The copper-dependent ACE1 transcription factor activates the transcription of the mco1 gene from the basidiomycete \textit{Phanerochaete chrysosporium}

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Paulina Bull and Rafael Vicuña

INTRODUCTION

The lignin-degrading basidiomycete \textit{Phanerochaete chrysosporium} has an impressive array of extracellular enzymes involved in lignocellulose degradation. Among them, the extensively characterized lignin and manganese peroxidases (LiP and MnP, respectively) have been described as true ligninases because of their high redox potential (Martinez et al., 2005; Kersten & Cullen, 2007). A third kind of enzyme also involved in lignin degradation, although not produced by \textit{P. chrysosporium}, is the phenol oxidase termed laccase (Larrondo et al., 2003; Baldrian, 2006). Laccases belong to the family of multicopper oxidases (MCOs), a large group of copper-containing proteins that include mammalian ceruloplasmin, plant ascorbate oxidases and fungal Fet3 ferroxidases (Solomon et al., 1996). Spectroscopic and X-ray crystallographic studies have revealed that MCOs contain at least one blue copper or type 1 site (T1) and a type 2/type 3 (T2/T3) trinuclear copper cluster as the minimal functional unit. These copper centres, located in the active site of these enzymes, play a key role in catalysis (Solomon et al., 1996; Baldrian, 2006).

In addition to the structural and catalytic roles played by copper, some reports have shown that this metal also affects the expression of some MCOs. For instance, copper increases mammalian ceruloplasmin mRNA levels in hepatoma cells through copper-induced promoter activation (Martin et al., 2005). Copper has also been shown to activate the transcription of \textit{CmA04}, a gene encoding ascorbate oxidase in the plant \textit{Cucumis melo} (Sanmartin et al., 2007), as well as to increase mRNA levels of \textit{fet3}, a gene encoding a ferroxidase involved in iron uptake in \textit{Saccharomyces cerevisiae} (Gross et al., 2000). Laccases are not an exception in this respect. Thus, copper regulates laccase mRNA levels in the fungi \textit{Trametes versicolor} (Collins & Dobson, 1997), \textit{Ceriporiopsis subvermispora} (Karahanian et al., 1998), \textit{Pleurotus ostreatus} (Palmieri et al., 2000), \textit{Pleurotus sajor-caju} (Soden & Dobson, 2001, 2003) and \textit{Trametes pubescens} (Galhaup et al., 2002).

Analysis of the promoter region of some of the MCO-encoding genes has shown the presence of a copper-dependent responsive element named ACE (activation of
cup1 expression), that was previously described in yeast as the recognition site for the ACE1 transcription factor (Evans et al., 1990). ACE1 is a member of a group of fungal transcription factors that contain a copper-fist cysteine-rich DNA-binding domain located in the N-terminal region (Hu et al., 1990). This trans-acting regulatory protein activates the transcription of the S. cerevisiae metallothionein-encoding genes cup1 (Thiele, 1988; Buchman et al., 1989; Culotta et al., 1989; Evans et al., 1990; Thorvaldsen et al., 1993) and crs5 (Culotta et al., 1994) and the superoxide dismutase gene (sod1) (Gralla et al., 1991; Carri et al., 1991) in response to copper. This metal is essential for the DNA-binding activity of ACE1 (Winge, 1998). Interestingly, we have recently identified the gene encoding ACE1 in P. chrysosporium (Pc-acel), the first basidiomycetal orthologue of the yeast ACE1 transcription factor (Polanco et al., 2006). However, until this work, the possible target gene(s) of Pc-ACE1 remained unknown.

Previously, we have shown that the genome database of P. chrysosporium (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html) lacks laccase-encoding sequences. Instead, we identified four clustered MCO genes (designated mco1 to mco4) distantly related to laccases (Larrondo et al., 2003, 2004). Heterologous expression of mco1-cDNA in Aspergillus nidulans showed that the substrate specificity of the recombinant MCO1 differs from that of laccases. For example, MCO1 has strong ferroxidase activity, with a Km value similar to Fet3 protein from S. cerevisiae (Larrondo et al., 2003). Although extracellular MCO1 shows biochemical and structural similarities with the membrane-bound Fet3 from yeast, we have recently identified and characterized Pc-fet3, the gene encoding the canonical ferroxidase involved in iron uptake in P. chrysosporium (Larrondo et al., 2007a). The presence of Pc-fet3 and the lack of a C-terminal transmembrane domain anchor in MCO1, which is distinctive of Fet3 proteins, supports the assertion that MCO1 and Fet3 play different roles in the cell (Larrondo et al., 2003, 2007a). A recent and detailed phylogenetic analysis of more than 350 MCOs, including those from P. chrysosporium, supports this statement (Hoegger et al., 2006). To date, the physiological function of MCO1 remains obscure.

In an effort to gain insight into the role of MCO1, we decided to analyze the transcriptional regulation of mco1 and the molecular mechanisms involved in it. The fact that copper affects the transcription of various MCO-encoding genes in different organisms, plus the presence of putative ACE elements in the promoter of mco1 (Polanco et al., 2006), led us to analyze the effect of copper on the expression of this novel group of multicopper oxidases recently discovered in P. chrysosporium (Larrondo et al., 2003). In this work we show that mco1 is a target gene of the recently described ACE1 transcription factor in P. chrysosporium.

**METHODS**

**Strain and culture conditions.** P. chrysosporium homokaryotic strain RP-78 was obtained from the Center for Mycology Research, Forest Products Laboratory, Madison, WI, USA. For solid-state incubations, P. chrysosporium spores were inoculated on potato-dextrose agar plates and grown for 1 week at 39 °C. Spores were collected by flooding the agar plates with 5 ml sterile water, and 2 × 109 spores were inoculated in 20 ml carbon-limited stationary liquid cultures as previously described (Brown et al., 1993). Cultures were harvested after 4 days of incubation. When indicated, they were supplemented with CuSO4 to a final concentration of 0.25 mM for the indicated times.

**Analysis of the promoter regions of mco genes.** Using the reported cDNA sequences of mco1, mco2, mco3 and mco4 (GenBank accession numbersAY225437, AY532139, AY532142 and AY532149, respectively) and by means of BLAST, mco genes were localized in the P. chrysosporium genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). Taking into consideration the presence of several gene models, each promoter was manually obtained and arbitrarily defined as the corresponding intergenic region. This procedure led to uncovering of promoter regions of 1407, 827 and 537 bp for mco1, mco2 and mco3, respectively. Since the closest upstream gene model for mco4 is located 5.2 kbp away, the promoter region was arbitrarily defined as the sequence 1500 bp upstream from the mco4 translation start site. These sequences were examined for the presence of putative ACE elements using the MatInspector software (http://www.genomatix.de/products/MatInspector/). The analysis was restricted to fungal transcription binding sites.

**RNA extraction.** After 4 days of growth, the mycelia obtained from five independent flasks (processed as a batch) were separated from the culture fluid by filtration through Miracloth (Calbiochem) and frozen in liquid nitrogen. The frozen mycelium was ground to a powder in a mortar containing liquid nitrogen. The powder was homogenized in the same mortar by the addition of 4 ml Tris/HCl buffer (0.2 M Tris/HCl pH 7.5, 0.5 M NaCl, 0.01 M EDTA, 1% SDS, 50 mM β-mercaptoethanol) and 4 ml phenol/chloroform/isooamyl alcohol (25: 24: 1, by vol.). Each sample was transferred to a 50 ml RNase-free tube and vortex-homogenized. In the same tube and using a sterile syringe and needle, the fluid was pipetted up and down several times to shear genomic DNA (gDNA). Thereafter, the fluid was transferred to four 2 ml Eppendorf tubes. After centrifugation (15 min at 14 000 r.p.m.), the aqueous phase was phenol-extracted in a clean tube and the RNA was obtained as described by Manubens et al. (2003). Ten micrograms of total RNA was fractionated by electrophoresis in a formaldehyde-agarose gel (1.2% w/v) and RNA integrity was verified by ethidium-bromide staining. Poly(A) mRNA was obtained from 100 μg total RNA using the mRNA DIRECT kit (Dynal), according to manufacturer’s instructions. To ensure the absence of gDNA, the poly(A) mRNA obtained was incubated at 37 °C for 30 min using RQ1 RNase-free DNase (Promega), according to the manufacturer’s instructions. For each sample, 2 μl treated mRNA was collected before proceeding with the retrotranscription step. To check for potential gDNA contamination, RT-minus reactions were carried out in real time using SYBR Green detection chemistry. No cycle threshold (Ct) values were obtained, at least under the cycle conditions used (see below). Only those mRNA samples that did not show gDNA contamination were reverse transcribed using the MMLV reverse transcriptase (Invitrogen) for 45 min at 42 °C, according to manufacturer’s instructions. The cDNA obtained was diluted twofold.

**Real-time quantitative RT-PCR (qRT-PCR).** Relative quantification of mco transcript levels was performed in real time using the Brilliant SYBR Green QPCR Master Reagent kit and the Mx3000P detection system (Stratagene). Primer sequences and predicted Tm values, as well as amplicon length, are shown in Table 1. Levels of mRNA from the glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene were used for normalization. The qRT-PCR mixture (25 μl)
Table 1. Oligonucleotides employed for qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Predicted T_m (°C)</th>
<th>Amplicon length (bp)</th>
</tr>
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<tbody>
<tr>
<td>mco1 Fw</td>
<td>CATACCGAGTGCGGAATACC</td>
<td>58.6</td>
<td>101</td>
</tr>
<tr>
<td>mco1 Rv</td>
<td>CGCTGTGATATTGGGATGC</td>
<td>59.3</td>
<td></td>
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<tr>
<td>mco2 Fw</td>
<td>CTCGCCGTAATATTTCCCATGT</td>
<td>58.9</td>
<td>107</td>
</tr>
<tr>
<td>mco2 Rv</td>
<td>ACCCGAGGGCGGAAGTGA</td>
<td>59.5</td>
<td></td>
</tr>
<tr>
<td>mco4 Fw</td>
<td>TGGCATAAACGTTGATTTGCACT</td>
<td>59.9</td>
<td>91</td>
</tr>
<tr>
<td>mco4 Rv</td>
<td>TATAGACGCGACCGGTAGGT</td>
<td>58.1</td>
<td></td>
</tr>
<tr>
<td>gapdh Fw</td>
<td>TGCTGATGCCTCCCATGT</td>
<td>60.0</td>
<td>81</td>
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<tr>
<td>gapdh Rv</td>
<td>CAGGACGCGTTCGAGATG</td>
<td>58.0</td>
<td></td>
</tr>
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In *vitro* transcription–translation of the *P. chrysosporium* ACE1 transcription factor (Pc-ACE1). The previously cloned Pc-ACE1 transcription factor (Polanco et al., 2006) was subcloned into the NdeI and BamHI sites of the bacterial expression vector pET21a (Novagen). Primers to obtain the *S. cerevisiae* ACE1 (Sc-ACE1) coding sequence (5'-CATATGTCGTAATTAAGGGGTTCA-3’, direct, and 5'-GGATCCCTTGTGAATGTTA-3’, reverse) were designed in accordance with the reported sequence (GenBank accession number AY557820). Because of the absence of introns, the Sc-ACE1 coding sequence was obtained by PCR using gDNA. The Sc-ACE1 transcription factor was also cloned into the NdeI and BamHI sites of the pET21a vector. Nucleotide sequences were determined with the ABI Prism Big Dye terminator cycle sequencing kit on ABI automated sequencers (Applied Biosystems). Bacterial heterologous expression was unsuccessful (data not shown). Therefore, taking into account the uncloned part of the gene, the probe was PCR-amplified using primers 5'-ATTAAATAGA-GGCCGTCGCCAGACC-3’ (direct) and 5'-AGAGTTTTAGAATGCAGG-CAAATGGAAGGATG-3’ (reverse). A specific PCR product was obtained, cloned into pGEM-T vector (Promega) and sequenced. The probe was PCR-amplified in the presence of [α-32P]dCTP using the obtained plasmid as a template. In addition, 32P-end-labelled double-stranded oligomers containing one of the ACE elements identified in the promoter region of *mco1* were used as probe. Radioactive labelled probes were purified by PAGE.

**Electrophoretic mobility-shift assays (EMSAs).** Standard binding reaction mixtures (30 μl) contained: 30 mM HEPES/KOH buffer pH 7.5, 2.0 mM EDTA, 0.1 mM ZnSO4, 0.2 mM MgCl2, 75 mM DTT, 12% glycerol, 3% PEG 4000, 30 μg poly(dl)–dc), and 300 ng Bluescript KSII plasmid. The concentrations of CuSO4, and ascorbic acid (included to reduce Cu2+ to Cu+) were 0.15 mM, unless otherwise specified. To each binding reaction, 2 μl rabbit reticulocyte lysate containing the synthesized Sc-ACE1 or Pc-ACE1 transcription factor was added, as well as 50 ng (20 000–40 000 c.p.m.) labelled DNA probe. After incubation for 15 min at room temperature, samples were separated by electrophoresis as described previously (Polanco et al., 2002). No DNA–protein interaction was observed when binding assays were carried out with 2 μl rabbit reticulocyte lysate in which the translation reaction was omitted (data not shown). Binding assays shown in Fig. 3(D) were carried out in the absence of AgNO3. For competition experiments, labelled probe was premixed with the unlabelled probe or the non-specific competitor.

**Pc-ACE1-mediated in *vitro* transcription.** Promoter activation mediated by Pc-ACE1 was studied using an in *vitro* transcription system. First, a 500 bp DNA fragment corresponding to the upstream sequence from the *mco1* translation start site containing one ACE element was PCR amplified using primers 5'-ATTTAAATAGA-GGCCGTCGCCAGACC-3’ (direct) and 5'-AAAGTTTATGAGAATGCAGG-CAAATGGAAGGATG-3’ (reverse). After this procedure, the 589 bp CMV promoter of the mammalian expression vector pEGFP-N1 (Clontech) was removed using Asel and HindIII restriction endonucleases and replaced with the aforementioned 500 bp promoter fragment. Similarly, the complete mco2 promoter (as defined above, ‘Analysis of the promoter regions of *mco* genes’; 827 bp) was PCR-amplified using primers 5'-ATTAAATAGAGGGCTGGTCCGTCTG-3’ (direct) and 5'-AAAGTTTATGAGAATGCAGG-CAAATGGAAGGATG-3’ (reverse) and cloned into the pEGFP-N1 vector as mentioned. In each case, the containing the respective *mco* promoter region was linked to an EGFP reporter gene. In *vitro* transcription mixtures (25 μl) contained 3.3 μl HeLa nuclear extract (Promega), 0.4 μl of each ribonuclease, 100 ng linearized plasmid DNA template, 40 units RNase OUT and 3 μl rabbit reticulocyte lysate containing the in *vitro*-synthesised Pc-ACE1 transcription factor in 20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 3 mM MgCl2, 20% (v/v) glycerol. When indicated, reaction mixtures were also supplemented with 25 μM CuSO4, 25 μM methionine. Thereafter, 1 μl of each translation product was separated by SDS-PAGE using standard procedures. The SDS-PAGE gel was fixed, dried and exposed on scientific autoradiographic imaging films (Kodak), thus allowing the confirmation of the predicted molecular mass of each protein (see Fig. S1).

**Preparation of labelled probes.** A probe containing two previously described ACE elements from the *S. cerevisiae* cap1 gene (Calutta et al., 1989) was PCR-amplified using primers 5'-GATCTCTGCTTGGCTTGATGAATGATT-3’ (direct) and 5'-GAGAATCTCATATTCGTTGATGAATGATT-3’ (reverse). A specific PCR product was obtained, cloned into pGEM-T vector (Promega) and sequenced. The probe was PCR-amplified in the presence of [α-32P]dCTP using the obtained plasmid as a template. In addition, 32P-end-labelled double-stranded oligomers containing one of the ACE elements identified in the promoter region of *mco1* were used as probe. Radioactive labelled probes were purified by PAGE.
ascorbate and 25 μM KCN. Control reactions were carried out with 3 μl rabbit reticulocyte lysate in which the translation reaction was omitted. Following transcription, reporter gene mRNA levels were measured by qRT-PCR using primers 5’-GAGAAGCCGGATCACATGGT-3’ (direct) and 5’-TTACTTGACAGCTGTCATGC-3’ (reverse), as described above.

RESULTS

Effect of copper on transcript levels of mco genes

The effect that copper exerts at the transcriptional level of mco genes in various organisms (see above), and the previous detection of putative ACE elements in the promoter of *mco1* from *P. chrysosporium* (Polanco et al., 2006), prompted us to examine whether this metal regulates the expression of *mco1*, *mco2* and *mco4*. As described previously, the *mco3* gene is transcriptionally inactive due to the insertion of an 8.14 kb gypsy-class retroelement within the 12th intron (Larrondo et al., 2007b). Therefore, it was not analysed in this study. As shown in Fig. 1, the addition of copper to the culture medium led to a 4-fold increase in *mco1*-transcript levels after 30 min, while *mco2* transcripts were increased 2.5-fold. In the case of *mco4*, no major change was observed during the period analysed.

Analysis of mco promoter regions

These results prompted us to examine the promoters of *mco* genes to look for the presence of ACE elements. The four clustered *mco* genes (*mco1* to *mco4*) are located on Scaffold 9 of the assembly version 2.0 of the *P. chrysosporium* genome database (Larrondo *et al.*, 2004). Their respective promoter regions were defined and searched for the presence of regulatory elements as described in Methods. As expected, two putative ACE elements were identified in the promoter of *mco1* (Polanco *et al.*, 2006) (Fig. 2A). Both adhere to the consensus sequence of the previously described ACE elements in the promoter regions of the *S. cerevisiae* *cup1* and *sod1* genes (Fig. 2B). The ACE sequences in the promoter of *mco1* are centred between positions −306 to −292 and −849 to −835 from the *mco1* translation start site (Fig. 2C). On the other hand, although *mco2*-transcript levels showed a slight increase (Fig. 1), no putative ACE elements were identified in its promoter. Likewise, no ACE-like sequences were found in the upstream regulatory region of *mco4*.

DNA-binding activity of Pc-ACE1

It seemed highly likely that the higher levels of *mco1* transcripts observed upon incubation of cultures with copper are mediated by Pc-ACE1. In order to test this hypothesis, as a first approach we characterized the DNA-binding activity of the *in vitro*-synthesized Pc-ACE1 protein to the two previously described ACE elements from the *S. cerevisiae* *cup1* gene (Culotta *et al.*, 1989). The EMSAs shown in Fig. 3 illustrate the specific binding of Pc-ACE1 to a DNA probe containing these sequences. In this experiment, two DNA–protein complexes were observed (Fig. 3A, lane 2). The lower-mobility complex appears as a slight band signal that shows some level of competition with the unlabelled probe. On the other hand, a stronger band corresponding to a higher-mobility DNA–protein complex was readily displaced when binding assays were carried out in the presence of a molar excess of unlabelled DNA probe (Fig. 3A, lanes 3–5). Displacement was much more limited when assays were conducted in the presence of non-specific DNA competitor (Fig. 3A, lanes 6–8). Similar results were obtained using the same DNA probe with the *in vitro*-synthesized ACE1 protein from *S. cerevisiae*, used as a positive control (see supplementary Fig. S1, available with the online version of this paper).

In order to determine whether Pc-ACE1 can specifically interact with a probe containing one of the ACE sequences identified in the promoter of *mco1*, a probe containing the element located between positions −306 and −292 was synthesized (Fig. 3B). As shown in Fig. 3(C), this 32P-labelled 40 bp oligomer forms an easily observed DNA–protein complex with Pc-ACE1. An equivalent result was also observed using a 100 bp DNA probe (data not shown). To confirm the specificity of this association, competition experiments were carried out using double-stranded unlabelled 40 bp DNA oligomers containing either the same ACE element or a mutant version of this ACE with five point mutations (Fig. 3B). Formation of the DNA–protein complex was completely inhibited by incubation...
with excess wild-type oligomer, whereas the mutant competitor was less effective (Fig. 3C). Although this result indicates that the mutated sequence is important for the observed protein–DNA interaction, some additional DNA sequences may also be involved in complex stabilization.

A further control of binding specificity was conducted by measuring the effect of copper on complex formation. As indicated in Methods, standard binding assays were conducted in the presence of this metal. No DNA–protein complex was observed in the absence of copper (Fig. 3D, lane 7). Addition of KCN, a specific chelator of Cu
+ inhibited the DNA–protein complex formation in a concentration-dependent manner (Fig. 3D, lanes 3–5). To avoid copper reduction, a binding assay containing 25 µM CuSO4 was also carried out, in the absence of ascorbic acid. As shown in Fig. 3(D), lane 6, a weak DNA–protein complex signal was observed under these conditions. These results indicate that Pc-ACE1, similar to Sc-ACE1 (Buchman et al., 1989), requires Cu
+ for binding to the DNA probe.

**Pc-ACE1 mediates the activation of ACE-containing promoters**

Three different lines of evidence strongly suggest that Pc-ACE1 mediates mco1 promoter activation: (a) the copper-dependent increase of mco1-mRNA; (b) the presence of two ACE elements in the promoter region of this gene and (c) the copper-dependent DNA-binding of Pc-ACE1 to one of these two ACE elements. In order to confirm that Pc-ACE1 is involved in the copper-dependent transcription induction of the mco1 promoter, a cell-free transcription system was employed. This consisted of a linearized plasmid containing the mco1 promoter region linked to an EGFP reporter gene, a HeLa nuclear extract providing RNA polymerase plus general transcription factors and the Pc-ACE1 protein (see Methods). The basal level of transcription was greatly increased by adding Pc-ACE1 in the presence of Cu
+ (Fig. 4, lanes 1 and 2). In contrast, no transcriptional activation was observed when ascorbate was omitted, to prevent reduction of Cu
+ to Cu
0 (Fig. 4, lane 3) or when Cu
0 was chelated with KCN (Fig. 4, lane 4). Finally, the transcriptional activity of the mco2 promoter was also tested. In this case, no transcriptional activation was observed (Fig. 4, lanes 5 and 6).

**DISCUSSION**

In this work, we report that copper increases mRNA levels of mco1 and mco2, but not of mco4. Among these genes, only the promoter of mco1 showed the presence of two copper-dependent cis-acting regulatory elements known as ACE. These had been originally identified in the promoter regions of the *S. cerevisiae* copper metallothioneins *cup1* and *crs5*, as well as in the promoter region of the superoxide dismutase gene *sod1* (Culotta et al., 1989, 1994; Gralla et al., 1991). This element is the recognition site for the ACE1 transcription factor, which activates the transcription of target genes in response to copper. Prior to the present work, we had reported the cloning and sequencing of Pc-ace1, the gene encoding an ACE1 transcription factor in *P. chrysosporium*. However, until...
**Fig. 3.** Competition analysis by EMSAs. (A) Top, schematic representation of the *S. cerevisiae* cup1 gene promoter and its related 100 bp DNA probe containing two ACE elements (white boxes); the position of each element (superscript numbers) is indicated with respect to the translation start site (ATG). Bottom, binding assays conducted with this probe (ACEcup1) and *in vitro*-synthesized Pc-ACE1 protein. Lanes contain: (1) free probe; (2) standard binding reaction without competitor; (3, 4, 5) binding assays in the presence of 10-, 25- or 50-fold excess of unlabelled specific DNA probe, respectively; (6, 7, 8) binding assays in the presence of 10-, 25- or 50-fold excess, respectively, of a 101 bp unrelated DNA as a non-specific competitor. (B) Synthetic oligonucleotides used as double-stranded competitors in the experiment shown in (C). Boxes denote the wild-type ACE element (WT) and the corresponding region within the mutant (MUT) competitor. Wild-type and substituted residues within the ACE consensus sequence are indicated in bold type. (C) Binding assays conducted with a 40 bp DNA probe containing the ACE element centred between positions −306 and −292 from the mco1 translation start site (Fig. 2C) and the *in vitro*-synthesized Pc-ACE1 transcription factor. Lanes contain: (1) free probe; (2) standard binding reaction without competitor; (3, 4, 5) binding assays in the presence of 10-, 25- or 50-fold excess unlabelled specific WT DNA probe, respectively; (6, 7, 8) binding assays in the presence of 10-, 25- or 50-fold excess unlabelled MUT competitor, respectively. (D) Binding assays conducted with the same DNA probe and Pc-ACE1 transcription factor. Lanes contain: (1) free probe; (2) standard binding reaction; (3, 4, 5) binding reactions in the presence of 25, 50 or 100 mM KCN, respectively, as Cu<sup>2+</sup>-specific chelator; (6) binding reaction in the presence of 150 μM Cu<sup>2+</sup> or (7) in the absence of copper.
ACE1 strain lacking a functional ACE1 transcription factor. In this paper, the possible target gene(s) of Pc-ACE1 remained unknown. Now, the possible target gene(s) of Pc-ACE1 remained unknown.

The Pc-ace1 cDNA complements in vivo a mutant yeast strain lacking a functional ACE1 transcription factor. In this ACE1Δ yeast strain, Pc-ACE1 was strictly required for growth in the presence of a high concentration of copper. Notably, Pc-ACE1 restored the copper inducibility of the yeast cup1 gene (Polanco et al., 2006). In order to characterize the DNA-binding activity of Pc-ACE1, EMSAs were carried out using the in vitro-synthesized Pc-ACE1 transcription factor. This protein was tested with DNA probes containing ACE elements either from yeast or from P. chrysosporium. As a positive control, we also synthesized the S. cerevisiae ACE1 (Sc-ACE1). EMSAs using a DNA probe containing the two previously described ACE elements from the promoter region of the cup1 gene from yeast (Culotta et al., 1989) showed specific DNA–protein interaction of both Sc-ACE1 (Fig. S1) and Pc-ACE1 (Fig. 3).

With each transcription factor, two DNA–protein complexes were obtained. Cysteine amino acids located within the copper-fist DNA-binding domain of Sc-ACE1 have been described as key residues that participate in DNA-binding activity and also as being involved in DNA target site specificity (Buchman et al., 1990). Since these cysteine residues are conserved in Pc-ACE1 (Polanco et al., 2006), a similar binding pattern of the two transcription factors was not unexpected. Two DNA–protein complexes had also been observed with Sc-ACE1 and a similar DNA probe (Hu et al., 1990). In that work, the authors observed that the lower-mobility complex was obtained only at high protein concentrations, whereas the higher-mobility complex was observed at both low and high protein concentrations. When the cup1 ACE elements are placed in separate probes, Sc-ACE1 binds efficiently, forming only one DNA–protein complex (Hu et al., 1990). Consequently, it has been proposed that when both elements are located within the same DNA molecule, the higher-mobility complex corresponds to the occupancy of one DNA-binding site, whereas the DNA–protein complex showing a lower migration in the gel results from the occupancy of both ACE elements (Hu et al., 1990). This result clearly indicates that Pc-ACE1, when interacting with and recognizing canonical ACE elements, behaves like Sc-ACE1. It also explains, at a molecular level, the reported copper-dependent Pc-ACE1-mediated induction of the yeast cup1 gene in an ACE1A background (Polanco et al., 2006). The presence of two ACE elements in the promoter of mco1 led us to examine the binding of Pc-ACE1 to at least one of them. The chosen element was centred between positions −306 and −292 from the mco1 translation start site, similar to one of the ACE elements of the yeast cup1 gene (see Fig. 3). Fig. 3(C) illustrates that Pc-ACE1 does interact with this ACE element. The specificity of this interaction was tested in competition experiments with the same unlabelled probe and with an unlabelled probe including five point mutations. Four of these five mutations were located within the invariant 5′-GCTG-3′ ACE core (Fig. 3B) (Gralla et al., 1991). Although some displacement of the complex was observed with the mutant probe, the complex was completely displaced with a 50-fold molar excess of the unlabelled probe possessing the wild-type ACE element. This result indicates that the complex was formed by sequence-specific interactions, as has also been demonstrated for Cuf1, a Schizosaccharomyces pombe transcription factor that has a DNA sequence binding specificity similar to the ACE1 protein from Saccharomyces cerevisiae (Beaudoin et al., 2003). The requirement for Cu⁺ in the incubation mixture is also indicative of the specificity of this binding (Fig. 3D). It has been described that this metal, when binding to the copper regulatory domain within Sc-ACE1, stabilizes a specific tertiary fold forming a tetracopper thiolate cluster (Winge, 1998).

In order to demonstrate that Pc-ACE1 can stimulate the transcriptional activity of an ACE-containing promoter, an in vitro transcription assay was developed. As shown in Fig. 4, Pc-ACE1 enhances the transcription of a reporter gene linked to a DNA fragment encompassing 500 bp upstream of the mco1 translation start site. This promoter fragment contains the same ACE element first studied by EMSA. Interestingly, no transcriptional activation was observed in the absence of Cu⁺, or when Cu⁺ was
chelated with KCN. These results may account for the higher mco1-mRNA levels observed in vivo in cultures of P. chrysosporium supplemented with copper.

We also observed higher mco2-mRNA levels when cultures of P. chrysosporium were supplemented with copper. The absence of ACE elements in the promoter of mco2 made this result difficult to interpret. We then decided to test whether Pc-ACE1 can stimulate the transcription of a reporter gene linked to the promoter of mco2. As described in Results, this stimulation does not take place, suggesting that mco2 may not be a direct target of Pc-ACE1. In this regard, through hierarchical clustering of microarray data, a group of six differentially expressed genes that are highly induced in response to copper have been observed in S. cerevisiae. Among these, only cup1 and crs5, but not the other four genes, contain ACE elements in their respective promoter regions (van Bakel et al., 2005). It is conceivable that copper may induce gene expression by a mechanism different to that mediated by ACE1 transcription factor, such as, for example, the generation of reactive oxygen species. Clearly, further work is necessary to clarify the molecular mechanisms underlying the increment in mco2-mRNA levels in response to copper.

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