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BRIEF COMMUNICATION

Differential proteomic analysis of cadmium-responsive proteins in wheat leaves

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Abstract

To gain a comprehensive understanding of plant response to Cd, physiological and proteomic changes in wheat (*Triticum aestivum* L.) leaves exposed to a range of Cd concentrations (10, 100 and 200 μM) were investigated. Leaf elongation was decreased, whereas the H_2O_2 and malondialdehyde content increased significantly at higher Cd concentrations. Changes in protein profiles were analyzed by two-dimensional electrophoresis. Twenty-one proteins which showed 1.5-fold change in protein abundance in response to Cd were identified. These proteins can be functionally grouped into three groups: *1*) oxidative stress response, *2*) photosynthesis and sugar metabolism and *3*) protein metabolism and others. These results provide a new insight into our understanding of the molecular basis of heavy metal response in plants.

Additional key words: antioxidative enzymes, heavy metal, oxidative stress, *Triticum aestivum*.

Despite cadmium is a non-essential element and even toxic at very low concentration, it is easily taken up by plant roots. Cd accumulation in plant causes various toxicity symptoms such as chlorosis, wilting, growth reduction and cell death (Sanità di Toppi and Gabbrielli 1999, Clemens 2006). The cellular toxicity can result from various effects of Cd, for instance, disturbing sugar metabolism, damaging photosynthetic system, interfering nutrients balance and inducing oxidative stress (Clemens 2006).

The identification of the functional genes or proteins that are involved in the responses to heavy-metal stress is a fundamental step in understanding the molecular mechanisms of stress responses. Such understanding could lead to the development of transgenic plants that have an enhanced tolerance to heavy-metal stress or plants capable of being used in phytoremediation. With the advent of functional genomic technologies such as microarray and two-dimensional electrophoresis (2-DE), integrated analysis of Cd response both at RNA and protein level have been initiated to investigate the mechanisms of plant response to Cd stress (Cebeci *et al*. 2008, Alvarez *et al*. 2009). To date, several studies have been conducted to elucidate the response of model plant such as *Arabidopsis* and rice under heavy metal stress at proteome level (Lee *et al*. 2010, Semane *et al*. 2010). However, the plant response to Cd is far from understanding, as there is very little overlap between the differentially expressed genes and proteins as revealed by previous reports (Semane *et al*. 2010).

Wheat is an important crop worldwide and is known to be a leading dietary source of Cd (Zhang *et al*. 1997). However, very few proteomic studies were conducted on wheat response to heavy metals. In addition to the high-level Cd responses studied by Ge *et al.* (2009), the present study focused on the effects of Cd at the

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Abbreviations: APX - ascorbate peroxidase; 2-DE - two-dimensional electrophoresis; GST - glutathione-S-transferase; MDA - malondialdehyde; RuBPC/O - ribulose-1,5-bisphosphate carboxylase/oxygenase; SOD - superoxide dismutase.

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proteome level of wheat leaves, using moderate Cd concentrations.

Wheat (*Triticum aestivum* L. cv. Yangmai 15) seeds were sterilized using 70 % ethanol for 2 min and 2 % sodium hypochlorite for 25 min. Having thoroughly washed with distilled water, wheat seeds were germinated in Petri dish on the filter paper with 5 cm^3 of one-fifth strength Hoagland nutrient solution supplemented with $CdCl_2$ at the final concentrations of 0 (control), 10, 100 and 200 μM. For each treatment, three different dishes were prepared and placed in a growth chamber (16-h photoperiod, irradiance of 300 μmol m^{-2} s^{-1}, dark/light temperature of 18/23 °C and constant relative humidity of 65 %). After one-week Cd treatment, leaves from treated and untreated 7-d-old wheat seedlings were collected.

To determine the Cd content, wheat leaves were mineralized with a HNO_3-$HClO_4$ (4:1, v/v) mixture. Cd content in the tissue extracts were measured by atomic absorption spectrophotometry. The content of chlorophyll was determined in 80 % acetone extract of leaf tissues as described by Hegedüs *et al*. (2001). H_2O_2 content in wheat leaves was measured by method of Hung and Kao (2004). The lipid peroxidation was determined in terms of malondialdehyde (MDA) contents, a product of lipid peroxidation, according to the method of Heath and Packer (1968).

2-DE and protein identification were performed as described by Wang *et al*. (2005). 450 μg of each protein extract was separated by iso-electrophoresis using IPG strips (pH 3 - 10, 17 cm) in the *Protean* system (*BioRad*, Hercules, USA). The second dimension was run on a 12.5 % polyacrylamide gel using *Ettan DALTsix* system (*GE Healthcare*, Piscataway, USA). Each treatment was repeated three times. Gels were visualized by Coomassie Blue staining and analyzed using *PDQuest* software (*version 7.2*; *Bio-Rad*). Spots showing 1.5-fold difference in volume when compared with the corresponding spots of control were excised from the gels and digested with trypsin. The resulting peptides were analyzed in a *MALDI-qTOF* mass spectrometer (*Ultraflex III*, *Bruker-Daltonics*, Bremen, Germany) and protein identification was performed using the *MASCOT* software. Wheat ESTs downloaded from *NCBI* were transformed into polypeptides by *cTrans* program (Xu *et al*. 2007). The peptide mass fingerprintings (PMF) were also used to search against wheat polypeptide database with localized *MASCOT* program for protein identification (Wang *et al*. 2005).

All results are presented as means ± standard error of at least three replicates. Statistical assays were carried out by one-way *ANOVA* using Student's *t*-test to compare control with different treated samples. Significance was determined at the $P < 0.05$ levels.

After one-week exposure, Cd content in wheat leaves increased concomitantly with the metal concentration in nutrient solution (Table 1). Cd accumulation was associated with the evident symptoms of phytotoxicity, as the decrease in leaf length and the reduction in chlorophyll content. A significant decrease in leaf length and chlorophyll content was observed at higher Cd concentration (100 and 200 μM), while the lower concentration (10 μM) had no remarkable effect as compared to the control (Table 1).

Treatment with Cd resulted in a dose-dependent H_2O_2 accumulation in wheat leaves. Significant increase of H_2O_2 was observed even at lowest Cd concentration. In contrast, the MDA content in wheat leaves was not significantly affected by 10 μM Cd, but increased dramatically in the case of higher Cd concentration (Table 1). The data obtained here suggested that Cd induced an oxidative stress and correlated with those announced by Dixit *et al*. (2001). Higher Cd concentrations (100 μM upwards) lead to obvious stress in wheat plants. To obtain better understanding of Cd responsive mechanism in wheat leaves, 100 μM Cd treated leaves were selected for further proteomic analysis using 2-DE. Each gel revealed more than 300 well-resolved protein spots (Fig. 1). Image analysis indicated that 43 protein spots showed 1.5-fold difference in abundance when compared with the corresponding spots of control. 21 of them were identified by MS analysis and querying PMF against protein database or wheat polypeptide database (Table 2). These identified proteins can be put into groups of those involved in oxidative stress responses, photosynthesis and sugar metabolism, protein metabolism and others.

Three up-regulated proteins belong to the group related to oxidative stress: ascorbate peroxidase (APX; spot 8 and 9), glutathione-S-transferase (GST; spot 10) and superoxide dismutase (SOD; spot 11). SOD catalyzes

Table 1. Effect of Cd treatment on Cd accumulation, leaf length, chlorophyll, H_2O_2 and MDA content. Data are means ± SE, $n = 20$ for leaf length, $n = 3$ for Cd, chlorophyll, H_2O_2 and MDA content. * indicate values that differ significantly from control at $P < 0.05$.

Treatment	Cd content [μg g^{-1}(d.m.)]	Leaf length [cm]	Chlorophyll content [mg g^{-1}(f.m.)]	H_2O_2 content [μmol g^{-1}(f.m.)]	MDA content [nmol g^{-1}(f.m.)]
Control	1.90±0.21	20.1±1.1	1.089±0.127	1.90±0.17	6.99±0.51
10 μM	13.29±1.08*	19.2±1.3	1.069±0.077	2.92±0.26*	8.32±0.97
100 μM	50.17±3.69*	15.0±0.9*	0.887±0.083*	7.02±0.41*	10.13±1.04*
200 μM	79.73±5.11*	14.3±0.6*	0.848±0.054*	8.79±0.63*	11.31±1.37*

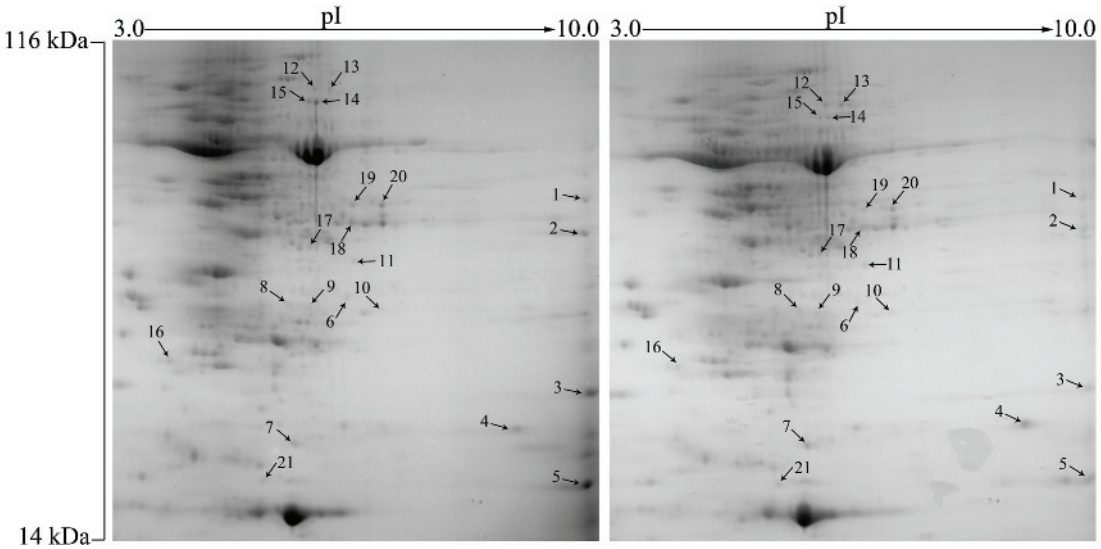

Fig. 1. Changes in protein profiles of wheat leaves induced by the treatment with 100 μM Cd (*on the right*) in comparison with control (*on the left*). *Arrows* indicate the proteins with at least 1.5-fold change after Cd treatment.

Table 2. Identification of proteins differentially expressed following Cd exposure in wheat leaves.

Spot No.	Mr /pI theoretical	Mr/pI observed	Fold change	Sequence coverage [%]	Matched peptides	Score	Accession No	Protein name
1	40.4/8.8	40.3/9.6	+2.38	30	7	90	BG904931	glycolate oxidase
4	22.0/10.2	18.3/8.5	+1.92	24	5	57	Q9BC10_9BRYO	ribosomal protein
8	27.5/5.9	29.3/5.5	+1.60	40	8	105	CAA06996	ascorbate peroxidase
9	27.5/5.9	28.4/5.6	+1.51	40	8	101	CAA06996	ascorbate peroxidase
10	25.0/6.0	28.8/6.7	+1.93	18	3	61	AAL47688	glutathione-S-transferase
11	25.3/7.9	33.5/6.3	+1.56	45	9	107	AAB68035	superoxide dismutase
12	55.3/6.1	66.1/5.8	+1.67	28	13	165	BAB47031	H^+-transporting two-sector ATPase α-chain
13	55.3/6.1	66.1/6.0	+2.63	31	15	155	BAB47031	H^+-transporting two-sector ATPase α-chain
17	42.1/9.0	35.6/5.9	+1.86	22	6	82	ACM78035	fructose 1,6-bisphosphate aldolase precursor
21	16.0/6.3	17.8/5.5	+1.65	37	4	76	BAF30986	glycine-rich RNA-binding protein
2	41.4/9.4	35.3/9.5	-2.63	55	14	160	S04783	protochlorophyllide reductase
3	22.0/9.8	30.6/9.6	-3.16	33	8	115	P36213	photosystem 1 reaction center subunit 2
5	21.1/9.7	16.9/9.6	-1.63	45	7	78	ABO70330	putative oxygen-evolving complex precursor
6	32.5/6.7	29.8/6.3	-2.52	32	8	74	BAA32786	VER2 protein
7	13.6/5.8	19.4/5.4	-1.50	23	3	61	BAB19810	RuBPC small chain
14	53.4/6.2	64.0/6.0	-3.53	23	12	120	AAP92166	RuBPC large chain
15	47.2/6.1	64.0/5.9	-2.75	30	13	132	AAV33293	RuBPC/O large subunit
16	18.9/4.6	24.2/3.7	-1.56	29	4	54	AAM34280	translationally-controlled tumor protein
18	36.7/7.7	37.9/6.3	-1.60	27	9	90	BAD22157	glyceraldehyde-3-phosphate dehydrogenase
19	36.6/6.7	40.1/6.3	-2.14	33	9	86	P26517	glyceraldehyde-3-phosphate dehydrogenase
20	84.8/5.7	40.1/6.7	-2.15	19	10	75	BAD34660	methionine synthase

the dismutation of superoxide into oxygen and H_2O_2, and the resulted H_2O_2 can be converted into H_2O by APX. Consistently with our results, it has been reported that increased expression of SOD and APX, both at RNA and protein level, was detected in Cd treated plants (Herbette *et al*. 2006, Ammar *et al*. 2008, Alvarez *et al*. 2009). GST is a kind of GSH-dependent detoxifying enzymes involved in protecting plants against environment stress (Marrs 1996). A role in the oxidative stress response emerges because they can catalyze GSH-dependent peroxidases and can conjugate, and thus detoxify metabolites arising from oxidative damage (Edwards *et al*. 2000). For example, over-expression of GST in *Arabidopsis* can reduce the salt stress-induced lipid peroxidation (Katsuhara *et al*. 2005). In this study, the increased expression of GST and enhanced lipid

peroxidation after Cd application are in agreement with these observations. Additionally, GST has been reported to be involved in the direct quenching of Cd ions, forming GSH-Cd complex, and play a crucial role in heavy metal detoxification in plant cells (Adamis *et al.* 2004, Mishra *et al*. 2009). Up-regulated expression of APX, SOD and GST addresses an important role for antioxidant enzymes under Cd stress.

Cd treatment resulted in decreased expression of several proteins related to photosynthesis, including the enzymes in chlorophyll synthesis pathway (protochlorophyllide reductase; spot 2), the proteins of photosystem (PS) 1 reaction center (subunit II; spot 3) and PS 2 (putative oxygen-evolving complex precursor; spot 5), and also some enzymes involved in Calvin cycle (ribulose-bisphosphate carboxylase small and large subunits; spots 7, 14, 15). It has been reported that a large number of photosynthesis-related genes markedly decreased after Cd treatment in *Arabidopsis* (Herbette *et al*. 2006). The decreased content of chlorophyll and reduced expression of photosynthesis-related proteins in this study correlated with Cd-induced damage to the photosynthetic apparatus (Siedlecka and Krupa 1996, Vassilev *et al*. 2004, Wang *et al*. 2009).

In contrast to the suppression of photosynthetic proteins, some enzymes dealing with photorespiratory pathway and glycolysis were induced by Cd treatment: glycolate oxidase (spot 1), H^+-transporting two-sector ATPase (spots 12, 13) and fructose 1,6-bisphosphate aldolase precursor (spot 14). The induction of these proteins may suggest that under Cd stress, the flow of sugars through these pathways is enhanced, possibly for supplying enough energy under reduced photosynthetic rate. The elevation of proteins involved in glycolytic pathway was also demonstrated by Lee *et al*. (2010) in Cd exposed *Arabidopsis*.

After Cd treatment, the abundance of several proteins involved in protein synthesis increased. Glycine-rich RNA-binding protein (spot 21) is known to be a kind of post-transcriptional regulator. An induced expression of glycine-rich RNA-binding protein has been documented in plants response to environmental stresses such as cold, drought or wounding (Kim *et al.* 2008). Recently, the *Arabidopsis thaliana* glycine-rich RNA-binding protein has been associated with the regulation of gene expression at the posttranscriptional level in response to oxidative stress (Schmidt *et al*. 2010). Ribosomal protein (spot 4) makes up the ribosomal subunits by conjunction with rRNA. The induced expression of these proteins indicated that protein synthesis might be essential in plant response to heavy metal stress (Semane *et al*. 2010).

Reduced expression of methionine synthase (spot 20) was observed during Cd stress. Methionine synthase is known to be an important enzyme in cysteine and methionine metabolism pathway, and can convert homocysteine into methionine (Wirtz and Droux 2005). It has been suggested that the increased biosynthesis of Cys and GSH was essential for fighting against heavy metal-induced toxicity (Cobbett 2000, Bačkor and Loppi 2009). Thus, the decreased expression of methionine synthase might contribute to maintaining high level of Cys and GSH in plant cells.

Other proteins such as VER2 protein (spot 6), translationally-controlled tumor protein (spot 16) and glyceraldehyde-3-phosphate dehydrogenase (spot 18, 19) exhibited decreased expression in the presence of Cd.

In conclusion, our data indicates that the exposure to 100 μM Cd strongly affects wheat physiology, inhibits plant growth and alters protein patterns of leaves. Physiological data showed a good correlation with the proteomic analysis. A total of 21 proteins were identified that were differentially expressed in response to Cd. Greater accumulation of antioxidants or of other stress-related proteins in wheat leaves upon exposure to Cd suggests that they might work together to establish a new homeostasis in response to Cd. A further study on the function of these proteins would lead to a deeper insight concerning the molecular mechanism of Cd response in plant cell.

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