Research Article

Genome-Wide Transcriptome Analysis of Cadmium Stress in Rice

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1. Introduction

Cadmium (Cd) is a widespread heavy metal pollutant that is highly toxic to living cells. Accumulation of the nonessential metal Cd in plants is a major agricultural problem. Specifically, Cd is absorbed by the roots from the soil and transported to the shoot, negatively affecting nutrient uptake and homeostasis in plants, even in very small amounts. Many agricultural soils have become contaminated with Cd through the use of phosphate fertilizers, sludge, and irrigation water containing Cd. Cd exposure inhibits root and shoot growth and ultimately reduces yield. Furthermore, Cd accumulation in the edible parts of plants such as seed grains places humans at a risk because of its highly toxic effects on human health. Reducing the Cd concentration in plants below the maximum level indicated by the Codex Alimentarius Commission of FAO/WHO [1] is necessary to avoid negative impacts on human health. Thus, it is important to study the mechanisms of plant responses and defenses to Cd exposure to overcome this problem.

Cd causes oxidative stress and generates reactive oxygen species, which can cause damage in various ways such as reacting with DNA causing mutation, modifying protein side chains, and destroying phospholipids [2]. Various biochemical and physiological processes associated with defense systems are active in plants under Cd exposure. Many genes such as glutathione S-transferase (GST) for detoxification and cysteine-rich metallothioneins (MT) for defense against Cd toxicity respond to Cd stress in plants and might confer Cd tolerance in rice. Transporters with heavy metal binding domains are key factors for root uptake of Cd from soil and efflux pumping of Cd at the plasma membrane; however, the manner in which these genes respond to low Cd concentrations has not been well investigated in rice.

In a previous study, we investigated the gene expression of rice plants (Oryza sativa L. cv. Nipponbare) under a high Cd concentration using the RNA-Seq platform. A clear and detailed view of the transcriptomic changes triggered by Cd exposure is important to understand the gene expression network of the basal response to Cd stress. This could not be
obtained from past studies using the microarray platform, but RNA-Seq can accurately quantify gene expression levels over a broad dynamic range with high resolution and sensitivity [3]. We found that drought stress signaling pathways were activated under Cd exposure through the responses of many drought-related genes [4]. Thus, the recently elucidated scaffolding mechanisms for Cd signaling pathways are complex but of great importance. In this study, we performed rice transcriptome analysis under different low Cd concentrations using the RNA-Seq platform to deepen our understanding of Cd responses.

2. Materials and Methods

2.1. Sample Preparation. Rice (Oryza sativa ssp. japonica cv. Nipponbare) seeds were germinated and grown by hydroponic culture in Yoshida’s solution [1.425 mM NH4NO3, 0.323 mM NaH2PO4, 0.513 mM K2SO4, 0.998 mM CaCl2, 1.643 mM MgSO4, 0.009 mM MnCl2, 0.075 mM (NH4)6 MoO4·4H2O, 0.019 mM H2BO3, 0.155 mM CuSO4, 0.036 mM FeCl3, 0.070 mM citric acid, and 0.152 mM ZnSO4] [5]. After 10 days, seedlings of uniform size and growth were subjected to Cd stress treatment by transferring them to a similar reagent solution containing Cd at different concentrations of 0.2, 1, or 50 μM Cd. These values were chosen based on a report that the total dissolved Cd in 64 fields [5]. After 10 days, seedlings were harvested and used for further analysis as described previously [4].

2.2. Identification of Differentially Expressed Transcripts. The biological replicates (2-3) for each set of conditions were highly correlated (coefficient > 0.95), so reads from the same treatment were merged for subsequent analysis. Trimming of Illumina adaptor sequences and low-quality bases (Q < 20) by Cutadapt [8] and mapping of preprocessed reads to the IRGSP-1.0 genome assembly (http://rapdb.dna.affrc.go.jp/) were performed as described previously [9]. To estimate the expression levels of each transcript, all preprocessed reads were mapped to the IRGSP-1.0 genome assembly by Bowtie with default parameters [10]. The expression level for each transcript was calculated as the RPKM- (Reads per Kilobase Exon Model per million mapped reads-) derived read count [11] based on the number of uniquely mapped reads that overlapped with exonic regions. A G-test was performed to detect differentially expressed transcripts in the control and Cd treatments based on the statistical null hypothesis that the proportions of mapped reads to the transcripts were the same between the two conditions. A false discovery rate (FDR < 0.01) was used in multiple-hypothesis testing to correct for multiple comparisons. When calculating fold changes, 1 was added to avoid division by 0.

2.3. Hierarchical Clustering and Gene Ontology Enrichment Analysis. The Cd-responsive transcripts in root and shoot were used for hierarchical clustering analysis. We used the heatmap.2 in the R package gplots (version 2.11.0) to perform clustering analyses of transcripts. The Z scores were used to compare significant changes in gene expression. A Gene Ontology (GO) term was assigned to each transcript based on the GO annotations for biological process, molecular function, and cellular component in RAP-DB. GO enrichment was evaluated by Fisher’s exact test with a FDR threshold of 5% for responsive transcripts in the biological process category of each cluster. The results were plotted as a heatmap.

2.4. qRT-PCR Analysis. The expression of Cd upregulated genes in root sample was confirmed by qRT-PCR analysis. Rice seeds were germinated and grown in water in a growth chamber. After 10 days, the seedlings were subjected to different stress treatments by transferring them to water containing different reagents. RNA was extracted from them and the CDNA was synthesized according to the manufacturer’s protocol and it is used for the further analysis as described previously [4]. The resulting cDNA was used for PCR amplification in the LightCycler 480 system (Roche, Basel, Switzerland) with each primer set (Os04g060300: 5’-GGCGCTCTG-AGAATCATTAC-C-3’, 5’-CATTCGGGAGCTCATTCTCG-3’, Os01g069210: 5’-ATTCAAGAGTCCGGATG-3’, 5’-CTCTCAACCAGTTACCCC-3’, Os12g0570700: 5’-GACCTCACTTCAAGCTTTTC-3’, 5’-GCAAGACATCTTCTTGG-3’, Os12g0571000: 5’-ATTTCCTGAAAGATTTA-3’, 5’-TTCCGAGGCGAGCTTA-3’). The detection threshold cycle for each reaction was normalized using Ubiquitin1 primers (5’-CCAGGACAAAGATGTCTGCCC-3’, 5’-AAGAAGCTGAGACATCCACGC-3’).

3. Results and Discussion

3.1. Low Cd Concentration Exposure of Rice Plants and Growth Retardation during the Treatment. We used rice plants grown in hydroponic culture, which enabled us to control the Cd exposure easily. High Cd concentration exposure has been previously shown to elicit robust physiological responses and gene expression as acute toxic responses in rice seedlings [12–14]. Growth retardation of the shoot was slightly visible after 1 day (data not shown), the leaves turned yellow and the leaf tips of the seedlings began to wilt after 4 d, and all leaf blades were curled completely and the seedlings were wilting after 10 d under high Cd concentration (50 μM) exposure (Figure 1). While no visible symptoms were observed in shoots under low Cd concentration exposure (0.2 and 1 μM Cd) after
Figure 1: Phenotypic changes in rice plants grown in culture medium with low concentrations of Cd (0.2, 1 μM) and a high concentration of Cd (50 μM) from 0 to 14 d.

1 d, growth retardation occurred gradually compared with the control, with symptoms starting to appear after 7 d. Plants in the same growth chamber exposed to different Cd concentrations showed clear growth differences after 10 d (Figure 1). Even after 28 d, the seedlings under low Cd concentration exposure did not show yellow leaves or wilting (data not shown). These results suggested that high Cd concentration exposure causes fatal damage to plants while low Cd concentrations lead to growth retardation (Figure 1), which is supported by the fact that plant detoxification processes are insufficient to cope with this toxic metal beyond a 10 μM dose [15].

3.2. Gene Expression Profiles under Low Cd Concentration Exposure in Rice. We next analyzed the transcriptome profiles of the response to Cd exposure using RNA-Seq during plant growth, at 1, 4, and 10 d after Cd treatment, and before treatment (0 d). For each set of conditions, an average of approximately 15.1 million (92.2%) quality-evaluated reads (total 211 million) were mapped to the rice genome sequence and used for further analysis (Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9739505). The number of upregulated transcripts ranged from 4,529 to 6,515, whereas the number of downregulated transcripts ranged from 2,359 to 8,734 under 0.2 μM Cd (Figure 2). Twelve transcripts including GST, MT, and DREB (drought responsive element binding protein) 1E were upregulated more than 20-fold among the upregulated transcripts in roots at 0.2 μM Cd.

The number of upregulated transcripts ranged from 5,830 to 7,271 whereas the number of downregulated transcripts ranged from 2,965 to 10,020 under 1 μM Cd (Figure 2). Fifty-one transcripts including GST, MT, Prx (peroxidase), and heat shock proteins were upregulated more than 20-fold among the upregulated transcripts in roots at 1 μM Cd (Table 1). Induction of detoxification enzymes against oxidation stress such as GST and Prx under Cd exposure might be associated with the defense system that confers Cd tolerance to plants [16–18] even at low Cd concentrations. The cysteine-rich MT might function as a ligand for chelation of metal ions to defend against Cd toxicity [19]. The DREB/C-repeat binding factor (CBF) specifically interacts with the DRE/CRT cis-acting element and controls the expression of many stress-inducible genes in plants [20]. The activation of gene expression in several drought stress signal pathways under Cd exposure has been reported [4].
Table 1: Cadmium-upregulated transcripts identified in roots by RNA-Seq analysis.

| Transcript | Description | Fold change | Root 1d | Root 4d | Root 10d | Shoot 1d | Shoot 4d | Shoot 10d |
|------------|-------------|-------------|---------|---------|----------|----------|----------|----------|
| **0.2 μM Cd** |             |             |         |         |          |          |          |          |
| Os1010527400-01 | Tau class GST protein 3 | 27.8 | 21.4 | 27.5 | 1.2 | 2.0 | 1.7 |
| Os0302830000-00 | In2-1 protein | 27.5 | 2.8 | 1.0 | 1.3 | 1.1 | 1.5 |
| Os0810156000-01 | Conserved hypothetical protein | 26.4 | 21.4 | 25.3 | 1.3 | 1.6 | 1.7 |
| Os0110627967-00 | Hypothetical protein | 26.1 | 16.5 | 24.1 | 1.5 | 1.9 | 1.4 |
| Os0410178300-02 | Syn-copalyl diphosphate synthase | 20.1 | 8.0 | 20.3 | 0.6 | 4.2 | 1.4 |
| Os0410305000-01 | HLH (helix-loop-helix) DNA-binding domain containing protein | 26.4 | 33.1 | 0.5 | 1.0 | 47.5 | 9.2 |
| Os0206768000-01 | DREBIE (drought responsive element binding protein 1E) | 0.9 | 28.7 | 0.9 | 1.2 | 10.9 | 2.0 |
| Os0210792000-00 | Glutamine amidotransferase class-I domain containing protein | 0.8 | 28.1 | 1.7 | 0.9 | 3.2 | 1.1 |
| Os1210154000-00 | RmlC-like jelly roll fold domain containing protein | 4.0 | 21.4 | 5.7 | 1.0 | 1.4 | 1.2 |
| Os1210570000-01 | MT (metallothionine)-like protein type 1 | 18.6 | 20.3 | 15.8 | 0.9 | 1.0 | 0.9 |
| Os0310836800-01 | IAA-amino acid hydrolase 1 | 4.3 | 6.5 | 33.6 | 1.0 | 1.0 | 1.0 |
| Os1010337000-00 | Plant disease resistance response protein domain containing protein | 9.7 | 6.0 | 21.6 | 1.0 | 1.0 | 1.0 |
| **1 μM Cd** |             |             |         |         |          |          |          |          |
| Os0410178300-02 | Syn-copalyl diphosphate synthase | 122.0 | 32.1 | 25.5 | 0.5 | 1.0 | 3.6 |
| Os0410178300-01 | Isoform 3 of Syn-copalyl diphosphate synthase | 109.8 | 27.8 | 21.5 | 0.5 | 0.9 | 3.1 |
| Os0410178400-01 | Cytochrome P450 CYP99A1 | 69.8 | 21.1 | 16.0 | 0.8 | 1.0 | 2.8 |
| Os0310267000-00 | Heat shock protein 180 | 57.5 | 7.7 | 10.9 | 1.2 | 0.7 | 0.7 |
| Os0310266900-01 | Heat shock protein 173 | 47.0 | 4.9 | 5.3 | 1.0 | 0.4 | 0.6 |
| Os0110156200-01 | Heat shock protein 1 | 43.7 | 3.9 | 1.3 | 1.0 | 1.0 | 1.0 |
| Os0710190000-00 | 1-Deoxy-D-xylulose 5-phosphate synthase 2 precursor | 42.4 | 11.5 | 8.6 | 0.7 | 1.1 | 3.9 |
| Os0710127500-01 | PR-1a pathogenesis related protein precursor | 40.0 | 5.6 | 5.0 | 0.8 | 0.8 | 2.1 |
| Os0710154100-00 | Viviparous-14 | 38.8 | 5.2 | 1.5 | 1.1 | 1.4 | 2.3 |
| Os0710154200-00 | Hypothetical gene | 37.7 | 4.7 | 1.3 | 1.0 | 1.3 | 2.1 |
| Os1210552000-01 | Probenazole-inducible protein PBZ1 | 37.7 | 13.5 | 10.9 | 0.3 | 0.5 | 2.2 |
| Os0610586000-01 | Conserved hypothetical protein | 37.6 | 9.3 | 6.5 | 0.6 | 0.9 | 1.4 |
| **Os1010527400-01** | Tau class GST protein 3 | 34.3 | 18.0 | 32.4 | 1.1 | 1.4 | 2.0 |
| Os1210555000-01 | Probenazole-inducible protein PBZ1 | 33.2 | 13.5 | 11.0 | 0.6 | 0.7 | 2.5 |
| Os0310277700-01 | Protein of unknown function DUF26 domain containing protein | 32.8 | 7.6 | 3.4 | 1.0 | 0.6 | 1.0 |
| Os1110687100-01 | von Willebrand factor (type A domain) | 32.5 | 4.1 | 13.8 | 0.7 | 0.7 | 2.3 |
| Os0510217000-00 | — | 28.8 | 1.4 | 1.2 | 1.0 | 1.0 | 1.0 |
| Os0610662550-01 | Conserved hypothetical protein | 28.5 | 7.8 | 8.8 | 0.8 | 0.8 | 1.6 |
| Os0110944000-02 | Conserved hypothetical protein | 28.4 | 6.3 | 9.8 | 0.5 | 0.6 | 1.7 |
| Os0610568600-01 | Ent-kaurene oxidase 1 | 27.1 | 28.1 | 11.0 | 0.6 | 1.4 | 4.7 |
| Os1210418600-01 | Hypothetical conserved gene | 26.7 | 2.0 | 1.3 | 1.0 | 1.0 | 1.0 |
| Os1210258000-01 | Cupredoxin domain containing protein | 26.2 | 14.7 | 10.6 | 0.7 | 1.1 | 7.1 |
| Os0110615000-01 | Subtilisin/chymotrypsin-like inhibitor | 25.6 | 9.5 | 7.9 | 0.7 | 1.0 | 1.8 |
| Os0410107900-02 | Heat shock protein 81-1 | 25.6 | 2.5 | 1.6 | 1.0 | 1.0 | 0.9 |
Table 1: Continued.

| Transcript | Description | 1 d | 4 d | 10 d | 1 d | 4 d | 10 d |
|------------|-------------|-----|-----|------|-----|-----|------|
| Os09t0493000-01 | Conserved hypothetical protein | 25.3 | 2.6 | 1.8 | 0.9 | 1.2 | 0.9 |
| Os01t0627967-00 | Hypothetical protein | 25.3 | 19.5 | 21.6 | 1.3 | 1.8 | 1.4 |
| Os01t0994400-03 | Conserved hypothetical protein | 25.2 | 4.6 | 6.3 | 0.6 | 0.6 | 1.8 |
| Os04t0180400-01 | Cytochrome P450 99A2 | 24.4 | 4.3 | 6.0 | 0.5 | 0.5 | 3.1 |
| Os04t0108101-00 | Hypothetical protein | 24.4 | 2.3 | 1.4 | 1.0 | 1.0 | 1.0 |
| Os02t0269600-00 | Subtilase | 22.6 | 7.8 | 4.1 | 0.3 | 1.2 | 6.0 |
| Os01t0360000-00 | Heat shock protein 175 | 22.5 | 3.1 | 1.2 | 1.0 | 1.4 | 1.2 |
| Os04t0180500-00 | Hypothetical protein | 22.2 | 4.0 | 5.4 | 0.5 | 0.6 | 3.1 |
| Os01t0946600-01 | Conserved hypothetical protein | 21.8 | 16.6 | 8.0 | 0.7 | 0.7 | 0.8 |
| Os09t0255400-02 | Indole-3-glycerolphosphatesynthase | 21.4 | 5.1 | 3.8 | 0.7 | 0.9 | 2.3 |
| Os01t0349900-01 | SA gene product | 21.2 | 6.5 | 8.9 | 0.1 | 0.1 | 0.2 |
| Os12t0491800-01 | Ent-kaurene synthase IA | 21.1 | 1.5 | 1.7 | 0.4 | 0.8 | 5.5 |
| Os01t0320000-01 | Wound-induced protease inhibitor | 21.0 | 8.8 | 11.6 | 1.6 | 0.5 | 0.2 |
| Os11t0592200-01 | Chitin-binding allergen Bra r 2 | 20.7 | 3.4 | 2.8 | 0.7 | 0.5 | 1.6 |
| Os01t0963000-01 | Prx (Peroxidase) BP 1 precursor | 20.6 | 3.8 | 4.4 | 0.7 | 1.1 | 1.3 |
| Os08t0894000-01 | Oryza sativa germin-like protein 8-7 | 20.6 | 11.5 | 6.7 | 2.1 | 1.5 | 0.8 |
| Os07t0496250-01 | Expansin-like BI | 20.5 | 2.2 | 2.2 | 1.5 | 1.2 | 4.5 |
| Os01t0963000-04 | Prx (Peroxidase) BP 1 precursor | 20.3 | 3.7 | 4.4 | 0.7 | 1.1 | 1.3 |
| Os09t0255400-01 | Indole-3-glycerolphosphatesynthase | 20.2 | 5.2 | 3.7 | 0.7 | 0.9 | 2.3 |
| Os11t0601950-01 | cDNA clone:002-114-B06 | 20.0 | 1.7 | 1.9 | 0.7 | 1.0 | 1.1 |
| Os03t0129400-01 | Hypothetical protein | 19.3 | 27.1 | 17.6 | 1.0 | 1.9 | 3.6 |
| Os01t0327700-01 | Nonprotein coding transcript | 12.2 | 25.5 | 15.7 | 0.9 | 1.3 | 2.5 |
| Os03t0129400-02 | EST AU078206 corresponds to a region of the predicted gene | 9.4 | 24.3 | 16.3 | 1.1 | 1.4 | 2.8 |
| Os12t0570700-01 | MT (metallothionein)-like protein type 1 | 16.7 | 21.2 | 17.7 | 0.8 | 0.8 | 3.1 |
| Os12t0571000-01 | MT (metallothionein)-like protein type 1 | 13.9 | 20.0 | 13.0 | 0.9 | 1.0 | 3.6 |
| Os08t0156000-01 | Conserved hypothetical protein | 15.4 | 17.9 | 26.0 | 1.1 | 1.5 | 1.6 |
| Os03t0836800-01 | IAA-amino acid hydrolase 1 | 0.7 | 4.0 | 23.7 | 1.0 | 1.0 | 1.0 |

Reads were mapped to the rice genome and responsive genes were identified by G-tests. Transcripts upregulated more than 20-fold in one or more treatments/time points in roots are shown. Transcripts in bold were upregulated under both 1 and 0.2 \( \mu \text{M} \) Cd exposure.

proteins (Hsps) were strongly upregulated in roots under 1 \( \mu \text{M} \) Cd, with the greatest relative expression at 1 d (Table 1). These genes may contribute to cellular homeostasis by protecting macromolecules such as enzymes, protein complexes, and membranes under Cd exposure. This result suggested that the roots of hydroponically cultured rice might be affected more directly and earlier by Cd exposure. There was a difference between the low Cd concentrations in that no Hsps were strongly upregulated in roots at 0.2 \( \mu \text{M} \) Cd (Table 1), suggesting that the effect of this condition might be small or show time lag. In shoots, 15 and 11 transcripts were upregulated more than 20-fold among the upregulated transcripts under 0.2 and 1 \( \mu \text{M} \) Cd, respectively (Table S2). Nine transcripts including Nramp1 (natural resistance-associated macrophage protein) were upregulated under both 0.2 and 1 \( \mu \text{M} \) Cd (Table S2). In Arabidopsis, Nramp1 localizes to the plasma membrane and functions as a high-affinity transporter for manganese (Mn) uptake [21], while OsNramp5 uptakes Mn and Cd [22]. Transporters with heavy metal binding domains are often capable of transporting several metals, such as Fe, Zn, Mn, and Cd, because of their low substrate specificity [23–26]. We found that upregulation of a HLH DNA-binding domain containing transcription factor (Os04g0301500) in both roots and shoots peaked at 4 d under 0.2 \( \mu \text{M} \) Cd; this protein may function as a regulatory factor under Cd exposure (Table 1, Table S2). The number of downregulated transcripts in roots peaked at 4 d after Cd exposure, while the number in shoots gradually increased under low Cd concentration exposure (Figure 2). A few dozen transcripts were downregulated less than 0.05-fold among the downregulated transcripts in roots and shoots under Cd exposure (Table S2). Therefore, a small part of transcripts were strongly
up- or downregulated among several thousand responsive transcripts under low Cd concentration exposure. Large-scale changes in gene expression occurred in rice under Cd exposure, even at low concentrations, possibly because Cd is a nonessential metal for the plant.

To obtain a functional annotation of responsive transcripts under Cd exposure, we used GO biological process categories. The responsive transcripts in shoot and root were clustered into several groups based on their expression patterns. GO enrichment analysis was performed using clustered transcripts assigned by GO terms in RAP-DB (The Rice Annotation Project Database [http://rapdb.dna.affrc.go.jp/]) (Supplementary Figure S1). Enriched GO terms significantly in each cluster may represent the functional categories in rice under Cd exposure. Enriched GO terms of gradually upregulated transcripts under Cd exposure include metal ion transport (GO:00030001) (cluster 3 in root under 0.2 μM Cd, cluster 4 in root under 1 μM Cd), which may function in Cd transport. Response to oxidative stress (GO:0006979) and responsive to oxidative stress (GO:0006979) were also included in cluster 3 and cluster 4, respectively. This suggested that they might function in defense against Cd. Enriched GO terms of gradually downregulated transcripts under Cd exposure include translation (GO:0006412), translation elongation (GO:0006414), DNA replication (GO:0006260), and DNA repair (GO:0006281) (cluster 1 in root under 0.2 μM Cd, cluster 2 in root under 1 μM Cd). Photosynthesis, light harvesting (GO:0009765), and photosynthesis (GO:0015979) were also included in both clusters. These may function in plant growth. Thus, these correspond to the observed changes in phenotype (Figure 1), which clearly validated the RNA-Seq expression profiling data obtained from rice tissue under Cd stress condition. However, the pattern of gene expression is quite complex and would require more detailed analysis.

3.3. Constitutively Expressed Genes Responded Differently under Low Cd Concentration to High Cd Concentration. As many genes responded to both low and high Cd concentrations [4], we assessed the effect of the stress degree on rice seedlings through the expression of constitutively expressed genes. We investigated the expression of 18 genes annotated by the RAP that were expressed constitutively in 39 tissues collected throughout the life cycle of the rice plant from two varieties according to 190 Affymetrix GeneChip Rice Genome Arrays, in addition to four genes annotated by the RAP that have frequently been used as internal controls in expression analyses [27]. The results showed that the expression of more than half of them fluctuated drastically (>2 or <2) in roots or shoots after 1 d of high Cd concentration exposure (Figure 3). This drastic response may be partly because RNA-Seq can accurately quantify gene expression levels over a broad dynamic range with high resolution and sensitivity [10, 28, 29]. However, our results suggest that their expression is greatly affected by strong stress, even though they are expressed constitutively across the developmental course. Note that a high Cd concentration can cause fatal damage to rice seedlings, such as by affecting homeostasis, which corresponds to the observed changes in phenotype (Figures 1 and 3).

3.4. Comparative Gene Expression Analysis between Low and High Cd Concentrations Reveals Novel Cd-Responsive Transporters. We investigated the expression of metal transporter genes containing metal ion binding Pfarats [PF01554 (MatE), PF08370 (PDR_assoc), PF01545 (Cation_efflux), PF02535 (Zip), PF00403 (HMA), and PF01566 (Nramp)] that may function in Cd transport under Cd exposure. The expression of 183 transport transcripts was compared between low and high Cd concentration treatments in roots and shoots at 1 d, because Cd uptake from the hydroponic culture and efflux pumping are initial responses to Cd exposure (Figure 4, Table S3). The transcripts tended to be more responsive in roots and shoots under higher Cd concentration exposure. This result indicated the potential of the RNA-Seq strategy to reveal novel Cd-responsive transporters by analyzing gene expression under exposure to different Cd concentrations. The responsive transcripts might function in roots at the early stage of Cd exposure. No transcripts were upregulated more than 3-fold in shoots under low Cd exposure (Figure 4, Table S3), suggesting that the effect takes more time to appear in shoots. Os03g0667500 (Zip, root) encoding iron-regulated transporter 1 (IRT1) was upregulated more than 5-fold under lower Cd concentrations but responded only slightly under the high Cd concentration. IRT1s often transport Cd because of their low substrate specificity [24–26, 30]. Os02g0585200 (HMA, root), Os03g0152000 (HMA, root), Os0g0584800 (HMA, root), Os01g0609900 (PDR_assoc, shoot), and Os01g0609300 (PDR_assoc, shoot) showed the highest (32-fold) upregulation under high Cd concentration exposure and responded only slightly to low Cd concentrations (Table S3). The balance between Cd and various other metal ions in the hydroponic culture might affect the expression of these genes, because specific systems for transporting Cd may have not developed in rice as it is a nonessential metal. The effects of other ions on the expression of transporters [4] and responsive genes associated with defense systems against Cd (Supplementary Figure S2) have been indicated.

4. Conclusions

We generated gene expression profiles for rice seedlings grown under low Cd concentrations. Phenotypic observations and constitutive gene expression indicated that low Cd concentrations cause growth retardation but are far from being fatal in rice. Several genes associated with defense systems were strongly upregulated; the expression of metal ion transporter genes tended to correlate with Cd concentration and GO enrichment analysis of the clustered genes based on their expression patterns, suggesting that our transcriptome profiles reflect responses to Cd in rice. Our data also suggest that it could be dangerous to eat plants that do not show specific Cd pollution symptoms growing in soil contaminated by small amounts of Cd. Establishing the exact composition and organization of the transcriptional network underlying the response to Cd exposure will provide a robust tool for improving crops in the future, for example, by creating low Cd uptake plants.
Figure 3: Response of constitutively expressed genes in roots and shoots to Cd exposure. The relative expression of constitutively expressed genes [27] in roots (a) and shoots (b) is shown under Cd exposure at each stress time point (1, 4, and 10 d) during 0.2 μM (white, grey, and black) and 1 μM (light blue, light green, and green) Cd exposure compared with nontreatment (0 d). The red bar shows the relative expression at 1 d under 50 μM Cd exposure. The x-axis shows the genes and the y-axis shows relative expression. Wang et al. [27] suggested the following genes as candidates for constitutive expression: glycine-rich RNA-binding protein (Os12g0632000), expressed protein (Os06g0686700), profilin (Os06g0152100), ADP-ribosylation factor (Os05g0489600), triosephosphate isomerase (Os01g0147900), glycine-rich RNA-binding protein (Os01g0670700), peptidyl-prolyl cis-trans isomerase (Os02g0121300), endothelial differentiation factor (Os08g0366100), ubiquitin monomer (Os06g0152100), protein translation factor SUI1 (Os07g0529800), GAPDH (Os02g0601300), polyubiquitin (Os02g0161900), protein elongation factor (Os03g0177500), ubiquitin-conjugating enzyme (Os01g0819500), GTP-binding nuclear protein (Os05g0574500), peptidyl-prolyl isomerase (Os02g0760300), and 60S ribosomal protein L31 (Os02g0717800). Their paper also introduced the following genes that have frequently been used as internal controls in expression analyses: elongation factor1-alpha (Os03g0177500), ubiquitin fusion protein (Os03g0234200), GAPDH (Os02g0601300), and tubulin beta-6 chain (Os01g0805900).
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Youko Oono and Takashi Matsumoto contributed valuable insights into the discussion and revision of the paper. Hirokazu Handa and Hiroyuki Kanamori analyzed the data and contributed to the experiments. Youko Oono, Takayuki Yazawa, and Takashi Matsumoto conceived and designed the experiments. Takashi Matsumoto performed sampling. Hiroyuki Kanamori, Harumi Sasaki, and Satomi Mori performed the experiments. Youko Oono, Takayuki Yazawa, and Hiroyuki Kanamori analyzed the data and contributed analysis tools. Youko Oono wrote the paper. Hirokazu Handa and Takashi Matsumoto contributed equally to this work.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Youko Oono and Takashi Matsumoto conceived and designed the experiments. Takashi Matsumoto performed sampling. Hiroyuki Kanamori, Harumi Sasaki, and Satomi Mori performed the experiments. Youko Oono, Takayuki Yazawa, and Hiroyuki Kanamori analyzed the data and contributed analysis tools. Youko Oono wrote the paper. Hirokazu Handa and Takashi Matsumoto contributed valuable insights into the discussion and revision of the paper. Youko Oono and Takayuki Yazawa contributed equally to this work.

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References

[1] CODEX, "Report of the 38th session of the CODEX Committee on Food Additives and Contaminants," ALINORM 06/29/12, Codex Alimentarius Commission, 2006.
[2] B. Halliwell and J. M. C. Gutteridge, "Oxygen-toxicity, oxygen radicals, transition-metals and disease," Biochemical Journal, vol. 219, no. 1, pp. 1–14, 1984.
[3] Z. Wang, M. Gerstein, and M. Snyder, "RNA-Seq: a revolutionary tool for transcriptomics," Nature Reviews Genetics, vol. 10, no. 1, pp. 57–63, 2009.
[4] Y. Oono, T. Yazawa, Y. Kawahara et al., "Genome-wide transcriptome analysis reveals that cadmium stress signaling controls the expression of genes in drought stress signal pathways in rice," PLoS ONE, vol. 9, no. 5, Article ID e96946, 2014.
[5] S. Yoshida, D. A. Forno, J. H. Cock, and K. A. Gomez, Laboratory Manual for Physiological Studies of Rice, International Rice Research Institute, Manila, Philippines, 3rd edition, 1976.
[6] S. Sauve, W. A. Norvell, M. McBride, and W. Hendershot, “Speciation and complexation of cadmium in extracted soil solutions,” *Environmental Science & Technology*, vol. 34, no. 2, pp. 291–296, 2000.

[7] Y. Kawahara, Y. Oono, H. Wakimoto et al., “TENOR: database for comprehensive mRNA-Seq experiments in rice,” *Plant and Cell Physiology*, vol. 57, no. 1, article e7, 2016.

[8] M. Martin, “Cutadapt removes adapter sequences from high-throughput sequencing reads,” *EMBnet Journal*, vol. 17, no. 1, pp. 10–12, 2011.

[9] Y. Oono, Y. Kawahara, H. Kanamori et al., “mRNA-seq reveals a comprehensive transcriptome profile of rice under phosphate stress,” *Rice*, vol. 4, no. 2, pp. 50–65, 2011.

[10] H. Li and R. Durbin, “Fast and accurate short read alignment with Burrows-Wheeler transform,” *Bioinformatics*, vol. 25, no. 14, pp. 1754–1760, 2009.

[11] A. Mortazavi, B. A. Williams, K. McCue, L. Schaeffer, and B. Wold, “Mapping and quantifying mammalian transcriptomes by RNA-Seq,” *Nature Methods*, vol. 5, no. 7, pp. 621–628, 2008.

[12] M. Zhang, X. Liu, L. Yuan et al., “Transcriptional profiling in cadmium-treated rice seedling roots using suppressive subtractive hybridization,” *Plant Physiology and Biochemistry*, vol. 50, no. 1, pp. 79–86, 2012.

[13] K. Lee, D. W. Bae, S. H. Kim et al., “Comparative proteomic analysis of the short-term responses of rice roots and leaves to cadmium,” *The Journal of Plant Physiology*, vol. 167, no. 3, pp. 161–168, 2010.

[14] K. Shah, R. G. Kumar, S. Verma, and R. S. Dubey, “Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings,” *Plant Science*, vol. 161, no. 6, pp. 1135–1144, 2001.

[15] L. Perfus-Barbeoch, N. Leonhardt, A. Vavasseur, and C. Forestier, “Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status,” *Plant Journal*, vol. 32, no. 4, pp. 539–548, 2002.

[16] R. Mittler, S. Vanderauwera, N. Suzuki et al., “ROS signaling: the new wave?” *Trends in Plant Science*, vol. 16, no. 6, pp. 300–309, 2011.

[17] C. Frova, “The plant glutathione transferase gene family: genomic structure, functions, expression and evolution,” *Physiologia Plantarum*, vol. 119, no. 4, pp. 469–479, 2003.

[18] C. Cosio and C. Dunand, “Specific functions of individual class III peroxidase genes,” *Journal of Experimental Botany*, vol. 60, no. 2, pp. 391–408, 2009.

[19] C. Cobbett and P. Goldsborough, “Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis,” *Annual Review of Plant Biology*, vol. 53, pp. 159–182, 2002.

[20] K. Yamaguchi-Shinozaki and K. Shinozaki, “Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses,” *Annual Review of Plant Biology*, vol. 57, pp. 781–803, 2006.

[21] R. Caliari, A. Schikora, J.-F. Briat, S. Mari, and C. Curie, “High-affinity manganese uptake by the metal transporter NRAMP1 is essential for Arabidopsis growth in low manganese conditions,” *Plant Cell*, vol. 22, no. 3, pp. 904–917, 2010.

[22] A. Sasaki, N. Yamaji, K. Yokosho, and J. F. Ma, “Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice,” *Plant Cell*, vol. 24, no. 5, pp. 2155–2167, 2012.

[23] N. Satoh-Nagasawa, M. Mori, N. Nakazawa et al., “Mutations in rice (Oryza sativa) heavy metal ATPase 2 (OsHMA2) restrict the translocation of zinc and cadmium,” *Plant and Cell Physiology*, vol. 53, no. 1, pp. 213–224, 2012.

[24] Y. O. Korshunova, D. Eide, W. G. Clark, M. L. Guerinot, and H. B. Pakrasi, “The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range,” *Plant Molecular Biology*, vol. 40, no. 1, pp. 37–44, 1999.

[25] N. E. Grossoehme, S. Akiles, M. L. Guerinot, and D. E. Wilcox, “Metal-binding thermodynamics of the histidine-rich sequence from the metal-transport protein IRT1 of Arabidopsis thaliana,” *Inorganic Chemistry*, vol. 45, no. 21, pp. 8500–8508, 2006.

[26] S. Lee and G. An, “Over-expression of OsIRT1 leads to increased iron and zinc accumulations in rice,” *Plant, Cell and Environment*, vol. 32, no. 4, pp. 408–416, 2009.

[27] L. Wang, W. Xie, Y. Chen et al., “A dynamic gene expression atlas covering the entire life cycle of rice,” *Plant Journal*, vol. 61, no. 5, pp. 752–766, 2010.

[28] G. M. He, X. P. Zhu, A. A. Elling et al., “Global epigenetic and transcriptional trends among two rice subspecies and their reciprocal hybrids,” *Plant Cell*, vol. 22, no. 1, pp. 17–33, 2010.

[29] T. T. Lu, G. J. Lu, D. L. Fan et al., “Function annotation of the rice transcriptome at single-nucleotide resolution by RNA-seq,” *Genome Research*, vol. 20, no. 9, pp. 1238–1249, 2010.

[30] P. Pedas, C. K. Ytting, A. T. Fuglsang, T. P. Jahn, J. K. Schjoerring, and S. Husted, “Manganese efficiency in barley: identification and characterization of the metal ion transporter HvIrt1,” *Plant Physiology*, vol. 148, no. 1, pp. 455–466, 2008.