Identification and functional expression of \( \text{tahA} \), a filamentous fungal gene involved in copper trafficking to the secretory pathway in \( \text{Trametes versicolor} \)

Andreas Uldschmid, Michael Engel, Renate Dombi and Karin Marbach

In this study, cDNA and genomic clones encoding a homologue of the yeast gene anti-oxidant 1 (\( \text{ATX1} \)) from the white-rot fungus \( \text{Trametes versicolor} \), a basidiomycete known to produce several laccase isoenzymes involved in lignin degradation, were identified. This gene, named \( \text{Trametes ATX} \) homologue (\( \text{tahA} \)), encodes a protein of 7-9 kDa with 56% identity to the yeast Atx1p sequence. Two different alleles of \( \text{tahA} \) were obtained that differed mainly in their intervening sequences and in a 425 nt insertion located 183 nt upstream of the transcription start site. \( \text{tahA} \) is present as one copy per haploid nucleus in \( \text{T. versicolor} \), as shown by Southern analysis. Expression of \( \text{tahA} \) cDNA restored high-affinity iron uptake in a \( \Delta \text{atx1} \) yeast strain and oxygen sensitivity in a \( \Delta \text{sod1} \) \( \Delta \text{sod2} \) yeast strain, showing that \( \text{tahA} \) is also a functional homologue of \( \text{ATX1} \). The inability of \( \text{tahA} \) to rescue the \( \Delta \text{sod1} \) phenotype on copper-deficient medium indicated that \( \text{tahA} \) function is copper-dependent. Sequence analysis of the \( \text{tahA} \) promoter revealed several motifs that were similar to the conserved motifs found in the copper-regulated metallothionein and Cu, Zn superoxide dismutase genes, \( \text{CUP1} \) and \( \text{SOD1} \), of \( \text{Saccharomyces cerevisiae} \), \( \text{Neurospora crassa} \) and \( \text{Candida glabrata} \). In contrast to its yeast homologue \( \text{ATX1} \), \( \text{tahA} \) is induced under elevated copper concentrations in the medium (\( \geq 25 \text{µM CuSO}_4 \)) and repressed under copper starvation. The transcription of \( \text{tahA} \) 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 \( \text{c} \) 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...
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 (Himelblau 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 tabA (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 xenobiotica, such as diaminobenzaldehyde (DABA), and of the redox-cycling drug paraquat, and discuss the results in relevance to conserved motifs found in the S’-non-coding regulatory sequence of tabA 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/MATa leu2-3/112/MATa/tryp289a/ura3-52/GAL), SL103 (isogenic to EG103 with Δatx1::LEU2) and SL133 (isogenic to EG103 with Δsod2::URA3, Δsod2::TRP1, Δatx1::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 µM and paraquat was added up to a concentration of 50 µM 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 tabA 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 adenylic acid, 0.15 g 4-amino-2-methylphenylalanine, 2.5 g L-tryptophan, 0.48 mg thiamin, 10 mg calcium phosphate, 10 mg ferrous 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 cm³) 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 µg 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 (Sac3A) 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 BamHI 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 tabA 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 XhoI site. After synthesis of the second strands, ligation of the EcoRI linkers and restriction with XhoI, the resulting cDNA was separated by gel filtration (using CL-Sepharose); 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 tabA fragments by PCR with degenerate oligonucleotides and for the isolation of full-length tabA clones.

A tabA-overexpressing yeast vector was constructed from pZEO-tah20, a pZErO (Invitrogen) derivative carrying the tabA cDNA in its multiple-cloning site. The tabA cDNA from pZEO-tah20 was excised with EcoRI and XhoI and directionally ligated into the yeast centromere plasmid pAH (Feldmann et al., 1996) via the EcoRI and Sall sites. The single-copy vector pAH is a derivative of pRS313 (Sikorski & Hieter,
1989) and carries 

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 of each primer tah (5'-GTAAGTGTCGTCGAATGTGTA-

and primer gapDH (5'-GTGGCGTGGAGAGTCTCCCAAG-

GAGGCGGTATGCGAGAAAGGCCGCAATTGTTTGAGGAGAAG-

GTTGATCAGCTGAGCGAGCTGATG-3') that had been end-labelled (\( ^\gamma \)P)ATP using polynucleotide kinase. The labelled primers (10^6 c.p.m.) were then added to 50 µg total RNA in 1 M NaCl/330 µM EDTA (pH 7.5)/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 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 5x SSC/10 mM NaOH for 2 h as recommended by Low & 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 versicolor cDNA library by PCR with degenerate primers.** For the isolation of the cDNA fragment of tahA, two ‘guesser’ primers were used: primer A (5'-GTC GNN ATG ACC TGC-3') is homologous to the region V(M)(D)(G)MSV(M)/S, 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 Tag polymerase at an annealing temperature of 42 °C in a Corbett 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 versicolor cDNA 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+w 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 vitro excision protocol of the supplier, the phages were subcloned into pBKCMV (Stratagene), and analysed by restriction enzyme and Southern

**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 mycelia (up to 400 mg 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 resuspended 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 vol 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 T[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 phosphor screen (Kodak) overnight. The screen was scanned with Storm 860 (Amersham) and analysed with the IMAGE-QUANT software (Amersham).

**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-BRL) according to the plant protocol. To remove the polysaccharides, the RNA was precipitated with a high-salt buffer also described in the Gibco

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analyses. Two full-length alleles of \textit{tahA}, 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.ncbi.nlm.nih.gov/). Alignments were generated using the CLUSTAL x 6.3b 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 \textit{tahA}**. To obtain full-length cDNA clones of \textit{tahA}, two primers were designed based on the information obtained from the partial \textit{tahA} fragment generated by the first PCR. The primer TCC-ord (5'-GCT GAA GAC GGA CGG TGT-3') is homologous to a region near the 5' end of the \textit{tahA} fragment obtained by 'guessmer' PCR. The primer TCC-rev (5'-GTC GTC GTA CGG AAT CGT GGC C-3') is the reverse complement to a region near the 3' end of this \textit{tahA} 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'-TGG AGA CTT GAC CAA ACC TCT G-3'), in such a way that the 5' and 3' ends of the cDNAs of \textit{tahA} were obtained. These fragments were overlapping, meaning a full-length sequence of \textit{tahA} 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 MgCl$_2$, 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 5' end) and in a parallel tube 100 pmol each of the primer TCC-rec and pJG-ord (for the amplification of the 3' 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 \textit{tahA}: the primers cTAH-FW (5'-ACC ATG TCC GAG CAC ACT TAC-3') and cTAH-RV (5'-GA TCA TAC CAC CGT CTC TTC-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 pZErO (Invitrogen), and five independent clones were sequenced.

**Complementation of yeast by \textit{tahA}**. The yeast strains SL103 (\textit{Δatx1}) and SL133 (\textit{Aso1d Aso2Δatx1}) 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 \textit{tahA}, 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 CuSO$_4$ 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 CuSO$_4$ 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). SI analyses were performed as described above. To test lower copper concentrations, the strains F6, harbouring allele \textit{tahA-1}, and SP4, harbouring allele \textit{tahA-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 CuSO$_4$ 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 \textit{tahA}**

Sequence comparisons between cytoplasmic copper chaperones from four different organisms, i.e. yeast \textit{A. fumigatus}, nematode \textit{C. elegans}, human \textit{H. sapiens} and plant \textit{C. chlamydocarpa}, 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 \textit{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 \textit{tahA} fragment was obtained with a \textit{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 \textit{tahA} 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 \textit{tahA}) were combined individually with one of two vector backbone-specific primers (located either in the \textit{GAL1} promoter or in the \textit{ADH1} terminator) to yield two overlapping \textit{tahA} sequences from which the full-length cDNA was obtained by a subsequent PCR.
The results of the complementation experiment (Fig. 1) suggest that the two different full-length genomic tahA 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 tahA 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 tahA per T. versicolor haploid genome and that the two different sequences were alleles (data not shown).

The two tahA 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. This, 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 tahA 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 ΔCC ATG. The nucleotide at position −3 is an A, which is in agreement with the findings of Kozak (1984), who defined a consensus ATG initiation 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 tahA 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 copper chaperonal ferrozine (Lin et al., 1997). To test whether tahA could complement the copper-chaperoning function of ATX1 in yeast, we constitutively expressed the full-length tahA-1 cDNA in a yeast atx1 mutant. The tahA-1 cDNA was expressed under the control of the strong ADH1 promoter carried on a low-copy vector in the Δatx1 yeast strain SL103. Serial dilutions of transformants were made on Ferrozine-containing media with and without supplementary iron (Fig. 1). Transformants expressing either tahA or ATX1 were able to grow on Ferrozine in the absence of iron. Expression of either ATX1 or tahA restored high-affinity iron uptake to Δatx1 mutant strain SL103 was transformed with vector pAH (empty vector, negative control), pAH-tah (tahA expression) or pAH-ATX1 (ATX1 expression, positive control). The vector pAH-ATX* carries a mutated ATX1 gene (Arg92 to Lys92) 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 μM ferrous ammonium sulfate (+iron). Plates were photographed after incubation at 30 °C for 4 days.

**Fig. 1** cDNA of tahA restores high-affinity iron uptake to Δatx1 mutant yeast. The Δatx1 mutant strain SL103 was transformed with vector pAH (empty vector, negative control), pAH-tah (tahA expression) or pAH-ATX1 (ATX1 expression, positive control). The vector pAH-ATX* carries a mutated ATX1 gene (Arg92 to Lys92) 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 μM ferrous ammonium sulfate (+iron). Plates were photographed after incubation at 30 °C for 4 days.
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Fig. 2. cDNA of tahA complements SOD-deficient yeast mutants. The ∆sod1 ∆sod2 ∆atx1 mutant strain SL133 was transformed with pAH (empty vector, negative control), pAH-ATX1 (ATX1 expression, positive control), pAH-tah (tahA expression) or pAH-ATX* (expression of mutated ATX1 gene). Both tahA and ATX1 restored aerobic methionine auxotrophy, demonstrated by growth on media without methionine (−Met aerob), and enabled growth on paraquat-containing (+paraquat) medium. This antioxidant effect was abolished on media containing 50 µM of the copper chelator BCS. The vector pAH-ATX* did not complement the SOD-deficient phenotype.

Fig. 3. Sequence of the IS from tahA-2. Upper sequence, tahA-2; lower sequence, tahA-1; IR 1-L or IR 1-R, inverted repeat 1 (left or right, respectively); IR 2-L or IR 2-R, inverted repeat 2 (left or right, respectively); dotted line above sequence, 60% identity to stealth virus. The IS is located 183 nt upstream of the transcription start site and 129 nt upstream of the putative TATA box. It does not have the potential to code for any polypeptide, but several sequences similar to yeast regulatory elements, i.e. GCN4, GCR1 and GAL4, are located within the IS. However, it remains to be elucidated whether any of these sequences has the potential to act as an upstream activating sequence. Several direct repeats were also found. No homology to known fungal ISs, transposons or enhancer elements has been identified. Unexpectedly, a FASTA search revealed a 197 nt overlap with 59-6% identity (63-6% ungapped) to the stealth virus, a double-stranded DNA virus that belongs to the group of Herpesviridae. The stealth virus has been reported to have a wide host range and is discussed as a natural vector in the transfer of genetic information between viral, bacterial and eukaryotic genomes (Martin, 1999).

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

Sequence analysis of the tahA 5′-regulatory sequence

An insertion sequence (IS) of 425 bp was found in the tahA-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).
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 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 RRTGACnmnGC, 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 synthesizes (Saríaslan, 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, MxCxxC, 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 change 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 pararquat 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 MIT 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 (Himmelsblau 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 Usulago spaerogena (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.
producers suited for large-scale laccase production. Further studies of \textit{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 \textit{Aspergillus} and \textit{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.

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