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FULL PAPER

ChMCO1 of *Cochliobolus heterostrophus* is a new class of metallo-oxidase, playing an important role in DHN-melanization

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Abstract A metallo-oxidase gene from a phytopathogenic filamentous fungus, *Cochliobolus heterostrophus* was cloned. Structural prediction of *ChMco1* indicated that this protein lacks a transmembrane helix and is soluble, whereas other known fungal metallo-oxidases including *Saccharomyces cerevisiae* FET3 are localized to the cell membrane. The results of searches in fungal genomic databases and phylogenetic analysis of fungal metallo-oxidases revealed that *ChMco1* and its allies are distinct homologues of *Fet3* and unique to filamentous ascomycetous species including *C. heterostrophus*. We performed a functional analysis of ChMCO1 by generating null mutants for the *ChMco1* gene. The *ChMco1* null (Δ*ChMco1*) mutants clearly had reduced melanization, although they showed normal growth and conidiation. Results also show that Δ*ChMco1* mutants lost laccase activity. These results suggest that ChMCO1 is a novel class of metallo-oxidase that is necessary for laccase activity and melanization.

Keywords Filamentous fungi · Laccase · Mco1 · Melanin

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Introduction

Organisms have various proteins that require copper as their cofactors and are used for various biochemical reactions. Copper-transporting ATPases are proteins that are localized to the trans-golgi network and supply copper to copper-containing proteins. In eukaryotes, it is known that most copper-containing proteins are produced at this site (Huffman and O'Halloran 2001). In previously reported work, we cloned and described a copper-transporting ATPase gene, *ChCcc2*, from a plant pathogen and ascomycetous filamentous fungus *Cochliobolus heterostrophus* (anamorph: *Bipolaris maydis*, the causal agent of Southern corn leaf blight) (Saitoh et al. 2009). We showed that melanization, growth, and conidiation were severely affected in *ChCcc2* mutants. These impairments would result from the inactivation of copper-containing proteins targeted by ChCCC2, for example multicopper oxidases (MCOs), amine oxidases, and tyrosinases. Functional analyses of these copper-containing proteins are necessary for comprehensive understanding of the importance of copper delivery mediated by copper-transporting ATPase in fungi.

The group of MCOs includes copper-containing proteins such as ascorbate oxidases, bilirubin oxidases, laccases, and metallo-oxidases (Nakamura and Go 2005). The metallo-oxidase subfamily is known to be able to oxidize Cu^{1+} to Cu^{2+} and Fe^{2+} to Fe^{3+} (Shi et al. 2003; Stoj and Kosman 2003). However, their biological roles remain unclear. To date, several reports have described metallo-oxidases in yeasts (Eck et al. 1999; Eichhorn et al. 2006). For example, genetic studies of *Saccharomyces cerevisiae* have established that oxidation of Fe^{2+} to Fe^{3+} by a metallo-oxidase FET3 is necessary for iron uptake (high-affinity iron uptake) (Eide 1998; Kosman 2003). In

contrast, few reports describe metallo-oxidases from filamentous fungi, although members of the Fungi kingdom are overwhelmingly filamentous fungi. In this study, we cloned and characterized a metallo-oxidase gene, *ChMco1*, from a phytopathogenic filamentous fungus *C. heterostrophus*. Phylogenetic analysis showed that *ChMco1* is a new class of genes for fungal metallo-oxidases that are unique to filamentous ascomycetes. Null mutants for *ChMco1* showed less melanization and lost laccase activity. We also found *Aspergillus fumigatus Abr1*, that is necessary for melanization (Tsai et al. 1999), is an orthologue of *ChMco1*, suggesting that the role in melanization is conserved in *ChMco1* and its orthologues of filamentous ascomycetes. These results allowed us to define *ChMco1* and its orthologues as "DHN melanization class", a new class of metallo-oxidase family.

Materials and methods

Cloning the metallo-oxidase gene from *C. heterostrophus*

The wild-type *C. heterostrophus* strain, HITO7711 (Tanaka et al. 1991), was used for this study. Growth conditions and DNA isolation procedures were performed as described previously (Nakada et al. 1994). Genetic nomenclature and styles of genetic symbols in this paper basically followed the convention proposed by Yoder et al. (1986) with the two-letter prefix from the binomial species name.

The metallo-oxidase gene from *C. heterostrophus* was amplified using two degenerate primers that encompassed highly conserved regions of the protein [ChMCO1-f1 (5′-TGGGTYYGGGCADCKCCNGATGGCTT-3′) and ChMCO1-r1 (5′-GTGNGAGTGRTACCAAWANGT-3′)]. The amplified fragment was cloned into the pZErO-2 vector (Invitrogen) and analyzed using sequence analysis. The complete nucleotide sequence of the metallo-oxidase gene from *C. heterostrophus* (*ChMco1*) was obtained via inverse PCR (Ochman et al. 1988) and primer walking analysis. The gene sequence of *ChMco1* was deposited in DDBJ/EMBL/Genbank under accession no. AB505220. The sequence of a *Fet3* orthologue of *C. heterostrophus* (*ChFet3*) was amplified using the 3′ RACE method. Poly(A)$^+$ RNA isolation and RT-PCR were performed as described by Yoshimi et al. (2004). A primer (5′-CGA CGGCCAGTGCCAAGCTTTTTTTTTTT-3′) was used for first-strand cDNA syntheses. To amplify *ChFet3*, a degenerate primer, ChFET3f (5′-GTKTGSYTGWTCCAC TGCCAYAT-3′), annealed to the sequence in the highly conserved region of proteins classified as *Fet3* orthologues was used along with an adaptor primer (5′-CGAC GGCCAGTGCCAAGC-3′). The amplified fragment was cloned into the pZErO-2 vector (Invitrogen) and its sequence was analyzed. The sequence obtained was identical with the genomic sequence whose protein ID 104814 is that of the *C. heterostrophus* C5 strain (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=CocheC5_1&tid=104814).

Phylogenetic analysis of fungal metallo-oxidases

We obtained the predicted amino acid sequences of fungal MCOs from databases maintained by the Broad Institute's Fungal Genome Initiative (FGI) (http://www.broad.mit.edu/annotation/fgi/) and NCBI Genbank. MCO sequences from *Aspergillus fumigatus*, *A. nidulans*, *Botrytis cinerea*, *Candida albicans*, *Coprinopsis cinerea*, *Cryptococcus neoformans* serotype A, *Fusarium graminearum*, *Magnaporthe grisea*, *Neurospora crassa*, *Phaeosphaeria nodorum* (anamorph: *Stagonospora nodorum*), *S. cerevisiae*, and *Ustilago maydis* were obtained from the FGI database using the "feature search" function with the Cu-oxidase_3 domain (PF07732). The MCO sequences reported by previous studies from *Eschelichia coli*, *Gaeumannomyces graminis* var. *graminis*, *Acremonium* sp. HI-25, *Melanocarpus albomyces*, *Ceriporiopsis subvermispora*, *Lentinula edodes*, *Pleurotus ostreatus*, *Pleurotus* sp. Florida, *Pycnoporus cinnabarinus*, *Trametes pubescens*, *T. versicolor*, *T. villosa*, *Trametes* sp. AH28-2, *Trametes* sp. C30, and *Volvariella volvacea* were obtained from NCBI Genbank. *A. fumigatus* XP_756088, *A. nidulans* P17489, *F. graminearum* EAA70221, EAA78539, and *P. nodorum* XP_001791088, XP_001792899, XP_0017 92912, XP_001803284 were also obtained from the NCBI Genbank. The MCO sequences of *Phanerochaete chrysosporium* were obtained from the DOE Joint Genome Institute (JGI) website (http://www.jgi.doe.gov/) using a blastP search with MCO sequences of *S. cerevisiae* SCRG_01950 (FET3) and *T. versicolor* AAL07440 (LAC1). Sequences obtained from genome databases were annotated with accession numbers and gene names. The Cu-oxidase_3 (PF07732) and Cu-oxidase_2 (PF07731) domains were identified within the retrieved sequences using Pfam 22.0 software (http://pfam.jouy.inra.fr/index.html). Alignments of the Cu-oxidase_3 domain sequences and Cu-oxidase_2 domain sequences were performed with the ClustalX 2.0 program (http://www.clustal.org/) using default settings for multiple alignments. Alignments were adjusted manually and combined to one. A phylogenetic tree was constructed using the neighbor-joining method of the ClustalX 2.0 program. A resultant tree was drawn using the NJ-Plot program (Perrière and Gouy 1996). As the outgroup, *E. coli* YP_001878931 (CueO) was used; bootstrap analysis was performed with 1,000 replications. The resultant tree is presented in Fig. S1 in the electric

supplementary materials available online. The MCO sequences included in the same cluster with *S. cerevisiae* FET3, *C. albicans* CaFET3, and *U. maydis* FER1 in the resultant tree (Fig. S1c) were selected and used for phylogenetic analysis of fungal metallo-oxidases. Methods for alignments and tree construction were as described above.

Generation of *ChMco1* null mutant

The pZEROdMCO1 vector, which contained the hygromycin B phosphotransferase gene (*Hph*) cassette flanked by upstream and downstream sequences of the *ChMco1* open reading frame (ORF), was constructed and used to delete the *ChMco1* gene from *C. heterostrophus*. Briefly, an approximately 1,100-base-pair DNA fragment of the upstream region of the *ChMco1* gene was amplified using the DMCO1-5f (5′-CGTGGCCAAATCGGATCGCATC-3′) and DMCO1-5r (5′-CAGCCTCAAGCTTGCGCGGAAGTCATG-3′) primers. An approximately 400-base-pair DNA fragment of the downstream region of the *ChMco1* gene was amplified using the DMCO1-3f (5′-CATCTTTCCTCGTCGCCAAGACAT-3′) and DMCO1-3r (5′-AACTACTTCGAGCTGACGGTGT-3′) primers. The obtained fragments of the upstream and downstream regions of the *ChMco1* gene were introduced, respectively, into *Sma*I and *Eco*RV sites located on both sides of the *Hph* cassette in pZErO-Hph2 vector (pZErO-2 derivative, of which multiple cloning sites were partially modified to *Eco*RI–*Pst*I–*Eco*RV–*Hph* cassette–*Sma*I–*Not*I–*Xho*I). Subsequently, PCR was used to identify the direction of inserts using a primer that was homologous to the vector and a primer homologous to the insert, i.e. using M13-20 (5′-CGACGTTGTAAAACGACGGCCAGT-3′) as the forward primer and DMCO1-5r as the reverse primer for the insert from the upstream region, and DMCO1-3f as the forward primer and M13-RVM (5′-GGAAACAGCTATGACCATGATTAC-3′) as the reverse primer for the insert from the downstream region. The resulting plasmid pZEROdMCO1 was linearized by digestion with *Xba*I. The approximately 6,300-bp DNA fragment obtained was used for transformation of the *C. heterostrophus* wild-type strain HITO7711.

Fungal protoplasts were prepared using methods described in a previous report (Shimizu et al. 1997). Briefly, a 5-µl aliquot of plasmid DNA (1 µg/µl) was added to a protoplast suspension (i.e., 5×10^7 protoplasts/100 µl) in STC (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris–HCl; pH 7.5) and incubated for 20 min at room temperature. Subsequently, 1 ml of PTC solution (60% (*w*/*v*) poly(ethylene glycol) dissolved in STC) was added to the suspension and incubated for 20 min at room temperature. After removing the PTC solution by centrifugation, protoplasts were resuspended in 100 µl STC and mixed with 10 ml regeneration medium (1 g tryptone, 1 g yeast extracts, 15 g agar, and 1.2 M sucrose, per liter). After regeneration and germination of the resulting protoplasts, the solution was overloaded with 10 ml selection medium (1 g tryptone, 1 g yeast extract, 15 g agar, and 200 mg hygromycin B, per liter). The resulting transformants were isolated and reinoculated on to complete medium agar (CMA; Tanaka et al. 1991) containing 100 µg/ml hygromycin B.

Southern blot analysis

DNA digestion and gel electrophoresis were performed using standard methods (Sambrook et al. 1989). The probe consisted of a DNA fragment obtained through PCR amplification using DMCO1-3f as forward primer and DMCO1-3r as reverse primer. The DNA probe was labeled using the AlkPhos Direct labeling kit (GE Healthcare). Hybridization and detection were conducted using the AlkPhos Direct detection system (GE Healthcare), according to the manufacturer's instructions.

Complementation tests of Δ*ChMco1* mutants in broth media supplemented with metal ions

Mycelia from the wild-type strain and Δ*ChMco1* mutants were incubated for 48 h at 27°C in 100 ml of CM broth or CM broth supplemented with $CuSO_4$, $FeCl_3$, $MnCl_2$, and $ZnSO_4$; the resulting melanization of mycelia in these strains was studied (Saitoh et al. 2009). The concentrations of $CuSO_4$, $FeCl_3$, and $MnCl_2$ were 100 µM each. The concentration of $ZnSO_4$ was 25 µM because the mycelial growth of the wild-type strain was severely inhibited in CM broth media supplemented with $ZnSO_4$ more than 50 µM.

Preparation of cell-free extracts

Cell-free extracts were prepared according to the method used by Tanaka et al. (1992), with slight modifications. Briefly, 1 ml conidial suspension (approximately 10^5 conidia/ml) was inoculated into 100 ml CM broth or CM broth supplemented with 100 µM $CuSO_4$ for 48 h on a rotary shaker operated at 120 rpm. The resulting mycelia were collected by filtration through four-layered gauze and mixed with 0.3 volumes of sea sand (particle size 420–840 µm). Particles were broken into fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed with 1% (*w*/*w*) polyvinylpolypyrrolidone, suspended in a minimal volume of ice-chilled extraction buffer (10 mM Tris–HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0), and ground again. The homogenate was centrifuged for 20 min at $12{,}000 \times g$. The resulting supernatant

was separated and used as the cell-free extract. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology).

Laccase assay

Laccase activity was assayed colorimetrically as described by Tanaka et al. (1992), with slight modifications. The standard assay mixture was generated by filling a cuvette with 70 µl 500 µM syringaldazine as a substrate, 430 µl MacIlvane buffer (35.7 mM citric acid, 125 mM Na_2HPO_4, pH 6.0) and 50 µl cell-free extracts in which the protein concentration had been adjusted to 2 µg/µl. Substrate oxidation was measured using a spectrophotometer (Du®-64; Beckman Coulter) at 30°C and 525 nm, with a 1 cm optical path.

Appressorium formation assay and pathogenicity test

Conidia were suspended in water. Aliquots of the resulting spore suspensions (1×10^4 conidia/ml) were placed on glass slides and incubated under moist conditions at 27°C. After 16 h, cultures were inspected microscopically (DMLB DIC microscope; Leica Microsystems); the appressorium formation of the wild-type strain and Δ*ChMco1* mutants was observed.

Pathogenicity assays were performed using drops of spore suspension (1×10^4 conidia/ml) obtained from 7-day-old cultures on CMA. The drops were placed on paper disks (6 mm diameter). The resulting disks were placed on corn leaves cut from 14-day-old corn plants. Then they were incubated for 3 days under moist conditions at 27°C.

Results

Cloning and characterization of *C. heterostrophus* ChMCO1

A putative metallo-oxidase gene from *C. heterostrophus* was obtained by degenerate and inverse PCR. The amplified gene was cloned and named as *C. heterostrophus Mco1* (*ChMco1*). This gene includes an ORF composed of 1,476 bp, interrupted by two introns. Our phylogenetic analysis using ChMCO1 and other 133 MCO polypeptide sequences from 12 ascomycetous and 15 basidiomycetous species showed that ChMCO1 belongs to the cluster including sequences known as metallo-oxidases such as *S. cerevisiae* FET3 (Fig. S1). The degree of overall sequence identity of putative polypeptide between ChMCO1 and FET3 of *S. cerevisiae*, CaFET3 of *C. albicans*, and FER1 of *U. maydis* are, respectively, approximately 32, 36, and 34%. A subsequent Pfam HMM search showed that ChMCO1 consisted of Cu-oxidase 3 (PF07732), Cu-oxidase (PF00394), and Cu-oxidase 2 (PF07731) domains. This result is typical in fungal MCOs including metallo-oxidases. However, structural analyses using the transmembrane helices (THs) prediction program TMHMM v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) revealed that ChMCO1 had no THs, suggesting that ChMCO1 is a soluble protein whereas FET3 and other known fungal metallo-oxidases have one TH in the C-terminal region and are localized on the cell membrane (Fig. 1). We also investigated whether *C. heterostrophus* have a *Fet3* orthologue (i.e. a cell membrane-localized metallo-oxidase). We performed the 3′-RACE PCR and cloned a *Fet3* orthologue gene from *C. heterostrophus* (*ChFet3*) (Fig. 2). These results suggest

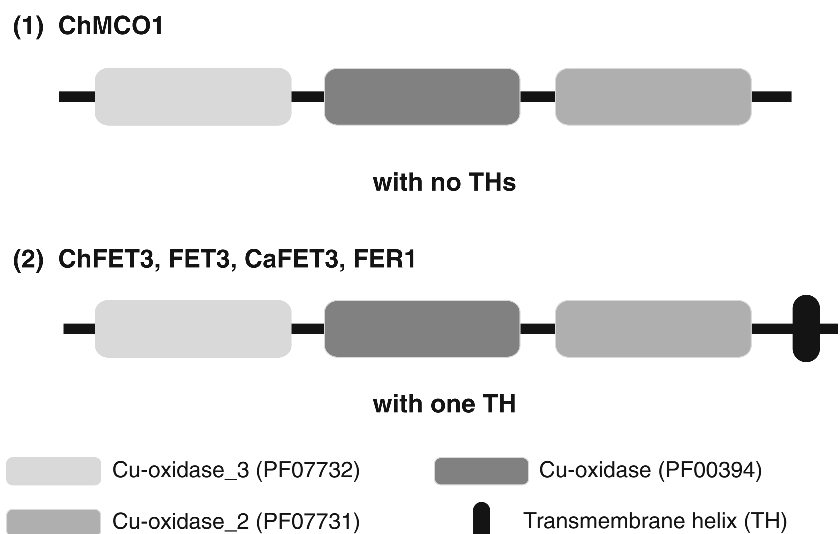

Fig. 1 Domain structures of fungal metallo-oxidase *C. heterostrophus* ChMCO1, *S. cerevisiae* FET3, *C. albicans* CaFET3, and *U. maydis* FER1

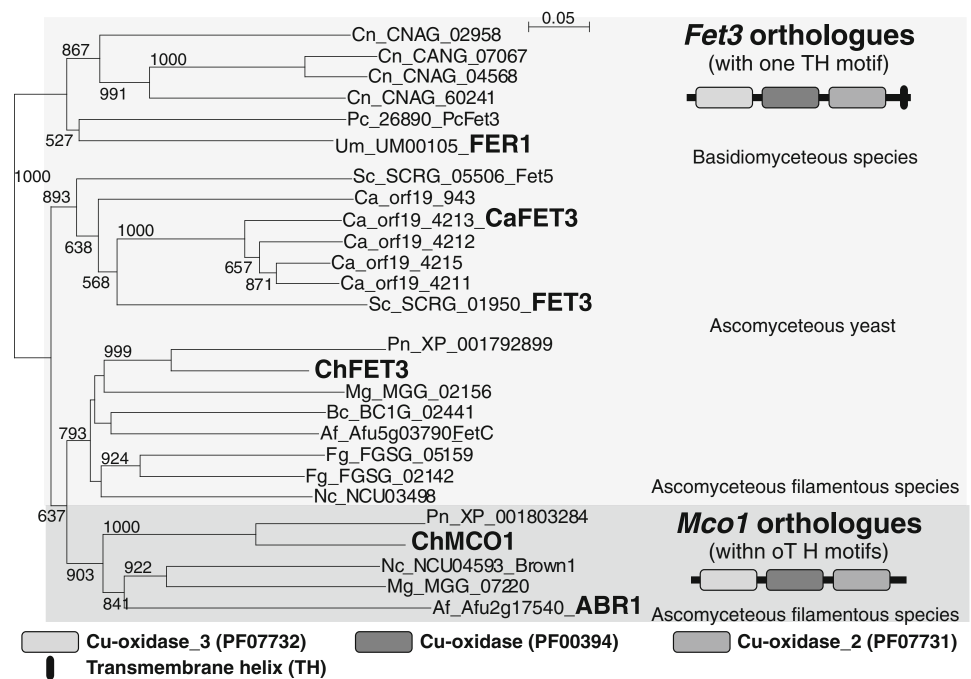

Fig. 2 Phylogenetic analysis of fungal metallo-oxidases. Bootstrap values are from 1,000 replications: only values ≥500 are shown. Species codes: *Af*, *Aspergillus fumigatus*; *Bc*, *Botrytis cinerea*; *Ca*, *Candida albicans*; *Cn*, *Cryptococcus neoformans* Serotype A; *Fg*, *Fusarium graminearum*; *Mg*, *Magnaporthe grisea*; *Nc*, *Neurospora crassa*; *Pc*, *Phanerochaete chrysosporium*; *Pn*, *Phaeosphaeria nodorum*; *Sc*, *Saccharomyces cerevisiae*; *Um*, *Ustilago maydis*

that *C. heterostrophus* have two metallo-oxidases, ChMCO1 and ChFET3.

ChMco1 represents a new class of fungal metallo-oxidase unique to ascomycetous filamentous species

We investigated the evolutionary relationship of *C. heterostrophus ChMco1* in *Fet3* homologues in fungi. Our phylogenetic and structural analyses of fungal metallo-oxidases suggest that *Fet3* orthologues (i.e. *Fet3* homologue with TH in the C-terminal of its product) are conserved across fungal species (Fig. 2). However, orthologues of *ChMco1* (i.e. *Fet3* homologue without TH, *Mco1* orthologues) are found in filamentous ascomycetous species only. Ascomycetous yeasts and basidiomycetous species have *Fet3* orthologues only, in general, whereas most filamentous ascomycetes have both *Fet3* and *Mco1* orthologues (Table 1). The *Fet3* and *Mco1* orthologues have identical functional domains other than THs in their products. *Mco1* orthologues are distinct homologues of *Fet3* metallo-oxidase genes, and can be classified as a new class. In addition, we report, for the first time, identification of *A. fumigatus Abr1* as a *Mco1* orthologue.

Deletion of the *ChMco1* gene

To investigate the function of ChMCO1, we generated null mutants for the *ChMco1* gene. For this purpose, a plasmid pZEROdMCO1 which contained the hygromycin B phosphotransferase gene (*Hph*) cassette flanked by upstream and downstream sequences of the *ChMco1* ORF was generated. First, pZEROdMCO1 was digested with *Xba*I, and the resulting DNA fragment was used for transformation of the *C. heterostrophus* wild-type strain HITO7711 (Fig. 3a). The *ChMco1* gene deletion of

Table 1 Number of *Fet3* and *Mco1* orthologues

Species	No. of genes encoding fungal metallo-oxidases	
	Fet3 orthologues	*Mco1* orthologues
Basidiomycetous species		
Phanerochaete chrysosporium	1	0
Ustilago maydis	1	0
Ascomycetous species		
Yeasts		
Candida albicans	5	0
Saccharomyces cerevisiae	2	0
Filamentous fungi		
Aspergillus fumigatus	1	1
Botrytis cinerea	1	0
Fusarium graminearum	2	0
Magnaporthe grisea	1	1
Neurospora crassa	1	1
Phaeosphaeria nodorum	1	1

Classification and count of fungal metallo-oxidase genes are based on the results presented in Fig. 2

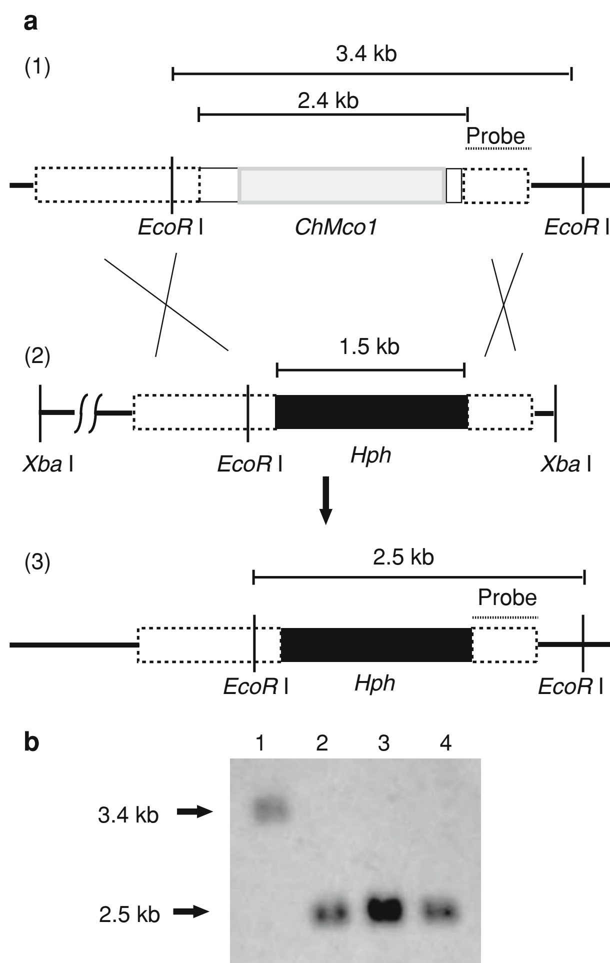

Fig. 3 Gene deletion of *ChMco1*. **a** Scheme used to delete the *ChMco1* gene with the DNA fragment containing 1.5 kb hygromycin B phosphotransferase gene (*Hph*) cassette as a selection marker between 1.1 kb upstream and 0.4 kb downstream sequences of *ChMco1* open reading frame. Maps show the *ChMco1* region in genomes of the wild-type strain HITO7711 (*1*), a DNA fragment used for the deletion of *ChMco1* ORF (*2*), and *ChMco1* null (Δ*ChMco1*) mutants DMCO1-1, DMCO1-2, and DMCO1-3 (*3*). **b** Southern blot analysis of *Eco*RI digests of genomic DNA from the wild-type HITO7711 and Δ*ChMco1* mutants DMCO1-1, DMCO1-2, DMCO1-3. *Lanes 1* HITO7711, *2* DMCO1-1, *3* DMCO1-2, *4* DMCO1-3

transformants was confirmed using Southern blot analysis, and three *ChMco1* null (Δ*ChMco1*) mutants—DMCO1-1, DMCO1-2, DMCO1-3—were obtained (Fig. 3b).

ChMCO1 plays an important role in normal melanization

Colony growth of the wild-type strain and Δ*ChMco1* mutants is portrayed in Fig. 4. Melanization in colonies of three Δ*ChMco1* mutants was clearly lower than that of the wild-type strain, suggesting that ChMCO1 plays an important role in melanization. No significant differences existed in growth and conidiation between Δ*ChMco1* mutants and the wild-type strain (data not shown).

Melanization of Δ*ChMco1* mutants is restored in copper-supplemented media

The Δ*ChMco1* mutants showed reduced melanization. The metallo-oxidase of *S. cerevisiae* FET3 seems to be necessary for iron uptake (Askwith et al. 1994; Yuan et al. 1995). To investigate whether abnormal melanization of Δ*ChMco1* mutants was caused by deficiency of intracellular iron ions, we cultivated Δ*ChMco1* mutants in media supplemented with $FeCl_3$ (Fig. 5a). No significant differences in melanization of Δ*ChMco1* mutants were found between normal media and $FeCl_3$-supplemented media. Furthermore, surprisingly, we observed the restoration of melanin production in Δ*ChMco1* mutants when cultivated in CM broth supplemented with $CuSO_4$ (Fig. 5a). Supplementation of $MnCl_2$ and $ZnSO_4$ did not restore the melanization of Δ*ChMco1* mutants, suggesting that an external supply of copper is necessary to restore melanization in Δ*ChMco1* mutants. The restoration of melanin production was moderate when Δ*ChMco1* mutants were grown on solid medium CMA supplemented with $CuSO_4$ (Fig. 5b).

Δ*ChMco1* mutants lost laccase activity

Laccases are important enzymes in fungal melanization because they function in the oxidative polymerization of melanin precursors such as naphthalene-1,8-diol (1,8-dihydroxynaphthalene, DHN) (Tanaka et al. 1991 1992). Laccases require copper ions as their cofactor. To investigate whether the activity of laccases was affected in Δ*ChMco1* mutants, the laccase assay was performed using cell-free homogenates extracted from mycelia of the wild-type strain and Δ*ChMco1* mutants (Fig. 6). Laccase activity was clearly observed in the cell-free homogenate of the wild-type strain, although Δ*ChMco1* mutants had no detectable laccase activity. Laccase activity of the Δ*ChMco1* mutants was restored to a similar level as that of the wild-type strain after growth in CM broth supplemented with $CuSO_4$. These results strongly suggest that the loss of laccase activity and subsequent decreased melanization are caused by reduced copper availability in Δ*ChMco1* mutants.

ChMCO1 is not related to pathogenicity

To determine whether the Δ*ChMco1* deletion interferes with invasion of the host plant, we performed an appressorium formation test. No clear differences were found in

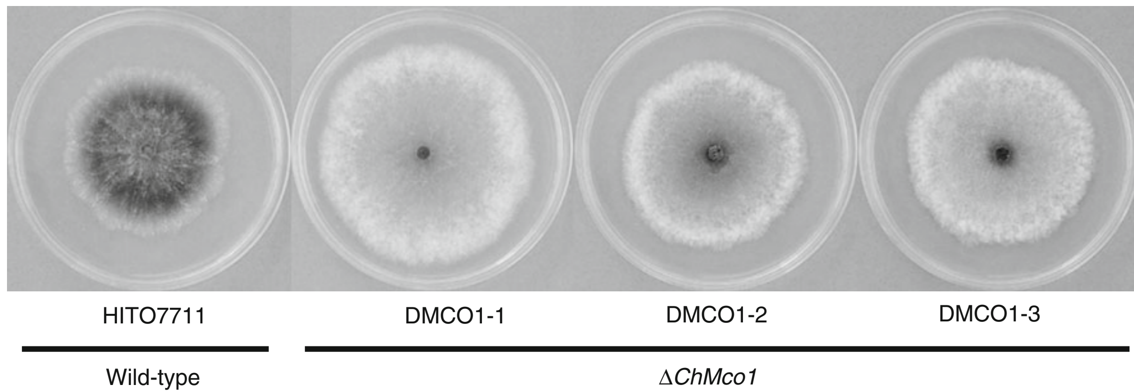

Fig. 4 Colony growth of the wild-type strain HITO7711 and the Δ*ChMco1* mutants DMCO1-1, DMCO1-2, and DMCO1-3 grown for 5 days on CMA

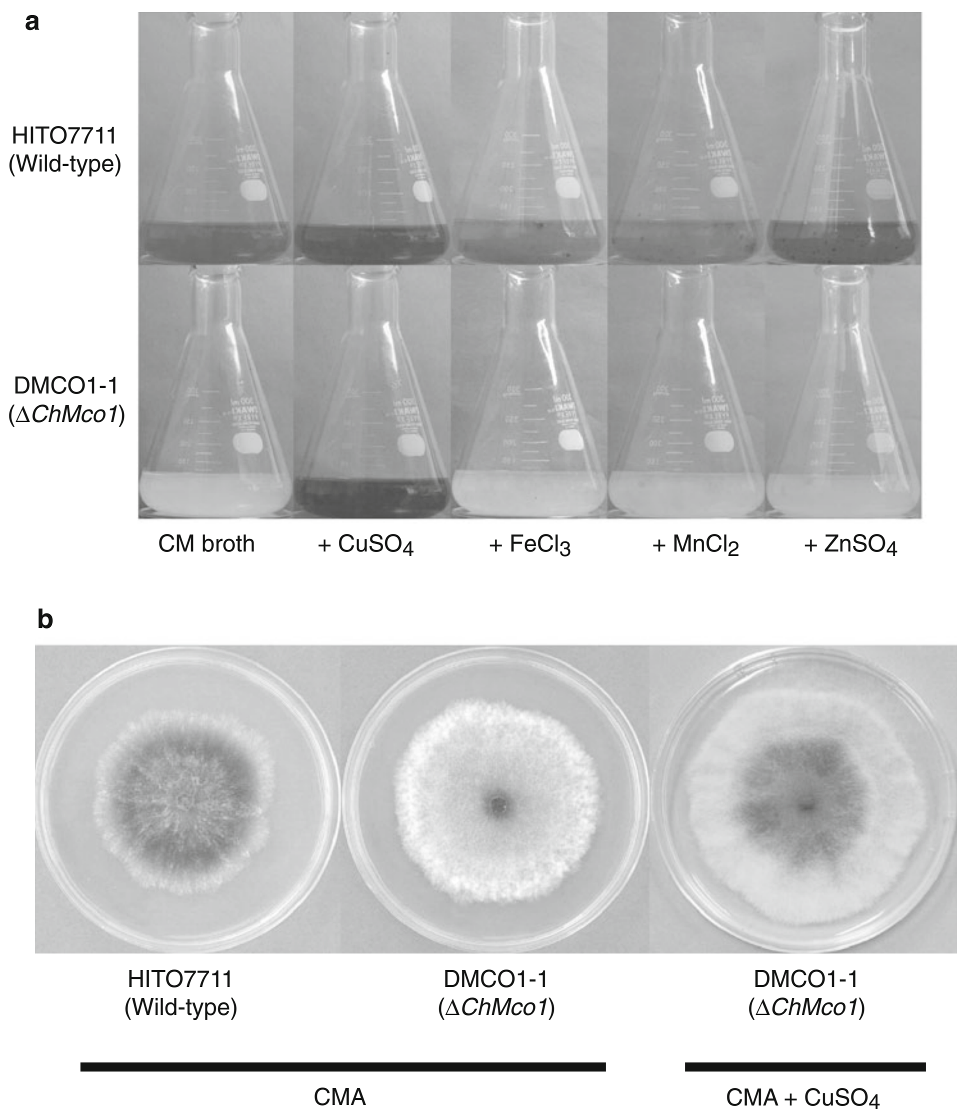

Fig. 5 Restoration for abnormal melanization of Δ*ChMco1* mutants in copper-supplemented media. **a** Mycelial growth of the wild-type strain HITO7711 and Δ*ChMco1* mutants DMCO1-1 in CM broth supplemented with 100 μM $CuSO_4$, 100 μM $FeCl_3$, 100 μM $MnCl_2$, and 25 μM $ZnSO_4$ for 48 h on a rotary shaker operated at 120 rpm. **b** Colonial growth of the wild-type strain HITO7711 and Δ*ChMco1* mutants DMCO1-1 on solid media CMA and CMA supplemented with 100 μM $CuSO_4$. Plates were grown for 5 days

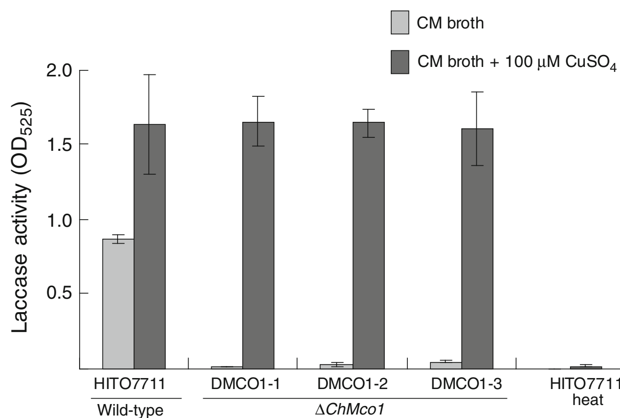

Fig. 6 Laccase activities in the cell-free homogenate of the wild type strain HITO7711 and Δ*ChMco1* mutants DMCO1-1, DMCO1-2, and DMCO1-3 cultivated in CM broth and CM broth supplemented with 100 μM $CuSO_4$. Samples of HITO7711 were heated at 100°C for 10 min and used as controls (HITO7711_heat). Syringaldazine was used as substrate. *Error bars* represent standard errors of means ($n = 3$)

appressorium formation between wild-type and Δ*ChMco1* mutant strains.

To investigate whether ChMCO1 was necessary for the pathogenicity of *C. heterostrophus*, we inoculated corn plants with conidial suspensions (i.e. using paper disks containing conidia of the wild-type and Δ*ChMco1* mutant strains). No differences in lesion formation by Δ*ChMco1* mutants or the wild-type strain were observed (data not shown).

Discussion

This study sought to find metallo-oxidase genes across fungal genome databases, and revealed that fungal metallo-oxidases are a large family of enzymes. Most fungal species including yeast and filamentous species have metallo-oxidase genes. Nevertheless, few phylogenetic analyses of fungal metallo-oxidase have been reported to date: the evolution of these enzymes in fungi remains unclear (Hoegger et al. 2006). Moreover, several reports have described studies of metallo-oxidases in yeast species, but few reports describe metallo-oxidases in filamentous species. Filamentous fungi and yeasts differ substantially in most aspects of their life cycles. Therefore, functional analyses of metallo-oxidases in filamentous fungi are needed to gain insight into the biological roles of these enzymes in fungi.

We cloned a metallo-oxidase gene, *Mco1*, from a phytopathogenic filamentous fungus *C. heterostrophus* (*ChMco1*). Metallo-oxidases characterized in yeast species to date have a transmembrane-helix (TH) in their C-terminal region and seem to be membrane-localized proteins, although ChMCO1 has no THs and seems to be a soluble protein. Our phylogenetic analysis revealed that *ChMco1* represents a new class of the genes for fungal metallo-oxidases (*Mco1* orthologue). In general, basidiomycetous fungi and ascomycetous yeasts have *Fet3* orthologues only, whereas ascomycetous filamentous fungi including *C. heterostrophus* have both *Mco1* and *Fet3* orthologues. Our functional domain analysis reveals that *Fet3* and *Mco1* orthologues have identical functional domains in their products, except for the lack of THs. These results suggest that *Fet3* orthologues might be ancestral and that *Mco1* orthologues might have arisen from gene duplication of an *Fet3* orthologue in ascomycetous filamentous species. The *Mco1* orthologues encode novel metallo-oxidases with characteristic structure, implying that products of *Mco1* orthologues are important in ascomycetous filamentous species and have biological roles, which differ from those of FET3.

We investigated the role of ChMCO1 by generating null mutants for the *ChMco1* gene. No significant impairment exists in growth, conidia formation, or pathogenicity of Δ*ChMco1* mutants. However, Δ*ChMco1* mutants showed clearly less melanization than the wild-type strain, suggesting that ChMCO1 is necessary for normal melanization of *C. heterostrophus*. FET3 of *S. cerevisiae* is known to be necessary for high-affinity iron uptake (Yuan et al. 1995). However, abnormal melanization of Δ*ChMco1* mutants was not restored by the supplementation with iron ion, suggesting that impairment of melanization did not result from the iron deficiency in Δ*ChMco1* mutants. We also showed that laccases were inactivated in *ΔChMco1* mutants. In *C. heterostrophus* and other ascomycetous fungal species, laccases seem to be necessary for oxidative polymerization of the melanin precursor, DHN (Tanaka et al. 1991, 1992). Therefore, the inactivation of laccases is thought to be a main cause of the impairment in DHN melanization in *ΔChMco1* mutants. As the metallo-oxidase FET3 is known to have laccase activities (Askwith and Kaplan 1998), there is still a possibility that ChMCO1 has a role as a major catalyst of oxidative polymerization of DHN. However, filamentous fungi are known to have several laccase genes in general (Hoegger et al. 2006; our unpublished data). Our phylogenetic analysis of fungal MCOs revealed that filamentous fungi *M. grisea*, *N. crassa*, or *P. nodorum* have five, eight, and three species of laccase genes, respectively (Fig. S1). Tanaka et al. (1992) also showed that at least four laccase isozymes are expressed in *C. heterostrophus*. Nevertheless, our result showed that Δ*ChMco1* mutants have no detectable laccase activity, suggesting that ChMCO1 is necessary for complete activity of laccase isozymes in *C. heterostrophus*. Results also showed that laccase activity of Δ*ChMco1* mutants was restored substantially when mutants were grown in broth

media supplemented with copper ions. Copper is known to serve as a cofactor for laccases, suggesting that the loss of laccase activity in Δ*ChMco1* mutants results from severely reduced availability of intracellular copper. Some external supply of copper would increase the intracellular copper concentration, which ensures the activity of laccases in Δ*ChMco1* mutants. Our results may imply that ChMCO1 plays a role in copper metabolism in *C. heterostrophus*.

In an earlier report, we described *ChCcc2*, which encodes a copper-transporting ATPase (Saitoh et al. 2009). Copper-transporting ATPases seem to deliver copper to various copper-containing proteins such as MCOs, including laccases and metallo-oxidases, tyrosinases, and amine oxidases in eukaryotes (Laliberté and Labbé 2006; Parisot et al. 2002; Petris et al. 2000; Yuan et al. 1995). The *ChCcc2* mutants show abnormal melanization and have no detectable laccase activity. We also showed that both growth and conidiation are severely affected in *ChCcc2* mutants. Inactivation not only of laccase but also of other diverse proteins targeted by ChCCC2 would result in pleiotropic effects in *ChCcc2* mutants. The metallo-oxidase ChMCO1 is also predicted to be a target of ChCCC2. Our results show that Δ*ChMco1* mutants showed abnormal melanization and lost laccase activity. On the other hand, Δ*ChMco1* mutants showed normal growth and conidiation, suggesting that the role of ChMCO1 in copper metabolism by *C. heterostrophus* is limited to the activation of laccases. Actually, ChMCO1 might play a regulatory role in the process by which laccases receive copper from ChCCC2.

Our phylogenetic analysis classifies *Abr1* of *A. fumigatus* as an *Mco1* orthologue. Tsai et al. (1999) reported that the *Abr1* mutant also shows the impairment in DHN melanization. Moreover, *Abr1* is known to be present in the melanin biosynthesis gene cluster. Our study also revealed that the *Mco1* orthologue of *M. grisea* (MGG_07220) is located near genes involved in DHN-melanin biosynthesis (unpublished data). These results suggest that products of the *Mco1* orthologue play important roles in DHN melanization, which is conserved in various ascomycetous filamentous species. Although the authors did not examine whether laccase activities are affected in the *Abr1* mutant of *A. fumigatus*, the *Abr1* mutant shows abnormal melanization, as *ΔChMco1* mutants did. Our results, combined with those of a study of the *Abr1* mutant, suggest that *Mco1* orthologues are necessary for laccase activities and subsequent DHN melanization in various ascomycetous filamentous fungi.

In conclusion, *ChMco1* represents a new class of fungal metallo-oxidases: the DHN melanization class. Most ascomycetous filamentous species have both orthologues of this class and *Fet3*. Our results reveal that the ChMCO1 is a functionally novel metallo-oxidase that is necessary for laccase activity and subsequent melanization in *C. heterostrophus*. Our results also suggest that the biological role of laccase activity and subsequent melanization is conserved in enzymes of the DHN melanization class. This study provides new insight into the evolution of fungal metallo-oxidases and melanin biosynthesis in ascomycetous filamentous fungi.

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