-exposed roots, respectively. The proteins responsive to Hg exposure are involved in antioxidative defense, sulfur and glutathione metabolism, carbohydrate and energy metabolism, programmed cell death, and pathogen defense. Chitinase and salt stress-induced proteins exhibited a greater differentitally expressed in response to MeHg stress compared to IHg stress. Hg-binding proteins were detected by the combined use of 1-DE, SRXRF, ESI-MS/MS. The results showed that Hg was bound with proteins of 15-25 kDa in rice roots under Hg stress. The Hg contents in the bandunder IHg stress were remarkably higher than that under MeHg.Hg binds with proteins, which leads to irreversible damage of root growth. Rice roots changed related proteins expression levels in response to Hg stress. These results may provide new insights into the mechanism of toxicity of IHg and MeHg in rice.

Introduction

Mercury (Hg), a highly toxic element for human, can cause toxic effects on the kidneys, livers, lungs and neurological systems.[1,2] Recent studies have shown that paddy soils in some areas of China have been severely polluted by Hg. For example, due to the irrigation with Hg-contaminated waters, the concentration of Hg in the paddy soils in Qingzhen, Guizhou of Southwestern China reached up to 236 ± 13 mg kg^{-1}, which far exceeds the Chinese national limit for paddy soils (1.5 mg kg^{-1}, GB15618-1995).[3] Accumulation of Hg in soil can seriously reduce the yield and quality of agricultural products.

Methylmercury (MeHg) is one of the most toxic chemical forms of Hg due to its capability of bioaccumulation, biomagnification in the food chain and its capability to cross the blood-brain barrier.[2,4,5] It can also cross the placenta barrier and cause adverse developmental effects to the fetuses and children.[4,6] MeHg could be produced from IHg by anaerobic organisms such as sulfate- and iron-reducing bacteria (SRB and IRB, respectively).[7-9] A strong correlation (r=0.91) was found between MeHg and fish consumption, implying that residents of the industrial area mainly exposed to MeHg through high-frequency fish consumption. Total daily intakes of MeHg for both children (696.8 ng/(kg·day)) and adults (381.3 ng/(kg·day)) from an electronic waste recycling area in Taizhou, China were far beyond the dietary reference dose (RfD) of 230 ng/(kg·day).[10,11] Daily dietary intake was thus the major MeHg exposure source. Rice serves as a main food staple for more than half the world's population. As reported in Wanshan, China, the contents of total Hg (THg) in rice were up to 569 μg kg^{-1} (145 μg kg^{-1} as MeHg), which is far beyond the maximum permissible limit in China for crops (20 μg kg^{-1}, GB 2762-2005).[12] The bioaccumulation factors (BAFs) of MeHg in rice grain were more than 800 times than those of IHg. MeHg constituted a large proportion of THg. Rice grain is thus an intensive bioaccumulator of MeHg.[13] Long-term dietary consumption of Hg-contaminated rice has become a primary source of MeHg intake for local residents, bringing potential health threat to them.

Plants can adapt to different environmental stresses by inducing changes in gene expression and protein synthesis.[14] Identification of the functional genes or proteins involved in the responses of plants to Hg stress is beneficial to understand the molecular mechanisms of Hg toxicity. This study focused

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on root tissues because root growth is sensitive to Hg toxicity and over 80% of the total Hg is blocked in rice roots.[3,15,] Because of its high affinity for sulfhydryl groups in biomolecules, Hg could bind with thiol groups in enzymes and proteins, which leaded to the disruption of cell structure and enzyme inactivation. Phytochelations (PCs) and metallothioneins are two classes of cysteine-rich peptides. PCs chelate Hg^{2+} and form complexes in the cytosol to prevent Hg^{2+} from binding with proteins. Metalloproteomic approach is powerful in studying plant tolerance to Hg stress. Chen and colleaguesreported biochemical and proteomic changes when rice seedlings exposed to Hg using two-dimensional gel electrophoresis (2-DE), coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS).[16] However, the Hg-containing proteins were not identified in their study. Recently, our group detected Hg-binding protein spots in Hg exposed *E. Coli.*with combined techniques of 2-DE andsynchrotron radiation X-ray fluorescence (SR-XRF).[17] The Hg binding protein spots were further studied using ESI-MS/MS. An absolute detection limit of 10^{-12}-10^{-15} g and relative detection of several μg g^{-1} can be achieved by SR-XRF. Besides, SRXRF allows a multi-elemental analysis of major, minor and trace elements in a microscopic region of a biological specimen or in protein bands after electrophoretic separation.[18] Therefore, Therefore, to achieve a better understanding of Hg stress responses in plants, we used a proteomic approach to investigate changes in rice (Oryza sativa) root. Hg-containing proteins were detected and identified through the combined use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 1-DE), SRXRF, ESI-MS/MS, and bioinformatics. It is beneficial to understand the uptake of Hg rice roots and the subsequent translocation of Hg to rice seeds.

Materials and methods

Rice cultivation and treatments

Rice (*Oryza sativa* L.GY1255) seeds are widely planted in Guizhou area. The Hg content is 4.68 ± 0.2 μg/kg, which is far below the maximum permissible limit for crops (20 μg kg^{-1}, GB 2762-2005) in China. They were sterilized with 1% (V/V) sodium hypochlorite for 15 min, washed thoroughly with ultrapure water (18.2 MΩ·cm). Then seeds were germinated in moist perlite for 1 week at 28 °C in the dark. The germinated seeds were transferred to plastic container containing 50% Hogland solution and placed in the manmade climate growth chamber with a 14 h light period (300–350 μmol·m^{-2} s^{-1}) and a 10 h dark period. The temperature and relative humidity were kept at 28 °C, 70% and 20 °C, 50% for day and night, respectively. The solution was renewed twice a week.

After two weeks, the seedlings were transferred into 50% Hogland solution without Hg (control group), 2.5μmol L^{-1} mercuric chloride ($HgCl_2$, IHg) or 2.5 μmol L^{-1} methylmercury chloride (MeHgCl, MeHg) for three weeks. Then the roots were harvested and stored at – 80 °C prior to analysis.

Protein extraction and separation

The roots proteins were extracted according to Yan *et al*. with some modifications.[19] Briefly, frozen root tissues (1 g) were grinded with a cold pestle into a fine powder with liquid nitrogen in the precooled mortar. Tissue powder was mixed with 15 mL pre-chilled protein extraction buffer containing 10% (w/v) trichloroacetic acid (TCA)/acetone and 0.07% dithiothretiol (DTT). After precipitating for 2 h at -20 °C, the mixture was centrifuged at 11000 r min^{-1} (Eppendorf, Germany) at 4 °C for 30 min. The pellet was rinsed with 10 mL pre-chilled acetone containing 0.07% DTT, precipitated 1 h at -20 °C, and then centrifuged at 11000 r min^{-1} for 30 min at 4 °C. This procedure was repeated three times and the protein extractions were freeze-dried. The dried powder was then dissolved in rehydration buffer (7 M urea, 2 M thioure, 1% dithiothreitol (DTT), 2% ampholyte (pH3-10) and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)). The supernatant was collected after centrifugation at 11000 r min^{-1} for 20 min. The protein concentration was quantified using Bradford assay with BSA as standard.

For SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a mini vertical gel system. The samples were separated by 12% (m/v) acrylamide separating gel and 5% (m/v) stacking gel. All the separated samples were diluted in 5×loading buffer, and then heated for 5 min at 95 °C. 30 μg amount of protein was loaded in each lane. Two gels run simultaneously with the same sample. The SDS-PAGE condition was 70 V for 30 min and 150 V for 1 h in an ice-water chamber. The gel was fixed with formaldehyde solution of 26% ethanol, 5% formaldehyde and 3% glycerol. The gel was then dried using Gel Drier (Bio-Rad) at 80 °C for 90min after electrophoresis to detect metal-containing bands. Another gel was stained with Coomassie brilliant blue (CBB) R-250 to analyze the molecular weight of metal-containing bands.

For 2-DE, 120 μg and 500 μg of proteins were loaded onto preparative and analytical gels, respectively. IPG dry strips (24 cm, pH 3-10 non-linear gradient; Bio-Rad, USA) were rehydrated passively for 12 h at 20 °C and then focused following a four-step program (500 V for 1 h, 1000V for 1 h, 8000 V for 3 h, 8000 V for 5.36 h) using a PROTEN IFR CELL (Bio-Rad). After IEF, the gel strips were equilibrated in 8 mL equilibration buffer (6 M urea, 2% SDS, 0.375% M Tris-HCl pH 8.8, 20% glycerol, 100 mM DTT) for 15min. Then it was equilibrated for another 15 min in the same buffer above except that 100 mM DTT was replaced with 250 mM iodoacetamide. Strips were transferred onto vertical slab 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. SDS-PAGE gels were carried out at 2 W for the first 60 min, followed by 17 W until the tracking dye reached the bottom of the gel. After electrophoresis, protein spots in analytical gels were visualized by a silver-staining method. The proteins in preparative gels were stained with colloidal Coomassie Brilliant blue G-250.

Analysis of differentially expressed of proteins

The gels were photographed using an image scanner (MICROTEK ScanMaker i800) at 300 dpi resolution. Imaging and data analyses of 2-DE were performed using ImageMaster 2D Platinum 5.0 software. The experiment was repeated three times for each sample. More than 2-fold change in protein concentration was recognized to represent differentially

expressed protein spots between control and Hg treated groups ($p < 0.05$ by one-way ANOVA and least significant difference test).

Detection of Hg -containing bands in SDS-PAGE gels with SRXRF

The Hg distribution in the SDS-PAGE gel was performed at the BL-4Ain Photon Factory, High Energy Accelerator Research Organization (KEK), Japan. The storage ring was working at 2.5 GeV with a maximum current intensity of about 350-450 mA. A spot size of 1×1 mm^2 and a monochromatic SR with photo energy of 13.5 keV was used to galvanize the sample. The samples were fixed on a precision motor-driven stage and the samples platform was moved along the horizontal and perpendicular direction with an interval of 1 mm for each step. The Si(Li)detector (PGT Inc. LS 30143-DS) wasutilized to collect X-ray fluorescence signals with live time of 10 s for each point. 6SCAs (single channel analyzers, Ortec 550) were used to record and analyze the fluorescence intensity of Hg, Fe, Cu, Zn and Compton scattering. The peak areas of Hg were normalized to the peak counts of Compton scattering with the purpose of correcting the effect of SR beam flux variation on the signal intensity. The relative contents of Hg were estimated by normalized peak counts.

In-gel digestion and Mass Spectrometry (MS) analysis

Selected protein spots and Hg-containing bands were manually excised from the preparative gels and SDS-PAGE gels. Each gel piece was detained and then incubated with 50 mM ammonium bicarbonate for 5 min. After dehydrating with acetonitrile (ACN), gel pieces were dried in a speed vacuum concentrator (Thermo, SC210A-115, USA). The gel species from 1-DE need reduce disulfides bonds and alkylate free cysteines by 100 mM DTT and 55 mMiodoacetamide. Then the proteins were digested using 20 µL 20 µg mL^{-1} trypsin solution with gentle shaking at 37 °C overnight. The peptides were extracted with 67% ACN and 0.1% trifluoroacetic acid (TFA). Tryptic peptide mixtures were analyzed at the nano liquid chromatography (LC)-ESI-Quadrupole time-of-flight MS/MS (NanoLC-ESI-MS/MS).A precolumn + analytical column setup (precolumn: Proxeon EASY-Column SC001, 100 µm × 20 mm, packed with Reprosil-Pur C18-AQ of 120 Å diameter packed with 5 µm particles; analytical column: Proxeon EASY-Column SC200, 75 µm × 100 mm, packed with Reprosil-Pur C18-AQ 120 Å 3 µm particles) were used to separate peptides at a flow rate of 200 nl/min with 90 min gradients as follows: pH 3–5 fractions: 8–36% solution B; pH 6 fraction: 8–35% solution B; pH 8 fraction: 5–33% solution B; pH 11 fraction: 2–30% solution B (A: 0.5% acetic acid; B: 0.5% acetic acid/80% acetonitrile).

Protein mass fingerprints (PMF)

Protein identification was performed using MASCOT program (http://www.matrixscience.com; London, UK). NCBInr and rice were selected as the database and taxonomy, respectively. Search parameters were defined as follows: trypsin enzymes; one missed cleavage; fixed modifications of cysteine as carbamidomethylated; variable modifications of methionine as oxidized; peptide tolerance of 0.1 Da; fragment tolerance of 0.1Da.

Results and discussion

Differentially expression of root proteins in Hg stress

In order to compare rice roots tolerance to different Hg species, two weeks old rice seedlings were treated with IHg and MeHg for 21 d. For the rice roots after the IHg and MeHg exposure, Hg contents were 462.61±42.14 mg/kg and 453.29±42.14 mg/kg, respectively. Total soluble proteins were extracted from the roots of control (without Hg treated), IHg-treated or MeHg-treated rice plants. Changes in spot intensity between untreated and Hg treated roots were quantified by image analysis software. The representative gel images stained by sliver nitrate are presented in Figure.1 (IHg treated) and Figure.2 (MeHg treated). Quantitative image analysis revealed that a total of 26 spots exhibited more than 2.0-fold differences in the intensity in response to IHg (Figure.1). 18 protein spots were up-regulated, while 8 were down-regulated. Compared to the control, 29 proteins spots were differentially expressed under MeHg stress and among them 16 proteins were induced and 13 proteins were repressed.

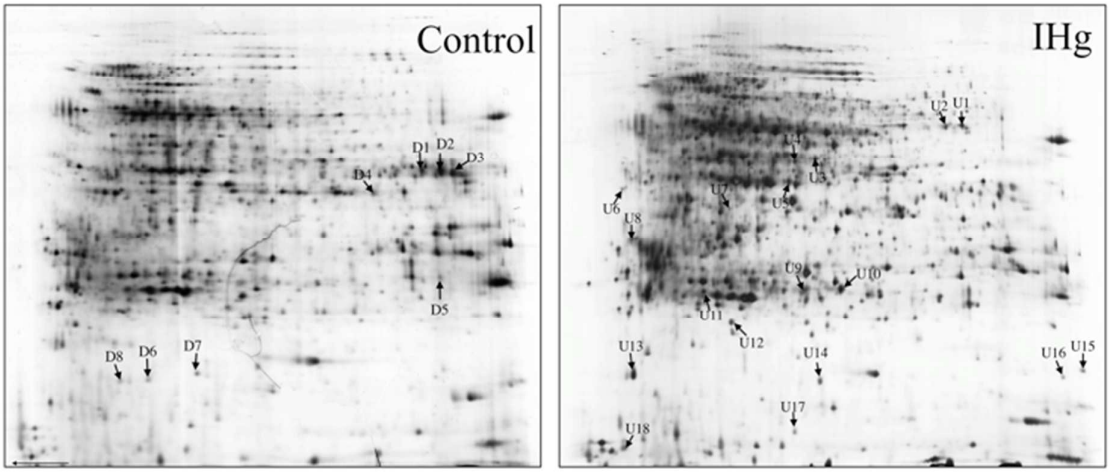

Figure 1. 2-DE images of rice roots untreated or treated with 2.5 μM $HgCl_2$ (IHg). 120 μg of proteins were loaded onto pH 3-10 IPG strips (24 cm, non-linear). SDS-PAGE was carried out with 12% gels. Differentially expressed protein spots are represented by arrows. Eight down-regulated spots (D1-D8) are indicated in the map of the control sample, and 18 up-regulated spots (U1-U18) are indicated on the map of IHg-treated sample.

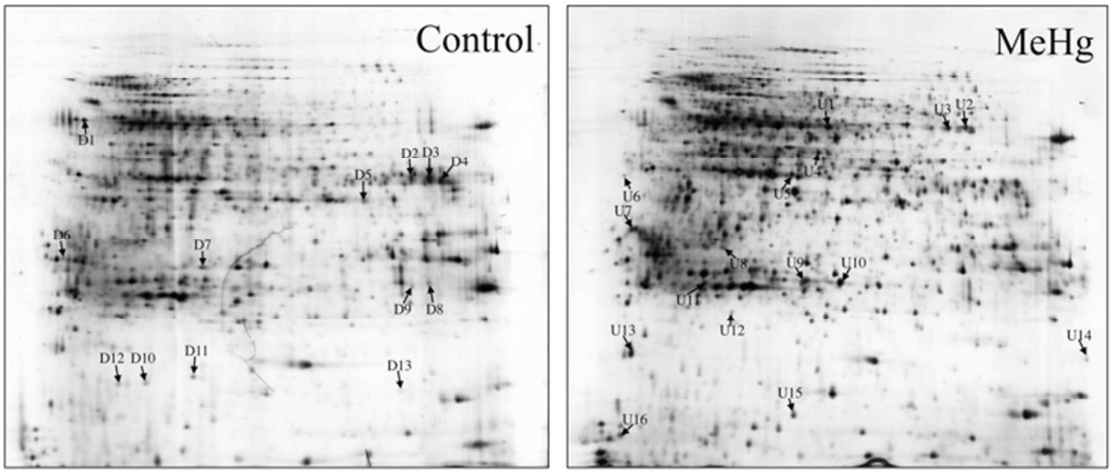

Figure 2. 2-DE images of rice roots untreated or treated with 2.5 μMMeHgCl (MeHg). 120 μg of proteins were loaded onto pH 3-10 IPG strips (24 cm, non-linear). SDS-PAGE was carried out with 12% gels. Differentially expressed protein spots are represented by arrows. 13 down-regulated spots (D1-D13) are indicated in the map of the control sample, and 16 up-regulated spots (U1-U16) are indicated on the map of MeHg-treated sample.

Hg responsive proteins identified by PMF

The 21 and 23differentially expressed protein spots induced by IHg and MeHg were analyzed byESI-MS/MS and further identified by PMF. Table 1 shows the identification of these proteins after database search. Hg responsive proteins were stored into five different functional classes based on their putative function reported: (1) enzymes with antioxidant properties, (2) sulfur and GSH metabolism, (3) carbohydrate metabolism, (4) programmed cell death, and (5) pathogenesis-related protein.

Hg, like other heavy metal, can cause oxidative damage to biological marcomolecules by inducing reactive oxygen species (ROS) production.[9, 16] The formation of ROS results in cellular structure disruption, lipid peroxidation, DNA and membrane damage.[20] Plants have developed a wide range of antioxidant systems to scavenge excessive ROS to protect against oxidative stress. Superoxidase peroxidase (SOD), as the first line of defense against superoxide (O_2^-), rapidly converts O_2^- to O_2 and H_2O_2.[16] Chen *et al*. reported that SOD activity enhanced quickly when rice roots were exposed to Hg at 1 h.[16] Germin-like proteins (GLPs) are involved in important aspects of cell wall remodeling and stress response. Some GLPs have been confirmed to possess SOD activity.[21] GLPs were bound to the cell wall and were induced within ROS-dependent pathway. This indicated GLPs played a role in plant defense.[22] Our proteomics analysis revealed that the treatment with IHg (U12) and MeHg (U12) significantly increased the expression of GLP 4-1. A marked decrease in the level of peroxidases (POD) in response to Hg stress was also detected, which is consistent with other studies that the activity of POD was inhibited by heavy metals in horseradish hairy roots.[23] POD is involved in the detoxification of H_2O_2 to H_2O[24] and plant POD is located mainly in cell wall and in vacuoles.[25] The accumulation of H_2O_2 is restricted by POD because POD is involved in the detoxification of H_2O_2 to H_2O.[24] Glutathione (GSH) is a non-enzymatic antioxidant in plants and is involved in detoxification mechanism of ROS scavenging.[8,25] An up-regulation of proteins responsive for GSH synthesis could alleviate oxidative stress and limit ROS production. The major function of cysteine synthase (CS) is to catalyze biosynthesis of cysteine in plants. Cysteine is the rate-limiting factor in cellular GSH synthesis.[26] Up-regulated expression of CS in rice roots increased plant tolerance to Al and Cu, and decreased ROS formation and oxidative damage.[24,27]

Mercuric ions (Hg^{2+} or CH_3Hg^+), as a class B metal, have high affinity with sulfur ligands. The cytotoxicity of Hg is regarded as binding with sulfhydryl groups in proteins.[16] Excessive accumulation of Hg in plants disrupts homeostasis of essential metals.[25] Over 80% of root growth inhibition was detected in the cad2-1 (with low glutathione content) and cad1-3 (unable to synthesis PCs), whereas wild type was inhibited by only 35% when *Arabidopsis thaliana* was exposed to 10 μmol Hg for 4 d.[28] Therefore, thiol-compounds play vital roles in plant defense. In this study, four proteins (CS, Cysteine proteinase rd21a, Glutathione S-transferase 2 and probable glutathione S-transferase GSTF2) related to sulfur assimilation and GSH biosynthesis showed a markedly increase in abundance after IHg and MeHg treatment. CS is a key enzyme in sulfur assimilation and is the precursor of metal chelators such as GSH, metallithionein (MT), phytochelations (PCs).[27] Free Hg^{2+} or CH_3Hg^+ ions are scavenged by binding of Hg to GSH.[25] Many studies demonstrate that a un-regulation of CS is important for plant adaptation of adverse environmental factors. Song *et al*. using a proteomic method showed that CS

expression was up-regulated in rice roots when rice exposed to Cu.[24] CS exhibited significantly higher in IHg treatment than MeHg treatment. It indicates that IHg is easier to combine with thiol groups than MeHg. Glutathione-S-transferase (GST) catalyzes the nucleophilic attack of the sulphur atom of GSH and the conjugation of the reductive GSH to cytotoxic substrates for the purpose of detoxification.[25] Therefore, GSTs are most often considered as detoxification enzymes. Biochemical studies have indicated that Cu ions interact with GSTs by directly binding to the peptides.[28] The expression of GSTs was upregulated when plants were exposed to Cu,[24,29] Zn,[30] Al,[26] and Cd.[31] Moreover, the cellular function of GSTs is associated with regulating levels of ROS.[32] The stress-induced up-regulation of GSTs observed in this study indicates a key role for GSTs in the tolerance to IHg and MeHg stress in rice.

Previous studies have shown the activities of proteins involved in glycolysis are generally inhibited under some stresses. Fructose-bisphosphatealdolase (FBPA) catalyzes the cleavage of fructose 1,6-biphosphate (F-1,6-BP) into the triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P) during glycolysis.[33] The treatment with IHg and MeHg significantly decreased the expression of FBPA, which suggests Hg treatment damages to glycolysis metabolism. Triosephosphateisomerase (TPI) plays an important role in glycolysis and is essential for efficient energy production.[27,34] The major reaction catalyzed by TPI is the reversible interconversion of DHAP and D-glyceraldehyde 3-phosphate (DG3P).[33] It was found that TPI increased in wheat roots and leaves after Al stress and salt stress.[28] Furthermore, the up-regulated of TPI were more pronounced in MeHg exposure than IHg. Up-regulation of TPI after MeHg treatment is probably due to a demand for extra energy of the detoxification.

Serine hydroxymethyltransferase (SHMT) is associated with metabolism and catalyzes the reversible interconversion of serine and glycine.[35] The enhanced abundance of SHMT in response to Hg stress suggests an Hg treatment induce the increase or activation of amino acid metabolism. Increased SHMT was reported to protect plants from oxidative stress in rice during drought[36] and cold acclimation.[37] Besides, an up-regulation of SHMT under Hg stress increased GSH contents because glycine is essential for GSH synthesis. GSH increases plant tolerance to Hg by alleviating oxidative stress damage and detoxifying by chelation. S-adenosylmethioninesynthetase (SAMs) is an enzyme that catalyzes the biosynthesis of S-adenosylmethionine (SAM) from methionine and ATP. SAM participates in ethylene production. Ethylene, a gas phytohormone, is involved in plant development and plant responses to both biotic and abiotic stress factors.[38] Genes related to ethylene metabolism were highly increased when alfalfa (*Medicago sativa*) seedlings treated with Hg, which suggested that ethylene participates in early transcriptional responses to Hg stress.[39] Moreover, ethylene could induce the expression of pathogen-related protein.[40] In present study, IHg and MeHg treatment induced the expression of SAMs. Therefore, overexpression of SAMs increases the production of ethylene, which severs as s signal that leads to the activation of other defense mechanism.

High levels of ROS are implicated in mediation of programmed cell death (PCD). Translationally-controlled tumor protein (TCTP) or homologous proteins were found to be present in a wide range of different organisms. TCTP is a cytoplasmic, growth-related and calcium-binding protein.[41] TCTP acts as a PCD protein inhibitor in mice.[42] In rice roots, it was found that the expression of TCTP was up-regulated after damage caused Al exposure.[43] High abundance of TCTP probably promotes the formation of a metal-histidine complex that can serve as a histamine-releasing factor.[43] Furthermore, TCTP, a calcium-binding protein, is a key protein involved in the maintenance of Ca homeostasis in plant cells. It was reported that essential Ca^{2+} concentration played key role in the amelioration of Al-caused damage.[43] Recent studies demonstrated that TCTP might have anti-oxidation function in parasite *Brugia malayi*.[44] H_2O_2 pre-treatment induced the accumulation of TCTP.[45] A more pronounced up-regulation of TCTP by IHg (spot U13) and MeHg (spot U13) was found in this study. Overexpression of TCTP increases the activities of antioxidant enzymes and decreases the Hg-induced H_2O_2 levels,[46] which indicates that TCTP may have an important role in the protection against oxidative stress and PCD induced by ROS.

Remarkable differentially expressed protein spot under MeHg stress was identified as chitinase and salt stress-induced proteins. The major function of chitinase is the catalysis the hydrolytic cleavage of the β-1,4-glycoside bond of N-acetylglucosamine.[47] Besides, it is also associated with a defense mechanism against a variety of pathogens as well as abiotic stresses, such as osmotic, dehydration, low temperature and wound stress. Oligosaccharide can act as a signal for defense responses in plants. Chitinase can work indirectly by releasing oligosaccharide to activate plant defense response.[48] It was found that transgenic tobacco and maize (overexpression of chitinases) got enhanced tolerance to abiotic stress caused by high concentrations of salt and metal ions in the growth substrate.[45, 49] DIP3 from the plant chitinase shares the highest identify with chitinase III.[47] Transcript levels of DIP3 changed greatly after rice exposed to low temperature, salt and drought stresses.[47] The expression of DIP3 down-regulated under IHg and MeHg stress. This indicates that chitinases and DIP3 function as a stress-induced protein involved in the regulation of plant tolerance to MeHg.

Hg-containing bands in SDS-PAGE gel and the speculation of Hg-containing proteins

Hg^{2+} formsstable covalent bondwith thiolic groups in biomolecules and Hg-thiol bonds could survive in the denaturing 2-DE process.[17] Therefore, it is feasible to simultaneously detect Hg-containing proteins using SR-XRF after a denatured electrophoresis. The SDS-PAGE gel of rice roots treated with no Hg, IHgor MeHgwas used for SRXRF elemental imaging after formaldehyde fixation. CBB image of SDS-PAGE and Hg distribution image are shown in Figure.3. It can be seen from Figure.3 that Hg cannot be detected in the control group. Hg-containing bands with a relative molecular

weight of 15~25 kDa are found with IHg or MeHg exposure. The Hg contents in the bandunder IHg stress are remarkably higher than that under MeHg. This may indicate that Hg^{2+}combine more easily with proteins than CH_3Hg^+ or Hg^{2+} binding proteins are more stable than CH_3Hg^+ binding proteins. The Hg-containing bands were digested with trypsin. The resulting peptides were extracted and analyzed by ESI-MS/MS, and the proteins were identified by PMF. Table 2 shows the results of identification.

Table 1Identification of the differentially expressed proteins in response to IHg and MeHg

Prote spot and fold-changes		Protein name	Score	Sequence coverage (%)	Accession number	Species	Nominal mass (Mr)	Calculated PI values
IHg	MeHg							
Proteins involved in antioxidative defense								
D4/3.62	D5/2.02	peroxidase	443	43	B8A753	O. sativa	38374	6.22
Proteins involved in stress response								
	D6/2.86	Chitinase (Fragment)	128	15	Q7XXQ0	O. sativa	30060	4.52
D7/2.08	D7/2.32	DIP3	320	32	Q5WMX0	O. sativa	32757	6.08
	U8	Salt stress-induced protein	237	45	ADM86855	O. sativa	15188	5.19
U12/1000	U12/1000	Germin-like protein 4-1	97	10	GL41	O. sativa	25813	6.25
Proteins involved in sulfur and GSH metabolism								
U1/1000	U2/1000	Serine hydroxymethyltransferase	374	26	A3CB05	O. sativa	49368	7.10
U2/3.79	U3/5.10	Serine hydroxymethyltransferase	342	25	A3CB05	O. sativa	49368	7.10
U7/2.76		Cysteine synthase	292	27	Q5JNB0	O. sativa	42104	6.28
U8/2.04	U7/2.26	Cysteine proteinase rd21a (Fragment)	175	16	A6N1K8	O. sativa	25925	4.81
U9/2.02	U9/2.34	Glutathione S-transferase 2	165	26	ABI17930.1	O. sativa	24860	6.59
U10/3.28	U10/3.21	Probable glutathione S-transferase GSTF2	165	27	O82451	O. sativa	23976	5.77
Proteins involved in carbohydrate and energy metabolism								
D1/2.87	D2/5.38	Fructose-bisphosphatealdolase	575	37	Q5N725	O. sativa	39141	8.35
D2/2.43	D3/2.79	Fructose-bisphosphatealdolase	446	44	Q5N725	O. sativa	39141	8.35
D3/3.49	D4/2.15	Fructose-bisphosphatealdolase	426	37	Q5N725	O. sativa	39141	8.35
U3/2.89	U4/4.97	S-adenosylmethionine synthase	269	21	Q1H9D0	O. sativa	43004	5.83
U4/2.08	U1/2.10	S-adenosylmethionine synthase (Fragment)	269	21	Q1H9D0	O. sativa	43004	5.83
U11/2.78	U11/3.57	Triosephosphateisomerase	328	37	Q69K00	O. sativa	32715	6.96
Proteins involved in programmed cell death								
U13/5.19	U13/3.67	Translationally-controlled tumor protein homolog	229	32	P35681	O. sativa	18991	4.51
Other proteins displaying various functions								
U5/3.16	U5/3.21	Putative uncharacterized protein	322	26	A1YRE2	O. sativa	39477	5.50
D6/4.72	D10/2.12	Os01g0173100	239	39	Q94E63	O. sativa	16250	5.10
D7/2.08	D11/2.19	Putative Acid phosphatase 1	113	10	Q5Z7F8	O. sativa	28408	4.88
U17/2.45	U15/5.93	Os03g0300400 protein	184	65	Q10M7	O. sativa	17277	5.85
U18/2.24	U16/2.09	60S acidic ribosomal protein P3	215	17	P56724.3	O. sativa	11894	4.35

Hg^{2+} has a particularly high affinity for thiol groups and coordination to cysteines is the dominant mechanism for Hg-protein interactions. The conserved motif of CXXC (where C is cysteine, X is any amino acid) is present in many metal-binding proteins and metal-transport proteins.[50] The CXXC is also found in two of the proteins of the bacterial mercury detoxification system including the periplasmic Hg-binding protein (MerP), and the enzyme mercuric reductase (MerA).[51] MerT and MerF of the detoxification system are membrane proteins, and were thought to be involved in the transport of Hg into the cell where it can be reduced by mercuric reductase.[52] Both sites in MerF and one in MerT have vicinal cysteines. Hg binds to CXXC and CCXX peptides with greater affinity than other metals. And the CCXX peptide with vicinal cysteine residues binds Hg(II) with more higher selectively than CXXC.[52] Therefore, the CCXX and CXXC could be a possible Hg binding sites in Proteins.

Among identified proteins, the amino acid sequences of RCc3 and pR1a protein include the CXXC and CCXXsequence.Therefore, RCc3 and pR1a protein were speculated to be two possible Hg-binding proteins. RCc3, a root-specific protein, was expressed in the elongation and

maturation zones of primary and secondary roots, as well as in root caps.[53] Hg binding with RCc3 may result in irreversible damage of root growth. Moreover, We also analyzed the differentially expressed proteins to verify the existence of Hg-containing proteins. The amino acid sequences of peroxidase, SHMT, SAMs, CS, cysteine proteinase rd21a (Fragment) and Os03g0300400 proteins include CXXC and CCXX sequence. These Hg-binding proteins may elude the SRXRF identification due to low expression and the formation of disulfidebonds.Cysteine proteinase is important players in plant immunity. Cysteine proteinase rd21a (cprd21a) has four Hg-binding sites. Four sites in cprd21a have vicinal cysteines. Therefore, Hg binds with cprd21a, which might lead to immunity damage to the Hg stress.

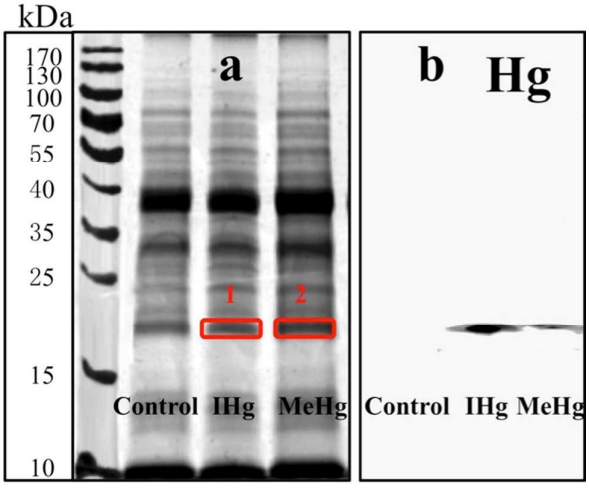

Figure. 3 Distribution of Hg in SDS-PAGE gel detected with SR-XRF. a: CBB image of SDS-PAGE; b: Hg distribution in SRXRF image; Red squares represent the stripes containing Hg in SDS-PAGE gels.

Hg tolerance mechanism in rice

Plants have developed many mechanisms to alleviate the adverse effects of Hg exposure include extracellular immobilization (cell wall and other carbohydrates), chelation in the cytosol and sequestration into the vacuoles, the activation of antioxidative systems and other defense metabolisms. A summarization of included mechanism is shown in Figure 4.

First, plants reduce heavy metals uptake into cell by extracellular immobilization.[54] Plant roots secrete exudates into the growth matrix. Root exudates have high chelating ability against heavy metals and reduce the uptake of them. Cell wall plays a vital role in immobilizing toxic heavy metals by providing pectic sites and hystidylgourps, and extra carbohydrates such as callose and mucilage, and thus restricts the uptake of them into the cytosol.

Second, the chelation of toxic heavy metals with thiol compounds in the cytosol is a very significant mechanism of heavy metal detoxification and tolerance.[55] These thiol compounds include Cys, GSH, metallothionein (MT) and PCs. The PCs-Hg complexes are sequestrated in the vacuole, thus reduce the toxic effects of heavy metals. Some organic acids such as citric, malic and histidine, as potential ligands for heavy metals, play a key role in plants tolerance and detoxification to heavy metals.[54]

Third, the balance of ROS production and removal is paramount in cellular homeostasis.[16] Hg induced the production of ROS. The activation of antioxidative systems is essential for plants to avoid the oxidative damage. ROS scavenging depends on the activity of nonenzymic reduced molecules, such as GSH, ascorbate, and enzymatic molecules, such as SOD, CAT, APX, GPX, POD. In this study, GLPs, GST, TCTP increased in abundance under Hg stress, which suggests that the activation of antioxidative systems and other defense-related metabolism are paramount to protect plants against oxidative damages.

Forth, Mercuric ions (Hg^{2+} or CH_3Hg^+) have high affinity with sulfur ligands. The cytotoxicity of Hg is regarded as binding with sulfhydryl groups in proteins. In present study, we observed that Hg binding with RCc3, peroxidase, SHMT, SAMs and csrd21a might lead to irreversible damage of root growth, sulfur metabolism, antioxidantive systems and immune system. Plants could alleviate the adverse effects by changing expression of related proteins.

Table 2 Identified proteins in SDS-PAGE gels

Associated element	Accession no	Protein name	Score	MW(Da)/pI Theoretical	emPAI	Species
Hg	AAM93438	putative type-1 pathogenesis-related protein	220	18743.06/9.10	1.09	O. sativa
	CAE02065	OJ000126_13.9	100	16419.81/10.45	0.53	O. sativa
	BAC56830	putative pathogenesis-related protein	87	19027.96/4.37	0.44	O. sativa
	AAB61213	glycine-rich protein	59	20645/9.51	0.18	O. sativa
	AAV59386	putative nucleoside diphosphate kinase	58	25937.72/8.88	0.31	O. sativa
	AAG60181	putative nucleoside diphosphate kinase	57	16779.37/6.84	0.23	O. sativa
	AAO37527	putative ribosomal protein S15	54	17328.36/10.19	0.22	O.

BAB21002	ribosomal protein S10	46	20253.80/9.68	0.19	O. sativa
AAB66886	ribosomal protein L12 homolog, partial	44	16313.57/5.32	0.24	O. sativa
ABF98987	Eukaryotic translation initiation factor 5A-2, putative, expressed	44	17712.99/5.87	0.21	O. sativa
CAC03571	PR1a protein	40	17560.16/4.55	0.21	O. sativa
AAA65513	RCc3	40	18360.98/8.61	0.28	O. sativa
AAA57045	Cyclophilin 2	35	18360.98/8.61	0.46	O. sativa

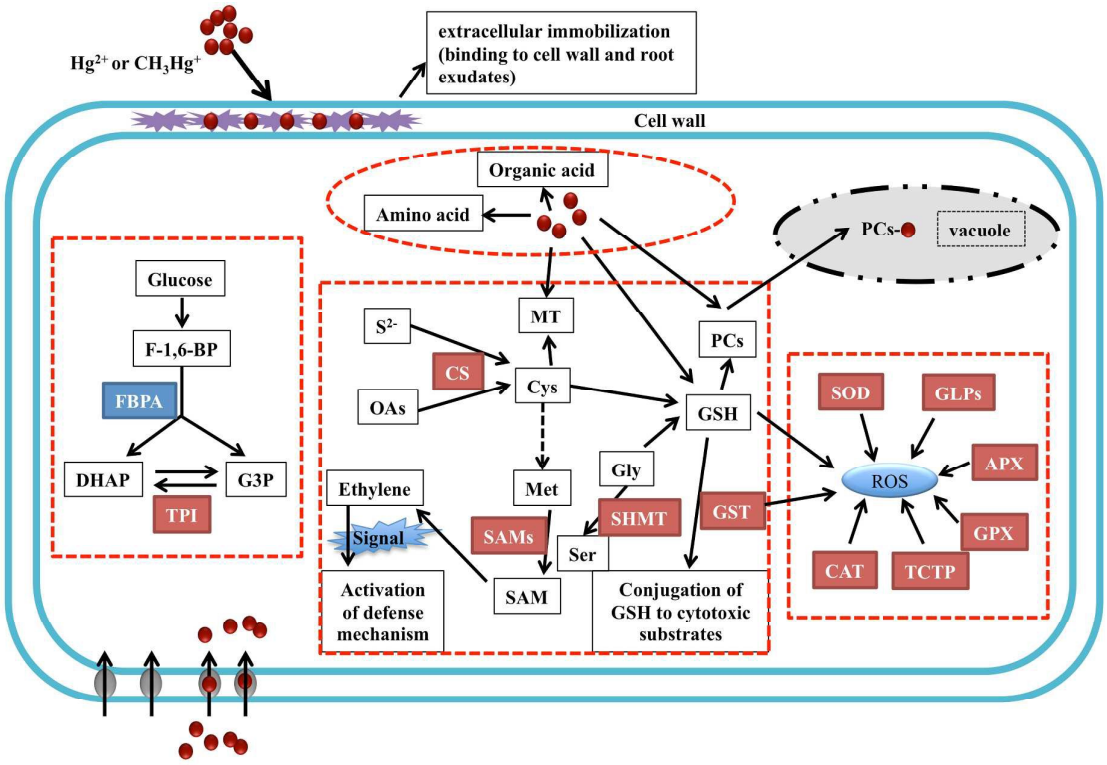

Figure 4. Proposed molecular mechanisms highlighted by proteomic investigations under IHg and MeHg stress. Up-regulated and down-regulated proteins identified in this study are indicated in the red and blue-shed box, respectively. They are showed on the corresponding pathways. Abbreviations are as follows: GST, glutathione S-transferase; APX, L-ascorbate peroxidase; SOD, Superoxide Dismutase; GLPs, Germin-like proteins; GPX, glutathione peroxidase; TCTP, Translationally-controlled tumor protein; CAT, catalase; SAMs, S-adenosylmethioninesynthetase; SAM, S-adenosylmethionine; CS, Cysteine; Gly, glycine; Ser, serine; Met, methionine; SHMT, Serine hydroxymethyltransferase; PCs, Phytochelatins; MT, metallothionein; FBPA, Fructose-bisphosphatealdolase; F-1,6-BP, fructose 1,6-biphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; TPI, Triosephosphateisomerase.

Conclusions

Rice is able to induce many defense mechanisms to enhance tolerance to IHg and MeHg. Induction of antioxidant enzymes, proteins linked to sulfur assimilation, carbon and energy metabolization system and programmed cell death protein inhibitor is the important defense mechanism of the cell against IHg and MeHg stress. Moreover, our results suggest that up-regulation of cysteine synthesis is more prominent in detoxifying IHg than MeHg. MeHg exposure strongly induces the differentially expression of pathogenesis related protein, chitinases and DIP3. Hg-containing bands with a relative molecular weight of 15~25 kDa are found with IHg or MeHgexposure.The Hg contents in the bandunder IHg stress are remarkably higher than that under MeHg. Hg binding with proteins may lead to irreversible damage of root growth.

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References

1 E. Guallar, M. I. Sanz-Gallardo, P. v. t. Veer, P. Bode, A. Aro, J. Gómez-Aracena, J. D. Kark, R. A. Riemersma, J. M. Martín-Moreno and F. J. Kok, *New.England.J.Med.*, 2002, **347**, 1747-1754.
2 T. W. Clarkson,*Crit.Rev.Cl.Lab.Sci.,* 1997, **34**, 369-403.
3 Y.-F. Li, J. Zhao, Y. Li, H. Li, J. Zhang, B. Li, Y. Gao, C. Chen, M. Luo and R. Huang, *Plant Soil*, 2015, 1-11.
4 T. Syversen and P. Kaur, *J.Trace.Elem.Med.Bio.,* 2012, **26**, 215-226.
5 T. Wei, J. Li, C. Bai, Q. Lin, H. Yao, Y. Xie and Y. Zhang, *Sci.China.Chem.*, 2013, **56**, 923-927.
6 S. Diez, *Rev. Environ. Contam. T.,* 2009,***198***, 111-132.
7 J. K. Schaefer, S. S. Rocks, W. Zheng, L. Liang, B. Gu and F. M. M. Morel, *P. Natl. Acad. Sci. USA*, 2011, **108**, 8714-8719.
8 S. J. B. Fretham, S. Caito, E. J. Martinez-Finley and M. Aschner, *Toxicol. Res.*, 2012, **1**, 32-38.
9 I. A. Adedara, D. B. Rosemberg, D. O. Souza, J. P. Kamdem, E. O. Farombi, M. Aschner and J. B. T. Rocha, *Toxicol. Res.*, 2015, **4**, 442-451.
10 W. Tang, J. Cheng, W. Zhao, W. Wang. *J. Environ. Sci.,* 2015, **34**, 107-115.
11 H. Peng, Michael S. Reid, X. Chris Le, *J. Environ. Sci.,* 2015, **38**, 83-86
12 M. Horvat, N. Nolde, V. Fajon, V. Jereb, M. Logar, S. Lojen, R. Jacimovic, I. Falnoga, L. Y. Qu, J. Faganeli and D. Drobne, *Sci. Total. Environ.*, 2003, **304**, 231-256.
13 H. Zhang, X. B. Feng, T. Larssen, L. Shang and P. Li, *Environ. Sci. Technol.*, 2010, **44**, 4499-4504.
14 J. Krasensky and C. Jonak, *J. Exp. Bot.*, 2012, **63**, 1593-1608.
15 Y. Li, J. Zhao, B. Zhang, Y. Liu, X. X, Y-F. Li, B. Li, Y.Gao, Z Chai. *Plant soil*, 2015, DOI 10.1007/s11104-015-2627-x
16 Y.-A. Chen, W.-C. Chi, T.-L. Huang, C.-Y. Lin, N. ThiThuyQuynh, Y.-C. Hsiung, L.-C. Chia and H.-J. Huang, *Plant Physiol.Biochem.*, 2012, **55**, 23-32.
17 Y. Gao, X. Peng, J. Zhang, J. Zhao, Y. Li, Y. Li, B. Li, Y. Hu and Z. Chai, *Metallomics*, 2013, **5**, 913-919.
18 Y. Gao, C. Chen and Z. Chai, *J. Anal. Atom.Spectrom.*, 2007, **22**, 856-866.
19 S. P. Yan, Z. C. Tang, W. Su and W. N. Sun, *Proteomics*, 2005, **5**, 235-244.
20 T. Karuppanapandian, J.-C. Moon, C. Kim, K. Manoharan and W. Kim, *Aust. J. Crop Sci.*, 2011, **5**, 709-725.
21 F. Bernier and A. Berna, *Plant. Physiol.Biochem.*, 2001, **39**, 545-554.
22 N. C. Soares, R. Francisco, J. M. Vielba, C. P. Ricardo and P. A. Jackson, *J. Proteome. Res.*, 2009, **8**, 2298-2309.
23 A. Nepovim, R. Podlipna, P. Soudek, P. Schroder and T. Vanek, *Chemosphere*, 2004, **57**, 1007-1015.
24 Y. F. Song, J. Cui, H. X. Zhang, G. P. Wang, F. J. Zhao and Z. G. Shen, *Plant Soil*, 2013, **366**, 647-658.
25 S. K. Yadav, *S. Afr. J. Bot.,* 2010, **76**, 167-179.
26 Q. Yang, Y. Wang, J. Zhang, W. Shi, C. Qian and X. Peng, *Proteomics*, 2007, **7**, 737-749.
27 M. W. Oh, S. K. Roy, A. H. M. Kamal, K. Cho, S.-W. Cho, C.-S. Park, J.-S. Choi, S. Komatsu and S.-H. Woo, *Mole. Bio. Rep.*, 2014, **41**, 671-681.
28 S. Carrasco-Gil, A. Alvarez-Fernandez, J. Sobrino-Plata, R. Millan, R. O. Carpena-Ruiz, D. L. Leduc, J. C. Andrews, J. Abadia and L. E. Hernandez, *Plant. Cell. Environ.*, 2011, **34**, 778-791.
29 S. S. Tang, C. C. Lin and G. G. Chang, *Free Radical Bio. Med.*, 1996, **21**, 955-964.
30 G. X. Yang, A. Inoue, H. Takasaki, H. Kaku, S. Akao and S. Komatsu, *J. Proteome Res.*, 2005, **4**, 456-463.
31 S. Gillet, P. Decottignies, S. Chardonnet and P. Le Marechal, *Photosynth. Res.*, 2006, **89**, 201-211.
32 L. Gao, X. Yan, X. Li, G. Guo, Y. Hu, W. Ma and Y. Yan, *Phytochemistry*, 2011, **72**, 1180-1191.
33 S. Komatsu, A. H. M. Kamal and Z. Hossain, *Front. Plant. Sci.*, 2014, **5**, 684.
34 S. Zhou, R. Sauve and T. W. Thannhauser, *J. Exp. Bot.*, 2009, **60**, 1849-1857.
35 B. J. Barkla, R. Vera-Estrella, M. Cristina Miranda-Vergara and O. Pantoja, *J. Proteomics*, 2014, **111**, 128-138.
36 G. M. Ali and S. Komatsu, *J. Proteome Res.*, 2006, **5**, 396-403.
37 T. Espevig, C. Xu, T. S. Aamlid, M. DaCosta and B. Huang, *J. Am. Soc. Hortic. Sci.,* 2012, **137**, 391-399.
38 H. E. Lee, D. Shin, S. R. Park, S.-E. Han, M.-J. Jeong, T.-R. Kwon, S.-K. Lee, S.-C. Park, B. Y. Yi, H.-B. Kwon and M.-O. Byun, *Biochem.Bioph. Res. Comm.*, 2007, **353**, 863-868.
39 M. Belen Montero-Palmero, A. Martin-Barranco, C. Escobar and L. E. Hernandez, *New Phytologist*, 2014, **201**, 116-130.
40 R. Rakwal, G. X. Yang and S. Komatsu, *Mol. Biol. Rep.*, 2004, **31**, 113-119.
41 M. Liao, Y. Li and Z. Wang, *Proteomics*, 2009, **9**, 2809-2819.
42 H. Liu, H. W. Peng, Y. S. Cheng, H. S. Yuan and H. F. Yang-Yen, *Mol. Cell. Bio.*, 2005, **25**, 3117-3126.
43 V. Ermolayev, W. Weschke and R. Manteuffel, *J. Exp. Bot.*, 2003, **54**, 2745-2756.
44 M. Gnanasekar and K. Ramaswamy, *Parasitol. Res.*, 2007, **101**, 1533-1540.
45 Y. Chen, X. Chen, H. Wang, Y. Bao and W. Zhang, *Proteome Sci.*, 2014, **12**:33.
46 Z. Q. Wang, G. Z. Li, Q. Q. Gong, G. X. Li and S. J. Zheng, *Bmc Plant Bio.*, 2015, **15**.
47 X. L. Guo, L. R. Bai, C. Q. Su, L. R. Shi and D. W. Wang, *Genet. Mol. Res.*, 2013, **12**, 6860-6870.
48 J.-S. Kim, J. Lee, C.-h. Lee, S. Y. Woo, H. Kang, S.-G. Seo and S.-H. Kim, *Plant Pathology. J.*, 2015, **31**, 195-201.
49 M. delas Mercedes Dana, J. A. Pintor-Toro and B. Cubero, *Plant Physiology*, 2006, **142**, 722-730.
50 S. J. Opella, T. M. DeSilva and G. Veglia, *Curr.Opin. Chem. Bio.*, 2002, **6**, 217-223.
51 T. M. DeSilva, G. Veglia, F. Porcelli, A. M. Prantner and S. J. Opella, *Biopolymers*, 2002, **64**, 189-197.
52 Y. Sone, R. Nakamura, H. Pan-Hou, T. Itoh and M. Kiyono, *Bio. Pharm. Bull.*, 2013, **36**, 1835-1841.
53 Y. Xu, W. G. Buchholz, R. T. Derose and T. C. Hall, *Plant Mol. Bio.*, 1995, **27**, 237-248.
54 J. L. Hall, *J. Exp. Bot.*, 2002, **53**, 1-11.
55 L. Zagorchev, C. E. Seal, I. Kranner and M. Odjakova, *Inte. J. Mol. Sci.,* 2013, **14**, 7405-7432