Differential Regulation by Organic Compounds and Heavy Metals of Multiple Laccase Genes in the Aquatic Hyphomycete *Clavariopsis aquatica*

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To advance the understanding of the molecular mechanisms controlling microbial activities involved in carbon cycling and mitigation of environmental pollution in freshwaters, the influence of heavy metals and natural as well as xenobiotic organic compounds on laccase gene expression was quantified using quantitative real-time PCR (qRT-PCR) in an exclusively aquatic fungus (the aquatic hyphomycete *Clavariopsis aquatica*) for the first time. Five putative laccase genes (*lcc1* to *lcc5*) identified in *C. aquatica* were differentially expressed in response to the fungal growth stage and potential laccase inducers, with certain genes being upregulated by, e.g., the lignocellulose breakdown product vanillic acid, the endocrine disruptor technical nonylphenol, manganese, and zinc. *lcc4* is inducible by vanillic acid and most likely encodes an extracellular laccase already excreted during the trophophase of the organism, suggesting a function during fungal substrate colonization. Surprisingly, unlike many laccases of terrestrial fungi, none of the *C. aquatica* laccase genes was found to be upregulated by copper. However, copper strongly increases extracellular laccase activity in *C. aquatica*, possibly due to stabilization of the copper-containing catalytic center of the enzyme. Copper was found to half-saturate laccase activity already at about 1.8 μM , in favor of a fungal adaptation to low copper concentrations of aquatic habitats.

Laccases (EC 1.10.3.2) belong to the multicopper oxidase protein family and are produced by many fungi, bacteria, plants, and insects (4, 18, 24, 32, 37). They couple the one-electron oxidation of numerous substrates to the reduction of molecular oxygen to form water (4, 18). Various functions have been attributed to fungal laccases, for example, the degradation of lignin and many xenobiotic compounds, morphogenesis, stress defense, and host-pathogen interactions (4, 18, 31, 37).

As yet, functions of fungal laccases and the regulation of their expression have predominantly been investigated in terrestrial fungi. In these organisms, heavy metals like copper, a structural component of the catalytic center of typical laccases, but also manganese and cadmium are known to differentially regulate gene transcript levels of individual laccase isoenzymes (18, 48). Enhanced extracellular laccase activity in the presence of zinc has also been reported (6, 22). Many natural and xenobiotic aromatic compounds, which are often structurally related to lignin or humic substances, were shown to induce laccase gene transcription in terrestrial basidio- and ascomycetes (18, 33, 44, 48). The endocrine disrupting chemical (EDC) nonylphenol is an example of a xenobiotic compound where laccase has been implicated in its fungal degradation (12, 29). Technical nonylphenol (tNP), which is a mixture of mainly *p*-substituted phenols with variously branched side chains, arises from incomplete biodegradation of nonylphenol ethoxylate surfactants in wastewater treatment plants. It enters the water cycle together with wastewater treatment plant effluents or contaminates soils through the use of tNP-containing sewage sludge as a fertilizer. Due to its endocrine activity, its demonstrated global occurrence, a largely uncertain environmental fate, and its resistance to biodegradation, tNP has increasingly gained attention (12, 57).

Aquatic hyphomycetes (AQHs), a particular group of exclusively aquatic mitosporic fungi, dominate the microbial decomposition of allochthonous plant detritus in rivers and streams and

are most prominent on the coarse particulate fractions of upper layers of stream bottom sediments (16, 30). Impoverished AQH communities were found to survive under strong heavy metal contamination of waters, indicating the maintenance of basic ecological functions even under such conditions (50). The demonstrated potential of AQHs to metabolize a variety of man-made chemicals such as tNP (26), polycyclic musk fragrances (35), pesticide metabolites (3), and synthetic dyes (25) suggests that these organisms may contribute to the elimination of xenobiotic water pollutants in natural aquatic environments. AQHs also produce laccases (1, 26). However, direct evidence for laccase involvement in bioconversion of water pollutants by AQHs was accumulated only recently. Laccase was implicated in oxidation of tNP (34, 49) and polycyclic musks by *Clavariopsis aquatica* (35), a frequently occurring AQH (50) with a teleomorph state belonging to the ascomycete genus *Massarina* (55).

So far, only one study has addressed the identification of laccase genes and factors controlling laccase gene expression in AQHs (49). In *C. aquatica*, the expression of two putative laccase genes was found to be only partly correlated with extracellular laccase activities in fungal culture supernatants under the influence of copper and organic compounds. This suggests the existence of additional laccase genes and/or a cell association of par-

Received 28 February 2012 Accepted 17 April 2012

Published ahead of print 27 April 2012

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Supplemental material for this article may be found at <http://aem.asm.org/>.

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doi:10.1128/AEM.00635-12

ticular laccase fractions, with the latter possibly impeding laccase detection in culture supernatants (49). Aims of the present study were to identify further putative laccase genes in *C. aquatica* and to quantify their expression under the influence of various, potentially laccase-inducing compounds of environmental relevance using a quantitative real-time PCR (qRT-PCR) approach. For this, copper, manganese, zinc, and cadmium were chosen as representatives of heavy metals usually found in the upper layers of bottom sediments of unpolluted rivers and springs at concentrations only in ranges of μmol to mmol kg^{-1} but found in sediments of freshwaters affected by historical mining activities at up to approximately 20- to 200-fold-higher concentrations (50). Vanillic acid was employed as a model for natural aromatic constituents of plant-derived AQH substrates. Vanillic acid concentrations of up to approximately $755 \mu\text{mol kg}^{-1}$ have been reported for freshwater sediments (23). tNP was used as a water pollutant representative where laccase degradation is relevant. Mean tNP concentrations of approximately 6.8 mmol kg^{-1} and $9.5 \mu\text{mol kg}^{-1}$ have been reported for sewage sludge and freshwater sediments, respectively (12). The influence of heavy metals and organic compounds on extracellular laccase activity and fungal biomass was comparatively assessed. The generated data are intended to advance our understanding of the molecular mechanisms controlling microbial activities that contribute to carbon cycling and concomitantly mitigate environmental pollution in freshwaters.

MATERIALS AND METHODS

Organism and culture conditions. The isolation, identification, and maintenance of the AQH *Clavariopsis aquatica* De Wild. strain WD(A)-00-01, which is available from the culture collection of the Department of Environmental Microbiology, Helmholtz Centre for Environmental Research-UFZ (Leipzig, Germany), were previously described (26).

Liquid cultivations of *C. aquatica* were carried out in Erlenmeyer flasks (250 ml) containing 75 ml of a 1% (wt/vol) liquid malt extract medium (pH 5.6 to 5.8) and inoculated with 1 ml of a mycelial suspension of the fungus prepared as previously described (26). Fungal cultures were agitated at 120 rpm and kept at 14°C in the dark.

In order to establish an effective concentration of the major laccase inducer copper (18) to be applied in subsequent experiments targeting laccase gene expression, the effects of different copper concentrations on extracellular laccase activity were assessed. Fungal cultures were supplemented with 0.5, 2.5, 5, 25, and $50 \mu\text{M}$ CuSO_4 on culture day 4 (early trophophase [26]). Without CuSO_4 supplementation, the cultivation medium contained a basal copper concentration of about $3 \mu\text{g liter}^{-1}$ (47 nM) (49). Laccase activities were recorded after 15 days of cultivation (onset of the idiophase and of maximal laccase production, as previously reported [26]). A dose-response model with variable Hill slope according to

$$A_L = A_{L1} + (A_{L2} - A_{L1}) / (1 + 10^{\log[\text{EC}_{50} - C] \times p})$$

where A_L is the measured laccase activity at a given copper concentration, A_{L1} is the laccase activity in the absence of copper (bottom asymptote, assumed to be zero), A_{L2} is the maximum laccase activity (top asymptote), C is the copper concentration, and p is the Hill slope, was used to estimate the copper concentration leading to half-maximal laccase activity (EC_{50}). Based on laccase activity versus copper concentration, an error-weighted nonlinear data fitting was performed using the software OriginPro 8G SR2 v8.0891 (OriginLab Corp., Northampton, MA) and yielding a coefficient of determination (COD) of >0.99 .

Fungal cultures used for identification of laccase gene fragments were supplemented with a mixture of $50 \mu\text{M}$ CuSO_4 and 1 mM vanillic acid at culture day 4 (26) and were harvested after 15 days of cultivation.

In order to study the effects of potential laccase inducers on laccase gene transcripts, extracellular laccase activity, and fungal dry masses, fun-

gal cultures were supplemented with the following compounds or mixtures thereof on culture day 4 (hereafter referred to as induction treatments): $50 \mu\text{M}$ CuSO_4 (treatment Cu), $50 \mu\text{M}$ CdSO_4 (treatment Cd), $50 \mu\text{M}$ ZnSO_4 (treatment Zn), $50 \mu\text{M}$ MnSO_4 (treatment Mn), 1 mM vanillic acid (treatment V), $25 \mu\text{M}$ tNP (treatment tNP), $50 \mu\text{M}$ CuSO_4 plus 1 mM vanillic acid (treatment Cu-V), $50 \mu\text{M}$ CuSO_4 plus $25 \mu\text{M}$ tNP (treatment Cu-tNP), and $50 \mu\text{M}$ CuSO_4 plus 1 mM vanillic acid plus $25 \mu\text{M}$ tNP (treatment Cu-V-tNP). Vanillic acid and tNP were aseptically added from methanolic stock solutions, always corresponding to a final methanol concentration of 1% (vol/vol) in tNP- and/or vanillic acid-containing fungal cultures. To improve the solubility of tNP, 0.1% (wt/vol) Tween 80 was additionally included in tNP-containing cultures. To assess potential effects of methanol and Tween 80 on laccase gene transcription and extracellular enzyme activity, additional fungal cultures contained either 1% methanol (treatment MeOH) or 0.1% Tween 80 (treatment Tween). Fungal cultures without potential laccase inducers served as controls. For each induction treatment and the controls, quadruplicate cultures were harvested on culture day 5 (early trophophase [26]), and triplicate cultures were harvested on culture days 10 (corresponding to the trophophase) and 15 (onset of the idiophase). Harvested cultures were used for laccase activity measurements and fungal dry mass determination, as well as for isolation of total RNA.

Laccase activity determinations. Extracellular laccase activities in supernatants of quadruplicate (culture day 5) and triplicate (culture days 10 and 15) liquid cultures were determined with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a substrate (26). Enzyme activities are expressed as units (U), where 1 U corresponds to $1 \mu\text{mol}$ of product formed per minute.

Determination of fungal dry masses. Mycelia were removed from quadruplicate (culture day 5) and triplicate (culture days 10 and 15) fungal cultures by filtration through Whatman no. 6 filter papers (Maidstone, United Kingdom) and washed with 50 ml of distilled water. Fungal dry masses were gravimetrically determined after the mycelia had been lyophilized in an Alpha 2-4 freeze dryer (Christ, Osterode, Germany) for 12 h.

Isolation of total RNA and cDNA synthesis. Triplicate lyophilized mycelia from identical induction treatments were combined and ground in a mortar, and 1 mg was used for total RNA isolation using TRIzol reagent (Invitrogen, Karlsruhe, Germany). Remaining traces of DNA were removed using the DNA-free kit (Ambion, Darmstadt, Germany) according to the protocol of the manufacturer. The quality of RNA was checked on agarose gels and the RNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Reverse transcription of $5 \mu\text{g}$ of DNA-free RNA was performed once per RNA sample, using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the protocol of the supplier.

Identification of 18S rRNA and laccase gene fragments. Amplification and sequencing of the 18S rRNA gene were performed according to reference 7. Fragments of putative laccase genes were amplified from cDNA using the degenerated primer pair Cu1AF/Cu3R, which targets gene fragments ranging from laccase copper binding regions (cbr) I to III (27) (purchased from Invitrogen) according to a previous study (36).

PCRs were performed on a Tetrad 2 gradient cyclor (Bio-Rad, Munich, Germany) in a total volume of $25 \mu\text{l}$, containing $12.5 \mu\text{l}$ of PCR master mix ($2\times$; Promega, Madison, WI), $1 \mu\text{l}$ of each primer ($100 \mu\text{M}$ stock solution), and $1.5 \mu\text{g}$ of cDNA in PCR-grade water. The PCR conditions were as follows: 3 min at 94°C , followed by 45 cycles (1 cycle consists of 30 s at 94°C , 30 s at 48°C , and 120 s at 72°C) and then a final elongation at 72°C for 10 min.

PCR products were cloned into the pCR4-Topo Vector (TOPO TA cloning kit; Invitrogen) by following the protocol of the manufacturer and transformed into TOP10 chemically competent *Escherichia coli* cells. Plasmids from positive clones were extracted from *E. coli* using the Perfect-prep plasmid minikit (Eppendorf, Hamburg, Germany) and sequenced on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Darmstadt,

Germany), using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the instructions of the manufacturer. Five putative laccase gene fragments were identified and are hereafter referred to as *lcc1*, *lcc2*, *lcc3*, *lcc4*, and *lcc5*.

The program BioEdit, version 7.0.9.0 (21), was used to edit sequences and was also used for pairwise comparisons of the deduced amino acid sequences corresponding to cbr I to III of the 5 putative *C. aquatica* laccase gene fragments. The Basic Local Alignment Research Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was employed to search for protein identities of *C. aquatica* laccase and 18S rRNA gene fragments (2).

Analysis of laccase gene expression. The 5 putative laccase gene fragments derived from application of the degenerated primers Cu1AF and Cu3R served as a basis for the development of gene-specific laccase primer pairs, which were used for qRT-PCR amplification and are listed in Table S1 in the supplemental material. Gene-specific primers used for amplification of the β -actin (49) and 18S rRNA genes, which were used as housekeeping genes, are also shown in Table S1 in the supplemental material.

For each gene, different concentrations of the respective forward and reverse primers were tested in order to lower the number of PCR cycles needed for detection. Forward and reverse primers were applied at final concentrations of 50, 300, and 900 nM and in all possible combinations thereof. A final concentration of 900 nM for the forward as well as for the reverse primer was always found to be most efficient.

qRT-PCR assays were always performed in triplicate per one cDNA sample (synthesized from one sample of total RNA, which has been isolated from previously pooled triplicate cultures). A total reaction mixture volume of 25 μ l contained cDNA generated from 125 ng of RNA, 1 μ l of each forward and reverse primer (added from 9 μ M stock solutions), and 12.5 μ l of IQ SYBR green Supermix (2 $\times$; Bio-Rad) in PCR-grade water. Real-time PCR was performed on an iCycler (Bio-Rad) under the following conditions: 95°C for 10 min, 45 cycles (1 cycle consists of 95°C for 40 s, 57°C for 40 s, and 72°C for 45 s), and 72°C for 15 min. PCR products were checked by melting-curve analysis. Cycle threshold (C_T) values intersecting the exponential parts of amplification curves of positive reactions were always determined at a constant fluorescence level of 500 relative units. For each gene, a standard curve was established with a 10-fold serial dilution of cDNA corresponding to a range of 2 ng to 2 μ g of RNA. PCR efficiencies (E) were calculated according to $E = 10^{(-1/\text{slope})}$, where the slopes were derived from linear regression of plots of C_T values versus log cDNA inputs (42). E values ranged from 1.807 (β -actin) to 1.997 (*lcc3*) (see Table S1 in the supplemental material). Gene expression analyses were performed with the iQ5 optical system software, version 2.0 (Bio-Rad), enabling correction of PCR efficiency and comparison to multiple reference genes. The housekeeping genes β -actin and 18S rRNA were both used as reference genes for the normalization of target gene expression according to reference 53, and the data derived thereof are referred to as normalized gene expression levels. Normalized laccase gene expression levels of fungal cultures treated with potential laccase inducers, which were set in relation to those obtained from fungal cultures without potential laccase inducers (controls) using the iQ5 optical system software mentioned before, are referred to as relative gene expression levels.

Statistical analyses. For estimating relevant laccase genes contributing to the recorded extracellular laccase activities, normalized *lcc1* to *lcc5* mRNA transcript levels and laccase activities of induction treatments and controls were fitted to a linear multivariate model based on the step algorithm using the Akaike's information criterion (AIC). AIC uses a stepwise goodness of fit which is derived from the penalized estimated residual inertia and constraint ranks. For estimating the active laccase genes involved, both the forward (adding) and backward (eliminating) procedures were used, starting from the null model followed by the addition of the different laccase genes to explain the laccase activities and starting from the full model followed by a stepwise reduction of nonrelevant laccase genes, respectively. The procedure stops when by addition or reduction of a laccase gene, respectively, the model does not gain or lose significant explanatory power (54). For estimating relevant interplay between

FIG 1 Extracellular laccase activities of 15-day-old *C. aquatica* cultures with dependence on CuSO_4 added to fungal cultures on culture day 4. Symbols indicate means $\pm$ standard deviations for triplicate cultures. The dashed line arises from data fitting to a dose-response model with variable Hill slope, with an EC_{50} of 1.82 ± 0.11 μM (mean $\pm$ standard error) calculated for copper.

extracellular laccase activities and normalized *lcc1* to *lcc5* mRNA transcript levels, direct multiple linear correlations were established. Correlation strengths were determined by calculation of the Pearson's correlation coefficient for all pairwise combinations of laccase activities and transcript levels of the individual laccase genes. The computational environment R, version 2.13.0 (43), was used for all of these calculations.

The OriginPro software mentioned before was used to perform Kruskal-Wallis, Levene's, and Dunn-Sidak tests as indicated. Outliers among parallel laccase activity data and C_T values of laccase and reference gene mRNA transcripts were identified using a Dean-Dixon test (14).

Nucleotide sequence accession numbers. The sequence of the 18S rRNA gene was submitted to GenBank and is accessible under accession no. [FJ804122](#). Sequences for *lcc1*, *lcc2*, *lcc3*, *lcc4*, and *lcc5* were submitted to GenBank and are available under accession no. [FJ940742](#), [FJ940743](#), [FJ804119](#), [FJ804120](#), and [FJ804121](#), respectively.

RESULTS

Effects of potential laccase inducers on extracellular laccase activity and fungal biomass. The influence of increasing copper concentrations essentially applied in the form of CuSO_4 (except a 47 nM copper background concentration of unknown nature contained in the malt extract medium [49]) on extracellular laccase activities of *C. aquatica* is shown in Fig. 1. An estimation of the total copper concentration leading to half-maximal laccase activity (EC_{50}) led to a remarkably low value of 1.82 ± 0.11 μM (mean $\pm$ standard error). A laccase activity-saturating CuSO_4 concentration of 50 μM (Fig. 1) was chosen for all further experiments employing this compound.

Extracellular laccase activities were not detectable in any type of fungal cultures (i.e., induction treatments and controls) just 1 day after the addition of potential laccase inducers (culture day 5; early trophophase [26]) but could be clearly recorded at culture days 10 (corresponding to the trophophase) and 15 (onset of the idiophase) (Fig. 2A and B and Table 1; see also Fig. S1 in the supplemental material). Laccase activities were based on fungal dry masses, since fungal biomasses significantly differed ($\alpha = 0.05$) between the tested types of cultures and over time according to a Kruskal-Wallis test chosen because of sometimes heteroscedastic variances of data (indicated by Levene's test at $\alpha = 0.05$; data not shown). Since treatment Cd strongly inhibited the growth of *C. aquatica* (only about 10% fungal dry mass, as related to the other types of fungal cultures on culture day 15) and laccase

FIG 2 Relative extracellular laccase activities (i.e., laccase activities of induction treatments divided by those of the respective controls) (A and B) and relative expression levels of the laccase genes *lcc1* to *lcc5* (i.e., normalized laccase gene expression levels of induction treatments divided by those of the corresponding controls, respectively) (C and D) in heavy metal- and/or organic-treated *C. aquatica* cultures on culture days 10 (A and C) and 15 (B and D). Relative laccase activities are means $\pm$ standard deviations (calculated according to Gaussian error propagation rules) for triplicate cultures (except for induction treatments Mn and Zn, for which only values from single fungal cultures were available on culture day 10, and for induction treatments Cu-V and Zn, for which duplicate cultures were considered since an outlier has been identified using a Dean-Dixon test and excluded from further analysis on culture day 15). Relative gene expression levels mostly are means $\pm$ standard deviations for triplicate analyses of one cDNA sample derived from previously pooled triplicate cultures (duplicate analyses in some cases where an outlier has been identified using a Dean-Dixon test and excluded from further analysis).

activities could not be detected (data not shown), it was excluded from further analyses. All other induction treatments and controls showed significant fungal growth over time, with the highest dry masses always observed on culture day 15 (verified using Dunn-Sidak tests at $\alpha = 0.05$). Laccase activities on culture day 15 com-

pared to culture day 10 were about 10-fold higher in control cultures and about 3-fold (treatment Zn) to roughly 29-fold (treatment Cu) higher in induction treatments (except treatment V; see below) (Fig. 2A and B, Table 1; see also Fig. S1 in the supplemental material). These higher laccase activities were signifi-

TABLE 1 Normalized gene expression levels^a and fungal dry mass-based extracellular laccase activities in *C. aquatica* control cultures

Culture day	Normalized gene expression (fold) ^b					Laccase activity (U g ⁻¹ dry mass) ^c
	<i>lcc1</i>	<i>lcc2</i>	<i>lcc3</i>	<i>lcc4</i>	<i>lcc5</i>	
10	0.58 $\pm$ 0.24	0.86 $\pm$ 0.33	1.40 $\pm$ 0.67	0.47 $\pm$ 0.16	0.26 $\pm$ 0.36	3.12 $\pm$ 0.64
15	0.27 $\pm$ 0.04	0.40 $\pm$ 0.07	0.28 $\pm$ 0.09	0.28 $\pm$ 0.03	0.13 $\pm$ 0.08	31.35 $\pm$ 7.28

^a The β -actin and 18S rRNA genes together were used as reference genes for the normalization of *lcc1* to *lcc5* mRNA transcript levels according to reference 53.

^b Values are means $\pm$ standard deviations from triplicate analyses (duplicate analyses for *lcc4* and *lcc5* on culture day 10, where an outlier has been identified using a Dean-Dixon test and excluded from further analysis) of one cDNA sample derived from previously pooled triplicate cultures.

^c Values are means $\pm$ standard deviations from triplicate cultures.

TABLE 2 Identity and similarity between amino acid sequences covering cbr I to III of putative laccase genes detected in *C. aquatica*

Laccase gene	% Identity (% similarity)				
	<i>lcc1</i>	<i>lcc2</i>	<i>lcc3</i>	<i>lcc4</i>	<i>lcc5</i>
<i>lcc1</i>	100 (100)	25 (35)	35 (48)	35 (55)	44 (64)
<i>lcc2</i>		100 (100)	21 (35)	22 (36)	24 (36)
<i>lcc3</i>			100 (100)	38 (54)	32 (49)
<i>lcc4</i>				100 (100)	39 (58)
<i>lcc5</i>					100 (100)

cant (Dunn-Sidak test at $\alpha = 0.05$) for most types of fungal cultures except for induction treatments MeOH, Mn, V, and Zn. Treatment V represents the only example where the laccase activity of culture day 15 (about 52 U g⁻¹) was, albeit insignificantly, lower than that of culture day 10 (about 62 U g⁻¹) (Fig. S1).

On culture day 10, laccase activities of all vanillic acid-containing induction treatments (with the rank order V > Cu-V > Cu-V-tNP) were more than 5-fold higher than the corresponding control value (Fig. 2A), which was significant (Dunn-Sidak test at $\alpha = 0.05$) for treatments Cu-V and V. Higher laccase activities in induction treatments than in controls were generally less pronounced on culture day 15 (Fig. 2B). Laccase activities more than 2-fold higher than in controls were recorded only for treatments Cu, Cu-V, and Cu-V-tNP (significant at $\alpha = 0.05$ according to Dunn-Sidak test for treatments Cu-V and Cu-V-tNP). Slightly (less than 2-fold) enhanced laccase activities, compared to controls, were observed for treatments Mn and V.

Identification of laccase and 18S rRNA gene fragments. Different PCR products were obtained on the mRNA level using the degenerated laccase primer pair Cu1AF/Cu3R, which targets gene fragments ranging from the laccase copper binding regions (cbr) I to III (27). Since *C. aquatica* strain WD(A)-00-1 represents an haploid stage of the organism (49), different nonallelic laccase genes are present in the genome. The use of this primer pair and cloning and sequencing of the resulting PCR products allowed an extension of the gene fragments *lcc1* and *lcc2* already identified in reference 49, as well as the identification of 3 additional putative laccase gene fragments (*lcc3*, *lcc4*, and *lcc5*). The deduced partial amino acid sequences of *lcc1*, *lcc2*, *lcc3*, *lcc4*, and *lcc5* cover a span of 334 (*lcc4*) to 382 amino acids (*lcc2*) and perfectly match the fungal laccase signature sequences L1 (H-W-H-G-X₉-D-G-X₅-QCPI), L2 (G-T-X-W-Y-H-S-H-X-Q-Y-C-X₃-D-G-L-X-G), and L3 (H-PXH-L-H-G-H) identified previously (32), hence indicating that *C. aquatica lcc1* to *lcc5* represent laccases sensu stricto (13, 31). The identities between the amino acid sequences corresponding to cbr I to III of the 5 putative laccase genes are rather low and range from 21 to 44% for pairwise comparisons of *lcc2* and *lcc3* and of *lcc1* and *lcc5* (Table 2). *lcc2* possesses the most divergent sequence and generally displays only low identities with the other laccase sequences, not exceeding 25%. *lcc1* and *lcc5* are the most identical (Table 2).

A BLAST search with the deduced amino acid sequences of *C. aquatica lcc1* to *lcc5* yielded identities of 59, 51, 34, 52, and 66% with laccases/multicopper oxidases from the ascomycetes *Phaeosphaeria halima* (accession no. AAN17291.1), *Glomerella graminicola* M1.001 (accession no. EFQ31500.1), *Fusarium oxysporum* (Lcc2; accession no. ABS19939.1), *Pyrenophora tritici-repentis* Pt-1C-BFP (laccase-1 precursor; accession no. XP_

001940410.1), and *Phaeosphaeria spartinicola* (accession no. AAN17282.1), respectively.

Laccase gene expression and correlation with extracellular laccase activities. The effects of the different induction treatments on the relative gene expression levels of *lcc1* to *lcc5* (i.e., relative to fungal control cultures) are summarized for culture days 10 and 15 in Fig. 2C and D, respectively. Table 1 displays the normalized *lcc1* to *lcc5* mRNA transcript levels of fungal control cultures, which were used as a calibrator for the calculation of relative laccase gene expression levels of induction treatments. Normalized laccase gene expression levels of all types of fungal cultures are compiled in Fig. S1 in the supplemental material. No sufficient amount of RNA was obtained from any type of fungal culture on culture day 5, and hence, laccase mRNA transcript levels could not be determined for this culture day.

Complex expression patterns of *lcc1* to *lcc5* in response to the tested compounds and the stage of cultivation were obtained, with a culture age-dependent upregulation of the transcription of certain laccase genes, e.g., recorded for treatments Mn, Zn, V, tNP, Cu-V, Cu-V-tNP, MeOH, and Tween (Fig. 2C and D). Surprisingly, laccase gene expression was not enhanced upon application of the well-established laccase inducer copper (18) (Fig. 2C and D, treatment Cu). Those induction treatments supplemented with the phenolics vanillic acid and/or tNP additionally contained methanol (treatments V and Cu-V) or methanol and Tween 80 in combination (treatments tNP, Cu-tNP, and Cu-V-tNP) to improve compound solubility. Therefore, their relative laccase gene expression levels were compared with the respective counterparts in treatments containing only methanol (treatment MeOH) or Tween 80 (treatment Tween) to discriminate between effects of the phenolic compounds and the solvent/detergent (see Table S2 in the supplemental material). A clearly inducing effect of vanillic acid when applied alone as well as in combination on the transcription of *lcc4* was recorded on culture day 10, with an induction treatment rank order of V > Cu-V-tNP > Cu-V (Fig. 2C and Table S2). Treatment Cu-V further caused an induction especially of *lcc3* transcription on culture day 15, with less pronounced effects on other laccase genes at this time point (Table S2). Treatment tNP led to a boost of the transcription of particularly *lcc5* on culture day 15 (Fig. 2D and Table S2). Further possible effects of vanillic acid, tNP, and any combinations thereof on laccase gene transcription remain ambiguous (Table S2). Methanol when applied alone (treatment MeOH) comparatively strongly induced especially *lcc5* and, albeit less pronounced, also *lcc1* transcription on culture day 10 (Fig. 2C and Fig. S1). Treatment Tween led to a considerable enhancement of the transcription of all laccase genes except *lcc4* particularly on culture day 15, with the rank order *lcc1* > *lcc3* > *lcc5* > *lcc2* (Fig. 2D and Fig. S1). Less pronounced but still detectable effects of Tween 80 on the induction of laccase genes were also observed on culture day 10 (Fig. 2D and Fig. S1).

Mixtures of potential laccase inducers led to clearly higher laccase gene expression levels than single components of mixtures, particularly for treatment Cu-V on culture day 15. In this case, the laccase gene expression levels (except that of *lcc2*) were about 2- to 9-fold higher than the corresponding sums of gene expression levels from induction treatments employing either copper (treatment Cu) or vanillic acid (treatment V) alone, whereas the expression level of *lcc2* equaled the sum of its expression levels in treatments Cu and V (Fig. 2D and Fig. S1). For treatments Cu, tNP, and Cu-tNP, laccase gene expression in the presence of the inducer

mixture was not remarkably higher than the highest value observed in the presence of a single constituent of the mixture on culture day 10 (Fig. 2C and Fig. S1) and was lower than the highest value recorded upon application of a single component of the mixture on culture day 15 (Fig. 2D and Fig. S1).

Normalized *lcc1* to *lcc5* mRNA transcript levels of fungal control cultures were roughly 2-fold (about 5-fold for *lcc3*) higher during the trophophase (culture day 10) than at the onset of the idiophase (culture day 15) (Table 1). Nearly 2-fold or even higher normalized expression levels of certain laccase genes on culture day 15 than on culture day 10 were observed for induction treatments Mn (*lcc2* and *lcc4*), tNP (*lcc1*, *lcc3*, and *lcc5*), Cu-V (*lcc2*, *lcc3*, and *lcc5*), and Tween (*lcc1*), whereas in all other cases the normalized laccase gene expression levels were either higher on culture day 10 or rather similar on both culture days (Fig. S1).

In order to identify the most relevant *C. aquatica* laccase gene(s) contributing to the measured extracellular laccase activities, a linear multiple regression stepwise model building algorithm, using Akaike's information criterion (AIC) as a selection criterion, was applied (54). For culture day 10, the laccase activities recorded in induction treatments and controls (Fig. 2A and Table 1) are best explained by *lcc4* gene expression (Fig. 2C, Table 1, and Fig. S1) as consistently obtained with the forward and the backward model procedures, both leading to the lowest AIC (43.32 for *lcc4* versus 64.64 for the null model and 50.03 for the full model) and a low residual sum of squares (392.4 for *lcc4* versus 3,270.7 for the null model and 378.8 for the full model), respectively. The most striking effects regarding a concomitant enhancement of extracellular laccase activities and upregulation of *lcc4* expression were observed with the vanillic acid-containing induction treatments V, Cu-V, and Cu-V-tNP (Fig. 2A and C). Multiple regression fitting following the method described above did not result in a sufficient alignment of the expression of *C. aquatica* laccase gene(s) to laccase activities for culture day 15 (Fig. 2B and D, Table 1, and Fig. S1).

Linear correlations estimates between all measured laccase activities and normalized *lcc1* to *lcc5* mRNA transcript levels (see Tables S3 and S4 in the supplemental material) were conducted. Strong significant correlation between laccase activity and *lcc4* gene expression (Pearson's correlation coefficient of 0.938) was found for culture day 10 (Table S3). No remarkable correlations between laccase activities and laccase gene expression levels were observed for culture day 15, as indicated by low Pearson's correlation coefficients, not exceeding a value of 0.006 (Table S4). Notably, *lcc1* and *lcc5* gene expression levels were particularly quite highly correlated on culture day 10 (Pearson's correlation coefficient of about 0.94), whereas all other pairwise comparisons of mRNA transcript levels of laccase genes yielded lower Pearson's correlation coefficients (Tables S3 and S4).

DISCUSSION

The occurrence of multiple laccase genes in one organism and their differential regulation in response to numerous external factors and the developmental stage are widely known from terrestrial asco- and basidiomycetes (10, 18, 31, 33). The present study on *C. aquatica* thus expands the knowledge to ascomycete-related freshwater fungi. Differentially expressed laccase genes of *C. aquatica* with dependence on the growth stage of this fungus (Fig. 2C and D and Table 1) corroborate related observations in terrestrial fungi, where such effects have been attributed to different

functions of laccases during the fungal life cycle (18). Maximal laccase expression during the earlier growth stages of basidiomycetes has been attributed to a role in lignin bioconversion expected to be required during the colonization of lignocellulosic substrates, whereas maximal laccase expression during the stationary phase has been linked to laccase functions in fungal morphogenesis of, e.g., fruiting bodies (13, 18, 31). Different functions of the *C. aquatica* laccases are also indicated by the observation that *lcc4* obviously accounts for the extracellular laccase activity recorded in the culture media during the trophophase to a major extent (see Table S3 in the supplemental material). The transcription of other *C. aquatica* laccase genes monitored during the growth phase of the organism (Fig. 2C and Fig. S1) may have resulted in enzymes remaining cell associated (51, 52) or in proteins being nonfunctional in laccase activity (28). For the stationary phase of *C. aquatica*, the obtained pattern of laccase activity and gene expression data (Fig. 2B and D, Fig. S1, and Table S4) is too complex to relate laccase genes to the measured laccase activities. Individual laccase activities of the different induction treatments observed at the onset of the stationary phase, which mostly greatly exceeded the corresponding activities recorded during the trophophase (Fig. 2A and B and Fig. S1), may partly be due to the upregulation of certain laccase genes with the onset of the stationary phase of the organism as observed for the induction treatments Mn, tNP, Cu-V, and Tween (Fig. 2C and D and Fig. S1). Enhanced laccase activities in culture supernatants may also result from an increasing release of cell-associated laccases with the onset of the stationary phase. The release of intracellular laccases into the culture medium due to cell lysis at the end of the trophophase has been reported for white rot fungi (8). A release from mycelia into agitated liquid culture media increasing at later cultivation stages may also apply to extracellular laccase forms normally (i.e., under natural conditions) staying associated with fungal cell surfaces, which have been demonstrated for both asco- and basidiomycetes (19, 40, 41). Extracellular laccase(s) remaining associated with fungal cells, e.g., within an extracellular polysaccharide sheath, would be favorable for the aquatic lifestyle of *C. aquatica*, where a loss of extracellular enzymes due to washout and dispersal by the water flow would have to be prevented (49).

Natural functions of ascomycete as well as basidiomycete laccases are not fully understood and potentially include, among others, lignocellulose degradation and oxidation of toxic phenolic compounds (31, 56). Lignin solubilization has been reported for diverse freshwater ascomycetes, but there is only scarce information regarding the abilities of AQHs to act on lignin, and extensive lignin degradation comparable to that caused by terrestrial white rot fungi is not known from AQHs (9, 30). Laccase-catalyzed oxidations can detoxify natural compounds such as low-molecular-weight phenolics arising from lignin depolymerization (39), antibiotics produced by microorganisms antagonistic to plant-pathogenic fungi (46), and antimicrobial plant compounds such as, e.g., flavonoids or phytoalexins (17, 31, 37), but also xenobiotics such as, e.g., various EDCs (29). The inducibility of *lcc4* by the lignocellulose breakdown product vanillic acid and vanillic acid-containing compound mixtures particularly during the trophophase of the saprotroph *C. aquatica* (Fig. 2B) would be in line with a role for the corresponding extracellular laccase during colonization of decaying leaves and woody debris serving as fungal substrates, perhaps contributing to the detoxification of plant-related phenolics. Induction of fungal laccase gene transcription by

lignin-related aromatic compounds has been widely reported (18). Other potential functions of *C. aquatica* laccase(s) may be related to competition or other interspecies interactions (4, 24, 31), which could be expected during successions of microbial communities on AQH substrates in aquatic environments (30), and to pigmentation/melanization (17, 24, 52) as fungal pellets of liquid *C. aquatica* cultures are turning from grayish into black color with increasing culture age (data not shown), indicating the formation of melanin-like pigments. The highly correlated transcription of the laccase genes *lcc1* and *lcc5* during the trophophase of *C. aquatica* (see Table S3 in the supplemental material) and their comparatively high degree of identity among the *C. aquatica* laccase genes (Table 2) suggest that the corresponding laccase enzymes share an as-yet-unknown function.

An enhanced transcription of laccase genes under the influence of the phenolic compound tNP, as particularly observed for *lcc5* on culture day 15 (Fig. 2D and Table S2), was also reported for the white rot fungus *Trametes versicolor* (29). The observed influence of methanol on *C. aquatica* laccase gene expression (Fig. 2C, Fig. S1, and Table S2) may indicate a general stress response to the compound and corroborates previous results obtained with asco- and basidiomycetes (38, 47). The induction of *C. aquatica* laccase gene transcription by Tween 80 (Fig. 2C and D, Fig. S1, and Table S2) confirms a regulatory role for this detergent in laccase production as already implied in previous studies (15).

None of the *C. aquatica* laccase genes seems to be upregulated by copper (Fig. 2C and D and Fig. S1). This result is quite unexpected since copper is known to strongly enhance the transcription of most genes of fungal laccases *sensu stricto* investigated so far (18, 31), despite the existence of some copper-independent laccase genes in ascomycetes (33) as well as basidiomycetes (48). Nevertheless, a regulatory role for copper in *C. aquatica* laccase gene expression is indicated, since in combination with organic laccase inducers copper can either enhance (compare *lcc3* expression in treatments Cu, V, and Cu-V on culture day 15 [Fig. 2D, Fig. S1, and Table S2]) or diminish (compare *lcc4* expression in treatments Cu, Cu-V, and V on culture day 10 and *lcc5* expression in treatments Cu, Cu-tNP, and tNP on culture day 15 [Fig. 2C and D, Fig. S1, and Table S2]) laccase gene expression. The reasons for such effects still remain to be explored. Synergistic effects of different factors on laccase expression have often been reported for terrestrial fungi (11, 18). Copper strongly increases extracellular laccase activities in *C. aquatica* (Fig. 1). Whereas an as-yet-unknown regulatory effect of copper on posttranscriptional or post-translational laccase modifications (31) seems possible in principle, perhaps a more likely explanation for the observed effect of copper on laccase activity could be a stabilization of the copper-containing catalytic center of the enzyme, e.g., via (partial) incorporation of excess copper as has previously been proposed for white rot fungi (11). The presence of copper-interacting amino acid ligands of all three types of laccase copper centers in the *C. aquatica* laccase proteins is indicated by the fungal laccase signature sequences L1 to L3 (24, 31, 32) found in the deduced partial amino acid sequences of *C. aquatica* *lcc1* to *lcc5*. In favor of possible effects of copper on the functionality of the *C. aquatica* laccase protein(s) are the increased laccase activities of the copper-containing induction treatments Cu-V and Cu-V-tNP on culture day 15 (i.e., at the onset of the idiophase of the organism, when the highest laccase activities in culture supernatants were observed). Comparatively lower laccase activities were observed in those

treatments where either copper or organic inducers were applied as single components (treatments Cu, V, and tNP [Fig. 2B and Fig. S1]). A quite low copper concentration, of about 1.8 μM (corresponding to about 114 $\mu\text{g liter}^{-1}$), found to half-saturate extracellular laccase activity in *C. aquatica* (Fig. 1) may indicate a fungal adaptation to low copper concentrations of the aquatic habitat of the organism. Copper concentrations below 20 $\mu\text{g liter}^{-1}$ and of 16 mg kg^{-1} , respectively, have been reported for water and sediments at the isolation site of the *C. aquatica* strain used in the present study (25, 50). Water copper concentrations below 20 $\mu\text{g liter}^{-1}$ and sediment copper concentrations not exceeding the three-digit mg kg^{-1} range were reported for other aquatic sites showing no or only moderate pollution, with *C. aquatica* being present at most of these sites (50). In contrast, water as well as sediment concentrations of manganese and zinc at these sites are up to 1 order of magnitude higher than the respective copper concentrations, whereas concentrations of cadmium about 1 order of magnitude lower than those of copper were reported (50). Interestingly, manganese and zinc cause a culture age-dependent upregulation of certain laccase genes in *C. aquatica*, although corresponding gene expression levels under the influence of zinc were comparatively weak (Fig. 2C and D and Fig. S1). Manganese regulation of laccase gene expression has repeatedly been demonstrated in white rot basidiomycetes (44, 48) and likely reflects the reported capability of fungal laccases to oxidize divalent manganese in the presence of appropriate fungal organic acids, thereby producing chelated trivalent manganese as an oxidant contributing to lignocellulose decay (20, 31, 45). Also, enhanced laccase production upon zinc exposure is known from basidiomycetes (6, 22). The high sensitivity of *C. aquatica* toward cadmium, where a strong growth inhibition was already observed at 50 μM , contrasts with results reported for white rot basidiomycetes, for which cadmium concentrations of up to the mM range still enabled growth and increased the laccase production (5). All in all, the observed effects of the investigated metals on laccase expression in *C. aquatica* reflect the habitat conditions of the organism very well and may be interpreted as a result of the fungal adaptation to freshwater environments.

ACKNOWLEDGMENTS

We are grateful to the DFG (German Research Foundation) research training group 416 (Adaptive Physiological and Biochemical Reactions to Ecological Important Substances) at the Martin-Luther-University Halle-Wittenberg (Germany) and to the Helmholtz Centre for Environmental Research-UFZ (Leipzig, Germany), research topic CITE (Chemicals in the Environment), for providing resources for this research.

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