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ORIGINAL ARTICLE

Cu(II)-induced molecular and physiological responses in the brown-rot basidiomycete *Polyporales* sp. KUC9061

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Keywords
brown-rot fungus, copper, differentially expressed genes, GeneFishing, oxalic acid, real-time PCR.

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Abstract

Aim: A potentially safe disposal method for copper-containing waste wood is bioremediation using brown-rot fungi. However, the mechanisms regulating brown-rot fungi copper tolerance are poorly understood. The objective of this study was to better understand the molecular and physiological changes in *Polyporales* sp. KUC9061 in response to Cu(II) using GeneFishing technology.
Methods and Results: The presence of Cu(II) in the malt extract agar (MEA) media decreased the brown-rot fungi's growth rate in a concentration-dependent manner, but the fungal biomass was significantly increased in part for the biosorption of Cu(II). Increased expression of the genes encoding for the GIS2 DNA-binding protein and the 40S ribosomal protein S3A appears to be involved in this process. Oxalic acid is not used as a defence mechanism against high copper exposure, and ATP citrate lyase is not directly involved in oxalic acid production in this fungus. Several Cu(II)-sensitive proteins showed stable gene expression, suggesting that mechanisms that do not rely on these genes are responsible for the Cu(II) tolerance of the fungus.
Conclusions: *Polyporales* sp. KUC9061 does not use oxalic acid to chelate excess Cu(II) and potentially has other mechanisms, including the increased production of mycelia, to regulate Cu(II) biosorption.
Significance and Impact of the Study: This study is the first effort to examine Cu(II)-induced differential gene expression and the related physiological changes in the brown-rot fungus, a potential degrader of copper-containing waste wood. The results of this study will help with using this fungus to safely dispose of waste wood safe.

Introduction

Copper is an essential micronutrient; however, copper ions are toxic to cells at high concentrations. Thus, copper has been widely used as one of the main components of wood preservatives (Richardson 1997). While alternative chemicals, such as borates and organic biocides, are available for wood treatment, copper is still the primary wood biocide (Lebow *et al.* 2004). Recently, the use of copper-based wood preservatives, such as chromated copper arsenate (CCA), has raised concerns because of the release of heavy metals, including chromium and arsenic, into the environment. Thus, the development of effective and environmentally friendly wood disposal methods is needed.

Copper-tolerant wood-decaying fungi could be used for the bioremediation of copper-containing waste wood (Humar *et al.* 2004). Copper toxicity in organisms can be circumvented via chelation or complexation through the action of proteins or metal-chelating agents, such as metallothioneins and phytochelatins (Freeman and McIntyre 2008). In addition, oxalic acid production has been linked to copper tolerance in brown-rot fungi, and many researchers have investigated this phenomenon (Munir *et al.* 2001; Clausen and Green 2003). It has been reported that oxalic acid, which has metal-chelating properties, is

the main mineral-transforming agent for converting toxic metals into oxalate complexes (Fomina *et al.* 2005). Kim *et al.* (2009) investigated the bioleaching potential of selected brown-rot fungi for the removal of heavy metals from CCA-treated wood waste. In their study, *Polyporales* sp. LAS6497 accumulated the highest amounts of oxalic acid during the biodegradation process and oxalic acid produced by *Polyporales* sp. LAS6497 demonstrated a robust removal of copper, chromium and arsenic.

When using a copper-tolerant fungus to bioremediate waste woods, it is important to understand the mechanisms that underlie the fungal response to copper. Investigating the regulation of gene expression in response to various culture conditions is a popular method for identifying both the induced physiological changes following exposure to copper and the potential mechanisms that underlie copper tolerance (Gorfer *et al.* 2009). GeneFishing, a technique that uses annealing control primers (ACP) (Kim *et al.* 2004), has been frequently used to identify differentially expressed genes (DEGs) in organisms at different developmental stages (Cui *et al.* 2005; Lee *et al.* 2006) and in response to toxic conditions, such as insecticides (Yoo *et al.* 2007) or wastewater (Jo and Jung 2008).

Recently, a copper-tolerant brown-rot fungus was isolated from decaying wood in Korea. This fungus, *Polyporales* sp. KUC9061, was selected as one of the most copper-tolerant fungi using a screening process. In this study, we examined the DEGs in this fungus using GeneFishing to characterize transcriptional changes, which was confirmed using real-time PCR and oxalic acid production in response to copper exposure. In addition, we discuss the potential roles of the DEGs in the mechanisms responsible for the fungal copper tolerance.

Materials and methods

Identification of the fungus

A brown-rot basidiomycete *Polyporales* sp. KUC9061 from the Korea University Culture Collection (KUC) was used in this study. To identify the fungus, DNA was extracted from mycelia grown on 2% malt extract agar (MEA), and the sequences of the 28S and ITS rDNA loci were analysed according to the method used by Jang *et al.* (2012). The obtained sequences for *Polyporales* sp. KUC9061 were deposited in GenBank under the accession numbers JQ927218 (ITS) and JQ927219 (28S rDNA).

Effect of copper on growth

To determine what copper concentrations were suitable for investigating differential gene expression, fungal growth was assessed on MEA supplemented with different concentrations of Cu(II) (0–130 mg l^{-1} copper in the form of $CuSO_4 \cdot 5H_2O$ in 10 mg l^{-1} increments). A mycelium plug was placed in the centre of each 90-mm dish, and the growth was measured for 30 days at 25°C.

Isolation of total RNA

The fungus was inoculated on MEA media supplemented with the selected concentrations of Cu(II) (0, 50 and 100 mg l^{-1}). The mycelium from each plate was then scraped from the surface using a sterile scalpel and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the isolated RNA were assessed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

ACP-based RT-PCR

Total RNA was extracted from the fungal hyphae and was used to synthesize cDNA. To detect the DEGs, we used the GeneFishing DEG 101 & 102 Premix Kit (Seegene, Seoul, Korea). First-strand cDNA was synthesized using the dT-ACP1 primer with MMLV reverse transcriptase (Beams Biotechnology, Kyounggi-do, Korea) and an RNase inhibitor (INtRON Biotechnology, Kyounggi-do, Korea) according to the manufacturer's instructions. The second-strand cDNA synthesis and the second-stage PCR amplification were performed using dT-ACP2 and one of twenty arbitrary ACPs according to the manufacturer's instructions. The PCR products were separated on a 2% agarose gel using electrophoresis, stained with ethidium bromide and detected under UV light. The differentially expressed bands were excised from the gel and purified using an Accuprep Gel Purification Kit (Bioneer, Seoul, Korea).

Cloning and analysis of the DEGs

Purified DNA fragments were cloned using the T&A Cloning Vector Kit (RBC, Kyounggi-do, Korea) and transformed into competent DH5α *Escherichia coli* cells. The colonies were grown at 37°C for 12–16 h on Luria broth agar containing ampicillin and X-gal for blue/white selection. The plasmids from the selected colonies were extracted using an Accuprep Plasmid Mini Extraction Kit (Bioneer), and the inserts were sequenced (Macrogen, Seoul, Korea). The resulting sequences were compared and identified using the GenBank database and the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/).

Oligonucleotide synthesis and specificity confirmation

The primers for quantitative real-time PCR were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on the sequences obtained from cloning, which are shown in Table 1. To assess the specificity of the designed primers, total RNA was extracted and cDNAs were synthesized using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Reverse transcription was performed with 3 μg of purified total RNA and an oligo$(dT)_{20}$ primer according to the manufacturer's instructions. The cDNA amplification reaction was performed in a MyCycler (Bio-Rad, Hercules, CA, USA) using an Accupower PCR premix kit (Bioneer) and the designed primers, including GAPDH. Approximately 1 μl of synthesized cDNA was used in a total reaction volume of 20 μl. The amplification conditions consisted of an initial cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension step of 72°C for 10 min. To assess the specificity of the amplification, the amplified products were subjected to gel electrophoresis on 1% agarose gels and visualized using ethidium bromide staining.

Table 1 Primer sequences used to assay gene expression by RT-PCR

Gene	Amplicon size (bp)	Primer sequence
Gene 1	143	Forward 5′-GCC CAT TGA AAT ACG CAG TC-3′ Reverse 5′-CGG TAA CAG CGG TAA CGA GT-3′
Gene 2	187	Forward 5′-TGT CTG AGG TAG GCA CGT TG-3′ Reverse 5′-GGG AGG GGA TTC TTA ACG TC-3′
Gene 3	158	Forward 5′-GAC ATC GAC ATC GAC ACT GG-3′ Reverse 5′-GGT CGT AGC ATC GGT TTC AT-3′
Gene 4	122	Forward 5′-ATA CAA TTC ACC GCC GAA AA-3′ Reverse 5′-GCC GCC TAA AAT TTG CTG TA-3′
Gene 5	175	Forward 5′-GTC TTC TTC ACC TGC GAT GG-3′ Reverse 5′-ACT GCC TCA CCA ACT TCC AC-3′
Gene 6	153	Forward 5′-AGC ATG AAA GGG GTG TTT TG-3′ Reverse 5′-GTT TGC TGA CGA CGA GTG AG-3′
Gene 7	188	Forward 5′-CAG GAC GTG AAG GAT CAA GG-3′ Reverse 5′-GAT GCC GTC AAG AAT TCG AT-3′
GAPDH	103	Forward 5′-TCA CAA AGT GGT CGT TGA GC-3′ Reverse 5′-GCG TAC ACG GAA GAC AAG GT-3′

Real-time PCR

To quantify the DEGs, the cDNA synthesized in the previous step was used with the synthesized primer pairs in Table 1. Real-time PCR was performed on a CFX96 Real-Time Detection System (Bio-Rad) using the Quanti-Tech SYBR Green PCR Kit (Qiagen, Hilden, Germany) with 2 μl of cDNA in a 50 μl reaction according to the manufacturer's instructions. GAPDH was used as a control to standardize the amount of RNA in each reaction. The real-time PCR amplification efficiencies of the DEGs and GAPDH were calculated as previously described (Rasmussen 2001) using the following formula:

$$\text{Amplification efficiency} = 10^{\overline{\text{slope}}^{-1}}$$

where the slope was obtained by plotting the threshold cycle (Ct) values against the input cDNA concentrations (serial dilutions). The Ct values reflect the cycle number at which the fluorescence generated within a reaction crosses an arbitrary threshold limit.

The relative expression levels of the DEGs were calculated as previously described (Pfaffl 2001) using the following formula:

$$\text{Relative expression ratio} = \frac{(E_{\text{target}})\Delta\text{Ct}_{\text{target}}(\text{control}-\text{treated})}{(E_{\text{ref}})\Delta\text{Ct}_{\text{ref}}(\text{control}-\text{treated})}$$

where E_{target} and E_{ref} are the amplification efficiencies of the target and reference genes, respectively. The $\Delta\text{Ct}_{\text{target}}$ (control–treated) and $\Delta\text{Ct}_{\text{ref}}$ (control–treated) values are the Ct deviations of the control minus the treated samples of the target and reference genes, respectively.

Oxalic acid production

To characterize the production of oxalic acid, the revised method from Havir and Anagnostakis (1983) was used. The fungus was grown on a 90-mm Petri dish using the three Cu(II) concentrations above. The oxalate content was determined using high-performance liquid chromatography (Waters, Milford, MA, USA) equipped with a Supelcogel C-610H column. The eluting agent was 0·1% phosphoric acid, and the flow rate was set to 1 ml min^{-1}. The peak area of oxalate was calculated using Waters Empower 2 software.

Statistical analyses

Statistical analyses were performed using SAS 8·2 (SAS Institute, Cary, NC, USA). The significance of the gene expression changes in response to various copper concentrations was determined using ANOVA with a post hoc Dunnett's test. The significance of the differences in the mycelial mass and the oxalic acid production was assessed using ANOVA with a post hoc Duncan's test.

Results

Effect of copper on growth

The growth of *Polyporales* sp. KUC9061 was characterized to determine what copper concentration resulted in no observable adverse effect on physiological processes and what the highest copper concentration that allowed growth. The fungal growth curves in response to different copper concentrations are shown in Fig. 1. Compared with the control (copper absent media), no differences were observed at <60 mg l^{-1} Cu(II); under these conditions, the dishes were covered with mycelia within 7–8 days after inoculation. Reduced growth was observed from 70 to 100 mg l^{-1} Cu(II), with the exception of 80 mg l^{-1} Cu(II), which showed similar growth to the control. At 110 mg l^{-1} Cu(II), the growth rate of the fungus was significantly reduced, and 10% of the plates (one of 10 plates) showed no growth. As the copper concentration increased, the number of plates with no growth also increased. However, the abundance of mycelia increased in the presence of copper. Even under conditions in which no differences in the growth rate were observed (0–60 mg l^{-1} of copper), the fungus did not produce many mycelia in the absence of copper. Thus, copper not only affects the fungal growth rate but also increases the number of mycelia in the fungus.

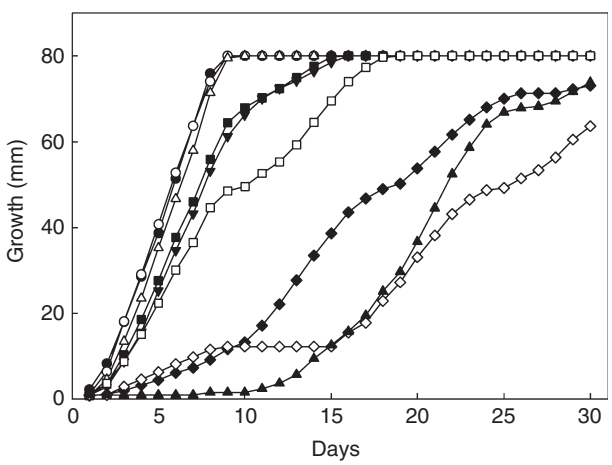

Figure 1 Effect of the copper concentration on the growth of *Polyporales* sp. KUC9061 on solid media malt extract agar at 25°C. The standard deviations of the measurements were omitted to avoid cluttering. Copper concentrations (mg l^{-1}) ●, 0; ○, 60; ▼, 70; △, 80; ■, 90; □, 100; ♦, 110; ◇, 120; ▲, 130.

Differential expression of *Cu(II)-induced fungal genes*

To understand the changes in gene expression in *Polyporales* sp. KUC9061 following exposure to copper, we selected three different concentrations of copper (0, 50 and 100 mg l^{-1}) based on the growth results and used ACP-based GeneFishing with a combination of 20 arbitrary primers. A total of seven differentially expressed bands were detected from ACPs 1, 3, 5, 6, 7, 9 and 17. A representative result is shown in Fig. 2. The extracted DNA was used for cloning and sequencing to identify DEGs. The sequences were similar to various predicted or hypothetical fungal proteins (Table 2), including oxalate synthesis-related gene ATP citrate lyase (ACL); the protein synthesis-related proteins 40S ribosomal protein S3A (RPS3A), dihydroxy-acid dehydratase (DAD), GIS2 DNA-binding zinc-finger protein (GIS2) and RNase III; the stress response protein heat shock protein 60 (HSP60); and the methyl-cycle-related gene for S-adenosyl-L-homocysteine hydrolase (SAHH).

Real-time PCR was performed using cDNAs from *Polyporales* sp. KUC9061 and gene-specific primers to confirm the differential expression of the identified genes. GAPDH was used as an internal control (Table 1). The genes were found to be differentially expressed in response to the Cu(II) concentration, and the results are shown in Table 2. The expression of three genes, ACL, GIS2 and RPS3A, was significantly different from the

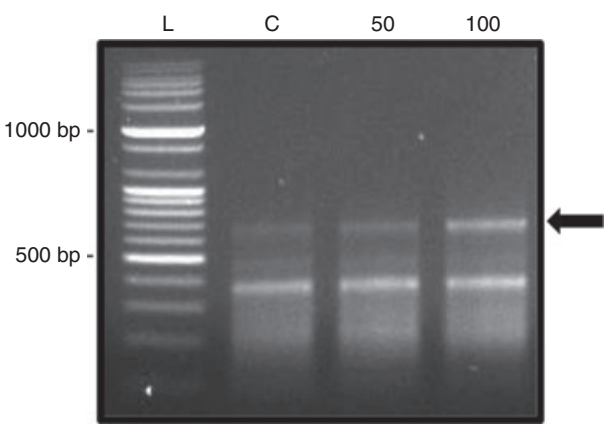

Figure 2 Agarose gel electrophoresis of annealing control primers (ACP)-based RT-PCR products using the ACP 7 primer. Differentially expressed gene (Gene 5) is indicated by black arrow. L, DNA marker (Bioneer 100 bp Plus DNA Ladder); C, control; 50, 50 mg l^{-1} Cu(II); 100, 100 mg l^{-1} Cu(II).

Table 2 Sequence characterization of the differentially expressed genes in *Polyporales* sp. KUC9061 following exposure to copper

Gene	ACPs	Length (bp)	GenBank accession no.	Putative identity† (Conserved domain)‡	Blast hit accession no. (Domain hit)	Score	E-value (Domain E-value)	Fold change§ in 50 mg l^{-1} Cu(II)	Fold change§ in 100 mg l^{-1} Cu(II)
Gene 1	ACP 1	412	JQ595380	RNase III (Double-stranded RNA binding motif)	EHB88461 (pfam00035)	47	3E-04 (2·33E-05)	0·97 ± 0·11	0·93 ± 0·18
Gene 2	ACP 3	752	JQ595381	S-adenosyl-L-homocysteine hydrolase (S-adenosyl-L-homocysteine hydrolase)	AAZ95180 (cd00401)	262	5E-83 (7·53E-58)	1·17 ± 0·32	0·93 ± 0·12
Gene 3	ACP 5	710	JQ595382	ATP citrate lyase (ATP citrate lyase)	XP 001839751 (PLN02522)	320	5E-99 (6·78E-50)	1·89 ± 0·36*	1·13 ± 0·27
Gene 4	ACP 6	461	JQ595383	GIS2 DNA-binding protein (Zinc-finger domain)	CCA68045 (cd10719)	158	4E-47 (4·79E-04)	1·83 ± 0·41*	2·11 ± 0·44*
Gene 5	ACP 7	587	JQ595384	40S ribosomal protein S3A (Ribosomal S3Ae family)	XP 002475466 (pfam01015)	327	6E-112 (1·89E-45)	1·59 ± 0·06	2·44 ± 0·28*
Gene 6	ACP 9	328	JQ595385	Heat shock protein 60 (Chaperonin)	XP 003334205 (PRK00013)	50·4	2E-05 (6·59E-03)	0·52 ± 0·26	0·84 ± 0·31
Gene 7	ACP 17	463	JQ595386	Dihydroxy-acid dehydratase (Dihydroxy-acid dehydratase)	XP 001835988 (PRK00911)	187	4E-54 (5·78E-37)	0·92 ± 0·10	0·83 ± 0·06

*Significantly different ($P > 0·05$) from basal gene expression according to Dunnett's test.
†The putative identity of each gene was searched by Basic Local Alignment Search (BLASTX) at GenBank, NCBI.
‡Conserved domain was also searched by BLASTX and shown in parenthesis.
§Relative gene expression ratio in response to Cu(II) compared to the basal gene expression. Numbers > 1 indicate up-regulation, and numbers < 1 indicate down-regulation.

control ($P < 0·05$) (Fig. 3). Compared with the control, the expression levels of ACL and GIS2 were 1·9-fold and 1·8-fold higher, respectively, at 50 mg l^{-1} Cu(II), whereas the expression levels of GIS2 and RPS3A were 2·1-fold and 2·4-fold higher, respectively, at 100 mg l^{-1} Cu(II). However, the expression of RNase III, SLHH, HSP60 and DAD was not significantly different at 50 or 100 mg l^{-1} Cu(II) relative to the control; therefore, these genes were not DEGs.

Oxalic acid production

When *Polyporales* sp. KUC9061 was grown under three different Cu(II) concentrations [0, 50 and 100 mg l^{-1}Cu (II)], both the oxalic acid production and the amount of mycelia in the media differed (Table 3). The total amount of oxalic acid in the media containing 50 mg l^{-1} Cu(II) (1·48 ± 0·02 μg) was higher than the other two conditions, but this difference was not significantly different according to Tukey's test ($\alpha = 0·05$). However, the mycelial weight increased with increasing copper concentrations. Thus, the amount of oxalic acid per mycelial weight decreased in the presence of copper. The oxalic acid concentration was not significantly different between the media lacking copper (0·54 ± 0·12 mg g^{-1}) and media containing 50 mg l^{-1} Cu(II) (0·48 ± 0·01 mg g^{-1}). The media containing 100 mg l^{-1} Cu(II) had the lowest oxalic acid production (0·2 ± 0·03 mg g^{-1}).

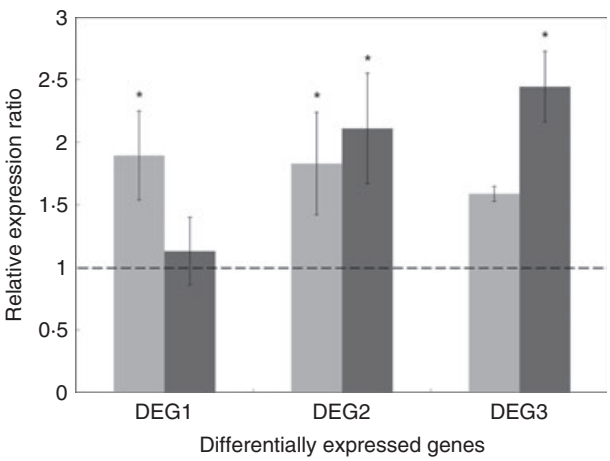

Figure 3 Relative expression ratios of the differentially expressed genes (DEGs) in *Polyporales* sp. KUC9061 exposed to 50 and 100 mg l^{-1} copper. DEG1: ATP citrate lyase, DEG2: GIS2 DNA-binding protein, DEG3: 40S ribosomal protein S3A. The data were analysed using Dunnett's test, and *indicates $P < 0·05$ when compared with the control. The error bars [Cu(II) 50 mg/l (▒); Cu(II) 100 mg/l (■)] represent the standard error ($n = 3$).

Discussion

We used *Polyporales* sp. KUC9061 because this fungal strain is one of the most copper-tolerant fungi and has the potential for remediating CCA-treated wood wastes. The growth rate, gene expression and oxalic acid

Table 3 Effect of the copper concentration on the oxalic acid production of *Polyporales* sp. KUC9061 in solid media malt extract agar at 25°C

Cu(II) (mg l^{-1})	Dry mycelial weight (g)	Total oxalic acid (μg)	Oxalic acid per dry weight (mg g^{-1})
0	1·45 ± 0·35 b	0·80 ± 0·36 a	0·54 ± 0·12 a
50	3·20 ± 0·00 a	1·48 ± 0·02 a	0·48 ± 0·01 ab
100	3·85 ± 0·07 a	0·80 ± 0·09 a	0·20 ± 0·03 b

Numbers followed by the same letter in each row are not significantly different (α = 0·05) according to Tukey's test.

production of the fungus were assessed to characterize the physiological changes in response to copper.

The expression level of the protein synthesis genes GIS2 and RPS3A was significantly increased. GIS2 has 74% identity to the *Piriformospora indica* GIS2 putative zinc-finger protein, and RPS3A has 92% identity to *Postia placenta* 40S ribosomal protein S3A. GIS2 in *Saccharomyces cerevisiae* contains seven zinc-finger motifs, which is responsible for DNA and potentially RNA interactions (Scherrer *et al.* 2011). Scherrer *et al.* (2011) showed that *S. cerevisiae* GIS2 was associated with mRNAs involved in ribosome biogenesis and controls the cell size of the yeast. In fact, a GIS2 over-expression *S. cerevisiae* mutant had a significantly larger cell size compared to normal cells. Kho *et al.* (1996) showed that elevated RPS3A expression increased protein synthesis in Rat-1 cells. In our study, the dry mycelial weight of the fungus increased approximately 2·1- and 2·7-fold in the presence of 50 and 100 mg l^{-1} of Cu(II), respectively (Table 3). Similarly, the expression of GIS2 increased approximately 1·8-fold and 2·1-fold at 50 and 100 mg l^{-1} Cu(II), respectively, and the expression of RPS3A increased approximately 1·6-fold and 2·4-fold at 50 and 100 mg l^{-1} Cu(II), respectively (Fig. 3). These results suggest that the expression of GIS2 and S3A is related to increased mycelial mass. Copper can be sorped by the hyphal cell wall and accumulate outside, inside (between the cell wall and cell membrane) or intracellular cell wall (Gonzalez-Chavez *et al.* 2002). The strain-specific sorption capacity was 2·8–13·8 mg g^{-1} in three *Glomus* spp. (arbuscular mycorrhizal fungi). How the increased mycelial mass is related to Cu(II) tolerance in *Polyporales* sp. KUC9061 is not fully understood, but it can in part be explained by the increased sorption of Cu(II) via the increased production of mycelia.

HSP60 was the most homologous to HSP60 in *Puccinia graminis* f. sp. *tritici* (89% identity), which possesses a chaperonin region that is involved in the productively folding proteins. Heat shock proteins are expressed under normal conditions and are induced at increased temperatures and under other stress conditions, such as increased levels of free oxygen radicals, heavy metals, ethanol or amino acid analogues (Maio 1999). However, compared with the control, HSP60 expression was not increased but rather decreased at 50 mg l^{-1} Cu(II), and the expression level of HSP60 at 100 mg l^{-1} Cu(II) was similar to expression in the control (Table 2). Clayton *et al.* (2000) obtained a similar result when characterizing HSP60 expression in the zebra mussel *Dreissena polymorpha* after exposure to copper in which no correlation between the expression of HSP60 and the concentration of copper was found.

SLHH and DAD expression was not related to the copper tolerance. SLHH was the most homologous to SLHH in *Volvariella volvacea* (89% identity), which is involved in the activation of the methyl cycle and is responsible for the reversible hydration of S-adenosyl-L-homocysteine into adenosine and homocysteine. It has been reported that SLHH in *Arabidopsis thaliana*, which has 56% similarity to SLHH of *Polyporales* sp. KUC9061, is a copper-binding protein (Kung *et al.* 2006). Li *et al.* (2004) suggested that copper might be a regulator of SLHH activity via reversibly binding to SLHH and inhibiting its catalytic activity. However, in our study, the presence of copper did not affect the expression of SLHH. DAD was the most similar to *Coprinopsis cinerea* DAD and has 83% identity. DAD plays a role in the conversion of 2,3-dihydroxy-3-methylbutanoate to 3-methyl-2-oxobutanoate and H_2O. According to Macomber and Imlay (2009), dehydratases, such as the *E. coli* DAD, form iron-sulfur clusters that are vulnerable to copper stress, and enzymatic activity is reduced through the suppression of *de novo* enzyme synthesis and activation. However, it has been suggested that dehydratases are protected against copper via chelation in eukaryotes. In our study, DAD showed similar expression levels at all Cu(II) concentrations. Therefore, it appears that DAD expression might not be affected by the copper concentration as a consequence of fungal defence mechanisms. A similar hypothesis could also explain the results observed with SLHH and RNase III, which have 35% identity to *Rothia mucilaginosa* RNase III.

ATP citrate lyase, with 94% identity to *C. cinerea* ACL, demonstrated increased expression at 50 mg l^{-1} Cu(II), and the expression returned to basal levels at 100 mg l^{-1} Cu(II). ACL catalyses the production of acetyl CoA and oxaloacetate via the hydrolysis of ATP. ACL is important for cell growth (Bauer *et al.* 2005) and has been detected

in both eukaryotes and prokaryotes (Attwood 1973). Oxalate can be produced from a number of pathways (Gentile 1954; Munir *et al.* 2001), one of which involves the conversion of oxaloacetate to oxalate via cytosolic oxaloacetase (Gadd 1999). However, no direct correlation between the expression of ACL and oxalic acid production was detected in *Polyporales* sp. KUC9061 (Fig. 3 and Table 3). Moreover, as shown in Table 3, the oxalic acid concentration per dry weight (mg g^{-1}) decreased in the presence of copper. Thus, it seems that ACL is not involved in oxalate production in *Polyporales* sp. KUC9061 and that oxalic acid is not involved in copper tolerance in this fungus. Clausen *et al.* (2000) showed similar results with no correlation between copper tolerance and oxalic acid production in fungi grown in artificial media. Sammons *et al.* (2011) also showed that brown-rot fungi mediate the decay of metal-enriched wood blocks without enhanced secretion of oxalate (Schilling and Jellison 2006).

Although we focused on gene expression in response to the concentration of copper, gene expression also changes over time. According to Ramesh *et al.* (2009), the expression of metallothioneins in the presence of copper was time dependent and showed peak levels of expression within 3 days in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. To fully understand the role of the genes identified in this study, an assessment of their expression levels as a function of time is needed. To thisend, efforts to identify additional DEGs using other ACPs and determine how gene expression changes in response to copper exposure over time are currently in progress.

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