Cloning and functional characterization of the gene encoding the transcription factor Ace1 in the basidiomycete *Phanerochaete chrysosporium*

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ABSTRACT

In this report we describe the isolation and characterization of a gene encoding the transcription factor Ace1 (Activation protein of *cup1* Expression) in the white rot fungus *Phanerochaete chrysosporium*. *Pc-ace1* encodes a predicted protein of 633 amino acids containing the copper-fist DNA binding domain typically found in fungal transcription factors such as Ace1, Mac1 and Haa1 from *Saccharomyces cerevisiae*. The *Pc-ace1* gene is localized in Scaffold 5, between coordinates 220841 and 222983. A *S. cerevisiae ace1* null mutant strain unable to grow in high-copper medium was fully complemented by transformation with the cDNA of *Pc-ace1*. Moreover, Northern blot hybridization studies indicated that *Pc-ace1* cDNA restores copper inducibility of the yeast *cup1* gene, which encodes the metal-binding protein metallothionein implicated in copper resistance. To our knowledge, this is first report describing an Ace1 transcription factor in basidiomycetes.

Key terms: Ace1, basidiomycete, copper, *Phanerochaete chrysosporium*.

INTRODUCTION

A small group of filamentous fungi, collectively known as white-rot fungi, has the unique ability to breakdown lignin, a highly recalcitrant polymer present in the plant cell walls. Lignin-degrading fungi secrete an array of oxidative extracellular enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP) and a copper-containing phenol oxidase called laccase (Gold and Alic, 1993; Cullen and Kersten, 2004). All these enzymes act nonspecifically through the generation of lignin free radicals, which undergo spontaneous cleavage reactions (Kirk and Farrell, 1987).

Although the involvement of laccase in ligninolysis has been well established (Eggert et al., 1997), some white rot fungi *(i.e. Phanerochaete chrysosporium)* do not produce this enzyme, suggesting that it may not be absolutely required for lignin degradation (Hatakka, 1994). Laccases belong to the large family of multicopper oxidases (MCOs) that also includes plant ascorbate oxidases, fungal Fet3 ferroxidases and mammalian ceruloplasmin, among other proteins (Solomon et al., 1996). A combination of detailed spectroscopic and X-ray crystallographic studies has revealed that all these enzymes contain at least one blue copper or T1 site and a type 2 - type 3 (T2/T3) trinuclear copper cluster as the

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minimal functional unit (Baldrian, 2006; Solomon et al., 1996). These copper centers, located in the active site of these enzymes, play a key role in catalysis. On the other hand, this metal also exerts an effect at the transcriptional level, as shown mainly with laccases. Thus, copper regulates transcription of laccase genes in *Trametes versicolor* (Collins and Dobson, 1997), *Ceriporiopsis subvermispora* (Karahanian et al., 1998), *Pleurotus ostreatus* (Palmieri et al., 2000), *Pleurotus sajor-caju* (Soden and Dobson, 2001; Soden and Dobson, 2003) and *Trametes pubescens* (Galhaup et al., 2002). Since dozens of closely related laccase genes have been characterized in lignin-degrading fungi (Kumar et al., 2003), it is highly likely that their regulation by copper could be a widespread phenomenon.

Little is known about the mechanism by which this metal activates laccase gene expression in basidiomycetes. Several transcription factors responding to copper, such as Ace1, Mac1, Cuf1 and Amt1, have been identified in Ascomycetes (Jungmann et al., 1993; Labbe et al., 1999; Thiele, 1988; Zhou and Thiele, 1991). However, there is no evidence that any of these transcription factors are present in basidiomycetes. Interestingly, putative Ace1 transcription factor-binding sites have been identified in the promoter region of the laccase genes in the basidiomycete PM1 (Coll et al., 1993), *C. subvermispora* (Karahanian et al., 1998) and *P. sajor-caju* (Soden and Dobson, 2003). This element was first described in the promoter regions of the *cup1* (Thiele, 1988) and *sod1* (Gralla et al., 1991) genes, which encode metallothionein and a copper-zinc superoxide dismutase in yeast, respectively. The *S. cerevisiae* Ace1 transcription factor binds its recognition sequence and activates the transcription of target genes in response to copper or silver, but not to zinc (Furst et al., 1988).

The fact that the laccase gene from *C. subvermispora (Cs-lcs)* is also activated by silver but not by zinc (Karahanian et al., 1998) prompted us to identify and isolate the Ace1 transcription factor in this fungus. We initially followed the strategy of identifying the protein by means of electrophoretic mobility-shift assays (EMSA), using a 95 bp DNA probe containing the *Cs-lcs* ACE-like element (Polanco et al., 2002). Although specific complexes were formed upon incubation of this DNA probe with crude nuclear extracts, attempts to isolate the Ace1 factor were unsuccessful. Taking a different approach, we used the publicly available information in the genome database of *P. chrysosporium* (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html), the first basidiomycete whose genome has been sequenced, to find out whether this fungus has a gene encoding for Ace1. In this work we report the finding of a presumed gene model and the subsequent confirmation of its identity by cDNA cloning, sequencing and complementation of a *S. cerevisiae ace1* null mutant strain.

METHODS

Strains, plasmids and culture conditions

P. chrysosporium homokaryotic strain RP-78 was obtained from the Center for Mycology Research, Forest Products Laboratory, Madison, Wisconsin. *P. chrysosporium* spores were collected by flooding the agar plates with 5 ml of sterile water. 10^7 spores were inoculated in 100 ml of defined media containing wood-derived crystalline cellulose (Avicel PH-101, Fluka Chemika) as the sole carbon source, as described by Wymelenberg et al. (2002). Cultures were incubated at 37 °C for 6 days with constant agitation (300 R.P.M.). *Saccharomyces cerevisiae* DTY7 strain (MATα, his6, leu-, ura3-52, CUP1 R-3), DTY59 strain (MATα, his6, leu-, ura3-52, CUP1 R-3, ace1-Δ225) and the plasmid p416GPD were kindly provided by Dr. D. J. Thiele (Duke University Medical School). These strains were maintained on YPD plates. DTY7 and DTY59 transformants were maintained on SC URA(-) plates (Sherman, 2002) at 30 °C not supplemented with copper. For Northern blot analysis and metal resistance test experiments, each transformant was grown on SC URA(-)

liquid cultures at 30 °C with constant agitation (200 R.P.M.). These cultures contained different concentrations of $CuSO_4$ as indicated in each experiment (see below). Bacterial strain DH5α (Stratagene) was used for the propagation of all plasmids. pGEM-T easy vector (Promega) was used for cloning experiments and sequencing.

RNA extraction

After six days of growth, *P. chrysosporium* mycelia were separated from the culture fluids by filtration through Miracloth (Calbiochem) and immediately frozen in liquid nitrogen. The frozen mycelia were ground to a powder in a mortar containing liquid nitrogen and total RNA was extracted as described by Manubens et al. (2003). Poly(A) mRNA was obtained from 100 μg of total RNA using the mRNA DIRECT micro kit (Dynal) according to the manufacturer's directions. For *S. cerevisiae* total RNA extraction, 20 ml cultures of each transformant were grown in 250 ml flasks as described above until reaching an OD_{600} = 1.0. Cultures were either harvested or exposed to $CuSO_4$ to a final concentration of 250 mM for 45 minutes. Each flask was pelleted in a 50 ml size RNase-free phenol-resistant centrifuge tube. Pellets were washed with 2 ml of DEPC-treated water and pelleted again. Thereafter, pellets were vortex-homogenized in the same tube by the addition of 0.7 ml Tris-HCl buffer (0.2M Tris-HCl pH 7.5, 0.5M NaCl, 0.01M EDTA, 1 % SDS, 50mM β-mercaptoethanol), 0.7 ml of phenol:cloroform:isoamyl alcohol (25:24:1) and 0.4 gr of acid-washed glass beads. After centrifugation, the aqueous phase was phenol-extracted in a clean tube and the RNA was obtained as described by Manubens et al. (2003).

Pc-ace1 *cDNA identification, cloning and analysis*

Pc-ace1 cDNA was obtained by reverse transcription using poly(A) mRNA and the Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 45 min at 42 °C. RT-PCR was conducted as described by Larrondo et al. (2003) using high fidelity DNA polymerase (Pfu, Stratagene). The RT-PCR amplification of the *Pc-ace1* cDNA was primed using the direct (5'-GTCATATCCAGCCATGGT-3') and reverse (5'- AGATTAGAATATCCGTGGAC -3') oligonucleotides. Both primers were designed to amplify the entire predicted coding region according to the genomic sequence. The RT-PCR product was subsequently cloned into the pGEM-T easy vector and nucleotide sequences were determined using the ABI Prism Big Dye terminator cycle sequencing kit on ABI automated sequencers (Applied Biosystems). The *Pc-ace1* cDNA was subcloned into the BamHI site of the p416GPD vector. Electrocompetent DTY7 and DTY59 cells were prepared as described by Becker and Guarente (1991). Both strains were transformed by electroporation with the empty p416GPD vector and with the vector containing the *Pc-ace1* cDNA (p416GPD-*Pc-ace1*) using a MicroPulser apparatus (Biorad) according to the manufacturer's instructions. The four transformants were denoted as DTY7-p416GPD, DYT7-p416GPD-*Pc-ace1*, DTY59-p416GPD and DYT59-p416GPD-*Pc-ace1*. Sequence editing and analysis employed DNAstar software (DNAstar). The *Pc-ace1* cDNA sequence had been deposited in the GenBank database under accession number DQ517293.

Northern-blot hybridization

For Northern blot hybridization studies, 10 μg of *S. cerevisiae* total RNA were fractionated by electrophoresis in a formaldehyde-agarose gel (1.2 % w/v) and blotted onto Hybond-membranes. Blots were prehybridised at 42 °C during 4h in a high stringency solution containing 50% formamide, 1% sodium dodecyl sulphate (SDS), 5X SSPE (Ambion Inc., Austin, TX), 5X Denhardt's solution and 100 μg/ml denaturated sheared nonhomologous DNA. Northern hybridization was carried out at 42 °C for 12 – 14h in the same solution containing 1×10^7 cpm/ml of [α-^{32}P] dCTP-labelled cDNA probe prepared with the direct (5'-TCAATCATCACATAAAATGTTC-3') and reverse (5'-CGTTTCATTTCCCAGAGCAG-3') oligonucleotides for *cup1*. After

hybridization, blots were washed in a 2X SSPE 1% SDS solution for 20 min at 42°C and then washed again in a 0.1X SSPE 1% SDS solution for 10 min at 42°C. Blots were exposed on scientific autoradiographic imaging film (Kodak) at –80°C for 24h. As a control, levels of mRNA from the glyceraldehyde-3-phosphate dehydrogenase gene (*tdh3*) were also monitored using cDNA probes generated with the direct (5'-CCAAGAAAGAGACCCAGC-3') and reverse (5'-CGGTTGGGACTCTGAAAG-3') specific oligonucleotides. All cDNA probes were prepared by PCR using [α-^{32}P] dCTP as described by Mertz and Rashtchian (1994).

Metal resistance test

To evaluate copper resistance, two different clones of each transformant were tested. Each experiment is expressed as the minimal inhibitory concentration (MIC). The copper-resistance test was conducted using tubes containing SC URA(-) broth either without or with $CuSO_4$ in a range between 50 to 1500 μM. The tubes were inoculated with 0.1 ml of each *S. cerevisiae* clone grown to an OD_{600} = 0.9-1.0, and then incubated 72h at 30 °C with constant agitation (200 R.P.M.) in the presence of different copper concentrations. Following incubation, visual turbidity was noted and the OD_{600} was measured and recorded. The MIC was defined as the lowest concentration of copper tested at which no growth was observed after a 72h incubation period.

Genome-wide in silico *search of possible Ace1 transcription factor-binding sites*

In an attempt to identify potential Pc-Ace1 target genes, we inspected the Gene Ontology database (http://www.geneontology.org) in order to uncover all the Gene Ontology identification numbers or IDs related to copper-associated molecular functions, biological processes and cellular components. The IDs obtained were used for a Gene Ontology search in the *P. chrysosporium* database (http://shake.jgi-psf.org/cgi-bin/ToGo?species=Phchr1). Using this approach, 25 gene models that exhibited a copper-associated biological function were identified and their promoter regions were manually obtained. These promoter sequences were finally analyzed for the presence of regulatory motifs using the MatInspector software (http://www.genomatix.de).

Multiple-sequence analysis

A multiple-sequence alignment was constructed by using the ClustalW method in the MegAlign software (DNAstar). Default gap opening and extension penalties were used to construct the alignment.

RESULTS AND DISCUSSION

Identification and characterization of Pc-ace1

The *P. chrysosporium* database was searched for the presence of a gene encoding a putative Ace1 transcription factor. Three gene models were identified in this genome. They are located in Scaffold 25 (between coordinates 30283 and 30513; protein ID 130363), Scaffold 7 (between coordinates 1969672 and 1970136; protein ID 136848) and Scaffold 5 (between coordinates 220893 and 221217; protein ID 131179). The later gene model showed the highest similarity to the *S. cerevisiae ace1* and therefore it was further characterized. Its identity was confirmed by isolation and sequencing of the corresponding cDNA from mycelia grown in Avicel medium, as described in Methods. Comparison of the cDNA with the genomic sequence showed the presence of 4 introns (Figure 1A), the first exon being just 3 nucleotides long. The deduced protein has 633 aa and according to InterProScan (www.ebi.ac.uk/InterProScan/) it possesses a copper-fist DNA-binding domain (IPR001083) comprising residues 1 through 39 of the N-terminal (Figure 1B). As expected, a manual inspection of the copper-fist domain signature led to the identification of a conserved array of zinc-binding residues (C-X2-C-X8-C-X-H, CETCIKGHRSSNCKH in Pc-Ace1, as shown in Figure 1B). Interestingly, the

codon for the first Cys in the conserved zinc-binding path is split by intron two (Figure 1B). A multiple aminoacidic general alignment was conducted with several well-characterized Ace1 related sequences present in NCBI (BLOSUM 62) using ClustalW. *Pc-ace1* encodes a polypeptide with an overall identity of about 10 % to other fungal Ace1-like transcription factors. The highest similarities resulted with the Haa1 from *S. cerevisiae* (15.3 %) and with the Crf1 protein from *Yarrowia lipolytica* (12.8 %) (data not shown). In spite of this low identity, the alignment also revealed that the copper-fist DNA-binding domain of Pc-Ace1 possesses a high degree of identity with those of other Ace1-like transcription factors. For example, the copper-fist DNA-binding domain of Pc-Ace1 shows 59.0, 48.7 and 51.3 % identity with the equivalent domains of *S. cerevisiae* Ace1, *S. cerevisiae* Mac1 and *Candida glabrata* Amt1 transcription factors, respectively (data not shown).

Pc-ace1 encodes a functional transcription factor

Due to the role of Ace1 in the activation of transcription of the *cup1* in response to copper in *S. cerevisiae*, the *ace1*Δ strain is unable to grow in copper-rich medium (Thiele, 1988). This observation was the basis for our complementation experiments addressed to the confirmation that *Pc-ace1* indeed encodes a functional transcription factor. *S. cerevisiae* DTY59 *ace1Δ* strain and the isogenic DTY7 wild-type strain were transformed with either p416GPD or

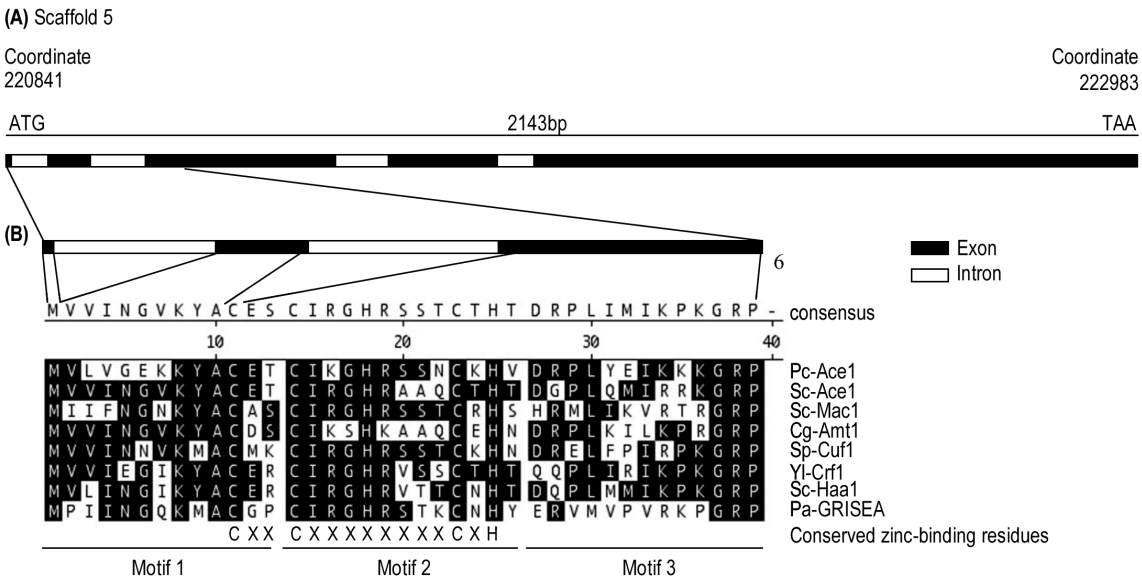

Figure 1: (A) Intron-exon composition of the gene encoding Ace1 in *Phanerochaete chrysosporium*. *Pc-ace1* was localized in Scaffold 5 between coordinates 220841 and 222983, as shown in the figure. (B) Representation of the N-terminal half of Pc-Ace1 that contains the copper-fist DNA-binding domain signature (M - [LIVMF](3) - x(3) - [KN] - [MY] - A - C - x(2) - C - [IL] - [KR] - x - H - [KR] - x(3) - C - x - H - x(8) - [KR] - x - [KR] - G - R – P), which comprises three-13 residues long motifs. The partial alignment shows the conserved residues (white letters in black boxes) among all the transcription factors analyzed (GenBank access numbers are given in brackets). *Phanerochaete chrysosporium* Ace1 (Pc-Ace1) [ABF60559], *Saccharomyces cerevisiae* Ace1 (Sc-Ace1) [NP_011349], *Saccharomyces cerevisiae* Mac1 (Sc-Mac1) [AAT92953], *Candida glabrata* Amt1 (Cg-Amt1) [XP_447430], *Schizosaccharomyces pombe* Cuf1 (Sp-Cuf1) [CAA90469], *Yarrowia lipolytica* Crf1 (Yl-Crf1) [XP_500631], *Saccharomyces cerevisiae* Haa1 (Sc-Haa1) [AAT92823] and *Podospora anserina* Mac1 homolog (Pa-GRISEA) [CAA61598]. Exons are indicated in black boxes while introns are denoted in white boxes.

p416GPD-*Pc-ace1* plasmids. Cells were plated on SC plates lacking uridine so that only transformants bearing a plasmid would be able to grow. Plasmid-rescue experiments were performed with some of the colonies in order to confirm successful transformation. All four transformants were able to grow on SC URA(-) plates not supplemented with copper sulfate (data not shown). As expected, the wild-type DTY7 strain but not the *ace1Δ* strain proliferated in SC URA(-) liquid medium in the presence of copper. As described in Methods, two different clones of each transformant were exposed to different concentrations of this metal. As shown in Figure 2, addition of $CuSO_4$ to a final concentration of 50 μM led to a dramatic decrease of culture growth of only the DTY59 mutant strain. The lack of growth of the *S. cerevisiae* null mutant was fully compensated by transformation with *Pc-ace1*. DYT59-p416GPD-*Pc-ace1* transformants behaved very similar to the wild-type strain, being able to grow in the same copper concentrations as transformants DTY7-p416GPD or DTY7-p416GPD-*Pc-ace1*. A fivefold increment of the MIC for copper of DTY59 *ace1Δ* strain (MIC≤200 μM) was observed after complementation with *Pc-ace1* cDNA. The MIC showed by DTY59-p416GPD-*Pc-ace1* strain was above 1000 μM, similar to that observed with transformants DTY7-p416GPD and DTY7-p416GPD-*Pc-ace1*.

cup1 mRNA levels in S. cerevisiae

To gain insight into the molecular mechanism that accounts for the phenotypes described above, the levels of *cup1* mRNA in the various yeast strains were analyzed by Northern blot hybridization experiments. As shown in Figure 3, the *cup1* transcripts were virtually undetected in cultures of DTY7 and

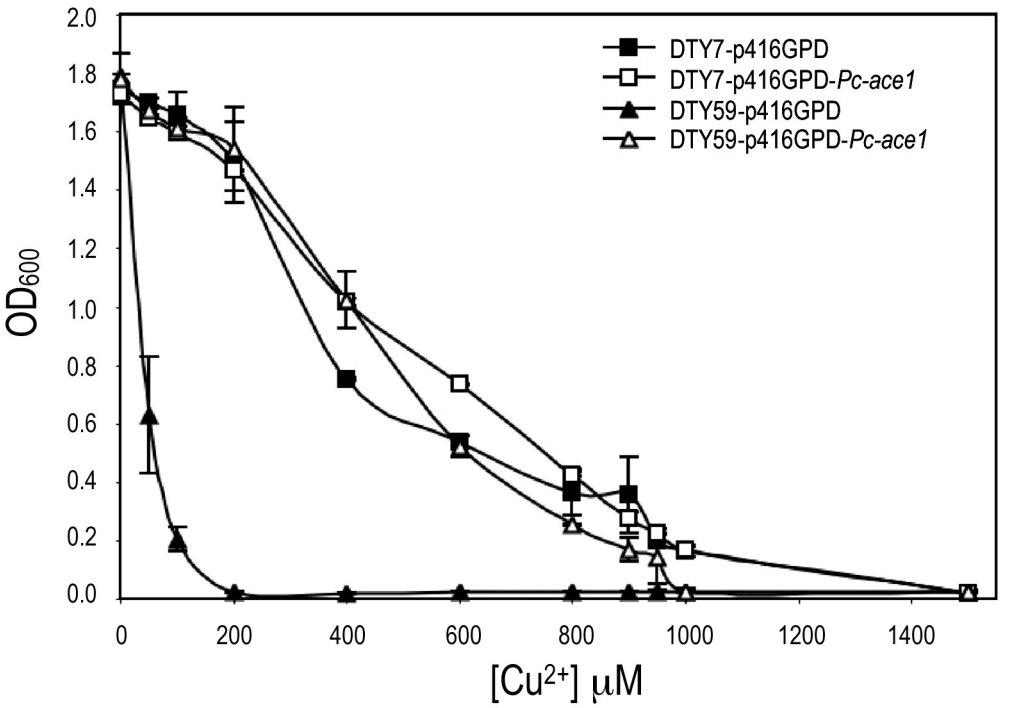

Figure 2: Copper resistance test. Two different clones of each transformant were grown on SC URA(-) liquid cultures not supplemented with copper until reaching an OD_{600} = 0.9-1.0, as described in Methods. A separate set of SC URA(-) containing tubes supplemented with different concentrations of copper was inoculated with the *S. cerevisiae* transformants. Cultures were incubated 72h at 30 °C with constant agitation (200 R.P.M.) and thereafter OD_{600} was measured. Values represent the mean of two independent cultures ± standard deviation.

DTY59 (*ace1Δ*) strains lacking exogenously added copper (Figure 3, lanes 1 to 4). As expected, addition of Cu^{2+} to a final concentration of 250 μM resulted in a dramatic increase in *cup1* mRNA levels in the DTY7 wild-type strain (Figure 3, lanes 5 & 6) but not in the *S. cerevisiae* DTY59 strain (Figure 3, lane 7). Notably, *Pc-ace1* cDNA restores copper inducibility of *cup1* expression in the latter strain (Figure 3, lane 8). These results strongly support the assertion that *Pc-ace1* is an ortholog of the gene encoding transcription factor Ace1 in yeast.

Possible target genes of transcription factor Pc-Ace1

The isolation of the cDNA encoding Ace1 in *P. chrysosporium* provides us with the opportunity to use it as a heterologous probe for the identification of the *ace1* encoding sequence in *C. subvermispora*. This goal, which motivated the work reported here, will be accomplished by thorough screening of the genomic (Karahanian et al., 1998) and cDNA (Lobos et al., 1998) libraries of this fungus that are available in our laboratory. At the same time, it poses the challenge of identifying possible target genes in *P. chrysosporium*. In this regard, we failed to identify a *cup1*-like sequence in the *P. chrysosporium* genome database and the *sod1*-like gene model found seems not to contain an ACE-like regulatory element in its promoter region, according to the MatInspector software (data not shown). In view of the absence of laccase genes in *P. chrysosporium* (Larrondo et al., 2003), we used the *in silico* approach described in Methods in an attempt to identify presumed Pc-Ace1 target genes in its genome. Even though this strategy only considered those genes that have been previously annotated, and among them, only those gene models with a copper associated biological function, we identified 7 gene models possessing at least one putative ACE regulatory element in their respective upstream regions. Among these are the genes encoding a copper-exporting P-type ATPase, subunit Va of cytochrome c oxidase, a copper amino oxidase and a multicopper oxidase 1 (Pc-Mco1). The latter corresponds to a new member of the multicopper oxidase family that possesses a strong ferroxidase activity (Larrondo et al., 2003). We are currently conducting experiments to confirm whether these genes are regulated by copper.

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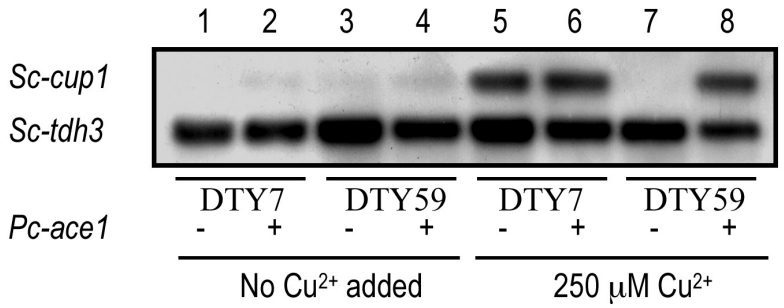

Figure 3: *cup1* mRNA levels in the wild type (DTY7) and the *ace1*Δ strain (DTY59). Representative clones of each strain were grown in SC URA(-) medium. Exponential phase cultures (OD_{600}= 0.9-1.0) were harvested (lanes 1 to 4) or exposed to 250 μM $CuSO_4$ for 45 min (lanes 5 to 8), as described in Methods. Total RNA was extracted and subjected to Northern blot hybridization.

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