

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

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1 **Title:**

2 A novel, highly conserved metallothionein family in basidiomycete fungi and  
3 characterization of two representative *SLMTa* and *SLMTb* genes in the ectomycorrhizal  
4 fungus *Suillus luteus*

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19 **characterization of two representative *SIMTa* and *SIMTb* genes in the**  
20 **ectomycorrhizal fungus *Suillus luteus***

21

22 SUMMARY

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

37

38

## 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

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

65 Fungi are ubiquitous in the natural environment and play important roles in  
66 decomposition, nutrient cycling and transformation of metals. Until recently, fungal  
67 MTs have been characterized in yeasts, a few other ascomycetes (*Neurospora crassa*,  
68 *Candida albicans*, *Candida glabrata*, *Yarrowia lipolytica*) and basidiomycetes  
69 (*Paxillus involutus*, *Laccaria bicolor*, *Hebeloma* spp., *Russula atropurpurea*,  
70 *Cryptococcus neoformans*). Among those identified, most are Cu-binding MTs (Fogel  
71 and Welch, 1982; Munger et al., 1987; Riggle and Kumamoto, 2000; Ding et al.,  
72 2011), although Ag (Osobová et al., 2011) and Zn (Leonhardt et al., 2014) binding  
73 MTs have also been found. Fungal MTs are involved in a variety of physiological  
74 processes, including Cu homeostasis and Cd detoxification (Ramesh et al., 2009), Ag  
75 hyperaccumulation (Osobová et al., 2011) and oxidative stress response (Reddy et al.,  
76 2014). They were identified as virulence factors of pathogens (Tucker et al., 2004;  
77 Ding et al., 2013), they were hypothesized to function as Cu-supplier for lignin  
78 degradation pathways of saprotrophs (Iturbe-Espinoza et al., 2016) and they might  
79 play a role in the development and functioning of symbiotic interactions (Lanfranco et  
80 al., 2002; Bergero et al., 2007; Reddy et al., 2016).

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

85 them belong to the subphylum Agaricomycotina) including many plant and animal  
86 pathogens, saprotrophs, and mycorrhizal fungi (Hibbett, 2006). The ectomycorrhizal  
87 (ECM) basidiomycete *Suillus luteus* is a common root symbiont of young pine trees.  
88 It has been reported to occur at various metal polluted sites in Europe (Colpaert et al.,  
89 2011; Op De Beeck et al., 2015). Some mechanisms of metal tolerance in this fungus  
90 have been studied before (Colpaert et al., 2000; Colpaert et al., 2005; Ruytinx et al.,  
91 2011) but metal chelation via MTs has not yet been reported. In this study, we  
92 identified and functionally characterized two novel MT coding genes of *S. luteus*, and  
93 searched for their homologs in other basidiomycete genomes.

94

## 95 **RESULTS**

### 96 **Identification of *SIMTa* and *SIMTb***

97 BLASTp using as a query the metallothionein CnMT2 (183 aa) of the human fungal  
98 pathogen *Cryptococcus neoformans* H99 (Ding et al., 2011) gave one positive hit with  
99 a 40 % identity to a hypothetical protein with ID 802625. By increasing the expected  
100 value (to  $E = 10^{-4}$ ) and re-BLASTp in the *S. luteus* genome using the protein with ID  
101 802625 as a query we found its paralog with protein ID 84059. None of the protein  
102 sequences were annotated. Both protein sequences contain multiple cysteine residues  
103 arranged in CXC or CXXC motifs (in which X is any other amino acids other than  
104 cysteine) typical for metallothioneins and therefore are named SIMTa and SIMTb. The  
105 respective genes are *SIMTa* and *SIMTb*. Both genes contain three exons and code for  
106 67 and 65 aa, respectively. The proteins show a particular arrangement of CXC and

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

### 110 **Homologs of SIMTa and SIMTb in Basidiomycota**

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

130 Seven cysteine-rich boxes can be distinguished and are indicated in Fig. 1 and 2. The  
131 abundance (15-16) and arrangement of cysteine residues of SIMT homologues are  
132 different from those of known MT families (Table 1; Binz and Kägi, 1999) and MTs  
133 previously characterized in Basidiomycetes (Fig. 2 and *Supplemental Table S1*).  
134 Neither they are built by repetitive units (of known or unknown families) as is the case  
135 for CnMT2 of *Cryptococcus neoformans* (7-cystein segments homologous to  
136 *Neurospora crassa* MT of family 8) and TmMT of *Tremella mesenterica*, the longest  
137 MT currently characterized (7 blocks of -CXCX<sub>3</sub>CSCPPGXCXCAXCP-, two  
138 fragments of six cysteins and three N-terminal cysteins). Nor they represent a  
139 homolog of the single building block of TmMT (*Supplemental Table S1*). In a  
140 phylogenetic tree, both SIMT's cluster with AsMT3 and apart from previously  
141 characterized and classified fungal MT's. Though, not all nodes are well supported as  
142 indicated by low bootstrap values (*Supplemental figure S2*).

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



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

### 159 **Functional complementation of *SIMTa* and *SIMTb* in yeast**

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

### 166 **Effects of exogenous Cu, Cd and Zn on the expression of *SIMTa* and *SIMTb***

167 We conducted a quantitative real-time PCR (RT-qPCR) experiment to determine  
168 changes in transcription of *SIMTa* and *SIMTb* when *S. luteus* was exposed to sublethal  
169 concentrations of Cu, Cd or Zn (Fig. 4). Transcription of *SIMTa* was induced by Cu  
170 after three and six hours of exposure. Transcription of *SIMTb* was induced only after  
171 six hours of exposure to 500  $\mu$ M Cu. Exposure to sublethal concentrations of Cd and  
172 Zn did not induce significant changes in expression of *SIMTa* and *SIMTb*.

### 173 **Promoter analysis of *SIMTa* and *SIMTb***

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

183

## 184 **DISCUSSION**

185 The present study aimed to identify MTs of the fungus *S. luteus*, an ECM symbiont  
186 known for its heavy metal tolerance and its ability in protecting young pine trees from  
187 different metal stresses (Adriaensen et al., 2005; Krznicaric et al., 2009; Colpaert et al.,  
188 2011). Identification of two novel MTs, SlMTa and SlMTb, in *S. luteus* lead to the  
189 discovery of ubiquitous, highly conserved homologs in other fungi in the subphylum  
190 Agaricomycotina. These two *S. luteus* genes were characterized and their ability to  
191 complement the Cu sensitivity of the  $\Delta cup2$  mutant of *S. cerevisiae* as well as their  
192 transcriptional response to Cu indicate that they are Cu-thioneins and play a role in Cu  
193 homeostasis.

194 Up to present, 15 MT families are classified (Binz and Kägi, 1999) showing the high  
195 heterogeneity in length and primary structure of the MT sequences. An extra MT  
196 family composed of environmental cysteine-rich proteins of unknown taxonomic  
197 origin and showing unique features was recently described (Ziller et al., 2017). In  
198 addition to the six fungal MT families (families 8 to 13) in the current classification,  
199 several functionally studied fungal MTs are not (yet) classified. Identification and  
200 classification of MTs is generally difficult with regular BLASTp because of this  
201 heterogeneity as well as the short sequence nature of the proteins. So far the longest  
202 MT known is found in fungal basidiomycetes: TmMT (257 aa) of *Tremella*  
203 *mesenterica*. In addition, the CnMT1 (122 aa) and CnMT2 (183 aa) of the pathogenic  
204 fungus *C. neoformans* were identified and characterized in many details (Ding et al.,  
205 2011; Ding et al., 2013). In Agaricomycotina, a number of MT coding genes have  
206 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

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

244 The complementation assay using the yeast *S. cerevisiae* metal sensitive mutants has  
245 been successfully used in characterizing a number of MTs in fungi (Bellion et al.,  
246 2007; Ding et al., 2011; Osobová et al., 2011). In this study, we could confirm the  
247 roles of *SIMTa* and *SIMTb* in Cu detoxification within the yeast system. Accordingly,  
248 exogenous Cu but no other metals at the tested concentrations induced the expression  
249 of the genes in *S. luteus*. In the yeast complementation assay, both *SIMTa* and *SIMTb*  
250 were under the control of a GAL1-inducible promoter; however, we found that  $\Delta cup2$   
251 transformants expressing *SIMTa* grew better those expressing *SIMTb* on all Cu  
252 concentrations tested. Transformants expressing *SIMTa* also slightly improved growth

253 of Cd sensitive *Δycf1* and *Δyap1* 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 SIMTa and SIMTb 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 SIMTa and SIMTb 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 SIMTb, 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<sub>2</sub>  
275 and CdCl<sub>2</sub>). 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 SIMT  
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*,

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



321 The subphylum Agaricomycotina contains about one-third of the described  
322 basidiomycete species and accommodates a diverse array of fungi, in size, lifestyle  
323 (unicellular yeasts, jelly fungi to mushroom-forming fungi) and ecology (wood-rots,  
324 litter decomposers, ectomycorrhizal fungi and a few pathogens) (Hibbett, 2006). The  
325 broad species distribution of the novel MTs indicates their contribution to  
326 fundamental and conserved cellular process(es) amongst those Cu homeostasis and  
327 detoxification are likely included. It is also noteworthy that Cu-containing fungicides  
328 and wood preservatives are commonly used all over the world. Extensive use of these  
329 compounds can be a threat for the environment in particular for microbial  
330 communities. However, there is some evidence that particular wood-rot fungi became  
331 tolerant to the metal-containing preservatives (Baldrian, 2003; Green Iii and Clausen,  
332 2003; Hastrup et al., 2005; Guillen et al., 2009). Also ECM fungi from Cu-polluted  
333 soils may develop Cu resistance and such ecotypes may be good candidates for  
334 bioremediation of Cu-polluted areas (Adriaensen et al., 2005; Colpaert et al., 2011;  
335 Silva et al., 2013). Therefore, understanding how these fungi cope with excess Cu and  
336 heavy metals in general will help to develop new technologies for the control and  
337 efficient use of these fungi in the future.

338

## 339 **EXPERIMENTAL PROCEDURES**

### 340 **Fungal strains and culture medium**

341 A *S. luteus* monokaryotic isolate (UH-Slu-Lm8-n1) obtained from a basidiospore  
342 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](http://genome.jgi-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<sub>2</sub>PO<sub>4</sub>, 0.4 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 μM CuSO<sub>4</sub>·5H<sub>2</sub>O, 20  
349 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 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 *SIMTa* and *SIMTb***

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 *SIMTa* and *SIMTb* genes**

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

365 CTACTTCGTTGCGCAACTGCACGCCTGC). PCR reactions were performed using  
366 the Advantage 2 DNA polymerase mix (Clontech, US) following the manufacturer's  
367 instructions. Amplicons were separated by electrophoresis and bands of approximately  
368 200 base pairs (bp) were purified using Qiaquick Gel extraction Kit (Qiagen, France).  
369 The purified PCR-products were cloned into the Gateway entry vector  
370 pCR8/GW/TOPO (Life technologies, Paisley, UK) and subsequently transferred by  
371 LR-Clonase into the yeast expression vectors pAG426GAL-ccdB (Alberti et al., 2007)  
372 for functional complementation tests. Bacterial transformations followed standard heat  
373 shock protocol into chemically competent TOP10 *E. coli* (Life Technologies, Paisley,  
374 UK) with cells being plated onto Luria-Bertani agar plates containing the appropriate  
375 selecting antibiotic. The inserts were sequenced in both directions to assure correct  
376 fusion.

377 The yeast strains used for heterologous expression of *SIMTa* and *SIMTb* were BY4741  
378 (MAT a; his3 $\Delta$ 1; leu2 $\Delta$ ; met15 $\Delta$ 0; ura3 $\Delta$ 0),  $\Delta$ *zrc1* (BY4741; MAT a; his3 $\Delta$ 1; leu2 $\Delta$ ;  
379 met15 $\Delta$ 0; ura3 $\Delta$ 0; YMR243c::kanMX4),  $\Delta$ *ycf1* (BY4741; MAT a; his3 $\Delta$ 1; leu2 $\Delta$ ;  
380 met15 $\Delta$ 0; ura3 $\Delta$ 0; YDR135c::kanMX4),  $\Delta$ *yap1* (BY4741; MAT a; his3 $\Delta$ 1; leu2 $\Delta$ 0;  
381 met15 $\Delta$ 0; ura3 $\Delta$ 0; YML007w::kanMX4) and  $\Delta$ *cup2* (BY4741; MAT a; his3 $\Delta$ 1;  
382 leu2 $\Delta$ 0; met15 $\Delta$ 0; ura3 $\Delta$ 0; YGL166w::kanMX4) obtained from Euroscarf  
383 (EUROSCARF, Frankfurt, Germany, [http: www.uni-](http://www.uni-frankfurt.de/fb15/mikro/euroscarf)  
384 [frankfurt.de/fb15/mikro/euroscarf](http://www.uni-frankfurt.de/fb15/mikro/euroscarf)). Yeast cells were transformed using the LiAC/PEG  
385 method as previously described (Gietz and Schiestl, 2007). Transformed yeast mutants  
386 and wild type were selected on agar plates containing SD medium without uracil (1.7  
387 g/L of yeast nitrogen base (Difco, BD, US), 5 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 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  $\mu$ l 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 cm<sup>2</sup> 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  $\mu$ M Cd; 0, 100, 500  
407  $\mu$ M 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<sub>2</sub>-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

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

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

#### 449 **Promoter analysis of *SIMTa* and *SIMTb* genes**

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

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

456

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460 The authors declare no conflicts of interest.

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647

648 **LEGENDS**

649 **Table 1.** Different families of the Binz and Kägi MT classification that contain fungal  
650 MTs. Conserved cysteines and histidines are indicated in an example sequence for  
651 each family, SIMTa and SIMTb.

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

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

662 **Fig. 3.** Functional complementation of *S. cerevisiae* mutants on selective media. Yeast  
663 mutant strains were transformed with the empty vector (EV) pAG426GAL or with  
664 vector containing coding sequence of *SIMTa* and *SIMTb*. Wild-type strain BY4741  
665 (WT) was transformed with EV as a control. Yeast cultures were adjusted to OD =  
666 1.0, and 10 µl of serial dilutions were spotted on SD medium with 2 % galactose  
667 and supplemented with Cu, Cd or Zn as indicated. Plates were incubated for five  
668 days at 30 °C.



669 **Fig. 4.** Relative expression of (a) *SIMTa* and (b) *SIMTb* 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  $\mu$ M, Cd: 20, 40  $\mu$ M, 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  $\pm$  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](http://genome.jgi-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.

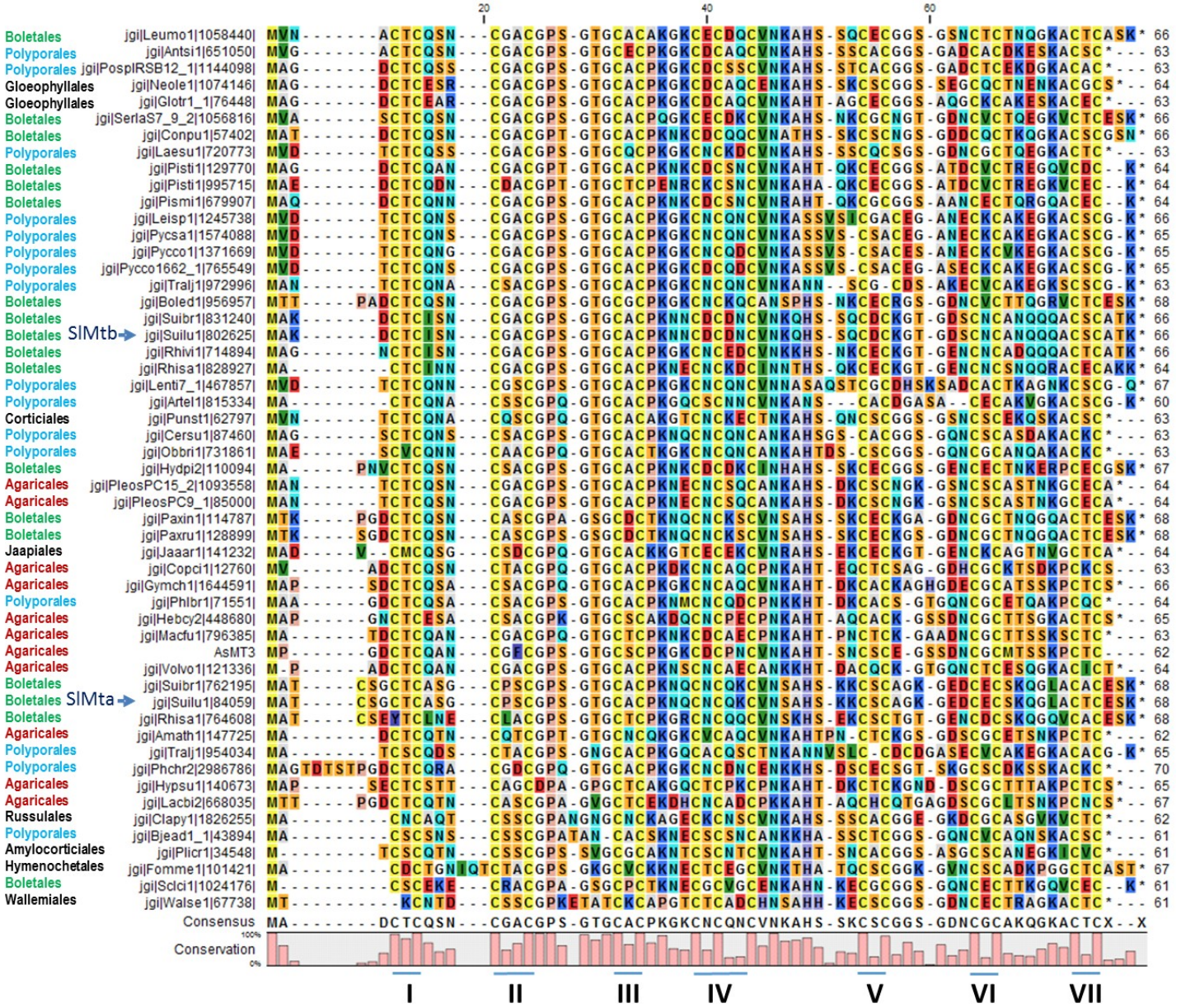
687 **Table S5.** Ranking and expression stability values of selected candidate reference  
688 genes for the different experimental set-ups included in this study as calculated by  
689 geNorm.

690 **Table S6.** Data-set containing C<sub>q</sub> 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* SIMTa, SIMTb 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) *SIMTa* and (B) *SIMTb*. Predicted TF binding sites are indicated:  
703 ARE (TGACNNNGC), STRE (CCCCT), CuSE (DDDHGCTGD), CuSE\*  
704 (DDHGCTGD), yMRE (HTHNNGCTGD), MRE (TGCRCNC).



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	SCASCCKCA	SG	37	
A_strobiliformis_MT1	MHSNVSPV -	- - - - - SNAT	CSC	LNKGGSC	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 - - KDCTC I	SNCGACGPSG	TGCACPKNNC	DCDNCVNKQH	SSQCDCKGTG	DSCNCANQQQ	ACSCATK	65	
A_strobiliformis_MT3	MP - - GDCTC Q	ANCGFCGPSG	TGCSCPCKGKC	DCPNCVNKAH	TSNCSCGSS	DNCGCMTSSK	PCTC - - -	62	
S_luteus_MTa	MATCSGCTCA	SGCPSCGPSG	TGCACPKNQC	NCQKCVNSAH	SKKCSAGKG	EDCECSKQGL	ACTCESK	67	
			I	II	III	IV	V	VI	VII

