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A novel, highly conserved metallothionein family in basidiomycete fungi and characterization of two representative *SlMTa* and *SlMTb* genes in the ectomycorrhizal fungus *Suillus luteus*

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SUMMARY

The basidiomycete *Suillus luteus* is an important member of the ectomycorrhizal community that thrives in heavy metal polluted soils covered with pioneer pine forests. This study aimed to identify potential heavy metal chelators in *S. luteus.* Two metallothionein (MT) coding genes, *SlMTa* and *SlMTb,* were identified. When heterologously expressed in yeast, both *SlMTa* and *SlMTb* can rescue the Cu sensitive mutant from Cu toxicity. In *S. luteus,* transcription of both *SlMTa* and *SlMTb* is induced by Cu but not Cd nor Zn. Several putative Cu-sensing and metal-response elements are present in the promoter sequences. These results indicate that SlMTa and SlMTb function as Cu-thioneins. Homologs of the *S. luteus* MTs are present in 49 species belonging to ten different orders of the subphylum Agaricomycotina and are remarkably conserved. The length of the proteins, number and distribution of cysteine residues indicate a novel family of fungal MTs. The ubiquitous and highly conserved features of these MTs suggest that they are important for basic cellular functions in species in the subphylum Agaricomycotina.

INTRODUCTION

Metallothioneins (MT) are small, low molecular weight proteins that bind heavy metals, such as Zn, Cu, Cd and Ag. They contain a high content of cysteine residues (20-30 %) that bind the metal ions through clusters of thiolate bonds (Kägi and Schaeffer, 1988; Kägi, 1991; Chen and Russell, 2015). Based on taxonomic criteria and the patterns of distribution of cysteine residues along the sequence, MTs are assigned to one of the 15 MT families proposed by Binz and Kägi (Binz and Kägi, 1999). Alternatively, MTs can be classified according to their Zn- or Cu-binding character ranging from genuine Zn-thioneins, with a clear preference for Zn/Cd binding to extreme Cu-thioneins, preferring Cu/Ag binding (Palacios et al., 2011). Metallothioneins are present in a vast range of taxonomic groups. Almost all groups of organisms from prokaryotes to eukaryotes contain multiple MTs and these proteins may exhibit different metal preferences (Palacios et al., 2011; Capdevila et al., 2012).

Since its discovery in 1954 in horse kidney (Margoshes and Vallee, 1957), many studies have been carried out to define the functions of MTs. As reviewed recently, most studies on MTs were conducted in mammals but also plant MTs are well studied. The main hypothesized functions of MTs are: (1) homeostasis of the essential trace metals Zn and Cu; (2) detoxification of the non-essential metals Cd and Ag; (3) carrier of essential metals to apo-metalloproteins; (4) free radical scavenging and protection against oxidative damage (Capdevila et al., 2012). Their metal specificity, production, and regulation in a variety of tissues are well studied. MTs are not only constitutively expressed, but the production of different types of MTs is stimulated by several endogenous and exogenous agents in both a temporally and spatially regulated manner

(Leszczyszyn et al., 2013). Accordingly, beside metal homeostasis and detoxification, MTs have been linked to a variety of biotic and abiotic stresses (Zhu et al., 2009), but also to embryogenesis, grain development and maturity (Hegelund et al., 2012).

Fungi are ubiquitous in the natural environment and play important roles in decomposition, nutrient cycling and transformation of metals. Until recently, fungal MTs have been characterized in yeasts, a few other ascomycetes (*Neurospora crassa*, *Candida albicans*, *Candida glabrata*, *Yarrowia lipolytica*) and basidiomycetes (*Paxillus involutus*, *Laccaria bicolor*, *Hebeloma* spp., *Russula atropurpurea*, *Cryptoccocus neoformans*). Among those identified, most are Cu-binding MTs (Fogel and Welch, 1982; Munger et al., 1987; Riggle and Kumamoto, 2000; Ding et al., 2011), although Ag (Osobová et al., 2011) and Zn (Leonhardt et al., 2014) binding MTs have also been found. Fungal MTs are involved in a variety of physiological processes, including Cu homeostasis and Cd detoxification (Ramesh et al., 2009), Ag hyperaccumulation (Osobová et al., 2011) and oxidative stress response (Reddy et al., 2014). They were identified as virulence factors of pathogens (Tucker et al., 2004; Ding et al., 2013), they were hypothesized to function as Cu-supplier for lignin degradation pathways of saprotrophs (Iturbe-Espinoza et al., 2016) and they might play a role in the development and functioning of symbiotic interactions (Lanfranco et al., 2002; Bergero et al., 2007; Reddy et al., 2016).

Thanks to the 1000 fungal genomes project (Grigoriev et al., 2011), a very high amount of genomic and transcriptomic data are now available, which greatly facilitates the identification and functional characterization of genes and proteins in fungi. The phylum Basidiomycota contains roughly 30,000 species (about 20,000 of

them belong to the subphylum Agaricomycotina) including many plant and animal pathogens, saprotrophs, and mycorrhizal fungi (Hibbett, 2006). The ectomycorrhizal (ECM) basidiomycete *Suillus luteus* is a common root symbiont of young pine trees. It has been reported to occur at various metal polluted sites in Europe (Colpaert et al., 2011; Op De Beeck et al., 2015). Some mechanisms of metal tolerance in this fungus have been studied before (Colpaert et al., 2000; Colpaert et al., 2005; Ruytinx et al., 2011) but metal chelation via MTs has not yet been reported. In this study, we identified and functionally characterized two novel MT coding genes of *S. luteus*, and searched for their homologs in other basidiomycete genomes.

RESULTS

Identification of *SlMTa* and *SlMTb*

BLASTp using as a query the metallothionein CnMT2 (183 aa) of the human fungal pathogen *Cryptococcus neoformans* H99 (Ding et al., 2011) gave one positive hit with a 40 % identity to a hypothetical protein with ID 802625. By increasing the expected value (to $E = 10^{-4}$) and re-BLASTp in the *S. luteus* genome using the protein with ID 802625 as a query we found its paralog with protein ID 84059. None of the protein sequences were annotated. Both protein sequences contain multiple cysteine residues arranged in CXC or CXXC motifs (in which X is any other amino acids other than cysteine) typical for metallothioneins and therefore are named SlMTa and SlMTb. The respective genes are *SlMTa* and *SlMTb*. Both genes contain three exons and code for 67 and 65 aa, respectively. The proteins show a particular arrangement of CXC and

CXXC motifs and spacers, different from the query sequence (CnMT2) and the 6 previously described fungal metallothionein families (Table 1, and *Supplemental Table S1*).

Homologs of SlMTa and SlMTb in Basidiomycota

Expanding our BLAST searches to 152 sequenced basidiomycetes in the JGI database we found a number of potential homologs of SlMTa and SlMTb. A list of 48 fungal species exhibiting a putative SlMTa and SlMTb homolog is provided in the *Supplemental information,* Table S2. BLAST searches in the NCBI nr protein collection indicate high homology with a recently characterized MT of *Amanita strobiliformis,* AsMT3. Fig. 1 shows 53 sequences that are most likely homologs of SlMTa and SlMTb obtained from 49 fungal species representing ten different orders of the subphylum Agaricomycotina. Among these, one protein AsMT3 of *A. strobiloformis* is functionally characterized as a MT transcriptionally induced by Zn and Cd and with the potential to detoxify Cu, Zn and Cd (Hlozková et al., 2016). Sequence alignment of 18 putative MTs of *S. luteus* and other Boletales was performed separately (*Supplemental figure S1*) to show that these MTs are intensively conserved among species in this order. A comparison of two related *Suillus* species revealed that SlMTa of *S. luteus* and its homolog in *S. brevipes* are different in only one amino acid and that SlMTb and SbMTb are completely identical (Fig. 1). Both sequence alignments show that the putative MTs, with few exceptions, share common features (1) absence of CC or CCC motifs, (2) absence of long spacers typical for plant MTs, (3) length of approximately 60-70 aa, (4) presence of 15-16 cysteine residues, (5) presence of one histidine and absence of other aromatic amino acid.

Seven cysteine-rich boxes can be distinguished and are indicated in Fig. 1 and 2. The abundance (15-16) and arrangement of cysteine residues of SlMT homologues are different from those of known MT families (Table 1; Binz and Kägi, 1999) and MTs previously characterized in Basidiomycetes (Fig. 2 and *Supplemental Table S1*). Neither they are built by repetitive units (of known or unknown families) as is the case for CnMT2 of *Cryptococcus neoformans* (7-cystein segments homologous to *Neurospora crassa* MT of family 8) and TmMT of *Tremella mesenterica*, the longest MT currently characterized (7 blocks of -$CXCX_3CSCPPGXCXCAXCP$-, two fragments of six cysteins and three N-terminal cysteins). Nor they represent a homolog of the single building block of TmMT (*Supplemental Table S1*). In a phylogenetic tree, both SlMT's cluster with AsMT3 and apart from previously characterized and classified fungal MT's. Though, not all nodes are well supported as indicated by low bootstrap values (*Supplemental figure S2*).

A few variations exist in the cysteine-rich motifs among species listed in Fig. 1: four Polyporales species (*Leiotrametes sp*., *Pycnoporus cinnabarinus*, *Pycnoporus sangguineus*, *Trametes ljubarskyi*) have CXXC instead of CXC in cysteine-rich box V (Fig. 1). Three Suilloid species (*S. luteus, S. brevipes, and Rhizopogon salebrosus*) have an additional cysteine residue in the cysteine-rich box I, whereas *Wallemia sebi* (Wallemiomycetes, Basidiomycotina) lacks one cysteine. Other than these 53 sequences, a few putative MTs with more variations were found in the genome of *Cortinarius glaucopus* and *Agaricus bisporus* (order Agaricales), as well as in *Botryobasidium botryosum*, *Tulasnella calospora* (order Cantharellales). We did not find any homologs of SlMTa and SlMTb in other orders of Agaricomycotina, neither

in the other two subphyla of Basidiomycotina: Pucciniomycotina and Ustilaginomycotina. Instead, putative MTs that count more than 70 aa and are homologous to the Cu-thioneins CnMT2 of *Cryptococcus neoformans* or TmMT of *Tremella mesenterica* were found in *Dioszegia cryoxerica* (all Tremellales, Agaricomycotina), *Rhodotorula sp.* (Pucciniomycotina), and *Sporisorium reilianum* (Ustilaginomycotina).

Functional complementation of SlMTa and SlMTb in yeast

To investigate the function of the two putative MTs of *S. luteus*, we carried out complementation experiments in *Saccharomyces cerevisiae* wild type and mutant strains. The Cu sensitive phenotype of the Δ*cup2* yeast mutant was complemented by *SlMTa* and *SlMTb* (Fig. 3A). Overexpression of *SlMTa* slightly improved growth of the Cd sensitive mutants Δ*ycf1* and Δ*yap1* (Fig. 3B and C). Overexpression of neither of the MT genes could restore the growth of the Zn sensitive mutant (Fig. 3D).

Effects of exogenous Cu, Cd and Zn on the expression of *SlMTa* and *SlMTb*

We conducted a quantitative real-time PCR (RT-qPCR) experiment to determine changes in transcription of *SlMTa* and *SlMTb* when *S. luteus* was exposed to sublethal concentrations of Cu, Cd or Zn (Fig. 4). Transcription of *SlMTa* was induced by Cu after three and six hours of exposure. Transcription of *SlMTb* was induced only after six hours of exposure to 500 µM Cu. Exposure to sublethal concentrations of Cd and Zn did not induce significant changes in expression of *SlMTa* and *SlMTb*.

Promoter analysis of *SlMTa* and *SlMTb*

A promoter analysis was performed for *SlMTa* and *SlMTb* of *S. luteus*. Promoter region of *SlMTa* contained different responsive elements including the general stress responsive element (STRE), an antioxidant response element (ARE), metal responsive (yMRE - "y" stands for "yeast" or Ace1 binding site) and copper sensing elements (CuSE) (*Supplemental figure S3*). The promoter region of *MTb* contained another type of metal responsive element (MRE), i.e. a binding site for MTF1 (metal responsive element-binding TF1). In addition, several CuSE-like elements (denoted as CuSE*), which differed from CuSE by one nucleotide outside the core region GCTG, were found in the promoter region of both genes (*Supplemental figure S3*).

DISCUSSION

The present study aimed to identify MTs of the fungus *S. luteus,* an ECM symbiont known for its heavy metal tolerance and its ability in protecting young pine trees from different metal stresses (Adriaensen et al., 2005; Krznaric et al., 2009; Colpaert et al., 2011). Identification of two novel MTs, SlMTa and SlMTb, in *S. luteus* lead to the discovery of ubiquitous, highly conserved homologs in other fungi in the subphylum Agaricomycotina. These two *S. luteus* genes were characterized and their ability to complement the Cu sensitivity of the Δ*cup2* mutant of *S. cerevisiae* as well as their transcriptional response to Cu indicate that they are Cu-thioneins and play a role in Cu homeostasis.

Up to present, 15 MT families are classified (Binz and Kägi, 1999) showing the high heterogeneity in length and primary structure of the MT sequences. An extra MT family composed of environmental cysteine-rich proteins of unknown taxonomic origin and showing unique features was recently described (Ziller et al., 2017). In addition to the six fungal MT families (families 8 to 13) in the current classification, several functionally studied fungal MTs are not (yet) classified. Identification and classification of MTs is generally difficult with regular BLASTp because of this heterogeneity as well as the short sequence nature of the proteins. So far the longest MT known is found in fungal basidiomycetes: TmMT (257 aa) of *Tremella mesenterica*. In addition, the CnMT1 (122 aa) and CnMT2 (183 aa) of the pathogenic fungus *C. neoformans* were identified and characterized in many details (Ding et al., 2011; Ding et al., 2013). In Agaricomycotina, a number of MT coding genes have been characterized (Fig. 2). Examples in Boletales include PiMT1 of *Paxillus*

involutus (Bellion et al., 2007) and PaMT1 of *Pisolithus albus* (Reddy et al., 2016). In Agaricales, *HcMT1* and *HcMT2* of *Hebeloma cylindrosporum* (Ramesh et al., 2009), *HmMT1-3* of *H. mesophaeum* (Sacky et al., 2014), *LbMT1* and *LbMT2* of *Laccaria bicolor* (Reddy et al., 2014) and *AsMT1-3* of *Amanita strobiliformis* (Osobova et al., 2011; Hlozkova et al., 2016) were characterized. Homologs of these MTs have not been found in *S. luteus*, except for AsMT3, which is homologous to SlMTa/SlMTb. In the genome of *H. cylindrosporum*, *P. involutus* and *L. bicolor*, ECM fungi in which several other MTs are already discovered, at least one homolog of SlMTa/SlMTb is detected (Fig. 1). The presence of different types of MTs indicates that these proteins might take part in different cellular processes in fungi. Here we would like to highlight the importance of this group of MTs because of their sequence conservation across species and omnipresence in the mushroom-forming fungi -- class Agaricomycetes, subphylum Agaricomycotina. It is also interesting to find a homolog of SlMTa/SlMTb in the order Wallemiales (the earliest diverging lineage of Agaricomycotina) and not in some other orders (Auriculariales, Sebacinales, Tremellales, Dacrymycetales, Filobasidiales). Wallemia is known for its ability to tolerate harsh environments, especially osmotic stress (Padamsee et al., 2012). Beside the high number of transporters present in its genome that are assumed to be involved in its xero-tolerance, it is possible that this MT also plays a role in metal ion homeostasis and osmotic stress tolerance of Wallemia.

The analysis of the SlMTa and SlMTb protein sequences revealed unique features when compared to the other known MTs. The distribution of cysteine residues clearly indicates that SlMTa and SlMTb do not belong to any of the MT families classified

previously (Binz and Kägi, 1999). The length of the SlMTa and SlMTb protein sequences and the distribution of cysteine residues are also different from CnMT1 and CnMT2 that were used as queries in our search. In addition to AsMT3 and 19 sequences (including *S. luteus* SlMTb) identified recently by Hlozkova et al. (2016), we identified 32 putative MTs homologous to SlMTa in fungi of the subphylum Agaricomycotina. Comparison of the primary structure of the MT homologs revealed that these proteins are very well conserved, all showing a length of 60-70 aa, seven conserved cysteine-rich boxes and one conserved histidine (Fig.1). We conclude that these MTs are ubiquitous in agaricomycetes and form a novel MT family. A neighbour joining tree of previously characterized and classified fungal MT's supports the classification of the SlMT's and AsMT3 in a novel MT family (*Supplemental figure S2*). Though also MT's of other families need to be inventoried and characterized within the agaricomycetes to infer a highly supported phylogenetic tree and to understand evolution and diversification of MT's within this taxonomic group.

The complementation assay using the yeast *S. cerevisiae* metal sensitive mutants has been successfully used in characterizing a number of MTs in fungi (Bellion et al., 2007; Ding et al., 2011; Osobová et al., 2011). In this study, we could confirm the roles of *SlMTa* and *SlMTb* in Cu detoxification within the yeast system. Accordingly, exogenous Cu but no other metals at the tested concentrations induced the expression of the genes in *S. luteus*. In the yeast complementation assay, both *SlMTa* and *SlMTb* were under the control of a GAL1-inducible promoter; however, we found that Δ*cup2* transformants expressing *SlMTa* grew better those expressing *SlMTb* on all Cu concentrations tested. Transformants expressing *SlMTa* also slightly improved growth

253 of Cd sensitive Δ*ycf1* and Δ*yap1* mutants but not completely restored their growth on
254 high Cd concentrations. These results suggest that there exist differences in the metal-
255 binding abilities of SlMTa and SlMTb proteins. It is widely accepted that cysteine
256 residues are responsible for metal binding ability of MT's. Therefore, one would
257 expect that MTs showing the same amount and distribution of cysteines are showing
258 the same metal-binding abilities, energetically favouring the same metal-thiolate
259 clusters. Within one species, differentiation in metal specificity of MT isoforms
260 exhibiting the same amount of perfectly conserved cysteine residues was reported
261 previously (Perez-Rafael et al., 2014). Cysteine residues are not the unique
262 determinants of metal-binding abilities, histidine residues can act as ligands in
263 metalloproteins and other small differences in primary MT structure could influence
264 protein folding, 3D structure and stability of the particular metal-MT complex
265 (holoprotein). Participation of chloride ions in the stabilisation of metal-MT
266 complexes has been reported as well (Palacios et al., 2011). The prediction of the
267 physiological function of MTs based on primary protein sequence is therefore difficult.
268 Subtle changes in primary protein sequence, even apart from cysteine residues can
269 result in altered metal binding properties and distinct physiological functions. AsMT3
270 of *A. strobiliformis*, a homologue of SlMTa and SlMTb was characterized recently as
271 a MT with Cu, Zn and Cd binding potential (Hlozkova et al., 2016). AsMT3 exhibits
272 exactly the same cysteine and histidine pattern as SlMTb, though both proteins differ
273 in 27 aa (Fig. 2) and analysis of Zn and Cd tolerance of AsMT3 overexpression yeast
274 mutants by Hlozkova et al. was done in the presence of excess Cl (exposed to $ZnCl_2$
275 and $CdCl_2$). In vivo metal binding potential of additional MTs of this novel family is

required to link primary protein sequence to function and to understand functional diversification and molecular evolution of these ubiquitous and well conserved MT's in Agaricomycotina.

It has been reported that the expression of MT genes is induced by metals and oxidative stress (Palmiter, 1994; Andrews, 2000; Ruttkay-Nedecky et al., 2013). In the basidiomycete *C. neoformans*, transcription factor (TF) Cuf1 is essential for activation of MT genes in response to excess Cu (Ding et al., 2011) and promoter regions of both MT genes of *C. neoformans* contain several CuSE-like motifs. Other than the Cuf1 of *C. neoformans*, there is yet any information on the participation of TFs in regulation of MT genes in basidiomycete fungi. However, potential TF binding sites (MRE, CuSE, STRE and MRE) have been found in the promoter regions of several basidiomycete MT genes (Ramesh et al., 2009; Ding et al., 2011; Eastwood et al., 2011). Likewise, promoter analysis of *SlMTa* and *SlMTb* shows the presence (co-existence) of different putative response elements (*Supplemental figure S3*); this indicates the complexity of *SlMTa* and *SlMTb* regulation. Transcription of both SlMT genes might be influenced by general stress related factors that bind to STRE and ARE, elements that are found in several copies in their promoter regions. Yet, despite the similarity in protein sequences, *SlMTa* and *SlMTb* might be regulated differently. Here we could only show that *SlMTa* transcription is more sensitive to Cu since it responded to Cu exposure earlier and at lower external concentrations than *SlMTb* (Fig. 4). Regulation of the expression of the two *SlMT* genes might be more differential in other conditions (for example, metal exposure time and doses, growth condition and developmental stages of the fungi, …). In *Saccharomyces cerevisiae*,

the TF Ace1 is responsible for the Cu-dependent transcription of target genes (containing a yMRE in their promoter sequence). Homologs of TF Ace1 in the basidiomycete saprotrophs *Phanerochaete chrysoporium* (Polanco et al., 2006; Canessa et al., 2008) and *Ceriporiopsis subvermispora* (Álvarez et al., 2009) have been characterized. These TFs respond to Cu and are activators of several multicopper oxidases (laccases) in the two Polyporales species. Putative Ace1 binding sites were detected in *SlMTa* promoter sequence and might explain its Cu sensitivity. On the other hand, the MTF1 in higher eukaryotes is the main activator of MT genes but MTF1 not only responds to Cu, Cd, Zn but also to hypoxia and oxidative stress (Günther et al., 2012). Though we could not detect any transcriptional response of *SlMTb*, which is proceeded by multiple putative MTF1 binding sites (i.e. MRE sites), on Cd nor Zn. In *A. strobiliformis*, a metal accumulating species with a characterized *SlMTa/b* homolog, two (*AsMT1/2*) out of three MT genes contain putative Ace1 binding sites, transcriptionally respond to excess Cu and have the potential to detoxify Cu. The third MT gene (*AsMT3*) homologous to *SlMTa/b* does not contain putative Ace1 binding sites, does not respond to excess Cu but to Cd/Zn and has the potential to detoxify Cu, Cd and Zn (Hlzokova et al., 2016). The presence in the genome of two other CuMTs (*AsMT1/2*) might have allowed the evolution of *AsMT3* towards a MT with Zn/Cd binding potential in this metal accumulating ECM species. However, characterization of additional homologs within this novel MT family is required to infer ancestral state and to comprehend evolution and functional diversification of MT genes in Agaricomycotina.

The subphylum Agaricomycotina contains about one-third of the described basidiomycete species and accommodates a diverse array of fungi, in size, lifestyle (unicellular yeasts, jelly fungi to mushroom-forming fungi) and ecology (wood-rots, litter decomposers, ectomycorrhizal fungi and a few pathogens) (Hibbett, 2006). The broad species distribution of the novel MTs indicates their contribution to fundamental and conserved cellular process(es) amongst those Cu homeostasis and detoxification are likely included. It is also noteworthy that Cu-containing fungicides and wood preservatives are commonly used all over the world. Extensive use of these compounds can be a threat for the environment in particular for microbial communities. However, there is some evidence that particular wood-rot fungi became tolerant to the metal-containing preservatives (Baldrian, 2003; Green Iii and Clausen, 2003; Hastrup et al., 2005; Guillen et al., 2009). Also ECM fungi from Cu-polluted soils may develop Cu resistance and such ecotypes may be good candidates for bioremediation of Cu-polluted areas (Adriaensen et al., 2005; Colpaert et al., 2011; Silva et al., 2013). Therefore, understanding how these fungi cope with excess Cu and heavy metals in general will help to develop new technologies for the control and efficient use of these fungi in the future.

EXPERIMENTAL PROCEDURES

Fungal strains and culture medium

A *S. luteus* monokaryotic isolate (UH-Slu-Lm8-n1) obtained from a basidiospore released by a sporocarp collected from a heavy metal polluted site in Lommel,

343 Belgium was used in this study. The genome of the strain was sequenced and can be
344 consulted through the *S. luteus* genome portal of the Functional Genomics Program of
345 the Department of Energy Joint Genome Institute (JGI) (http://genome.jgi-
346 psf.org/Suilu1/Suilu1.home.html) (Grigoriev et al., 2012; Kohler et al., 2015). The
347 fungus is maintained on solid modified Fries medium (28 mM glucose, 5.4 mM
348 ammonium tartrate, 1.5 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 5 µM $CuSO_4 \cdot 5H_2O$, 20
349 µM $ZnSO_4 \cdot 7H_2O$, 0.1 µM biotin, 0.5 µM pyridoxine, 0.3 µM riboflavin, 0.8 µM
350 nicotinamide, 0.7 µM p-aminobenzoic acid, 0.3 µM thiamine, 0.2 µM Ca-
351 pantothenate and 0.8 % agar; pH-adjusted to 4.8).

352 **Identification of *SlMTa* and *SlMTb***

353 To identify genes encoding for MTs in *S. luteus*, BLASTp, tBLASTn and BLASTn
354 was performed at the JGI genome portal. Protein sequences of known MTs of
355 different organisms and their corresponding coding sequences were used as queries
356 (*Supplemental table S3)* Sequence alignment and construction of a Neighbor joining
357 tree were performed with the CLC main workbench 7.7.3 (http://www.clcbio.com).

358 **Cloning of *SlMTa* and *SlMTb* genes**

359 A cDNA library was made using the SMARTer PCR cDNA synthesis kit (Clonetech,
360 US) following the manufacturer's instructions. Specific primers were designed to
361 amplify full-length coding sequences of *SlMTa* (F:
362 ACAAAAACCATAATGGCGACCTGCAG; R:
363 TCACTTTGACTCGCAGGTACATGCTAGA), *SlMTb* (F:
364 GCGCTCTGCATCAACATGGCTAAAGAC; R:

CTACTTCGTTGCGCAACTGCACGCCTGC). PCR reactions were performed using the Advantage 2 DNA polymerase mix (Clontech, US) following the manufacturer's instructions. Amplicons were separated by electrophoresis and bands of approximately 200 base pairs (bp) were purified using Qiaquick Gel extraction Kit (Qiagen, France). The purified PCR-products were cloned into the Gateway entry vector pCR8/GW/TOPO (Life technologies, Paisley, UK) and subsequently transferred by LR-Clonase into the yeast expression vectors pAG426GAL-ccdB (Alberti et al., 2007) for functional complementation tests. Bacterial transformations followed standard heat shock protocol into chemically competent TOP10 *E. coli* (Life Technologies, Paisley, UK) with cells being plated onto Luria-Bertani agar plates containing the appropriate selecting antibiotic. The inserts were sequenced in both directions to assure correct fusion.

The yeast strains used for heterologous expression of *SlMTa* and *SlMTb* were BY4741 (MAT a; his3Δ1; leu2Δ; met15Δ0; ura3Δ0), Δ*zrc1* (BY4741; MAT a; his3Δ1; leu2Δ; met15Δ0; ura3Δ0; YMR243c::kanMX4), Δ*ycf1* (BY4741; MAT a; his3Δ1; leu2Δ; met15Δ0; ura3Δ0; YDR135c::kanMX4), Δ*yap1* (BY4741; MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YML007w::kanMX4) and Δ*cup2* (BY4741; MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL166w::kanMX4) obtained from Euroscarf (EUROSCARF, Frankfurt, Germany, http: www.uni-frankfurt.de/fb15/mikro/euroscarf). Yeast cells were transformed using the LiAC/PEG method as previously described (Gietz and Schiestl, 2007). Transformed yeast mutants and wild type were selected on agar plates containing SD medium without uracil (1.7 g/L of yeast nitrogen base (Difco, BD, US), 5 g/L $(NH_4)_2SO_4$, 2 % (w/v) D-glucose or

388 galactose, 0.77 g/L CSM-URA, 2 % agar in case of solid medium, pH 5.6-5.8). Plates
389 were incubated at 30 °C for five days before imaging.

390 For the drop test, one yeast colony was grown in liquid SD medium to mid log phase
391 (OD_{600nm} between 1-1.5). Cells were collected by centrifugation and re-suspended in
392 sterile distilled water and adjusted to OD_{600nm} = 1. Subsequently a 1/10 dilution series
393 was prepared and 10 µl of each dilution was plated out on agar plates that contained
394 SD medium with 2 % galactose (to initiate gene expression) and metals as indicated.
395 The drop test was repeated three times using different yeast colonies.

396 **Cultivation of *S. luteus* for metal treatments and gene expression assay**

397 *S. luteus* inocula of 0.5 cm² were initially grown for eight days on cellophane-covered
398 solid Fries medium. One gram fresh weight of mycelium was subsequently collected,
399 blended aseptically with a kitchen mixer and transferred to 150 ml of Fries liquid
400 medium without agar. The cultures were incubated at 23 °C on a shaker (120 rpm) for
401 eight days. In order to obtain regular growth and uniform fungal spheres half of the
402 medium was replaced every two days with fresh medium. Fresh mycelial spheres
403 (approximately 100 mg fresh weight) were transferred to Petri dishes containing 10 ml
404 of liquid Fries medium and were grown further for 24 h with shaking (120 rpm).
405 Metals were added as sulphates ($CuSO_4 \cdot 5H_2O$, $3CdSO_4 \cdot 8H_2O$ and $ZnSO_4 \cdot 7H_2O$) to
406 the sphere cultures to obtain final concentrations: 0, 20, and 40 µM Cd; 0, 100, 500
407 µM Cu or 0, 0.5, or 1 mM Zn. The cultures were placed at 23 °C on a shaker
408 incubator (70 rpm). After three and six hours, the spheres were collected in four

409 replicates, immediately frozen in liquid nitrogen and stored at -80 °C until RNA
410 extraction.

411 Total RNA was extracted using the RNeasy Plant Kit (Qiagen, France), according to
412 the manufacturer's instructions. DNase treatment with the TURBO DNA-free™ Kit
413 (Ambion, Life Technologies, Paisley, UK) was performed to eliminate possible
414 genomic DNA contamination. RNA concentration and purity was evaluated
415 spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, Wilmington,
416 DE, USA). One μg of the treated RNA per sample was converted to single stranded
417 cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied
418 Biosystems, Life Technologies, Paisley, UK) according to the manufacturer's
419 instructions. A 5-fold dilution of the cDNA was prepared in 1/10 diluted TE buffer (1
420 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0; Sigma–Aldrich, Belgium) and stored at
421 −20°C.
422 Quantitative real-time PCR was performed in a 96-well optical plate with the ABI
423 PRISM 7500 Fast Real-Time PCR System (Life Technologies, Paisley, UK) using
424 SYBR Green chemistry, fast cycling conditions (20 s at 95°C, 40 cycles of 1 s at 95°C
425 and 20 s at 60°C) and followed by the generation of a dissociation curve to verify
426 amplification specificity. Reactions contained 2.5 μL diluted cDNA template (or
427 RNase-free water for the 'no template controls'), 5 μL 2x Fast SYBR® Green Master
428 Mix (Life Technologies, Paisley, UK), forward and reverse primers (300 nM each)
429 and 1.9 μL RNase-free water in a total volume of 10 μL. Gene-specific forward and
430 reverse primers were designed via the Primer-BLAST (Ye et al., 2012). All primer
431 pairs were evaluated for specificity using the dissociation curve and primer efficiency

was evaluated before use as recommended in the 7500 Fast Real-Time PCR System manual (Life Technologies, Paisley, UK). Reference gene primers were described and evaluated previously by Ruytinx et al. (2016).

All primer sequences are provided in the *Supporting information* (Table S4) with JGI protein ID or GenBank accession number deposited previously (Ruytinx et al., 2011). Gene expression was calculated relative to the sample with the highest expression (relative expression = $2^{-(Cq(sample)-Cq(min)}$), normalized to four reference genes using a normalization factor (geometric mean of relative expression levels of the reference genes, Vandesompele et al., 2002) and rescaled to the non-exposed control (fold changes). The reference genes TUB1, GR75621, AM085168 and AM085168 were selected (out of 10 candidates) and their stability of expression was validated for individual experimental set-ups according to Ruytinx et al., 2016 (*Supporting information, Table S5*). Mean values of four biological repeats were calculated, and error bars represent the standard error of the means. Data were analyzed statistically using the one-way ANOVA procedure and Dunnett's test was used to compare different treatments with a control. Transformations were applied when necessary to approximate normal distribution of the data.

Promoter analysis of *SlMTa* and *SlMTb* genes

Upstream DNA sequences, approximately 1500 bp from the start codon, of *MTa* and *MTb* of *S. luteus* were retrieved from the JGI genome database. Putative transcription factor binding sites were searched manually using their consensus sequences as follow: ARE (TGACNNNGC), STRE (CCCCT), CuSE (DDDHGCTGD), CuSE*

(DDHGCTGD), yMRE (HTHNNGCTGD), MRE (TGCRCNC), in which D = A, G or T; H = A, C or T; N = any nucleotides; R = A or G.

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The authors declare no conflicts of interest.

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647

LEGENDS

Table 1. Different families of the Binz and Kägi MT classification that contain fungal MTs. Conserved cysteines and histidines are indicated in an example sequence for each family, SlMTa and SlMTb.

Fig. 1. Sequence alignment of 53 putative MTs found in basidiomycete fungi. Sequences were retrieved from the JGI genome database and JGI protein IDs are provided. The three largest orders Agaricales, Boletales, Polyporales are indicated in red, blue, and green, respectively. The other orders are indicated in black. Protein sequence alignment was performed with CLC main work-bench 7.0.2 (http://www.clcbio.com). Seven cysteine-rich boxes are indicated (I to VII).

Fig. 2. SlMTa, SlMTb and previously functional characterized MTs of basidiomycete fungi. Sequences were retrieved from NCBI protein database and can be divided in three groups based on their length, number and position of cysteine-rich boxes (underlined) and conserved histidines (boxed).

Fig. 3. Functional complementation of *S. cerevisiae* mutants on selective media. Yeast mutant strains were transformed with the empty vector (EV) pAG426GAL or with vector containing coding sequence of *SlMTa* and *SlMTb*. Wild-type strain BY4741 (WT) was transformed with EV as a control. Yeast cultures were adjusted to OD = 1.0, and 10 µl of serial dilutions were spotted on SD medium with 2 % galactose and supplemented with Cu, Cd or Zn as indicated. Plates were incubated for five days at 30 °C.

669 **Fig. 4.** Relative expression of (a) *SlMTa* and (b) *SlMTb* in a *S. luteus* monokaryon
670 (UH-Slu-Lm8-n1) exposed to excess Cu, Cd or Zn. Treatments were started by
671 adding the metals to the medium (Cu: 100, 500 µM, Cd: 20, 40 µM, or Zn: 0.5, 1
672 mM) and incubated for three (■) and six (■) hours. Gene expression was measured
673 by RT-qPCR and presented as fold changes (metal-exposed relative to non-exposed
674 control). Data are represented as means ± SE of four biological replicates. Statistics
675 were performed separately for each metal and time point. Dunnett's comparison
676 was performed to test for significant difference of each treatment with the non-
677 treated control; (*) indicate significant difference at $p<0.05$.

678 **Table S1.** Protein sequences of previously characterized CnMT1, CnMT2 and
679 TmMT1. Conserved cysteines are indicated in bold.

680 **Table S2.** List of 48 fungal species used in the study. Species are listed in alphabetical
681 order and hyperlink to the MycoCosm genome portal of the Functional Genomics
682 Program of the Department of Energy Joint Genome Institute (http://genome.jgi-
683 psf.org/programs/fungi/index.jsf).

684 **Table S3.** List of previously characterized metallothioneins and their protein
685 sequences used as blast queries in this study.

686 **Table S4.** Primer sequences used in this study.

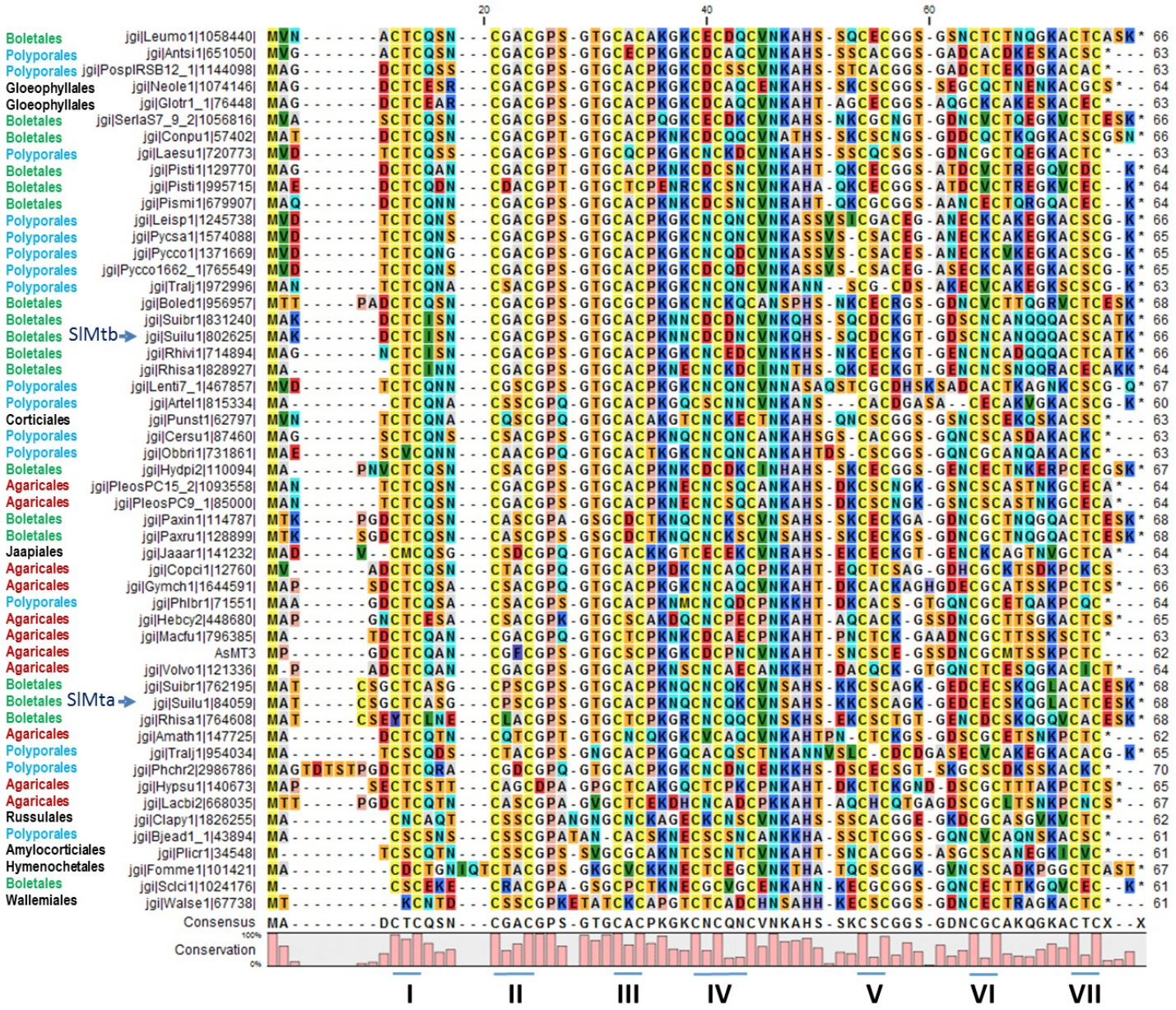
687 **Table S5.** Ranking and expression stability values of selected candidate reference
688 genes for the different experimental set-ups included in this study as calculated by
689 geNorm.

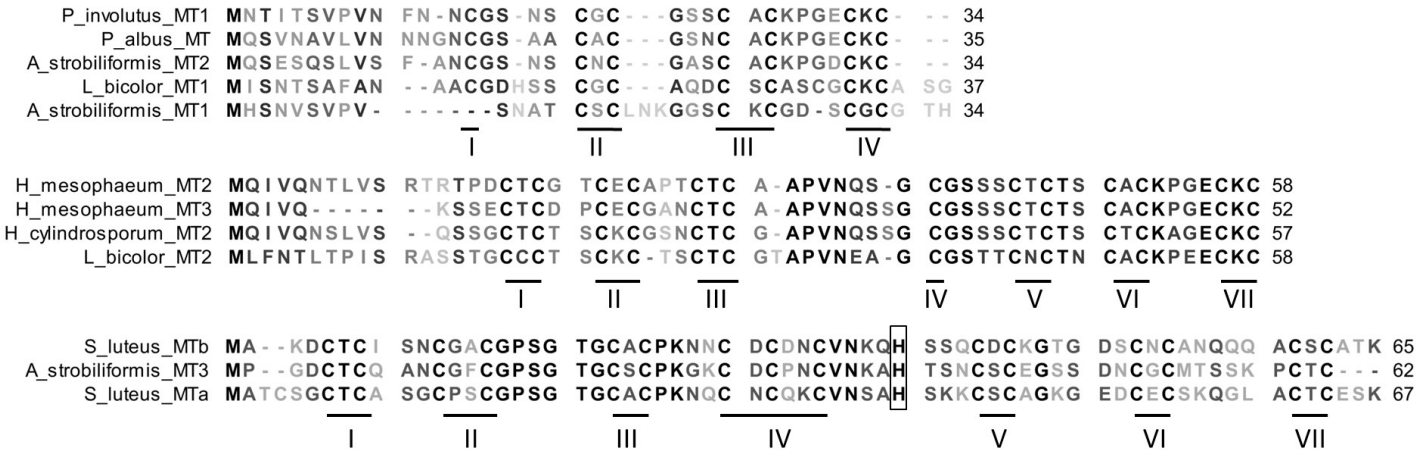
690 **Table S6.** Data-set containing Cq values determined by qPCR for different genes in
691 different samples.

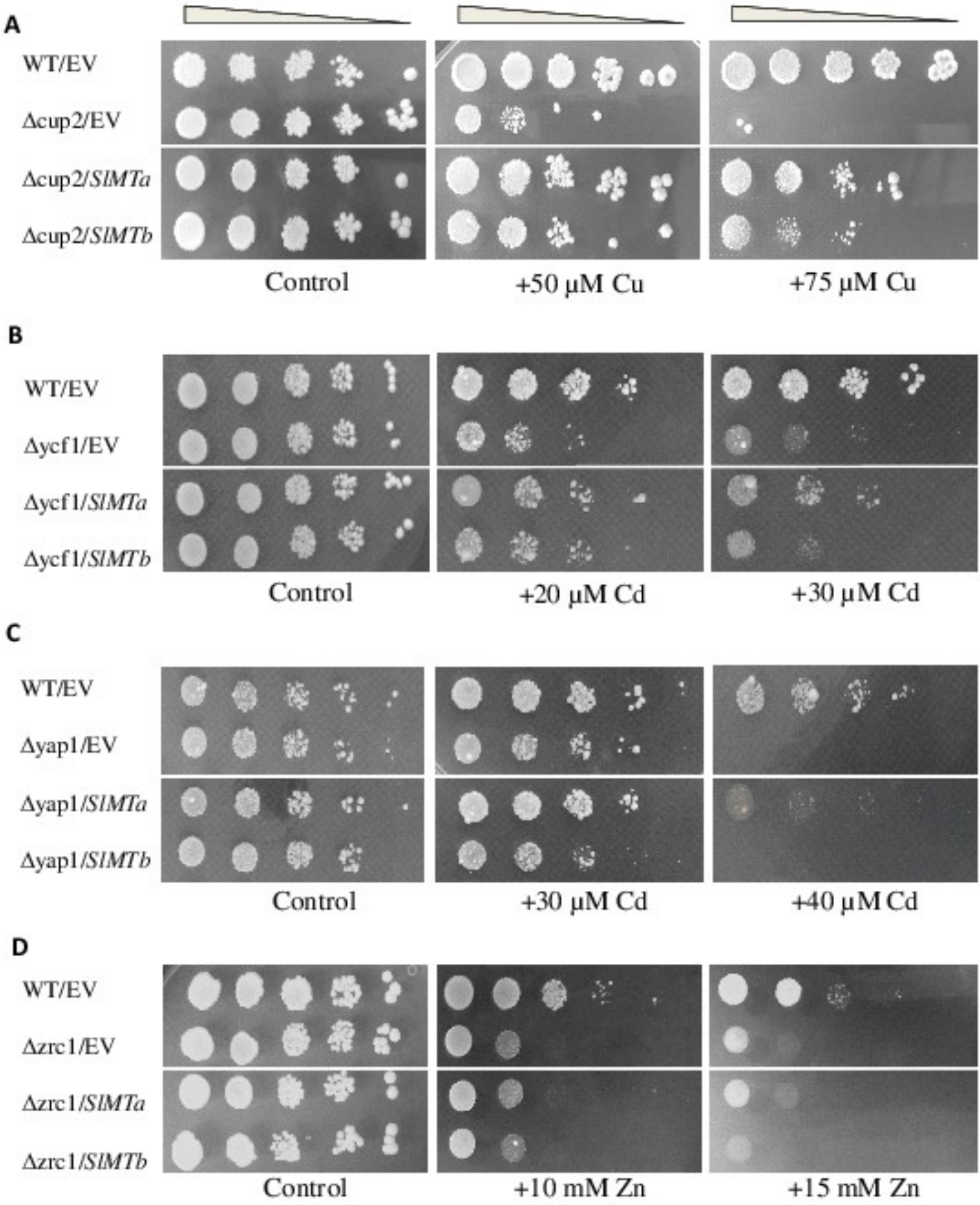
692 **Figure S1.** Sequence alignment of 18 putative MTs found in 14 species belonging to
693 the order Boletales. Sequences were retrieved from the JGI genome database and
694 JGI protein IDs are provided. Seven cysteine-rich boxes are indicated (I to VII).

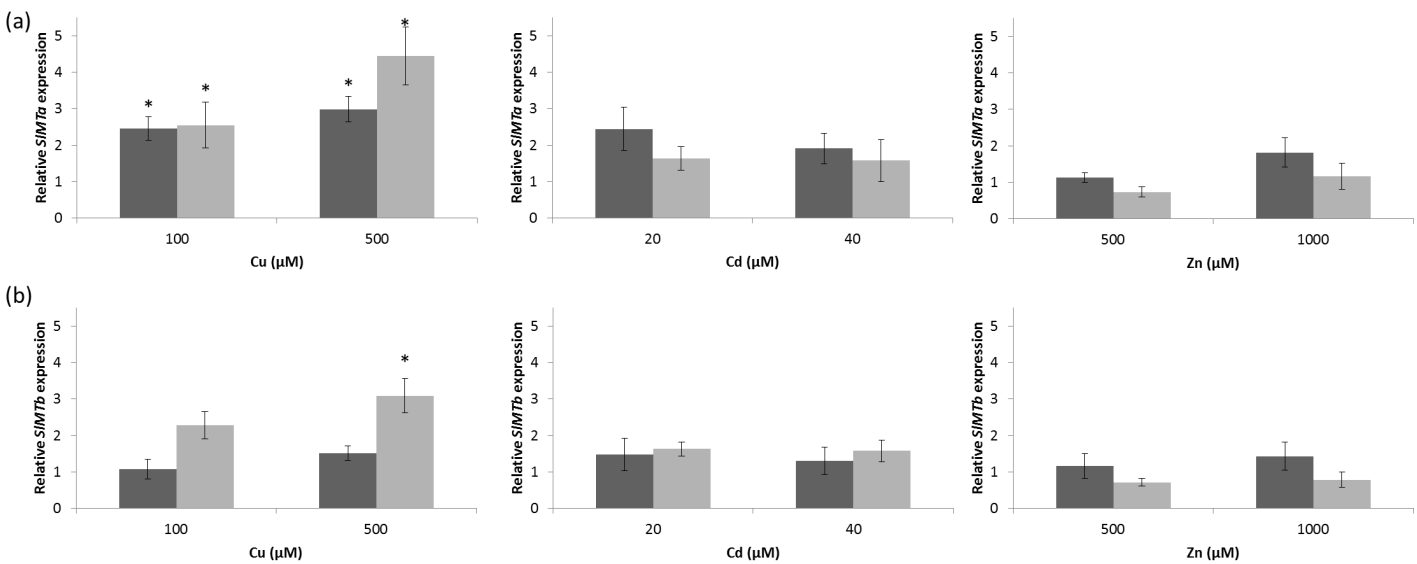
695 **Figure S2.** An unrooted Neighbor-Joining-based phylogenetic tree (Jukes-Cantor
696 protein distance measure) generated by CLC main workbench after sequence
697 alignment. *S. luteus* SlMTa, SlMTb and their homolog AsMT3 of *A. strobiliformis*
698 cluster together and apart of other previously characterized or classified MT's.
699 Bootstrap values (%; 10 000 replicates) are indicated. Branch lengths are
700 proportional to phylogenetic distance.

701 **Figure S3.** Promoter regions of approximately 1500 bp upstream of the translation
702 start codon of (A) *SlMTa* and (B) *SlMTb*. Predicted TF binding sites are indicated:
703 ARE (TGACNNNGC), STRE (CCCCT), CuSE (DDDHGCTGD), CuSE*
704 (DDHGCTGD), yMRE (HTHNNGCTGD), MRE (TGCRCNC).


20
40
60
Boletales jgi|Leumo1|1058440|
Polyporales jgi|Antsi1|651050|
Polyporales jgi|PospIRSB12_1|1144098|
Gloeophyllales jgi|Neole1|1074146|
Gloeophyllales jgi|Glotr1_1|76448|
Boletales jgi|SerlaS7_9_2|1056816|
Boletales jgi|Conpu1|57402|
Polyporales jgi|Laesu1|720773|
Boletales jgi|Pisti1|129770|
Boletales jgi|Pisti1|995715|
Boletales jgi|Pismi1|679907|
Polyporales jgi|Leisp1|1245738|
Polyporales jgi|Pycsa1|1574088|
Polyporales jgi|Pycco1|1371669|
Polyporales jgi|Pycco1662_1|765549|
Polyporales jgi|Tralj1|972996|
Boletales jgi|Boled1|956957|
Boletales jgi|Suibr1|831240|
SlMtb
Boletales jgi|Suilu1|802625|
Boletales jgi|Rhivi1|714894|
Boletales jgi|Rhisa1|828927|
Polyporales jgi|Lenti7_1|467857|
Polyporales jgi|Artel1|815334|
Corticiales jgi|Punst1|62797|
Polyporales jgi|Cersu1|87460|
Polyporales jgi|Obbri1|731861|
Boletales jgi|Hydpi2|110094|
Agaricales jgi|PleosPC15_2|1093558|
Agaricales jgi|PleosPC9_1|85000|
Boletales jgi|Paxin1|114787|
Boletales jgi|Paxru1|128899|
Jaapiales jgi|Jaaar1|141232|
Agaricales jgi|Copci1|12760|
Agaricales jgi|Gymch1|1644591|
Polyporales jgi|Phlbr1|71551|
Agaricales jgi|Hebcy2|448680|
Agaricales jgi|Macfu1|796385|
Agaricales AsMT3
Agaricales jgi|Volvo1|121336|
Boletales jgi|Suibr1|762195|
SlMta
Boletales jgi|Suilu1|84059|
Boletales jgi|Rhisa1|764608|
Agaricales jgi|Amath1|147725|
Polyporales jgi|Tralj1|954034|
Polyporales jgi|Phchr2|2986786|
Agaricales jgi|Hypsu1|140673|
Agaricales jgi|Lacbi2|668035|
Russulales jgi|Clapy1|1826255|
Polyporales jgi|Bjead1_1|43894|
Amylocorticiales jgi|Plicr1|34548|
Hymenochetales jgi|Fomme1|101421|
Boletales jgi|Sclci1|1024176|
Wallemiales jgi|Walse1|67738|
Consensus
Conservation
100%
0%
I
II
III
IV
V
VI
VII


P_involutus_MT1 MNTITSVPVN FN-NCGS-NS CGC---GSSC ACKPGECKC- -- 34
P_albus_MT MQSVNAVLVN NNGNCGS-AA CAC---GSNC ACKPGECKC- -- 35
A_strobiliformis_MT2 MQSESQSLVS F-ANCGS-NS CNC---GASC ACKPGDCKC- -- 34
L_bicolor_MT1 MISNTSAFAN --AACGDHSS CGC---AQDC SCASCGCKCA SG 37
A_strobiliformis_MT1 MHSNVSVPV- ------SNAT CSCLNKGGSC KCGD-SCGCG TH 34
I II III IV
H_mesophaeum_MT2 MQIVQNTLVS RTRTPDCTCG TCECAPTCTC A-APVNQS-G CGSSSCTCTS CACKPGECKC 58
H_mesophaeum_MT3 MQIVQ----- --KSSECTCD PCECGANCTC A-APVNQSSG CGSSSCTCTS CACKPGECKC 52
H_cylindrosporum_MT2 MQIVQNSLVS --QSSGCTCT SCKCGSNCTC G-APVNQSSG CGSSSCTCTS CTCKAGECKC 57
L_bicolor_MT2 MLFNTLTPIS RASSTGCCCT SCKC-TSCTC GTAPVNEA-G CGSTTCNCTN CACKPEECKC 58
I II III IV V VI VII
S_luteus_MTb MA--KDCTCI SNCGACGPSG TGCACPKNNC DCDNCVNKQH SSQCDCKGTG DSCNCANQQQ ACSCATK 65
A_strobiliformis_MT3 MP--GDCTCQ ANCGFCGPSG TGCSCPKGKC DCPNCVNKAH TSNCSCEGSS DNCGCMTSSK PCTC--- 62
S_luteus_MTa MATCSGCTCA SGCPSCGPSG TGCACPKNQC NCQKCVNSAH SKKCSCAGKG EDCECSKQGL ACTCESK 67
I II III IV V VI VII


A
WT/EV
Δcup2/EV
Δcup2/SlMTa
Δcup2/SlMTb
Control
+50 μM Cu
+75 μM Cu
B
WT/EV
Δycf1/EV
Δycf1/SlMTa
Δycf1/SlMTb
Control
+20 μM Cd
+30 μM Cd
C
WT/EV
Δyap1/EV
Δyap1/SlMTa
Δyap1/SlMTb
Control
+30 μM Cd
+40 μM Cd
D
WT/EV
Δzrc1/EV
Δzrc1/SlMTa
Δzrc1/SlMTb
Control
+10 mM Zn
+15 mM Zn


(a)
Relative SlMTa expression
0
1
2
3
4
5
*
*
*
*
100
500
Cu (μM)
20
40
Cd (μM)
500
1000
Zn (μM)
(b)
Relative SlMTb expression
*
100
500
Cu (μM)
20
40
Cd (μM)
500
1000
Zn (μM)