

Proteomic Approach for Discovery of Potential Biomarkers for Cd Toxicity in Rice

Yoon Kyung Kim¹ & Mi Young Lee¹

¹Department of Medical Biotechnology, SoonChunHyang University, Asan, Chungnam 336-600, Korea
Correspondence and requests for materials should be addressed to M.Y. Lee (miyoung@sch.ac.kr)

Accepted 7 August 2009

Abstract

We have used proteomic approaches to identify differentially expressed proteins in rice roots in response to CdCl₂. More than 750 protein spots that were detected by 2-dimensional gel electrophoresis showed reproducible abundance. 29 of these proteins showed significant changes after Cd treatment. Most prominent alteration was about 5 fold increase of putative glucan endo-1,3- β -D-glucosidase, while pathogen-related protein, glucanase and β -1,3-glucanase were dramatically reduced. Newly induced glutathione-S-transferase and the increase in methione synthase, fructose-bisphosphate aldolase and triosephosphate isomerase suggest their role in defense against Cd toxicity. Cd-induced alterations in specific isozyme patterns of ascorbate peroxidase, glutathione-S-transferase and peroxidase on the activity stained gels were in accordance with those of proteomic data.

Keywords: Proteomics, Cd, Toxicity, Biomarker, Rice

Introduction

Cadmium (Cd) is an important environmental pollutant with high toxicity to plants and animals. Cd has been reported to interact with photosynthetic, respiratory and nitrogen metabolism in plants, resulting in poor growth and low biomass accumulation¹. Cd also produces oxidative stress possibly by generating free radicals and active oxygen species. These species react with lipids, proteins, pigments and nucleic acids, and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus affecting cell viability. Cd has recently been reported to inhibit photoactivation of photosystem II by competitive binding to the Ca²⁺ site². It has also been hypothesized that photochemistry might be somehow altered in the presence

of Cd, with the consequent formation of chlorophyll triplets under high light.

Several of the detoxification processes activated in the cells play very important roles during the exposure of plants to Cd. These are complexing of the metal by phytochelatin, compartmentalization in vacuole, immobilization at the cell wall, exclusion through plasma membrane, and synthesis of stress-related proteins^{3,4}. Despite recent progress in understanding individual aspects of Cd accumulation, the cellular and molecular defense mechanisms against Cd toxicity are only partially understood.

The functions of proteins depend considerably on post-translational modification and protein-protein interaction which cannot be deduced from nucleic acid data. Present proteomic research aims at both identifying new proteins in relation to their function and ultimately at unraveling how their expression is controlled within regulatory networks⁵. On the rice plant, there have been some classical works dealing with the construction of proteomes from complex origins, such as the leaf, embryo, endosperm, root, stem, shoot and callus⁶⁻⁸. With the increases in the nucleotide and protein sequence database, the transcriptomic and proteomic analyses to date have mainly focused on the changes that are triggered by environmental factors⁹⁻¹¹, such as abiotic stressors, UV irradiation, copper chloride, ozone and drought. However, the studies on the biomarkers for Cd toxicity in rice have not been examined at the level of proteome. In order to investigate the candidates of biomarkers for Cd toxicity in rice, the differentially expressed proteins following Cd treatment in the presence of light were examined in this investigation.

Results and Discussion

Proteomic Analysis of Differentially Expressed Proteins Following Cd Treatment

Proteomic analysis of rice roots following CdCl₂ treatment was performed in the presence of light. Proteins extracted from 0.1, 0.5 and 1 mM CdCl₂-treated roots were displayed on 2-dimensional gel electrophoresis (2-DE) gels. Cd induced-profound changes of protein expression patterns on 2-DE gels were shown in Figure 1. Over 750 proteins in the control sample were detected with computer-aided image ana-

lysis. In particular, 29 protein spots showed notable differences in protein expression patterns between con-

trol and Cd treated samples. These differentially expressed 29 protein spots, which were highly reproducible in triple experiments, were shown with high-magnification view (Figure 2).

As shown in Table 1, spot 3 was identified as a methionine synthase, and about 2 fold increase of methionine synthase was shown at 1 mM CdCl₂ (Figure 2). Actually this protein catalyzes the methylation of C-homocysteine to generate methionine, which is very important for methylation reaction and synthesis of polyamine and ethylene. It has also been proposed that polyamine and ethylene biosynthesis are important in the defense against environmental stresses¹². Spot 20 was identified as a putative glucan endo-1,3-β-D-glucosidase. Remarkably, this enzyme increased about 5 fold at 0.1 mM CdCl₂ over the control. Glucan endo-1,3-β-D-glucosidase has been reported to exist as structural isoforms that differ in size, pI, cellular localization and regulation pattern. Glucan endo-1,3-β-D-glucosidase has often been called as a β-1,3-glucanase in seed-plant species. However, another β-1,3-glucanase was induced in a β-1,3-glucanase deficient mutant after pathogen attack in tobacco¹³. Moreover, proteomic analysis in this study revealed that the response

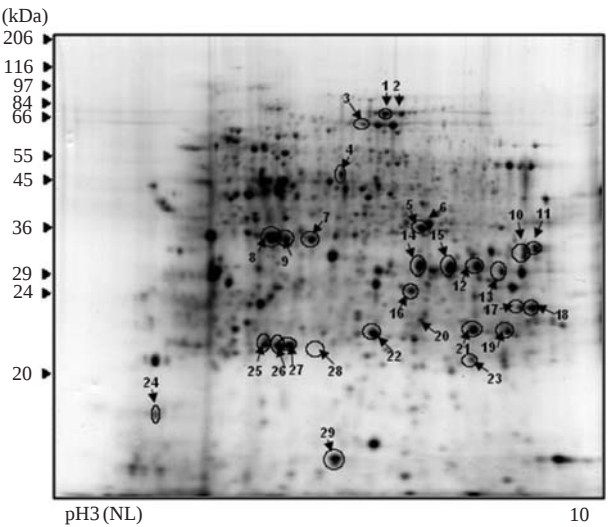


Figure 1. 2-Dimensional gel electrophoretic separation of proteins from rice seedling roots after CdCl₂ treatment. Numbered arrowheads indicate changes in proteins after Cd treatment.

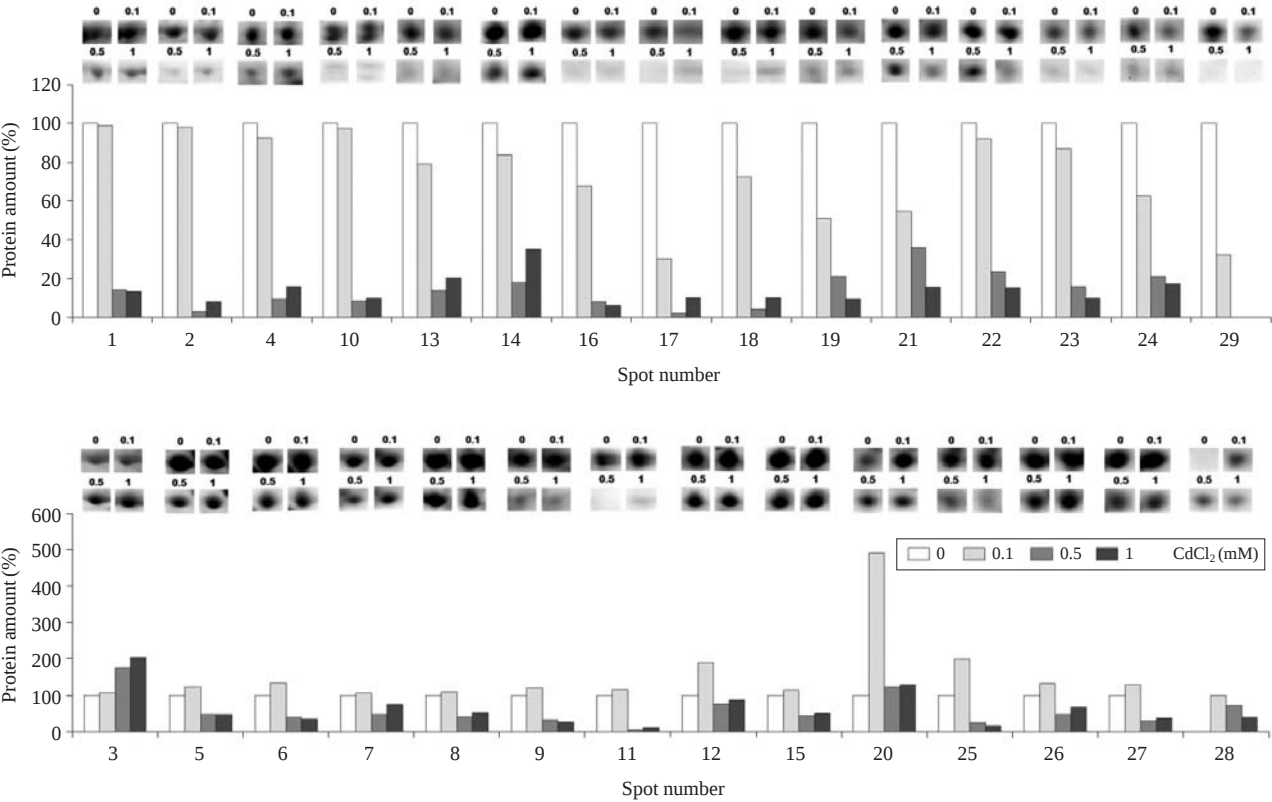


Figure 2. High magnification views of the differentially expressed proteins by CdCl₂. Protein expression levels were determined by relative intensity using image analysis. Normalized spot intensities of the CdCl₂ versus control group were compared. Mean spot intensities on individual gels are shown. P < 0.05 by Student's t-test.

Table 1. List of differentially expressed proteins from green roots of rice seedlings following CdCl₂ treatment.

Spot No.	Identified protein	Accession No.	Cov. %	Matching peptide No.	Tr/M.W/pI	CdCl ₂ (mM)		
						0.1	0.5	1
1	Sucrose synthase 1 (Sucrose-UDP glucosyltransferase 1)	P30298	17	16	92,610/6.0	—	—	—
2	Sucrose synthase 1 (Sucrose-UDP glucosyltransferase 1)	P31924	22	17	93,400/5.9	—	—	—
3	Methionine synthase	AAL33589	18	11	84,780/5.7	+	+	+
4	Serine/threonine kinase receptor precursor like protein	BAC57305	7	4	76,750/8.9	—	—	—
5	Glutamate dehydrogenase	AAO37984	24	9	44,610/6.1	+	—	—
6	Ferredoxin-NADP ⁺ reductase enzyme	BBA02248	19	6	35,830/7.7	+	—	—
7	Putative glyceraldehydes-3-phosphate dehydrogenase	CAD79700	20	8	42,380/6.4	+	—	—
8	Receptor protein kinase-like protein	BAB93286	14	7	46,550/7.8	+	—	—
9	Putative cytochrome P450	AAG59665	17	5	39,570/5.8	+	—	—
10	Fructose-bisphosphate aldolase, isoenzyme C-1, cytosolic-rice	S65073	28	11	39,150/8.7	—	—	—
11	Fructose-bisphosphate aldolase, isoenzyme C-1, cytosolic-rice	S65073	39	11	29,150/8.7	+	—	—
12	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	Q42977	33	10	36,650/6.6	+	—	—
13	Putative glyceraldehyde-3-phosphate dehydrogenase	CAD79700	15	7	42,380/6.4	—	—	—
14	Putative glyceraldehyde-3-phosphate dehydrogenase	CAD79700	18	7	42,380/6.4	—	—	—
15	Putative glyceraldehydes-3-phosphate dehydrogenase	CAD79700	35	13	42,380/6.4	+	—	—
16	β -1,3-glucanase	BAA77784	36	8	35,910/6.8	—	—	—
17	Putative pentatricopeptide (PPR) repeat containing protein	BAC79788	8	7	82,930/7.3	—	—	—
18	Hypothetical protein	CAD41354	8	4	62,640/9.0	—	—	—
19	Voltage-dependent anion channel	CAD82853	14	3	29,200/7.1	—	—	—
20	Putative glucan endo-1,3- β -D-glucosidase	BAC15774	30	7	37,030/7.0	+	+	+
21	dnaK-type molecular chaperone hsp70-rice (fragment)	S53126	18	12	71,490/5.1	—	—	—
22	Glucanase	AAK16694	20	4	34,760/5.9	—	—	—
23	Putative polyprotein	AAM22019	9	7	92,320/9.2	—	—	—
24	Peroxidase	AAC49820	33	8	32,880/8.0	—	—	—
25	L-ascorbate peroxidase	T03595	37	8	27,250/5.4	+	—	—
26	Triosephosphate isomerase, cytosolic (TIM)	P48494	25	6	27,270/5.4	+	—	—
27	Hypothetical protein	BAB86270	15	9	82,490/6.9	+	—	—
28	Putative glutathione-S-transferase OsGSTU6	AAG32469	24	5	25,700/5.8	ON	+	+
29	Pathogen-related protein	AAL27005	38	6	17,270/5.8	—	OFF	OFF

+: Increased protein, —: Decreased protein, OFF: Disappeared protein, ON: Newly induced protein

of glucan endo-1,3- β -D-glucosidase to Cd was completely different from that of β -1,3-glucanase. As shown in Figure 2, glucanase (spot 22) and β -1,3-glucanase (spot 16) were drastically diminished following Cd treatment, while glucan endo-1,3- β -D-glucosidase notably increased. This result indicates that glucan endo-1,3- β -D-glucosidase in rice roots might be separate and different from glucanase or β -1,3-glucanase. Taken together, about 5 fold enhancement of glucan endo-1,3- β -D-glucosidase might indicate the defensive role of the enzyme against Cd toxicity. The significant-

ly diminished spot 29 was identified as a pathogen-related protein (PR protein). One of the plant defense mechanisms has been the rapid expression of PR proteins, in response to microbial attack and tissue wounding¹⁴. Strong induction of PBZ1 gene (encoding a PR protein) in rice by UV and CuCl₂ suggested gene PBZ1 product's role against abiotic stress as well as biotic stress. Although the functions of all PR proteins have not been fully defined, they are thought to include the β -1,3-glucanase activity. β -1,3-glucanase was thought to contribute to the defense capability by hydrolyzing

various types of β -glucans, which are the major cell wall components of fungi¹⁵. However, β -1,3-glucanase and PR protein did not seem to strongly contribute to the defense against Cd toxicity, because they diminished to the extent that they almost disappeared at 1 mM CdCl₂. Spot 9 (putative cytochrome P450, CYPs) is a heme-containing monooxygenase that catalyzes numerous enzymatic reactions involved in detoxification. Due to the diversity of pathways requiring monooxygenase activity, the expression of CYPs has been reported to be modulated by a variety of environmental factors such as heavy metals, light, fungal elicitors and wounding¹⁶. Spot 7, 13, 14 and 15 were determined to be putative glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and spot 12 was determined to be cytosolic GAPDH. 4 putative and 1 cytosolic GAPDH were reduced probably due to Cd toxicity, while GAPDH was reported to increase with ozone stress in rice seedlings earlier¹⁷. GAPDH is a catalytic enzyme commonly known to be involved in glycolysis. Apart from playing a key role in glycolysis, this ubiquitously expressed enzyme also displays other activities unrelated to its glycolytic function, such as DNA replication, DNA repair, nuclear RNA export, membrane fusion and microtubule bundling in response to various signals¹⁸. Spot 6 (Ferredoxin-NADP⁺ reductase; FNR) increased slightly with 0.1 mM Cd, and then it was decreased with 0.5 and 1 mM CdCl₂. FNR is a flavoenzyme related to the terminal electron transfer to NADP⁺ in the photosynthetic electron transport system¹⁹. Cytosolic fructose-bisphosphate aldolase was decreased notably with 0.5 and 1 mM CdCl₂ treatment

and only about 10% of the enzyme remained at 1 mM CdCl₂, showing severe damages on the carbohydrate metabolism. This enzyme, which has at least four loci in the rice genome²⁰, plays vital roles in the carbohydrate metabolism and in the production of triosephosphates. Ozone fumigated rice was reported to have an increased fructose-bisphosphate aldolase²⁰. Decreased cytosolic triosephosphate isomerase (spot 26) by 0.5 and 1 mM CdCl₂ might play important roles in triosephosphate-involving metabolic cascades and photosynthetic carbon dioxide fixation. Notably, spot 25 increased about 2 fold at 0.1 mM CdCl₂ and this was identified to be L-ascorbate peroxidase (APX). APX is an important component of the ascorbate-glutathione pathway, which operates to scavenge reactive oxygen species produced through photosynthesis²¹. Thus, rice seedlings exposed to 0.1 mM CdCl₂ seemed to have a highly reinforced antioxidant scavenging system. However, this enzyme could not overcome the stress generated from high concentrations of Cd, because about 85% of the enzyme was diminished at 1 mM CdCl₂. Spot 24, corresponding to peroxidase (POD), was significantly lower in Cd-treated sample compared to that seen in the control. Peroxidase activities and isozyme patterns were changed in response to a variety of stressors. In particular, changes in specific isozyme levels were previously reported to be correlated with the defense signals responding to various stressors²². The prominent alteration by Cd in rice roots was the induction of putative glutathione-S-transferase (GST; Spot 28). This protectant protein, GST, might be newly induced in Cd-treated rice roots to counteract deleteri-

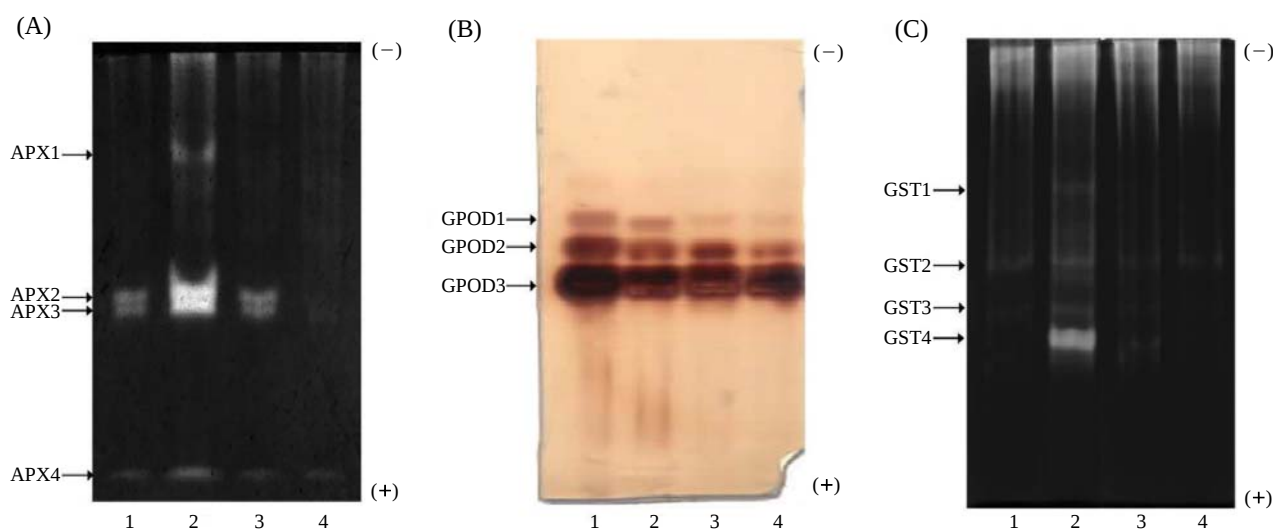


Figure 3. Electrophoregram of in situ activity staining for ascorbate peroxidase (A), guaiacol peroxidase (B) and glutathione-S-transferase (C) from rice seedling roots. 1: control, 2: 0.1 mM CdCl₂, 3: 0.5 mM CdCl₂, 4: 1 mM CdCl₂.

ous effects of oxidative injury incurred by Cd. Plant GSTs have been known for their role in herbicide detoxification, stress-induced metabolism and apoptosis²³. Pentatricopeptide repeat (PPR) containing protein that was notably down-regulated by CdCl₂ might be related to the RNA metabolism and is predicted to target to chloroplasts or mitochondria²⁴.

In Situ Activity Staining of Several Antioxidant Enzymes

Plants can change their proteomic expression to counteract a variety of environmental stresses^{25,26}. The roots are the primary locations that sense and react against various environmental stresses^{27,28}. Thus, the stress-responsive genes and antioxidant enzymes seem to be more strongly induced in root than in other organs. The alterations of antioxidant enzyme patterns by CdCl₂ on the activity stained gels showed good correlations with those on the 2-dimensional gels as shown in Figure 3. Ascorbate peroxidase (APX) increased about 2 times in the green roots at 0.5 mM Cd, and a marked enhancement of isozyme APX2 or APX3 contributed to this increase as measured by the band intensity. APX was already reported to increase in response to jasmonic acid and ozone in rice leaf²¹. Cd caused a gradual and drastic reduction in guaiacol peroxidase (GPOD) level in this condition, this being due to the decrease of isozyme GPOD1 or GPOD2 on the activity stained gel. In case of glutathione-S-transferase (GST), only GST4 isozyme was newly induced in response to Cd on the activity stained gel, while the residual isozymes were not inducible.

Conclusions

This is the systematic study of the extensive changes in rice green seedling roots following Cd treatment. Over 750 proteins in the control sample were detected, and 29 protein spots showed notable differences in protein expression patterns between control and Cd treated samples. In particular, this study provides strong evidence for the specific and rapid accumulation of glucan endo-1,3- β -D-glucosidase as a defense to Cd toxicity. However, pathogen-related protein, glucanase and β -1,3-glucanase showed decreased expression levels in response to Cd. Oxidative stress tolerance might be represented by the induction of glutathione-S-transferase and the increase in ascorbate peroxidase. Specific isozymes of stress-related enzymes, such as ascorbate peroxidase APX2 and APX3 and glutathione-S-transferase GST4 were induced notably under Cd stress on the activity-stained gels.

Materials and Methods

Material

Seeds of rice (*Oryza sativa* L. cv. Dong-Jin) were germinated in the dark for 3 days at 27°C. Green seedlings were grown under 3,000 lux illumination with 12 hrs light period/day. 5 day old plants grown in Hoagland's nutrient solution were further grown in the same solution, but containing 0.1, 0.5 and 1 mM CdCl₂ for 48 hrs. The roots of the seedlings were immediately stored at -70°C until analyzed for changes in protein profiles by proteomics.

Preparation of Crude Protein

For preparation of crude protein extracts, approximately 500 mg of rice roots were grounded in liquid nitrogen and homogenized with 1 mL of a homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM EDTA, 1 mM DTT and 1% Triton X-100. The homogenate was centrifuged at 15,000x g for 10 min and the supernatant was replaced in a new tube. The proteins were precipitated with 10 % trichloroacetic acid on ice for overnight, and the pellet was suspended in 250 μ L of sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 0.5% ampholytes.

2-Dimensional Gel Electrophoresis

2-Dimensional gel electrophoresis (2-DE) was carried out as described in our previous reports^{29,30}. For analytical and preparative gels, the 13 cm IPG strips (non-linear pH 3-10) were rehydrated for 12 hrs with 250 μ L of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS and 0.5% ampholytes) containing 500 μ g protein in a strip holder (Amersham Pharmacia Biotech, Uppsala, Sweden). IEF was conducted at 20°C with a Pharmacia IPGphor (Amersham Pharmacia Biotech). The running condition was as follows: 200 V for 1 hr, followed by 500 V for 1 hr, 1,000 V for 1 hr, and finally 8,000 V for 16 hrs. The focused strips were equilibrated twice for 15 min in 5 mL equilibration solution. The first equilibration was performed in a solution containing 6 M urea, 30% w/v glycerol, 2% w/v SDS, 1% w/v DTT and 50 mM Tris-HCl buffer, pH 8.8. The second equilibration was performed in a solution modified by the replacement of DTT by 2.5% w/v iodoacetamide. Second dimensional electrophoresis was performed on 11% linear gels of 18 $\times$ 16 cm gel cell, and gels were stained with colloidal CBB by incubation of the gels in 34% methanol, 0.1% Coomassie G-250, 17% ammonium sulfate and 3% phosphoric acid followed by destaining in 5% glacial acetic acid. The CBB stained gels were scanned by using

a UMAX PowerLook 1100, and data were analyzed by using Image master 2D Elite software (Amersham Pharmacia Biotech, Uppsala, Sweden).

Enzymatic In-gel Digestion and MALDI-TOF Mass Analysis

Protein spots were enzymatically digested in a manner similar to that previously described^{29,30}. Protein analysis was performed by using a Ettan MALDI-TOF (Amersham Biosciences). Peptides were evaporated with a N₂ laser at 337 nm, and they were accelerated with 20 Kv injection pulse for time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak m/z (842.510, 2211.1046) as internal standards.

In Situ Enzyme Activity Staining

In situ activity stainings of guaiacol peroxidase, ascorbate peroxidase and glutathione-S-transferase on the non-denatured gel were performed as reported in our previous investigations³¹⁻³³.

References

- Sanita di Toppi, L. & Gabbriellini, R. Response to cadmium in higher plants. *Environ. Exp. Bot.* **41**, 105-130 (1999).
- Faller, P., Kienzler, K. & Krieger-Liszkay, A. Mechanism of Cd²⁺ toxicity: Cd²⁺ inhibits photoactivation of Photosystem II by competitive binding to the essential Ca²⁺ site. *Biochim. Biophys. Acta* **1706**, 158-164 (2005).
- Lee, M.Y. & Shin, H.Y. Cadmium-induced changes in antioxidant enzymes from the marine alga *Nannochloropsis oculata*. *J. Appl. Phycol.* **15**, 13-19 (2003).
- Mahto1, S.K., Yoon, T.H. & Rhee, S.W. Characterizing cell death events using a microfluidics-based method. *BioChip J.* **2**, 242-247 (2008).
- Oh, Y.H., Kim, Y.P. & Kim, H.S. SUMO (Small Ubiquitin-like Modifier) chip for analysis of SUMO-conjugation to a target protein. *BioChip J.* **1**, 28-34 (2007).
- Komatsu, S., Muhammad, A. & Rakwal, R. Separation and characterization of proteins from green and etiolated shoots of rice (*Oryza sativa* L.): Towards a rice proteome. *Electrophoresis* **20**, 630-636 (1999).
- Komatsu, S., Rakwal, R. & Li, Z. Separation and characterization of proteins in rice (*Oryza sativa*) suspension cultured cells. *Plant Cell Tissue Organ Cult.* **55**, 183-192 (1999).
- Tsugita, A. *et al.* Separation and characterization of rice proteins. *Electrophoresis* **15**, 708-720 (1994).
- Rakwal, R., Tamogami, S. & Kodama, O. Role of jasmonic acid as a signaling molecule in copper chloride-elicited rice phytoalexin production. *Biosci. Biotech. Biochem.* **60**, 1046-1048 (1996).
- Agrawal, G.K., Rakwal, R., Yonekura, M., Kubo, A. & Saji, H. Proteome analysis of differentially displayed proteins as a tool for investigating ozone stress in rice (*Oryza sativa* L.) seedlings. *Proteomics* **2**, 947-959 (2002).
- Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. & Bennett, J. Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics* **2**, 1131-1145 (2002).
- Rzewuski, G. *et al.* OsMTN encodes a 5'-methylthioadenosine nucleosidase that is up-regulated during submergence-induced ethylene synthesis in rice (*Oryza sativa* L.). *J. Exp. Bot.* **58**, 1505-1514 (2007).
- Beffa, R.S., Neuhaus, J. & Meins, F. Jr. Physiological compensation in antisense transformants: specific induction of an "ersatz" glucan endo-1,3-beta-glucosidase in plants infected with necrotizing viruses. *Proc. Natl. Acad. Sci. USA* **90**, 8792-8796 (1993).
- Dixon, R.A. & Lamb, C.J. Molecular communication in interactions between plants and microbial pathogens. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 339-367 (1990).
- Wessels, J.G.H. Tansley Review No. 45 Wall growth, protein excretion and morphogenesis in fungi. *New Phytol.* **123**, 397-413 (1993).
- Mizutani, M., Ward, E. & Ohta, D. Cytochrome P450 superfamily in *Arabidopsis thaliana*: isolation of cDNAs, differential expression, and RFLP mapping of multiple cytochromes P450. *Plant Mol. Biol.* **37**, 39-52 (1998).
- Pillai, M.A., Lihuang, Z. & Akiyama, T. Molecular cloning, characterization, expression and chromosomal location of OsGAPDH, a submergence responsive gene in rice. *Theor. Appl. Genet.* **105**, 34-42 (2002).
- Kim, C.I., Lee, S.H., Seong, G.J., Kim, Y.H. & Lee, M.Y. Nuclear translocation and overexpression of GAPDH by the hyper-pressure in retinal ganglion cell. *Biochem. Biophys. Res. Commun.* **341**, 1237-1243 (2006).
- Paladini, D.H., Musumeci, M.A., Carrillo, N. & Ceccarelli, E.A. Induced fit and equilibrium dynamics for high catalytic efficiency in ferredoxin-NADP(H) reductase. *Biochemistry* **48**, 5760-5768 (2009).
- Nakamura, H. *et al.* Genomic structure of the rice aldolase isozyme C-1 gene and its regulation through a Ca²⁺-mediated protein kinase-phosphatase pathway. *Plant Mol. Biol.* **30**, 381-385 (1996).
- Asada, K. Ascorbate peroxidase-a hydrogen peroxide-scavenging enzyme in plants. *Physiol. Plant.* **85**, 235-241 (1992).
- Yi, K.W. & Lee, M.Y. Environmental stress-induced extracellular isoperoxidase RC3 from rice. *J. Environ. Biol.* **24**, 17-22 (2003).

23. Dixon, D.P., Laphorn, A. & Edwards, R. Plant glutathione transferases. *Genome Biol.* **3**, reviews30004.1-reviews30004.10 (2002).
24. Williams, P.M. & Barkan, A. A chloroplast-localized PPR protein required for plastid ribosome accumulation. *Plant J.* **36**, 675-686 (2003).
25. Hashimoto, M., Toorchi, M., Matsushita, K., Iwasaki, Y. & Komatsu, S. Proteome analysis of rice root plasma membrane and detection of cold stress responsive proteins. *Protein Pept. Lett.* in press
26. Feng, H., Xu, W.Z., Lin, H.H. & Chong, K. Transcriptional regulation of wheat VER2 promoter in rice in response to abscisic acid, jasmonate and light. *J. Genet. Genomics* **36**, 371-377 (2009).
27. Akiyama, T., Yoshida, M., Hoshino, T., Opasiri, R. & Ketudat Cairns, J.R. Expression of an endo-(1,3;1,4)-beta-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings. *J. Plant Physiol.* in press
28. Xiong, J. *et al.* Cadmium decreases crown root number by decreasing endogenous nitric oxide, which is indispensable for crown root primordia initiation in rice seedlings. *Planta* **230**, 599-610 (2009).
29. Son, B.S. *et al.* Toxicoproteomic analysis of differentially expressed proteins in rat liver by DEHP. *Mol. Cell. Toxicol.* **3**, 299-305 (2007).
30. Sim, Y.J. & Lee, M.Y. Identification of proteins in human follicular fluid by proteomic profiling. *Mol. Cell. Toxicol.* **4**, 253-259 (2008).
31. Lee, M.Y. Effects of Na₂SO₃ on the activities of antioxidant enzymes in geranium seedlings. *Phytochemistry* **59**, 493-499 (2002).
32. Park, S.K., Jin, E.S. & Lee, M.Y. Expression and antioxidant enzymes in *Chaetoceros neogracile*, an antarctic alga. *CryoLetters* **29**, 351-361 (2008).
33. Lee, M.Y. & Kim, S.S. Characteristics of six isoperoxidases from Korean radish root. *Phytochemistry* **35**, 287-290 (1994).