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Title:

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

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.

39 INTRODUCTION

40 Metallothioneins (MT) are small, low molecular weight proteins that bind heavy
41 metals, such as Zn, Cu, Cd and Ag. They contain a high content of cysteine residues
42 (20-30 %) that bind the metal ions through clusters of thiolate bonds (Kägi and
43 Schaeffer, 1988; Kägi, 1991; Chen and Russell, 2015). Based on taxonomic criteria
44 and the patterns of distribution of cysteine residues along the sequence, MTs are
45 assigned to one of the 15 MT families proposed by Binz and Kägi (Binz and Kägi,
46 1999). Alternatively, MTs can be classified according to their Zn- or Cu-binding
47 character ranging from genuine Zn-thioneins, with a clear preference for Zn/Cd
48 binding to extreme Cu-thioneins, preferring Cu/Ag binding (Palacios et al., 2011).
49 Metallothioneins are present in a vast range of taxonomic groups. Almost all groups of
50 organisms from prokaryotes to eukaryotes contain multiple MTs and these proteins
51 may exhibit different metal preferences (Palacios et al., 2011; Capdevila et al., 2012).

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

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 *SIMTa* and *SIMTb*. The respective genes are *SIMTa* and *SIMTb*. 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 previously described fungal metallothionein families (Table 1, and *Supplemental Table S1*).

Homologs of SIMTa and SIMTb in Basidiomycota

Expanding our BLAST searches to 152 sequenced basidiomycetes in the JGI database we found a number of potential homologs of SIMTa and SIMTb. A list of 48 fungal species exhibiting a putative SIMTa and SIMTb 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 SIMTa and SIMTb obtained from 49 fungal species representing ten different orders of the subphylum Agaricomycotina. Among these, one protein AsMT3 of *A. strobiliformis* 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 SIMTa of *S. luteus* and its homolog in *S. brevipes* are different in only one amino acid and that SIMTb 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 SIMT 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-cysteine segments homologous to *Neurospora crassa* MT of family 8) and TmMT of *Tremella mesenterica*, the longest MT currently characterized (7 blocks of -CXCX₃CSCPPGX₃CAXCP-, two fragments of six cysteines and three N-terminal cysteines). Nor they represent a homolog of the single building block of TmMT (*Supplemental Table S1*). In a phylogenetic tree, both SIMT'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 sanguineus*, *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 SIMTa and SIMTb 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 $\Delta cup2$ yeast mutant was complemented by *SLMTa* and *SLMTb* (Fig. 3A). Overexpression of *SLMTa* slightly improved growth of the Cd sensitive mutants $\Delta ycf1$ and $\Delta yap1$ (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 μ M 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 $\Delta cup2$ 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*

207 *involutus* (Bellion et al., 2007) and PaMT1 of *Pisolithus albus* (Reddy et al., 2016). In
208 Agaricales, *HcMT1* and *HcMT2* of *Hebeloma cylindrosporum* (Ramesh et al., 2009),
209 *HmMT1-3* of *H. mesophaeum* (Sacky et al., 2014), *LbMT1* and *LbMT2* of *Laccaria*
210 *bicolor* (Reddy et al., 2014) and *AsMT1-3* of *Amanita strobiliformis* (Osobova et al.,
211 2011; Hlozkova et al., 2016) were characterized. Homologs of these MTs have not
212 been found in *S. luteus*, except for AsMT3, which is homologous to SIMTa/SIMTb. In
213 the genome of *H. cylindrosporum*, *P. involutus* and *L. bicolor*, ECM fungi in which
214 several other MTs are already discovered, at least one homolog of SIMTa/SIMTb is
215 detected (Fig. 1). The presence of different types of MTs indicates that these proteins
216 might take part in different cellular processes in fungi. Here we would like to
217 highlight the importance of this group of MTs because of their sequence conservation
218 across species and omnipresence in the mushroom-forming fungi -- class
219 Agaricomycetes, subphylum Agaricomycotina. It is also interesting to find a homolog
220 of SIMTa/SIMTb in the order Wallemiales (the earliest diverging lineage of
221 Agaricomycotina) and not in some other orders (Auriculariales, Sebaciniales,
222 Tremellales, Dacrymycetales, Filobasidiales). Wallemia is known for its ability to
223 tolerate harsh environments, especially osmotic stress (Padamsee et al., 2012). Beside
224 the high number of transporters present in its genome that are assumed to be involved
225 in its xero-tolerance, it is possible that this MT also plays a role in metal ion
226 homeostasis and osmotic stress tolerance of Wallemia.

227 The analysis of the SIMTa and SIMTb protein sequences revealed unique features
228 when compared to the other known MTs. The distribution of cysteine residues clearly
229 indicates that SIMTa and SIMTb 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 $\Delta cup2$ transformants expressing *SlMTa* grew better than those expressing *SlMTb* on all Cu concentrations tested. Transformants expressing *SlMTa* also slightly improved growth

of Cd sensitive *Δycf1* and *Δyap1* mutants but not completely restored their growth on high Cd concentrations. These results suggest that there exist differences in the metal-binding abilities of SIMTa and SIMTb proteins. It is widely accepted that cysteine residues are responsible for metal binding ability of MT's. Therefore, one would expect that MTs showing the same amount and distribution of cysteines are showing the same metal-binding abilities, energetically favouring the same metal-thiolate clusters. Within one species, differentiation in metal specificity of MT isoforms exhibiting the same amount of perfectly conserved cysteine residues was reported previously (Perez-Rafael et al., 2014). Cysteine residues are not the unique determinants of metal-binding abilities, histidine residues can act as ligands in metalloproteins and other small differences in primary MT structure could influence protein folding, 3D structure and stability of the particular metal-MT complex (holoprotein). Participation of chloride ions in the stabilisation of metal-MT complexes has been reported as well (Palacios et al., 2011). The prediction of the physiological function of MTs based on primary protein sequence is therefore difficult. Subtle changes in primary protein sequence, even apart from cysteine residues can result in altered metal binding properties and distinct physiological functions. AsMT3 of *A. strobiliformis*, a homologue of SIMTa and SIMTb was characterized recently as a MT with Cu, Zn and Cd binding potential (Hlozkova et al., 2016). AsMT3 exhibits exactly the same cysteine and histidine pattern as SIMTb, though both proteins differ in 27 aa (Fig. 2) and analysis of Zn and Cd tolerance of AsMT3 overexpression yeast mutants by Hlozkova et al. was done in the presence of excess Cl (exposed to ZnCl₂ and CdCl₂). In vivo metal binding potential of additional MTs of this novel family is

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

279 It has been reported that the expression of MT genes is induced by metals and
280 oxidative stress (Palmiter, 1994; Andrews, 2000; Ruttkay-Nedecky et al., 2013). In the
281 basidiomycete *C. neoformans*, transcription factor (TF) Cuf1 is essential for activation
282 of MT genes in response to excess Cu (Ding et al., 2011) and promoter regions of both
283 MT genes of *C. neoformans* contain several CuSE-like motifs. Other than the Cuf1 of
284 *C. neoformans*, there is yet any information on the participation of TFs in regulation
285 of MT genes in basidiomycete fungi. However, potential TF binding sites (MRE,
286 CuSE, STRE and MRE) have been found in the promoter regions of several
287 basidiomycete MT genes (Ramesh et al., 2009; Ding et al., 2011; Eastwood et al.,
288 2011). Likewise, promoter analysis of *SIMTa* and *SIMTb* shows the presence (co-
289 existence) of different putative response elements (*Supplemental figure S3*); this
290 indicates the complexity of *SIMTa* and *SIMTb* regulation. Transcription of both SMT
291 genes might be influenced by general stress related factors that bind to STRE and
292 ARE, elements that are found in several copies in their promoter regions. Yet, despite
293 the similarity in protein sequences, *SIMTa* and *SIMTb* might be regulated differently.
294 Here we could only show that *SIMTa* transcription is more sensitive to Cu since it
295 responded to Cu exposure earlier and at lower external concentrations than *SIMTb*
296 (Fig. 4). Regulation of the expression of the two *SIMT* genes might be more
297 differential in other conditions (for example, metal exposure time and doses, growth
298 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 chrysosporium* (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 preceded 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 (Hlzkova 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,

Belgium was used in this study. The genome of the strain was sequenced and can be consulted through the *S. luteus* genome portal of the Functional Genomics Program of the Department of Energy Joint Genome Institute (JGI) (<http://genome.jgi-psf.org/Suilu1/Suilu1.home.html>) (Grigoriev et al., 2012; Kohler et al., 2015). The fungus is maintained on solid modified Fries medium (28 mM glucose, 5.4 mM ammonium tartrate, 1.5 mM KH₂PO₄, 0.4 mM MgSO₄·7H₂O, 5 μM CuSO₄·5H₂O, 20 μM ZnSO₄·7H₂O, 0.1 μM biotin, 0.5 μM pyridoxine, 0.3 μM riboflavin, 0.8 μM nicotinamide, 0.7 μM p-aminobenzoic acid, 0.3 μM thiamine, 0.2 μM Ca-pantothenate and 0.8 % agar; pH-adjusted to 4.8).

Identification of *SIMTa* and *SIMTb*

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

Cloning of *SIMTa* and *SIMTb* genes

A cDNA library was made using the SMARTer PCR cDNA synthesis kit (Clontech, US) following the manufacturer's instructions. Specific primers were designed to amplify full-length coding sequences of *SIMTa* (F: ACAAAAACCATAATGGCGACCTGCAG; R: TCACTTTGACTCGCAGGTACATGCTAGA), *SIMTb* (F: 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; his3 Δ 1; leu2 Δ ; met15 Δ 0; ura3 Δ 0), Δ *zrc1* (BY4741; MAT a; his3 Δ 1; leu2 Δ ; met15 Δ 0; ura3 Δ 0; YMR243c::kanMX4), Δ *ycf1* (BY4741; MAT a; his3 Δ 1; leu2 Δ ; met15 Δ 0; ura3 Δ 0; YDR135c::kanMX4), Δ *yap1* (BY4741; MAT a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; YML007w::kanMX4) and Δ *cup2* (BY4741; MAT a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; YGL166w::kanMX4) obtained from Euroscarf (EUROSCARF, Frankfurt, Germany, [http: www.uni-frankfurt.de/fb15/mikro/euroscarf](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 (NH₄)₂SO₄, 2 % (w/v) D-glucose or

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

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

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

S. luteus inocula of 0.5 cm² were initially grown for eight days on cellophane-covered solid Fries medium. One gram fresh weight of mycelium was subsequently collected, blended aseptically with a kitchen mixer and transferred to 150 ml of Fries liquid medium without agar. The cultures were incubated at 23 °C on a shaker (120 rpm) for eight days. In order to obtain regular growth and uniform fungal spheres half of the medium was replaced every two days with fresh medium. Fresh mycelial spheres (approximately 100 mg fresh weight) were transferred to Petri dishes containing 10 ml of liquid Fries medium and were grown further for 24 h with shaking (120 rpm). Metals were added as sulphates (CuSO₄·5H₂O, 3CdSO₄·8H₂O and ZnSO₄·7H₂O) to the sphere cultures to obtain final concentrations: 0, 20, and 40 µM Cd; 0, 100, 500 µM Cu or 0, 0.5, or 1 mM Zn. The cultures were placed at 23 °C on a shaker incubator (70 rpm). After three and six hours, the spheres were collected in four

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

Total RNA was extracted using the RNeasy Plant Kit (Qiagen, France), according to the manufacturer's instructions. DNase treatment with the TURBO DNA-free™ Kit (Ambion, Life Technologies, Paisley, UK) was performed to eliminate possible genomic DNA contamination. RNA concentration and purity was evaluated spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, Wilmington, DE, USA). One µg of the treated RNA per sample was converted to single stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Paisley, UK) according to the manufacturer's instructions. A 5-fold dilution of the cDNA was prepared in 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM Na₂-EDTA, pH 8.0; Sigma–Aldrich, Belgium) and stored at -20°C.

Quantitative real-time PCR was performed in a 96-well optical plate with the ABI PRISM 7500 Fast Real-Time PCR System (Life Technologies, Paisley, UK) using SYBR Green chemistry, fast cycling conditions (20 s at 95°C, 40 cycles of 1 s at 95°C and 20 s at 60°C) and followed by the generation of a dissociation curve to verify amplification specificity. Reactions contained 2.5 µL diluted cDNA template (or RNase-free water for the 'no template controls'), 5 µL 2x Fast SYBR® Green Master Mix (Life Technologies, Paisley, UK), forward and reverse primers (300 nM each) and 1.9 µL RNase-free water in a total volume of 10 µL. Gene-specific forward and reverse primers were designed via the Primer-BLAST (Ye et al., 2012). All primer 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 = $2^{-(Cq(\text{sample}) - Cq(\text{min}))}$), 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 *SIMTa* and *SIMTb* 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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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, SIMTa and SIMTb.

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. SIMTa, SIMTb 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 *SIMTa* and *SIMTb*. 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.

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

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

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

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

Table S4. Primer sequences used in this study.

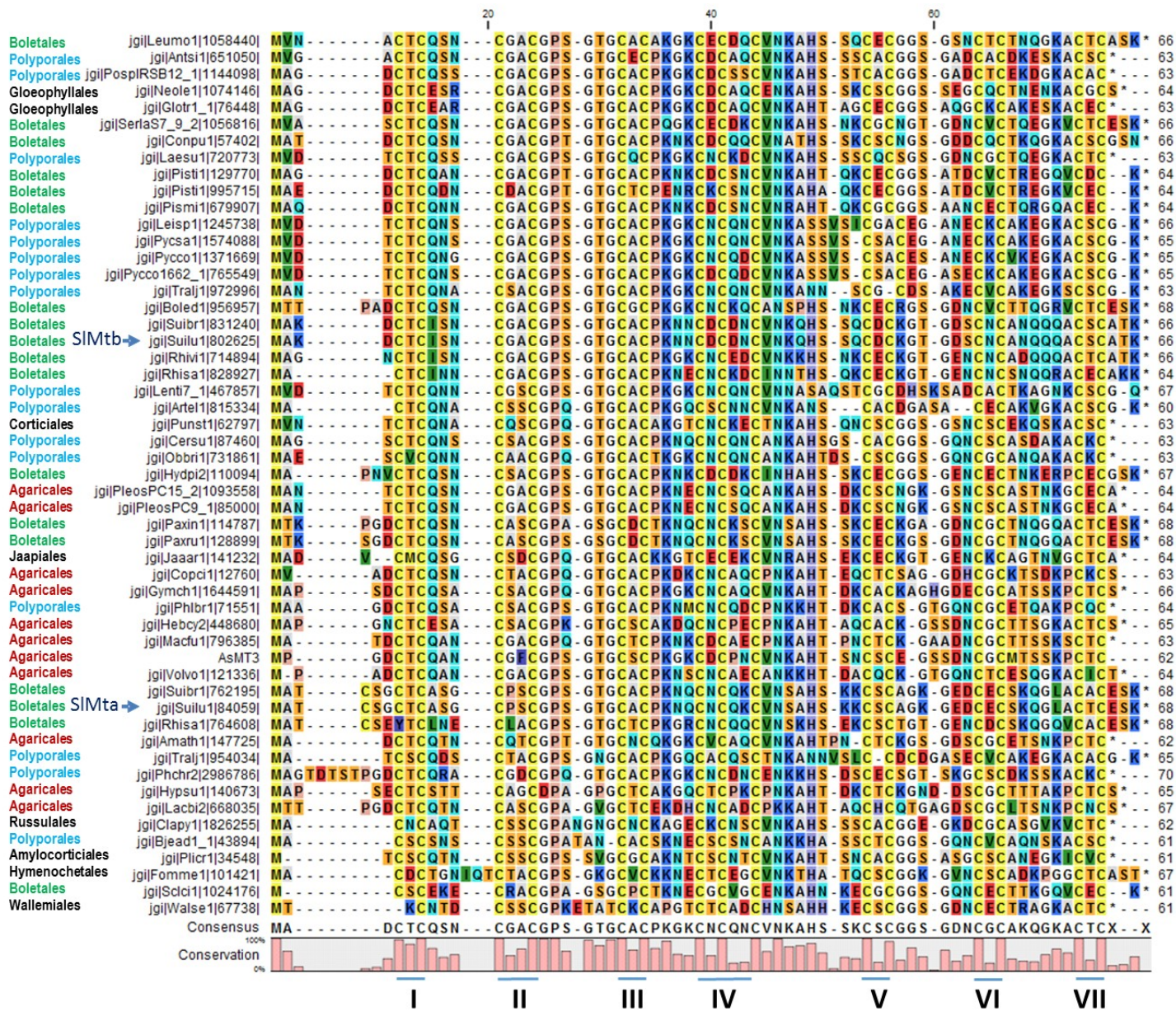
Table S5. Ranking and expression stability values of selected candidate reference genes for the different experimental set-ups included in this study as calculated by geNorm.

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

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

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

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



P_involutus_MT1	MNTITSVPVN	FN-NCGS-NS	CGC--GSSC	ACKPGECK-	--	34
P_albus_MT	MQSVNAVLVN	NNGNCGS-AA	CAC--GSNC	ACKPGECK-	--	35
A_strobiliformis_MT2	MQSESQSLVS	F-ANCGS-NS	CNC--GASC	ACKPGDCK-	--	34
L_bicolor_MT1	MISNTSAFAN	--AACGDHSS	CGC--AQDC	SCASCCKCA	SG	37
A_strobiliformis_MT1	MHSNVSPV-	-----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	MLFNTLTPI S	RASSTGCCCT	SCKC-TSCTC	GTAPVNEA-G	CGSTTCNCTN	CACKPEECKC 58
		I	II	III	IV	V VI VII
S_luteus_MTB	MA--KDCTC I	SNCGACGPSG	TGCACPKNNC	DCDNCVNKH	SSQCDCKGTG	DSCNCANQQQ ACSCATK 65
A_strobiliformis_MT3	MP--GDCTCQ	ANCGFCGPSG	TGCSCPCKGC	DCPNVCNKAH	TSNCSCGESS	DNCGCMTSSK PCTC-- 62
S_luteus_MTa	MATCSGCTCA	SGCPSCGPSG	TGCACPKNQC	NCQKCVNSAH	SKKCSACGKG	EDCECSKQGL ACTCESK 67
		I	II	III	IV	V VI VII

