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Time course analysis of gene regulation under cadmium stress in rice

Ippei Ogawa · Hiromi Nakanishi · Satoshi Mori ·
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Abstract To elucidate the regulation of gene expression in response to cadmium (Cd) stress in rice (*Oryza sativa*), transcriptional changes in roots and shoots were investigated using a 22 K microarray covering 21,495 genes. Rice plants were exposed to 10 µM $CdCl_2$ for 3 h or 1 µM $CdCl_2$ for 24, 48, and 72 h, and 8 days in hydroponic culture. In roots, 1,207 genes were up-regulated, whereas 519 genes were down-regulated by more than twofold under 10 µM Cd stress for 3 h. Compared with roots, the shoots had fewer Cd-responsive genes. The expression of genes such as those encoding cytochrome P450 family proteins, heat shock proteins, and glutathione S-transferase was strongly induced. Genes encoding proteins involved in signal transduction, including transcription factors such as DREB and NAC, and protein kinases, were also induced. Genes involved in photosynthesis were mainly down-regulated after 3 h of stress. Genes for the synthesis of nicotianamine and 2′-deoxymugineic acid were induced in roots under 1 µM Cd stress for 8 days, suggesting the occurrence of iron deficiency under longer-term Cd stress. Cd-regulated transporter genes included PDR and MATE family transporters, which were strongly up-regulated in roots, especially under 10 µM Cd stress, suggesting their role in Cd detoxification via export of Cd from the cytoplasm. Their modification may potentially lead to the development of low-Cd rice, which contributes to human health as well as high-Cd rice useful for phytoremediation.

Keywords Cadmium · Rice · Microarray · Gene regulation

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Introduction

In recent years, accumulation of cadmium (Cd) in crop plants has become a major threat to agriculture and human health. Many of the world's areas of arable soils have become moderately contaminated with Cd through the use of phosphate fertilizers, sludge, and irrigation water containing Cd (Sanità di Toppi and Gabbrielli 1999; McGrath et al. 2001; Kikuchi et al. 2007). Considerable Cd accumulation in edible parts of crops, including rice, occurs in these areas (Arao and Ae 2003; Arao et al. 2003; Ishikawa et al. 2005). Cd causes toxic effects on all living organisms, and humans are at risk because it enters our bodies through the food chain. Crop plants uptake

Cd from soil and accumulate it in the edible parts ingested by humans or domesticated animals. Generally, higher concentrations of Cd in the soil result in higher concentrations of Cd in the plant body, and phytoremediation is one solution to this problem. Milyang 23 was selected as a promising cultivar of rice for the phytoextraction of Cd in paddy soils (Murakami et al. 2008). Breeding or biotechnology may provide effective methods for either producing food plants with low Cd content or sequestering Cd for phytoremediation; however, little is known about the molecular mechanisms of Cd distribution in the plant body or the response to Cd stress.

Cd has been reported to cause oxidative stress in cells (Sandalio et al. 2001; Romero-Puertas et al. 2004), although the details of the stress mechanism are still largely unknown. Unlike iron (Fe) or copper (Cu), Cd does not directly catalyze the generation of reactive oxygen species (ROS). Cd-caused toxicity has been reported to result from a disturbance in the balance of essential metals such as Fe and Cu in metalloenzymes (Stohs and Bagchi 1995).

Some genes and/or proteins regulated by excess Cd have been identified from transcription and proteomic analyses of *Arabidopsis thaliana* (Suzuki et al. 2001; Herbette et al. 2006; Sarry et al. 2006) and Indian mustard (*Brassica juncea*) (Fusco et al. 2005). One well-characterized mechanism of Cd detoxification is the chelation of Cd by glutathione (GSH) or phytochelatins (PCs) and the sequestration of the complexes in locations where their effects are less toxic (Grill et al. 1985; Howden et al. 1995a, b; Cobbett et al. 1998). In yeast, glutathione-conjugated Cd (GS-Cd) is sequestered in the vacuole by export from the cytoplasm via a glutathione-conjugated complex (GS-X)-transporting protein localized at the tonoplast (Li et al. 1997). Metal-lothioneins, which are gene-encoded peptides, also form complexes with heavy metals and are involved in detoxification or translocation (Clemens et al. 2002; Benavides et al. 2005). In *Arabidopsis*, root-to-shoot or shoot-to-root translocation of PCs, mostly via phloem, has been reported, suggesting that long-distance Cd translocation between the root and shoot is mediated by PCs (Gong et al. 2003).

Thlaspi caerulescens and *Arabidopsis halleri* are able to accumulate zinc (Zn) and Cd at high concentrations and are thus referred to as hyperaccumulators (Robinson et al. 1998; Bert et al. 2000). In these plants, highly expressed Zn transporters such as the ZIP family transporters (Grotz et al. 1998) are involved in Zn influx into the cytoplasm (Pence et al. 2000). However, the mechanism of Cd uptake and accumulation has not been well defined in these hyper-accumulators. Often, Fe or Zn transporters, owing to their low substrate specificity, are also involved in Cd transport. Among the ZIP family of *Arabidopsis* and rice, AtIRT1, OsIRT1, and OsIRT2, which are Fe transporters, and OsZIP1, which is a Zn transporter, have been shown to transport Cd (Korshunova et al. 1999; Nakanishi et al. 2006). In the heavy metal P-type ATPase (HMA) family, one of the P_{1B}-ATPase family transporters is involved in Cd detoxification. Over-expression of these genes conferred Cd resistance and resulted in changes in the amount of Cd accumulated in the plant body (Gravot et al. 2004; Verret et al. 2004). Other ATPases, cation diffusion facilitator (CDF) transporters, and natural resistance-associated macrophage protein (NRAMP) family transporters have been reported to transport Cd (Thomine et al. 2000). Pleiotropic drug resistance (PDR) family proteins are involved in Cd tolerance via export out of the cytoplasm (Kim et al. 2007). Transporter genes in rice should be studied further to clarify Cd transport and tolerance mechanisms.

In this study, we focused on determining the candidate genes involved in Cd transport for detoxification or translocation and on the identification of the molecular determinant/signals/network regulating Cd stress in rice. Using an Agilent 22 K rice oligomicroarray kit (Agilent Technologies, Tokyo, Japan), we analyzed Cd-regulated stress-related genes in rice roots and shoots, as well as some of the metabolic pathways and transporters putatively involved in Cd translocation or tolerance. The results provide an overview of molecular responses to Cd stress and identify potential target genes that may contribute to the reduction of Cd content or may be useful for the accumulation of large amounts of Cd in rice for phytoremediation.

Materials and methods

Plant material

Rice (*Oryza sativa* var. japonica. cv. Nipponbare) was grown in 20-L plastic containers containing a nutrient

solution with the following composition: 0.35 mM $(NH_4)_2SO_4$, 0.18 mM Na_2HPO_4, 0.27 mM K_2SO_4, 0.36 mM $CaCl_2$, 0.46 mM $MgSO_4$, 18 μM H_3BO3, 4.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 1.0 μM Na_2MoO_4, and 45 μM Fe(III)-EDTA. The solution was maintained at pH 5.0–5.5 with 1 M KOH. After 24 days of growth, half of the plants were transferred to a nutrient solution containing 10 μM $CdSO_4$ and grown for 3 h, or containing 1 μM $CdSO_4$ and grown for 24 h, 48 h, 72 h, or 8 days. The remaining plants were transferred to a nutrient solution without Cd, as a control. At each time point, nine plants from Cd-treated and control groups respectively were harvested for microarray analysis.

Oligo-DNA microarray analysis

A rice 22 K oligo-DNA microarray (Agilent Technologies), which contains 21,495 oligonucleotides synthesized based on sequence data of the rice full-length cDNA project (http://cdna01.dna. affrc.go.jp/cDNA/), was used. Total RNA was prepared from whole roots or shoots using the RNeasy Plant Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The yield and RNA purity were determined spectrophotometrically. The integrity of the RNA was checked using an Agilent 2100 Bioanalyzer, and total RNA (400 ng) was labeled with Cy-3 or Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit. To assess the reproducibility of the signals, the experiment was repeated by dye swapping. Fluorescently labeled targets were hybridized to Agilent rice 22 K oligo DNA microarrays. The hybridization process was performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray Scanner. Feature Extraction software (Agilent Technologies) was used for image analysis and data extraction processes. The fold changes were calculated (signal intensity of Cd-treated plants/signal intensity of control plants) at each time point. Genes showing fold changes >2 or <0.5 and with P-values<0.05 were considered to be significantly up- or down-regulated, respectively. For clustering and imaging of the data, Cluster and Treeview (Eisen lab; http://rana.lbl.gov/) were used.

Results

Shoot growth was inhibited and chlorophyll content (SPAD value) of the largest leaves did not increase after the addition of either 1 μM or 10 μM Cd (Fig. 1). After 2 days of Cd exposure, growth was significantly inhibited. The growth inhibition by Cd was nearly identical at both concentrations.

The number of genes whose expression was changed by Cd stress was analyzed (Fig. 2). In the case of treatment with 10 μM of Cd for 3 h, 1,207 (in roots, 5.6% of the total) and 211 (in shoots, 1.0%) genes were up-regulated by Cd. Under the same conditions 519 (in roots, 2.4%) and 375 (in shoots, 1.7%) genes were down-regulated by more than twofold. When rice was exposed to the lower concentration (1 μM) for 24 h, 48 h, 72 h, and 8 days, the number of Cd-regulated genes was much lower than that for 10 μM Cd. It seems that dramatic changes in the level of gene expression occurred in the roots exposed to 10 μM Cd for 3 h. In roots, the number of up-regulated genes was always greater than the number down-regulated, although this was not true in the shoots. The number of genes regulated in roots was larger than that regulated in the corresponding shoots except when exposed to 1 μM Cd for 8 days. In shoots exposed to 1 μM Cd for 24–72 h, changes in the expression of only a few genes were greater than twofold. This suggested that the shoot is less affected by Cd stress than the roots, although an early response to Cd at 24 h and possibly a secondary effect of Cd after 8 days of treatment appeared to exist.

When Cd-regulated genes showing the most dramatic regulation are ordered according to their relative change in expression in roots exposed to 10 μM Cd for 3 h, the top 50 up-regulated genes (Table 1) comprise six zinc-finger domain-containing protein genes including a ZIM domain-containing protein gene and a ZPT2-14 gene, three heat shock protein or precursor genes, three cytochrome P450 family protein genes, *OsDREB1A*, *OsDREB1B*, an MYB domain protein gene, a NAC domain-containing protein gene, an AP2 domain-containing protein, a WRKY transcription factor gene, glutathione S-transferase (GST) gene, genes for enzymes such as isocitrate lyase and allene oxide synthase, which are related to specific metabolic pathways, and some genes for transporter proteins such as the PDR transporter and the

Fig. 1 Changes in shoot length and chlorophyll content of rice before/after Cd stress treatment. a Shoot length. b Chlorophyll content measured as the soil plant analysis development (SPAD) value. Control (blue), 1 µM Cd (orange), 10 µM Cd (red). Arrows show the addition of Cd. n=3 (after Cd treatment) or 5 (before Cd treatment). Significant differences compared with controls were analyzed using a t-test (*, P<0.05). Error bars indicate SD
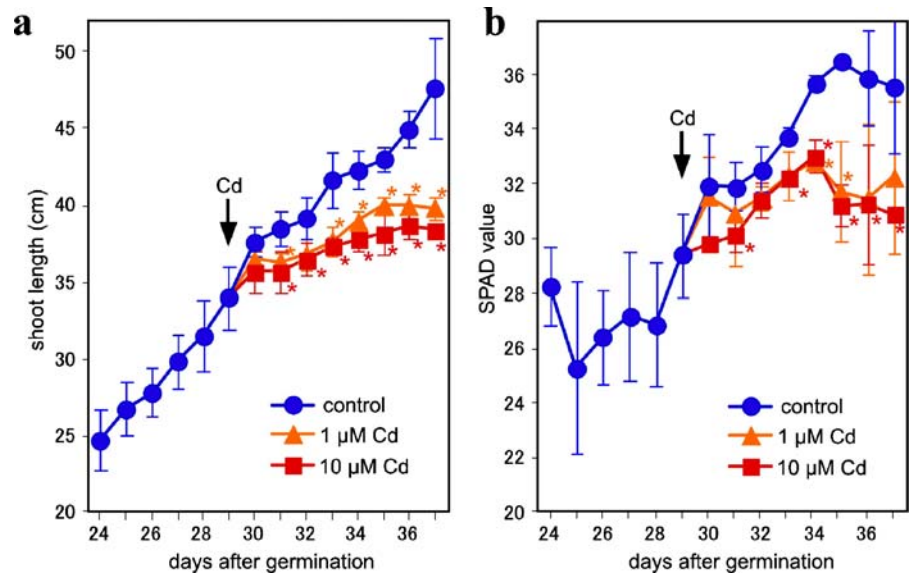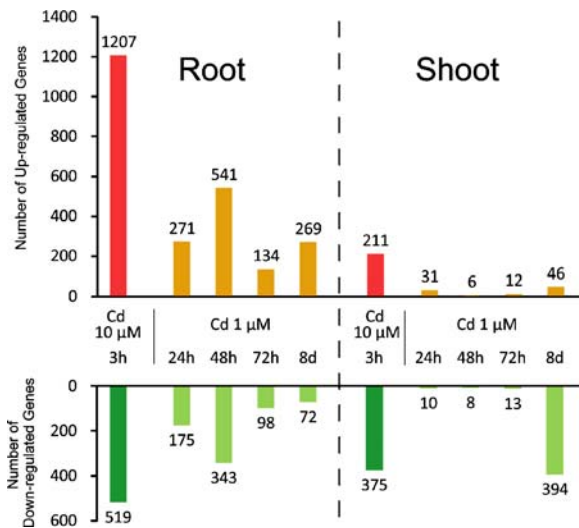
multidrug and toxin extrusion (MATE) family proteins.

Discussion

Significantly regulated genes and their possible functions in Cd stress

The inhibition of shoot growth by treatment with both 1 µM and 10 µM Cd demonstrated nearly equivalent

Fig. 2 The number of genes up- or down-regulated by Cd by more than twofold under each set of conditions (P<0.05)

trends (Fig. 1); however, 3-h treatment with 10 µM Cd induced a greater number of genes than did 24-h treatment with 1 µM Cd (Fig. 2). Table 1 lists the Cd-regulated genes showing the most dramatic regulation. The strong induction by Cd in a short time was confirmed by semi-quantitative reverse transcription PCR using the different plant samples from those used for microarray (Supplemental Fig. 1). The up-regulation of heat shock protein genes was consistent with other organisms such as yeast (Momose and Iwahashi 2001; Vido et al. 2001) and Arabidopsis (Vierling 1991; Sarry et al. 2006). Protein misfolding caused by Cd stress may have led to the induction of heat shock proteins. P450 enzymes have a wide spectrum of enzymatic activities; they are able to metabolize many xenobiotics and one of their orthologs in Drosophila was also up-regulated by Cd stress (Yepiskoposyan et al. 2006). GST catalyzes the conjugation of GSH to a variety of harmful compounds. Involvement of these genes, along with the P450 enzymes, in the Cd response was also observed in Drosophila, suggesting that involvement of P450 enzymes and GSTs are a well-conserved mechanism of the Cd response in a wide range of organisms. Transcription factors OsDREB1A and OsDREB1B have been reported to respond to cold stress and salt stress, and their constitutive over-expression in plants resulted in tolerance to freezing and high-salt (Dubouzet et al. 2003). Other transcription factors such as NAC proteins have been reported

Table 1 The 50 genes with the greatest relative expression in roots of 10 μM Cd-treated (3 h) plants

	Gene location	RAP-DB description	Fold Change
1	Os01g0373700	Conserved hypothetical protein	62.68
2	Os03g0180900[a]	ZIM domain containing protein	52.15
3	Os01g0699600[a]	Protein kinase-like domain containing protein	44.16
4	Os03g0820300[a]	Similar to ZPT2-14	43.27
5	Os01g0389700	Protein of unknown function DUF679 family protein	40.16
6	Os10g0517500[a]	Cys/Met metabolism pyridoxal-phosphate-dependent enzymes family protein	37.59
7	Os01g0373800	Conserved hypothetical protein	35.63
8	Os01g0609300[a]	OsPDR9	34.85
9	Os11g0151400[a]	Cytochrome P450 family protein	32.61
10	Os07g0529000[a]	Similar to Isocitrate lyase (Fragment)	30.12
11	Os10g0392400[a]	ZIM domain containing protein	28.78
12	Os01g0661800	Conserved hypothetical protein	28.72
13	Os09g0243200	Zinc finger, RING-type domain containing protein	28.59
14	Os03g0437200[a]	Zinc finger, C2H2-type domain containing protein	27.96
15	Os03g0760200[a]	Cytochrome P450 family protein	27.73
16	Os08g0120600	Similar to fructose-bisphosphate aldolase, cytoplasmic isozyme (EC 4.1.2.13)	27.07
17	Os09g0367700	OsGSTU5	25.49
18	Os02g0758000	Similar to low molecular weight heat shock protein precursor	23.78
19	Os01g0952900	Conserved hypothetical protein	23.56
20	Os04g0419100	Conserved hypothetical protein	23.28
21	Os04g0405300	Similar to Stem secoisolariciresinol dehydrogenase (Fragment)	23.07
22	Os09g0522000[a]	OsDREB1B	22.26
23	Os12g0564100	Similar to R2R3MYB-domain protein (Fragment)	22.04
24	Os01g0816100[a]	Similar to NAC domain protein	21.43
25	Os04g0180400[a]	Similar to cytochrome P450 CYP99A1 (EC 1.14.-.-) (Fragment)	21.27
26	Os04g0107900[a]	Similar to heat shock protein 80	21.09
27	Os07g0605800[a]	Similar to STF-1 (Fragment)	19.94
28	Os01g0106900	Similar to 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Fragment)	19.82
29	Os09g0522200	OsDREB1A	19.13
30	Os04g0339400[a]	Aldo/keto reductase family protein	18.73
31	Os03g0676400	VQ domain containing protein	18.50
32	Os10g0345100[a]	Multi antimicrobial extrusion protein MatE family protein	18.22
33	Os09g0385700	Zinc finger, AN1-type domain containing protein	18.06
34	Os07g0550600	Transferase family protein	17.95
35	Os08g0140300	Similar to tryptophan decarboxylase	17.80
36	Os08g0474000[a]	Similar to AP2 domain containing protein RAP2.6 (Fragment)	17.69
37	Os07g0638100	TolB, C-terminal domain containing protein	17.61
38	Os02g0181300	WRKY transcription factor 71 (Transcription factor WRKY09)	17.52
39	Os01g0639600	Protein of unknown function DUF1645 family protein	17.43
40	Os11g0643100	Transferase family protein	17.32
41	Os03g0225900[a]	Allene oxide synthase	17.19
42	Os01g0699500	Protein kinase-like domain containing protein	17.00
43	Os07g0526600	Esterase/lipase/thioesterase domain containing protein	16.75
44	Os01g0172100	Similar to triose phosphate/phosphate translocator	16.20

Table 1 (continued)

	Gene location	RAP-DB description	Fold Change
45	Os10g0370500	Actin-crosslinking proteins family protein	16.15
46	Os04g0516600	Aromatic amino acid beta-eliminating lyase/threonine aldolase domain containing protein	15.76
47	Os06g0586000	Conserved hypothetical protein	15.74
48	Os11g0244200	Similar to pisum sativum 17.9 kDa heat shock protein (hsp17.9) (Fragment)	15.30
49	Os06g0215600[a]	Similar to oxo-phytodienoic acid reductase	15.24
50	Os01g0510200	Conserved hypothetical protein	15.16

[a] Induction by Cd was confirmed by semi-quantitative reverse transcription PCR (Supplemental Fig. 1)

to mediate viral resistance (Xie et al. 1999) or to be involved in abiotic stress responses and tolerance by responding to drought, high salinity, abscisic acid (ABA), and methyl jasmonic acid (Fujita et al. 2004). Although the involvement of these transcription factors in response to Cd has not been previously documented, the novel finding that these are also up-regulated by Cd suggests a common responsive pathway between Cd and other abiotic stresses.

Up-regulated transporters with a possible involvement in Cd transport in roots

Analyzing Cd-regulated transporter genes is important because their modification may affect the level of accumulation of substrates, which can then be applied to bioengineering of crops with either significantly lesser or greater amounts of Cd, which is advantageous for food or phytoremediation, respectively. Most of the Cd-regulated transporters were affected within 3–48 h of Cd stress, suggesting that these transporters are affected relatively rapidly by Cd (Table 2). These include genes belonging to transporter families such as the ABC transporter superfamily [PDR, MDR, and multidrug resistance protein (MRP) subfamilies] and the MATE, CDF, HMA, and ZIP families. PDR, MDR, MRP, MATE, CDF, and HMA all have a high possibility of involvement in export of Cd from the cytoplasm because some orthologs of these transporters have been reported to be involved in Cd transport or detoxification activities (Li et al. 2002; Gravot et al. 2004; Verret et al. 2004; Kim et al. 2007). For example, one of the PDR transporters in *Arabidopsis*, AtPDR8, whose expression is up-regulated by Cd or Pb, is a Cd/Pb extrusion pump conferring tolerance to these metals (Kim et al. 2007). AtPDR8 is localized to the plasma membrane

primarily in the root hair and epidermal cells. AtPDR8-over-expressing plants have lower Cd contents than the wild type, and plants whose AtPDR8 function is mutated by RNAi or T-DNA show lower Cd contents. As for the MDR proteins, both the human and bacterial MDR genes confer Cd resistance to *E. coli* by extruding Cd (Achard-Joris et al. 2005). One of the MRP proteins in yeast, YCF1, confers Cd tolerance by transporting $(GSH)_2$–Cd^{2+} complexes to vacuoles, which have a low metabolic activity, resulting in a greater capacity to accumulate toxic compounds (Li et al. 1997). One of the MRP proteins in *Arabidopsis*, AtMRP3, whose gene expression was up-regulated by Cd treatment (Bovet et al. 2003), has been reported to partially restore the Cd resistance of a complemented *ycf1* yeast mutant (Tommasini et al. 1998). One of the MATE proteins in *Arabidopsis*, AtDTX1, confers Cd tolerance to the drug-sensitive *E. coli* mutant strain KAM3 (Li et al. 2002). One of the CDF proteins in *Bacillus subtilis*, CzcD, is involved in the efflux of Zn, Cd, and Co. Some HMA proteins in *Arabidopsis* have been reported to be involved in Cd tolerance, accumulation, and root-to-shoot translocation via export of Cd from the cytoplasm (Eren and Argüello 2004; Gravot et al. 2004; Verret et al. 2004; Mills et al. 2005). An up-regulated ZIP family transporter, OsIRT1, is an Fe importer that has been reported to also transport Cd (Nakanishi et al. 2006). Further investigation is needed to explain its role in the response to Cd.

Cluster analysis revealed time-dependent regulation of responsive genes

We performed clustering of the 3,596 genes shown in Fig. 2 (genes that were up- or down-regulated by more than twofold under at least one of the

Table 2 Transporter genes possibly involved in the Cd stress response whose expression was induced or reduced under Cd stress in roots

Gene location	Gene product	Fold change (Root)[a]				
		10 μM Cd	1 μM Cd			
		3 h	24 h	48 h	72 h	8 d
Os01g0609300[b]	OsPDR9	**34.9**	1.8	**2.0**	1.0	0.5
Os08g0544400	OsPDR1	**9.7**	**2.2**	**4.7**	1.4	1.6
Os08g0384500[b]	OsPDR17	**7.5**	1.6	0.9	1.2	0.9
Os07g0522500[b]	OsPDR5	**6.0**	1.8	1.4	1.0	1.0
Os01g0609900	OsPDR8	**2.8**	1.4	1.8	1.0	0.6
Os01g0342700	OsPDR16	**2.0**	1.4	1.6	1.3	0.9
Os01g0695800	MDR	**4.6**	1.6	1.7	1.2	1.0
Os04g0209200	MRP	**3.6**	1.2	1.2	1.1	0.8
Os10g0345100[b]	MATE efflux family protein	**18.2**	1.9	**0.4**	0.9	1.2
Os10g0190900	MATE efflux family protein	**3.5**	1.0	0.8	0.8	1.2
Os01g0504500	MATE efflux family protein	**2.9**	1.4	1.1	1.1	1.2
Os01g0684900	MATE efflux family protein	**2.4**	1.0	1.0	1.0	0.9
Os01g0837800	CDF	**2.0**	1.3	1.2	0.9	0.9
Os04g0556000	OsHMA5	**2.0**	1.2	1.4	1.0	1.2
Os03g0667500	OsIRT1	**2.2**	1.2	1.3	1.2	1.8

[a] Fold change values greater than 2 or less than 0.5 are indicated in bold letters

[b] Induction by Cd was confirmed by semi-quantitative reverse transcription PCR (Supplemental Fig. 1)

conditions) into 10 clusters (Fig. 3). Clustering allowed us to see the regulation patterns of genes at different time points during Cd stress in each tissue (root or shoot).

Unexpectedly, the Cd regulation of most of the Cd-regulated genes were time point-specific; in other words, most of the genes were regulated under only one of the conditions, although some genes were regulated under more than one condition in cluster 3 (up-regulated from 3 to 48 h in roots), 5 (up-regulated from 48 h to 8 days), and 8 (down-regulated from 3 to 48 h). In addition, only a few genes were similarly regulated in roots and shoots. These results suggest that gene regulation is dramatically different in the Cd-treated roots and shoots of rice plants.
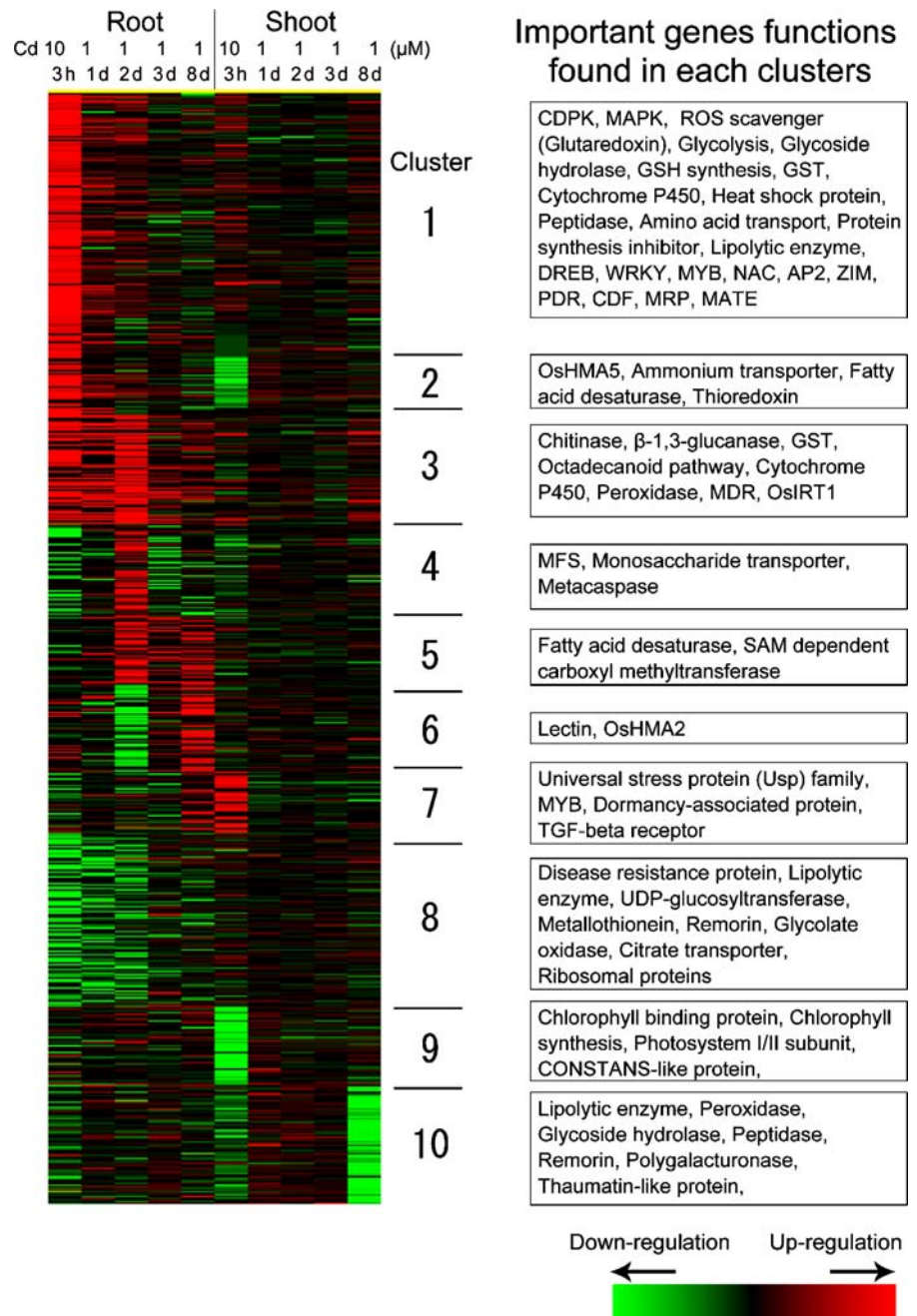
Based on the gene expression data, rice plants respond to Cd as follows (Fig. 4). (i) Stress signal transduction is activated by protein kinases, transcription factors, and ROS within a few hours after Cd exposure mainly in the roots. (ii) Stress-resistance mechanisms involving transporters, chelators, and antioxidants are activated soon after Cd exposure, mainly in the roots. (iii) The photosynthetic pathways are inactivated soon after Cd exposure in shoots, whereas in roots, protein synthesis is inactivated, and glycolysis and protein degradation are activated soon after Cd exposure. These may contribute to the energy and nutrient demands of the stress-resistance mechanisms. (iv) After 8 days of Cd exposure, Fe-acquisition mechanisms are activated to restore the Fe homeostasis damaged by Cd.

Stress signal transduction

The data showed that genes specifically up-regulated in roots during the earliest phase of the response (Cluster 1 in Fig. 3) encoded calcium-dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPKs), and transcription factors DREB protein, WRKY, NAC, MYB, and AP2. CDPKs and

Fig. 3 Cluster analysis of 3,596 genes whose expression changed by more than twofold after Cd exposure. Genes were classified in 10 clusters according to the patterns of their regulation of gene expression. The luminosity of the *red* or *green color* indicates the intensity of up- or down-regulation of each gene under each set of conditions. Gene functions shown in the right half represent those frequently found in each cluster and that are thought to be important in the response to Cd

Cluster 1: CDPK, MAPK, ROS scavenger (Glutaredoxin), Glycolysis, Glycoside hydrolase, GSH synthesis, GST, Cytochrome P450, Heat shock protein, Peptidase, Amino acid transport, Protein synthesis inhibitor, Lipolytic enzyme, DREB, WRKY, MYB, NAC, AP2, ZIM, PDR, CDF, MRP, MATE

Cluster 2: OsHMA5, Ammonium transporter, Fatty acid desaturase, Thioredoxin

Cluster 3: Chitinase, β-1,3-glucanase, GST, Octadecanoid pathway, Cytochrome P450, Peroxidase, MDR, OsIRT1

Cluster 4: MFS, Monosaccharide transporter, Metacaspase

Cluster 5: Fatty acid desaturase, SAM dependent carboxyl methyltransferase

Cluster 6: Lectin, OsHMA2

Cluster 7: Universal stress protein (Usp) family, MYB, Dormancy-associated protein, TGF-beta receptor

Cluster 8: Disease resistance protein, Lipolytic enzyme, UDP-glucosyltransferase, Metallothionein, Remorin, Glycolate oxidase, Citrate transporter, Ribosomal proteins

Cluster 9: Chlorophyll binding protein, Chlorophyll synthesis, Photosystem I/II subunit, CONSTANS-like protein,

Cluster 10: Lipolytic enzyme, Peroxidase, Glycoside hydrolase, Peptidase, Remorin, Polygalacturonase, Thaumatin-like protein,

MAPKs are involved in biotic and abiotic stress responses controlling the activation of defense mechanisms (Sheen 1996; Kovtun et al. 2000). The MAPK and CDPK pathways are thought to engage in cross talk with ROS production activities (Ren et al. 2002; Kobayashi et al. 2007). The expression of some genes belonging to the WRKY, NAC, MYB, and AP2 families are reported to be induced by biotic and abiotic stresses, and to play a role in the tolerance to those stresses. Expression of some of them is reported to be enhanced by ROS (Vandenabeele et al. 2003). Therefore, signal transduction involving ROS under biotic and abiotic stresses is suggested to also function in Cd stress.
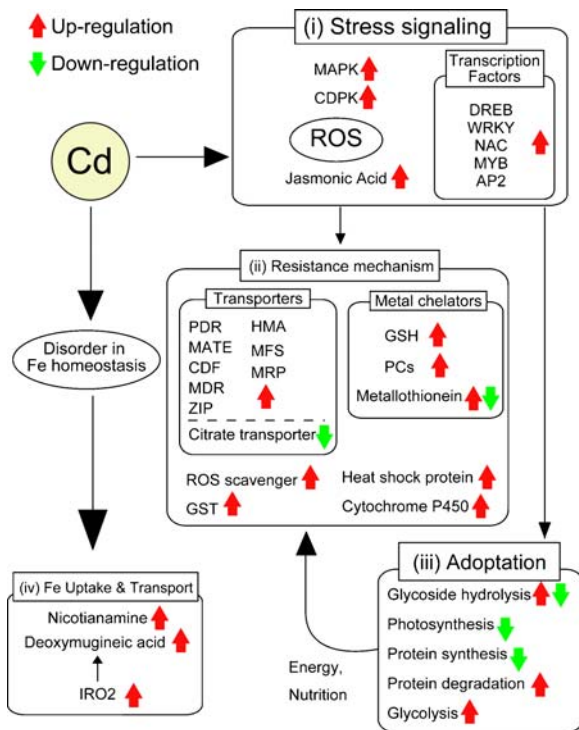
Fig. 4 The response to Cd in rice as deduced by gene expression. *Red* or *green arrows* indicate up- or down-regulated genes, respectively

Stress-resistance mechanism

Many kinds of stress-resistance mechanisms appeared to be up-regulated in roots soon after the initiation of Cd stress. For example, transporter genes belonging to transporter families such as PDR and MATE, genes for enzymes that may be involved in GSH synthesis (including those involved in cysteine, glutamate, and glycine, e.g., cysteine synthase, ATP sulfurylase, NADH-dependent glutamate synthase, serine hydroxymethyltransferase, and glutathione synthetase), genes for GST, heat shock proteins, cytochrome P450, and antioxidants such as glutaredoxin and thioredoxin were up-regulated (Cluster 1 and 2 in Fig. 3). GST has a role in the removal of ROS and also in the conjugation of toxic compounds with GSH because GSH also functions as a chelator. GSH-conjugated Cd is less toxic than Cd per se, and in yeast, it is exported to the vacuole where Cd is less toxic to the cell.

As Cd causes oxidative stress to cells (Romero-Puertas et al. 1999; Dixit et al. 2001; Romero-Puertas et al. 2002), scavenging of ROS is important for the

detoxification of Cd. Although ROS have a role in signal transduction for stress recognition, their production should be tightly regulated, or damage to the cells will result. Glutaredoxin, thioredoxin, and GSH may be responsible for such regulation by removing ROS in rice under Cd stress.

Activation or inactivation of metabolism, which leads to more efficient energy use and contributes to stress-resistance mechanisms

Genes involved in the photosynthetic pathways were down-regulated, probably to avoid acceleration of oxidative damage because these pathways are major sources of ROS (Cluster 9 in Fig. 3). The inactivation of photosynthetic pathways is thought to liberate nutrient assimilates that become available for the synthesis of proteins involved in Cd resistance.

Down-regulation of many genes for ribosomal proteins suggests that protein synthesis, which is a major consumer of ATP and nutrients, is inactivated (Cluster 8 in Fig. 3). This may also liberate nutrient assimilates and contribute to Cd-resistant proteins. In addition, protein degradation is activated by the up-regulation of genes for peptidases, which leads to nutrient recycling for the synthesis of Cd-resistant proteins (Cluster 1 in Fig. 3).

Up-regulation of genes involved in glycolysis, the pentose-phosphate pathway, and the tricarboxylic acid cycle by Cd stress, also reported to occur in *Arabidopsis* (Sarry et al. 2006), might be necessary to meet the demand for energy and reducing molecules present as, for example, ATP, NADH, and NADPH in Cd-resistance mechanisms (Cluster 1 in Fig. 3).

Fe-acquisition mechanisms

After 8 days of Cd exposure in roots, the expression of genes involved in the synthesis of nicotianamine (NA) and 2′-deoxymugineic acid (DMA), which are chelators of heavy metals were up-regulated nearly twofold. These genes include NA synthase genes (*OsNAS1*, *OsNAS2*, and *OsNAS3*) and the DMA synthetase gene (*OsDMAS1*). In addition, genes involved in the methionine cycle (Fig. 5) (Kobayashi et al. 2005) and the Fe-deficiency-responsive transcription factor *OsIRO2* gene (2.93 fold up-
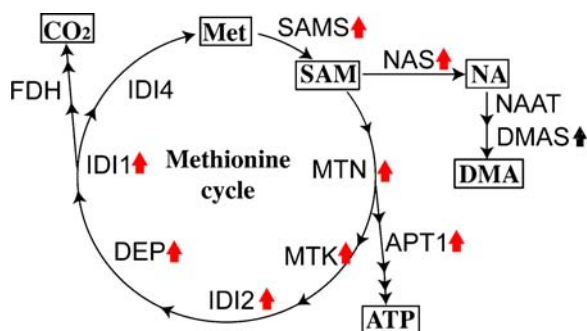
Fig. 5 The biosynthetic pathway of MAs and the methionine cycle in rice. *Red arrows* indicate up-regulation of the proteins under Cd stress. NA, nicotianamine; DMA, 2′-deoxymugineic acid; Met, methionine; SAM, S-adenosyl-Met; SAMS, SAM synthetase; NAS, NA synthase; NAAT, NA aminotransferase; DMAS, DMA synthase; MTN, methylthioadenosine/S-adenosyl homocysteine nucleosidase; MTK, methylthioribose kinase; IDI2, eukaryotic initiation factor 2B-like methylthioribose-1-phosphate isomerase; DEP, methylthioribulose-1-phosphate dehydratase-enolase-phosphatase; IDI1, 2-keto-methylthiobutyric-acid-forming enzyme; IDI4, putative aminotransferase catalyzing the synthesis of Met from 2-keto-methylthiobutyric acid; FDH, formate dehydrogenase; APT, adenine phosphoribosyltransferase

regulation) were also up-regulated after 8 days of Cd exposure. These results and the fact that the longer-term Cd stress worsened the effect on leaf chlorophyll content (Fig. 1b) suggest that Fe availability is affected after longer periods of Cd stress.

Comparison of gene regulation between rice and *Arabidopsis* under Cd stress, or other abiotic stresses in rice

As a comparative analysis of gene regulation in different species is useful for discovering important genes, we compared our data with the data on Cd stress in *Arabidopsis* reported by Herbette et al. (2006). In addition, we compared the results of gene regulation by other abiotic stresses (drought, cold, salt, or ABA) reported by Rabbani et al. (2003). Supplemental Table 1 summarizes the rice genes and their orthologs in *Arabidopsis* that were similarly up- or down-regulated in both species, or under certain stresses. Many orthologs up- or down-regulated in *Arabidopsis* were also similarly up- or down-regulated in rice. For example, similarities were observed in the down-regulation of genes involved in photosynthesis and cell expansion,

although the down-regulation of cell expansion in rice occurred after only 3 h in roots and 8 days in shoots, unlike in *Arabidopsis*. Down-regulation of cell expansion may contribute to Cd resistance through rigidification of plant cell walls. Cell wall structure has been said to be strengthened under stress conditions through rigidification to reduce plant growth (Braam et al. 1997; Lu and Neumann 1998). In addition, the metal-binding properties of the cell wall are proposed to be related to metal tolerance (Hall 2002).

Genes involved in the biosynthesis of jasmonic acid were up-regulated in both rice and *Arabidopsis*. These data indicate that this pathway is important in signal transduction under Cd stress in both plant species.

Cd causes oxidative stress to cells, which is also true for other abiotic stresses such as drought, salt, cold, and ABA. Therefore, some similarities in gene regulation may exist among these stress responses. Some genes showed common up-regulation in roots among Cd and other abiotic stresses, suggesting common regulatory pathways of genes and stress defense mechanisms among these stress responses. Genes up-regulated in roots under Cd stress for 3 h include those for carbohydrate metabolism-related enzymes (isocitrate lyase and phosphorylmutase family protein, UDP glucose 4-epimerase-like protein, trehalose-6-phosphate phosphatase, and pyruvate dehydrogenase kinase isoform), the aldehyde dehydrogenase family protein, thioredoxin, and regulatory proteins, that is, proteins involved in signal transduction or the regulation of gene expression (e.g., zinc finger, bZIP, NAC, WRKY, and protein kinase/phosphatase). These genes may be involved in tolerance to a wide variety of abiotic stresses. For example, the up-regulation of carbohydrate metabolism-related enzymes may contribute to reducing molecules and producing energy and various kinds of sugars, facilitating the regulation of osmotic pressure.

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