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

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Received 7 April 2008; Revised 24 June 2008; Accepted 2 July 2008

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 CuCl2, 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 (|log2F|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 susceptible to Cu toxicity...
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-response 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 \(\mu\)M, 45 \(\mu\)M, or 130 \(\mu\)M CuCl\(_2\). Treatment with the standard rice hydroponic solution containing 0.3 \(\mu\)M 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 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) at the position of the leaf in the chamber. CO\(_2\) and H\(_2\)O 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 \(^\text{TM}\) 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 \(\mu\)M-1, 2, 3; 45 \(\mu\)M-1, 2, 3; 130 \(\mu\)M-1, 2, 3) was within the range \(5 \times 10^7\) to \(1 \times 10^8\); the \(F\) of triplicate samples under the 130 \(\mu\)M (130 \(\mu\)M-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 \(\mu\)M) 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 \(\mu\)M.
>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 \( F \) (EC50s) and the amplitude of expression change with the 130 μM treatment (|log₂F|130) were determined. EC50s 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 ABI Prism 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 CuCl₂ 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 CuCl₂ at ambient CO₂ 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 CO₂ concentration and stomatal conductance (data not shown), suggesting that intercellular CO₂ diffusion was inhibited as a result of stomatal

![Fig. 1.](image_url)
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 CuCl₂-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 ($\text{r}^2=0.717$, 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. CuCl₂ treatment increases production of the rice phytoalexins sakuranetin and momilactone A.
(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 CuSO4 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 (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/

\[ \text{Sensitivity of Cu-responsive genes} \]

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 (EC50s) and fluctuation of expression in the highest Cu concentration (llog2F130) (Supplementary Table S1 at JXB online). EC50s varied from 4.86 μM to 230 μM with a mean value of 97.9 μM. llog2F130 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 \(\mu M\) 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 (\(\mu M\)) |
|----------|------------------|----------|-------------|---------------------------|-------------------------|
| A_71_P105870 | AK060724 | LOC_Os02g41630 | Phenylalanine ammonia-lyase | 1.02 1.70 2.01 | 10 45 130 |
| A_71_P105867 | AK068993 | LOC_Os02g41680 | Phenylalanine ammonia-lyase | 1.01 1.42 5.01 | 10 45 130 |
| A_71_P105871 | AK102817 | LOC_Os02g41630 | Phenylalanine ammonia-lyase | 1.19 1.82 2.26 | 10 45 130 |
| A_71_P113211 | AK067801 | LOC_Os04g43800 | Phenylalanine ammonia-lyase | 1.34 1.78 4.61 | 10 45 130 |
| A_71_P126860 | AK099443 | LOC_Os11g02440 | Chalcone-flavonone isomerase | 1.38 1.89 2.19 | 10 45 130 |
| A_71_P104485 | AK070746 | LOC_Os02g08420 | Dihydroflavonol-4-reductase | 1.07 1.32 2.23 | 10 45 130 |
| A_71_P119630 | AK065515 | LOC_Os08g38910 | Caffeoyl-CoA O-methyltransferase 2 | 1.19 2.12 3.18 | 10 45 130 |
| A_71_P115157 | AK104994 | LOC_Os05g04500 | Trans-cinnamate 4-mono-oxygenase | 1.21 1.42 2.43 | 10 45 130 |
| A_71_P123533 | AK069301 | LOC_Os04g43800 | Phenylalanine ammonia-lyase | 1.34 1.78 4.61 | 10 45 130 |
| A_71_P119630 | AK065515 | LOC_Os08g38910 | Caffeoyl-CoA O-methyltransferase 2 | 1.19 2.12 3.18 | 10 45 130 |
| A_71_P115157 | AK104994 | LOC_Os05g04500 | Trans-cinnamate 4-mono-oxygenase | 1.21 1.42 2.43 | 10 45 130 |
| A_71_P119630 | AK065515 | LOC_Os08g38910 | Caffeoyl-CoA O-methyltransferase 2 | 1.19 2.12 3.18 | 10 45 130 |
| A_71_P115157 | AK104994 | LOC_Os05g04500 | Trans-cinnamate 4-mono-oxygenase | 1.21 1.42 2.43 | 10 45 130 |
| A_71_P119630 | AK065515 | LOC_Os08g38910 | Caffeoyl-CoA O-methyltransferase 2 | 1.19 2.12 3.18 | 10 45 130 |

Sudo et al.
of 1.52 (Fig. 6). Compared with the average value of all Cu-responsive genes, the EC50F and |log2F|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.
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-glucanase (β-1,3-glucanase), Bowman–Birk-type 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 CuCl₂.

Acknowledgements

We thank N Makita for her assistance to register our microarray data. This work was supported by a grant for a Leading Project (a project to design sustainable management and recycling systems of biomass, general and industrial wastes) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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