Differential expression of metallothioneins in response to heavy metals and their involvement in metal tolerance in the symbiotic basidiomycete

**Laccaria bicolor**

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Cysteine-rich peptides such as metallothioneins (MTs) are involved in metal homeostasis and detoxification in many eukaryotes. We report the characterization and expression of two MT genes, *LbMT1* and *LbMT2* from the ectomycorrhizal fungus *Laccaria bicolor* under metal stress conditions. *LbMT1* and *LbMT2* differ with respect to the length of the encoded peptides (58 versus 37 aa, respectively) and also by their expression patterns in response to metals. The expression levels of both *LbMT1* and *LbMT2* increased as a function of increased external Cu concentration, the expression levels for *LbMT2* were always significantly higher compared with those of *LbMT1*. Only *LbMT1*, but not *LbMT2*, responded to Cd supply in the range of 25–100 μM while Zn did not affect the transcription of either *LbMT1* or *LbMT2*. Both genes also responded to oxidative stress, but to a lesser extent compared to their responses to either Cu or Cd stress. Heterologous complementation assays in metal-sensitive yeast mutants indicated that both *LbMT1* and *LbMT2* encode peptides capable of conferring higher tolerance to both Cu and Cd. The present study identified *LbMTs* as potential determinants of the response of this mycorrhizal fungus to Cu and Cd stress.

**INTRODUCTION**

Ectomycorrhizal fungi enhance the growth and fitness of their host plant mostly by improving their mineral nutrition (Smith & Read 1997). They also increase their host plant’s tolerance to heavy metals (Bellion et al., 2006; Colpaert & van Assche, 1993; Dixon & Buschena, 1988; Howe et al., 1997), and as a consequence, improve the survival and growth of trees in ecosystems contaminated by heavy metals (Jacob et al., 2004). This capability is of key ecological importance because heavy metals are continuously being dispersed into the biosphere by natural processes or human activity and can constitute a serious environmental hazard (Ayres, 1992).

Metal tolerance is a result of different mechanisms such as induction of oxidative stress response enzymes and elevation of the antioxidant glutathione (GSH) levels (Jacob et al., 2004), metal-efflux systems (Colpaert et al., 2011; Ruytinx et al., 2013) and sequestration of excess metal by intracellular complexation or compartmentalization. The three major classes of intracellular peptides chelating metal ions are GSH, phytochelatins (PCs), and metallothioneins (MTs). Jacob et al. (2004) showed that complexation of Cd by MTs is a key mechanism for Cd tolerance in the ectomycorrhizal fungus *Paxillus involutus*. MTs are ubiquitously present in eukaryotes and also occur in some prokaryotic organisms (Cobbett & Goldsbrough, 2002). They are characterized by their small size (usually below 7 kDa), high cysteine content (up to 33 %) and a high degeneracy in the remaining amino acid residues. They are often encoded by a multigene family and contain metal-binding Cys-rich domains (Cobbett & Goldsbrough, 2002). MTs differ from other metalloproteins by their high affinity towards both the essential (e.g. Cu and Zn) and non-essential metal ions (e.g. Cd, Hg and Ag) and their large metal-binding capacities (Loebus et al., 2013). Generally, MTs are involved in metal homeostasis and detoxification (Gadd, 1993; Cobbett & Goldsbrough, 2002). Transcription of MTs is typically induced by the same metal ion(s) that bind to the protein, thus providing a direct

**Abbreviations:** MT, metallothionein; PC, phytochelatins.

Two supplementary figures are available with the online version of this paper.
Plant and animal MTs are generally induced by a wide array of metals and stress conditions (Cobett & Goldsborough, 2002). By contrast, fungal MT genes are either induced by a single or multiple metal signals and the range of inducing metals could correspond to the metal specificity of the corresponding MTs. It has been observed that in Neurospora crassa and Podospora anserina, MTs are induced only by Cu and not by other metals (Cobine et al., 2004; Averbeck et al., 2001), while in Candida glabrata, MTs were induced by Cu and Ag but not by Cd (Mehra et al., 1989). Two MTs have been identified in Saccharomyces cerevisiae, the first one, encoded by the CUP1 gene, is induced only by Cu and Ag, (Butt et al., 1984; Okuyama et al., 1999) while CRS5, the second MT, is regulated by Cu, Zn and oxidative stress (Pagani et al., 2007; Culotta et al., 1994). Cd-inducible MTs have been identified in different fungi (Bellion et al., 2007; Courbot et al., 2004; Hwang & Kolattukudy, 1995; Jacob et al., 2004; Lanfranco et al., 2002; Ramesh et al., 2009; Loebus et al., 2013). Cd inducibility of MTs is usually accompanied by a similar inducibility by either Cu or Zn and further heavy metals (Loebus et al., 2013). Lanfranco et al. (2002) and Bellion et al. (2007) reported that the Cd-inducible MTs of the glomeromycete Gigaspora margarita and the basidiomycete P. involutus are also induced by Cu. Ramesh et al. (2009) showed HcMT1, one of the MTs of the ectomycorrhizal fungus Hebeloma cylindrosporum, induced by Cu and Cd. Sácký et al. (2014) identified three MTs (HmMT1, HmMT2 and HmMT3) from Hebeloma mesophaeum, where HmMT1 was induced by Zn, Cu and Cd, while HmMT2 and HmMT3 were strongly induced by Ag. Osobová et al. (2011) reported three isoforms of MTs (AsMTs) from Amanita strobiliformis that sequester intracellular Ag in its fruit bodies and mycelium. In addition to the direct toxicity of the metals at elevated concentrations, some of the metals (Cu and As) can also cause oxidative stress e.g. via Fenton and Heber–Weisse-like reactions in the case of Cu. Hence, it is essential to know whether the MTs are triggered by metal ion stress or oxidative stress.

The focus of the present study was to identify MTs of the ectomycorrhizal fungus Laccaria bicolor and to obtain a deeper insight into the regulation and detoxification mechanisms of toxic metals by the organism. Six different genes encoding Cys-rich putative MTs were identified in the genome of L. bicolor (Martin et al., 2008) (Figs S1 and S2, available in the online Supplementary Material). Five of the proteins were less than 50 aa in length (range, 33–48); the sixth one was longer with 58 residues. Each of the five shorter ORFs was interrupted by two introns at conserved positions (Fig. S1). Their encoded proteins contained between seven and nine mostly conserved Cys residues, several of them arranged as C-x-C doublets, which could participate to metal chelation (Fig. S1). Sequence homology between several of the short MT genes extended outside of their coding sequences. Protein-coding genes, accession numbers 399685, 388087, 388066 and 399683, all located on scaffold 10 of the genome assembly, shared sequence similarities extending 350–520 nt upstream of their start codons and their transcriptions may therefore be controlled by similar environmental clues. The longest protein (accession no. 396313), might be derived from the smaller ones as its C-terminal moiety contained all of the conserved Cys residues observed in the short proteins and its gene has retained the 3′ intron observed in the shorter genes (Fig. S1). Length of this protein resulted from the acquisition of an N-terminal extension of unknown origin containing seven cysteines including a rare C-C-C triplet. All five ‘short’ L. bicolor MTs and the C-terminal part of the longest one could be aligned over their entire length to short MTs from unrelated Agaricales species (Fig. S2), several of them characterized experimentally (Bellion et al., 2007; Ramesh et al., 2009; Osobová et al., 2011; Sácký et al., 2014). Interestingly, as illustrated for the H. mesophaeum AHA31880 protein (Sácký et al., 2014) other Agaricales species also present long MTs whose homology to the shorter ones is limited to their C-terminal ends. However, no obvious sequence similarities could be detected between the N-terminal end of the long L. bicolor 396313 MT and the N-terminal ends of other long MTs from Agaricales.

In the present study, we choose to study in more detail the regulation patterns and metal specificities of MTs 399685 (LbMT1) and 396313 (LbMT2), taken as representative of the short and long MTs respectively.

**METHODS**

**Organisms, culture media and conditions.** The ectomycorrhizal basidiomycete fungus Laccaria bicolor (haploid strain S238) was used in this study. It was maintained on modified Melin’s medium (Melin, 1953) supplemented with Heller’s micronutrients (Gay, 1990). To evaluate the effect of different heavy metals and H$_2$O$_2$, mycelial discs (7 mm diameter) were placed in the centre of Petri plates containing modified Melin’s agar medium supplemented with different concentrations of Cu (0, 100, 200, 300 and 400 µM as CuSO$_4$·5H$_2$O), Cd (0, 25, 50, 75 and 100 µM as CdSO$_4$·8H$_2$O), Zn (0, 1, 2, 4, 6, 8 and 10 mM as ZnSO$_4$·7H$_2$O) and H$_2$O$_2$ (0, 5, 10, 15, 20 and 25 mM) overlaid with a cellophane sheet. After 3 weeks of growth at 25 °C, the mycelium was harvested, dried at 48 °C and the biomass was determined.

DNA manipulations in E. coli DH5α were performed according to standard protocols (Sambrook & Russell, 2001). The Saccharomyces cerevisiae strains used for complementation assays were: two Cu-sensitive strains (DTY3; MATa, leu2-3, leu2-112 his3A1, trpl-1, ura3-50, gal1 CUP1 and DTY4; MATa, trpl-1, leu2-3, leu2-112, gali, his3A1, ura3-50, cup1A::URA3) and 396313 (MATa, his3D1, leu2D0, met15D0, ura3D0, YML007w (=YAP1):::kanMX4) derived from the WT strain BY4741 (MATa, his3D1, leu2D0, met15D0, ura3D0). The S. cerevisiae YAP1 locus encodes a transcription factor related to the mammalian AP-1 complex that positively controls various genes involved in metal and more generally, oxidative stress tolerance in yeast (Kuge & Jones 1994). Although yeast MT genes are not direct targets of such an activator, yap1 mutants are particularly sensitive to Cd and are thus suitable to highlight tolerant phenotypes induced by the expression of foreign cDNAs (Wu et al., 1993). In
S. cerevisiae, CUP1 encodes an MT that is induced by Cu and binds to Cu. The Cu-tolerance threshold is reduced to 75 μM CuSO₄ in the cup1¹ strain (cup1: URA3), which has no functional copies of CUP1 compared with 300 μM in the corresponding WT strain (Hamer et al., 1985).

cDNA synthesis and cloning of MT genes. Total RNA was extracted from mycelia using Trizol reagent (Invitrogen). cDNA was synthesized using approximately 5 μg of total RNA using a SuperScript first strand cDNA synthesis kit (Invitrogen) as per the manufacturer’s instructions. PCR amplification of LbMT1 was performed with primers LbMT1F (5’-ATGATCTCCAACACCT-CTGCTC-3’) and LbMT1R (5’-TTATCAGATGCGCAGTCTTTA-AG-3’) and LbMT2 using LbMT2F (5’-ATGCTTTAATACCT-TCACCCCTATAC-3’) and LbMT2R (5’-TTAGCACTTGCATGCC-TCCAGG-3’). PCR were carried out in a final volume of 50 μl containing 1× reaction buffer, 2.5 U Taq polymerase (Invitrogen), primers (0.2 μM), MgCl₂ (1.5 mM), deoxy nucleotide triphosphates (dNTPs, 100 μM), template DNA (100 ng) and nuclease free water. The PCR program was as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 1 min at 92 °C, 1 min at 55 °C, 1 min at 72 °C and a final extension at 72 °C for 5 min. PCR products were separated on 1.2 % (w/v) agarose gels and visualized by ethidium bromide staining. The PCR products were purified and directly cloned into pGEM-T Easy Vector (Promega). DH5α E. coli competent cells were transformed and plated onto Luria agar with ampicillin medium containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and (isopropyl-beta-D-thiogalactopyranoside (IPTG). The transformants were randomly selected and plasmid DNA was prepared with the Qiagen Plasmid mini kit. DNA sequences were determined using T7 and SP6 primers and ABI310 DNA sequence (ABI). Sequence analysis was performed with the BLAST server (Altschul et al., 1997) using the nucleic acid and predicted amino acid sequences deposited in multiple databases and were aligned using the MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/).

Real-time PCR (RT-PCR). RT-PCR was used to study the expression of MT genes in response to heavy metals and oxidative stress. Mycelia discs (7 mm diameter) were placed in the centre of Petri plates containing with modified Melin's agar medium overlaid with cellophane sheet. After 2 weeks of growth at 25 °C, the cellophane along with mycelium was transferred to Petri plates containing Melin's liquid medium (10 ml) supplemented with different concentrations of Cu, Cd, Zn or H₂O₂ as mentioned above. After 48 h, the mycelia were harvested and ground in liquid nitrogen. Total RNA was isolated from the metal treated mycelium using the Trizol method. Reverse transcription was performed with total RNA as mentioned above. LbMT1 and LbMT2 mRNA accumulation was quantified by RT-PCR performed using Master Mix SYBR ROX (5 prime, GmbH, Hamburg) on a Realplex 4 RT-PCR system (Eppendorf AG, Hamburg). Primers used were LbMT1F and LbMT1R for LbMT1 and LbMT2F and LbMT2R for LbMT2. The cycling program was as follows: 95 °C for 2 min (one cycle), 95 °C for 20 s, 54 °C for 20 s and 68 °C for 30 s (45 cycles). In a final volume of 25 μl, 15 ng cDNA, 2 μM of each primer, and 11.0 μl of 2.5X real mastermix were mixed. The relative quantification of gene expression between samples was calculated using the comparative threshold (CT) method (Heid et al., 1996). The value was calculated for each sample and then the comparative expression level of the gene was given by the formula 2⁻ΔΔCT where ΔΔCT was calculated by subtracting the baseline’s ACT to the sample’s ACT and where the baseline represents the expression level of the control. All RT-PCR measurements were performed on independent biological samples from three replicate experiments in three technical replicates.

Yeast functional complementation assays. The full-length LbMT1 and LbMT2 sequences were amplified using MT1F (5’-CGGATCCATGATCTCCAACACCT-CTGCTC-3’) and MT1R (5’-CCGGAATTC TTATCAGATGCGCAGTCTTTA-AG-3’) and MT2F (5’-CGGATCCATGCTTTAATACCT-TCACCCCTATAC-3’) and MT2R (5’-CCGGAATTC TTATCAGATGCGCAGTCTTTA-AG-3’) primers introducing BamHI and EcoRI sites (sites underlined). The PCR products were digested with BamHI and EcoRI and ligated into the yeast expression vector p424 (Mumberg et al., 1995) under the transcriptional control of the yeast GPD (glyceraldehyde 3-phosphate dehydrogenase) promoter. The p424 vector also contains the CYC1 terminator, the 2μ replication origin and the TRP1 gene as selection marker. Vector p424 and the construct p424-LbMT1 and p424-LbMT2 were introduced into yeast CUP1 and YAPI mutant cells using lithium acetate procedure (Stearns et al., 1990) and transformed cells were selected by their capacity to grow in complete synthetic medium (SD), without Trp.

For the functional complementation experiments, cultures of cup1¹ and yap1 yeast cells carrying either p424 or p424-LbMT1 and p424-LbMT2 were grown in SD-Trp media at 30 °C and 220 r.p.m. for 24 h. Yeast cultures were adjusted to OD₆₀₀=1.0 and 5 μl serial dilutions were spotted on SD plates supplemented or not with either 150 μM CuSO₄ or 40 μM CdSO₄. Plates were incubated for 3 days at 30 °C and photographed. In parallel experiments, Falcon tubes containing 20 ml of fresh SD-Trp media were inoculated with mid-exponential pre-cultures of cup1¹ and yap1 cells containing p424, p424-LbMT1 and p424-LbMT2 to attain a starting optical density of 0.02 at 600 nm. Cells were grown at 30 °C in a rotary shaker (220 r.p.m.); CuSO₄ (150 μM) and CdSO₄ (40 μM) were added 5 h after inoculation. The OD of the cultures were measured at 2–3 h intervals for 42 h.

Data were analysed by ANOVA and the means were compared with Tukey’s test at P<0.05. All the analysis was performed by using Graph Pad Prism 5.1 software.

RESULTS

Metal tolerance of L. bicolor
The growth of L. bicolor was adversely affected with increasing concentrations of metals and of H₂O₂. Increasing concentration of Cu, Cd and H₂O₂ significantly decreased the growth of the fungus. Fifty per cent growth inhibition was observed at 300 μM Cu and at 75 μM Cd (Fig. 1a, b). L. bicolor showed higher tolerance to Zn where the growth was not much affected at 10 mM (Fig. 1c). With H₂O₂, a 50 % growth inhibition was observed at 10 mM (Fig. 1d).

Sequence analysis of LbMT1 and LbMT2
Two representative MT genes, LbMT1 and LbMT2 (GenBank accession no. KJ652245 and KJ652246) were studied with respect to their regulation patterns and metal specificities of the encoded proteins. The LbMT1 cDNA contains a 114 bp ORF encoding 37 aa (Fig. 2) with a predicted molecular mass of 3.523 kDa and isoelectric point of 5.19. Other characteristics of LbMT1, in addition to its small size, are the presence of eight Cys residues (representing about 22 % of the total amino acid content) with two aromatic residues (Phe-3 and His-16). Most of
the Cys residues are part of the C-x-C (where x is any amino acid) motif. LbMT2 cDNA has an ORF of 174 bp encoding a polypeptide containing 58 aa with calculated a molecular mass of 5.9 kDa and isoelectric point of 7.35. The LbMT2 sequence contains 24.1 % Cys with only one aromatic residue (Phe-3) and six C-x-C motifs.

As mentioned in the Introduction, LbMT1p is a prototype of short MTs identified in different Basidiomycete species with which it likely shares a common ancestor. All these MTs (e.g. AsMTp from A. strobiliformis, PiMTp from P. involutus or GlMT from Ganoderma lucidum) are of a similar length and share three C-x-C motifs (Fig. S1).

Fig. 1. Effect of different concentrations of (a) Cu, (b) Cd (c) Zn and (d) H$_2$O$_2$ on the mycelial growth of *Laccaria bicolor* harvested after 3 weeks of growth. The metals were added 3 days after fungal inoculation to avoid immediate stress and also for the fungus to initiate growth. Error bars are ± SEM.

Fig. 2. Multiple sequence alignment of MT proteins of basidiomycetous fungi sequences. Gaps introduced to provide the best alignment are indicated by dashes. Fully conserved Cys residues are highlighted in blue; Accession numbers are: *Amanita strobiliformis* (AsMT), AGO04615; *Paxillus involutus* (PiMT), AAS19463; *Lentinula edodes* (LeMT), CO501612; *Piriformospora indica* (PsMT), ACT83730; *Pisolithus tinctorius* (PtMT), EST 11A2; *Ganoderma lucidum* (GlMT), ABP02008; *Laccaria bicolor* (LbMT1 & 2), KJ652246, KJ652246; *Hebeloma cylindrosporum* (HcMT1 & 2), EU049884, EU049885; *H. mesophaeum* (HmMT1 & 2) KF278558, KF278559; *Agaricus bisporus* (AbMT1), CAC85298.
Furthermore, their coding genes are interrupted by two introns at conserved positions. As for LbMT2p, its C-terminal moiety seems to derive from the above-mentioned short MTs as they share all conserved Cys residues and the LbMT2 gene has conserved the 3′ intron found in short MTs. LbMT2p N-terminal moiety contains additional Cys residues (one C-C-C triplet and two C-x-C motives) and is of uncertain origin. The alignment presented in Fig. 2 showed that LbMTs share similarities with other known MTs of basidiomycetes.

**Induction of LbMTs under stress conditions**

To document the regulation of LbMT1 and LbMT2, the effect of various Cu, Cd, Zn and H2O2 concentrations on the expression of these genes were studied. The mRNA accumulation of LbMT1 increased as the concentration of Cu increased and the maximum accumulation was recorded at 200 μM where it was almost 30 times higher than the control (Fig. 3a). The induction of LbMT2 also significantly increased as a function of Cu concentration. It was more than 108 fold over control when Cu concentration was 300 μM. Cd-induced transcription of LbMT1 increased as a function of metal concentration up to a maximum of 50 μM. The induction levels of LbMT1 were very low at higher concentrations of Cd (75 and 100 μM) compared to lower ones. The induction of LbMT2 was not significant in all the concentrations of Cd tested (Fig. 3b). The expression levels of both LbMT1 and LbMT2 were not significantly different from those measured in control in response to different concentrations of Zn (Fig. 3c), although the highest Zn concentration inhibited the fungal growth (Fig. 1c). Expression levels of LbMT1 increased due to H2O2 treatment (oxidative stress) compared to control. Maximum expression levels (sixfold) for LbMT1 was observed at 10 mM H2O2. The induction of LbMT2 also increased with increasing H2O2 concentration and maximum induction (12-fold) was observed at 15 mM (Fig. 3d). However, the induction levels were not as high as those recorded under Cu and Cd stress, suggesting the expression of LbMT1 and LbMT2 is more specific in response to metals than oxidative stress.

**Yeast functional complementation assays**

To validate the functional role of the LbMT1 and LbMT2 protein products as heavy metal-binding ligands, these genes were expressed in two S. cerevisiae metal-sensitive mutant strains and the growth of the resulting transformants was monitored on media with and without metal supplements. As shown in Fig. 4(a), 150 μM CuSO4 inhibited the growth of p424 cup1D cells, whereas the same cells carrying p424-LbMT1 and p424-LbMT2 were able to grow at a rate similar to parental cup1s cells. The yap1
strain transformed with the empty vector (p424) was unable to grow at 40 μM Cd, whereas the yap1 cells expressing either LbMT1 or LbMT2 were able to grow similar to the WT strain BY4741 (Fig. 4b). Further, the restoration of Cd and Cu tolerance by expression of LbMT1 and LbMT2 were confirmed in liquid culture assays (Fig. 4c, d).

**DISCUSSION**

The present study aimed to characterize MTs of the ectomycorrhizal fungus *L. bicolor* and to study the specificity of their induction in an attempt to determine their possible functional role in heavy metal detoxification. Two representative MT genes from *L. bicolor* genome LbMT1 (encoding a ‘short’ peptide) and LbMT2 (encoding a longer one) were selected in this study. The sequence features of *L. bicolor* LbMT1 can be regarded as region-specific traits of known short MTs from basidiomycetes. Indeed, intron distribution, peptide length and the distribution of C-x-C motifs indicate a close relationship of LbMT1 with several MTs from basidiomycetes such as *A. strobiliformis* and *Hebeloma* spp. (Fig. 2). Osobová et al. (2011) suggested that MTs preceded by a stretch of 10–13 amino acids lacking Cys residues could be regarded as a specific feature of MTs from the ectomycorrhizal basidiomycetes. Though LbMT1 qualifies to this trait along with MTs of *A. strobiliformis* and *P. involutus*, this is not true for other MTs of ectomycorrhizal basidiomycetes including LbMT2. Also, the functional importance of this feature, if any, is unknown. LbMT2 contains two domains consisted of three C-x-C motifs as reported in HcMT2 of *H. cylindrosporum* (Ramesh et al., 2009) with rare C-C-C triplet at its N-terminal domain.

Ramesh et al. (2009) showed that HcMT1, one of the MTs of the ectomycorrhizal fungus *H. cylindrosporum* was induced by Cu and Cd, while HcMT2, a second MT gene from the same species responded to Cd alone. In the present study, LbMT2 was induced by both Cu and Cd while LbMT1 responded to Cd alone. These results showed that both the genes responded more strongly to Cu than to Cd. One possible explanation could be that Cd-detoxification also involves other mechanisms such as GSH chelation. Since the concentrations of GSH in ectomycorrhizal fungi are in the millimolar range (Courbot et al., 2004) and GSH readily binds to Cd (Leverrier et al., 2007), it may constitute a first and transient protective barrier against the Cd ions entering the cytoplasm. Although some MTs of plants and animals have been shown to bind Zn (Cobbett & Goldsborough, 2002), there are only few reports of Zn-inducible MTs in fungi (Pagani et al., 2007). In the present study, both LbMTs were not significantly induced in response to different concentrations of Zn. Based on the levels of induction of MTs by different metals, Capdevila & Atrian (2011) proposed the concept of MT evolution, which suggests that functional adaptations of MT genes.
reflect the precise demands of the cells on the expressed levels and metal specificity of a particular MT peptide.

Agents capable of mediating formation of free radicals are also known to induce MT gene transcription (Bauman et al., 1991). For example, a plant type 2 MT was reported to respond to oxidative stress by Mir et al. (2004). González-Guerrero et al. (2007) also reported the role of GintMT1 in the regulation of the redox status of the extraradical mycelium of *Glomus intraradices* through either its metal chelation activity or its thiol groups. LbMT2 and, to a lesser extent, LbMT1 were also induced to some extent by H$_2$O$_2$. This suggests a role for *L. bicolor* MTs in maintaining the local redox balance either by sequestering Cu and preventing potentially deleterious Fenton chemistry reactions (Zhang et al., 1999).

To demonstrate the roles of *LbMT1* and *LbMT2* in metal detoxification, yeast complementation assay was performed in this study. Heterologous complementation assays in yeast demonstrate that *LbMT1* and *LbMT2* encode functional peptides capable of conferring increased tolerance against Cu- and Cd-sensitive yeasts, thereby confirming that *LbMT1* and *LbMT2* peptides may confer defence against metal ions.

In conclusion, the results show that ectomycorrhizal fungus *L. bicolor* encodes different MTs. One of them, *LbMT2*, is highly induced by Cu and may be involved in the specific detoxification of this metal, while *LbMT1* exhibits a more pleiotropic role. Further studies are required for a better understanding of heavy metal tolerance of this fungus and subsequently for the determination of its ability to detoxify and improve heavy metal tolerance of its host plants.

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**REFERENCES**


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