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RESEARCH PAPER

Gene expression and sensitivity in response to copper stress in rice leaves*

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Abstract

Gene expression in response to Cu stress in rice leaves was quantified using DNA microarray (Agilent 22K Rice Oligo Microarray) and real-time PCR technology. Rice plants were grown in hydroponic solutions containing 0.3 (control), 10, 45, or 130 μM of $CuCl_2$, and Cu accumulation and photosynthesis inhibition were observed in leaves within 1 d of the start of treatment. Microarray analysis flagged 305 Cu-responsive genes, and their expression profile showed that a large proportion of general and defence stress response genes are up-regulated under excess Cu conditions, whereas photosynthesis and transport-related genes are down-regulated. The Cu sensitivity of each Cu-responsive gene was estimated by the median effective concentration value (*EC50*) and the range of fold-changes (*F*) under the highest (130 μM) Cu conditions ($|\log_2 F|_{130}$). Our results indicate that defence-related genes involved in phytoalexin and lignin biosynthesis were the most sensitive to Cu, and that plant management of abiotic and pathogen stresses has overlapping components, possibly including signal transduction.

Key words: Copper-sensitivity, DNA microarray, excess copper stress, gene expression, *Oryza sativa* L.

Introduction

Copper is an essential element for plants as a cofactor of enzymes such as plastocyanin, cytochrome *c*, and Cu/Zn-superoxide dismutase (Cu/Zn-SOD). Cu has a long history in agriculture as an antifungal agent, but in recent years it has been extensively released into the environment by human activities, such as industrial processes, pesticide application, and mining, that often cause environmental pollution. Exposure to excess Cu causes phytotoxicity by inhibiting key cellular processes, including photosynthesis and electron transport, lipid peroxidation, and disruption of protein functions due to Cu-binding to sulphhydryl groups (Sandmann and Böger, 1980; Yruela *et al*., 1993; Babu *et al*., 2001). Cu also induces the formation of reactive oxygen species (ROS) based on the Fenton or Haber–Weiss reactions (Halliwell and Gutteridge, 1989; Bartosz, 1997). A positive correlation between Cu exposure and the accumulation of hydroxy radicals has been reported in *Arabidopsis* (Drążkiewicz *et al*., 2004). However, plants have ROS scavenging systems that prevent or reduce cellular injury that can be caused by the generation of ROS in response to heavy metal stresses. Some ROS scavenging enzymes (e.g. SOD, CAT, APX) change their activities or transcription levels in response to excess Cu exposure (Luna *et al*., 1994; Weckx and Clijsters, 1996; Kurepa *et al*., 1997; Lombardi and Sebastiani, 2005).

Toxic concentrations of heavy metals can, in some cases, be reduced by chelation with metal ligands, or metal ions can be effluxed or sequestered, resulting in lower toxicity (Clemens, 2001; Hall, 2002). Metallothioneins and phytochelatins are well-known metal-binding peptides. Guo *et al*. (2003) reported that *Arabidopsis* metallothioneins play a role in Cu tolerance, homeostasis, and long-distance transport for sequestration.

Susceptibility to excess Cu stress varies with plant species. For instance, alfalfa and barley are highly tolerant to Cu stress, but rice and potato are less tolerant (Jones, 1998). In addition, rice is more susceptable to Cu toxicity

* Microarray data have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database under accession number GSE11021.

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than to other heavy metals, such as Ni, Co, and Zn (Chino, 1981). Although plant responses to heavy metal exposure have been widely investigated, it is still not completely understood how excess Cu affects the plant, nor how the plant copes with that stress at the gene expression level. Thus, a better understanding of how Cu stress affects gene expression in rice is important for providing an overall understanding of how higher plants adapt to heavy metal stress.

DNA microarrays are one of the most powerful tools for providing an overview of gene expression under various environmental conditions. Weber *et al*. (2006) examined transcriptome changes upon Cd^{2+} and Cu^{2+} exposure in roots of the Cd^{2+}-hypertolerant metallophyte *Arabidopsis halleri*. Keinänen *et al*. (2007) identified genes that are up-regulated by $CuSO_4$ exposure in a Cu-tolerant birch clone using macroarrays. The search for genes whose expression is modified by Cu stress has yielded a number of valuable tools that have been used to understand the Cu stress response. Completion of the rice genome sequence has made the comprehensive identification of Cu stress-responsive genes in this model monocot plant possible. The aim of this study is to identify genes which are affected directly or indirectly by toxic levels of Cu, some of which may be involved in ameliorating heavy metal, oxygen radical or other stress damage. Therefore, the effects of $CuCl_2$ doses on rice leaf gene expression were examined using an Agilent 22K Rice Oligo Microarray. Three hundred and five Cu-responsive genes were selected which were either up- or down-regulated depending on $CuCl_2$ dose, and the Cu sensitivity of the genes was analysed to determine what kind of functional genes and pathways might be critically involved in response to excess Cu.

Materials and methods

Plant culture

Rice plants (*Oryza sativa* L. cv. Nipponbare) were grown hydroponically (Kamachi *et al*., 1991) in an environment-controlled greenhouse with a photoperiod of 12 h light (25–28 °C) for 6–7 weeks. The basal nutrient solution was prepared as described by Kamachi *et al*. (1991) and the pH was adjusted to 5.5. Three rice plants were grown in each 500 ml plastic pot containing the nutrient solution, which was renewed once a week. Rice plants whose 8th leaf was fully expanded were used for experimental treatments.

Experimental design

Rice plants which had been grown as described above were treated with hydroponic solutions containing 10 μM, 45 μM, or 130 μM $CuCl_2$. Treatment with the standard rice hydroponic solution containing 0.3 μM Cu was performed simultaneously as a control. Gas exchange measurements were performed using the fully expanded 8th leaf 24–30 h after the start of treatment, after which the leaves were harvested for RNA extraction. In addition, 8th leaf blades, the remainder of the shoot, and roots were separately collected for examining Cu contents.

Gas exchange measurements

Gas exchange was measured using a CIRAS-1 portable system (PP-system, Hitchin, Herts, UK). Measurements were made at a leaf temperature of 28 °C, and a PPFD of 800 μmol quanta m^{-2} s^{-1} at the position of the leaf in the chamber. CO_2 and H_2O partial pressures of the air exiting from the chamber were maintained at 38 Pa and 2.3 kPa, respectively. Irradiance was provided by a halogen lamp attached to an exclusive light unit (PP-system). Gas exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981).

Measurement of Cu in rice tissues

For analyses of Cu concentrations in rice tissues, inductively coupled plasma mass spectrometry (ICP-MS) (Elan6100DRC; Perkin Elmer, Norwalk, CT, USA) was used. Rice plant tissues were dried for more than 3 d at 60 °C, followed by wet microwave digestion in 8 ml of concentrated HNO_3 using a microwave sample preparation system (MultiWave-3000, Perkin Elmer). The digested samples were brought up to a volume of 50 ml with Milli-Q water and filtered through 5B filter paper (Advantec, Tokyo, Japan). For ICP-MS analysis, a portion of the filtered samples of leaf blade, sheath, and root were diluted 5-, 100-, and 100-fold with Milli-Q water, respectively.

RNA extraction and synthesis of Cy3- and Cy5-labelled cRNA

Total RNA was extracted from three different leaf samples per treatment using an RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). Cy3- and Cy5-labelled cRNA was prepared from 400 ng of total RNA from rice leaves, using a Low RNA Input Linear Amplification Kit (Agilent Technologies, Inc., Palo Alto, CA, USA) and Cy3- and Cy5-CTP (Perkin Elmer). Labelled cRNA was purified with RNeasy mini spin columns (Qiagen).

Microarray experiment and data analysis

A 22K Rice Oligo Microarray kit (Agilent Technologies) was used for microarray analysis. One microgram of Cy3-labelled cRNA was mixed with the same amount of Cy5-labelled cRNA and used for subsequent hybridization. Hybridization was carried out for 17 h with rotation at 60 °C. After washing, slides were scanned using a GenePix 4000A scanner (Axon Instruments Inc., Foster City, CA, USA) with 550 V and 680 V of PMT voltage for Cy3 and Cy5 detection, respectively, and quantified by Microarray Suite 2.0 (IPLab Spectrum Software, Scanalytics, Fairfax, VA, USA). Subsequent analysis was performed using GeneSpring 7 software (Agilent Technologies).

Genes which were up- or down-regulated with increasing Cu exposure concentration were selected as candidate Cu-responsive genes. Signal intensity, amplitude of expression fluctuation, and standard error of the mean F (F=the ratio of normalized data between experiment and control) were also considered. First, Cu-responsive genes meeting the criteria were selected as follows: the average signal intensity of the control RNA in the nine experiments (10 μM-1, 2, 3; 45 μM-1, 2, 3; 130 μM-1, 2, 3) was within the range 5×10^3 to 1×10^7; the F of triplicate samples under the 130 μM (130 μM-1, 2, 3) treatment were all significantly higher or lower than 1 ($P < 0.01$); and standard errors divided by the mean F in each treatment (10, 45, and 130 μM) were all less than 1. Second, up-regulated Cu-responsive genes were selected which met three additional criteria: the F value in each treatment was 130 μM

>45 μM >10 μM; *F* was >2 in the 130 mM treatment; and *F* was >1 in both the 10 μM and 45 μM treatments. Third, down-regulated Cu-responsive genes were selected if they met the following additional criteria: *F* in each condition was 130 μM <45 μM <10 μM; *F* was <0.5 in the 130 μM treatment, but <1 in both the 10 μM and 45 μM treatments.

For estimating the Cu sensitivity of each Cu-responsive gene, median effective concentrations for *F* ($EC50_F$), and the amplitude of expression change with the 130 μM treatment ($|\log_2 F|_{130}$) were determined. $EC50_F$s were calculated by probit analysis (Finney, 1978).

Descriptions of each Cu-responsive gene were annotated according to the TIGR database (http://www.tigr.org/tdb/e2k1/osa1/). In addition, Cu-responsive genes were classified into rough functional categories based on the Gene Ontology Classification database (http://www.geneontology.org/).

Quantitative real-time PCR

Total RNA was prepared using an RNeasy® plant Mini Kit (Qiagen) with RNase-free DNase I (Qiagen). Primers for each gene were designed using OLIGO Primer Analysis Software (Takara Bio Inc., Otsu, Japan). Primer sequences for the genes examined are summarized in Table 1. Accumulation levels of the target transcripts were analysed by real-time PCR with an ABIPRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by monitoring amplification with SYBR-Green I dye (Applied Biosystems) as described in Takei *et al.* (2004).

Statistical analyses

Data were analysed by Dunnett's multiple comparison tests using SPSS software version 14.0J (SPSS Japan Inc., Tokyo, Japan).

Results and discussion

Effect of Cu treatment on Cu accumulation and photosynthesis in leaves

Application of $CuCl_2$ to rice roots caused significant increases in Cu concentrations in the leaf blades, and shoots, as well as in the roots (Fig. 1). These results demonstrated that some of the Cu in hydroponic solution was absorbed by the roots and transported to the leaves. Photosynthetic and transpiration rates were significantly affected at 130 μM of $CuCl_2$ at ambient CO_2 levels (Fig. 2). The results confirm that Cu exposure above 45 μM is toxic to rice leaves. The photosynthetic decline at 130 μM (Fig. 2) was accompanied by a decrease in both the intercellular CO_2 concentration and stomatal conductance (data not shown), suggesting that intercellular CO_2 diffusion was inhibited as a result of stomatal

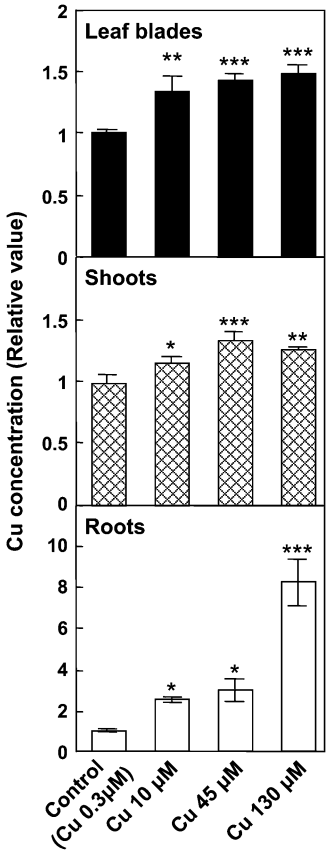

Fig. 1. The relative concentrations of Cu in the leaf blades, shoots, and roots of rice. Values are means ±SD of three individual samples. Actual Cu concentrations in the control leaf blades, shoots, and roots are 139±3, 165±13, and 2180±90 μg g^{-1} dry weight, respectively. The statistical significance was determined by Dunnett's multiple comparison tests. Asterisks indicate a significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 1. *List of primers used for quantitative real-time PCR*

Genes	Forward primers	Reverse primers
AK060724	5′-GCCGTTTGGTTTATAGTG-3′	5′-CCAAAATACAGTTTAGCGAC-3′
AK062653	5′-CAAACTGCTCCTGCGGAAAG-3′	5′-CACACCCAGCACGACGG-3′
AK099241	5′-CCTCTTCACGTCGGACCAC-3′	5′-ACCATGGCCTTCACGAACTT-3′
AK058896	5′-CCAGCGTGAACTAATCTG-3′	5′-CAAGATACAAAGCGTGAGAC-3′
AK101836	5′-TGGCCGTGTTGGAGCAATAC-3′	5′-CCAAAGCTTCTCGGAATGGG-3′
AK070467	5′-ACAGCGGACGACACCACGAC-3′	5′-CGGCAGCCTCACGATGTTG-3′
AK062796	5′-ACGAGCTACCAGTACCACTA-3′	5′-CGGCAACATGACATACAT-3′
AK058551	5′-AGTGGCATTGTTACCGTGAT-3′	5′-CGCCTGGTGCTCGTC-3′
AK060904	5′-TGCTGGCTTTTGTGGGTTTC-3′	5′-CGTGCCAAGCTCAAGGGTAG-3′
AK065381	5′-CGATTTGGCGTGACGTGT-3′	5′-AATGCGCCACAAGATACCTG-3′
AK067353	5′-CTGTTGATCCAGCGTTCTAC-3′	5′-TGAACCCGACGATAGCA-3′
AK107472	5′-CGGTCGCAGGTGACGCT-3′	5′-TGATGAGGAGGGCGAACTTG-3′

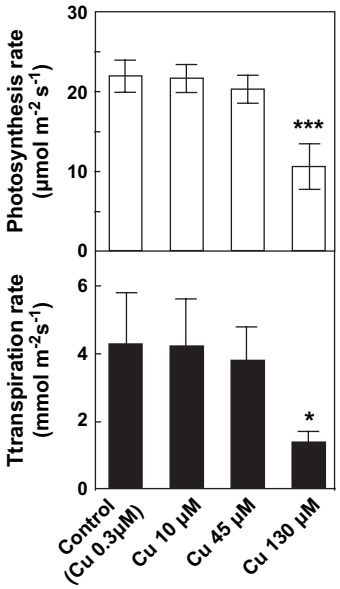

Fig. 2. Photosynthetic and transpiration rates after $CuCl_2$ treatment. Values are the means ±SD of three individual leaves. The statistical significance was determined by Dunnett's multiple comparison tests. Asterisks indicate a significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

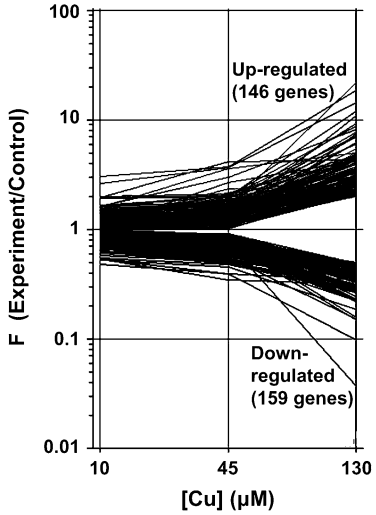

Fig. 3. Expression profiles of Cu-responsive genes under excess Cu conditions.

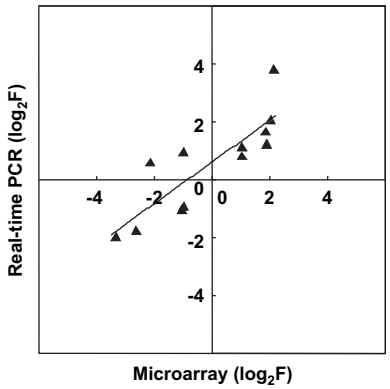

Fig. 4. Confirmation of microarray signal ratios by real-time PCR. Real-time PCR analysis of 12 genes selected from Cu-responsive genes was performed with RNA extracted from rice leaves under control or 130 μM Cu treatment: $y=0.718x + 0.605$, $r^2=0.717$.

closure. Compared with tissue Cu concentration (Fig. 1), the profile of photosynthetic activity under toxic conditions was consistent with root Cu content (Figs 1, 2). Root-to-shoot stress signalling via chemical components has been widely reported (e.g. ABA, Davies and Gowing, 1999; Sauter *et al*., 2001). ABA and other compounds may thus provide a mechanism by which root stress induced by excess Cu affects leaf photosynthetic activity by modulating stomatal apertures.

Selection of Cu-responsive genes with DNA microarray analysis

To gain insight into how excess Cu damages cellular processes in rice, a DNA microarray analysis was performed with RNA extracted from $CuCl_2$-treated leaves. 146 genes were up-regulated and 159 were down-regulated in a dose-response manner (Fig. 3).

Verification of microarray results by real-time PCR

To verify the microarray results, real-time PCR was performed on 12 genes randomly selected from the Cu-responsive genes using the same RNA samples as were used in the microarray hybridization. There was a positive correlation between F from the 130 μM treatment and real-time PCR amplification ($r^2=0.717$, Fig. 4), indicating that the microarray data are valid with respect to Cu dose response.

Cu-responsive genes

The Cu-responsive genes showed some notable features, and both up- and down-regulated Cu-responsive genes are in each functional category (Fig. 5; a complete list is given in Supplementary Table S1 at *JXB* online). The number of defence and stress response genes greatly outnumbered the down-regulated genes (Fig. 5). Most of the defence-related genes are involved in the phenylpropanoid pathway for flavonoid, phytoalexin, and lignin biosynthesis (Table 2). Flavonoid accumulation in response to UV-B (Reddy *et al*., 1994), cold (Christie *et al*., 1994), and drought stresses (Balakumar *et al*., 1993) were previously reported. Flavonoids function as scavengers of ROS, and also prevent ROS formation by chelating metals (Scalbert, 1991; Ferrali *et al*., 1997; Heim *et al*., 2002). Phytoalexin and lignin biosynthesis are key responses to pathogen attack. $CuCl_2$ treatment increases production of the rice phytoalexins sakuranetin and momilactone A

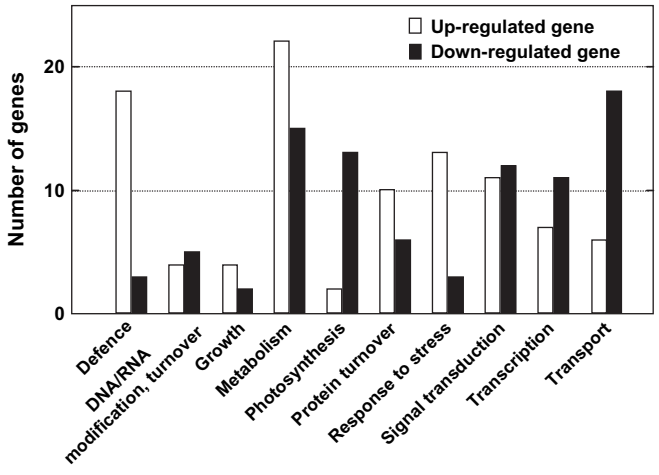

Fig. 5. Functional classification of Cu-responsive genes. Up-regulated genes are represented by empty bars and down-regulated genes by filled bars.

(Rakwal *et al*., 1996). Our observation of up-regulated defence genes in response to Cu confirms its role as an abiotic elicitor (Graham, 1980).

Plants synthesize metal-binding polypeptides, such as metallothionein and phytochelatin, whose apparent function is to maintain cellular metal concentration homeostasis by sequestering and detoxifying excess metal ions. In this study, two genes encoding metallothionein-like proteins were up- and down-regulated by excess Cu (AK062653 and AK062796, respectively; Table 2). At present, the physiological meaning of the differential response of the two genes to excess Cu is not clear. The gene products could be different in their ligand affinity or specificity, and thus functionally specialized to respond to different levels of Cu stress. Cu homeostasis may also be regulated by Cu-containing proteins which act as Cu sinks under excess Cu conditions. Abdel-Ghany *et al.* (2005) reported that $CuSO_4$ treatment enhanced the production of Cu/Zn-SOD and plastocyanin proteins in *Arabidopsis*. In this study, the set of Cu-responsive genes contained monocopper oxidase-like protein and L-ascorbate oxidase, which were both up-regulated (see Supplementary Table S1 at *JXB* online) by excess Cu treatment.

Our results also showed the up-regulation of genes which are known to respond to abiotic stresses such as drought, salt or heat shock (Table 2), suggesting a partial overlap of the signal transduction pathways coping with metal exposure, drought, heat shock or salinity. The dehydration-responsive element (DRE) is involved in response to drought, salt, and cold stresses in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994), and over-expression of the *trans*-acting factor DREB confers tolerance to these stresses in transgenic *Arabidopsis* (Nakashima and Yamaguchi-Shinozaki, 2006). Our results imply that DREB genes may also play a role in Cu tolerance in rice leaves. Because a gene encoding ABA/WDS-induced protein was also up-regulated by excess Cu, metal ions like Cu may also affect the ABA-dependent signal transduction pathway.

The number of photosynthesis and transport-related genes, on the other hand, greatly outnumbered the up-regulated genes (Fig. 5). Generally, photosynthesis-related genes are induced by light and are influenced by circadian rhythms. However, they were often down-regulated under abiotic stresses such as low temperatures (Hahn and Walbot, 1989), heat and/or drought (Rizhsky *et al*., 2002), salinity (Allakhverdiev *et al*., 2002; Kore-eda *et al*., 2004), excess light (Teramoto *et al*., 2002), or by signal transduction factors, including ROS (Vandenabeele *et al*., 2003; op den Camp *et al*., 2003), jasmonate (JA) (Reinbothe *et al*., 1993) and hexose (Sheen, 1994). These results demonstrate that excess Cu also represses the photosynthetic system at the genetic level (Fig. 5; Table 2) as well as at the physiological level (Fig. 2). Schiavon *et al*. (2007) reported that excess Cu decreases transcript levels of plastocyanin in *Arabidopsis*, an observation which is supported by our results.

Cu treatment also repressed transport-related genes (Fig. 4). Transport systems are indispensable for keeping metal concentrations in equilibrium in plant species. Metal homeostasis is maintained by chelating, effluxing or sequestering the potentially toxic ions (Clemens, 2001; Hall, 2002). Sancenón *et al*. (2003) identified a five-member family of Cu transporters (CORT1 to CORT5) in *Arabidopsis*. In addition, some of the P_{1B}-type heavy metal ATPases (HMAs) have a role in Cu transport in rice (Williams and Mills, 2005; Lee *et al*., 2007). Excess $CuSO_4$ decreased *Arabidopsis* transcription of *PAA1* and *PAA2* (Schiavon *et al*., 2007), both of which function as Cu transporters for Cu delivery in chloroplasts (Abdel-Ghany *et al*., 2005). In our results, the genes encoding amino acid and peptide transporters were conspicuously down-regulated (Table 2). Wintz *et al*. (2003) reported that AtOPT3, a potential oligopeptide transporter of *Arabidopsis*, is involved in Cu transport. It is, however, still unclear whether the amino acid and peptide transporter genes among the Cu-responsive genes are involved in Cu homeostasis.

Sensitivity of Cu-responsive genes

Each of the Cu-responsive genes responds distinctively to Cu concentration, and the fluctuation range of Cu-responsive expression also varied under the 130 μM Cu treatment conditions. These variations can be attributed to 'Cu-sensitivity', which can be calculated from the median effective concentration values ($EC50_F$) and fluctuation of expression in the highest Cu concentration ($|\log_2 F|_{130}$) (see Supplementary Table S1 at *JXB* online). $EC50_F$s varied from 4.86 μM to 230 μM with a mean value of 97.9 μM. $|\log_2 F|_{130}$ ranged from 1.00 to 4.75, with a mean

Table 2. *Expression profiles of Cu-responsive genes under excess Cu treatment conditions (10, 45, and 130 μM of $CuCl_2$)*

Values are means of fold-change (*F*) calculated from triplicate data of different leaves. The descriptions of each gene were annotated according to the TIGR database (http://www.tigr.org/tdb/e2k1/osa1/), and were classified into rough functional categories based on the Gene Ontology Classification database (http://www.geneontology.org/).

Probe ID	Full length cDNA	Locus_ID	Description	*F* (experiment/control)		
				Cu-exposure (μM)		
				10	45	130
Defence (up-regulated)						
A_71_P105870	AK060724	LOC_Os02g41630	Phenylalanine ammonia-lyase	1.02	1.70	2.01
A_71_P105867	AK068993	LOC_Os02g41680	Phenylalanine ammonia-lyase	1.01	1.42	5.01
A_71_P105871	AK102817	LOC_Os02g41630	Phenylalanine ammonia-lyase	1.19	1.82	2.26
A_71_P113211	AK067801	LOC_Os04g43800	Phenylalanine ammonia-lyase	1.34	1.78	4.61
A_71_P126860	AK099443	LOC_Os11g02440	Chalcone-flavonone isomerase	1.38	1.89	2.19
A_71_P104485	AK070746	LOC_Os02g08420	Dihydroflavonol-4-reductase	1.07	1.32	2.23
A_71_P119630	AK065515	LOC_Os08g38910	Caffeoyl-CoA *O*-methyltransferase 2	1.19	2.12	3.18
A_71_P115157	AK104994	LOC_Os05g25640	*Trans*-cinnamate 4-mono-oxygenase	1.21	1.42	2.43
A_71_P123533	AK069308	LOC_Os10g02880	*O*-methyltransferase ZRP4	1.17	1.19	4.27
A_71_P122641	AK072740	LOC_Os09g17560	*O*-methyltransferase ZRP4	1.03	1.59	21.92
A_71_P111602	AK065090	LOC_Os04g59190	Peroxidase 2 precursor	1.38	1.79	7.16
A_71_P113417	AK106200	LOC_Os05g04500	Peroxidase 63 precursor	1.62	2.97	8.13
A_71_P117837	AK072862	LOC_Os07g47990	Peroxidase 2 precursor	1.34	1.37	3.50
A_71_P103756	AK099241	LOC_Os01g22370	Peroxidase 1 precursor	1.22	1.48	4.33
A_71_P120304	AK069503	LOC_Os08g02110	Peroxidase 47 precursor	1.20	1.31	3.30
A_71_P117839	AK073202	LOC_Os07g48020	Peroxidase 2 precursor	1.18	1.69	9.18
A_71_P103305	AK107822	LOC_Os01g72170	Glutathione *S*-transferase	1.21	1.24	2.07
A_71_P125246	AK062653	LOC_Os11g47809	Metallothionein-like protein 1	1.37	1.48	4.06
Defence (down-regulated)						
A_71_P103051	AK103129	LOC_Os01g53330	Anthocyanidin 5,3-*O*-glucosyltransferase	0.80	0.58	0.29
A_71_P119739	AK067868	LOC_Os08g07880	Phosphopantothenate-cysteine ligase	0.61	0.48	0.43
A_71_P103162	AK062796	LOC_Os01g74300	Metallothionein-like protein type 2	0.92	0.82	0.16
Response to stress (up-regulated)						
A_71_P112980	AK100788	LOC_Os04g34600	ABA/WDS induced protein	1.47	1.88	2.17
A_71_P115472	AK107775	LOC_Os06g07030	Dehydration responsive element binding protein	1.17	1.64	4.35
A_71_P126985	AK062422	LOC_Os09g35010	Dehydration-responsive element-binding protein 1B	1.24	1.86	2.27
A_71_P118699	AK106022	LOC_Os07g44250	Disease resistance response protein 206	1.08	1.40	3.35
A_71_P111503	AK071013	LOC_Os04g41680	Endochitinase A precursor	1.09	1.14	2.45
A_71_P114512	AK060312	LOC_Os05g42230	ER6 protein	1.03	1.16	2.37
A_71_P124122	AK065000	LOC_Os10g22520	Glucan 1,3-β-glucosidase precursor	1.14	1.40	4.57
A_71_P121735	AK061896	LOC_Os09g30418	Heat shock protein 81-3	1.37	1.78	2.39
A_71_P126129	AK066682	LOC_Os12g14440	Jasmonate-induced protein	1.63	1.99	11.92
A_71_P103425	AK062520	LOC_Os01g24710	Salt stress-induced protein	1.16	1.28	6.20
A_71_P114369	AK070138	LOC_Os05g28740	Universal stress protein	1.54	1.55	2.54
A_71_P114262	AK065866	LOC_Os05g15770	Xylanase inhibitor protein 2 precursor	2.01	2.09	4.34
A_71_P114261	AK062114	LOC_Os05g15770	Xylanase inhibitor protein 2 precursor	1.66	2.01	4.20
Response to stress (down-regulated)						
A_71_P117292	AK099477	LOC_Os06g47800	Disease resistance protein RGA3	0.77	0.61	0.27
A_71_P118794	AK065027	LOC_Os07g01630	Disease resistance response protein 206	0.95	0.77	0.49
A_71_P122593	AK060664	LOC_Os09g37600	*Erwinia*-induced protein 1	0.94	0.76	0.43
Photosynthesis (up-regulated)						
A_71_P114297	AK100910	LOC_Os05g50380	Glucose-1-phosphate adenylyltransferase large subunit, chloroplast precursor	1.39	1.47	3.93
A_71_P116411	AK101836	LOC_Os06g49110	Δ-Aminolevulinic acid dehydratase, chloroplast precursor	1.39	1.73	2.03
Photosynthesis (down-regulated)						
A_71_P105099	AK062994	LOC_Os02g51470	ATP synthase delta chain, chloroplast precursor	0.96	0.87	0.45
A_71_P115841	AK060904	LOC_Os06g21590	Chlorophyll *a-b* binding protein 6A, chloroplast precursor	0.81	0.71	0.48
A_71_P125058	AK061295	LOC_Os11g13890	Chlorophyll *a-b* binding protein M9, chloroplast precursor	0.70	0.63	0.37
A_71_P118301	AK109399	LOC_Os07g37550	Chlorophyll *a-b* binding protein of LHCII type III, chloroplast precursor	0.69	0.58	0.40

Table 2. *Continued*

Probe ID	Full length cDNA	Locus_ID	Description	*F* (experiment/control)		
				Cu-exposure (μM)		
				10	45	130
A_71_P121584	AK109203	LOC_Os09g32620	Chloroplastic quinone-oxidoreductase	0.76	0.73	0.48
A_71_P101901	AK066307	LOC_Os12g10604	Cytochrome *b*/b6/petB family protein	0.61	0.54	0.32
A_71_P126393	AK059037	LOC_Os12g08770	Photosystem I reaction centre subunit N, chloroplast precursor	0.73	0.67	0.40
A_71_P114565	AK066345	LOC_Os05g43310	Photosystem II reaction centre W protein, chloroplast precursor	0.76	0.76	0.40
A_71_P108389	AK058858	LOC_Os03g55720	Plastoquinol-plastocyanin reductase	0.94	0.73	0.36
A_71_P117917	AK069170	LOC_Os07g36080	Oxygen-evolving enhancer protein 3-1, chloroplast precursor	0.93	0.80	0.30
A_71_P117916	AK058793	LOC_Os07g36080	Oxygen-evolving enhancer protein 3-1, chloroplast precursor	0.70	0.61	0.29
A_71_P120166	AK058551	LOC_Os08g25734	Glucose-1-phosphate adenylyltransferase small subunit, chloroplast precursor	0.86	0.84	0.47
A_71_P124217	AK110705	LOC_Os06g39730	Ribulose bisphosphate carboxylase large chain, catalytic domain containing protein	0.76	0.73	0.50
Transport (up-regulated)						
A_71_P105105	AK108711	LOC_Os02g34580	Ammonium transporter 2	1.06	1.35	2.47
A_71_P119764	AK065217	LOC_Os08g03350	LHT1	1.19	1.36	2.46
A_71_P117869	AK105311	LOC_Os07g33780	PDR-like ABC transporter	1.09	1.17	2.61
A_71_P127448	AK108393	LOC_Os05g27010	Peptide transporter PTR2	1.17	1.33	2.22
A_71_P103242	AK063835	LOC_Os01g45640	Tat pathway signal sequence family protein	1.02	1.04	7.43
A_71_P100920	AK103784	LOC_Os01g31980	Transparent testa 12 protein	1.10	1.43	2.56
Transport (down-regulated)						
A_71_P106018	AK100650	LOC_Os02g44980	Amino acid transport protein	0.99	0.76	0.45
A_71_P116013	AK107472	LOC_Os06g12320	Amino acid/polyamine transporter II	0.80	0.68	0.22
A_71_P115705	AK072617	LOC_Os06g03700	Oligopeptide transporter 9	0.87	0.84	0.24
A_71_P104541	AK065840	LOC_Os02g46460	Peptide transporter PTR2	0.74	0.57	0.29
A_71_P122896	AK066937	LOC_Os10g42900	Peptide transporter PTR2	0.77	0.73	0.49
A_71_P114702	AK070558	LOC_Os05g34010	Peptide transporter PTR2	0.90	0.77	0.48
A_71_P102553	AK066793	LOC_Os01g50616	Phosphatidylinositol transporter/ transporter	0.75	0.68	0.44
A_71_P119359	AK066067	LOC_Os07g46780	Tyrosine-specific transport protein	0.67	0.64	0.41
A_71_P123937	AK111957	LOC_Os10g38910	ABC-type Co^{2+} transport system, permease component	0.91	0.73	0.44
A_71_P115940	AK105826	LOC_Os06g30730	ATPase, coupled to transmembrane movement of substances	0.99	0.75	0.48
A_71_P100064	AK065048	LOC_Os01g17214	Carbohydrate transporter/sugar transporter/transporter	0.86	0.63	0.22
A_71_P123327	AK071193	LOC_Os10g35140	Permeases of the drug/metabolite transporter	0.82	0.68	0.50
A_71_P104342	AK071338	LOC_Os02g56510	Phosphate transporter 1	0.59	0.54	0.42
A_71_P117558	AK067110	LOC_Os06g29790	Phosphate transporter 1	0.55	0.48	0.27
A_71_P112325	AK070018	LOC_Os04g38026	Sugar transport protein 5	0.74	0.63	0.37
A_71_P108667	AK067353	LOC_Os03g09930	Sulphate transporter 2.1	0.53	0.39	0.10
A_71_P112060	AK072809	LOC_Os04g55800	Sulphate transporter 3.3	0.90	0.84	0.49
A_71_P116372	AK063490	LOC_Os06g36450	Transporter like protein	0.92	0.66	0.39

of 1.52 (Fig. 6). Compared with the average value of all Cu-responsive genes, the $EC50_F$ and $|\log_2 F|_{130}$ of defence-related genes are significantly lower and higher than others, respectively, at $P < 0.05$ (Fig. 6), indicating that the defence-related genes are highly Cu-sensitive to lower concentrations of Cu, and that their expression varies greatly with exposure to Cu.

Within the defence-related genes, phytoalexin and lignin biosynthesis pathway genes (phenylalanine ammonia-lyase, caffeoyl-CoA *O*-methyltransferase, *trans*-cinnamate 4-mono-oxygenase, *O*-methyltransferase ZRP4, peroxidase) were particularly sensitive (Table 3). Although one gene encoding a metallothionein-like protein was up-regulated, and the other was down-regulated, their Cu-sensitivities were both higher than many other defence-related genes (Table 3; see Supplementary Table S1 at *JXB* online). Thus, sequestering mechanisms for heavy metals are also acutely responsive to Cu.

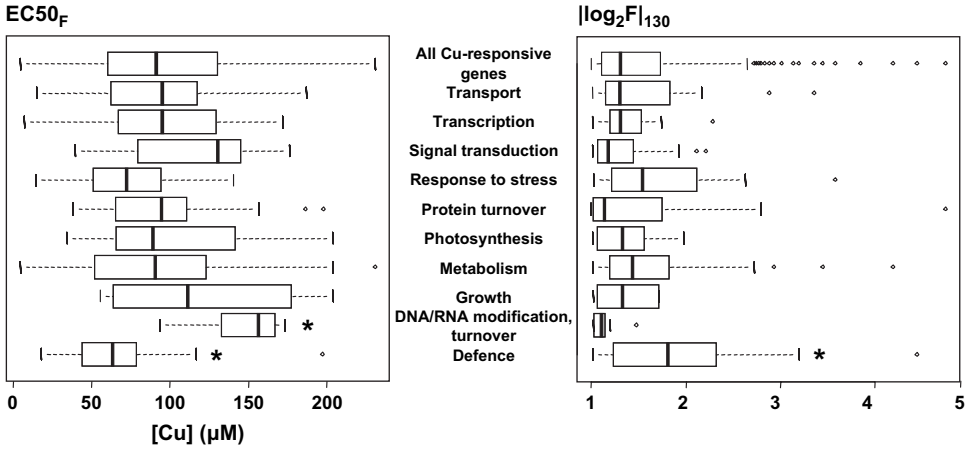

Fig. 6. Boxplots of $EC50_F$ (left panel) and $|\log_2 F|_{130}$ (right panel) in each functional category. The empty box indicates the interquartile (25–75%) range. Bars across the boxes represent the median value. Whiskers below and above the box indicate the range of values within 1.5 times the value of the upper or lower edge of the box. Circles represent outliers. The statistical significance of differences was tested by Dunnett's multiple comparison tests. Asterisks indicate significant differences with average values of all Cu-responsive genes ($*P < 0.05$).

Table 3. *Cu-sensitivity of defence-related genes and some expected genes involved in pathogen resistance mechanisms among the up-regulated Cu-responsive genes*

Probe ID	Full length cDNA	Locus_ID	Description	$EC50_F$	$\|\log_2 F\|_{130}$
Genes categorized into 'defence'					
A_71_P105870	AK060724	LOC_Os02g41630	Phenylalanine ammonia-lyase	96.84	1.01
A_71_P105867	AK068993	LOC_Os02g41680	Phenylalanine ammonia-lyase	66.59	2.32
A_71_P105871	AK102817	LOC_Os02g41630	Phenylalanine ammonia-lyase	79.62	1.17
A_71_P113211	AK067801	LOC_Os04g43800	Phenylalanine ammonia-lyase	39.45	2.20
A_71_P126860	AK099443	LOC_Os11g02440	Chalcone-flavonone isomerase	75.43	1.13
A_71_P104485	AK070746	LOC_Os02g08420	Dihydroflavonol-4-reductase	116.44	1.16
A_71_P119630	AK065515	LOC_Os08g38910	Caffeoyl-CoA *O*-methyltransferase 2	50.11	1.67
A_71_P115157	AK104994	LOC_Os05g25640	*Trans*-cinnamate 4-mono-oxygenase	100.41	1.28
A_71_P123533	AK069308	LOC_Os10g02880	*O*-methyltransferase ZRP4	77.19	2.09
A_71_P122641	AK072740	LOC_Os09g17560	*O*-methyltransferase ZRP4	44.06	4.45
A_71_P111602	AK065090	LOC_Os04g59190	Peroxidase 2 precursor	32.57	2.84
A_71_P113417	AK106200	LOC_Os05g04500	Peroxidase 63 precursor	18.11	3.02
A_71_P117837	AK072862	LOC_Os07g47990	Peroxidase 2 precursor	65.73	1.81
A_71_P103756	AK099241	LOC_Os01g22370	Peroxidase 1 precursor	54.26	2.12
A_71_P120304	AK069503	LOC_Os08g02110	Peroxidase 47 precursor	78.35	1.72
A_71_P117839	AK073202	LOC_Os07g48020	Peroxidase 2 precursor	39.26	3.20
A_71_P103305	AK107822	LOC_Os01g72170	Glutathione *S*-transferase	197.47	1.05
A_71_P125246	AK062653	LOC_Os11g47809	Metallothionein-like protein 1	50.71	2.02
Expected genes involved in pathogen resistance mechanism					
A_71_P126555	AK066737	LOC_Os12g37260	Lipoxygenase 2.1, chloroplast precursor	4.86	4.20
A_71_P107746	AK061537	LOC_Os03g57970	Lipid transfer protein	17.29	2.32
A_71_P125078	AK061288	LOC_Os11g24070	Non-specific lipid-transfer protein 1 precursor	76.80	1.42
A_71_P125472	AK058896	LOC_Os11g02369	Non-specific lipid-transfer protein 2 precursor	32.98	1.84
A_71_P115043	AK062463	LOC_Os05g47700	Non-specific lipid-transfer protein precursor	25.88	2.24
A_71_P101377	AK067257	LOC_Os01g03340	Bowman–Birk-type bran trypsin inhibitor precursor	38.00	2.80
A_71_P101369	AK070467	LOC_Os01g03310	Bowman–Birk-type bran trypsin inhibitor precursor	62.48	1.89
A_71_P111503	AK071013	LOC_Os04g41680	Endochitinase A precursor	140.75	1.29
A_71_P124122	AK065000	LOC_Os10g22520	Glucan 1,3-β-glucosidase precursor	60.04	2.19
A_71_P126129	AK066682	LOC_Os12g14440	Jasmonate-induced protein	21.62	3.58
A_71_P114262	AK065866	LOC_Os05g15770	Xylanase inhibitor protein 2 precursor	14.42	2.12
A_71_P114261	AK062114	LOC_Os05g15770	Xylanase inhibitor protein 2 precursor	25.71	2.07

In gene categories other than defence-related, Cu-sensitivity did not differ significantly from the average of all Cu-responsive genes, but DNA, RNA modification, and turnover category genes had relatively lower Cu sensitivity.

Sensitivity of defence mechanisms to pathogens and their roles under excess Cu stress

Our results showed that defence-related genes are strikingly up-regulated, with the highest Cu-sensitivity.

Considering that Cu is an abiotic elicitor that induces resistance against pathogen attack (Graham, 1980), this result is understandable. According to van Loon and van Strien (1999), there are 14 families of PR proteins (PR-1–14), including β-1,3-glucanase, chitinase, peroxidase, proteinase-inhibitor, and lipid-transfer protein. High Cu sensitivity was also evident in genes encoding glucan β-1,3-glucosidase (β-1,3-glucanase), Bowman–Birk-type bran trypsin inhibitor, lipid-transfer protein, and xylanase inhibitor (Table 3). Furthermore, the sensitivity of JA-induced protein and chloroplast-located lipoxygenase were extraordinarily high (Table 3). Thus, the responses of general defence mechanism genes to Cu treatment suggest either some role in handling Cu stress, or that signal transduction is shared by the stress-response systems. In analysing the Cu-tolerant birch, Keinänen *et al*. (2007) isolated genes which were suggested to contribute to Cu tolerance mechanisms, including genes encoding HR-induced protein, chitinase, and lipoxygenase. This indicated the involvement of disease defence mechanisms in Cu tolerance.

Concluding remarks

Genome-wide analysis using DNA microarray technology demonstrated the broad response of rice genes to excess Cu. Our results suggest that Cu treatment particularly affected genes involved in defence, various abiotic stresses, photosynthesis, and transport. Further analysis demonstrated the range of defence-related genes for Cu-sensitivity, which suggests one aspect of the Cu-responsive mechanism, and that the defence response has an essential role in the stress response to excess Cu treatment. Defence-related genes could thus be effective targets for increasing tolerance to Cu. Alternatively, the role of Cu as an antifungal agent may act in part by inducing defence-response genes, as well as by inhibiting the pathogen.

Recently, gene expression profiles have been used as indicators of various kinds of stressors, such as environmental pollutants (Lettieri, 2006). The potential use of Cu-responsive genes as an indicator of environmental Cu-pollution was reported previously (Sudo *et al*., 2006). This study suggests the additional potential of using defence-related genes as biomarkers for very small amounts of Cu-pollution because of their acute sensitivity.

In this study, the focus was on analysing expression profiles in leaves 1 d after inducing Cu stress. Thus, early events, which are indicative of a direct response to some systemic signal that is expressed *de novo*, or triggered in roots in response to the increase of heavy metal ion concentrations, or to the direct effects of leaf intracellular concentrations, might have been overlooked. Further analysis, including a time-course covering this earlier period, could provide us with information which complements our new understanding of the gene regulatory events that occur in the 1 d timeframe for adaptation to Cu stress.

Supplementary data

Supplementary data for this article are available at *JXB* online.

Table S1. Expression profiles of all Cu-responsive genes grown with 10, 45, or 130 μM of $CuCl_2$.

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