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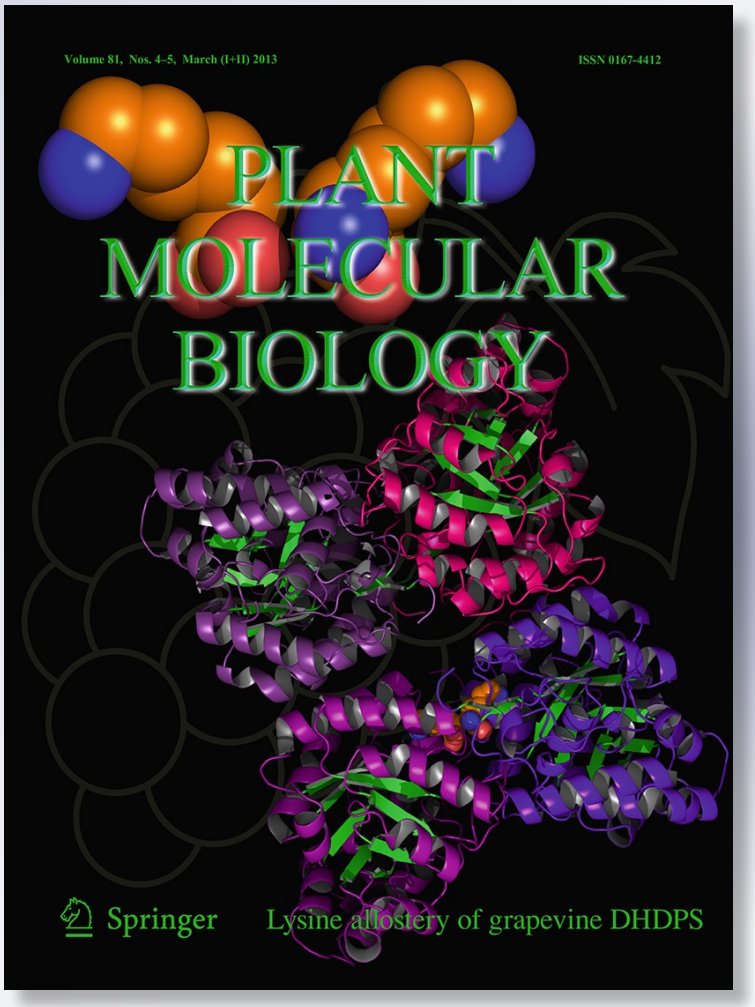

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Abstract The phytotoxic effects of copper (Cu) and cadmium (Cd) on plant growth are well documented. However, Cu and Cd toxicity targets and the cellular systems contributing to acquisition of tolerance are not fully understood at the molecular level. We aimed to identify genes and pathways that discriminate the actions of Cu and Cd in rice roots (*Oryza sativa* L. cv. TN67). The transcripts of 1,450 and 1,172 genes were regulated after Cu and Cd treatments, respectively. We identified 882 genes specifically respond to Cu treatment, and 604 unique genes as Cd-responsive by comparison of expression profiles of these two regulated gene groups. Gene ontology analysis for 538 genes involved in primary metabolism, oxidation reduction and response to stimulus was changed in response to both metals. In the individual aspect, Cu specifically altered levels of genes involved in vesicle trafficking transport, fatty acid metabolism and cellular component biogenesis. Cd-regulated genes related to unfolded protein binding and sulfate assimilation. To further characterize the functions of vesicle trafficking transport under Cu stress, interference of excytosis in root tissues was conducted by inhibitors and silencing of *Exo70* genes. It was demonstrated that vesicle-trafficking is required for mediation of Cu-induced reactive oxygen species (ROS) production in root tissues. These results may provide new insights into understanding the molecular basis of the early metal stress response in plants.

Keywords Copper · Cadmium · VIGS · Vesicle trafficking transport · *Oryza sativa* · Transcriptome

Abbreviations

VIGS	Virus-induced gene silencing
TRV	Tobacco rattle virus
ROS	Reactive oxygen species
DAB	3,3′-Diamninobenzidine
NBT	Nitroblue tetrazolium

Introduction

Contamination of crop plant and water with heavy metals such copper (Cu) and cadmium (Cd) is a potent environmental threat to human health. In living organisms, Cu acts as a cofactor in various enzymes involved in electron transfer, such as in pyruvate metabolism, the tricarboxylic acid cycle, and the respiratory chain (Himelblau and Amasino 2000; Yruela 2005). Although Cu is essential for normal plant growth and development, elevated Cu concentrations in the soil can lead to toxicity symptoms and stunted growth in most plants (Drążkiewicz et al. 2004). The toxic effects of Cu can be attributed to interact with cellular nucleic acids and enzyme active sites (Cervantes

Chung-Yi Lin and Ngoc Nam Trinh contributed equally to this work.

The microarray data described in this study have been deposited in the Gene Expression Omnibus under the accession number (GSE: 34895).

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and Gutierrez-Corona 1994). Cd is often considered to be biologically non-essential for all living organisms. Chronic exposure to Cd inhibits mismatch repair by reducing the capacity for post-replication mismatch repair (MMR) of small misalignments and base–base mismatches (Jin et al. 2003). The accumulation of Cd in plants causes various toxicity symptoms such as grey-green leaf color and growth inhibition. It can replace some essential elements due to its high affinity for sulphhydryl groups (Larsson et al. 1998; Haag-Kerwer et al. 1999; Moulis 2010).

The one of the most important toxicity mechanisms related to Cu and Cd is the increase accumulation of reactive oxygen species (ROS), which may cause lipid peroxidation, enzyme inactivation, and DNA and membrane damage (Hall 2002; Boominathan and Doran 2003). Redox-active metal, Cu, is known to be capable of inducing ROS production such as superoxide and hydroxyl redicaks via Haber–Weiss and Fenton reacions (Elstner et al. 1988). Non-redox metal, Cd, can indirectly enhance oxidative stress by depleting free-radical scavengers, or via the activation of ROS-producing enzymes like NADPH oxidases (Romero-Puertas et al. 2004). ROS can directed damage protein, amino acids and nucleic acids, and cause lipid peroxidation, enzyme inactivation, and membrane damage (Hall 2002; Boominathan and Doran 2003). It is also apparent that plant cells generate such reactive species as signaling molecules, produced at controlled levels and leading to tolerance responses (Maksymiec 2007; Lin and Aarts 2012).

Recent research has focused on identifying the mechanisms that allow organisms to adapt to or alleviate damage due to metal stress. Current knowledge of basic metal tolerance indicates that plants share several common mechanisms that prevent metal hazards rather than resist excess metal effects. These mechanisms involve extracellular exudates, reduced uptake or efflux pumping of metals, repair of damaged protein, chelation of metals and compartmentation to decrease free metal concentration in the cell (Hall 2002; Mendoza-Cózatl et al. 2005; Ahsan et al. 2009; Lin and Aarts 2012). Metal-tolerant plants usually take these strategies to maintain metal homeostasis. Plants have developed mechanisms that can reduce nonessential and toxic metals and metalloids influx by inhibiting their transport and enhance metal removal from the cytosol (Wysocki and Tamás 2010). Several transport proteins involved in acquisition, distribution and homeostasis of metals in plants include heavy metal ATPases; ATP-binding cassette transporters (ABC) (Verrier et al. 2008); natural resistance associated macrophage proteins; the cation diffusion facilitator family (CDF) (Williams et al. 2000); ZRT, IRT-like proteins (Guerinot 2000); cation antiporters (Gaxiola et al. 2002); and the Cu transporter family (Yuan et al. 2011). Metal transporter proteins (MTPs), the member of the plant CDF family which mediate bivalent cation efflux from the cytosol, is highly overexpressed in leaves of Zn/Ni hyper accumulators *Thlaspi goesingense* (Kim et al. 2004). OsMTP1 is a bivalent cation transporter localized in the cell membrane, which is necessary for efficient translocation of Zn and Cd (Yuan et al. 2012). Thus, plants have developed a precise transporter system to control the uptake, transport, storage, and use of the metal ions.

Although extrusion of toxic compounds by efflux transporters was thought to be one of the route for detoxification, most plant cells possess large vacuoles that can be a disposal compartment for toxic compounds. In contrast to metal efflux proteins, these proteins also transport endogenously produced substrates and confer a moderate level of tolerance. Numerous studies reported that phytochelatin (PCs) produced by sulfate assimilation are involved in metal detoxification, and resulting complexes may be recognized as substrates by transporters for export or trafficking for vacuolar sequestration (Cobbett 2000a, b; Cobbett and Goldsbrough 2002; Hall 2002; Mendoza-Cózatl et al. 2005). PCs are glutathione (GSH)-derived metal-binding peptides, increased PCs concentration by overexpression of genes involved in GSH synthesis increased Cd tolerance in *Brassica juncea* (Zhu et al. 1999). Recent studies revealed details of the transporters responsible for active transport of PC-metal complexes into plant vacuoles. The AtABCC1 and AtABCC2 are major vacuolar PC transporters that confer tolerance to metal stress (Song et al. 2010; Park et al. 2012).

Metallothioneins (MTs) are members of a family of cysteine-rich low molecular weight polypeptides which play an important role in heavy metal detoxification and homeostasis of intracellular metal ions. MTs can bind Cd, Cu and Zn in plants (Cobbett and Goldsbrough 2002; Cobbett 2003). A role of soybean MTs in Cd accumulation as one of the main responses to an overload of this metal is suggested (Pagani et al. 2012). MTs are believed to serve as a chaperone for long-distance transport of copper during leaf senescence (Cobbett and Goldsbrough 2002). In addition, Cu is an activator of PC biosynthesis but PC-deficient mutants show little sensitivity to Cu, indicating potential involvement of MT-mediated detoxification. These mechanisms appear to be involved in avoiding the build-up of toxic concentrations within the cell and thereby preventing the damaging effects (Hall 2002).

Analyses of transcriptomes and proteomes in plants have revealed transcripts or networks of proteins related to Cu and Cd responses. For *Arabidopsis*, these studies have focused mainly on the transcriptional level. Herbette et al. (2006) used whole-genome arrays to analyze the transcriptomic response to Cd stress in *Arabidopsis* roots. Weber et al. (2006) and Zhao et al. (2009) performed comparative transcriptomic analysis with Cu and Cd ions in the root of *A. thaliana* and the Cd-hypertolerant

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metallophyte *Arabidopsis halleri*. In recent years, Sudo et al. (2008) clarified the effect of Cu on gene expression in rice leaf by using an Agilent 22K Rice Oligo Microarray. Recently, Ogawa et al. (2009) clarified the effect of Cd on gene expression in rice shoots and roots. In order to understand the functions of such gene expression responding specifically to each metal, it is important to distinguish those genes induced as part of the common stress responses from those specific to individual stressors. These provide the useful approaches for determining the characteristics of Cu and Cd in plants.

Here, we used a custom Agilent 44K rice microarray to understand the mechanisms of heavy metal toxicity and cellular detoxification and protection pathways in the early stress response to Cu and Cd in rice roots. We found mechanisms associated with vesicle trafficking, fatty acid metabolic process, flavonoid biosynthesis and cellular component biogenesis in the Cu-specific pathway and unfolded protein binding and sulfate assimilation pathway in the Cd pathway. In addition, interference of vesicle trafficking by inhibitors and virus-induced gene silencing (VIGS) in roots demonstrated its functions in response to Cu stress. Differential expression of transporter genes may contribute to the metal-specific responses.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. cv. TN67) seeds were surface disinfected with 2.5 % (v/v) sodium hypochlorite (Katayama, Japan) for 15 min, then thoroughly washed in distilled water, and placed in 9 cm Petri dishes containing 25 ml distilled water at 37 °C in darkness. After 3 days of incubation, uniformly germinated seeds were selected and transferred to Petri dishes over filter paper discs (Advantec No. 1) moistened with 10 ml distilled water. Each Petri dish contained 15 germinated seeds grown at 26 °C in darkness for 3 days. Once the roots reached 3–4 cm in length, they were used for $CuCl_2$ and $CdCl_2$ exposure experiments under sterile conditions in the same Petri dish. The rice seedlings, including the coleoptiles and roots, were exposed to the solutions containing the heavy metals. $CuCl_2$ and $CdCl_2$ were added to final concentrations between 0 and 100 μM for treatment durations as described in the following sections. The root tips were excised from the heavy metal-treated rice seedlings and subjected to RNA isolation.

Root length determination

Germinated rice seeds were kept at 26 °C in dark for 3 more days, and once roots reached 3–4 cm length, they were exposed to different concentrations (0–100 μM) of $CuCl_2$ or $CdCl_2$. Root length was measured after 3 days of incubation at 26 °C in darkness. Mean root length was obtained from 15 individual seedlings from at least 3 separate experiments.

Purification of total RNA

For microarray analysis and semi-quantitative RT-PCR, root samples (100 mg) treated with 5 μM $CuCl_2$ or 25 μM $CdCl_2$ for different time points were harvested for total RNA extraction. Total RNA extraction involved use of the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) with some modification. The concentrations of total RNA samples were measured by the use of NanodropND 2000 (Nanodrop Technologies, Wilmington, DE, USA). The purity of RNA samples was determined by $OD_{260/280}$ and $OD_{260/230}$. RNA samples more than 2 g/l with high purity ($OD_{260/280} > 2$, $OD_{260/230} > 2$) underwent microarray analysis and semi-quantitative RT-PCR.

Microarray preparation

Extraction of RNA samples to analyze the early transcriptomic changes was performed as described with minor modifications (Desikan et al. 2001). The 6-day-old rice roots after exposure to 5 μM $CuCl_2$ or 25 μM $CdCl_2$ for 1 and 3 h were collected for total RNA extraction. The RNA isolated from the rice roots with 1- and 3 h metal treatment was to maximize gene discovery and examine rapid changes in global patterns of gene expression. Three biological replicate samples underwent RNA labeling and microarray hybridization. Briefly, 0.5 μg total RNA was amplified by use of a Fluorescent Linear Amplification Kit (Agilent Technologies, USA) and labeled with Cy3-CTP (control samples) or Cy5-CTP (metal-treated) (CyDye, PerkinElmer, USA) during in vitro transcription. RNA was labeled with Cy3 or Cy5. In total, 0.825 μg Cy-labeled cRNA was fragmented to an average size of about 50–100 nt by incubation with fragmentation buffer (Agilent Technologies, USA) at 60 °C for 30 min. The fragmented labeled cRNA was then pooled and hybridized to the Rice Oligo DNA Microarray 44K RAP-DB (G2519F#15241; Agilent Technologies) at 60 °C for 17 h. After washing and blow-drying with a nitrogen gun, microarrays were scanned with use of an Agilent microarray scanner (Agilent Technologies, USA) at 535 nm for Cy3 and 625 nm for Cy5. Scanned images were analyzed by use of Feature Extraction v9.5.3 (Agilent Technologies, USA), which quantifies signal and background intensity for each feature and normalizes data by rank-consistency-filtering with LOWESS intensity normalization.

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Microarray data analysis and organization

Microarray analysis involved Agilent Oligo DNA Microarray Hybridization protocols with the Rice Oligo DNA Microarray 44K RAP-DB (G2519F#15241; Agilent Technologies) for 3 biological replicates and color swap experiments for each replicate. The hybridized slides were scanned by use of a DNA microarray scanner (Agilent Technologies). Signal intensities were extracted by use of Feature Extraction v9.5.3. For statistical analysis, we excluded genes with signal intensities <100 in all experiments after correction of the dye effect by averaging the 2 color swaps. Statistical analysis involved unpaired *t* test with use of GeneSpringGX11 (Agilent Technologies). The Benjamini-Hochberg FDR method was used to obtain corrected P values (false discovery rate, FDR) for multiple testing. The fold change of each probe after metal treatment was calculated with the mean of 3 biological replicates. We selected genes with >twofold change in expression (cutoff by FDR $\leq$ 0.1) regulated by Cu and Cd (Supplemental Table 1).

Descriptions of each Cu- and Cd-responsive gene were annotated according to the RAP-DB [Rice Annotation Project Data Base: http://rapdb.lab.nig.ac.jp/ (Rice Annotation Project 2007, 2008)] and the TIGR Rice Genome Annotation Resource [http://www.tigr.org/tdb/e2k1/osa1/index.shtml (Ouyang et al. 2007)]. Cu- and Cd- responsive genes were classified into functional categories by AgriGO functional enrichment analysis (http://bioinfo.cau.edu.cn/agriGO/).

MapMan display

The averaged signals for a given treatment (3 biological replicates for 5 μM $CuCl_2$ and 25 μM $CdCl_2$ for 1 + 3 h) were expressed relative to those for control samples, converted to a $\log_2$ scale and displayed by use of MapMan v3.5.1 (http://gabi.rzpd.de/projects/MapMan/) (Thimm et al. 2004). The downloadable installers include (1) Experimental Data Files, (sample experimental data, or data imported by the user) (2) Pathways (Biological Pathways or processes), (3) Chromosome Views (Display of genes on Chromosomes, only for sequenced plants) and (4) Mappings (Files classifying transcripts, metabolites into functional classes i.e. BINS). *O. sativa* mapping files were imported into MapMan. These contents structure the rice genes represented on the Rice Oligo DNA Microarray into BINS and sub-BINS for display on the schematic maps of metabolism and cellular processes.

VIGS in *Nicotiana benthamiana*

A 568 bp *NbExo70* cDNA fragment was amplified by PCR with primer pair 5′-AGAGTTGGAGCATCAAGAGAG-3′ and 5′-AGCCTGAACTCTAATTGACTCC-3′, annealing between position 81 and 650 of *NbExo70* gene. The generated PCR product was then cloned into EcoRI site of pTRV2-empty to generate pTRV2-*NbExo70*. For VIGS experiment, pTRV1, pTRV2-empty and pTRV-*NbExo70* were introduced into *Agrobacterium* strain GV3101. Virus infection on *N. benthamiana* was performed as described by Liu et al. (2002) with some minor modifications. A 5 mL culture was grown overnight at 28 °C in 50 mg L^{-1} kanamycin in Luria–Bertani medium and used to inoculate 50 mL of Luria–Bertani medium containing the same antibiotics. After an overnight culture at 28 °C, the cells were harvested by centrifugation and re-suspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone), adjusted to an optical density at 600 nm of 2.0, and left at room temperature for 3–4 h. Equivalent aliquots of GV3101-pTRV1 and -pTRV2 were mixed immediately before inoculation. Ten-old-day *N. benthamiana* plants with three leaves were infiltrated by syringe infiltration. 10 days after infiltration, post-transcriptional gene silencing was confirmed by RT-PCR employing three primers (5′-CGATGGAAGCTGAA TCTCTT-3′ as forward primer, 5′-TGGTCAACGGCGAG TCAA-3′ and 5′-AATCATTCGCTCGGCGATG-3′ as reverse primers), annealing between positions 1 and 81 or 1 and 206 of *NbExo70* ORF. 20 days after infiltration, *N. benthamiana* plants were treated with 100 μM $CuCl_2$. After a 5 day treatment, leaves were used for in situ detection of ROS.

Agrodrench-mediated VIGS in *N. benthamiana* roots

Nicotiana benthamiana seeds were germinated in soil. Five-old-day seedlings were transplanted to 3.5 cm diameter round pots, containing vermiculite, with one plant per pot. Growth chamber conditions were kept at 27 °C and 70 % humidity under 16 h extended day with supplemental lighting with 50–100 μE s^{-1} m^2 light intensity. Ten-day-old plants were used for silencing experiments as described (Ryu et al. 2004). For Agrodrench, mixture of *Agrobacterium* strains containing pTRV1 and pTRV2-NbExo70 was drenched, 3 ml each, into the crown part of each plant. *Agrobacterium* strain containing pTRV2-empty vector was used as a control. 3 weeks after inoculation, plants were treated by 100 μM $CuCl_2$ solution. 5 days after treatment, roots were collected by washing under water flowing out a faucet for in situ detection of ROS.

ROS detection in roots and leaves

Three-day-old seeds were used to localize the generation of ROS in rice roots. The roots were exposed to 5 μM Cu and 25 μM Cd for 1 and 3 h. Then, the roots were labeled with

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10 μM 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydro- fluorescein diacetate, acetyl ester (CM-H2DCFDA) redox probe (Invitrogen, USA) for 30 min. A Leica MPS60 fluorescent microscope equipped with a green fluorescent protein filter (excitation 450–490 nm, emission 500–530 nm) was used for fluorescence images. Autofluorescence was not observed in unstained controls at the exposure time used. Images were captured with a CoolSNAP Cooled CCD Camera (CoolSNAP 5.0, north Reading, MA, USA).

Hydrogen peroxide and superoxide anion were visually detected by treating with 3,3′-diamninobenzidine (DAB), and with nitroblue tetrazolium (NBT), respectively. For detection of hydrogen peroxide, leaves and roots were placed in a solution of 1 mg mL^{-1} DAB, pH 3.8 and vacuum infiltrated at 700 mmHg for 10 min. Leaves and roots were immersed in DAB solution for 8 h in darkness. For detection of superoxide, leaves and roots were vacuum infiltrated at 700 mmHg for 5 min with 0.05 M phosphate-buffered saline (pH 7.4) containing 0.5 mM NBT and 10 mM NaN_3. Leaves and roots were left in NBT solution at room temperature for 20 min and the reaction was stopped with 95 % ethanol. Subsequently, treated leaves were decolorized by boiling ethanol. The roots were placed on a microscope slide and observed under a stereo microscope.

Histochemical staining for lipid peroxidation

Histochemical detection of lipid peroxidation involved use of Schiff's reagent (Pompella et al. 1987). In brief, freshly harvested rice roots were stained with Schiff's reagent for 60 min, which detects aldehydes originating from lipid peroxides. Then roots were rinsed with potassium sulphite solution (0.5 % [w/v] $K_2S_2O_5$ prepared in 0.05 m HCl) and maintained in the solution.

Treatment of rice roots with vesicle-trafficking inhibitors

Six-day-old rice roots pretreated with 10 μM brefeldin A (BFA) for 30 min were exposed to $CuCl_2$ (5 μM) over a period of 3 h. After exposure to Cu stress, superoxide and hydrogen peroxide accumulation in rice roots were detected as previously described (Jabs et al. 1996).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR contained 0.5 μl of first-strand cDNA solution with 30 cycles of denaturation at 95 °C for 2 min, annealing at 50–55 °C for 60 s, and extension at 72 °C for 1 min. The primers for amplifying rice *OxExo70*, *OsArf*, *OsSyp, sHsp* (small heat shock protein) and tobacco *Exo70* genes are as follows. *OsExo70FX8* (*Os11g0572200*), F: 5′-gcaagcctggtgaagatgat-3′and R: 5′-atttgattgaccgggtggat-3′; *OsExo70FX14* (*Os01g0905300*), F: 5′-gagatcaaccccaagctgtc-3′ and R: 5′-ctggagcttggggtgattct-3′; *OsExo70F4* (*Os08g0530300*), F: 5′-tgcagaagatgatggtggag-3′ and R: 5′-cgaggctgtacttgatgtgc-3′; *OsArf* (*Os02g0699300*), F: 5′-gagatgaggtcgtgatgct-3′ and R: 5′-aaccacttctttccttctttaatgc-3′; *OsSyp132* (*Os06g0168500*), F: 5′-cgcgttttcactgtcactgg-3′ and R: 5′-acctgaaacctgtgactctatattg-3′; *OsHsp20-15* (*Os02g0782500*), F: 5′-acatccaggtgacgctggag-3′ and R: 5′-acttggtcttcttctccggc-3′; *OsHsp20-18* (*Os03g0266300*), F: 5′-cggatcgactggaaggagac-3′ and R: 5′-gttctccatggacgccttga-3′; *NbExo70*, F: 5′-atggagtccaccacattctcc-3′ and R: 5′-tgctgctcgtatccatctacg-3′. One of the rice tubulin isoforms (*Os03g0726100*) was used as an internal control (F: 5′-tcgcagcatcaacccaatc-3′ and R: 5′-gcaaccagtcctcacctcat-3′). In tobacco, *elongation factor 1-α gene (EF1-α)*, a ubiquitously expressed gene, served as a control for cDNA synthesis and PCR efficiency in the different samples (F: 5′-tggtgtcctcaagcctggtatggttgt-3′ and R: 5′- acgcttgagatcctta accgcaacattctt-3′). The annealing temperature and the number of PCR cycles were optimized. Amplicons were analyzed by agarose gel electrophoresis (1 %). Experiments were repeated at least twice and reproducibility was confirmed.

Statistical analyses

Data are presented as mean ± SE from at least 3 separate experiments. Data analysis involved use of Microsoft Excel (Microsoft Corp., Redmond, WA).

Results

Effect of metal stress on root growth of rice seedling

Roots are the first and most critical organ to contact heavy metal stresses which leading to accumulate metal amounts in root tissues and inhibit root growth. To evaluate the toxic effects of heavy metals in rice seedlings, dose–response experiments revealed similar toxic effects with Cu (5 μM) or Cd (25 μM): a significant decrease (55–70 %) in root length, which demonstrates an iso-toxic effect (Supplemental Fig. 1).

Genes that were commonly regulated by exposure to Cu and Cd in rice roots

To investigate the rice in response to Cu and Cd treatment, we analyzed 6-day-old rice roots exposed to 5 μM $CuCl_2$ or 25 μM $CdCl_2$ for 1 and 3 h. Microarray RNA pool was prepared by combining rice root samples collected at the following time points: 1 and 3 h. We used Agilent Rice Oligo 44K DNA Microarray to identify Cu- and Cd-regulated genes with FDR ≤ 0.1 and twofold change in gene expression. Cu- and Cd-regulated gene sets were compared

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by Venn diagrams to identify the genes with expression controlled exclusively by Cu, Cd, or both (Fig. 1, Supplemental Table 2). Cu and Cd treatment altered the expression of 1,450 and 1,172 genes, respectively: 568 of these genes were altered with both treatments.

Among the 568 common genes, 530 genes and 38 genes were upregulated and downregulated, respectively. The common upregulated genes were grouped into 9 functional categories, mainly biological regulation, metabolic process, oxidation reduction pathway, localization, response to stimulus, transporter activity, transcription regulator activity, catalytic activity, and binding (Table 1). A set of common downregulated genes are classified to 2 gene families related to metabolic process and hydrolase activity.

Gene expression specifically in response to Cu and Cd

We used AgriGO functional enrichment analysis to find gene function from Cu-regulated gene group (Supplemental Table 1). Cu-specific functional categories that were significantly upregulated in our data set were related to cellular localization and protein transport, fatty acid metabolic process, lipoxygenase activity, ATPase activity and cellular component biogenesis (selected functions in Table 2; complete results in Supplemental Table 3). In the cellular localization and protein transport groups, 5 genes encoding OsExo70 proteins were found to be enriched in Cu treatment (Supplemental Fig. 2). Three genes encoding SNARE proteins have been implicated in mediating vesicle fusion in exocytosis. The Arf and Rab GTPases of two of the RAS-related subfamilies that function in regulating vesicle trafficking were also induced by Cu stress. In the fatty acid metabolism process and lipoxygenase activity, genes encoding lipoxygenase, NADH oxidase and Oxophytodienoic acid reductase were also identified. Five genes encoding ATP-dependent transporters were shown to be specifically upregulated under Cu stress. The functional group which included downregulated genes responsive to Cu is cellular component biogenesis. Six genes encoding ribosomal protein, armadillo (ARM) repeat-containing protein, Arp2/3 complex, WD40 protein, xyloglucan fucosyltransferase and 65 kDa microtubule-associated protein (MAP65) revealed significant down-regulation under Cu stress.

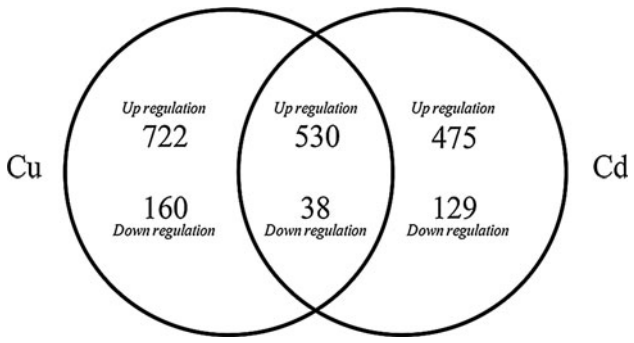

Fig. 1 Transcription profiles of rice roots with 5 μM Cu and 25 μM Cd treatment by Venn diagram. Shows genes with significant change at false discovery rate ≤0.1 and twofold change in expression

Genes encoding function in unfold protein binding were enriched in the Cd-upregulated data set including heat shock protein and DnaJ protein (Table 2). The transcription upon exposure to Cd of heat shock protein and DnaJ protein revealed that protein denaturation is one of the effects of Cd toxicity.

Comparison of Cu and Cd responses using MapMan

With microarray data visualization and overlaying the Cu-regulated data on a secondary metabolism map (Fig. 2a), within the flavonoids domain, 3 genes encoding the chalcone synthases (*Os07g0526400*, *Os07g0525900* and *Os07g0525500*) and 1 gene encoding the chalcone-flavonone isomerase (*Os11g0116300*) were strongly and rapidly induced by Cu. Two genes encoding flavonols, flavonol synthase/flavanone 3-hydroxylase (*Os03g0122300* and *Os09g0353400*), and 6 genes encoding dihydroflavonols (*Os10g0536400*, *Os06g0651000*, *Os02g0180700*, *Os08g0277200*, *Os02g0812000*, and *Os09g0441400*) were also regulated. In addition, Cu treatment significantly and immediately regulated 15 genes encoding ATP-dependent transporters (*Os04g0588700*, *Os11g0134900*, *Os09g0472200*, *Os01g0121600*, *Os01g0609200*, *Os02g0208300*, *Os12g0132500*, Os01g0695800, Os09g0472100, *Os04g0209200, Os04g0194500*, *Os07g0522500*, *Os01g0609300*, *Os06g0589300,* and *Os08g0384500*) (Fig. 2b).

MapMan analysis of the Cd-regulated genes revealed a substantial and coordinated upregulation of genes encoding glutathione S-transferase (GST). For example, 16 genes belonging to the TAU family were upregulated (Fig. 2c, Supplemental Table 4). Cd treatment upregulated 6 sulfate-assimilation pathway genes (*Os04g0111200*, *Os02g0222100*, *Os01g0720700*, *Os03g0185000*, *Os03g0196600*, and *Os05g0533500*) (Fig. 2d) and 2 genes encoding sulfate transporter, *Os09g0240500* and *Os03g0195500* (Fig. 2b).

Inhibition of Cu-induced ROS by interference of vesicle-trafficking in rice roots

Microarray analysis identified the Cu- and Cd-specific pathways in rice roots (Table 2). The Cu-induced expression of exocytosis-related genes such as *OsExo70FX14* and *OsExo70FX15* was further validated in rice roots. (Fig. 3a). These results implied the potential involvement of exocytosis in rice roots responding to Cu stress. To better

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Table 1 Gene ontology analysis of 568 genes commonly regulated by Cu and Cd in rice

GO ID	GO term	Query item[a]	Backgroup item[b]	FDR[c]
Upregulared GO/pathway				
Biological process				
Biological regulation				
GO:0050789	Regulation of biological process	61	429	1.30E-44
GO:0050794	Regulation of cellular process	60	410	1.30E-44
Metabolic process				
GO:0019222	Regulation of metabolic process	54	326	2.40E-43
GO:0044238	Primary metabolic process	152	1308	2.20E-92
GO:0044237	Cellular metabolic process	131	1338	1.10E-71
GO:0043170	Macromolecule metabolic process	114	1103	1.50E-65
GO:0009058	Biosynthetic process	76	689	7.60E-47
GO:0006807	Nitrogen compound metabolic process	78	731	6.70E-47
Catabolic process				
GO:0055114	Oxidation reduction	20	142	1.60E-15
Localization				
GO:0051234	Establishment of localization	25	232	2.30E-16
GO:0006810	Transport	25	232	2.30E-16
Response to stimulus				
GO:0006950	Response to stress	27	103	3.60E-28
GO:0042221	Response to chemical stimulus	17	133	1.30E-12
Molecular function				
Transporter activity				
GO:0022892	Substrate-specific transporter activity	10	85	2.20E-07
GO:0022857	Transmembrane transporter activity	12	95	4.90E-09
Transcription regulator activity				
GO:0003700	Transcription factor activity	31	116	3.40E-32
Catalytic activity				
GO:0016491	Oxidoreductase activity	47	280	4.40E-38
GO:0016829	Lyase activity	10	52	1.90E-09
GO:0016740	Transferase activity	66	507	1.50E-45
GO:0016787	Hydrolase activity	59	521	1.40E-37
Binding				
GO:0003676	Nucleic acid binding	58	596	1.80E-33
GO:0043167	Ion binding	79	175	4.50E-101
GO:0001882	Nucleoside binding	51	467	4.10E-32
GO:0000166	Nucleotide binding	56	501	1.60E-35
GO:0005515	Protein binding	28	92	5.00E-31
Cellular component				
Macromolecular complex				
GO:0043234	Protein complex	12	133	4.10E-07
Cell part				
GO:0044425	Membrane part	18	88	1.30E-16
Downregulared GO/pathway				
Biological process				
Metabolic process				
GO:0044238	Primary metabolic process	9	1308	2.90E-05
GO:0044237	Cellular metabolic process	5	1338	1.60E-02
GO:0043170	Macromolecule metabolic process	5	1103	9.30E-03

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Table 1 continued

GO ID	GO term	Query item[a]	Backgroup item[b]	FDR[c]
Molecular function				
Catalytic activity				
GO:0016787	Hydrolase activity	8	521	1.20E-07

[a] Query item: Number of query list

[b] Backgroup item: Number of Background

[c] FDR: false discovery rate

characterize the functional significance of exocytosis in response to Cu, the roots of rice seedlings were treated with a vesicle-trafficking inhibitors, BFA. It was observed that Cu stress had profound effects on ROS generation and ROS-induced lipid peroxidation in rice roots (Supplemental Fig. 3). We tested the roles of vesicle-trafficking in Cu-induced ROS production. The rice roots were pre-treated with BFA, and the generation of ROS was assayed immediately after exposure to Cu stress. The rice roots were stained with NBT (a histochemical reagent for superoxide anion) and DAB (a histochemical reagent for H_2O_2) to detect the ROS. As shown in Fig. 3b, the ROS level was elevated in the rice roots at the first 30 min after treatment with Cu. An increase in the ROS level was detected over a time period of 3 h after exposure to Cu stress. The production of ROS was observed in two regions of the root, the root tip and the elongation zone. Pre-treatment of rice roots with BFA strongly suppressed ROS production during Cu stress (Fig. 3b). At 3 h after exposure to Cu, the production of ROS was induced in both root regions, while in the BFA-treated roots it remained low. The result suggested that vesicle-trafficking is required for mediation of Cu-induced ROS production in rice roots.

Functional analysis of *Exo70* gene by VIGS in the leaves and roots of *N. benthamiana* plants

To gain more insight into the roles of *Exo70* in response to Cu stress, we extended the transcriptomic and physiological studies to examine its functions in *N. benthamiana* by VIGS strategies. In *N. benthamiana*, expression of *NbExo70* was found to be significantly increased in Cu-treated leaf tissues when compared to the control (Fig. 4a). The leaf and root tissues with suppression of endogenous *NbExo70* transcripts were generated by VIGS. The NbExo70 protein sequence showed significant homology to *O. sativa* Exo70s (Rice Annotation Project Database RAP-DB ID; Os09g0439600 and Os08g0455700) and *Arabidopsis* Exo70s (Arabidopsis Genome Initiative AGI number; At1g72470, At1g54090 and At3g14090) (Supplemental Fig. 4). The *NbExo70* cDNA fragment containing the partial sequence of ORF was clone into the TRV-based VIGS vector (Fig. 4b). The young leaves were infiltrated with the pTRV:*NbExo70* construct to initiate VIGS. In addition, silencing of *NbExo70* gene expression in roots was triggered by Agrodrench. The effect of VIGS on endogenous *NbExo70* mRNA levels was examined by semi-quantitative RT-PCR using leaf RNA samples (Fig. 4b). After 5 days treatment with 100 μM Cu, the Cu-induced *NbExo70* gene expression was compromised in the *NbExo70*-silenced plants (*NbExo70-1* and *NbExo70-2*) in comparison with the wild-type and TRV control plants. To dissect the roles of *NbExo70* gene in ROS generation during Cu-stress response of *N. benthamiana* plants, we stained leaves (Fig. 4c) and roots (Fig. 4d) with NBT and DAB for in situ detection of ROS. Surprisingly, *NbExo70*-silenced plants with Cu treatment greatly inhibited the Cu-induce ROS production in both leaves and roots (Fig. 4c, d). This result suggested that the silencing of vesicle-trafficking related *NbExo70* may alleviate the oxidative burst in response to Cu toxicity.

Validation of microarray data by semi-quantitative RT-PCR

To further detect dynamic changes in transcriptome beyond the time points (1 and 3 h) selected for microarray analysis, we exposed the rice roots to 3, 6 and 12 h of Cu and Cd treatments. Semi-quantitative RT-PCR measured the transcript abundance of 7 genes differentially upregulated by Cu and Cd: OsExo70FX8 (*Os11g0572200*), OsExo70FX14 (*Os01g0905300*), OsExo70F4 (*Os08g0530300*), OsArf9 (*Os02g0699300*), SNARE (OsSyp132) (*Os06g0168500*), sHsp20–15 (*Os02g0782500*) and sHsp 20–18 (*Os03g0266300*) (Supplemental Fig. 2). We found Cu-induced dynamic changes in the transcript abundance of Exo70, ARF and SNARE as early as 3 h for most them and kept high expression after 6 h Cu exposure. We also found the high expression pattern of Hsp genes in rice tissue for each time points after Cd treatment.

Discussion

Global genome expression analysis is increasingly used to understand the plant defense mechanism against excess

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Table 2 Gene ontology/pathway terms for selected genes regulated ≥twofold with 5 μM Cu and 25 μM Cd in rice roots

	Type[a]	Cu		Cd		Description
		FC[b]	FDR[c]	FC[b]	FDR[c]	
Cu-specific upregulated						
Cellular localization	P					
Os12g0560300		1.767	0.06	0.631	ns[d]	NTGB2 (OsArf42)
Os03g0787000		1.658	0.07	1.353	0.08	Qa SNARE, syntaxin-like (OsSyp121)
Os06g0168500		2.779	0.05	0.309	ns	Qa SNARE, syntaxin-like (OsSyp132)
Os01g0905300		3.501	0.05	1.465	ns	OsExo70FX14
Os06g0255900		1.615	0.08	−0.174	ns	OsExo70F5
Os05g0475300		1.343	0.05	0.917	0.05	VHS domain containing protein
Os02g0699300		1.663	0.06	0.340	ns	NTGB1 (OsArf9)
Os01g0905200		1.828	0.05	0.117	ns	OsExo70FX15
Os08g0530300		1.696	0.06	−0.704	ns	OsExo70F4
Os11g0572200		1.992	0.05	0.309	ns	OsExo70FX8
Protein transport	P					
Os12g0560300		1.767	0.06	0.631	ns	NTGB2 (OsArf42)
Os02g0437200		2.445	0.04	0.364	ns	Qb + c SNARE, SNAP25-like (OsSNAP11)
Os03g0787000		1.658	0.07	1.353	0.08	Qa SNARE, syntaxin-like (OsSyp121)
Os06g0168500		2.779	0.05	0.309	ns	Qa SNARE, syntaxin-like (OsSyp132)
Os05g0475300		1.343	0.05	0.917	0.05	VHS domain containing protein
Os02g0699300		1.663	0.06	0.340	ns	NTGB1 (OsArf9)
Os03g0191400		1.704	0.05	0.087	ns	Ras-related protein Rab-6A (OsRab14)
Fatty acid metabolic process	P					
Os08g0508800		5.877	0.10	0.453	ns	Lipoxygenase
Os01g0369900		1.654	0.07	0.191	ns	NADH:flavin oxidoreductase/NADH oxidase family protein
Os06g0216300		1.578	0.05	3.575	0.05	Oxo-phytodienoic acid reductase
Os04g0447100		1.928	0.08	1.479	0.07	Lipoxygenase
Os03g0738600		1.811	0.07	0.325	ns	Lipoxygenase L-2
Os06g0216200		1.558	0.04	3.556	0.05	Oxo-phytodienoic acid reductase
Os08g0509100		3.485	0.10	0.565	ns	Lipoxygenase
Lipoxygenase activity	F					
Os08g0508800		5.877	0.10	0.453	ns	Lipoxygenase
Os03g0738600		1.811	0.07	0.325	ns	Lipoxygenase L-2
Os03g0700700		2.005	0.06	0.715	0.06	Lipoxygenase
Os08g0509100		3.485	0.10	0.565	ns	Lipoxygenase
Os04g0447100		1.928	0.08	1.479	0.07	Lipoxygenase
ATPase activity, coupled	F					
Os03g0326000		1.280	0.06	1.582	0.06	Phospholipid-transporting ATPase 1
Os09g0533400		1.451	0.07	0.487	0.08	Chaperonin clpA/B family protein
Os04g0209200		2.943	0.08	0.612	ns	Glutathione-conjugate transporter
Os10g0418100		3.419	0.05	0.460	ns	Calcium-transporting ATPase 8
Os03g0203700		2.793	0.06	0.586	0.09	Plasma membrane Ca^{2+}-ATPase
Os01g0695800		5.035	0.05	0.970	ns	Multidrug resistance protein 1 homolog
Cu-specific down regulated						
Cellular component biogenesis	P					
Os11g0105400		−1.279	0.05	−0.942	0.05	Ribosomal protein L10 family protein
Os11g0580000		−1.169	0.06	−0.535	ns	ARM repeat fold domain containing protein
Os04g0512300		−1.557	0.05	−1.996	0.09	Arp2/3 complex, 34 kDa subunit p34-Arc family protein
Os06g0644600		−1.181	0.07	−0.821	0.07	Utp21 specific WD40-associated domain containing protein

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Table 2 continued

	Type[a]	Cu		Cd		Description
		FC[b]	FDR[c]	FC[b]	FDR[c]	
Os04g0449200		−2.342	0.07	−0.513	0.09	Xyloglucan fucosyltransferase family protein
Os02g0720200		−1.117	0.05	−0.305	ns	65kD microtubule associated protein
Cd-specific upregulated						
Unfolded protein binding	F					
Os04g0107900		3.215	0.07	6.480	0.06	Similar to Heat shock protein 82
Os08g0500700		0.759	ns	1.704	0.06	Similar to Heat shock protein 82
Os03g0293000		0.009	ns	1.607	0.05	Similar to DnaJ domain containing protein
Os05g0562300		0.077	ns	2.022	0.06	Similar to DnaJ-like protein
Os03g0787300		0.888	0.06	1.378	0.08	Similar to DnaJ homolog
Os06g0650900		2.673	ns	2.501	0.08	Heat shock protein DnaJ family protein

[a] Type: P, biological process F, molecular function

[b] FC: log2 fold change

[c] FDR: false discovery rate

[d] ns: non-significant

metal stress. Transcriptome analysis can be used to assess changes in gene expression under single stress conditions, but comparative approaches including quantitative and qualitative analyses of gene expression with 2 stresses are important. We aimed to compare the early effect of Cu and Cd stress on transcriptome regulation in rice roots by a bioinformatics approach. Cu and Cd elicited similar responses in rice roots. Analysis of the general overview of the global changes in the gene expression in response to Cu and Cd stress shows about 27 % of regulated genes was commonly to 2 stresses. These common regulated genes included a large number of genes involved in biological regulation, metabolism, oxidation reduction, localization, and response to stimulus (Table 1). These findings is similar with those by Zhao et al. (2009) for *Arabidopsis* about 40 % of genes with expression change common to Cu and Cd stress in roots, indicating that common stress responsive genes appear to exist in different plant species.

The Cu- or Cd-mediated responses such as sulfate assimilation (Fig. 2), metabolism of flavonoids (Fig. 2), induction of GST genes (Supplemental Table 4) and regulation of sHsp (Supplemental Table 8) confirmed the earlier observations of heavy metal-related transcriptome and proteome studies (Herbette et al. 2006; Weber et al. 2006; Sudo et al. 2008; Villiers et al. 2011). Metabolically, sulfur metabolism is a core pathway for the synthesis of molecules required for heavy metal tolerance in plants. Induction of genes involved in sulfur assimilation and glutathione (GSH) metabolism was observed in *Arabidopsis* roots exposed to Cd (Herbette et al. 2006). Genes involved in the phenylpropanoid pathway for flavonoids were regulated in rice leaves exposed to Cu stress (Sudo et al. 2008). The role of plant GSTs in cellular detoxification from oxygen toxicity has been proposed to act as glutathione peroxidases and prevents apoptosis (Kampranis et al. 2000). The induction of GSTs by Cd was described in soybean and poplar, and correlated with an important increase in transcript level and their activity (Villiers et al. 2011). The upregulation of GSTs has also been noted in transcriptomic and proteomic analysis of plant roots under arsenic (As) stress (Ahsan et al. 2008; Norton et al. 2008). Small heat shock proteins (Hsps) can bind selectively nonnative protein, prevent their aggregation, and maintain them in a state competent for ATP-dependent refolding by other chaperones (Nakamoto and Vígh 2007). A dominance of genes encoding sHsps were induced in Cd-hypertolerant facultative metallophyte *A. halleri* by comparative transcriptomic analysis (Weber et al. 2006). Taken together, these results suggest the important roles of sulfate assimilation, flavonoids, GST and sHsp in plants responding to heavy metal stresses.

In mammalian cells, Cu-induced vesicle trafficking of P-type ATPases is key for maintaining Cu homeostasis. With increased intracellular Cu levels, the localization of P-type ATPases shifts to vesicular compartments close to the plasma membrane, where their primary role is removal of Cu from the cell. Vesicle trafficking transport involves 3 major protein families: SNARE family proteins, which participate in vesicle fusion; ARF family GTPases, involved in vesicle formation; and Rab family GTPases, involved in vesicle targeting (Sorkin 2000; Béraud-Dufour and Balch 2002). In addition, vesicle trafficking to the membrane requires the exocyst, a conserved complex of 8 proteins (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70,

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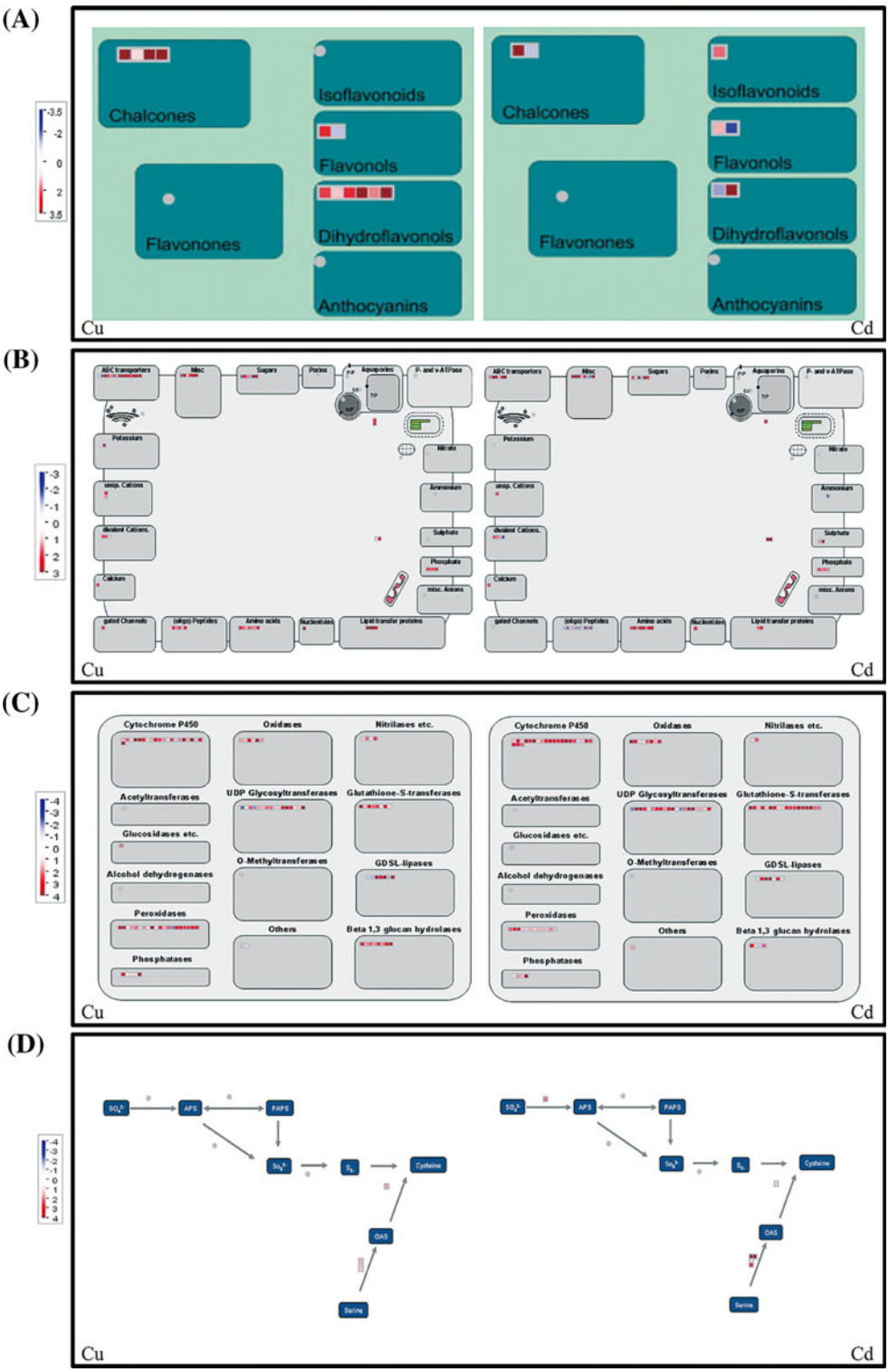

Fig. 2 MapMan analysis of genes involved in **a** flavonoid metabolism and **b** transport, **c** large enzymes, and genes involved in **d** sulfate assimilation. Each BIN or subBIN is represented as a *block*, with each transcript displayed as a *square*, *red* for upregulation or *blue* for downregulation

and Exo84) (TerBush et al. 1996; Kee et al. 1997). We found that Cu specifically upregulated genes involved in cellular localization and protein transport (Table 2). Within these 2 groups were genes encoding Exo70 (*Os01g0905200, Os01g0905300, Os06g0255900, Os08g0530300, Os11g0572200*), SNAREs (*Os02g0437200*, *Os03g0787000*, *Os06g0168500*), and 2 RAS-related subfamilies, ARF (*Os02g0699300*, *Os12g0560300*) and Rab (*Os03g0191400*), of the vesicle trafficking transport pathway (Supplemental Table 5). Recently, Lee et al. (2007) reported OsHMA9, a P-type ATPase, as a Cu efflux transporter in rice. Here, we found two P-ATPase genes specifically

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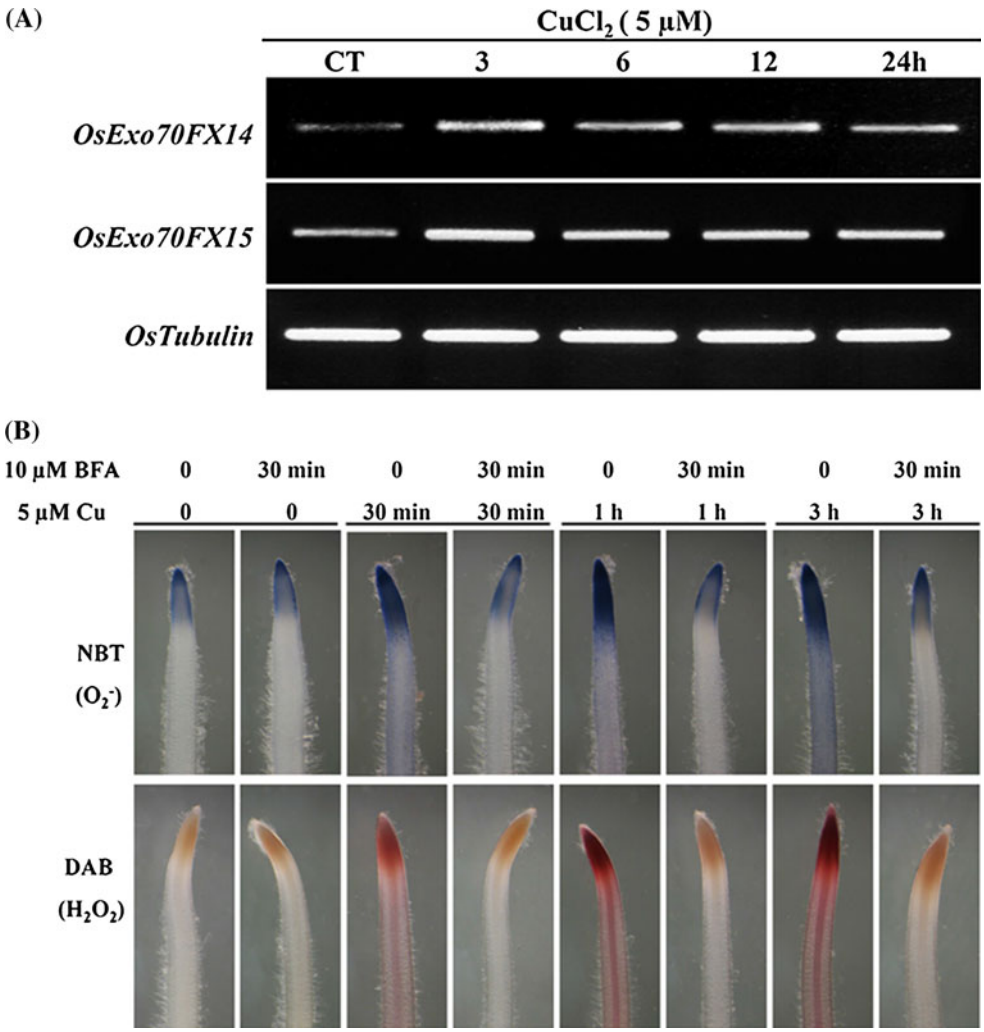

Fig. 3 Analysis of the roles of vesicle trafficking in rice roots exposed to Cu stress. **a** Cu-induced expression of *OsExo70* genes in rice roots. The mRNA levels of *OsExo70FX14* and *OsExo70FX15* were examined by semi-quantitative RT-PCR using gene-specific primers. The *OsTubulin* was used as an internal loading control. **b** Inhibition of Cu-induced production of ROS by vesicle trafficking inhibitors. Roots from 6-day-old rice seedlings were pretreated with brefeldin A (BFA) and exposed to 5 μM Cu. The root samples were collected at different time points after exposue to Cu. *In situ* detection of O_2^- and H_2O_2 was performed by NBT (*upper*) and DAB (*lower*) staining of rice roots with various treatments. Images were taken under a stereomicroscope. Each experiment was repeated at least three times with identical results

induced by Cu treatment (Supplemental Table 6). Thus, the results implied that trafficking of Cu pumping ATPases may play a role in plants responding to the heavy metal stress.

Less study has been performed on the relation between vesicle trafficking and tolerance to heavy metal stress. Based on the comparative transcriptomic analysis, genes involved in vesicle trafficking such as *OsExo70 s* was regulated specifically by Cu stress (Table 2). In contrast to a single gene in yeast and most animals, plants have greatly evolved numerous *Exo70* genes in their genomes, with unknown functions (Li et al. 2010a). *Arabidopsis thaliana* and *O. sativa* are predicted to have 23 and 41 *Exo70* genes, respectively (Chong et al. 2010). Keinänen et al. (2007) observed that expression of *Exo70* in birch (*Betula pendula*) was increased by the damaging effect of Cu. Increase of trehalose, a stress factor, induced the transcript of Exo70 protein (Bae et al. 2005). To understand the functional significance of *Exo70* in response to Cu stress, interference of vesicle trafficking was achieved by treatment of the rice roots with BFA. Indeed, suppression of vesicle trafficking compromised the Cu-induced ROS production in rice roots (Fig. 3). Further investigation using the *Exo70*-silencing plants supported this observation in rice roots after exposure to Cu stress. Silencing of the *NbExo70* gene decreased the Cu-induced oxidative burst in the leaves and roots (Fig. 4). It has been reported in *Arabidopsis* that the vesicle trafficking leads to the intracellular activation of the NADPH oxidase and generation of ROS that act in signaling of the salt tolerance responses (Leshem et al. 2006). In this study, exposure of rice roots to the Cu stress induced the expression of *Exo70* genes, which plays a central role in vesicle trafficking. Therefore, it is suggested that Cu-induced ROS production and transmission via vesicle trafficking may trigger downstream signal transduction and lead to tolerance to the heavy metal stress.

Genes involved in cellular component biogenesis were downregulated by Cu stress (Table 2). Rice contains 200 potential *OsWD40* genes and transcripts of *OsWD40* are preferentially up or down-regulated in different tissues

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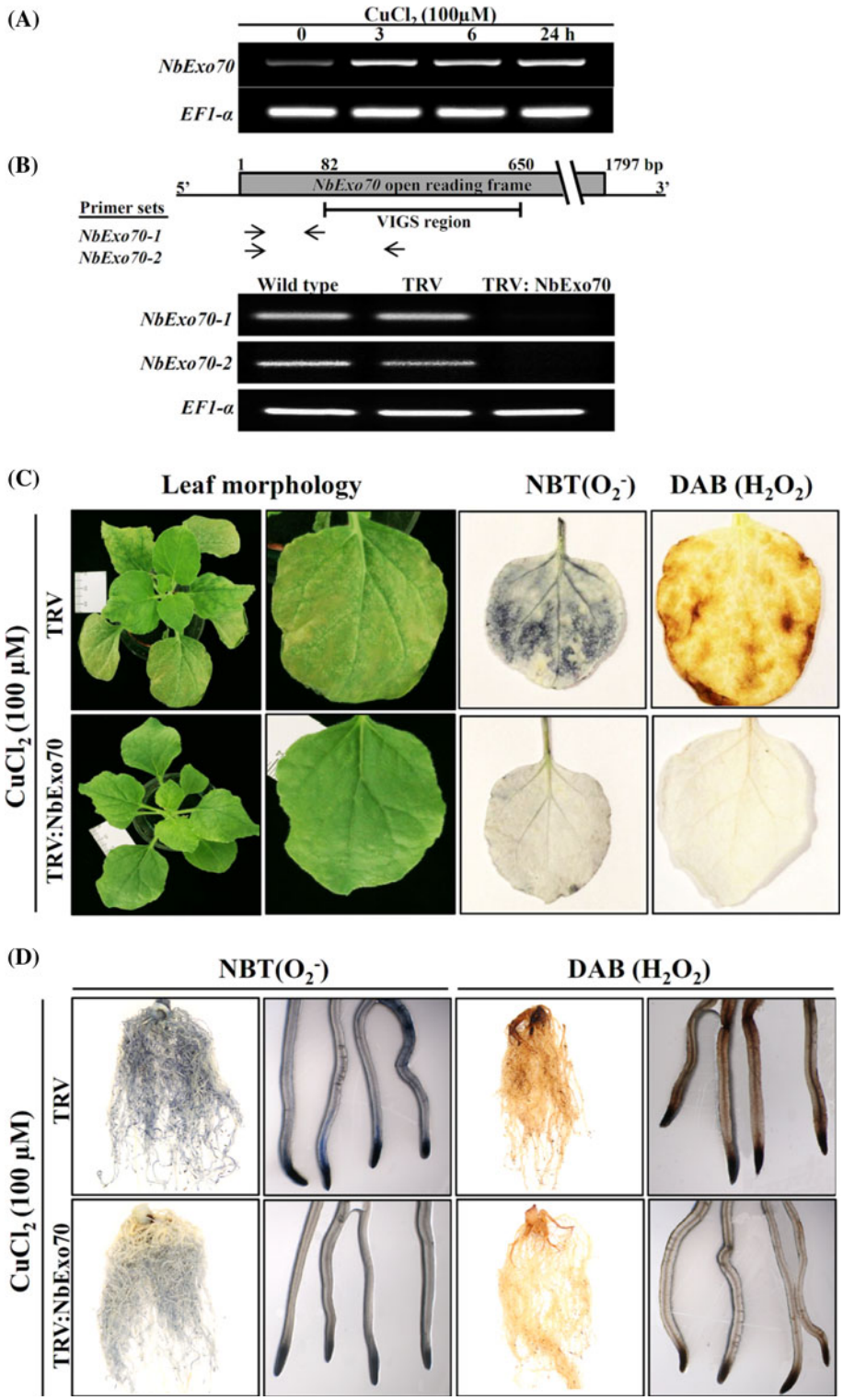

Fig. 4 Functional analysis of *NbExo70* in *N. benthamiana* plants by VIGS. **a** Expression of *NbExo70* in *N. benthamiana* in response to 100 μM Cu treatment. **b** Schematic of *NbExo70* cDNA structure and the VIGS construct containing the cDNA fragment. The *box* indicates the ORF region of *NbExo70*. The primer sets (NbExo70-1 and NbExo70-2) used to check the knockdown efficiency by RT-PCR. Characterization of the *NbExo70*-silenced leaf tissue in the tobacco plants by VIGS. Knockdown effects of *NbExo70* gene expression were determined at 5 days after treatment with 100 μM $CuCl_2$. The wildtype and TRV control plants served as control as compared with the *NbExo70*-silenced plants. **c** Reduce of leave necrosis and ROS accumulation in *NbExo70*-silenced tobacco plants after 100 μM Cu treatment for 5 days. Detached leaves were stained with NBT and DAB solution as described previously. **d** Effects of silencing of *NbExo70* gene expression on Cu-induced ROS production in root tissues. Suppression of *NbExo70* gene in root tissues was achieved by VIGS using Agrodrench. The *NbExo70*-silencing root tissues were treated with 100 μM $CuCl_2$ for 5 days and subjected for in situ detection of O_2^- and H_2O_2. The results shown are from one representative experiment of at least three independent experiments

(Ouyang et al. 2012). Cu-downregulated *OsWD40* gene were belonged to histone-related genes and acted as important cellular components in nucleus and membrane-bounded organelle. ARM repeat is a motif known to mediate protein–protein interactions and most of these characterized proteins are implicated as E3 ubiquitin ligases with U-box in plants (Mudgil et al. 2004; Zeng et al. 2004). It was found that genes encoding for ribosomal protein were downregulated by Cu stress as compared with the previous study with arsenite stress (Li et al. 2010b). Genes involved in cell wall modification (xyloglucan fucosyltransferase) and cell expansion (MAP65) (Smertenko

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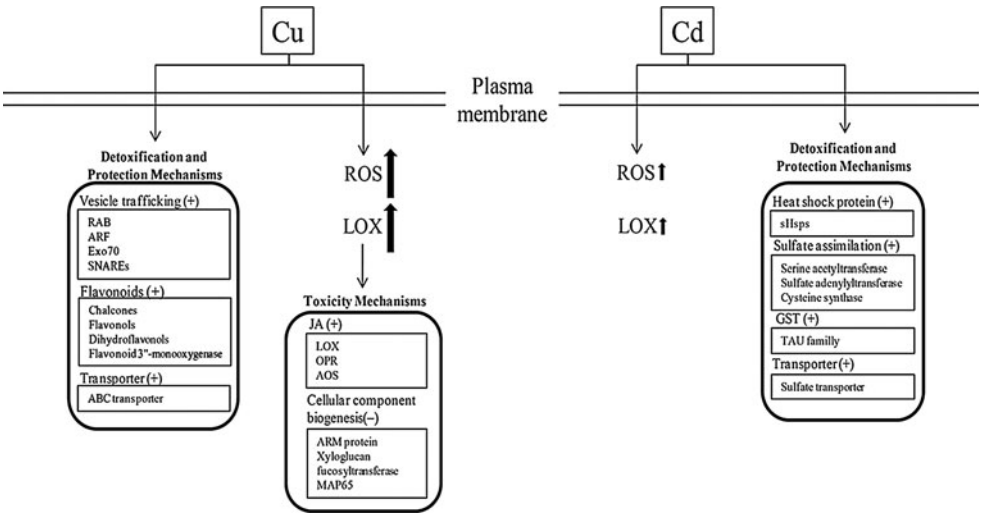

Fig. 5 Schematic representations of 2 different regulation pathways with Cu and Cd stress in rice roots. The *plus* (+) and *minus* (-) symbols indicate that the genes involved in pathway were up- or downregulated, respectively

et al. 2004; Takehisa et al. 2012) were also repressed by Cu stress. Taken together, the results suggested that Cu stress repressed genes involved in the several functional components to inhibit root growth.

Early studies demonstrated the content of jasmonic acid (JA) and its biosynthesis-related genes changed in response to metal stress (Maksymiec et al. 2005; Skórzyńska-Polit et al. 2006). Maksymiec (2007) noted that JA signaling is one of the crucial elements in the plant response to various metal stresses. Our microarray results revealed greater change with Cu than Cd in expression of JA biosynthesis genes such as phospholipase, lipoxygenases, 12-Oxo-PDA-reductase (OPR), allene oxidase synthase (AOS) and jasmonate Zim-domain (JAZ) in rice roots (Supplemental Table 7). In addition, we observed ROS production and an increased level of lipid peroxidation in Cu-treated roots (Supplemental Fig. 3). Formation of lipid peroxides may be a prolonged consequence of heavy metal-induced oxidative stress and may act as an activation signal for plant defense genes through increase of the octadecanoid pathways (Maksymiec 2007). Sasaki-Sekimoto et al. (2005) reported that JA upregulates the expression of antioxidant and defence-related genes. Tamari et al. (1995) found that JA induces flavonoid gene expression in *Petunia corollas*. Our MapMan analysis revealed the induction of flavonoid biosynthesis pathway genes involved in Cu responses (Fig. 2a). Activation of the flavonoid biosynthesis and defence-related genes mediated by JA may provide resistance to Cu toxicity in rice roots.

Induction of sulfate assimilation and Hsps by Cd has been demonstrated in plants (Mendoza-Cózatl et al. 2005; Ogawa et al. 2009). Compared with Cu stress, our MapMan analysis revealed sulfate assimilation metabolism genes upregulated early with Cd stress but with only slight expression with Cu stress. Moreover, among the 16 sulfate transporters identified in *O. sativa*, only 2 showed changed expressions in response to Cd. This result is consistent with a mechanism in which synthesis of the heavy metal-chelating molecules GSH and PCs and Cd acts as a potent activator (Cobbett and Goldsbrough 2002). In addition, we found 19 Hsp and 6 transcription factors (Hsf) genes induced by Cd stress (Supplemental Table 8); only 9 Hsp and 3 Hsf genes were upregulated with Cu stress. The most distinctive expression response with early Cd exposure was observed with sHsps. This early response of sHsps to Cd treatment may have an important role in controlling Cd stress tolerance in rice root. Moreover, induction of these sHsps may reflect divergent functions in response to distinct metal stresses at the early stage.

A possible model of cellular mechanisms involved in Cu and Cd heavy metal detoxification and protection in rice was displayed (Fig. 5). Cu increases intracellular transport, such vesicle trafficking and ABC transport, and induces a flavonoid-mediated detoxification pathway. Small Hsps, sulfate assimilation and GST are specifically upregulated with Cd stress. In addition, the toxicity mechanisms such as JA biosynthesis and cellular component biogenesis were regulated in responded to Cu stress. Therefore, Cu and Cd induce distinct cellular detoxification mechanisms at the early stage of heavy metal stress in rice. Further investigation on the specific pathways will address the questions why different metals induced the distinct mechanisms to counter metal stresses.

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