Identification and functional expression of *tahA*, a filamentous fungal gene involved in copper trafficking to the secretory pathway in *Trametes versicolor*

Andreas Uldschmid, Michael Engel, Renate Dombi and Karin Marbach

Author for correspondence: Karin Marbach. Tel: +49 89 2180 6952. Fax: +49 89 2180 6999.
e-mail: marbach@lmb.uni-muenchen.de

Institute for Biochemistry,
Ludwig Maximilians
Universitaet Muenchen,
Feodor-Lynen-Strasse 25,
D-81377 Munich, Germany

In this study, cDNA and genomic clones encoding a homologue of the yeast gene anti-oxidant 1 (*ATX1*) from the white-rot fungus *Trametes versicolor*, a basidiomycete known to produce several laccase isoenzymes involved in lignin degradation, were identified. This gene, named *Trametes ATX* homologue (*tahA*), encodes a protein of 7.9 kDa with 56% identity to the yeast *Atx1p* sequence. Two different alleles of *tahA* were obtained that differed mainly in their intervening sequences and in a 425 nt insertion located 183 nt upstream of the transcription start site. *tahA* is present as one copy per haploid nucleus in *T. versicolor*, as shown by Southern analysis. Expression of *tahA* cDNA restored high-affinity iron uptake in a $\Delta atx1$ yeast strain and oxygen sensitivity in a $\Delta sod1 \Delta sod2$ yeast strain, showing that *tahA* is also a functional homologue of *ATX1*. The inability of *tahA* to rescue the $\Delta sod1$ phenotype on copper-deficient medium indicated that *tahA* function is copper-dependent. Sequence analysis of the *tahA* promoter revealed several motifs that were similar to the conserved motifs found in the copper-regulated metallothionein and Cu, Zn superoxide dismutase genes, *CUP1* and *SOD1*, of *Saccharomyces cerevisiae*, *Neurospora crassa* and *Candida glabrata*. In contrast to its yeast homologue *ATX1*, *tahA* is induced under elevated copper concentrations in the medium ($> 0.25 \mu\text{M CuSO}_4$) and repressed under copper starvation. The transcription of *tahA* was analysed in response to copper and iron, and after adding xenobiotica. The results are discussed in relevance to laccase expression.

Keywords: white-rot fungi, metallochaperone, laccase

INTRODUCTION

The micronutrient copper (Cu) functions as an important co-factor in numerous enzymes that participate in redox reactions. These include proteins involved in the detoxification of oxygen radicals, for example, the (cytoplasmic) Cu, Zn superoxide dismutase (SOD), electron-transport proteins, such as (mitochondrial) cytochrome *c* oxidase, and proteins with oxidase activity, such as fungal laccase, a secreted phenoloxidase, or plant ascorbate oxidase.

Although being an essential co-factor, copper can be toxic even at low concentrations: Cu(I) and Cu(II) ions may bind with high affinity to inappropriate sites in non-copper proteins (Predki & Sakar, 1992) and they can participate in the aerobic generation of oxygen radicals, thus catalysing the auto-oxidation of biomolecules such as lipids, proteins and nucleic acids (Halliwell & Gutteridge, 1984). Organisms possess several mechanisms to maintain intracellular copper concentrations at adequate levels. These include various copper homeostasis factors that control the uptake, distribution and sequestration of this metal inside the cell. For example, under high copper concentrations, cells express metallothionein-like proteins that bind the metal tightly. Under normal cellular copper concentrations, estimated for the yeast cell model to be less than one atom of free copper

Abbreviations: BCS, bathocuproinedisulfonic acid; DABA, diamino-benzaldehyde; Ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid; IS, insertion sequence; SOD, superoxide dismutase.

per cell (Rae *et al.*, 1999), cells express so-called copper metallochaperones that guide and protect the copper ions, and facilitate their proper delivery to their different destinations, e.g. to mitochondria, to the secretory pathway and to cytoplasmic enzymes (O'Halloran & Culotta, 2000).

In the yeast *Saccharomyces cerevisiae* there is an overlap between copper homeostasis and oxygen-radical metabolism (Culotta *et al.*, 1995; Tamai *et al.*, 1993). The yeast gene encoding the copper metallochaperone Atx1p (anti-oxidant) was originally isolated as a gene conferring protection against oxidative stress in yeast (Lin & Culotta, 1995). Atx1p is a cytoplasmic copper chaperone that binds copper and delivers it to an intracellular copper-transporting P-type ATPase, Ccc2p, located in the Golgi compartment of the secretory pathway (Pufahl *et al.*, 1997). Ccc2p pumps copper into the lumen of the Golgi, where copper is then inserted into secreted copper-dependent enzymes. The protein factors involved in cellular copper homeostasis are highly conserved between eukaryotes: copper metallochaperones homologous to Atx1p and to the copper-transporting P-type ATPases have been described in yeast, mammal, nematode and plant systems (Himmelblau *et al.*, 1998; Hirayama *et al.*, 1999; Hung *et al.*, 1997; Klomp *et al.*, 1997; Payne & Gitlin, 1998; Wakabayashi, 1998). However, these factors seem to be regulated differently in the various systems.

In filamentous fungi there are no factors described to date that mediate copper supply to the secretory pathway (i.e. copper chaperone and P-type ATPase). As these factors appear to play an important role in the biogenesis of the copper-dependent laccases thought to participate in lignin degradation, we decided to characterize them in a fungal system and study their regulation.

Our organism of choice, *Trametes versicolor*, is a white-rot fungus capable of secreting substantial amounts of laccases. Here we describe the identification of the gene *tahA* (*Trametes* ATX homologue) from the basidiomycete *T. versicolor*, which encodes a protein of 72 aa with 56% sequence identity to yeast Atx1p. We characterized the gene and its product on the structural, biochemical and functional levels. We studied its transcriptional regulation when exposed to different concentrations of metals, such as copper or iron, of xenobiotics, such as diaminobenzaldehyde (DABA), and of the redox-cycling drug paraquat, and discuss the results in relevance to conserved motifs found in the 5'-non-coding regulatory sequence of *tahA* and to the expression or biogenesis of laccases.

METHODS

Organisms and culture conditions. The *Escherichia coli* strain used in this study was XL-1 Blue MRF'. The *S. cerevisiae* strains used were EG103 (MATa/MAT α leu2-3/his3-112 Δ 1/trp289a/ura3-52/GAL⁺), SL103 (isogenic to EG103 with Δ atx1::LEU2) and SL133 (isogenic to EG103 with Δ sod::URA3, Δ sod2::TRP1, Δ atx1::LEU2); they were obtained from Valeria Culotta, John Hopkins University School

of Public Health, Baltimore, MD, USA. *S. cerevisiae* strains were grown in yeast nitrogen base (YNB) in liquid and on solid medium. Carbon sources were used at a concentration of 2%. 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid (Ferrozine) was added up to a concentration of 2.5 mM, bathocuproinedisulfonic acid (BCS) was added up to a concentration of 50 μ M and paraquat was added up to a concentration of 50 μ M to solid medium.

The basidiomycete *T. versicolor* belongs to the ecological group of white-rot fungi. In this study, the dikaryotic strain TV-1 [deposited in the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1B, D-38124 Braunschweig, Germany) as DSM 11523] and two monokaryotic strains, F6 and Sp4, derived from TV-1 via sporulation and harbouring distinct alleles of *tahA* were used. *T. versicolor* was maintained at 4 °C on malt agar plates (3% malt extract, 0.3% peptone, 1.5% agar, pH 5.0). Liquid cultures were grown at 30 °C in minimal medium comprising 20 g glucose, 1 g potassium dihydrogen phosphate, 0.5 g magnesium sulfate, 0.1 g disodium hydrogen phosphate, 27.5 mg adenine, 0.15 g DL-phenylalanine, 2.5 g L-asparagine, 0.48 mg thiamin, 10 mg calcium phosphate, 10 mg ferric sulfate, 2 mg cupric sulfate, 1 mg zinc sulfate and 1 mg manganese sulfate per litre. Three agar plugs (diameter, 7 mm) of a fungal colony (grown for up to 7 days on malt agar) were used to inoculate 100 ml liquid standing cultures in 550 ml (182 cm²) tissue-culture flasks. These pre-cultures were homogenized with an UltraTurrax apparatus (3500 r.p.m., 1 min) and used as the inoculum for the main culture. The main cultures of *T. versicolor* were grown in 300 ml minimal medium on a rotary shaker (110 r.p.m.) at 28 °C. Laccase expression was induced by adding 20 μ g DABA ml⁻¹ to a 4-day-old culture.

Plasmids and DNA libraries. A genomic DNA library from *T. versicolor* was obtained from Dr Rupert Pfaller. The library was constructed with partially (*Sau*3A) digested total *T. versicolor* (TV-1) DNA that had been separated on a preparative gel. Fragments of between 5 and 25 kb in size were cloned into the *Bam*HI site of the vector Lambda ZAP Express (Stratagene) and amplified in XL-1 Blue according to the supplied protocol.

A *Trametes* cDNA library that we had previously constructed in the two-hybrid vector pJG4-5 was used as a template for the isolation of *tahA* by PCR. For the construction of the *T. versicolor* cDNA library in pJG4-5, the purified mRNA was converted into cDNA using the cDNA Synthesis Kit (Stratagene). The first strands were synthesized as described in the protocol supplied, with a linker-primer that contained a *Xho*I site. After synthesis of the second strands, ligation of the *Eco*RI linkers and restriction with *Xho*I, the resulting cDNA was separated by gel filtration (using CL-Sepharose); the 0.2–5 kb fragments were pooled and used for directional ligation into the yeast two-hybrid vector pJG4-5. As tested with restriction analysis of inserts from different clones, the cDNA library contains inserts ranging from 0.1 to 3.5 kb in size. This library was used for the isolation of *tahA* fragments by PCR with degenerate oligonucleotides and for the isolation of full-length *tahA* clones.

A *tahA*-overexpressing yeast vector was constructed from pZER0-tah20, a pZER0 (Invitrogen) derivative carrying the *tahA* cDNA in its multiple-cloning site. The *tahA* cDNA from pZER0-tah20 was excised with *Eco*RI and *Xho*I and directionally ligated into the yeast centromere plasmid pAH (Feldmann *et al.*, 1996) via the *Eco*RI and *Sall* sites. The single-copy vector pAH is a derivative of pRS313 (Sikorski & Hieter,

1989) and carries *ars*/*CEN* sequences and the *HIS3* gene for maintenance and selection in yeast. The resulting yeast yCp plasmid, pAH-*tah*, harbours the *tahA* cDNA under the control of the yeast *ADH1* promoter and terminator, and carries the *HIS3* gene for selection in yeast. The identity of *tahA* was verified by DNA sequencing. As a positive control, the yeast *ATX1* gene was cloned into pAH. To create an *EcoRI* site at the 5' end and an *XhoI* site at the 3' end of *ATX1*, two primers (ATX-Eco-FW, 5'-GGA ATT ACC ATG GCA GAG ATA AAA CAT TA-3', and ATX-Xho-RV, 5'-CG CTC GAG TCA CAA TTG TTT GCC AGA T-3') were used in a PCR with *Pwo* polymerase (Roche) and yeast genomic DNA. The *ATX1* gene obtained was directionally ligated into pAH, resulting in the vector pAH-*ATX* as confirmed by DNA sequencing. This vector served as a positive control in drop-test experiments. The original vector, pAH, was used as the negative control.

Preparation of *Trametes* DNA and Southern blot analysis. For preparation of DNA, mycelia of *T. versicolor* were separated from the culture medium by filtration through a double layer of cheese cloth, washed with distilled water, dried between paper towels and frozen in liquid nitrogen. Large amounts of cells (up to 5 g of semi-dried mycelia) were disrupted by grinding with a pestle and mortar in liquid nitrogen. Smaller amounts of mycelia (up to 400 mg of semi-dried mycelia) were disrupted using the Micro-Dismembrator U (B. Braun Biotech). The ground mycelia were then dissolved in 5 ml extraction buffer (200 mM Tris/HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) (g mycelia)⁻¹. Proteins were separated from DNA by phenol extraction according to Sambrook *et al.* (1989). The DNA was precipitated from the aqueous phase with 0.54 vols 2-propanol, washed with 70% ethanol and then dissolved in TE buffer (10 mM Tris/HCl, pH 8, 1 mM EDTA).

For Southern blot analyses, DNA samples of the diploid (dikaryon TV-1) and the haploids (monokaryons F2, F6, SP4 and SP17) were digested with *XhoI* and *XbaI*. In each lane, 10 µg DNA was digested with 10 U restriction enzyme (µg DNA)⁻¹ for 12 h. The DNA was separated by 1% agarose gel electrophoresis and transferred to a nylon membrane by capillary blotting. For the probe, cDNA of *tahA* was cut out of the yeast expression vector pAH (Feldmann *et al.*, 1996) and labelled with [³²P]dATP using the Random Primers Labelling System (Gibco). The blot was hybridized with the probe according to Church & Gilbert (1984) overnight at 65 °C, washed and then exposed to a phosphorous screen (Kodak).

Isolation of mRNA from *T. versicolor* for library construction. Total RNA from *T. versicolor* was prepared according to Logemann *et al.* (1987), starting with 2 g of semi-dried mycelium. The frozen mycelium together with 7 ml Z6 buffer (8 M guanidium hydrochloride, 20 mM MES, pH 7.0, 20 mM EDTA) and 1% (v/v) β-mercaptoethanol was ground with a pestle and mortar in liquid nitrogen. After phenol extraction and ethanol precipitation, total RNA was dissolved in up to 1 ml diethylpyrocarbonate (DEPC)-treated water. The amount of total RNA obtained ranged from 1 to 1.5 mg. Isolation of mRNA from total RNA was performed using the mRNA Purification Kit (Pharmacia) according to the manufacturer's instructions.

Isolation of total RNA from *T. versicolor* for Northern and S1-protection analyses. Mycelia (500 mg) were frozen in liquid nitrogen and then ground with the Micro-Dismembrator U (B. Braun Biotech). RNA from the ground cells was isolated with 5 ml TRIZOL-Reagent (Gibco-BLR) according to the plant protocol. To remove the polysaccharides, the RNA was precipitated with a high-salt buffer also described in the Gibco

protocol. The resulting RNA was then dissolved in 100 µl H₂O for further use. The S1-protection assay was performed according to Weaver & Weissmann (1979) with 2 pmol each of primer *tah* (5'-*GTAAGTGTGCTCGGACATGGTGTA-TATCGGTTCAAGAGGCGGGGGATCGAACGGGCAG-GGGGTGCGGAGGCAGAAAGTCAGTCAGCA-3') and primer *gapDH* (5'-*GTGGGCGTGCGAGGAGTCCCAAG-GAGGCCGTTGAATGCGGCAGAAAGGCCCGCCATT-CAGTCAGCA-3') that had been end-labelled (*) with 150 µCi (5.55 MBq) [^γ-³²P]ATP using polynucleotide kinase. The labelled primers (10³ c.p.m.) were then added to 50 µg total RNA in 1 M NaCl/330 µM EDTA (pH 8)/160 mM HEPES (pH 7.5), heated to 75 °C for 10 min and then hybridized at 55 °C overnight. S1-nuclease digestion was carried out with 150 U S1 nuclease (Amersham) in 270 µl of the provided buffer. The reaction was stopped after 30–60 min by adding 3 µl of 0.5 M EDTA followed by precipitation of the RNA with 0.7 ml ethanol. The pellet was dried and after resuspending it in 95% formamide/0.025% SDS/0.025% bromophenol blue/0.025% xylene-xanol blue, the fragments were heated to 90 °C for 2 min and then analysed by 8% denaturing PAGE. The detection of the signal was carried out as described for Northern blots (see below).

For Northern analyses, 10 µg total RNA was separated on a horizontal agarose gel and then transferred to a nylon membrane (Hybond-N⁺; Amersham) by capillary transfer, according to Sambrook *et al.* (1989) and Wahl *et al.* (1987). RNA was transferred under mildly alkaline conditions using 5× SSC/10 mM NaOH for 2 h as recommended by Löw & Rausch (1994). Hybridization with the radioactive probes was carried out overnight according to Church & Gilbert (1984). The blot was then washed and exposed to a phosphor screen (Kodak) overnight. The screen was scanned with Storm 860 (Amersham) and analysed with the IMAGE-QUANT software (Amersham).

Generation of a *tahA* fragment from a *Trametes* cDNA library by PCR with degenerate primers. For the isolation of a cDNA fragment of *tahA*, two 'guessmer' primers were used: primer A (5'-GTC GNN ATG ACC TGC-3') is homologous to the region V(M)G(D/V)MT(S)C, near the N-terminal copper-binding motif MTCxxC, and primer B (5'-CTT RCC GGT CTT-3') is the reverse complement to the lysine-rich C-terminal motif KTGK found in all four proteins of the different organisms described in Fig. 4 (the one-letter code of nucleotides is also explained in the legend for Fig. 4). As a template, the pJG4-5 *Trametes* cDNA library was used. PCR was performed with *Taq* polymerase at an annealing temperature of 42 °C in a Corbet Research FTS Capillary Fast Thermal Sequencer. A PCR fragment of 170 bp was obtained, subcloned in pCR2.1 (Invitrogen) and sequenced.

Isolation of genes from a *Trametes* genomic library. The genomic *Trametes* library was screened as recommended in the protocol supplied with the lambda ZAP Express cloning system (Stratagene). In a first screening round, *E. coli* XL-1 Blue MRF' was cultivated on 10 Petri dishes and infected with 50000 phages per Petri dish. After an overnight incubation at 37 °C, the phages were transferred onto nylon membranes (Amersham) according to the protocol of the supplier (Stratagene). The filters were hybridized with the 170 bp *tah* cDNA fragment (obtained from the PCR described above) using the Rapid Hybridization buffer (Amersham) at 65 °C. Cross-reacting clones were picked and further purified by two additional screening rounds. Nine independent cross-reacting clones were obtained. Using the *in vivo* excision protocol of the supplier, the phages were subcloned into pBKCMV (Stratagene), and analysed by restriction enzyme and Southern

analyses. Two full-length alleles of *tabA*, each with their 5'- and 3'-regulatory sequences, were obtained from five overlapping clones.

Sequence analysis. Clones were sequenced completely from their 5' and 3' ends by cycle sequencing on an automated sequencer (Laboratorium für molekulare Biologie, Abt. Genomics, München). Database searches with DNA-fragment-derived protein sequence data were performed with the program BLASTX (National Institutes of Health; <http://www.nih.gov/>). Alignments were generated using the CLUSTAL x.63B Multiple Sequence Alignment Program, a multiple alignment tool developed by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Further sequence analysis and data processing were performed with the GENE INSPECTOR 1.5 program (Textco).

Isolation of full-length cDNA clones of *tabA*. To obtain full-length cDNA clones of *tabA*, two primers were designed based on the information obtained from the partial *tabA* fragment generated by the first PCR. The primer TCC-ord (5'-GCT GAA GAA GAC GGA CGG TGT-3') is homologous to a region near the 5' end of the *tabA* fragment obtained by 'guessmer' PCR. The primer TCC-rev (5'-GTC GTC GTA CGG AAT CGT GCC-3') is the reverse complement to a region near the 3' end of this *tabA* fragment. Each primer was combined in a separate PCR with either one of two vector-specific primers, pJG-ord (5'-TTG CTG AGT GGA GAT GCC TCC-3') or pJG-rev (5'-TCC AGA CTT GAC CAA ACC TCT G-3'), in such a way that the 3' and 5' ends of the cDNAs of *tabA* were obtained. These fragments were overlapping in the centre, meaning a full-length sequence of *tabA* cDNA (two different alleles) could be obtained. *Taq* polymerase was used according to the supplier's instructions (Roche). The reaction volume was 18 µl and contained 10 ng pJG4-5 cDNA library, buffer, 1 U *Taq* polymerase, 1.5 mM MgCl₂, 0.2 mM each of the four dNTPs and 100 pmol each of the primers TCC-ord and pJG-rev (for the amplification of the 3' end) and in a parallel tube 100 pmol each of the primer TCC-rec and pJG-ord (for the amplification of the 5' end). The PCR conditions used were 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 10 s at 70 °C and 1 min at 72 °C. Under these conditions, PCR products of approximately 450 bp (for the 3' end) and 300 bp (for the 5' end) in size were obtained. The PCR fragments were purified by gel electrophoresis and cloned into the vector pCR2.1 (Invitrogen); from each fragment, five independent clones were sequenced. The information obtained from these overlapping sequences was used to generate primers for the isolation of full-length cDNAs of *tabA*: the primers cTAH-FW (5'-ACC ATG TCC GAG CAC ACT TAC-3') and cTAH-RV (5'-GA TCA TCC CAC CGT CTC TCC-3') were used in a PCR with *Pwo* polymerase (Roche) and the pJG4-5 cDNA library as a template. The PCR conditions were the same as described, except that the annealing temperature was 65 °C instead of 70 °C. The resulting DNA band of 224 bp was purified by gel electrophoresis, subcloned into pZER0 (Invitrogen), and five independent clones were sequenced.

Complementation of yeast by *tabA*. The yeast strains SL103 (Δ atx1) and SL133 (Δ sod1 Δ sod2 Δ atx1) were transformed with pAH-tah, pAH-ATX (positive control) or pAH (negative control), using the high-efficiency lithium acetate method (Gietz & Schiestl, 1995). To test for the restoration of iron uptake, SL103 transformants were grown on synthetic glucose medium (without histidine) (YNB without amino acids; Difco) buffered with 50 mM Mes/Tris (pH 5.2), and with 2.5 mM Ferrozine (Sigma), with or without 350 µM ferrous ammonium sulfate for 3–5 days at 30 °C. To test for the

reversal of lysine and methionine auxotrophy, the SOD1-deficient SL133 transformants were grown on synthetic glucose medium without lysine and on synthetic glucose medium without methionine, under aerobic and anaerobic conditions. Anaerobic conditions were generated with the Anaerocult A system (Merck). To test for reversal of paraquat toxicity, SL133 transformants were grown on synthetic glucose medium containing 50 µM paraquat. Growth of the different transformants was monitored by drop tests, where serial dilutions of yeast cultures were spotted onto agar plates (Fig. 2).

Metal-dependent gene expression. For copper-dependent expression analysis of *tabA*, the dikaryon TV-1 was initially grown on medium without extra copper for 4 days. For induction with different copper concentrations, 0, 2, 5, 10, 100 or 500 µM CuSO₄ was added to each culture flask. To achieve extreme copper starvation, 100 µM BCS was added to one flask one day before induction (negative control). As a positive control, 200 µM CuSO₄ was added to a culture containing 100 µM BCS. After induction (1.5 h) the cells were harvested and frozen in liquid nitrogen, followed by total RNA preparation (Wahl *et al.*, 1987). S1 analyses were performed as described above. To test lower copper concentrations, the strains F6, harbouring allele *tabA-1*, and SP4, harbouring allele *tabA-2*, were used. Culture conditions were as above, but the copper concentrations were 10-fold lower (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25 or 100 µM). Two controls with either 50 µM BCS or 50 µM BCS + 100 µM CuSO₄ were included, and expression was measured using Northern blots.

Induction under iron-starvation conditions was tested by growing cells in minimal medium for 2–4 days and then adding 2.5 mM Ferrozine to the cultures. After 1.5 h, cells were harvested and frozen in liquid nitrogen, followed by preparation of RNA. DABA and paraquat induction were tested by adding either 40 µg DABA ml⁻¹ or 100 µM paraquat to cultures growing in minimal medium. In each expression experiment, two independent parallel cultures were analysed; each experiment was repeated three times.

RESULTS

Identification and characterization of *tabA*

Sequence comparisons between cytoplasmic copper chaperones from four different organisms, i.e. yeast Atx1p, nematode Cuc1p, human Hah1p and plant Cchp, revealed two short conserved regions, which were used as rough templates for designing the degenerate primers A and B. Using these primers, designed such that only those codons frequently used by *T. versicolor* were chosen (i.e. a high G + C content with preference for C or G over U or A), a 170 bp PCR *tabA* fragment was obtained with a *Trametes* cDNA library (as described in Methods). This partial sequence encoded a peptide that showed homology to other copper chaperones. The 5' and 3' ends of *tabA* were obtained in two parallel PCR experiments, where two gene-specific primers (either homologous to the 5' end or complementary to the 3' end of *tabA*) were combined individually with one of two vector backbone-specific primers (located either in the *GAL1* promoter or in the *ADH1* terminator) to yield two overlapping *tabA* sequences from which the full-length cDNA was obtained by a subsequent PCR.

Two slightly different full-length genomic *tabA* clones (GenBank AY166609 and AY166608) including their 5'- and 3'-regulatory sequences were obtained from a genomic *Trametes* phage library by using the 170 bp *tabA* fragment as a probe. Southern blot analysis with genomic DNA from dikaryotic (TV-1) and monokaryotic (F2, F6, SP4 and SP17) *T. versicolor* strains confirmed that there was a single copy of *tabA* per *T. versicolor* haploid genome and that the two different sequences were alleles (data not shown).

The two *tabA* alleles differ in nine nucleotides between the start codon ATG and the stop codon TGA, of which eight are located within (non-consensus) intron sequences. The nucleotide exchange in exon III (ACG to ACA) is conservative, as both codons code for threonine. Thus, both alleles, although different at the DNA level, code for identical polypeptides. The five introns are located in the same positions, as confirmed by comparing the genomic sequences with the cDNA sequences. The overall consensus of the 5' and 3' splice sites in *tabA* is 5'-GTRVKK-----YAG-3', which fits well with the consensus described for filamentous fungi (Ballance, 1986). The size of the introns ranges from 57 to 66 nt and is typical for filamentous fungi. The translation initiation environment is ACC ATG. The nucleotide at position -3 is an A, which is in agreement with the findings of Kozak (1984), who defined a consensus ATG environment for filamentous fungi. These sequences are presumably involved in the recognition of the correct AUG by the ribosome and are thought to play an important role in the efficiency of translation initiation in eukaryotes (Kozak, 1986). The G + C content of the coding region is 56.5%, which is also reflected in the codon usage.

The 7.9 kDa protein encoded by *tabA* is 56% identical and 76% similar to yeast Atx1p, 41% identical and 66% similar to plant Cch1p, and 43% identical and 63% similar to human Hah1p (data not shown). TahAp contains the conserved copper-binding motif MxCxxC (Lin & Culotta, 1995) near the N terminus, which plays a role in copper binding and is also found in the copper-transporting P-type ATPases Ccc2p from *S. cerevisiae*, Mnkp and Wndp from humans, and CopA from *Enterococcus hirae*. TahAp also contains the highly conserved sequence KTGK near the C terminus, which was shown to be important for copper trafficking in Atx1p (Portnoy *et al.*, 1999; not shown).

Complementation of *atx1* and *sod1* mutant yeast strains

In yeast, Atx1p is involved in copper trafficking to the secretory pathway, where copper is inserted into the copper-dependent ferroxidase Fet3p, which is essential for the high-affinity iron uptake at the plasma membrane (Pufahl *et al.*, 1997). Thus, an *atx1* mutant is deficient in the high-affinity iron transport system and cannot take up iron from media containing the iron chelator Ferrozine (Lin *et al.*, 1997). To test whether *tabA* could complement the copper-chaperoning function of ATX1

Fig. 1. cDNA of *tabA* restores high-affinity iron uptake to $\Delta atx1$ mutant yeast. The $\Delta atx1$ mutant strain SL103 was transformed with vector pAH (empty vector, negative control), pAH-tah (*tabA* expression) or pAH-ATX1 (ATX1 expression, positive control). The vector pAH-ATX* carries a mutated ATX1 gene (Arg₆₈ to Lys₆₈) that fully restores growth on Ferrozine but not on paraquat (Fig. 2). Strain EG103 + pAH is the wild-type with the empty vector used as an additional positive control. Both media contained 2.5 mM of the iron chelator Ferrozine without iron (-iron) or with 350 μ M ferrous ammonium sulfate (+iron). Plates were photographed after incubation at 30 °C for 4 days.

in yeast, we constitutively expressed the full-length *tabA-1* cDNA in a yeast *atx1* mutant. The *tabA-1* cDNA was expressed under the control of the strong *ADH1* promoter carried on a low-copy vector in the $\Delta atx1$ yeast strain SL103. Serial dilutions of transformants were made on Ferrozine-containing media with and without supplementary iron (Fig. 1). Transformants expressing either *tabA* or ATX1 were able to grow on Ferrozine in the absence of iron. Expression of either ATX1 or *tabA-1* complemented the iron-deficient growth phenotype of the SL103 mutant. The growth of *tabA*- and ATX1-overexpressing cells was comparable (Fig. 1).

The copper chaperone gene ATX1 was originally isolated by complementation of a $\Delta sod1 \Delta sod2$ deletion mutant (Culotta *et al.*, 1995) due to its anti-oxidant activity. We thus tested whether *tabA* could also rescue the growth defects of a $\Delta sod1 \Delta sod2$ mutant on paraquat-containing media or on lysine- or methionine-deficient media. The same expression constructs as in the previous complementation experiment (Fig. 1) were transformed into the *sod* deletion mutant SL133. Only transformants overexpressing either *tabA-1* or ATX1 were able to grow aerobically on lysine- or methionine-deficient media (Fig. 2). A similar result was observed on media containing 50 μ M of the redox-cycling drug paraquat (Fig. 2).

The anti-oxidant effect of *tabA-1*-overexpressing $\Delta sod1 \Delta sod2$ strains was abolished on media made copper-deficient with 50 μ M of the copper chelator BCS,

In summary, *tabA* fully restores *ATX1* functions in yeast, i.e. copper chaperoning to the secretory pathway and protection against oxygen toxicity.

An insertion sequence (IS) of 425 bp was found in the *tabA-2* allele. This IS is flanked by inverted repeats 29 nt in length. Another pair of inverted repeats, each 16 nt long, localized between positions 150 and 173 of the IS are not separated by any nucleotides (Fig. 3). Whether these inverted repeats induce secondary structure in the DNA, and thus might enhance transcription or recombination, remains to be determined. It was estimated by Southern hybridization that four to six copies of this IS are present per haploid nucleus (data not shown).

The main transcription start site is located 116 nt upstream of the start codon ATG, as all independent cDNA clones isolated begin with the same nucleotide. The transcription start site resides in the middle of a pyrimidine-rich sequence, which is typical for genes of filamentous fungi. The main site for poly A addition occurs 111 nt downstream from the stop codon, behind a C. No sequences with homology to known 3'-terminal consensus sequences were found.

-385	ATAGAGCTGT	-376
-455	CTGTGGCTGA	-446
-710	GTCTCGCTGA	-701
-763	GTACCGCTGT	-754
TV CONSENSUS	VTVBVGCTGW	
MRE (S.c./C.g.)	HTHNNGCTGD	
CuSE (S.p.)	DWDDHGCTGD	

Fig. 4. Comparison of putative copper-sensing sequences. The four putative copper-sensing sequences from the *T. versicolor* copper chaperone gene *tahA-1* are compared with the Ace1 and Amt1 consensus MREs (metal-responsive elements) from *Saccharomyces cerevisiae* and *Candida glabrata*, and with the Cuf1 consensus CuSE (copper-signalling element) binding sites from *Schizosaccharomyces pombe*. V, G/C/A; B, G/T/C; W, A/T; K, G/T; H, C/A/T; D, G/A/T; N, any; S.c., *Saccharomyces cerevisiae*; C.g., *C. glabrata*; S.p., *Schizosaccharomyces pombe*.

Fig. 5. S1-protection assay with total RNA from the dikaryon TV-1. TV-1 was induced with 0–500 μM copper. P, undigested primer mix. BCS (100 μM) for copper starvation (negative control) and 100 μM BCS with 200 μM copper (positive control; BCS+) were also tested. (a) Autoradiogram of the S1 PAGE. (b) Bar graph evaluation of the S1 assay normalized to the *gapDH* signal.

Apart from the 425 nt IS, the 5' regulatory sequences of the two *tahA* alleles differ in 67 out of 693 nt. Screening the 5'-non-coding region of *tahA-1* for fungal or yeast upstream activating sequences, which bind regulatory proteins and consequently control the level of transcription, revealed four sequences at positions –385, –455, –710 and –763 (Fig. 4) that showed homology to the consensus sequence, HTHnnGCTGD, of metal-responsive elements (MREs) (Macreadie *et al.*, 1994). This suggests that the expression of *tahA* might be controlled by the availability of metal ions, with copper

Fig. 6. Northern analyses of *tahA* induction with copper. The two strains with the different *tahA* alleles (F6 and SP4) were either not induced (–) or induced with copper concentrations up to 100 μM. As controls, either 50 μM BCS or 50 μM BCS supplemented with 100 μM copper (BCS+) were tested. *gapDH* was used as the internal standard. (a) Autoradiogram of the Northern blot. (b) Composite bar graph showing relative induction of *tahA* normalized to the *gapDH* gene signal. Solid bars, SP4; open bars, F6.

being the most probable candidate since *tahA* possesses the copper-binding motif MxCxxC. The two MRE-like sequences closest to the transcription start site are mutated in allele *tahA-2*. Furthermore, one sequence with homology to the anti-oxidant-responsive element (ARE), with the consensus sequence RRTGACnnnGC, was found at position –536.

tahA expression

To find out whether *tahA* transcription is regulated by either metals or the toxic compound DABA, we performed S1-protection assays and Northern blot analysis. The dikaryon and both alleles of *tahA* (with and without IS) were tested under different copper and iron concentrations, and on DABA and paraquat. Fig. 5 shows that copper-dependent transcription of *tahA* in the dikaryon TV-1 is already fully induced at 2 μM copper. Transcription of *tahA* is clearly downregulated under copper starvation, where only 10% of fully induced levels are observed. Lower copper concentrations were tested with F6, harbouring the *tahA-1* allele, and SP4, harbouring the *tahA-2* allele, as described in Methods. Northern blot analysis of the RNA showed that *tahA* was induced at concentrations of 0.25 μM CuSO₄ and repressed when no copper was available, i.e. by adding BCS (Fig. 6). The different alleles showed little difference in their copper regulation, except that the IS-containing allele (*tahA-2*) showed a basal transcription at copper concentrations

from 0.1 to 1.0 μM and a 1.5-fold increase of signal at 2.5 μM copper, whereas the *tahA-1* allele (without IS) had a basal induction from 0 to 10 μM copper and a twofold increase of signal at 25 μM copper (Fig. 6).

Iron starvation did not seem to have a significant effect on *tahA* transcription in *T. versicolor*, independent of allele type. Similarly, DABA and paraquat did not affect *tahA* transcription, as determined by Northern blots (data not shown).

DISCUSSION

Laccases contain four copper ions per molecule, and not only are regarded as potential pathogenic factors produced by fungi but are also of great interest in biotechnology, particularly since they are capable of oxidizing a wide variety of phenolic compounds. For example, they may be used in chlorine-free paper bleaching processes (Call & Mücke, 1997), detoxification of xenobiotic compounds, organic syntheses (Sariaslani, 1989; Rosenau *et al.*, 1996), dye bleaching or binding of wood composites (Felby *et al.*, 1997). In *T. versicolor*, *Aspergillus nidulans*, *S. cerevisiae* and *Pichia pastoris*, the formation of (functional) laccases depends on elevated copper concentrations in the medium (our observations). However in general, almost nothing is known about the copper-homeostasis factors that participate in the trafficking of copper to laccases in higher fungi.

We identified two genomic alleles of a gene called *tahA* from *T. versicolor* that encodes a polypeptide of 7.9 kDa. The TahA protein displays homology to copper chaperones from other organisms, including a metal-binding motif, MxCxC, which is typical for diverse metal-binding proteins. Moreover, TahA proved not only a structural but also a functional homologue of ATX1, since expression of *tahA* cDNA could both complement the growth defect of Δatx1 yeast mutants on iron-limiting media and protect Δsod1 Δsod2 yeast mutants from oxygen toxicity. Interestingly, a chance point mutation of ATX1 (ATX1*; amino acid substitution Arg₆₈ to Lys₆₈), although in a non-conserved region of Atx1, impairs the SOD-like activity of Atx1. In other words, ATX1* restored high-affinity iron uptake to Δatx1 yeast, but not the aerobic methionine auxotrophy or the paraquat toxicity to Δsod1 Δsod2 yeast, indicating that the anti-oxidant function of the protein but not its copper chaperone function is impaired.

Since TahA can replace Atx1p in high-affinity iron transport, TahA can probably interact with Ccc2p, resulting in transport of copper into the lumen of the post-Golgi vesicles of yeast cells. In fact, it was shown (A. Uldschmid & K. Marbach, unpublished data) that *T. versicolor* expresses a gene homologous to CCC2. Thus, it is very likely that the transport of copper to laccase uses this copper chaperone–copper ATPase route in *T. versicolor*.

We observed that *tahA* in *T. versicolor* is moderately upregulated when copper is present (>0.25 μM) and

downregulated when no copper is available (addition of BCS). In the 5'-regulatory region of *tahA*, we found four sequences with similarity to metal-responsive elements. Since the gene regulation between *S. cerevisiae* and basidiomycetes is probably quite different, we did not expect to find any homology to *S. cerevisiae* Ace1p upstream activating sequence sites. Indeed, we were astonished to find sequences that not only correlated with the *S. cerevisiae* consensus but were also located upstream of the MT gene of *Neurospora crassa* (Munger *et al.*, 1985).

These four putative copper-sensing sequences were compared with the consensus of the *S. cerevisiae* Ace1p binding sites in *CUP1* and *SOD1* (Thiele & Hamer, 1986; Gralla *et al.*, 1991), of the *Candida glabrata* Amt1 binding sites in *MTI* and *MTII* (Zhou *et al.*, 1992) and of the *Schizosaccharomyces pombe* Cuf1 binding site in *ctr4* (Beaudoin & Labbé, 2001). The similarities of the sequences suggest that they are likely candidate target sequences for a copper-sensing transcriptional activator or repressor controlling the expression of copper-regulated genes. However, it remains to be elucidated by further experiments whether any or all of these putative copper-sensing sequences of the *tahA* promoter are indeed functional. The fact that the sequences were similar to Ace1 upstream activating sequence provides a starting point for further analyses of the relevant sites and factors involved in *tah1* induction.

Transcriptional regulation of ATX1 in yeast is not influenced by copper but is influenced by iron and oxygen (Lin & Culotta, 1995). In contrast, *tahA* is not regulated by iron in *T. versicolor* but by copper. This difference might be explained by the fact that basidiomycetes are strictly aerobic fungi, in contrast to yeasts, which also grow via fermentation. It is thought that basidiomycetes take up iron mainly via siderophores, again in contrast to yeasts, where iron uptake is accomplished via the Fet3p–Ftr1 complex (and also non-self-made siderophores when present). In *Arabidopsis thaliana*, however, the copper chaperone CCH was shown to be downregulated by copper treatment in contrast to metallothionein (Himelblau *et al.*, 1998), thus showing a different regulation compared to *T. versicolor* and yeasts.

It is not known whether *Trametes* uses a Fet3 protein for iron uptake. However, since it has been demonstrated that the basidiomycete *Ustilago spaeerogena* (Eckert & Emery, 1983) possesses two different iron uptake systems – one reductive and one non-reductive (i.e. via siderophores) – it is very likely that *Trametes* also contains a Fet3 protein, although we have, as yet, been unable to detect any sequence homologues in extensive cDNA library screening (not shown). Regulation of ATX1 and FET3 by iron makes sense in yeast, but in *Trametes* the structural homologues of yeast Fet3p are apparently the laccases, secreted proteins that require copper. Even if Fet3p should prove to be present, it has been shown that *Trametes* cultures produce five to six different laccase isoenzymes that can be secreted in very high quantities

(i.e. between 0.1 and 1 mg l⁻¹; Yaver & Golightly, 1996; Yaver *et al.*, 1996). Thus, in *Trametes* it makes sense that the laccases are primarily provided with copper by the TahAp-encoding gene *tahA*. Further studies of *tahA* and a better understanding of copper-homeostasis factors in these organisms could reveal the basis for the hierarchy of copper distribution inside the cells (mitochondria cytoplasm Golgi), which is observed under copper-starvation conditions.

Finally, basidiomycetes are good candidates for the large-scale industrial production of technical enzymes that require co-factors such as copper and iron. Indeed, basidiomycetes are better producers of laccases than the usual sources that employ *Aspergillus* and *Pichia* systems, although the reason for this is currently unknown. In addition to their many known and potential uses in biotechnology, discussed above, the laccases are involved in a wide variety of different cellular reactions, for example, detoxification of toxic compounds, morphogenesis and melanin formation. A better understanding of copper-homeostasis factors, copper-chaperone routes and copper trafficking in these higher fungi could help to generate strains that are better laccase producers suited for large-scale laccase production.

ACKNOWLEDGEMENTS

This work was supported financially by the Consortium für elektrochemische Industrie, München, Germany. We thank Dr Rupert Pfaller from the Consortium für elektrochemische Industrie, München, for his very helpful cooperation. We warmly thank Valeria Culotta for providing the Δ *atx1* yeast strain and the Δ *sod* knock-out strains *SL103* and *SL13* used in the complementation assays.

REFERENCES

- Ballance, D. J. (1986).** Sequences important for gene expression in filamentous fungi. *Yeast* **2**, 229–236.
- Beaudoin, J. & Labbé, S. (2001).** The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. *J Biol Chem* **276**, 15472–15480.
- Call, H. P. & Mücke, I. (1997).** History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). *J Biotechnol* **53**, 163–202.
- Church, G. M. & Gilbert, W. (1984).** Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991–1995.
- Culotta, V. C., Joh, H. D., Lin, S. J., Slekar, K. H. & Strain, J. A. (1995).** Physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. *J Biol Chem* **270**, 29991–29997.
- Eckert, D. J. & Emery, T. (1983).** Iron uptake from ferrichrome A and iron citrate in *Ustilago sphaerogena*. *J Bacteriol* **155**, 616–622.
- Felby, C., Pedersen, L. S. & Nielsen, B. R. (1997).** Enhanced auto adhesion of wood fibers using phenol oxidases. *Holzforschung* **51**, 281–286.
- Feldmann, H., Driller, L., Meier, B., Mages, G., Kellermann, J. & Winnacker, E. L. (1996).** HDF2, the second subunit of the Ku homologue from *Saccharomyces cerevisiae*. *J Biol Chem* **271**, 27765–27769.
- Gietz, R. D. & Schiestl, R. H. (1995).** Transforming yeast with DNA. *Methods Mol Cell Biol* **5**, 255–269.
- Gralla, E. B., Thiele, D. J., Silar, P. & Valentine, J. S. (1991).** ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. *Proc Natl Acad Sci USA* **88**, 8558–8562.
- Halliwell, B. & Gutteridge, J. M. C. (1984).** Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219**, 1–14.
- Himelblau, E., Mira, H., Lin, S. J., Culotta, V. C., Penarrubia, L. & Amasino, R. M. (1998).** Identification of a functional homolog of the yeast copper homeostasis gene *ATX1* from *Arabidopsis*. *Plant Physiol* **117**, 1227–1234.
- Hirayama, T., Kieber, J. J., Hirayama, N. & 7 other authors (1999).** RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. *Cell* **97**, 383–393.
- Hung, I. H., Suzuki, M., Yamaguchi, Y., Yuan, D. S., Klausner, R. D. & Gitlin, J. D. (1997).** Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 21461–21466.
- Klomp, L. W., Lin, S. J., Yuan, D. S., Klausner, R. D., Culotta, V. C. & Gitlin, J. D. (1997).** Identification and functional expression of *HAH1*, a novel human gene involved in copper homeostasis. *J Biol Chem* **272**, 9221–9226.
- Kozak, M. (1984).** Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res* **12**, 857–872.
- Kozak, M. (1986).** Point mutations define a sequence flanking the AUG initiation codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292.
- Lin, S. J. & Culotta, V. C. (1995).** The *ATX1* gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc Natl Acad Sci USA* **92**, 3784–3788.
- Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V. & Culotta, V. C. (1997).** A role for the *Saccharomyces cerevisiae* *ATX1* gene in copper trafficking and iron transport. *J Biol Chem* **272**, 9215–9220.
- Logemann, J., Schell, J. & Willmitzer, L. (1987).** Improved method for the isolation of RNA from plant tissues. *Anal Biochem* **163**, 16–20.
- Löw, R. & Rausch, T. (1994).** Sensitive, nonradioactive northern blots using alkaline transfer of total RNA and PCR-amplified biotinylated probes. *BioTechniques* **17**, 1026–1030.
- Macreadie, I. G., Sewell, A. K. & Winge, D. R. (1994).** Metal ion resistance and the role of metallothionein in yeast. In *Metal Ions in Fungi. Mycology Series*, pp. 279–310. Edited by G. Winkelmann & D. R. Winge. New York: Marcel Dekker.
- Martin, W. J. (1999).** Bacteria-related sequences in a simian cytomegalovirus-derived stealth virus culture. *Exp Mol Pathol* **66**, 8–14.
- Munger, K., German, U. A. & Lerch, K. (1985).** Isolation and structural organization of the *Neurospora crassa* copper metallothionein gene. *EMBO J* **4**, 2665–2668.
- O'Halloran, T. V. & Culotta, V. C. (2000).** Metallochaperones, an intracellular shuttle service for metal ions. *J Biol Chem* **275**, 25057–25060.
- Payne, A. S. & Gitlin, J. D. (1998).** Functional expression of the Menkes disease protein reveals common biochemical mechanisms among the copper-transporting P-type ATPases. *J Biol Chem* **273**, 3765–3770.

- Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O'Halloran, T. V. & Culotta, V. C. (1999). Structure-function analyses of the ATX1 metallochaperone. *J Biol Chem* **274**, 15041–15045.
- Predki, P. F. & Sakar, B. (1992). Effect of replacement of 'zinc finger' zinc on estrogen receptor DNA interactions. *J Biol Chem* **267**, 5842–5846.
- Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. & O'Halloran, T. V. (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853–856.
- Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C. & O'Halloran, T. V. (1999). Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805–808.
- Rosenau, T., Potthast, A., Chen, C. L. & Gratzl, J. S. (1996). A mild, simple and general procedure for the oxidation of benzyl alcohols to benzaldehydes. *Synth Commun* **26**, 315–320.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sariaslani, F. S. (1989). Microbial enzymes for oxidation of organic molecules. *Crit Rev Biotechnol* **9**, 171–257.
- Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.
- Tamai, K. T., Gralla, E. B., Ellerby, L. M., Valentine, J. S. & Thiele, D. J. (1993). Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. *Proc Natl Acad Sci U S A* **90**, 8013–8017.
- Thiele, D. J. & Hamer, D. H. (1986). Tandemly duplicated upstream control sequences mediate copper-induced transcription of the *Saccharomyces cerevisiae* copper-metalllothionein gene. *Mol Cell Biol* **6**, 1158–1163.
- Wahl, G. M., Meinkoth, J. L. & Kimmel, A. R. (1987). Northern and Southern blots. *Methods Enzymol* **152**, 572–581.
- Wakabayashi, T., Nakamura, N., Sambongi, Y., Wada, Y., Oka, T. & Futai, M. (1998). Identification of the copper chaperone, CUC-1, in *Caenorhabditis elegans*: tissue specific co-expression with the copper transporting ATPase, CUA-1. *FEBS Lett* **440**, 141–146.
- Weaver, R. F. & Weissmann, C. (1979). Mapping of RNA by a modification of the Berk-Sharp procedure: the 5' termini of 15 S β -globin mRNA precursor and mature 10 s β -globin mRNA have identical map coordinates. *Nucleic Acids Res* **7**, 1175–1193.
- Yaver, D. S. & Golightly, E. J. (1996). Cloning and characterization of three laccase genes from the white-rot basidiomycete *Trametes villosa*: genomic organization of the laccase gene family. *Gene* **181**, 95–102.
- Yaver, D. S., Xu, F., Golightly, E. J. & 7 other authors (1996). Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. *Appl Environ Microbiol* **62**, 834–841.
- Zhou, P., Szczypka, M. S., Sosinowski, T. & Thiele, D. J. (1992). Expression of a yeast metallothionein gene family is activated by a single metalloregulatory transcription factor. *Mol Cell Biol* **12**, 3766–3775.

Received 10 May 2002; revised 17 June 2002; accepted 5 September 2002.