Environmental Science and Pollution Research (2022) 29:14430–14442
https://doi.org/10.1007/s11356-021-16776-0

RESEARCH ARTICLE

Identification and characterization of eight metallothionein genes involved in heavy metal tolerance from the ectomycorrhizal fungus *Laccaria bicolor*

Binhao Liu[1] · Pengcheng Dong[1] · Xinzhe Zhang[1] · Zhihang Feng[1] · Zhugui Wen[3] · Liang Shi[1,2] · Yan Xia[1] · Chen Chen[1] · Zhenguo Shen[1,2] · Chunlan Lian[4] · Yahua Chen[1,2,4]

Received: 2 August 2021 / Accepted: 23 September 2021 / Published online: 6 October 2021

Abstract

Metallothioneins (MTs) are small, cysteine-rich, heavy metal-binding proteins involved in metal homeostasis and detoxification. The increasing numbers of available genomic sequences of ectomycorrhizal (ECM) fungi enable deeper insights into the characteristics of MT genes in these fungi that form the most important symbiosis with the host trees in forest ecosystems. The aim of this study was to establish a comprehensive, genome-wide inventory of MT genes from the ECM fungus *Laccaria bicolor*. Eight MT genes in *L. bicolor* were cloned, and the expression patterns of their transcripts at various developmental stages based on expressed sequence tag (EST) counts were analyzed. The expression levels of four MTs were significantly increased during symbiosis stages. Quantitative real-time PCR (qRT-PCR) analysis revealed that transcripts of *LbMT1* were dominant in free-living mycelia and strongly induced by excessive copper (Cu), cadmium (Cd), and hydrogen peroxide (H_2O_2). To determine whether these eight MTs functioned as metal chelators, we expressed them in the Cu- and Cd-sensitive yeast mutants, *cup1*Δ and *yap1*Δ, respectively. All LbMT proteins provided similar levels of Cu(II) or Cd(II) tolerance, but did not affect by H_2O_2. Our findings provide novel data on the evolution and diversification of fungal MT gene duplicates, a valuable resource for understanding the vast array of biological processes in which these proteins are involved.

Keywords Evolution and diversification · Gene duplication · *Laccaria bicolor* · Metallothioneins · qRT-PCR · Yeast mutants

Introduction

Metallothioneins (MTs) are low molecular weight, cysteine (Cys)-rich, metal-binding proteins. The first MT discovered was a cadmium (Cd)-binding peptide found in horse kidney (Margoshes and Vallee 1957). Subsequently, intensive studies on this small polypeptide revealed its wide distribution across taxonomic groups, from prokaryotes to eukaryotes (Babula et al. 2012; Cicatelli et al. 2010; Divya et al. 2018; Hassinen et al. 2011; Le Croizier et al. 2018; Sigel et al. 2009). MT sequences are highly heterogeneous in protein length and the distribution of Cys residues and do not show general homology, which has resulted in a classification system of 15 families encompassing taxa from bacteria to humans (Binz and Kägi 1999). In mammals, four tandemly clustered genes (MT1 to MT4) are known, and these four major genes originated through a single duplication event prior to the radiation of mammals (Moleirinho et al. 2011). A recent study suggested a likely association between MT functional divergence and duplication events in vertebrates (Serén et al. 2014). Iturbeespinoza et al. (2016) discovered the longest fungal MT in *Tremella mesenterica*, a saprophytic ascomycete. The *TmMT* gene has 10 exons, and it yields a 779-bp mature transcript encoding a 257-residue protein, built of repeated fragments.

Responsible Editor: Diane Purchase

✉ Yahua Chen
yahuachen@njau.edu.cn

[1] College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China

[2] Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource, Nanjing Agricultural University, Nanjing 210095, China

[3] Jiangsu Coastal Area Institute of Agricultural Sciences, Yancheng 224002, Jiangsu, China

[4] Asian Natural Environmental Science Center, The University of Tokyo, 1-1-8 Midoricho, Nishitokyo, Tokyo 188-0002, Japan

Compared with MT genes in other taxa, fungal MT genes have higher heterogeneity (Binz and Kägi 1999) *CUP1*, the first identified fungal MT gene, found in the yeast *Saccharomyces cerevisiae*, is mainly involved in copper (Cu(II)) detoxification and induced by Cu(II) and silver (Ag(I)) (Butt et al. 1984; Okuyama et al. 1999). Another MT gene, *CRS5*, was also found in *S. cerevisiae* but is not homologous to *CUP1*. CRS5 was shown to exist mainly in the form of a Cu(II)/zinc (Zn(II)) complex in *S. cerevisiae* and to play a crucial role in maintaining cytoplasmic Zn homeostasis (Culotta et al. 1994; Pagani et al. 2007). In addition, four MT-like genes were identified in the yeast *Yarrowia lipolytica* by whole genome analysis that were predicted to have evolved through recent gene duplication events (Dujon et al. 2004). Similar MT-like proteins were also identified in ascomycete fungi, including *Colletotrichum gloeosporioides*, *Pyrenopeziza brassicae*, *Podospora anserine*, *Heliscus lugdunensis*, and *Tuber melanosporum* (Averbeck et al. 2001; Hwang and Kolattukudy 1995; Jaeckel et al. 2005; Loebus et al. 2013; Singh and Ashby 1998). In basidiomycete fungi, most small Cu-binding Cys-rich peptides, presumably MTs, were isolated from the ectomycorrhizal (ECM) fungus *Laccaria laccata* and *Paxillus involutus* (Howe et al. 1997). Subsequently, a gene encoding the 34-amino acid (aa) metallothionein PiMT1 was identified as a potential determinant in the responses of *P. involutus* to Cu(II) and Cd(II) stresses (Bellion et al. 2007; Jacob et al. 2004). Two MTs in *Hebeloma cylindrosporum*, which share only 40% identity, exhibit different expression responses to Cu(II) and Cd(II) overload stresses (Ramesh et al. 2009). Three MT isoforms isolated from the Ag-hyperaccumulator *Amanita strobiliformis* were shown to play vital roles in the sequestration of intracellular Ag(I) in the fruit body and mycelia (Osobová et al. 2011). Nguyen et al. (2017) identified two more MT coding genes: *SlMTa and SlMTb* in the ECM fungus *Suillus luteus*. Reddy et al. (2016) reported the expression levels of *PaMT1* in *Pisolithus albus*, which encodes a 35 amino acid long polypeptide, increased higher with Cu(II) than Cd(II).

MTs have also been characterized in the arbuscular mycorrhizal (AM) fungi *Gigaspora rosea*, *Gigaspora margarita*, and *Glomus intraradices* (González-Guerrero et al. 2007; Lanfranco et al. 2002; Stommel et al. 2001). In addition to their pivotal role in the detoxification of heavy metals, increasing lines of evidence indicate a role of MTs or MT-like proteins in the development of fungi and plant-fungus interactions (Hwang and Kolattukudy 1995; Lanfranco et al. 2002; Johansson et al. 2004). Formation of the appressorium is essential for the penetration of *C. gloeosporioides* into its host, the avocado fruit (Hwang and Kolattukudy 1995). Hwang and Kolattukudy (1995) also found that the expression levels of *CgMT1* and *CgMT2* were significantly upregulated in the early phase of appressorium formation. The study of ECM fungi has similar results. Johansson et al. (2004) found that when *P. involutus* formed a symbiotic relationship with ECM fungus *Betula pendula*, the expression levels of *P. involutus* MT genes were significantly upregulated.

With the increasing number of fully sequenced fungal genomes (Floudas et al. 2012; Grigoriev et al. 2011; Kohler et al. 2015; Martin et al. 2008), MTs from various taxonomic groups can be easily retrieved. However, knowledge of the full range of fungal MT functions are unclear. As the first ECM fungus with a completely sequenced genome and the ability to form symbioses with a variety of host plants, *Laccaria bicolor* has become a model species for studies of symbiotic relationships between ECM fungi and woody plants and their processes of intrinsic signal recognition, signal transduction, and other molecular mechanisms (Martin et al. 2008). In this study, we identified eight MT genes in *L. bicolor*, present an overview of ECM fungal MTs, and described their genetic structure, phylogeny, evolution, and expression patterns in *L. bicolor*. In addition, we present results from complementary experiments testing the eight MTs identified in the *L. bicolor* genome in metal-deficient yeast mutants to determine their effects on Cu(II), Cd(II), and hydrogen peroxide (H_2O_2) tolerance.

Materials and methods

Organisms and growth conditions

The *L. bicolor* (Maire) P.D. Orton S238N strain used in this study was donated by Prof. A. G. Pardo (University of Quilmes, Argentina). The mycelia were kept in modified Melin-Norkrans (MMN; Marx 1969) medium and cultured in a dark incubator at 25 °C.

For the *L. bicolor* resistance to different heavy metals, a solid MMN medium was prepared, autoclaved at 121 °C for 20 min, and then heavy metal solutions were filtered by 0.22 μm filter and were, respectively, added to form different concentration gradients (Cu(II): 0, 10, 20, 50, 100, 150, 200 μM; Cd(II): 0, 1, 2, 5, 10, 20, 40 μM; Zn(II): 0, 0.5, 1.0, 1.5, 3.0, 4.5, 9.0 mM; H_2O_2: 0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 mM; Ag(I): 0, 5, 10, 20, 40, 80, 150 μM). The activated fungal plugs of *L. bicolor* with an initial diameter of 0.9 cm were separately cultivated in the solid MMN media with different treatments in the dark at 25 °C for 28 days, and then the pictures were taken.

For transcriptional induction experiments, fungal mycelia were added to liquid MMN medium and incubated for 21 days. Three plugs of 9-mm diameter were transferred to 50 mL of fresh medium for 3 days (adaption period). External metal ions (Cu(II): 50, 100, 150 μM; Cd(II): 5, 10, 20 μM; Zn(II): 0.5, 1.5, 3.0 mM; Ag(I): 10, 20, 40 μM) and H_2O_2 (0.5, 1.0, 2.0 mM) were added to the medium, and the mycelia

were harvested separately after 6, 12, 24, and 48 h incubation and then washed twice with distilled water.

The yeast strains DTY4 (*cup1*::*URA3*; donated by Prof. Dennis J. Thiele from Duke University, USA) and WYU (*yap1*::*URA3*; donated by Prof. Shusuke Kuge from Tohoku Pharmaceutical University, Japan), which were derived from the wild-type strain DTY3 (*MATα leu2-3*, *112 his3Δ1 trp1-1 ura3-50 gal1 CUP1*^{S}) (Longo et al. 1996) and W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3*) (Kuge and Jones 1994), were used for heterologous expression analyses, respectively. Transformed yeasts were grown at 30 °C on URA^{+}-selective SD agar medium containing (w/v) 0.7% yeast nitrogen base (Difco Laboratories, Inc., Franklin Lakes, NJ, USA), 0.005% adenine hemisulfate, 2% glucose, and 0.003% (each) essential amino acids (Sigma-Aldrich, St. Louis, MO, USA).

DNA and RNA isolation and cDNA synthesis

Fungal mycelia were frozen in liquid nitrogen, and then 100 mg of mycelia were ground for DNA/RNA extraction. Genomic DNA and total RNA were isolated using the E.Z.N.A. Plant DNA Mini Kit and E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek, Inc., Norcross, GA, USA), respectively. For RNA purification, DNA removal was achieved by incubating samples with RNase-free DNase (Omega Bio-tek) per the manufacturer's recommendations. Total RNA concentration was determined by measuring absorbance at 260 nm, and RNA integrity was checked by formaldehyde agarose gel electrophoresis.

RNA was converted to cDNA using a RevertAid First-strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The first cDNA strand was reverse-transcribed in a 12-μL reaction from 1 μg of total RNA using anchored oligo(dT) 18 primers.

Cloning of MTs in *L. bicolor*

Basic Local Alignment Search Tool (BLAST) searches with reported *P. involutus* and *H. cylindrosporum* MT sequences against *L. bicolor* expressed sequence tags (ESTs) and genomic sequences were performed using the Joint Genome Institute (JGI) *L. bicolor* genome portal v2.0 (https://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html). Eight unique genes (*LbMT1*, *LbMT2a*, *LbMT2b*, *LbMT3a*, *LbMT3b*, *LbMT3c*, *LbMT4*, and *LbMT5*) representing MT homologs were detected in both the genome and the EST library. These potential MTs were subsequently cloned from both genomic DNA (as a control) and cDNA from *L. bicolor*. PCR amplification was performed with primer sets introduced a *Bam*HI and an *Eco*RI site at the 5' and 3' ends, respectively (Table S2). The PCR program was as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were purified using a MiniBEST Agarose Gel DNA Extraction Kit (Takara Bio, Inc., Tokyo, Japan) and then directly cloned into the pMD19-T vector (Takara) and transformed into competent *Escherichia coli* cells. DNA sequences were determined using M13 RV and M4 primers (Thermo Fisher Scientific). The full-length cDNA sequences of eight *L. bicolor* MTs were submitted to the DDBJ/EMBL/NCBI DNA databank under the accession numbers KJ095785, KJ095786, KJ095787, KJ095788, KJ095789, KJ095790, KJ095791, and KJ095792.

Organ-specific expression count and promoter analysis

The upstream sequences (1,500 bp upstream of the start codon) of eight MT genes were cloned from *L. bicolor* genomic DNA and sequenced. Potential transcription factor binding sites (TFBSs) were predicted using Genomatix software (Genomatix GmbH, Munich, Germany) and the BIOBASE TRANSFAC database (Matys et al. 2003).

The relevant sequences of the eight cloned MTs genes were extracted from the *L. bicolor* EST database published on NCBI and their abundances at different growth and development stages were counted to represent these eight relative expression different developmental stages of *L. bicolor*. The selected plant was Douglas fir associated.

Sequence alignment and phylogenetic analysis

Multiple protein sequence alignments were performed using MUSCLE alignment software (Edgar 2004), and gaps and ambiguously aligned sites were manually removed. Phylogenetic reconstructions were performed using a maximum likelihood method using MEGA X software (Tamura et al. 2011) and PhyML 3.1 (Guindon et al. 2010) software with a bootstrap of 1000 pseudoreplicates. The appropriate substitution model was determined in MEGA X by applying the model estimator.

Real-time quantitative PCR of *L. bicolor* MT genes

The cDNA was normalized by dilution and amplified by qPCR using an iQ SYBR GREEN Supermix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions and run on a MiniOpticon Real-Time PCR System (Bio-Rad). *Laccaria* tubulin-1B alpha chain gene (protein ID: 192524) was used as reference gene for normalization (Kemppainen et al. 2009). Primers are presented in Table S3. The qPCR program was as follows: 95 °C for 10 min, then 40 cycles at 95 °C for 10 s, followed by 65 °C for 30 s. Samples were run in three biological replicates with two technical replicates each. Fold changes in gene expression between exogenous stressed mycelia and free-living mycelia

were calculated with the ΔΔCt method (Pfaffl 2001). All statistical analyses were performed using the IBM SPSS 21.0 software package (IBM Corp., Armonk, NY, USA). The average value of expression multiples is ≥ 2.0 can be considered as differentially expressed. A Student's two-tailed independent *t* test was used to determine the significance of the results ($P < 0.05$).

Yeast complementation assay

The full coding regions of the eight MT genes of *L. bicolor* were subcloned from pMD19-T clones into the yeast expression vector P424 (Mumberg et al. 1995). Transcription was controlled by the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and the cytochrome C oxidase gene (*CYC1*) terminator of *S. cerevisiae*. Empty vector P424 and the P424-LbMT constructs were introduced into *cup1*Δ and *yap1*Δ cells together with the parental strains using a lithium acetate procedure (Gietz and Schiestl 2007). Transformants were utilized for serial dilution spot assays. Briefly, strains were first cultured in liquid SD-Trp medium for 24 h at 30 °C. Cell density was adjusted to an OD_{600} value of 1.0, and 5.0-μL serial dilutions were spotted onto SD-Trp agar plates in the presence or absence of heavy metals (Cu(II): 100 μM; Cd(II): 60 μM) and H_2O_2 (2.0 mM). Growth was examined by visual inspection and digital image recording after incubation at 30 °C for 3 days.

Results

Effect of heavy metals and oxidative on the growth of *L. bicolor*

As shown in Figure S1, Cu(II), Cd(II), Zn(II), Ag(I), and H_2O_2 adversely affected the growth of *L. bicolor*. Zn(II) had the least effect, followed by H_2O_2, Cu(II), and Ag(I), because *L. bicolor* could still grow under 9.0 mM Zn(II) stress. Cd(II) had the most obvious inhibitory effect on strain growth due to *L. bicolor* could only tolerate Cd(II) up to 40 μM, which was almost 1/225 of Zn(II). *L. bicolor* stopped growing under the Ag(I) stress of 150 μM, which was the lowest value other than Cd(II).

MT gene family in *L. bicolor*

ESTs of the eight *LbMT* genes were obtained from the NCBI EST database as counts per million transcripts for each of the given tissue and displayed as $\log_2$ transcripts per million.

To identify the potential MT-like genes in *L. bicolor*, MTs from *P. involutus* and *H. cylindrosporum* were selected as query sequences for BLAST searches. The whole genome (JGI *L. bicolor* v 2.0) revealed the presence of 8 potential MT genes and 15 protein products in *L. bicolor*, which were further subdivided into 5 types (Table 1). Analysis of DNA arrangements of MT exons and introns demonstrated that all eight MT genes contain three or four exons, the first of which is extremely short (only nine nucleotides; Figure 1A). Sequence alignment revealed high similarity between *LbMT2a* and *LbMT2b*, *LbMT3a*, *LbMT3b*, *LbMT3c*, *LbMT4*, and *LbMT5* also had high sequence similarity. *LbMT1* had particularly high sequence similarity with *PiMT1*, isolated from *P. involutus* (Figure 1B).

The eight metallothionein-like genes of *L. bicolor* were located on three different pseudochromosomes corresponding to linkage groups (LGs) within the genome (Labbé et al. 2008). *LbMT2a* and *LbMT2b* are clustered on LG 7, separated by 51 kb. *LbMT3a*, *LbMT3b*, *LbMT3c*, *LbMT4*, and *LbMT5* are located in a cluster on LG 10 (Figure 2). *LbMT1*, which is a single-copy gene, is on scaffold 15. The close sequence homology among a number of proteins encoded by MT genes (Figure S2) suggests that the MTs in *L. bicolor* may have been generated by gene duplication. Transposable elements (TEs), reported to be widely distributed throughout the *L. bicolor* genome, may be major contributors to the genesis of new genes through gene duplication via mechanisms such as ectopic recombination, transposition molecular domestication, and gene retrotransposition. We analyzed the presence of TEs within the vicinity of each MT gene using an online database (https://mycor.nancy.inra.fr/IMGC/LaccariaGenome/download.php?select=anno). The highest density of TEs was found on LG 10 clustered around *LbMT3a*, *LbMT3b*, *LbMT3c*, *LbMT4*, and *LbMT5*, and there was also a dense cluster of TEs adjacent to *LbMT2a* and *LbMT2b*. No TEs were found within 60 kb up- or downstream of *LbMT1*.

Expression profiles of the *LbMT* genes

Because gene duplication often results in diversified spatio-temporal expression patterns in duplicate family members (Moleirinho et al. 2011; Byrne and Wolfe 2007; Greer et al. 2000; Gu et al. 2004), we collected EST sequences derived from various developmental stages of *L. bicolor*. According to the EST pattern abundance, all eight MT genes revealed diverse expression preferences among different development stages (Figure 3A). The majority of MTs (*LbMT1*, *LbMT2a*, *LbMT3a*, *LbMT3b*, *LbMT3c*, and *LbMT4*) were highly expressed in free-living mycelia. However, during the formation of ECM symbioses with poplar roots, *LbMT2a* and *LbMT2b* were expressed more highly than the other MT genes in the symbiotic mycelia, indicating an important role for these two paralogous genes in host root colonization. In Douglas fir-associated fruiting bodies of *L. bicolor*, *LbMT3c* and *LbMT4* were observed to be the most highly expressed MT genes and presumably participated in metal ion

Table 1 Members of metallothionein family (protein IDs based on Joint Genome Institute annotations) in *L. bicolor*.

Gene	Protein name	Tigr ID	Locus	Coordinate	Amino acids
LbMT1	LbMT1	AHI43933.1	AHI43933 33 aa linear	Scaffold 15	MISTINVPVSQTCGSSSCNCGESCACKPGECKC
LbMT2a	LbMT2a	AHI43934.1	AHI43934 58 aa linear	LG 7	MLFNTLTPISRASSTGCCCTSCKCTSCTCGTAPVNEAGC GSTTCNCTNCACKPEECKC
LbMT2b	LbMT2b	AHI43935.1	AHI43935 58 aa linear	LG 7	MLFNTFTPISRVSSTGCSCTSCKCTSCTCGTAPVNEAGC GSTTCNCTNCACKPEECKC
LbMT3a	LbMT3a	AHI43936.1	AHI43936 37 aa linear	LG 10	MISNTSALANAACGDHSSCGCAQDCSCASCGCKCASG
LbMT3b	LbMT3b	AHI43937.1	AHI43937 37 aa linear	LG 10	MISNTSAFANAACGDHSSCGCAQDCSCASCGCKCASG
LbMT3c	LbMT3c	AHI43938.1	AHI43938 36 aa linear	LG 10	MISNTSALANAACDHTSCGCAQDCSCASCGCKCASG
LbMT4	LbMT4	AHI43939.1	AHI43939 39 aa linear	LG 10	MISNTFALANDACDHTKCNCTDTCSCASCGCEQCCKPGK
LbMT5	LbMT5	AHI43940.1	AHI43940 36 aa linear	LG 10	MISNTSALINFGCGNDACSCGDACGCAQGGCKCNSN

homeostasis during fruiting body formation. The basal mRNA expression of *LbMT5* remained relatively constant at low levels in all three stages.

The cloned *LbMT1* exists as a single copy in the *L. bicolor* S238N genome and has high homology with the previously reported MT gene *PiMT1* in *P. involutus* (Figure 1B).

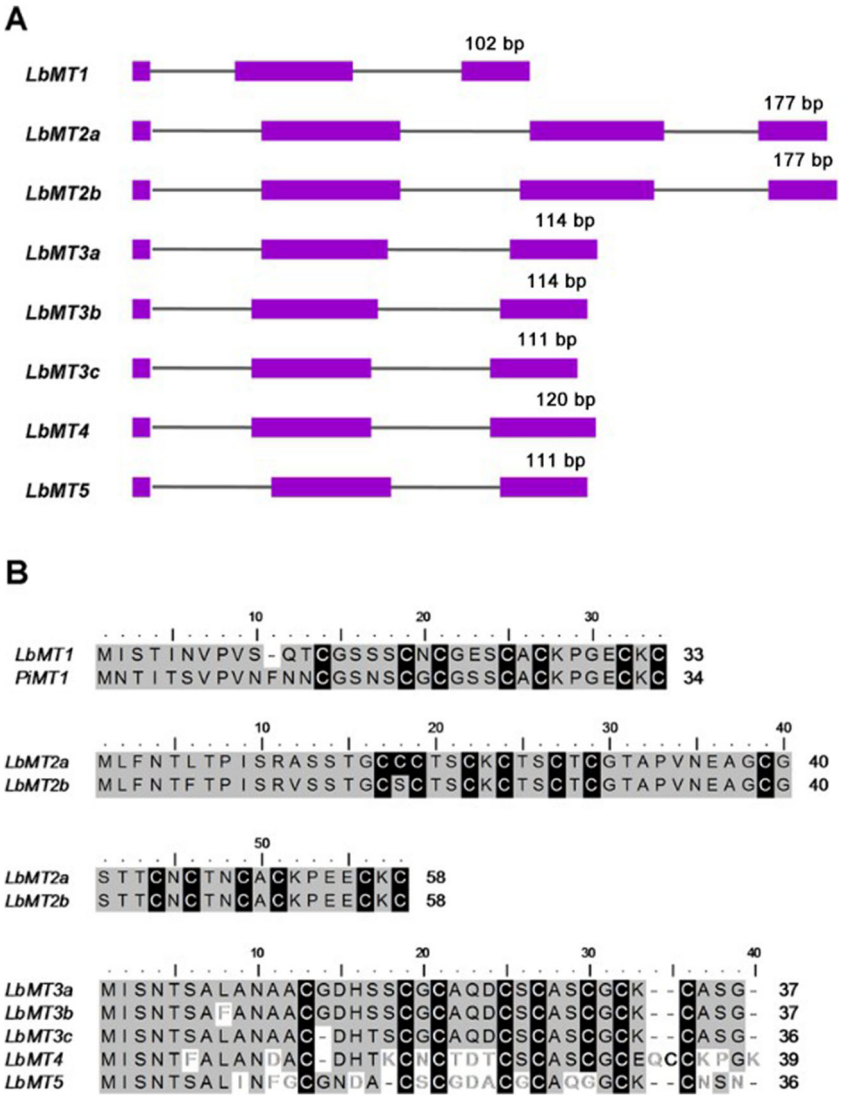

Fig. 1 Structures of *L. bicolor* metallothionein (MT) genes. A Exon-intron gene organization of the eight MT genes discovered in the *L. bicolor* genome. Exons and introns are indicated with purple boxes and lines, respectively. B Alignment of eight LbMT protein sequences. To indicate sequence similarities, *MT2a* and *MT2b* are aligned together, and *MT3a*, *MT3b*, *MT3c*, *MT4*, and *MT5* are aligned in a second group. *MT1* is presented alone and shows high homology with a functional MT identified in *Paxillus involutus*

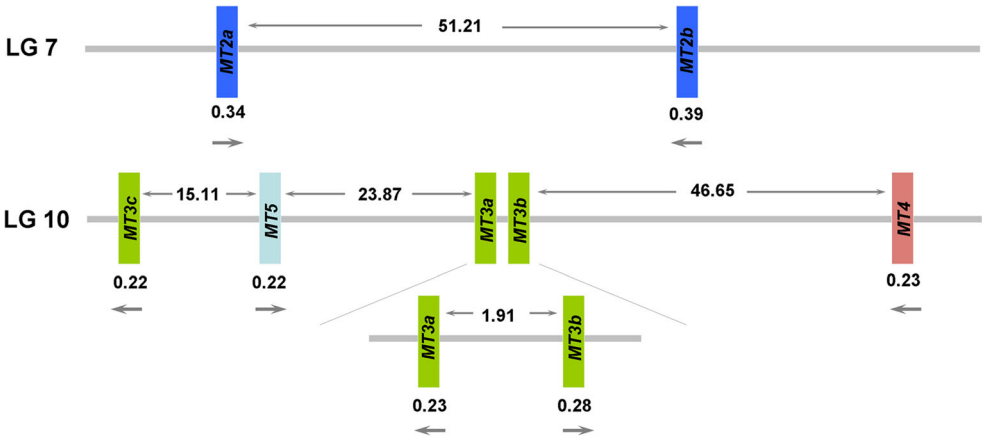

Fig. 2 Schematic illustration of the MT gene family in the *L. bicolor* genome. The transcriptional direction of each gene is indicated by an arrow. Genes are colored as follows: *LbMT2a* and *LbMT2b*, dark blue; *LbMT3a*, *LbMT3b*, and *LbMT3c*, green; *LbMT4*, red; and LbMT5, light blue. *LbMT1* is a single-copy gene on scaffold 15 and is not shown in this figure. The sizes of genes and intergenic regions are given in kilobases (kb)

Therefore, in this study, *LbMT1* was selected as the research object, and its transcription level changes under different heavy metals and H_2O_2 stress were detected by real-time PCR (Figure 4, Figure S3). The effects of different heavy

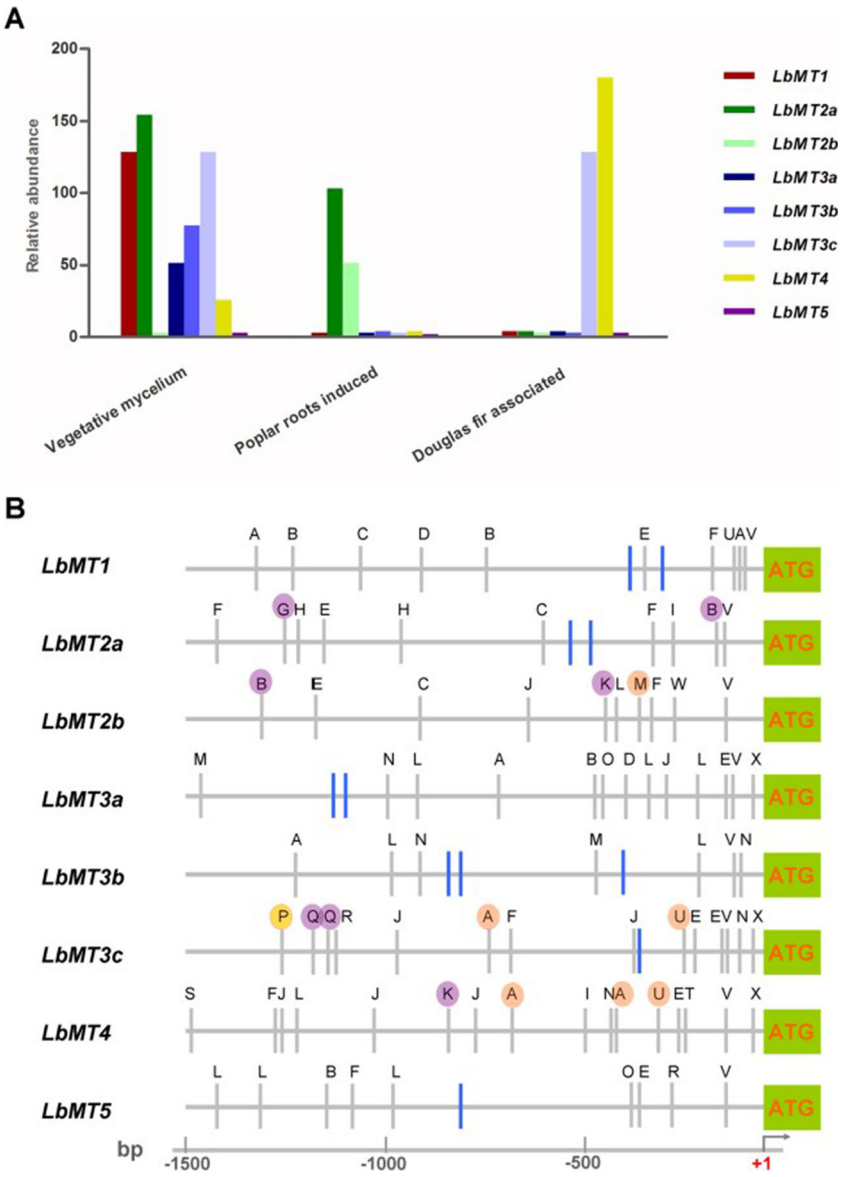

Fig. 3 Expression profiles and promoter analysis of *L. bicolor* MT genes. **A** Expression of the eight MT genes at different development stages. **B** In silico analysis of the promoter regions (~ 1500 bp) of the eight *LbMTs* genes. Letters above the rectangles indicate various transcription factor binding sites (TFBSs; Table S1), and blue rectangles represent metal response elements. Nutrient-related TFBSs in symbiosis-associated MTs (*LbMT2a*, *LbMT2b*, *LbMT3c*, and *LbMT4*) are colored as follows: carbon-responsive element, purple; nitrogen-response element, orange; and phosphorus-response element, yellow

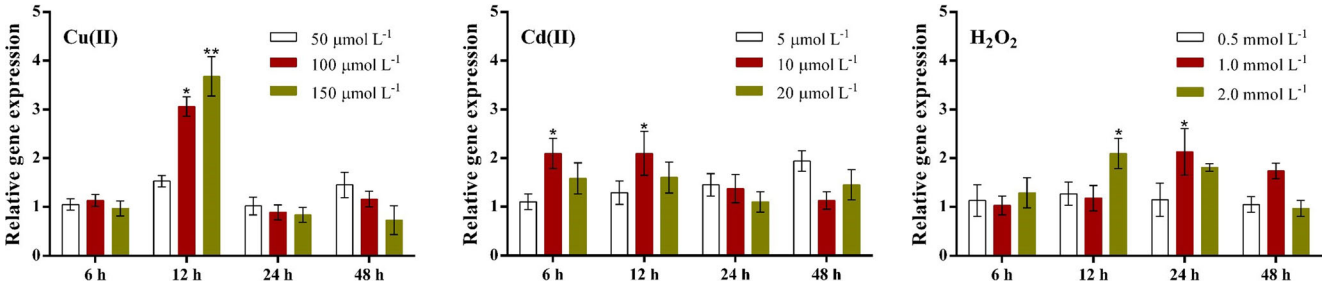

Fig. 4 Impacts of heavy metal ions (Cu(II) and Cd(II)) and oxidative(H_2O_2) stress on *LbMT1* expression. All expression values are described as fold differences from expression in free-living mycelia ± standard deviations. * indicates significant up- or down- regulation of MT gene expression at different processing times (6, 12, 24, or 48 h) under the same treatment concentration ($P < 0.05$)

metals and H_2O_2 on the expression of *LbMT1* were shown in Figure 4. Under the treatments of 100 μM and 150 μM Cu(II), the expression of *LbMT1* significantly increased at 12 hours and then decreased in the following time. For Cd(II), the expression of *LbMT1* increased significantly under the stress of 10 μM Cd(II) at 6 h and 12 h and declined at 24 h. For H_2O_2, the expression level of *LbMT1* increased when treated by 2.0 mM H_2O_2 for 12 h and 1.0 mM H_2O_2 for 24 h, respectively, and there was no significant change in other treatments. The expression of *LbMT1* did not change significantly under different Zn(II) and Ag(I) treatments during the experiment (Figure S3).

Promoters analysis

To investigate the regulatory mechanisms underlying the observed expression pattern differences, we performed in silico analyses of the respective upstream promoter regions of the *LbMT* genes. Differences in the types and locations of potential TFBSs in promoter regions were observed even among closely related MTs (Figure 3B; Table S1). Classic metal response elements were identified in the upstream regions of all *LbMT* genes except *MT2b* and *MT4*. Additionally, the *LbMT2a* and *LbMT2b* promoters contained several TFBSs potentially related to carbon and nitrogen utilization. Similar observations were found in the promoter regions of *LbMT3c* and *LbMT4* (Figure 3B). A phosphate starvation response element was also found upstream of *LbMT3c*.

Heterologous expression of *LbMTs* in yeast

To further document the impact of MT secondary structures on their capacity to bind metal ions and detoxify oxidative stress, we expressed *L. bicolor* MTs in mutant *S. cerevisiae* strains. The *S. cerevisiae cup1* locus encodes a Cu-thionein that plays a dominant role in Cu(II) sequestration. The wild-type strain DTY3 harbors a single copy of the MT gene, whereas the *cup1*Δ mutant strain exhibits a greater sensitivity to Cu(II) due to complete disruption of the MT gene. Figure 5 shows that the *cup1*Δ yeast transformed with empty vector exhibited no growth at 100 μmol L^{-1} Cu(II). In contrast, the Cu-sensitive phenotype of the *cup1*Δ mutant was fully complemented by each of the eight *L. bicolor* MTs. *Yap1* is a transcription factor related to the mammalian AP-1 complex which positively controls various genes involved in metal tolerance and oxidative stress tolerance in yeast. Complete disruption of this transcription factor renders the yeast mutant highly sensitive to Cd(II) and H_2O_2 (Kuge and Jones 1994). As with the results seen in the *cup1*Δ mutant, expression of each of the *LbMT* genes in *yap1*Δ yeast resulted in a full complementation of Cd-sensitive cells, enabling them to tolerate higher concentrations of Cd(II) compared with the wild-type W303B. In contrast, the presence of MTs in *yap1*Δ had no influence on cell growth with or without excess H_2O_2(Figure. 5).

Discussion

The current availability of several genomic sequences enables the study of the evolutionary steps underlying the expansion of gene families by detailed characterization of lineage-specific expansions. Due to the high heterogeneity of MT sequences in fungi, they are generally omitted in genome sequencing projects. Using ESTs in BLAST searches querying reported basidiomycete MTs, we identified eight MT-like genes in both the genomic and EST datasets of the first fully genome-sequenced ECM fungus, *L. bicolor* (Martin et al. 2008). Among the eight putative *LbMT* genes, the conserved arrangement of exon-intron positions and high protein identities suggest that these MTs originated from gene duplications. Consistent with this finding, abundant TEs were identified around *MT2a* and *MT2b* on LG7, as well as *MT3a*, *MT3b*, *MT3c*, *MT4*, and *MT5* on LG10. The *L. bicolor* S238N genome encodes a high percentage of TEs, which could be a major contributor to its greater quantity of multi-gene families compared with other fungal genomes (Labbe et al. 2012), as TEs are associated with gene duplication and exon shuffling (Bennetzen 2005; Morgante et al. 2005). These data are in agreement with previous observations on MT duplication in mammals (Moleirinho et al., 2011). After a duplication event, the genes that can follow distinct evolutionary paths: if a gene

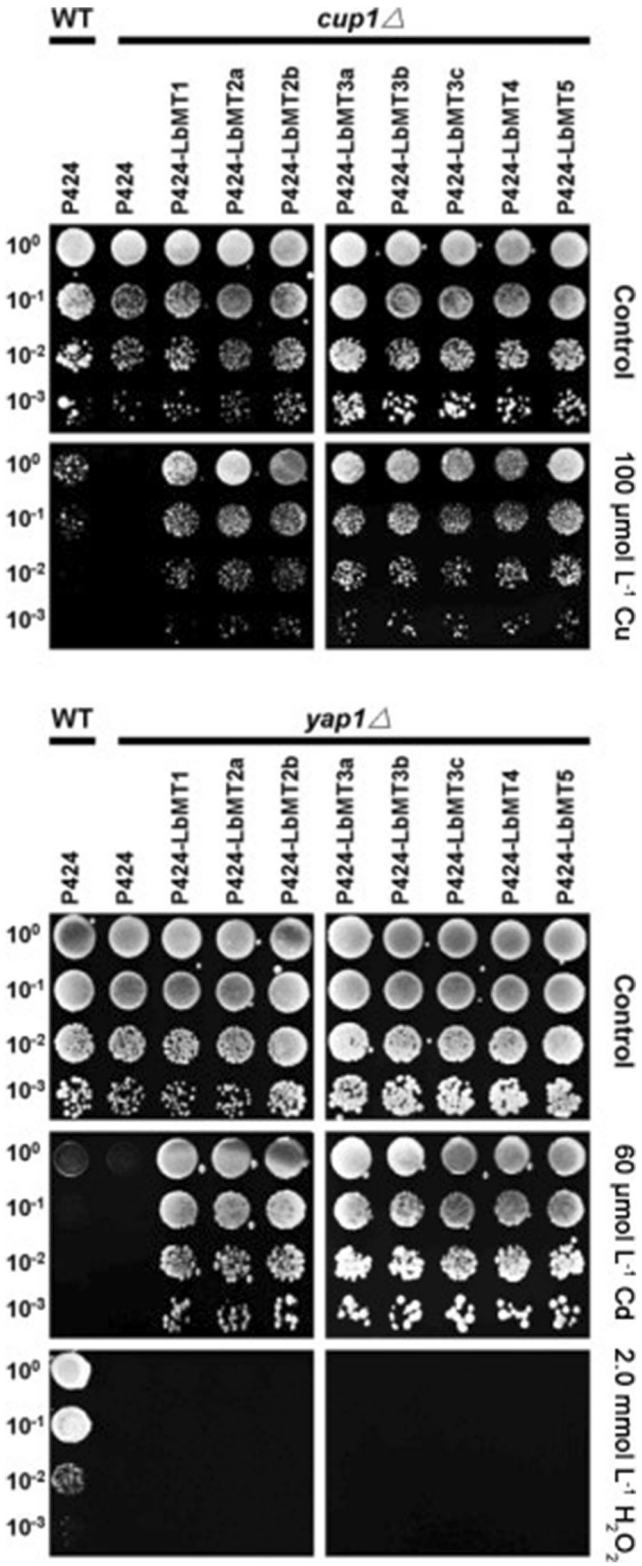

Fig. 5 Functional complementation in yeast mutants on selective medium. Cellular tolerance toward Cu(II), Cd(II), and H_2O_2 following overexpression of eight *LbMTs* genes in *cup1* and *yap1* mutant yeast. Mutants and the corresponding wild-type (WT) strains were transformed with empty vector p424 as the negative and positive controls. Serially diluted cells were spotted on SD-Trp medium, and yeast growth was assessed after 3 days of incubation at 30 °C

is actively maintained, redundant duplicates can escape purifying selection and begin accumulating loss-of-function mutations, resulting in pseudogenization; or, less frequently, particular replacements may direct this gene into novel functions (Nikolaos et al. 2021; Audrey et al. 2017). Subfunctionalization can also occur if parents and duplicates retain their functions, but become distinct and complementary in their spatiotemporal expression patterns (Zhang 2003).

Considering the large MT gene family in *L. bicolor* in the context of the discovery that gene duplication often results in diversified spatiotemporal expression patterns in duplicate family members, EST abundance can be utilized as a valuable metric for the basal expression patterns of the corresponding genes. Specifically, the expression levels of some MTs in *L. bicolor* are higher than others during the formation of ECM symbiosis, indicating an important role in plant-fungus associations. Supporting the results obtained with the EST datasets, a series of nutrient-associated TFBSs that primarily may participate in carbon, nitrogen, and phosphorus starvation responses were identified in the promoter regions of these genes. Symbiosis-regulated MTs were also discovered in both mycorrhizal fungi and certain plants (Lanfranco et al. 2002; Johansson et al. 2004; Flores-Monterroso et al. 2013; Voiblet et al. 2001). Previous studies have shown that MTs play critical roles in fungal development and plant-fungus interactions. For instance, two MT I-type genes in the plant pathogen *C. gloeosporioides* were uniquely expressed during appressorium formation by contact with host surface wax (Hwang and Kolattukudy 1995). Similarly, in the ascomycete fungus *Magnaporthe grisea*, known for causing rice blast disease, an unusual MT-like protein (22 aa long with only six Cys residues) was identified be play a novel role in the biochemical differentiation of the appressorium cell wall (Tucker et al. 2004). Furthermore, MTs were also differentially expressed during the symbiotic mycorrhizal period. Transcriptome analysis of ECM roots formed between *P. involutus* and *B. pendula* shown that a series of genes, including a fungal MT, were upregulated compared with the saprotrophic growth condition (Morel et al. 2005). In contrast, a 65-aa MT from the AM fungus *G. margarita* appeared to be downregulated in the symbiotic mycelia (Lanfranco et al. 2002).

Metallothionein is a low molecular weight, cysteine-rich metal binding protein (Audrey et al. 2017). MTs in different species generally have the ability to chelate heavy metals, maintain the balance of intracellular metal ion concentration, and scavenge reactive oxygen species (Sutherland and Stillman 2011). By chelating heavy metals, it reduces the accumulation and distribution of biological heavy metals to help organisms deal with heavy metal stress in the environment. For example, *OsMT1e* in *Oryza sativa* can reduce the accumulation of Cd in root and shoot (Rono et al. 2021). In ECM fungi, MTs also have a similar effect. Sacky et al. (2014) cloned three MTs genes in *Hebeloma mesophaeum*. Among

them, *HmMT1* is mainly responsible for the complexation of Zn(II) and Cd(II) in the cell and is finally transported in vacuoles and intracellular vesicles. *HmMT2* and *HcMT3* chelate with Ag(I) in the cytoplasm, reducing the toxic effect of Ag(I) on cells. Higher eukaryotic MTs are generally induced by a wide array of metals and stress conditions (Cobbett and Goldsbrough 2002). In contrast, each of the fungal MTs studied thus far is induced by a limited number of heavy metals, with most being induced by Cu(II) (Averbeck et al. 2001; Ramesh et al. 2009; Cobine et al. 2004; Kumar et al. 2005). This was also the case for the MTs characterized in this work, and the single-copy gene *LbMT1* was chosen for further expression investigations. In contrast with *PiMT1* found in *P. involutus* (Bellion et al. 2007), the expression of *LbMT1* remained constant at high expression levels in the free-living mycelium (FLM) of *L. bicolor* and was induced by relatively high Cu(II) and Cd(II) concentrations, giving rise to the hypothesis that *LbMT1* may play a housekeeping role in Cu(II) and Cd(II) homeostasis (Figure 4). In the study of Reddy et al. (2016), the *PaMT1* gene identified from *Pisolithus albus* can be induced by Cu(II) or Cd(II), and the expression of *PaMT1* gene under Cd(II) stress is lower than that under Cu(II) stress. This is similar to our results. In our study, *LbMT1* can be induced by high concentration of Cu(II) and medium to high concentration of Cd(II), and the expression level of *LbMT1* under Cd(II) stress is lower than that under Cu(II) stress. The similar results in *Suillus himalayensis* were also observed by Kalsotra et al. (Kalsotra et al. 2018). The expression levels of *ShMT1* and *ShMT1* genes of *S. himalayensis* under Cu(II) stress are higher than under Cd(II) stress. In the study of Courtois et al. (2020), Ag(I) was used to treat *Eisenia fetida* for 5 weeks, and no changes in the expression of Cd-related metallothionein and the oxidative stress-related genes were observed. We also found no effect of Ag(I) stress on the expression of *LbMT1*. Zn(II) is less toxic to *L. bicolor* (Figure S1), so it may not be more toxic to fungus. The expression of *LbMT1* is also not induced by Zn(II). Liu et al. (2005) shown that the inducing activity of MTs on Cd(II) in yeast cells is mediated by oxidative stress, which is similar with our result, and it may be due to the oxidative stress caused by H_2O_2 after inducing the expression of *LbMT1*. The function of metallothionein (MT) is currently characterized as chelating metals in cells and as a free radical scavenger (Chatterjee et al. 2020). Some reagents that can induce the formation of free radicals and can also increase the expression of MTs (Bauman et al. 1991). Pakdee et al. (2019) showed that oxidative stress can increase the expression of *PpMT1.2a*. At the same time, some MTs also have the effect of removing H_2O_2 (Mierek-Adamska et al., 2019). In our study, the expression level of *LbMT1* was increased when treated by 2.0 mM H_2O_2 for 12 h and 1.0 mM H_2O_2 for 24 h. This indicated that *LbMT1* may be involved in the detoxification of *L. bicolor* under high-concentration short-term or low-concentration long-term H_2O_2 stress. Many MTs can be induced by Cu(II), but some MTs can be induced by Cu(II) and Cd(II), and the expression level under Cu(II) stress is higher than Cd(II) stress (Reddy et al. 2016; Kalsotra et al. 2018). These results were similar to our study. Both Cu(II) and Cd(II) can increase the expression of *LbMT1*, but the expression of *LbMT1* under Cu(II) stress was higher than under Cd(II) stress. One possible reason is that Cd(II) has other chelating substances in the cell, such as GSH (Courbot et al. 2004). However, *LbMTs* still have the ability to improve the Cd(II) tolerance of *L. bicolor*. Yeast experiments have shown that all yeasts can grow under 60 μM Cd(II) stress after being transformed into *LbMTs*, but WT and *yap1*Δ cannot. However, replenishing the eight *LbMTs* genes does not improve the H_2O_2 tolerance of yeast. This may be because the main role of MT is to chelate heavy metals in the environment (Hauser-Davis et al. 2021), so it has no obvious replenishing effect on H_2O_2 stress. The sulfhydryl group of MT has nucleophilic properties and is easy to act on some electrophilic substances, especially easy to combine with free radicals, which can achieve the effect of eliminating free radicals (Chatterjee et al. 2020). Many MTs can eliminate oxidative damage (Jin et al. 2017; Zhou et al. 2014). Many studies have shown that MTs can be induced by H_2O_2 (Mierek-Adamska et al. 2019). Therefore, when the fungus is subjected to oxidative stress, the expression of MT will be increased to cope with the oxidative stress.

Under 50–100 μM Cu(II) or 1.0–2.0 mM H_2O_2 stress, the growth of *L. bicolor* was inhibited to a similar degree (Figure S1); therefore, there was no much difference in the expression level of *LbMT1* under these four stresses, because MT's scavenging effect on oxidative stress is weaker than that of metal chelation (Figure 5). Under Cu(II) stress, the expression level of *LbMT1* was slightly higher than H_2O_2 stress. Under the stress of 10 μM Cd(II), the growth of *L. bicolor* was inhibited to a great extent (Figure S1), and excessively high concentrations of Cd(II) will inhibit the expression of most genes in ECM fungi (Ruytinx et al. 2011). Therefore, although the expression of *LbMT1* was increased under 10 μM Cd(II) stress, it was still no much different from the former, even slightly lower than Cu(II) stress.

Previous studies have found that the expression of MTs genes in ECM fungi is significantly up-regulated or down-regulated in the process of ECM fungi interacting with host plants (Johansson et al.,2004). This is similar to our results. *LbMT2a*, *LbMT2b*, *LbMT3c*, and *LbMT4* play a certain role in the symbiosis process of *L. bicolor* and plants. Their specific functions are still unclear. It is speculated that the nutrient exchange between them occurs during symbiosis with *L. bicolor* and plants. Based on the levels of induction of MTs by different metals, Capdevila and Atrian (2011) proposed the concept of MT evolution, which suggests that functional adaptations of MT genes reflect

specific cellular demands for the expression level and metal specificity of a particular MT peptide.

It has been suggested that MT sequences preceded by a stretch of 10–13 aa lacking Cys residues may be a specific feature of the ECM basidiomycetes (Osobová et al. 2011). The functional importance of this feature, if any, is unknown. As for the LbMT2s, the C-terminal moiety seems to derive from other short LbMTs, as they all share conserved Cys residues, and the *LbMT2* gene has conserved the 39-bp intron found in all of the short MTs in this study. Shared characteristics of LbMT1, LbMT3a, LbMT3b, LbMT3c, LbMT4, and LbMT5 proteins include their small size and the presence of 7−9 Cys residues (representing about 22% of the total aa content) with two aromatic residues. The short MTs identified in different basidiomycete species likely share a common ancestor (Reddy et al. 2014). To test the roles of *L. bicolor* MTs after gene duplication, we performed a yeast complementation assay. Heterologous complementation assays in yeast demonstrated that the eight *LbMT* genes encode functional peptides capable of conferring increased tolerance against Cu(II) and Cd(II), thereby confirming that LbMT peptides may defend fungal cells against metal ions. The data are in agreement with the results of many related studies. For example, *SlMTa* and *SlMTb* can endow *S. luteus* Cd(II) and Cu(II) tolerance (Nguyen et al. 2017). Previous experiments in *Drosophila melanogaster* demonstrated that the number of functional MT gene duplications correlates directly with Cu(II) and Cd(II) resistance (Moleirinho et al. 2011). Clustered MT genes were coregulated and conferred tolerance to multiple abiotic stressors in rice (Kumar et al. 2012). In contrast, in our study, H_2O_2-elicited oxidative stress handling was not affected by the expression of *LbMTs* in mutant yeast, suggesting that *LbMTs* expression responds more directly to metals than to oxidative stress.

Conclusions

In conclusion, eight MTs genes were identified and cloned from the ECM fungus *L. bicolor* S238N. Genomic analysis found that, except for *LbMT1*, there were a large number of transposon elements in the upstream and downstream 60 kb range of the other 7 *LbMTs* genes with high similarity. It is speculated that these MTs are formed by gene tandem duplication. Through complementary expression in the corresponding yeast mutants, it was found that it has a strong chelating ability to heavy metals Cu(II) and Cd(II), but has no replenishing ability to H_2O_2 stress. At the transcriptional level, the expression levels of *LbMT2a*, *LbMT2b*, *LbMT3c*, and *LbMT4* in the symbiosis process of *L. bicolor* and plant roots were significantly higher than other *LbMTs*. Further bioinformatics analysis of the 1500 bp upstream region of these 8 genes revealed that the promoter regions of the 4 *LbMTs* genes have multiple transcription factor binding sites related to nutrient element response. In addition, the expression of single copy gene *LbMT1* under the stress of Cu(II), Cd(II), Zn(II), Ag(I), and H_2O_2 were studied. *LbMT1* transcript is relatively abundant in vegetative mycelium cells, and its expression is significantly increased under high concentration of Cu(II) or H_2O_2 stress and low concentration of Cd(II) stress.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-16776-0.

Acknowledgments We thank Prof. F. Martin (INRA, France) and Prof. A. G. Pardo (University of Quilmes, Argentina) for providing the fungus material *Laccaria bicolor* S238N. We thank Prof. Dennis J. Thiele (Duke University, USA) for the gift of yeast strains DTY3 and DTY4 and Prof. Shusuke Kuge (Tohoku Pharmaceutical University, Japan) for strains W303B and WYU. We thank Prof. Z. H. Ren (Shandong Agricultural University, China) for the gift of the vector P424-GPD.

Author contribution Binhao Liu, Pengcheng Dong, and Zhihang Feng involved in cloning of MTs in *L. bicolor*, organ-specific expression count and promoter analysis, sequence alignment and phylogenetic analysis, real-time quantitative PCR of *L. bicolor* MT genes, and yeast complementation assay. Xinzhe Zhang tested the heavy metal tolerance of *L. bicolor*. Binhao Liu, Pengcheng Dong, Yan Xia, and Zhenguo Shen were four major contributors in writing the manuscript. Liang Shi, Chen Chen, Zhugui Wen, Chunlan Lian, and Yahua Chen provided experimental programs and ideas. All authors read and approved the final manuscript.

Funding This study was supported by the National Natural Science Foundation of China (31901180; 31800525; 41571307); the China Agriculture Research System of MOF and MARA (CARS-10-B24, CARS-23-A03); China Postdoctoral Science Foundation (2019M651845); and Special Fund Project of Fundamental Scientific Research Funds for Central Universities of Nanjing Agricultural University (KYQN202061).

Declarations

Ethical approval and consent to participate Ethics approval and consent to participate was not required for this research.

Consent for publication The data in this article has been approved by all authors for publication.

Conflict of interest The authors declare no competing interests.

References

Audrey MVA, Jolly S, Howard SJ (2017) Gene gain and loss, and transcriptional remodeling cause divergence in the transcriptomes of Phytophthora infestans and Pythium ultimum during potato tuber colonization. BMC Genomics 18:1–28

Averbeck N, Borghouts C, Hamann A, Specke V, Osiewacz HD (2001) Molecular control of copper homeostasis in filamentous fungi: increased expression of a metallothionein gene during aging of Podospora anserina. Mol Gen Genet 264:604–612

Babula P, Masarik M, Adam V, Eckschlager T, Stiborova M, Trnkova L, Skutkova H, Provaznik I, Hubalek J, Kizek R (2012) Mammalian metallothioneins: properties and functions. Metallomics. 4:739–750

Bauman JW, Liu J, Liu YP, Klaassen CD (1991) Increase in metallothionein produced by chemicals that induce oxidative stress. Toxicol Appl Pharmacol 110:347–354

Bellion M, Courbot M, Jacob C, Guinet F, Blaudez D, Chalot M (2007) Metal induction of a *Paxillus involutus* metallothionein and its heterologous expression in *Hebeloma cylindrosporum*. New Phytol 174:151–158

Bennetzen (2005) Transposable elements, gene creation and genome rearrangement in flowering plants. Curr Opin Genet Dev 15:621–627

Binz PA, Kägi JH (1999) Metallothionein: molecular evolution and classification. In Metallothionein Iv, Springer. 7-13.

Butt TR, Sternberg EJ, Gorman JA, Clark P, Hamer D, Rosenberg M, Crooke ST (1984) Copper metallothionein of yeast, structure of the gene, and regulation of expression. Proc Natl Acad Sci 81:3332–3336

Byrne KP, Wolfe KH (2007) Consistent patterns of rate asymmetry and gene loss indicate widespread neofunctionalization of yeast genes after whole-genome duplication. Genetics. 175:1341–1350

Capdevila M, Atrian S (2011) Metallothionein protein evolution: a miniassay. JBIC J. Biol Inorg Chem 16:977–989

Chatterjee S, Kumari S, Rath S, Priyadarshanee M, Das S (2020) Diversity, structure and regulation of microbial metallothionein: metal resistance and possible applications in sequestration of toxic metals. Metallomics. 12:1637

Cicatelli A, Lingua G, Todeschini V, Biondi S, Torrigiani P, Castiglione S (2010) Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal-contaminated soil, and this is associated with upregulation of foliar metallothionein and polyamine biosynthetic gene expression. Ann Bot 106:791–802

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159–182

Cobine PA, McKay RT, Zangger K, Dameron CT (2004) Armitage, I. M., Solution structure of Cu6 metallothionein from the fungus *Neurospora crassa*. Eur. J. Biochem. 271(21):4213–4221

Courbot M, Diez L, Ruotolo R, Chalot M, Leroy P (2004) Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Appl Environ Microbiol 70:7413–7417

Courtois P, Rorat A, Lemiere S, Guyoneaud R, Attard E, Longepierre M, Rigal F, Levard C, Chaurand P, Grosser A, Grobelak A, Kacprzak M, Lors C, Richaume A, Vandenbulcke F (2020) Medium-term effects of Ag supplied directly or via sewage sludge to an agricultural soil on *Eisenia fetida* earthworm and soil microbial communities. Chemosphere. 269:128761

Culotta VC, Howard WR, Liu XF (1994) CRS5 encodes a metallothionein-like protein in *Saccharomyces cerevisiae*. J Biol Chem 269(41):25295–25302

Divya T, Chandwadkar P, Acharya C, Nmt A (2018) A novel metallothionein of Anabaena sp. strain PCC 7120 imparts protection against cadmium stress but not oxidative stress. Aquat Toxicol 199:152–161

Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De Montigny J, Marck C, Neuvéglise C, Talla E (2004) Genome evolution in yeasts. Nature. 430(6995):35–44

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5):1792–1797

Flores-Monterroso A, Canales J, de la Torre F, Ávila C, Cánovas FM (2013) Identification of genes differentially expressed in ectomycorrhizal roots during the *Pinus pinaster–Laccaria bicolor* interaction. Planta. 237(6):1637–1650

Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martínez AT, Otillar R, Spatafora JW, Yadav JS (2012) The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science. 336(6089):1715–1719

Gietz RD, Schiestl RH (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2(1):31–34

González-Guerrero M, Cano C, Azcón-Aguilar C, Ferrol N (2007) GintMT1 encodes a functional metallothionein in *Glomus intraradices* that responds to oxidative stress. Mycorrhiza. 17(4):327–335

Greer JM, Puetz J, Thomas KR, Capecchi MR (2000) Maintenance of functional equivalence during paralogous Hox gene evolution. Nature. 403(6770):661–665

Grigoriev IV, Cullen D, Goodwin SB, Hibbett D, Jeffries TW, Kubicek CP, Kuske C, Magnuson JK, Martin F, Spatafora JW (2011) Fueling the future with fungal genomics. Mycology. 2(3):192–209

Gu Z, Rifkin SA, White KP, Li WH (2004) Duplicate genes increase gene expression diversity within and between species. Nat Genet 36(6):577–579

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59(3):307–321

Hassinen V, Tervahauta A, Schat H, Kärenlampi S (2011) Plant metallothioneins–metal chelators with ROS scavenging activity? Plant Biol 13(2):225–232

Hauser-Davis RA, Silva-Junior DR, Linde-Arias AR, Vianna M (2021) Cytosolic and metallothionein-bound hepatic metals and detoxification in a sentinel teleost, Dules auriga, from Southern Rio de Janeiro, Brazil. Biol Trace Elem Res 199:744–752

Howe R, Evans R, Ketteridge S (1997) Copper-binding proteins in ectomycorrhizal fungi. New Phytol 135(1):123–131

Hwang CS, Kolattukudy PE (1995) Isolation and characterization of genes expressed uniquely during appressorium formation by *Colletotrichum gloeosporioides* conidia induced by the host surface wax. Mol Gen Genet 247(3):282–294

Iturbeespinoza P, Gilmoreno S, Lin W, Calatayud S, Palacios Ò, Capdevila M, Atrian S (2016) The fungus *Tremella mesenterica* encodes the longest metallothionein currently known: gene, protein and metal binding characterization. PLoS One 11(2):e0148651

Jacob C, Courbot M, Martin F, Brun A, Chalot M (2004) Transcriptomic responses to cadmium in the ectomycorrhizal fungus *Paxillus involutus*. FEBS Lett 576(3):423–427

Jaeckel P, Krauss G, Menge S, Schierhorn A, Rücknagel P, Krauss GJ (2005) Cadmium induces a novel metallothionein and phytochelatin 2 in an aquatic fungus. Biochem Biophys Res Commun 333(1):150–155

Jin SM, Xu C, Li GL, Sun D, Li Y, Wang XW, Liu SK (2017) Functional characterization of a type 2 metallothionein gene, SsMT2, from alkaline-tolerant *Suaeda salsa*. Sci Rep 7:17914

Johansson T, Le Quéré A, Ahren D, Söderström B, Erlandsson R, Lundeberg J, Uhlén M, Tunlid A (2004) Transcriptional responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. Mol Plant-Microbe Interact 17(2):202–215

Kalsotra T, Khullar S, Agnihotri R, Reddy MS (2018) Metal induction of two metallothionein genes in the ectomycorrhizal fungus *Suillus himalayensis* and their role in metal tolerance. Microbiology. 164:868–876

Kemppainen M, Duplessis S, Martin F, Pardo AG (2009) RNA silencing in the model mycorrhizal fungus *Laccaria bicolor*: gene knockdown of nitrate reductase results in inhibition of symbiosis with Populus. Environ Microbiol 11(7):1878–1896

Kohler A, Kuo A, Nagy LG, Morin E, Barry KW, Buscot F, Canbäck B, Choi C, Cichocki N, Clum A (2015) Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nat Genet 47(4):410–415

Kuge S, Jones N (1994) YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. EMBO J 13(3):655

Kumar KS, Dayananda S, Subramanyam C (2005) Copper alone, but not oxidative stress, induces copper–metallothionein gene in *Neurospora crassa*. FEMS Microbiol. Lett. 242(1):45–50

Kumar G, Kushwaha HR, Panjabi-Sabharwal V, Kumari S, Joshi R, Karan R, Mittal S, Pareek SL, Pareek A (2012) Clustered metallothionein genes are co-regulated in rice and ectopic expression of OsMT1e-P confers multiple abiotic stress tolerance in tobacco via ROS scavenging. BMC Plant Biol 12(1):107

Labbé J, Zhang X, Yin T, Schmutz J, Grimwood J, Martin F, Tuskan GA, Le Tacon F (2008) A genetic linkage map for the ectomycorrhizal fungus *Laccaria bicolor* and its alignment to the whole-genome sequence assemblies. New Phytol 180(2):316–328

Labbe J, Murat C, Morin E, Tuskan GA, Le Tacon F, Martin F (2012) Characterization of transposable elements in the ectomycorrhizal fungus *Laccaria bicolor*. PLoS One 7(8):e40197

Lanfranco L, Bolchi A, Ros EC, Ottonello S, Bonfante P (2002) Differential expression of a metallothionein gene during the presymbiotic versus the symbiotic phase of an arbuscular mycorrhizal fungus. Plant Physiol 130(1):58–67

Le Croizier G, Lacroix C, Artigaud S, Le Floch S, Raffray J, Penicaud V, Coquillé V, Autier J, Rouget ML, Le Bayon N (2018) Significance of metallothioneins in differential cadmium accumulation kinetics between two marine fish species. Environ Pollut 236:462–476

Liu JH, Zhang YM, Huang DJ, Song G (2005) Cadmium induced MTs synthesis via oxidative stress in yeast *Saccharomyces cerevisiae*. Mol Cell Biochem 280:139–145

Loebus J, Leitenmaier B, Meissner D, Braha B, Krauss GJ, Dobritzsch D, Freisinger E (2013) The major function of a metallothionein from the aquatic fungus *Heliscus lugdunensis* is cadmium detoxification. J Inogra Biochem 127:253–260

Longo VD, Gralla EB, Valentine JS (1996) Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae* mitochondrial production of toxic oxygen species in vivo. J Biol Chem 271(21):12275–12280

Margoshes M, Vallee BL (1957) A cadmium protein from equine kidney cortex. J Am Chem Soc 79(17):4813–4814

Martin F, Aerts A, Ahrén D, Brun A, Danchin E, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature. 452(7183):88–92

Marx D (1969) Influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. II. Production, identification, and biological activity of antibiotics produced by *Leucopaxillus cerealis* var. piceina. Phytopathology.

Matys V, Fricke E, Geffers R, Gößling E, Haubrock M, Hehl R, Hornischer K, Karas D, Kel AE, Kel-Margoulis OV (2003) TRANSFAC: transcriptional regulation, from patterns to profiles. Nucl Acids Res 31(1):374–378

Mierek-Adamska A, Kotowicz K, Goc A, Boniecka J, Dąbrowska GB (2019) Potential involvement of rapeseed (*Brassica napus* l.) metallothioneins in the hydrogen peroxide-induced regulation of seed vigour. J Agron Crop Sci 205:598–607

Moleirinho A, Carneiro J, Matthiesen R, Silva RM, Amorim A, Azevedo L (2011) Gains, losses and changes of function after gene duplication: study of the metallothionein family. PLoS One 6(4):e18487

Morel M, Jacob C, Kohler A, Johansson T, Martin F, Chalot M, Brun A (2005) Identification of genes differentially expressed in extraradical mycelium and ectomycorrhizal roots during *Paxillus involutus-Betula pendula* ectomycorrhizal symbiosis. Appl Environ Microbiol 71(1):382–391

Morgante M, Brunner S, Pea G, Fengler K, Zuccolo A, Rafalski A (2005) Gene duplication and exon shuffling by helitron-like transposons generate intraspecies diversity in maize. Nat Genet 37(9):997–1002

Mumberg D, Müller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene. 156(1):119–122

Nguyen H, Rineau F, Vangronsveld J, Cuypers A, Colpaert JV, Ruytinx J (2017) A novel, highly conserved metallothionein family in basidiomycete fungi and characterization of two representative SlMTa and SlMTb genes in the ectomycorrhizal fungus *Suillus luteus*. Environ Microbiol 19(7):2577–2587

Nikolaos V, Anne-Ruxandra C, Aoife M (2021) Synteny-based analyses indicate that sequence divergence is not the main source of orphan genes. ELIFE. 9:e53500

Okuyama MA, Kobayashi Y, Inouhe M, Tohoyama H, Joho M (1999) Effect of some heavy metal ions on copper-induced metallothionein synthesis in the yeast *Saccharomyces cerevisiae*. BioMetals. 12(4): 307–314

Osobová M, Urban V, Jedelský PL, Borovička J, Gryndler M, Ruml T, Kotrba P (2011) Three metallothionein isoforms and sequestration of intracellular silver in the hyperaccumulator *Amanita strobiliformis*. New Phytol 190(4):916–926

Pagani A, Villarreal L, Capdevila M, Atrian S (2007) The *Saccharomyces cerevisiae* Crs5 metallothionein metal-binding abilities and its role in the response to zinc overload. Mol Microbiol 63(1):256–269

Pakdee O, Songnuan W, Panvisavas N, Pokethitiyook P, Yokthongwattana K, Meetam M (2019) Functional characterization of metallothionein-like genes from physcomitrella patens: expression profiling, yeast heterologous expression, and disruption of *ppmt1.2a* gene. Planta 250(2):427–443

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29(9):e45–e45. https://doi.org/10.1093/nar/29.9.e45

Ramesh G, Podila G, Gay G, Marmeisse R, Reddy M (2009) Different patterns of regulation for the copper and cadmium metallothioneins of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Appl Environ Microbiol 75(8):2266–2274

Reddy MS, Prasanna L, Marmeisse R, Fraissinet-Tachet L (2014) Differential expression of metallothioneins in response to heavy metals and their involvement in metal tolerance in the symbiotic basidiomycete *Laccaria bicolor*. Microbiology. 160(10):2235–2242

Reddy MS, Kour M, Aggarwal S, Ahuja S, Marmeisse R, Fraissinet L (2016) Metal induction of a *Pisolithus albus* metallothionein and its potential involvement in heavy metal tolerance during mycorrhizal symbiosis. Environ Microbiol 18(8):2446–2454

Rono JK, Wang LL, Wu XC, Cao HW, Zhao YN, Khan IU, Yang ZM (2021) Identification of a new function of metallothionein-like gene *OsMT1e* for cadmium detoxification and potential phytoremediation. Chemosphere. 265:129136

Ruytinx J, Craciun AR, Verstraelen K, Vangronsveld J, Colpaert JV, Verbruggen N (2011) Transcriptome analysis by cdna-aflp of *Suillus luteus* Cd-tolerant and Cd-sensitive isolates. Mycorrhiza. 21(3):145–154

Sacky J, Leonhardt T, Borovicka J, Gryndler M, Briksi A, Kotrba P (2014) Intracellular sequestration of zinc, cadmium and silver in *Hebeloma mesophaeum* and characterization of its metallothionein genes. Fungal Genet Biol 67:3–14

Serén N, Glaberman S, Carretero MA, Chiari Y (2014) Molecular evolution and functional divergence of the metallothionein gene family in vertebrates. J Mol Evol 78(3-4):217–233

Sigel A, Sigel H, Sigel RK (2009) Metallothioneins and related chelators. R Soc Chem 5.

Singh G, Ashby AM (1998) Cloning of the mating type loci from *Pyrenopeziza brassicae* reveals the presence of a novel mating type gene within a discomycete MAT 1-2 locus encoding a putative metallothionein-like protein. Mol Microbiol 30(4):799–806

Stommel M, Mann P, Franken P (2001) EST-library construction using spore RNA of the arbuscular mycorrhizal fungus *Gigaspora rosea*. Mycorrhiza. 10(6):281–285

Sutherland DEK, Stillman MJ (2011) The "magic numbers" of metallothionein. Metallomics. 3:444–463

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Tucker SL, Thornton CR, Tasker K, Jacob C, Giles G, Egan M, Talbot NJ (2004) A fungal metallothionein is required for pathogenicity of *Magnaporthe grisea*. Plant Cell 16(6):1575–1588

Voiblet C, Duplessis S, Encelot N, Martin F (2001) Identification of symbiosis-regulated genes in *Eucalyptus globulus–Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. Plant J 25(2):181–191

Zhang JZ (2003) Evolution by gene duplication: an update. Trends Ecol Evol 18(6):292–298

Zhou BR, Yao WJ, Wang SJ, Wang XW, Jiang TB (2014) The metallothionein gene, *TaMT3*, from *Tamarix androssowii* confers Cd^{2+} tolerance in tobacco. Int J Mol Sci 15:10398–10409