Journal of Biotechnology **14**(4): 629-644, 2016

TRANSCRIPTOME ANALYSIS OF COPPER STRESS RESPONSE IN RICE SEEDLING USING DNA MICROARRAY

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Received: 18.9.2015
Accepted: 30.6.2016

SUMMARY

Heavy metal contamination along with the increase in food demand are a primary concern in Vietnam and all over the world. In order to enhance crop tolerance to unfavorable cultivation conditions including heavy metal toxicity, understanding of plant response system under the effect of heavy metals is necessary. In the current study, physiological, biochemical and transcriptomic changes of rice seedings (*Oryza sativa* L. cv. IR64) were investigated under copper (Cu) stress. Root elongation and root fresh weight were decreased whereas accumulation of copper in root was enhanced significantly with increasing copper concentration from 2.5 to 15 μM. In addition, copper induced endogenous reactive oxygen species (ROS) generation and activated isoenzymes of superoxide dismutase (SOD) and catalase (CAT). The molecular mechanism of rice roots in response to copper toxicity at mRNA expression level was analyzed by microarray technique. Functions and roles of genes were also analyzed by bioinformatic tools AgriGO and MapMan. Gene ontology analysis revealed that 1900 Cu responsive genes were involved in phytohormones, reactive oxygen species, signaling pathways, transcription factors, transport activities, antioxidant defense systems. Through phytohormones and reactive oxygen species, Cu may inhibit rice root growth. Phytohormones and reactive oxygen species can also be signal molecules in signaling pathways with the participation of mitogen-activated protein kinase (MAPK) cascades, and transcription factors in response to Cu stress. Detoxification and protection mechanisms may involve transport activities and antioxidant defense systems during Cu treatment. These results may provide new insights into mechanisms of rice plant to tolerate with Cu toxicity conditions.

Keywords: *Copper toxicity, microarray, Oryza sativa, phytohormones, reactive oxygen species, signaling transduction, transcription factors*

INTRODUCTION

Heavy metal toxicity is one of the major environmental problems to the present world (Thounaojam *et al.*, 2012). Even in trace concentration, they could cause serious problems to living organisms. Soil pollution owing to heavy metals affects 235 million ha, and this area is still growing (Giordani *et al.*, 2005). In this context, copper (Cu) has emerged as a serious pollutant because of its excessive use in the manufacturing and agricultural industries (Bouazizi *et al.*, 2011; Cambrollé *et al.*, 2013). Cu, as a co-factor of numerous proteins and enzymes, is an essential micronutrient for normal plant growth, development and protective mechanisms (Himelblau, Amasino, 2000; Yruela, 2009). There are more than 100 different Cu-containing proteins in plants (Yruela, 2009). However, Cu concentrations need to be kept at low concentrations since this element is extremely toxic in view of its high redox properties. At elevated concentrations (above 20 μg g^{-1} dry weight), Cu becomes toxic to plant and alters membrane permeability, chromatin structure, enzyme activities photosynthesis and respiratory processes and may induce senescence (Srivastava *et al.*, 2006). One of the most serious toxicity incident under Cu stress is to induce a high accumulation of reactive oxygen species (ROS), causing lipid peroxidation, enzyme inactivation, DNA and membrane damage (Hall, 2002). Due to its high redox properties, Cu catalyzes the production of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and

hydroxyl radicals (OH^-), via Haber-Weiss and Fenton reactions (Aust *et al.*, 1985). Cu toxicity also causes abnormal root morphology, chlorosis, necrosis, and rolling in leaves, all of which hinder plant growth and development, and ultimately lead to reduced crop productivity (Burkhead *et al.*, 2009; Xu *et al.*, 2006).

Like all other organisms, plants have evolved various mechanisms to adapt to or mitigate the damage of heavy metal stress when being exposed to toxic concentrations. The mechanism, which is most common among plants, is to prevent or reduce uptake such metals into root cells by restricting metal ions to the apoplast, binding them to the cell wall or to cellular exudates, or by inhibiting long distance transport. If this fails, metals already absorbed in the cell can be chelated, trafficked, transported and sequestered in specific compartments to detoxify their toxicity. When this is overload, plants can activate oxidative stress mechanisms and the synthesis of stress-related proteins and signaling molecules.

Rice is one of the world's most important food crops. But due to the increasing heavy metal contamination including Cu toxicity, rice crop productivity is reduced, threatening global food security. Therefore, understanding of plant mechanisms response to heavy metal toxicity is necessary for us to cope with the environmental problems in order to maintain and improve the crop productivity. Analyses of transcriptomes and proteomes in plants have revealed transcripts or networks of proteins related to Cu and other heavy metals responses. Sudo *et al.,* (2008) clarified the effect of Cu on gene expression in rice roots 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 this study, we used a custom Agilent 44K rice microarray to understand the mechanisms of heavy metal toxicity and cellular and protection pathways in the early stress response to Cu in rice roots. We found mechanisms associated with phytohormones, ROS, signaling pathways, transcription factors, transport activities, and antioxidant defense systems.

MATERIALS AND METHODS

Plant materials

Rice (*Oryza sativa* L. cv. IR64) seeds were surface disinfected with 2.5% (v/v) sodium hypochlorite (Katayama, Japan) for 15 minutes, then thoroughly washed in distilled water, and placed in 9 cm Petri dishes which contained 25 ml distilled water at 37°C in darkness. After 3 days of incubation, uniformly germinated seeds were transferred to Petri dishes over filter paper discs moistened with 10 ml distilled water and grown at 26°C in darkness for 3 days. Once the roots reached 3–4 cm in length, they exposed to different concentrations (0-15 μM) of $CuCl_2$. Root length was measured after 3 days of incubation at 26°C in darkness. The roots were excised from the Cu-treated rice seedlings were taken to determine ROS production, in-gel enzymatic activity and microarray assays.

For quantification of Cu content, 6-day-old rice seedlings were treated to 5 μM $CuCl_2$ for 24 h. The content of Cu were analyzed by ICP-MS Agilent 7500 ce (Agilent, USA) (Lin *et al.*, 2007). The analyses were performed at Center of analytical services and experimentation of Ho Chi Minh City.

In situ detection of reactive oxygen species (ROS)

Rice roots were treated with different concentrations (0-15 μM) of $CuCl_2$ for 1 or 3 h. H_2O_2 levels in rice roots during Cu treatment were detected by staining with 3,3'-diaminobenzidine (DAB) (Thordal-Christensen *et al.*, 1997). Roots were soaked in a 10 mM MES buffer, pH 3.8 with 1 mg ml^{-1} DAB for 8 h in darkness. The stained roots were observed using an Olympus SXZ16 stereomicroscope (Olympus, Tokyo, Japan).

Protein extraction and antioxidant enzyme activity assays

Approximately 1 gram rice roots was ground in protein extract buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 10 mM NaF, 10 mM Na_3VO_4, 1 mM sodium-tartrate, 10% v/v glycerol, 50 mM $Na_2S_2O_5$, 1% SDS, 1 mM PMSF). Homogenized root tissue was centrifuged at 15,700 g for 10 min at room temperature, and the supernatant was collected. Protein concentration was determined by the BioRad Dc Protein Assay (Bio-Rad, Hercules, CA, USA) at OD_{750}.

The isoenzymes of superoxide dismutase (SOD) and catalase (CAT) were separated on discontinuous polyacrylamide gels (stacking gel 4.5% and separating gel 10%) under the non-denaturing and non-reducing conditions. Proteins were electrophoretically separated at 4°C and 80 V in the stacking gel, then 120 V in the separating gel. Isoenzyme of SOD and CAT were indicated as described by Wang and Yang (2005). The antioxidant enzyme activities of SOD and CAT were

estimated as previously described Kumar *et al.* (2008). Each assay was replicated at least three times per sample.

Purification of total RNA

For microarray analysis, root samples (100 mg) treated with 5 μM $CuCl_2$ for 3 h were harvested. Total RNA extraction involved use of the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) with several modifications. 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.

Microarray preparation

Extraction of RNA samples to analyze the early transcriptomic changes was performed as described by Trinh *et al.,* (2014). 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 nucleotides by incubation with fragmentation buffer (Agilent Technologies, USA) at 60^{o}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^{o}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. The microarray analysis was performed by the DNA Microarray Core Laboratory at the Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan.

Microarray data analysis and organization

Microarray analysis involved Agilent Oligo DNA Microarray Hybridization protocols with the Rice Oligo DNA Microarray 44K Rice Annotation Project Data Base (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 GeneSpring GX11 (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 expression fold-change ≥ 2 or ≤ 0.5 (cut off by FDR < 0.1) regulated by Cu.

Descriptions of each Cu-responsive gene were annotated according to the RAP-DB and the TIGR Rice Genome Annotation Resource (Ouyang *et al.*, 2007)]. Annotation and functional information of genes were obtained from various sources such as Rice Kinase Database (Dardick *et al.*, 2007), the Database of Rice Transcription Factors (Gao *et al.*, 2006), the peroxidase database (Passardi *et al.*, 2007), the TransportDB (Ren *et al.*, 2004).

In addition to the aforementioned annotation and information, Cu-responsive genes were also classified into functional categories by AgriGO functional enrichment analysis (Du *et al.*, 2010). Furthermore, the averaged signals for a given treatment (3 biological replicates for 5 μM $CuCl_2$ treatment for 3h) were expressed relative to those for control samples, converted to a $\log_2$ scale and displayed by the use of MapMan version 3.6.0RC1 (Usadel *et al.*, 2005). Rice genes represented on the Rice Oligo DNA Microarray were organized by BINS and sub-BINS for display on the schematic map of transport overview. Gene expression was analyzed by the Wilcoxon Rank Sum test with uncorrected *p* value.

Expression analysis by semi-quantitative RT-PCR

Total RNA was extract from Cu-treated rice roots as described. Cu-regulated genes with significant fold change were selected and subjected

to semi-quantitative RT-PCR. Oligonucleotide primers are listed in table 1. A gene encoding *α-tubulin* (*Os03g0726100*) was used as a reference gene. Amplicons were analyzed by agarose gel electrophoresis (1.5%), and PCR products were sequenced. For each gene expression analysis, two or three biological replicates were performed and showed nearly identical results.

Table 1. The forward and reverse primer sequences used in semi-quantitative RT-PCR for detecting gene expression.

Gene name	RAD-DB locus ID	Forward primer (5'→ 3')	Reverse primer (5'→3')	Amplicon (bp)
OsPrx	*Os03g0234500*	GATGCAGAGCGAGAAGAACG	GCTGATGTTGCCCATCTTGAC	688
ABC transporter	*Os08g0384500*	CCAACACAAGGAGAAATGCTG	GGTCACAAGAATGGTGTCCTA	813
AP2/ERF	*Os03g0860100*	TGAGCACCACCAACAGCTCA	TGATATCGATGGGCTGAGAGG	507
CDPK	*Os03g0788500*	ATCAGCAAGAACGGCTTCTTC	TCGGTGACCAGCTTCCGCTT	488
MAPKKK	*Os05g0545400*	TACCAGATGTTCCTCGAGTTC	CGGTGTCGGACTCCCACAAT	637
RLCK-Os	*Os08g0374600*	ATCAGCCACATCGACATCATC	AGCAGGATGTTGGACAGCTT	629
OsDAD	*Os08g0143600*	AGCAACAGCAGCAACAGGGT	TCGTACTTTGTCAGTAGCCT	845
α-tubulin	*Os03g0726100*	TCGCAGCATCAACCCAATC	GCAACCAGTCCTCACCTCAT	272

RESULTS

Changes of growth and Cu uptake of rice seedling

To evaluate the toxic effects of Cu stress in rice seedlings, we performed a dose–response experiment with rice roots. A gradual decrease in length and fresh weight of seminal roots was observed with the increase in Cu concentration treatment (Fig. 1A-1C). Half-maximum inhibition was occurred at 5 μM $CuCl_2$ after 3 day with respect to control. At 15 μM $CuCl_2$, growth was completely inhibited. Cu content in root increased gradually with increasing Cu concentrations in the treatment (Fig. 1D).

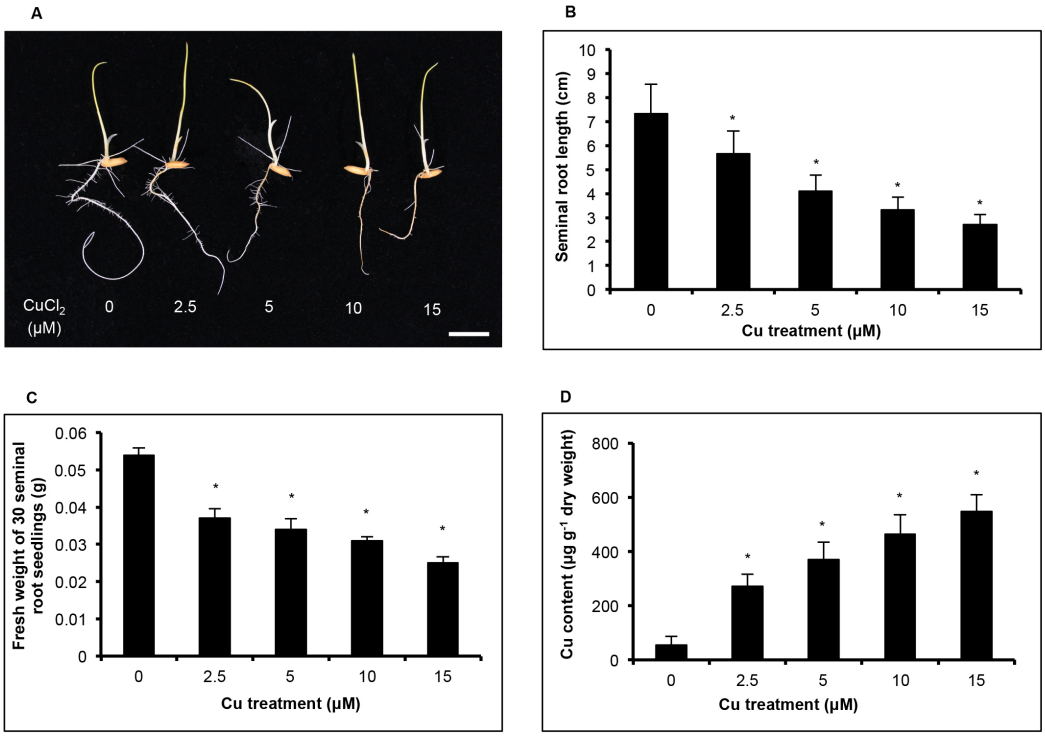

Figure 1. Effect of Cu treatment on growth of rice seedlings. Rice roots were exposed with different concentrations of Cu (0, 2.5, 5, 10, 15 μM) for 3 d, and seminal root lengths (A and B), seminal root fresh weight (C) and Cu content (D) were determined. Data are mean ± SD. *Significantly different from the control at $p<0.05$ by paired *t* test.

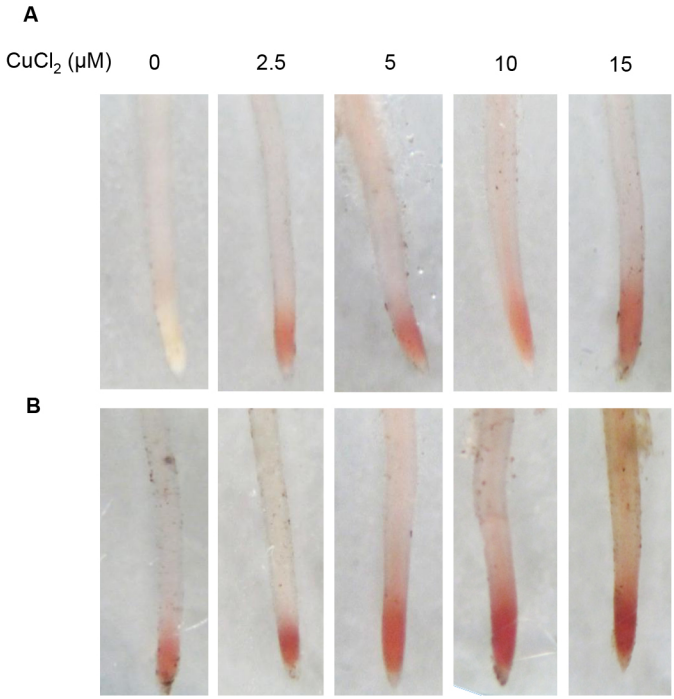

Figure 2. Cu-induced ROS accumulation in rice roots. Root samples were exposed with 5 μM Cu for 1 h (A) to 3 h (B). H_2O_2 was determined by staining roots with DAB.

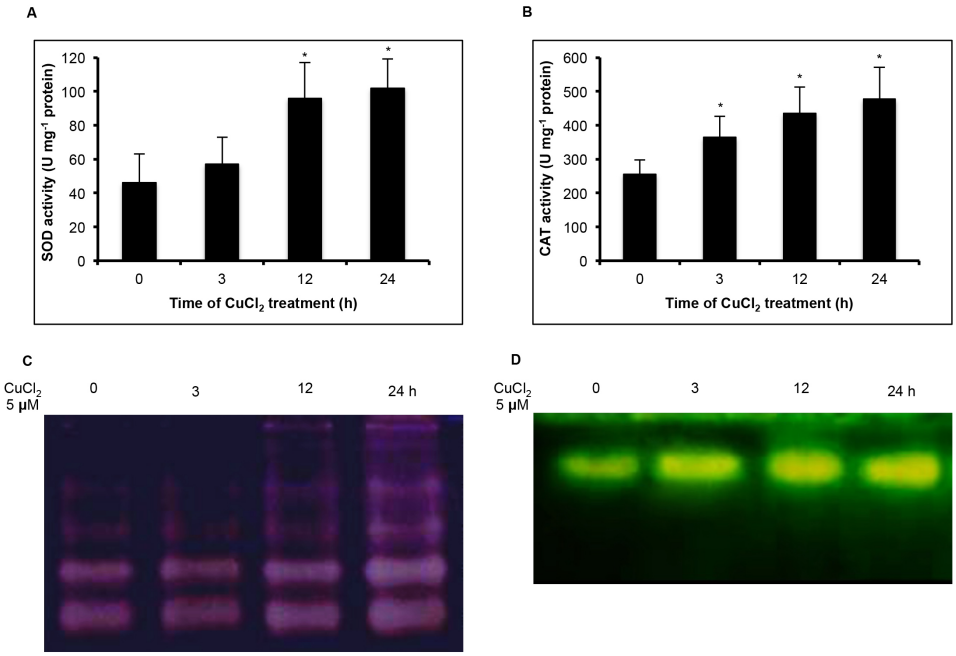

Figure 3. Effect of Cu on the activities and isoenzyme patterns of SOD (A and C) and catalase (B and D) in rice root. Data are mean ± SD. *Significantly different from the control at $p<0.05$ by paired *t* test.

Cu-induced ROS generation in rice roots

To determine whether Cu treatment induced ROS generation, rice roots were treated with 0, 2.5, 5, 10, 15 μM $CuCl_2$ for 1 and 3 hours. The levels of ROS accumulation increased progressively in root with the increase of Cu concentration and duration of treatment (Fig. 2A and 2B). These results suggest that Cu treatment triggered ROS generation in rice roots.

Antioxidant enzymes activity

In the present study, it is found that SOD activity increases more 2-fold after Cu treatment for 12 h and continued to increase up to 24 h (Fig. 3A). As shown in the result (Fig. 3B), catalase activity gradually increased from 3 to 24 h. Using non-denaturing PAGE, we determined Cu-treated rice roots mainly contained 4 distinct SOD isoforms. Cu induced a significant increase in all SOD isoform activity from 12 to 24 h (Fig. 3C). There was one isoenzyme of catalase detected in rice root (Fig. 3D).

Microarray expression profiling

To learn about the molecular mechanism of rice in response to Cu stress, we used Agilent Rice Oligo 44K DNA Microarray to identify Cu stress-regulated genes. The result indicated that there were 1.459 up-regulated genes (FDR < 0.1 and fold-change ≥ 2) and 441 down-regulated genes (FDR < 0.1 and fold-change ≤ 0.5) in response to 5μM $CuCl_2$ treatment for 3 h. In addition, Cu-responsive genes were functionally classified into several categories by AgriGO functional enrichment analysis (Table 2). The major biological categories of the Cu-up-regulated genes were cellular metabolic process, response to stimulus, post-translational protein modification. For molecular function, the significant gene ontology terms were binding, catalytic and electron carrier activity.

Table 2. Gene ontology analysis of 1900 Cu-regulated genes in rice root. [a]Query item: Number of query list; [b]Backgroup item: Number of Background; [c]FDR: False discovery rate

GO ID	GO term	Query item[a]	Background item[b]	FDR[c]
Biological process				
Metabolic process				
GO:0055114	Oxidation reduction	51	85	8.67e-34
GO:0000272	Polysaccharide catabolic process	12	68	0.0094
Response to stimulus				
GO:0006979	Response to oxidative stress	27	251	0.0029
Post-translational protein modification				
GO:0006468	Protein amino acid phosphorylation	108	1308	4.6e-09
Molecular function				
Binding				
GO:0005509	Calcium ion binding	53	309	1.4e-14
GO:0070279	Pyroxidal phosphate binding	10	15	8.7e-07
GO:0046906	Heme binding	42	250	2.3e-11
GO:0046983	Protein dimerization activity	8	45	0.038
Catalytic activity				
GO:0070001	Aspartic-type endopeptidase activity	15	75	9.3e-05
Electron carrier activity				
GO:0009055	Electron carrier activity	59	80	2.7e-43

Expression profiles of phytohormone-related genes induced by Cu stress

The rice genome has approximately 324 phytohormone-related genes. Here, we identified 298 genes on our arrays in which there were 42 genes significantly up-regulated by Cu (Table 3). Among them, transcripts of 2 ABA (*Abscisic acid*) biosynthesis genes and 7 ABA signaling genes were

up-regulated. In addition, there were 7 genes related to JA (*Jasmonic acid*) biosynthesis and 8 JA signaling genes were also up-regulated. Other phytohormones such *as GA (Gibberellin) deactivation* genes, *ethylene biosynthesis* gene showed increased expression in hormone signaling pathways.

Table 3. Cu-responsive transcripts related to phytohormones.

Functional categories	In genome	On array	Detected	Increased	Decreased
ABA					
Deactivation	3	3	1	0	0
Biosynthesis	13	11	8	2 (*OsNCED1, OsNCED1*)	0
Signaling	39	37	25	7 (*OsbZIP12, OsPLDα6, OsPLDα7, OsPP2C2, OsPP2C4, OsPP2C5, OsVP1*)	0
JA					
Biosynthesis	27	23	15	7 *(OsAOC, OsAOS1, OsAOS2, OsDAD1;2, OsDAD1;3, OsLOX2;1, OsLOX2;3)*	0
Signaling	11	11	10	8 *(OsJAZ1, OsJAZ2, OsJAZ3, OsJAZ4, OsJAZ5, OsJAZ6, OsJAZ7, OsJAZ9)*	0
GA					
Biosynthesis	11	11	4	1 *(GA20ox2)*	0
Deactivation	15	15	6	2 *(GA2ox3, GA2ox9)*	0
Signaling	10	10	7	*2 (GID1, SLRL1)*	0
Ethylene					
Biosynthesis	13	12	9	3 *(OsACO4, OsACO5, OsACS2)*	0
Signaling	16	16	13	*1 (OsEIN3;3)*	0

Table 4. Cu-responsive transcripts related to reactive oxygen species.

Functional categories	In genome	On array	Detected	Increased	Decreased
OsPrx (Rice class III peroxidase)	215	128	93	18	6
GT1 (Glycosyltransferase 1)	211	167	108	13	0
GST (Glutathione S-transferase)	147	65	51	10	0
OsMT1 (Rice Metallothionein 1)	36	11	9	3	0
AOX (Alternative oxidase)	4	4	3	2	0
MDHAR (Monodehydroascorbate reductase)	28	14	7	2	0
Rboh (respiratory burst oxidase homolog)	17	9	6	2	0
Trx (Thioredoxin)	30	29	24	2	0
GPX (Glutathione peroxidase)	15	5	4	1	0

Expression profiles of ROS-related genes induced by Cu stress

Like many metal, treatment with Cu generates ROS. We analyzed the global expression profiles of genes in ROS-related gene families. There are approximately 825 genes classified into these families. Among 490 ROS-related genes detected on our arrays, we identified 53 significantly up-regulated genes and classified predominantly into 3 families: *OsPrx* (*Rice class III peroxidase*), *GT1* (*Glycosyltransferase 1*) and *GST* (*Glutathione S-transferase*) (Table 4). In addition, we also identified genes belonging to *AOX* (Alternative oxidase), *MDHAR* (*Monodehydroascorbate reductase*), *Trx* (*Thioredoxin*), *GPX* (*Glutathione peroxidase*) and *rboh* (*respiratory burst oxidase homolog – NADPH oxidase*) families. NADPH oxidase enzyme participates in ROS generation on the cell membrane.

Expression profiles of genes related to signaling transduction under Cu stress

Stress signal perception and transmission are likely important aspects of plant response to heavy metal stress. The rice genome contains an estimated 1690 genes related to protein kinase classified into 6 groups: *GMGC* (including *CDK*, *MAPK*, *GSK3* and *CLK* families), *calcium/CaM dependent protein kinase* (*CAMK*), *casein kinase 1* (*CK1*), *tyrosine kinase-like* [*TKL*; *including interleukin1 receptor-associated kinase* (*IRAK*) family and both receptor and cytoplasmic kinases], *AGC* (*PKA*, *PKG* and *PKC*) and *STE* (homologs of yeast sterile 7, sterile 11 and sterile 20 kinases) (Dardick, Ronald, 2006; Manning *et al.*, 2002; Shiu *et al.*, 2004). Of 1166 genes represented on our microarray, we identified 111 significantly up-regulated genes under the effect of Cu stress (Table 5). Nearly all of these genes were associated with *TKL* group (93 genes). *TKL* group is the largest and includes the aforementioned *IRAK* and both receptor and cytoplasmic kinases. There are 69 subfamilies which belong to *IRAK* family based on phylogenetic analyses and organization of extracellular domain (Dardick, Ronald, 2006; Shiu *et al.*, 2004). Among 91 genes belong to *IRAK* family, we identified 6 important subfamilies including: *receptor like cytoplasmic kinase (RLCK)-VII*, *RLCK-IV*, *RLCK-Os*, *Leucine rich repeat receptor kinase (LRR)-VIII*, *Domain unknown function 26 (DUF26)* and *Leucine rich repeat kinase* (*LRK) 10-2* (Fig. 4). In addition, our microarray data revealed a number of calcium regulation-related genes belonging to *CAMK* group that were up-regulated by Cu treatment, such as 4 *Calcium-dependent protein kinase (CDPK)* genes including *OsCPK4, OsCPK13, OsCPK15, OsCPK10* and 1 *CBL interacting protein kinase (CIPK)* gene *OsCIPK15*. We also found increased abundance of 6 *MAPKKK* genes and 1 *MAPKK* gene belonging to *STE* group under the effect of Cu stress.

Table 5. Cu-responsive transcripts related to signaling transduction.

Functional categories	In genome	On array	Detected	Increased	Decreased
Calcium signaling cascades					
CAM and *CML (Calmodulin* and *CAM like*)	37	34	23	7	1
CBL (Interacting protein kinase)	10	9	9	0	0
CDPK (Calcium-dependent protein kinase)	29	29	19	4	1
CIPK (CBL interacting protein kinase)	30	29	23	1	1
IQD (IQ67-domain)	28	27	22	0	1
MAPK cascades					
MAPK (Mitogen-activated protein kinase)	16	16	15	3	1
MAPKK (MAPK kinase)	8	6	6	1	0
MAPKKK (MAPKK kinase)	75	70	59	9	0

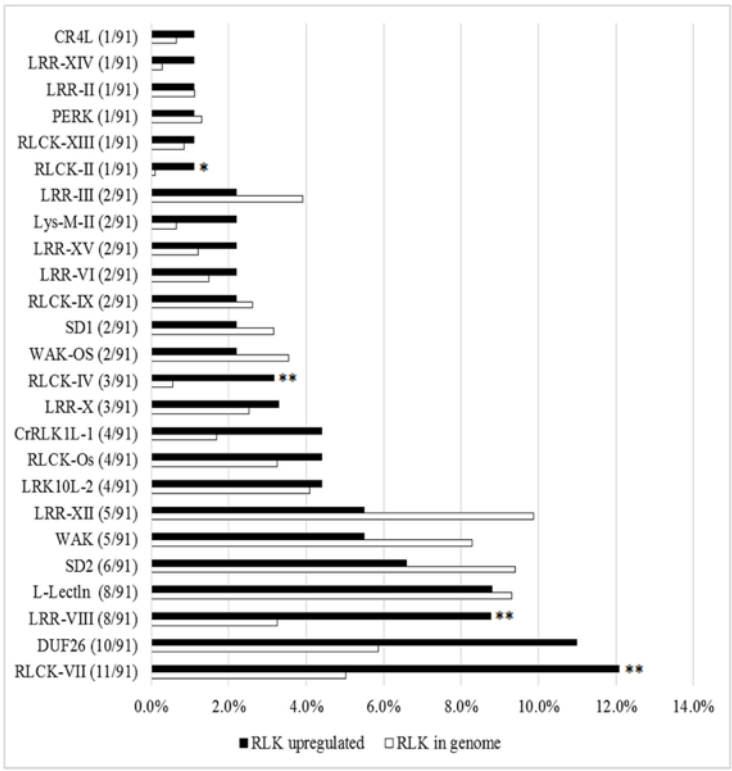

Figure 4. Subfamilies of *receptor–like kinase (RLK)* belong to the IRAK family showing alteration in expression with Cu treatment in rice roots. White bars indicate total number of genes of the functional category in the rice genome. Black bars indicate total number of up-regulated genes within the functional category. The data are percentages. *RLK* subfamilies that are significantly overrepresented in the functional category are shown with ** ($P < 0.01$) or * ($P < 0.05$). **$P < 0.01$ and *$P < 0.05$ are significantly overrepresented (*Chi*-square test).

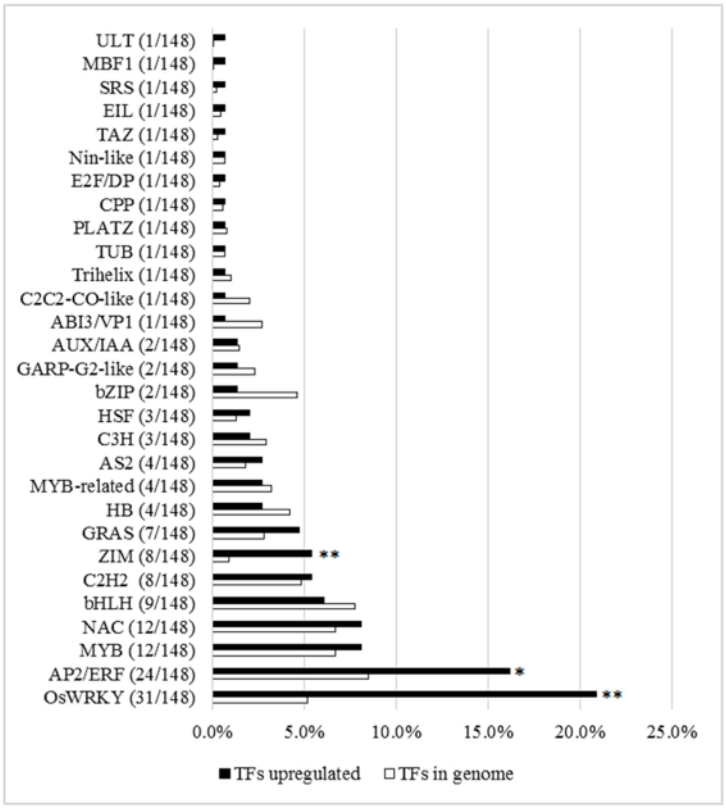

Figure 5. Families of transcription factors showing alteration in expression with Cu treatment in rice roots. White bars indicate total number of genes of the functional category in the rice genome. Black bars indicate total number of up-regulated genes within the functional category. The data are percentages. TF families that are significantly overrepresented in the functional category are shown with **($P < 0.01$) or * ($P < 0.05$). **$P < 0.01$ and *$P < 0.05$ are significantly overrepresented (*Chi*-square test).

Expression profiles of genes related to transcription factors induced by Cu stress

The rice genome contains approximately 1930 transcription factor (TF) genes which are classified into 63 families. Our microarray data indicated that 148 TF genes were significantly up-regulated with Cu treatment. Most of the genes belong to the following families: *WRKY*, *APETALA2/ethylene response factor (AP2/ERF)*, *Myeloblastosis (MYB)*, *NAM-ATAF-CUC (NAC)*, *basic helix loop helix (bHLH)*, *Cys2His2 zinc finger (C2CH2)*, *Zinc-finger protein expressed in inflorescence meristem (ZIM)* and *GAI-RGA-SCR (GRAS)* (Fig. 5). More than 20% of the genes belong to *WRKY* family. In *AP2/ERF* family we identified that 7 genes belonging to subfamily *ERF* that were up-regulated by Cu stress. Moreover, our result also showed that 3 *Heat shock factor (HSF)* genes were up-regulated significantly.

Expression profiles of genes related to transporter

There are approximately 1343 genes related to transporter in the rice genome classified into 4 distinct types based on mode of transport and energy coupling mechanisms: ATP-dependent (primary active) transporters, secondary transporters, ion channels and unclassified transporters. Several types of transporters showed differential expression with Cu treatment. We found 1131 transporter genes on our arrays and 68 were significantly up-regulated with Cu treatment (Table 6). Nearly all of the 68 genes were secondary transporter and ATP-dependent transporters. Several types of transporters showed differential expression with Cu treatment. Genes encoding transporters for ATP-binding cassette-type (*ABC*) and sugars were differentially regulated in response to Cu (Fig. 6).

Table 6. Cu-responsive transcripts related to transporter.

Functional categories	In genome	On array	Detected	Increased	Decreased
ATP-dependent					
ABC (ATP-binding cassette superfamily)	139	117	73	15	2
H^+PPase (H^+-translocating pyrophosphatase family)	7	7	6	1	0
P-ATPase (P-type ATPase superfamily)	47	43	34	4	0
Ion channels					
VIC (Voltage gated ion channel superfamily)	31	27	13	1	1
MIP (Major intrinsic protein family)	37	33	24	0	1
Secondary transporter					
MFS (Major facilitator superfamily)	155	134	89	15	8
AAAP (Amino acid/auxin permease family)	65	53	38	7	2
DMT (Drug/metabolite transporter superfamily)	128	107	66	6	2
MOP (Multidrug/ oligosaccharidyl lipid/polysaccharide flippase superfamily)	59	45	27	4	0
POT (Protein-depedent oligopeptide transporter family)	94	76	42	3	4
MC (Mitochondrial carrier family)	65	60	53	3	0
ZIP (Zinc (Zn2+)-iron (Fe2+) permase family)	19	17	12	2	0
Unclassified					
FP (Ferroportin family)	1	1	0	0	0
PPI (Peroxisomal Protein Importer family)	3	3	3	0	0

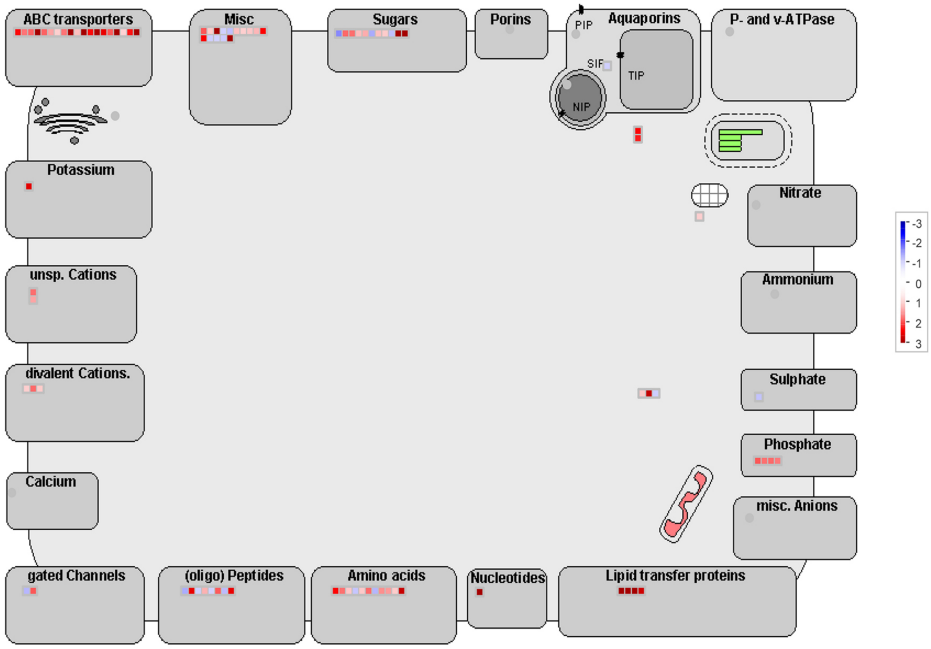

Figure 6. MapMan analysis of genes involved in transport. Microarray RNA pool was combined a fixed volume of rice root samples collected from 3 h time point. Rice genes represented on the Rice Oligo DNA Microarray were organized by BINS and sub-BINS for display on the schematic map of transport overview. Each BIN or subBIN is represented as a block, with each transcript displayed as a square, red for gene up-regulated and blue for those down-regulated. Functional bins identified by the Wilcoxcon rank sum statistic as being significantly changed with Cu stress are in green.

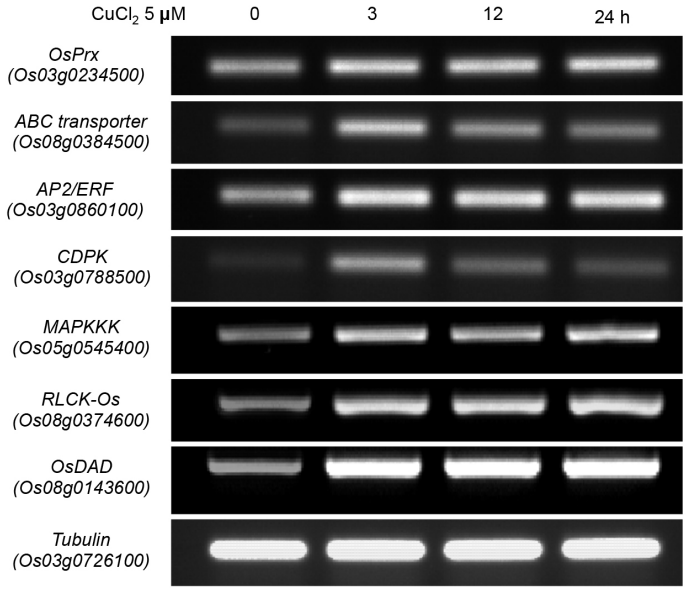

Figure 7. Validation of microarray-based gene expression profiles of rice roots in response to 5 µM Cu treatment for 0, 3, 12 and 24 h by semi-quantitative RT-PCR analysis. *α-tubulin* was an internal control.

Semi-quantitative RT-PCR analysis of selected genes

To verify our microarray studies we analyzed RNA instruction in response to Cu in rice root by semi-quantitative RT-PCR. As shown in fig. 7, time-course analysis revealed that induction of genes related to hormone signaling (*OsDAD-Rice decreased apical dominance)*, ROS (*OsPrx*), signaling transduction (*CDPK, MAPKKK*), transporter (*ATP-dependent transporter*), protein kinase (*RLCK-Os*) and transcription factor (*AP2/ERF*) occurs very rapidly, with high expression within 3 h.

DISCUSSION

Copper toxicity can inhibit plant growth by damaging or disrupting the function of roots (Thounaojam *et al.*, 2012). Global genome expression analysis is increasingly used to understand the plant defense mechanism against excess metal stress. We aimed to determine the early effect of Cu stress on transcriptome regulation in rice roots by a bioinformatics approach. In this study, we report a rapid molecular response to Cu surrounding the root of 6-day-old rice: 1900 genes are differentially expressed after 3 h of exposure to 5 μM Cu. The expression patterns obtained by semi-quantitative RT-PCR were consistent with those obtained by microarray analysis.

Plant growth and plant response to stresses are controlled by phytohormones (Bogatek, Gniazdowska, 2007). Our microarray results revealed the expression of many genes related to biosynthesis, signaling and deactivation of phytohormones under Cu stress. Seven genes related to JA biosynthesis and 8 genes implicated in JA signaling were significantly up-regulated by Cu treatment. Although JA plays a role as signaling molecule in tolerance response to stress, it is also associated with slowing down the growth (Maciejewska, Kopcewicz, 2002). ABA inhibits *Arabidopsis* root growth by signaling through ethylene response pathway 1 (Ghassemian *et al.*, 2000). Our microarray data showed that 8 *ABA signaling* genes and 1 gene encoding an important enzyme in ABA biosynthesis were significantly up-regulated under Cu stress. Recently, gibberellins (GAs) have been shown to have an important role in growth regulation during stress conditions (Achard *et al.*, 2006). GA2-oxidase encoded by *GA2ox* genes is related to GA deactivation and was found to be induced by stress to decrease GA content leading to growth repression (Achard *et al.*, 2008; Hedden, Thomas, 2012). We found transcripts for two *OsGA2ox* genes were strongly increased by Cu treatment of rice roots. Furthermore, heavy metal stress could also induce ethylene production (Vassilev *et al.*, 2004). Growth inhibition induced by ethylene treatment and enhancement of root elongation when being treated with ethylene perception inhibitors were discovered (Gallie *et al.*, 2009). Our microarray data showed that 1 *OsACS* gene encoding ACC synthase enzyme and 2 *OsACO* genes encoding ACC oxidase enzyme which are important for ethylene production, were induced by Cu treatment. Therefore, JA, ABA, GA and ethylene may be involved in Cu stress-induced rice root growth inhibition.

Protein kinases are vital components in signaling transduction. By perceiving or sensing the extracellular signals, receptor-like kinase (RLK) activates the downstream signaling pathway by phosphorylating specific targets (Becraft, 2002). Nearly 75% rice kinases belong to *TKL* group in which *RLK* family is contained. Six important *RLK* subfamilies including *RLCK-VII*, *RLCK-IV*, *RLCK-Os*, *LRR-VIII*, *DUF26* and *LRK10-2* were identified in our data. Recent studies showed that *RLCK-VII*, *RLKCK-IV* and *RLCK-Os* play a role in stress response (Lehti-Shiu *et al.*, 2009; Vij *et al.*, 2008). In plants, LRR-RLK participates in MAPK cascades in innate immune response (Gómez-Gómez, Boller, 2000). Here, we found 8 genes related to the subfamily *LRR-VIII* up-regulated with Cu treatment.

Many studies have indicated that MAPK cascades are the major downstream receptor transducing extracellular stimuli in plant intracellular responses (Tena *et al.*, 2001; Zhang, Klessig, 2001). The mRNA levels of rice *MAPKKK* and *MAPK* were found increased under various stresses (Fu *et al.*, 2002; Kim *et al.*, 2003). MAPK responsiveness may differ depending on the type of metals and ROS involved (Thapa *et al.*, 2012). Our microarray data showed that 3 *MAPK* genes, 1 *MAPKK* gene and 9 *MAPKKK* genes were up-regulated under the effect of Cu stress. Protein kinases and MAPK cascades therefore may play an important role in signaling pathways in response to Cu stress.

TFs are proteins that act together with other transcriptional regulators, including chromatin remodeling/modifying proteins, to employ or obstruct RNA polymerases to the DNA template (Udvardi *et al.*, 2007). TFs interact with cis-elements the promoters of stress-related genes and thus stimulate the expression of many downstream genes in response to abiotic stress (Agarwal, Jha, 2010). We identified *WRKY*, *AP2/ERF*, *MYB*, *NAC*, *bHLH*, *C2CH2*, *ZIM* and *GRAS* families as significantly participating in transcriptional regulation with Cu treatment in rice root. The WRKY, include more than 100 *WRKY* members in rice genome, plays a crucial role in plant abiotic response and in various stress signaling pathways (Eulgem, Somssich, 2007; Ramamoorthy *et al.*, 2008; Zou *et al.*, 2004). Our microarray data indicated that 31 *WRKY* genes were up-regulated under the effect of Cu stress. The participation of AP2/ERF in abiotic stress response was reported (Yamaguchi-Shinozaki, Shinozaki, 2006). Here, we found 24 *AP2/ERF* genes were up-regulated under the effect of Cu stress. The *AP2/ERF* family is divided into 4 subfamilies: *AP2*, *RAVE*, *DREB* and *ERF* (Sakuma *et al.*, 2002). *ERF* are involved in responses to drought, cold, salt or ABA treatment in and pathogen infection in *Arabidopsis* (Park *et al.*, 2001). In total 24 *AP2/ERF* genes, we identified 7 genes related to *ERF* subfamily. As a result, *AP2/ERF*, especially *ERF* can also be involved in the Cu stress response.

ROS overproduction is a common effect induced by heavy metal stress which can be toxic to plant tissues and trigger cell growth inhibition and cell death. In addition, ROS may act as signal molecules involved in triggering stress adaptation and development regulation (Apel, Hirt, 2004). We found 53 ROS-related genes were up-regulated under the effect of Cu stress in which there were 2 genes belonging to *rboh* family. These results indicated that Cu treatment does evoke ROS generation in rice roots.

To cope with ROS toxicity, plants activate many enzymatic and non-enzymatic antioxidant defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS. Many enzymatic defense systems related genes were found in our microarray data. In addition, in-gel activity assay showed that SOD and catalase activity were strongly induced under Cu stress.

CONCLUSION

Our microarray results suggest that Cu-stress may have a significant effect on inhibiting root elongation in rice through phytohormones such as JA, ABA, GA, ethylene and increasing ROS levels. On the other side, phytohormones and ROS may act as signal molecules in signaling pathways with the additional participation of protein kinases, MAPK cascades, TFs in response to Cu-stress in rice roots. Detoxification and protection mechanisms against Cu toxicity may involve transport activities carried out by transporters and enzymatic antioxidant defense systems.

Acknowledgments: *We would like to thank Professor Huang Hao Jen, Department of Life Sciences, National Cheng Kung University, Taiwan for the kind help and support.*

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PHÂN TÍCH HỆ PHIÊN MÃ Ở CÂY MẦM LÚA TRONG QUÁ TRÌNH ĐÁP ỨNG VỚI STRESS ĐỒNG BẰNG KỸ THUẬT DNA MICROARRAY

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TÓM TẮT

Sự tạp nhiễm kim loại nặng cùng với sự gia tăng nhu cầu về lương thực, thực phẩm là những vấn đề lo ngại hàng đầu không chỉ riêng Việt Nam mà của cả thế giới hiện nay. Ô nhiễm kim loại nặng ảnh hưởng nghiêm trọng đến năng suất mùa vụ. Với mục tiêu làm gia tăng khả năng đề kháng với độc tố kim loại nặng, giúp ổn định và nâng cao năng suất của cây trồng, sự hiểu biết đầy đủ về cơ chế cũng như các hệ thống đáp ứng của cây trồng với tác động của kim loại nặng là hết sức cần thiết. Nghiên cứu này được thực hiện để khảo sát những thay đổi về sinh lý, sinh hóa và phiên mã ở mầm lúa (*Oryza sativa* L. cv. IR64) trong điều kiện stress đồng (Cu). Kết

quả nghiên cứu cho thấy, sự kéo dài và trọng lượng tươi của rễ suy giảm trong khi hàm lượng Cu tích lũy trong rễ gia tăng theo sự tăng của nồng độ Cu xử lý, từ 2.5 đến 15 μM. Ngoài ra, Cu đã cảm ứng sự sản sinh gốc tự do (reactive oxygen species-ROS) nội sinh và cảm ứng các isoenzyme của enzyme superoxide dismutase (SOD) và catalase (CAT). Cơ chế phân tử của sự đáp ứng với độc tính của đồng (Cu) ở cây lúa được xác định thông qua phân tích sự biểu hiện của hệ gen khi xử lý với stress Cu bằng kỹ thuật phân tích microarray. Hơn thế, vai trò và định vị chức năng của các gen trong hệ gen còn được xác định bằng công cụ tin sinh học AgriGO và MapMan. Phân tích bản thể gen (Gene ontology) đã xác định được 1900 gen đáp ứng với stress Cu. Những gen này liên quan đến điều hoà sinh tổng hợp hormone, gốc tự do, con đường dẫn truyền tín hiệu tế bào, yếu tố điều hòa phiên mã, hoạt động chuyển vận, hệ thống đề kháng chống oxy hóa. Thông qua các hormone và ROS, Cu ức chế sự sinh trưởng của rễ lúa. Các hormone thực vật và ROS còn hoạt động như những phân tử tín hiệu trong các con đường truyền tín hiệu, đặc biệt trong hệ thống tín hiệu Ca, mitogen-activated protein kinase (MAPK) nhiều bậc và hệ thống các yếu tố điều hòa phiên mã trong đáp ứng với stress Cu. Cơ chế khử độc và bảo vệ tế bào trong stress Cu được xác định có liên quan đến hoạt động chuyển vận và hệ thống đề kháng chống oxy hóa. Như vậy, những kết quả của nghiên cứu này góp phần vào việc làm sáng tỏ cơ chế kháng đối với độc tố Cu ở rễ lúa.

***Từ khoá**: Độc tính đồng, gốc tự do, hormone thực vật, microarray, nhân tố điều hoà phiên mã, Oryza sativa, sự truyền tín hiệu*