universiteit hasselt
KNOWLEDGE IN ACTION

2016 | Faculty of Sciences

DOCTORAL DISSERTATION

Functional characterization of heavy metal transporting P-type ATPases and metallothioneins of the ectomycorrhizal fungus *Suillus luteus*

Doctoral dissertation submitted to obtain the degree of
doctor of Science: Biology, to be defended by

Hoai Thi Thu Nguyen

Promoter: Prof. Dr Jan Colpaert

D/2016/2451/2

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and support of many people. First and foremost I would like to thank Prof. Dr Jaco Vangrondsveld and Prof. Dr Jan V Colpaert for giving me the opportunity to conduct research at the Centre for Environmental Sciences at Hasselt University.

I would like to thank my supervisor Prof. Dr Jan V Colpaert for his great guidance and support throughout my research. I would like to thank my committee members Prof. Dr Ann Cuypers, Dr Annegret Kohler, Dr Elena Martino, Prof. Dr François Rineau, Dr Joske Ruytinx and Prof. Dr Nathalie Verbruggen for their constructive comments.

I would like to thank members of the Mycorrhiza group: Dr Mark Smiths, Dr Michiel Op De Beeck, Jan Wevers and Laura Coninx for sharing their expertise in mycorrhiza research. Special thanks go to Dr Joske Ruytinx for her help with lab work and insightful suggestions on this project from the very beginning.

I would like to thank Prof. Dr Marcel Ameloot for the permission to use the confocal microscopy facility at the Biomedical Research Institute and Dr Rik Paesen for his expertise in confocal microscopy.

I would like to thank Carine Put and Ann Wijgaerts for all administrative and technical support. I would like to thank all my colleagues at CMK for their support, friendship and for creating a pleasant working environment.

Last but not least, I would like to thank my family and friends for their love, support and encouragement.

SAMENVATTING

(Summary in Dutch)

De basidiomyceet *Suillus luteus* is een belangrijke ectomycorrhizaschimmel van jonge dennenbomen. Het frequent voorkomen van *S. luteus* in pioniersbossen op door zware metalen vervuilde sites in Europa heeft geleid tot meerdere studies waarbij metaaltolerantie van deze schimmel centraal stond. Op toxische bodems werden ecotypes van *S. luteus* gevonden die verhoogde toleranties voor zink, cadmium en koper vertonen. In eerdere studies werd aangetoond dat metaaltolerante ecotypes effectief zijn in het reduceren van de toxische effecten van zware metalen op jonge dennenplanten. Tot op heden zijn de mechanismen betrokken bij de metaalhomeostase en detoxificatie in *S. luteus* nog grotendeels onbekend.

Deze studie heeft als doel om in *S. luteus,* de werking van een familie van zware metalen transporters, de P1B-type ATPases, en van de metaal-chelerende metallothioneïnes (MTs), te identificeren en te onderzoeken met een speciale focus op koperhomeostase.

De P1B-type ATPases of HMA's zijn een onderfamilie van de P-type ATPases die gekend zijn voor het transport van zware metalen (Cu, Zn, Cd, Co, Ag en Pb). Deze HMA's zijn teruggevonden in bacteriën, archaea en eukaryoten en worden beschouwd als vroeg geëvolueerd in de evolutie. In schimmels werden met uitzondering van de metaal transporters die werden teruggevonden in de modelgisten, slechts enkele HMA's geïdentificeerd. In de laatste jaren zijn de volledige genomen van meerdere fungi waaronder *S. luteus* gradueel beschikbaar geworden. Bio-informatica tools werden bij deze studie ingezet om bij 345 mogelijke HMA proteïne sequenties van 132 basidiomyceten en 17 vroeg divergerende schimmels (Hoofdstuk 2) de aanwezigheid en diversiteit van de HMA's te evalueren evenals hun functies te voorspellen. De bekomen gegevens hebben aangetoond dat de HMA's in de basidiomyceten onderverdeeld kunnen worden in 3 verschillende clusters. Deze 3 clusters zijn CCC2-, CRD1-, en PCA1-type HMA's die vertegenwoordigers hebben die respectievelijk homoloog zijn aan

de *Saccharomyces cerevisiae* Ccc2p, Pca1p transporteiwitten en de *Candida albicans* Crd1p transporters.

Het onderzoek toont aan dat HMA genen veelvoorkomend aanwezig zijn in de geanalyseerde schimmelgenomen. Van deze 3 types zijn er 2 types (de CRD1- en PCA1-types) die specifiek zijn voor schimmels en nooit eerder werden geanalyseerd (met uitzondering van de modelgisten). Ons onderzoek toont aan dat de *S. luteus* HMA's van het CRD1-type betrokken zijn bij koper- en cadmiumhomeostase en detoxificatie (Hoofdstuk 3). Daarnaast toonden de bekomen gegevens aan dat meerdere PCA1-type HMA's waarschijnlijk zinktransporterende ATPases zijn waarvan eerder aangenomen werd dat deze laatste groep niet voorkomt in fungi.

Een belangrijke vaststelling is dat de evolutionair meest geconserveerde HMAs in basidiomyceten behoren tot het CCC2-type. De CCC2-type vertegenwoordiger in *S. luteus* (SlHma1) is experimenteel bevestigd als een koper exporter gelokaliseerd in het ER-Golgi, gelijkaardig aan zijn *S. cerevisiae* ortholoog dat een essentiële component is van het secretiesysteem in gist (Hoofdstuk 3).

Een nieuwe, evolutionair sterk geconserveerde familie van metallothioneïnen werd tijdens dit onderzoek ontdekt in *S. luteus* en in een aantal andere basidiomycete schimmels (Hoofdstuk 4). De twee metallothioneïne genen *SlMTa* en *SlMTb* van *S. luteus* werden experimenteel bestudeerd en vertonen de typische kenmerken van een kopermetallothioneïne. De sterk geconserveerde aminozuursequenties van deze familie van metallothioneïnen is een indicatie dat deze metallothioneïnen een essentiële functie vervullen in de Basidiomycota.

Vervolgexperimenten zijn nodig om de invloed van metallothioneïnen op de verschillende biologische processen en in verschillende ontwikkelingsstadia van *S. luteus* en andere basidiomyceten te bepalen. Daarenboven openen de resultaten van deze studie nieuwe perspectieven voor verder onderzoek. De mogelijke koperchaperones en metaal responsieve transcriptiefactoren geïdentificeerd in Hoofdstuk 3 zijn waarschijnlijk betrokken bij het functioneren en de regulatie van de HMA transporters. Dit kan worden nagegaan door verdere karakterisering.

Resultaten van deze studie suggereren ook een verband tussen koper en ijzer transport via de functie van de CCC2-type HMA's (SlHma1 in *S. luteus*) en tussen koper en cadmium via de metaal bindende capaciteit van de CRD1-type HMAs (SlHma2 and SlHma3 in *S. luteus*).

Alles bij elkaar beschouwd brengt deze studie meer duidelijkheid over de rol van drie P1B-type ATPase transporters en twee nieuwe metallothioneïnen in koperhomeostase en, tot op zekere hoogte, cadmiumdetoxificatie in *S. luteus*. Daarnaast wordt ook gewezen op de mogelijke betrokkenheid van verschillende types van de P1B-type ATPases in metaalhomeostase in de Basidiomycota. Er werd een nieuwe familie van metallothioneïnen ontdekt die van nut zal zijn voor verder onderzoek naar metaalhomeostase en –tolerantie in deze grote groep van fungi.

SUMMARY

The basidiomycete *Suillus luteus* is an important ectomycorrhizal fungus of young pine trees. The high occurrence of *S. luteus* in pioneer forests on heavy metal polluted sites in Europe prompted a number of studies on metal tolerance in the fungus. Ecotypes of *S. luteus* that are tolerant to zinc, cadmium and copper have been found on highly toxic soils. These ecotypes have been shown to be effective in ameliorating the effects of metal toxicity on the host plant. Until now, the mechanisms that are involved in metal homeostasis and detoxification in *S. luteus* are largely unknown.

This study aimed to identify and investigate the function of a family of heavy metal transporters — the P1B-type ATPases — and of heavy metal chelators — metallothioneins (MTs) — in *S. luteus* with special focus on Cu homeostasis.

The P1B-type ATPases or HMAs are a subfamily of P-type ATPases that are involved in the transport of heavy metals (Cu, Zn, Cd, Co, Ag and Pb). HMAs are found in bacteria, archaea and eukarya and are suggested to have evolved early in evolution. In fungi, except for those found in the model yeast species, only few HMAs have been identified. In the last few years, the complete genomes of numerous fungi including *S. luteus* gradually have become available. We applied bioinformatic approaches using computational predictions, homology and motif analysis to evaluate HMAs diversity, abundance and to predict their functions in 345 putative HMA protein sequences of 132 basidiomycetes and 17 early diverging fungi (Chapter 2). The data revealed that the HMAs in Basidiomycetes subdivide to form three distinct clusters. The three are: CCC2-, CRD1-, and PCA1-type HMAs which have members that are homologous to the *Saccharomyces cerevisiae* Ccc2p (copper-), Pca1p (cadmium-) and the *Candida albicans* Crd1p (copper–transporting) proteins. We show that HMAs are ubiquitously present in most fungi analyzed. Among the three types, the CCC2-type is the most evolutionary conserved; the CRD1- and PCA1-types are specific for fungi and have never been investigated (except in the model yeasts). We show later in Chapter 3 that the CRD1-type representatives in *S. luteus* are

involved in Cu and Cd homeostasis and detoxification. Our data revealed that several PCA1-type HMAs are likely Zn-transporting ATPases. The latter group was thought not to exist in fungi.

More important, three genes coding for the CCC2- and CRD1-type HMAs in *S. luteus* were further studied in Chapter 3. A yeast (*S. cerevisiae*) expression system was used to investigate the function and sub-cellular localization of the corresponding proteins. Expression of the genes in *S. luteus* exposed to different metals was analyzed using quantitative real-time PCR. The CCC2-type representative in *S. luteus* (SlHma1) is confirmed as a Cu exporter localized in the ER-Golgi similar to its *S. cerevisiae* ortholog that is an essential component of the secretory pathway. Heterologous expression of SlHma1 complements *ccc2* mutant yeast. Expression of *SlHMA1* gene increases when *S. luteus* is exposed to high Cu. Heterologous expression of CRD1-type HMAs (SlHma2 and SlHma3) in yeast does not result in functional Cu transport proteins. However, expression of the two HMAs' metal binding domain rescues both Cu and Cd sensitive mutants. Moreover, transcription of *SlHMA2* and *SlHMA3* strongly increases when *S. luteus* is exposed to high Cu and Cd conditions. These results indicate that SlHma2 and 3 are involved in Cu homeostasis and Cd detoxification in *S. luteus*.

In the present study, we discovered a novel, highly conserved family of metallothioneins that is present in *S. luteus* and a number of basidiomycete fungi (Chapter 4). The two metallothionein genes *SlMTa* and *SlMTb* of *S. luteus* were experimentally studied; they show typical Cu-thionein characteristics. The highly conserved amino acid sequences of this family of MTs indicates that these MTs must have essential functions. It will be interesting in the subsequent experiments to investigate the roles of these MTs in different biological processes and developmental stages of Basidiomycota.

Results of this study open new opportunities for future research. The putative copper chaperones and metal responsive transcription factors identified in Chapter 3 are likely involved in the function and the regulation of HMA transporters; this can be verified by further characterization. Results from this study also suggest a link between Cu and Fe transport via the function of the

CCC2-type HMAs (SlHma1 in *S. luteus*) and between Cu and Cd via the metal binding ability of the CRD1-type HMAs (SlHma2 and SlHma3 in *S. luteus*).

All together, the present study highlights the role of three P1B-type ATPase transporters and two novel metallothioneins in Cu homeostasis and, to a certain extent, Cd detoxification in *S. luteus*. Moreover, we call attention to the involvement of different types of P1B-type ATPases in metal homeostasis in basidiomycete fungi. We discovered a novel family of metallothioneins that will be useful in further research on metal homeostasis and tolerance of basidiomycete fungi.

LIST OF ABREVIATIONS

ABC	ATP-binding cassette transporter
Ace1p	synonym Cup2p *S. cerevisiae* copper-binding transcription factor; activates transcription of the metallothionein genes *CUP1-1* and *CUP1-2*
Amt1	Copper-sensing transcription factor of *Candida glabrata*; binds to the yeast metal responsive element (yMRE)
ARE	Antioxidant response element
Atx1p	Antioxidant; *S. cerevisiae* copper chaperone
SlAtx	*S. luteus* putative homolog of Atx1p
BLAST	Basic local alignment search tool
BLASTp	Protein BLAST
Bxa1	cyanobacterium *Oscillatoria brevis* P1B-type ATPase; responses to both mono (Cu^{+1} and Ag^{+1}) and divalent (Zn^{+2} and Cd^{+2}) heavy metals
Ccc2p	Cross complement calcium 2 protein; in *S. cerevisiae* transports copper into Golgi network
ccc2	Deletion mutant of *S. cerevisiae CCC2* gene
CCC2-type	Group of proteins homologous to Ccc2p
CCS1	*S. cerevisiae* copper chaperone to the Cu, Zn-superoxide dismutase; no homologs found in *S. luteus*
cDNA	Complementary DNA
CLC	CLC main workbench; software for advanced DNA, RNA, and protein analyses; www.clcbio.com
Cmt1, 2	*Cryptococcus neoformans* metallothionein 1, 2
Cox11p	Cytochrome *c* oxidase; *S. cerevisiae* mitochondrial inner membrane protein required for delivery of copper to cytochrome *c* oxidase Cox1p
Cox17p	Cytochrome *c* oxidase; *S. cerevisiae* copper chaperone that transfers copper to Sco1p and Cox11p; eventual delivery to cytochrome *c* oxidase
SlCox	*S. luteus* putative homolog of Cox17p

Crd1	Copper resistance determinant; synonym Crp1 (copper resistance protein 1); *Candida albicans* copper exporter at the plasma membrane
CRD1-type	Group of proteins homologous to Crd1p
CSM-URA	Complete supplement mixture without uracil
Cuf1	Copper-sensing transcription factor of *Schizosaccharomyces pombe*; binds to copper-sensing element CuSE
Cup1p	*S. cerevisiae* metallothionein protein
cup1	Deletion mutant of *S. cerevisiae CUP1* gene
Cup2p	synonym Ace1p *S. cerevisiae* copper-binding transcription factor; activates transcription of the metallothionein genes *CUP1-1* and *CUP1-2*
cup2	Deletion mutant of *S. cerevisiae CUP2* gene
CuSE	Copper-sensing element; binding site for transcription factor Cuf1 of *S. pombe*
CDF	Cation diffusion facilitator
Crs5p	*S. cerevisiae* copper-resistant suppressor 5; copper-binding metallothionein
ECM	Ectomycorrhiza
EGFP	Enhanced green fluorescent protein
EV	Empty vector
ER	Endoplasmic reticulum
Ferrozine	3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*'-disulfonic acid monosodium salt hydrate; a chelator of ferrous ion (Fe^{2+})
Fet3p	*S. cerevisiae* ferrous transport 3 protein; Ferro-O_2-oxidoreductase; multicopper oxidase that oxidizes ferrous (Fe^{2+}) to ferric iron (Fe^{3+}) for subsequent cellular uptake by transmembrane permease Ftr1p; required for high-affinity iron uptake and involved in mediating resistance to copper ion toxicity
FM4-64	N-(3-triethylammoniumpropyl)-4- (p-diethylaminophenyl-hexatrienyl) pyridinium dibromide; a vital stain for visualizing *S. cerevisiae* vacuolar membrane
Ftr1p	*S. cerevisiae* iron transporter; high affinity iron permease

GAL1	Galactose inducible promoter
GFP	Green fluorescent protein
GI	Genbank ID
GSH	Reduced glutathione
HMA	Heavy metal associated protein; heavy metal ATPase
HMA1-8	*Arabidopsis thaliana* heavy metal transporters (P1B-type ATPases)
SlHma1-3	*S. luteus* P1B-type ATPase 1-3 transporters
JASPAR	JASPAR core database contains a curated, non-redundant set of profiles, derived from published collections of experimentally defined transcription factor binding sites for eukaryotes
JGI	Joint Genome Institute
LiAc/PEG	Lithium acetate/ Polyethylene glycol; high-efficiency yeast transformation method
M	Metal
Mac1p	Metal binding activator; *S. cerevisiae* copper-sensing transcription factor; involved in regulation of genes required for high affinity copper transport
MRE	Metal responsive element
MSN2	*S. cerevisiae* stress responsive TF; binds DNA at stress response elements of responsive genes; paralog of MSN4
MSN4	*S. cerevisiae* stress responsive TF; binds DNA at stress response elements of responsive genes; paralog of MSN2
MT	Metallothionein
MTF1	Metal response element-binding TF-1; TF of higher eukaryotes
MTF1-BD	Binding site for MTF1
NCBI	National Centre for Biotechnology Information
NRAMP	Natural resistance-associated macrophage protein
Pca1p	Probable calcium transporting ATPase protein; *S. cerevisiae* cadmium exporter at the plasma membrane
PCA1-type	Group of proteins homologous to Pca1p
OD_{600}	Optical density measured at a wavelength of 600 nm
RT-qPCR	Quantitative real-time PCR
ROS	Reactive oxygen species

Sco1p	Suppressor of cytochrome oxidase deficiency; *S. cerevisiae* copper-binding protein of mitochondrial inner membrane; required for cytochrome *c* oxidase activity and respiration; may function to deliver copper to cytochrome *c* oxidase
SD	Synthetic dextrose
SE	Standard error
SKN7	Suppressor of Kre null; *S. cerevisiae* transcription factor; involved in osmotic and oxidative stress responses
SKO1	Suppressor of kinase overexpression; *S. cerevisiae* transcription factor; involved in osmotic and oxidative stress responses
SNP	Single nucleotide polymorphism
Sod1p	Cu, Zn-superoxide dismutase 1 protein
SlHma1-3	*S. luteus* heavy metal-associated proteins; P1B-type ATPase 1-3
SlMta, b	*S. luteus* metallothionein a, b
SlMTa, b	*S. luteus* metallothionein genes a, b
SlZnt1, 2	Zinc transporter 1, 2; belong to CDF transporter family
STB5	Sin three binding protein; *S. cerevisiae* transcription factor; involved in regulating multidrug resistance and oxidative stress response
STRE	Stress responsive element
TM	Transmembrane domain
TMHMM	Tied Mixture Hidden Markov Model; server for prediction of transmembrane helices in proteins
TF	Transcription factor
WT	Wild type
yap1	Deletion mutant of *YAP1* (yeast AP-1); *S. cerevisiae* transcription factor; required for oxidative stress tolerance; mediates resistance to cadmium
ycf1	Deletion mutant of *YCF1* (yeast cadmium factor); *S. cerevisiae* vacuolar glutathione S-conjugate transporter; ATP-binding cassette transporter; role in detoxifying metals (Cd, Hg, As)
yMRE	yeast metal regulatory element
ZiaA	cyanobacterium *Synechocystis* PCC6803 zinc exporter
ZIP	ZRT/IRT-like proteins

ZntA	*Escherichia coli* Zn^{+2}, Cd^{+2}, Pb^{+2} and Hg^{+2} exporter
zrc1	*S. cerevisiae* zinc resistance conferring; deletion mutant; vacuolar membrane zinc transporter; transports zinc from cytosol to vacuole for storage

TABLE OF CONTENT

CHAPTER 1

Introduction

1.1. MYCORRHIZA

The term 'Mycorrhiza' coined by Frank (1885), literally means 'fungus-root' and it is used to describe symbiotic associations between fungi (Greek = mikes) and the roots (Greek = rhiza) of higher plants ((Frank, 2005), for a translation).

It is often stated that 90 % of plant species are mycorrhizal. About 6000 recognized species of soil fungi form mycorrhizal symbiosis with the plant roots. The majority of mycorrhizal symbiosis provide substantial benefits to both plants and fungi. The plant supplies the carbohydrates obtained via photosynthesis to the fungus. In return, the mycorrhizal symbiont has a better access to water and nutrients and can transform organic matter into inorganic nutrients to supply to the plant. The fungal hyphae are much smaller in diameter than the roots of the plant. Extraradical hyphae can extend into the soil and serve as extensions of the root system (Figure 1.1). Fungal hyphae are more efficient for water and nutrient absorption than the root itself, in particular in nutrient poor soils (Peterson *et al.*, 2004). In such conditions, mycorrhizal plants grow much better than non-mycorrhizal plants, thus they also gain increased resistance to disease, drought, salinity and heavy metal stresses …

Mycorrhizas are currently classified into seven types: arbuscular, ectomycorrhiza, ectendomycorrhiza, arbutoid, ericoid, monotropoid, and orchid mycorrhiza (Smith and Read, 2010). Mycorrhizas are also commonly classified into two broad subdivisions the ectomycorrhiza and the others are endomycorrhizas by the fact that the hyphae of ectomycorrhizal fungi do not penetrate individual plant cells within the root, while the hyphae of endomycorrhizal fungi penetrate the cell wall and invaginate the cell membrane (Figure 1.1).

Endomycorrhizas include arbuscular mycorrhiza, orchid mycorrhiza, ericoid, ectendomycorrhiza, arbutoid and monotropoid types. The arbuscular mycorrhiza type is the most common whereas other endomycorrhizas are restricted to certain plant families: Orchidaceae, Ericaceae and sub-families.

Arbuscular mycorrhizas are the prominent and oldest type of mycorrhiza in land plants (Wang and Qiu, 2006). They are formed in an enormously wide variety of host plants by obligately symbiotic fungi of the phylum Glomeromycota (Smith and Read, 2010). Arbuscular mycorrhizal hyphae enter into the plant cells, producing vesicles or arbuscules (Peterson *et al.*, 2004). The fungal hyphae do not penetrate the protoplast but invaginate the cell membrane. The arbuscular structure greatly increases the contact surface between the hyphae and the cell cytoplasm to facilitate the transfer of nutrients between both partners. Extraradical hyphae colonise the soil surrounding the roots.

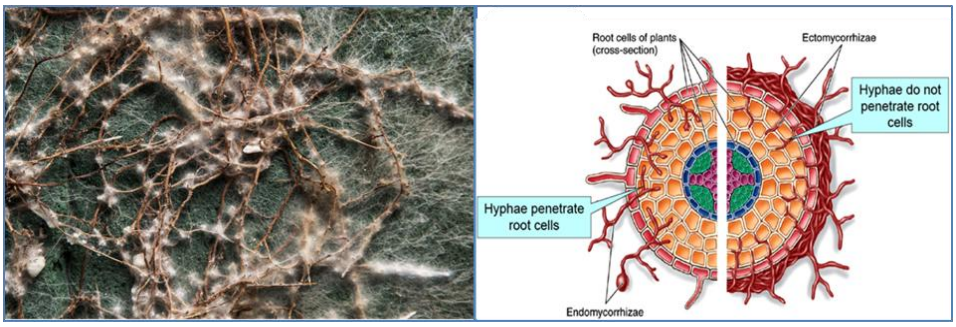


Figure 1.1. (Left) Pine roots colonized by *Suillus luteus.* (Right) Diagram showing the differences between endomycorrhiza and ectomycorrhiza.

Ectomycorrhizas consist of an intimate association between a living root tip and a basidiomycete or ascomycete fungus. The fungus forms a mantle (or sheath), which encloses the rootlet (Smith and Read, 2010). From the mantle, hyphae or rhizomorphs (root-like structures of certain fungi consisting of a dense mass of hyphae) radiate outwards into the soil. This extraradical mycelium increases the absorbing surface of the plant and forms the main interface between the soil solution and the plant (Rousseau *et al.*, 1994). Hyphae also penetrate inwards between the cortex cells of the root to form a network of hyphae called the Hartig net. The complex branching of hyphae in

the Hartig net increases the contact surface for the efficient exchange of nutrients (Smith and Read, 2010). The mantle is a unique part of the symbiosis because it makes contact with the root and the outer mantle is surrounded by soil. In addition to the Hartig net, the inner mantle might also be involved in the nutrient exchange. A compact mantle physically shields the plant roots from the soil therefore also minimizes water loss, reduces external stress and protects plant roots against pathogens or pollutants (Peterson *et al.,*2004).

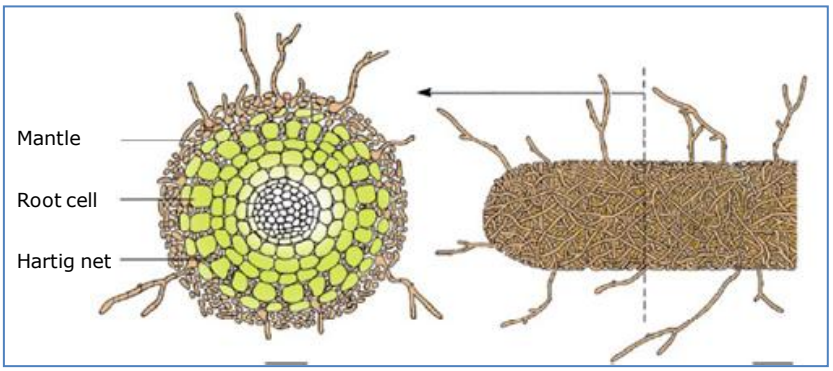


Figure 1.2. The structure of ectomycorrhizas. Ectomycorrhizas consist of an intimate association between a living root tip and a fungus. The fungus forms a mantle of fungal material around the root tip and penetrates the intercellular space between cortical root cells, forming a Hartig net (Landeweert *et al.*, 2001)

1.2. THE ECTOMYCORRHIZAL FUNGUS *Suillus luteus* AND HEAVY METAL TOLERANCE STUDIES

Suillus luteus is an ectomycorrhizal fungus in the genus *Suillus* within the order of Boletales (Figure 1.3). *Suillus luteus* is a typical pioneer fungus that forms symbiosis with the roots of young pine trees. The basidiocarp of *Suillus luteus* has a yellowish stalk with a distinctive ring and a slimy brown cap.

The fungus is relatively easy to culture in the laboratory. *Suillus luteus* mycelia can be grown in liquid or on solid synthetic media (Figure 1.4). Spores collected from basidiocarps from the field can germinate into monokaryotic strains.

Monokaryotic strains can be kept as such or can be crossed to generate dikaryotic strains. Dikaryotic strains often grow faster than monokaryotic strains.

The occurrence of *S. luteus* associated with pine trees in the pioneer forests at heavy metal polluted sites in Europe prompted a number of studies on the fungus metal tolerance (Colpaert *et al.*, 2000, Adriaensen *et al.*, 2005, Colpaert *et al.*, 2005, Krznaric *et al.*, 2009; Krznaric *et al.*, 2010). A number of ecotypes of *S. luteus* that are tolerant to zinc and cadmium have been found in heavy metal contaminated sites in the north-eastern part of Belgium (Krznaric *et al.*, 2009; Krznaric *et al.*, 2010). This area is heavily polluted with non-ferrous metals emitted by four pyrometallurgical zinc smelters that were active during the period between 1888 and 1974 (Colpaert *et al.*, 2004). Copper tolerant ecotypes have been isolated from a former copper mine in Central Norway (Adriaensen *et al.*, 2005). These metal tolerant ecotypes can effectively protect their host plants from metal toxicity both by sustaining mineral nutrition and by reducing metal transfer to the host.

Mechanisms of zinc and cadmium tolerance such as metal uptake, compartmentalization and exclusion have been addressed in several studies. Zinc and cadmium tolerance in *S. luteus* partially relies on a metal exclusion mechanism (Colpaert *et al.*, 2005; Colpaert *et al.*, 2011). Zinc tolerance and the function of two CDF (cation diffusion facilitator) transporters SlZnt1 and SlZnt2 have been studied, in which SlZnt1 might be responsible for transfer of zinc into the vacuole and SlZnt2 gene copy number seems to be correlated with zinc tolerance in *S. luteus* (Ruytinx, 2013a). Several candidates of the ZIP (zinc regulated transporter/iron-regulated transporter [ZRT/IRT1]-related protein), family of transporters in *S. luteus* are in the process of functional analysis (*personal communication*).

Molecular mechanisms underlying copper and cadmium homeostasis and detoxification of *S. luteus* especially the transporters and chelators are not yet known. In the last few years, the genomes of *S. luteus*, *S. brevipes* and some other Boletales species (Figure 1.5) gradually became available thanks to the 1000 fungal genomes project and the mycorrhizal genomics initiative of the Functional Genomics Program of the Department of Energy Joint Genome

Institute (JGI)(Grigoriev *et al.*, 2011; Grigoriev *et al.*, 2012; Kohler *et al.*, 2015). Based on this vast amount of bioinformatic resources, studies on molecular mechanisms of metal tolerance in *S. luteus* can progress further. The current study aimed to identify and investigate the function of several heavy metal transporters and chelators in *S. luteus* with special focus on copper homeostasis; when relevant, mechanisms of cadmium detoxification are discussed.

The *S. luteus* genome has only been assembled to the scaffold level. The genome assembly is composed of 649 scaffolds including 488 scaffolds of >= 2 Kbp and the three largest scaffolds of 1.38, 1.27, and 0.85 Mbp. The genome size and average number of coding genes of *S. luteus* are 37.01 and 18136, respectively (Figure 1.5). The *Suillus luteus* genome is compact in comparison to most boletales' genomes (Figure 1.5). The average genome sizes and number of genes of 69 Basidiomycota species analyzed by Mohana and Bae (2015) are 46.48 and 15431, respectively. The model ascomycete yeast *S. cerevisiae* genome size is smaller (~12 Mbp, ~6000 genes, 16 scaffolds).

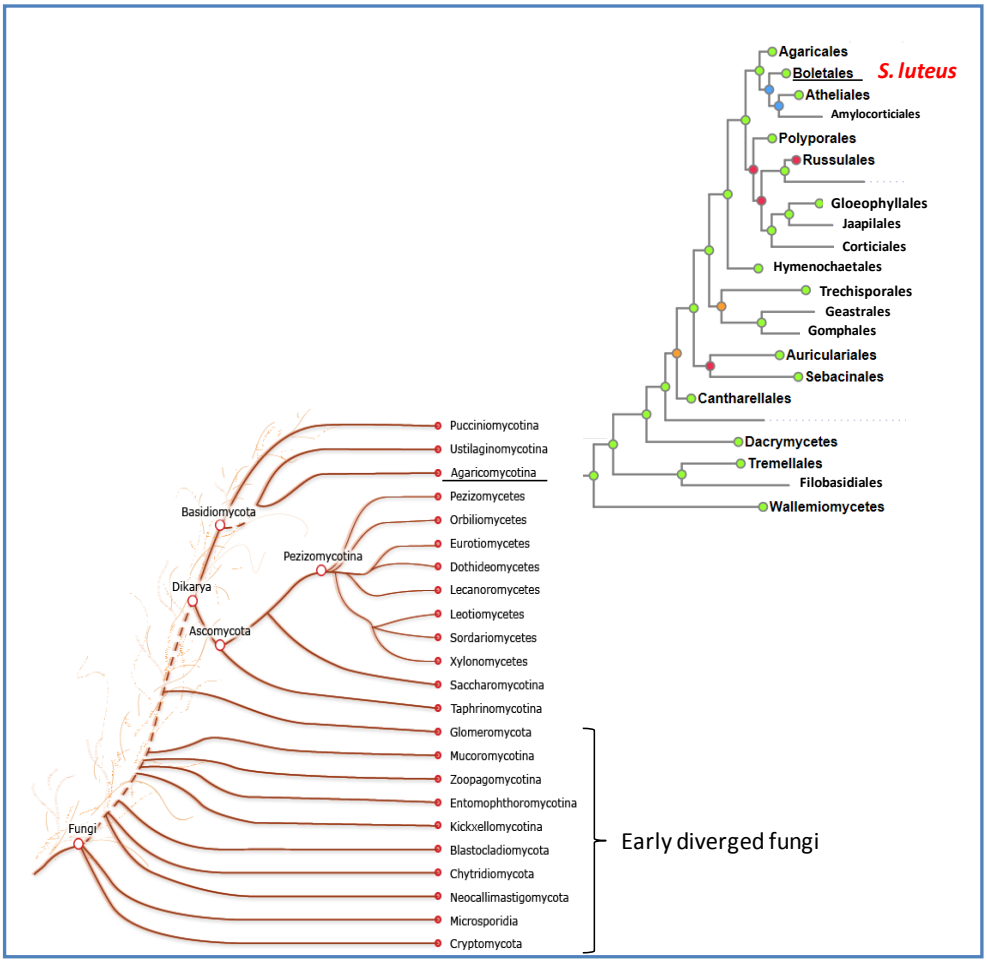


Figure 1.3. Phylogenetic tree showing the taxonomic position of *S. luteus.* The Kingdom fungi consists of the early diverged fungal lineages and the subkingdom Dikarya that includes the two phyla Ascomycota and Basidiomycota. Taxonomical orders in the subphylum Agaricomycotina are indicated; *Suillus luteus* belongs to the Boletales order. Phylogenetic trees were re-drawn from MycoCosm web portal of the Fungal Genomics Program of the Department of Energy Joint Genome Institute.

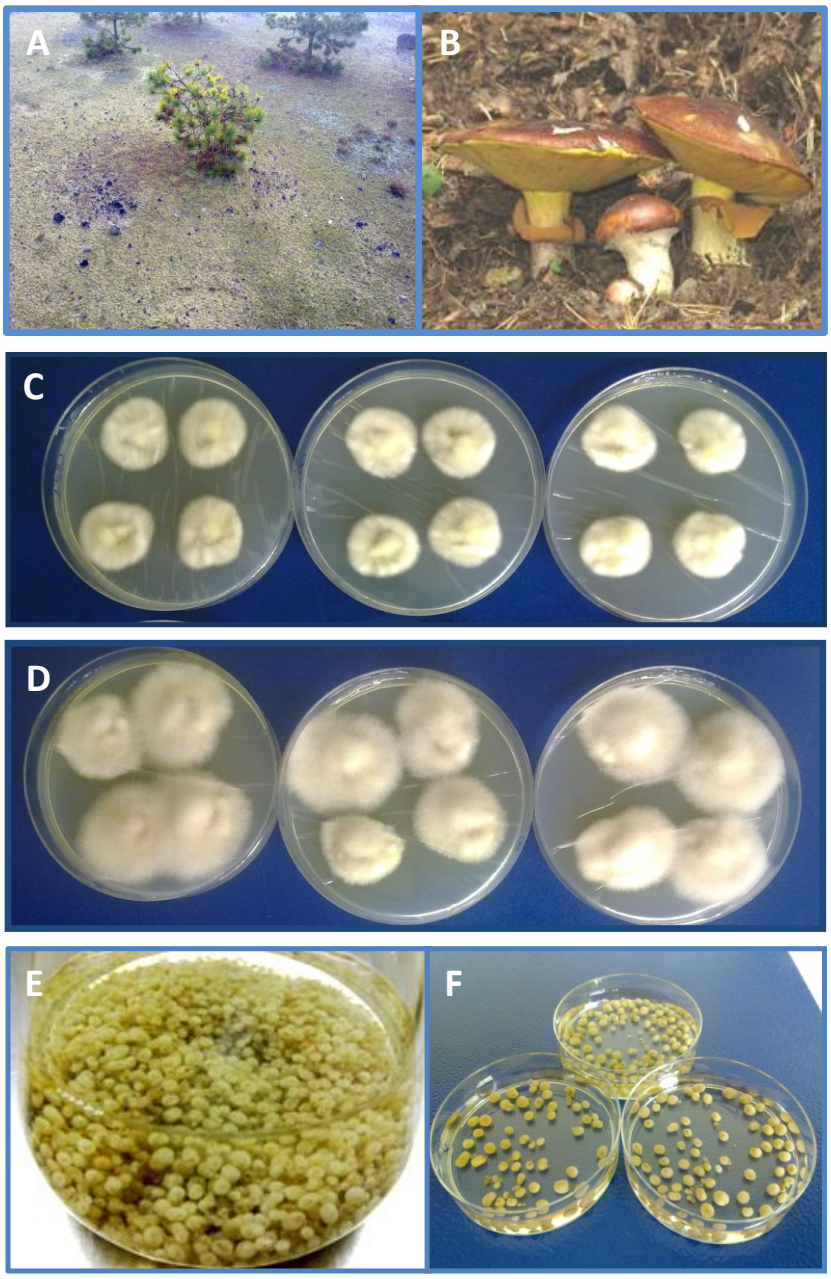
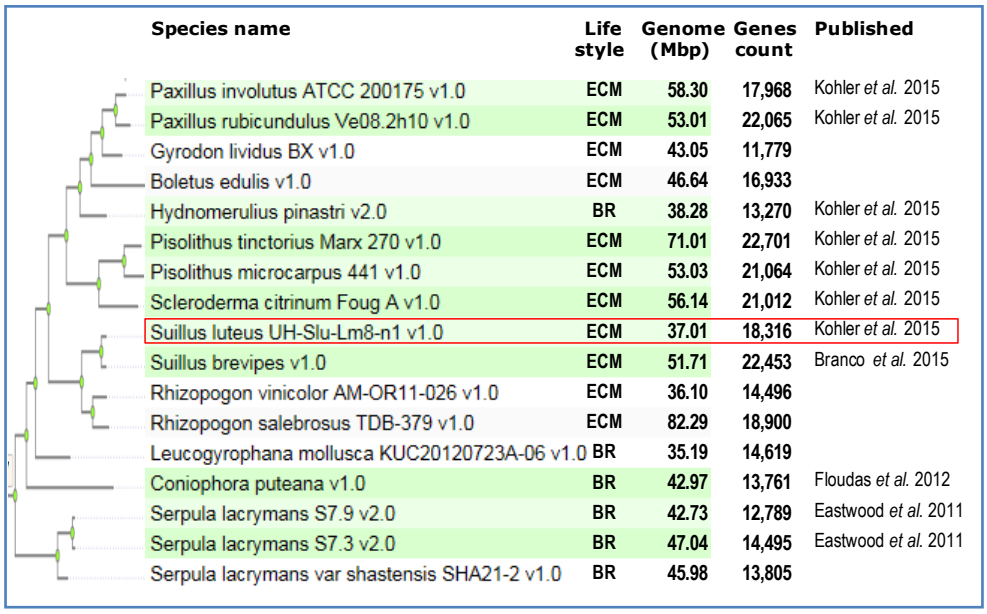


Figure 1.4. *Suillus luteus.* (A) a heavy metal polluted field in Lommel-Maatheide, north-eastern Belgium; (B) *S. luteus* sporocarps; (C) an 8-day-old monokaryon strain UH-Slu-Lm8-n1 and (D) dikaryon strain UH-Slu-Lm8 n+n on solid Fries medium; (E, F) 8-day-old fungal spheres in liquid Fries medium (E, F)



Species name	Life style	Genome (Mbp)	Genes count	Published
Paxillus involutus ATCC 200175 v1.0	ECM	58.30	17,968	Kohler *et al.* 2015
Paxillus rubicundulus Ve08.2h10 v1.0	ECM	53.01	22,065	Kohler *et al.* 2015
Gyrodon lividus BX v1.0	ECM	43.05	11,779	
Boletus edulis v1.0	ECM	46.64	16,933	
Hydnomerulius pinastri v2.0	BR	38.28	13,270	Kohler *et al.* 2015
Pisolithus tinctorius Marx 270 v1.0	ECM	71.01	22,701	Kohler *et al.* 2015
Pisolithus microcarpus 441 v1.0	ECM	53.03	21,064	Kohler *et al.* 2015
Scleroderma citrinum Foug A v1.0	ECM	56.14	21,012	Kohler *et al.* 2015
Suillus luteus UH-Slu-Lm8-n1 v1.0	ECM	37.01	18,316	Kohler *et al.* 2015
Suillus brevipes v1.0	ECM	51.71	22,453	Branco *et al.* 2015
Rhizopogon vinicolor AM-OR11-026 v1.0	ECM	36.10	14,496	
Rhizopogon salebrosus TDB-379 v1.0	ECM	82.29	18,900	
Leucogyrophana mollusca KUC20120723A-06 v1.0	BR	35.19	14,619	
Coniophora puteana v1.0	BR	42.97	13,761	Floudas *et al.* 2012
Serpula lacrymans S7.9 v2.0	BR	42.73	12,789	Eastwood *et al.* 2011
Serpula lacrymans S7.3 v2.0	BR	47.04	14,495	Eastwood *et al.* 2011
Serpula lacrymans var shastensis SHA21-2 v1.0	BR	45.98	13,805	

Figure 1.5. Genome size and predicted number of coding genes of Boletales fungi. The *S. luteus* genome assembly is composed of 649 scaffolds (including 488 scaffolds of >= 2Kbp and the three largest scaffolds of 1.38, 1.27, and 0.85 Mbp). For comparison, the model yeast *S. cerevisiae* genome size and number of coding genes are 12 Mbp and ~6000 genes. ECM: ectomycorrhiza; BR: brown rot.

Source: MycoCosm web portal of the Fungal Genomics Program of the Department of Energy Joint Genome Institute.

1.3. COPPER, CADMIUM AND ZINC IN CELLS

Organisms require essential heavy metals including copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), cobalt (Co) and molybdenum (Mo) to carry out biological functions. Other heavy metals including cadmium (Cd), lead (Pb), chromium (Cr), mercury (Hg) and arsenic (As) have no known physiological activity and are non-essential. Elevated levels of both essential and non-essential heavy metals result in toxicity symptoms.

Copper is a cofactor for a number of key enzymes that are essential for most living organisms. This redox-active metal forms the active site of the respiratory protein cytochrome *c* oxidase that is responsible for ATP production, of the superoxide dismutase that detoxifies the superoxide radical, and of a family of multicopper oxidases including ferroxidases that mediate the transport of iron across membranes. Fungi have additional multicopper oxidases including amine oxidases and laccases (Kues and Ruhl, 2011); the later have a crucial role in fungal morphogenesis, pathogenesis, stress defence, melanin biosynthesis and lignin degradation (Schulze and Rodel, 1989; Thurston, 1994; Walton *et al.*, 2005; Upadhyay *et al.*, 2013)

Moreover, a number of copper responsive transcription factors exist that regulate expression of a repertoire of transporters and metallothioneins that are responsible for copper homeostasis in various organisms from bacteria to humans (Rutherford and Bird, 2004; Rademacher and Masepohl, 2012). Some of these copper responsive transcription factors are also involved in homeostasis and detoxification of other metals such as iron via regulation of iron uptake in yeasts (Georgatsou *et al.*, 1997; Labbe *et al.*, 1999), or zinc and cadmium via regulation of certain metallothioneins that do not have strict metal specificity.

On the other hand, at high concentrations, copper is toxic to most living organisms. Copper has a high affinity for metal binding sites within proteins and can displace other metals such as iron or zinc (Macomber and Imlay, 2009; Waldron and Robinson, 2009). Copper binds to the thiol group of glutathione (GSH), the major antioxidant of the cell; it can cause glutathione oxidation and depletion hereby generating an oxidative environment (Bi *et al.*, 2007). Redox cycling between Cu^{+1} and Cu^{+2} produces the highly reactive hydroxyl radicals

(Speisky *et al.*, 2008). The production of these radicals leads to damage to lipids, proteins, DNA and other molecules (Halliwell and Gutteridge, 1985; Harrison *et al.*, 2000).

Cadmium is a non-essential heavy metal and a human carcinogen. Cadmium strongly interferes with cellular metabolism: it binds thiol groups, altering the activity of several enzymes. It causes deficiency of other essential metals by competing with them for the same carriers. Cadmium replaces zinc in zinc-finger proteins (found in transcription factors, DNA repair proteins, …) results in interference with various cellular processes involved in gene expression, growth regulation, and maintenance of the genomic integrity (Hartwig, 2001).

Cadmium toxicity is often associated with oxidative damage even though it is not a redox-active metal and does not directly produce reactive oxygen species. It has been proposed that cadmium initially binds to protein thiols in the inner mitochondrial membrane, inhibits respiratory chain reaction, therefore generates reactive oxygen species in mammalian and plant cells (Dorta *et al.*, 2003; Wang *et al.*, 2004a; Heyno *et al.*, 2008). Similar to copper, cadmium binds to thiol group of glutathione, leads to depletion of the reduced form of glutathione therefore generating an oxidative environment (Ercal *et al.*, 2001; Jozefczak *et al.*, 2012). Cadmium also competes with copper for binding sites on metallothioneins (Foster and Robinson, 2011; Palacios *et al.*, 2011) and replaces iron in proteins such as ferritin and transferrin (Casalino *et al.*, 1997; De Smet *et al.*, 2001) consequently increasing the amount of free redox-active iron and copper in the cells.

Zinc is an essential metal for all organisms, playing an important structural and catalytic role in a number of proteins (Eide, 2006; Porcheron *et al.,* 2013). Zinc is the second most abundant transition metal ion in living organisms after iron (Porcheron *et al.*, 2013). The approximation of intracellular zinc concentrations is ranging from 0.1-0.5 mM and the vast majority of zinc is bound to proteins (Eide, 2006). Physiologically important zinc binding proteins include zinc-finger proteins that are abundant and have diverse functions in cells such as DNA recognition, transctriptional activation, protein folding, … (Laity *et al.*, 2001). Another example is Cu/Zn superoxide dismutase that is an important antioxidant

protecting cellular components from reactive oxygen species (Fridovich, 1995). Although zinc is not redox active, excess zinc can be toxic to cells (MacDiarmid *et al.,* 2002). Zinc toxicity may be mediated via binding of the cation to inappropriate sites in proteins or cofactors (Eide, 2006). In fungi and plant cells, the major site of zinc sequestration and detoxification is the vacuole (Simm *et al.*, 2007; Ruytinx *et al.*, 2013b). Zinc sequestered within the vacuole also serves as a storage pool of zinc that can be mobilized under zinc-deficient conditions for use by the cell (Simm *et al.*, 2007).

1.4. HEAVY METAL TRANSPORTERS IN CELLS; THE P1B-TYPE ATPASE FAMILY IN THE PLANT GENUS *Arabidopsis*

Heavy metal transporters in cells: in order to prevent the potential toxicity of metal ions while maintaining the physiological concentrations, all living organisms possess mechanisms to regulate the distribution of metal ions in cellular compartments and to minimize the damage from exposure to non-essential ones. The main cellular mechanisms of heavy metal homeostasis and detoxification include mechanisms that reduce uptake into the cytosol by extracellular chelation or binding onto cell-wall components, efflux from the cytosol, intracellular chelation of metals in the cytosol by a range of ligands such as glutathione or metallothioneins, or efflux from the cytosol into sequestering compartments such as vacuoles in plants and fungi (Clemens, 2001; Hall, 2002; Bellion *et al.*, 2006, summarized in Figure 1.6). These mechanisms require the participation of different families of metal transporters, chaperones and metal chelators. A number of membrane transport protein families have been involved in metal transport; these include the heavy metal P1B-type ATPases (or CPx-type ATPases), the NRAMPs (natural resistance-associated macrophage proteins), the CDF (cation diffusion facilitator), the ZIP family (zinc regulated transporter/iron-regulated transporter [ZRT/IRT1]-related protein), CaCA/CAX (Ca^{2+}/ cation antiporter/ cation exchanger), CCX (calcium cation exchanger), OPT/YSL (oligopeptide/yellow-stripe-like transporter), MATE (multidrug and toxic compound extrusion), COPT/Ctr (copper transporter), … (Williams *et al.*, 2000; Clemens, 2001; Hall, 2002). Besides, ATP-binding cassette (ABC) transporter

family is involved in metal efflux into the vacuoles via transporting of conjugates of cadmium with glutathione (GSH) or with phytochelatins in model yeasts (Li *et al.*, 1996*;* Ortiz *et al.*, 1992).

Genes encoding homologs of diverse families of metal transporters are present in *S.luteus* genomes (Kohler *et al.*, 2015). All transporter families might be involved in heavy metal transport and tolerance to certain extent depending on metal specificity, localization, direction of the transporters, ... Our interest focuses on the P1B-type ATPase family. This transporter family have been found in a wide range of organisms from bacteria, achaea to eukarya (Axelsen and Palmgren, 1998; Williams *et al*., 2000). In fungi, only few P1B-type ATPase transporters have been found. Except the Pca1 in *S. cerevisiae* that exports cadmium at the yeast plasma membrane (Adle *et al.*, 2007), other transporters are involved in copper transport in the model yeasts, the basidiomycete *Trametes versicolor* (Ulschmid *et al*., 2003), in the basidiomycete and human pathogen *Cryptoccocus neoformans* (Walton *et al.*, 2005), and in a few ascomycete pathogens (Parisot *et al.*, 2002; Saitoh *et al.*, 2009; Saitoh *et al.*, 2010; Upadhyay *et al.*, 2013).

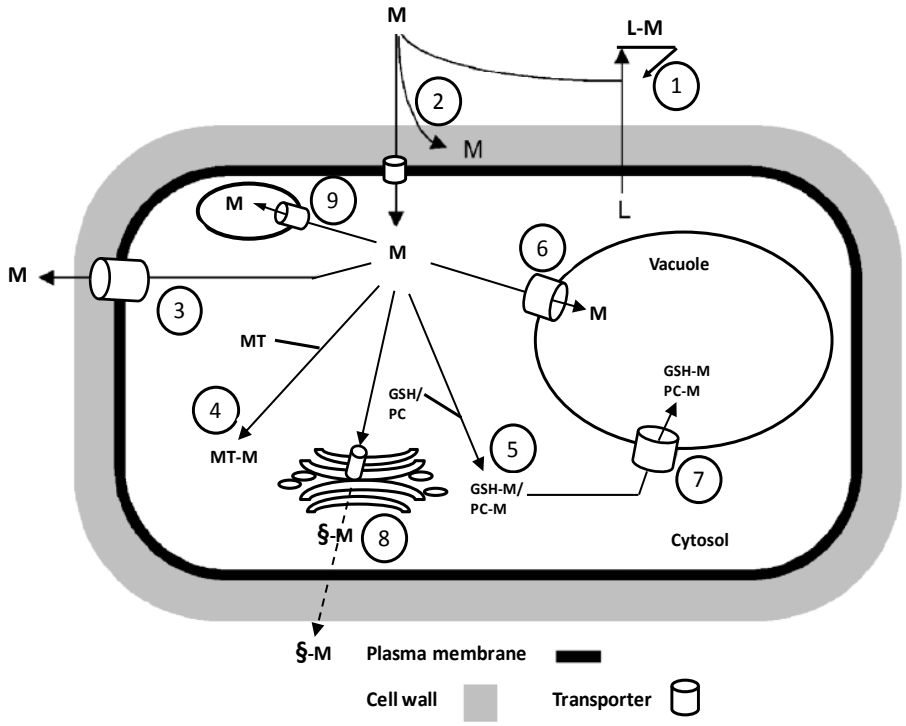


Figure 1.6. Cellular mechanisms involved in the traffic of metals in a cell showing the involvement of different types of transporters and chelators. M, metal-ion; 1, extracellular chelation by excreted ligands (L); 2, cell-wall binding; 3, metal efflux; 4, intracellular chelation by metallothionein (MT); 5, intracellular chelation by glutathione (GSH) or phytochelatins (PC); 6, vacuolar compartmentation; 7, vacuolar compartmentation of GSH-M or PC-M complex; 8, exportation into the Golgi to incorporation into secretory proteins; 9, compartmentation in other internal compartments. *Modified after Hall et al., 2002* and *Bellion et al., 2006.*

The P1B-type ATPase family of transporters in *Arabidopsis*: in the model plant *Arabidopsis thaliana,* P1B-type ATPase transporters are abundant and have been studied intensively. The *A. thaliana* transporters are introduced here as examples of the different types of transporters, their metal specificity and localization.

P-type ATPases form a large superfamily of enzymes which use ATP to pump a wide range of cations (and possibly phospholipids) against their electrochemical gradient across membranes (Axelsen and Palmgren, 1998; Williams *et al.*, 2000). They are called P-type because of a shared enzymatic mechanism involving a phosphorylated reaction cycle intermediate. The enzymes are also known as E1-E2 ATPases because they appear to interconvert between at least two different conformations, denoted by E1 and E2 (Figure 1.7).

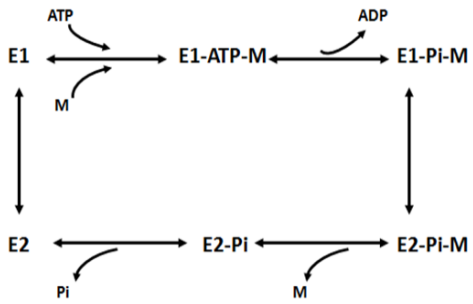


Figure 1.7. Catalytic mechanisms of P1B-type ATPases. The first step is the binding of ATP and metal ion (E1-ATP-M state of the enzyme). The transfer of the phosphate of ATP to an Aspartic residue in the protein induces a conformational change (intermediate state E1-Pi-M) which allows the metal ion into the intramembranous channel created by the transmembrane helices. The protein undergoes a further conformational change to the E2-Pi-M state, which allows the release of the metal ion on the other side of the intramembranous channel. After the release of the metal ion, the protein is left in its E2-Pi state. The protein is then de-phosphorylated leading to E2 state.

The P1B-type ATPase subfamily is involved in the transport of heavy metals (Cu, Zn, Co, Cd, Ag and Pb), and therefore, the ATPases in this subfamily have also been referred to as HMAs (Williams and Mills, 2005). The model plant *A. thaliana*

has eight HMAs (Figure 1.8). Monovalent metal ions, such as Cu^{+1} and Ag^{+1}, are transported by HMA5–8 (Andres-Colas *et al.*, 2006; Binder *et al.*, 2010; Sautron *et al.*, 2015), whereas divalent metal ions, such as Zn^{+2} and Cd^{+2}, are transported by HMA2, 3 and 4 (Eren and Argüello, 2004; Mills *et al.*, 2005). *A. thaliana* HMA3 also transports Co^{+2} and Pb^{+2} (Morel *et al*, 2009) The HMAs of *A. thaliana* function at different subcellular locations and efflux heavy metal ions from the cytosol, either into the apoplast, the vacuole, or other organelles; HMAs are therefore involved in numerous physiological processes in the plant.

Moreover, studies on the *A. thaliana* sister species and Zn/Cd hyperaccumulator *A. halleri* have shown that the plant Zn/Cd hypertolerance was associated with enhanced *HMA4* gene expression that was the result of tandem gene triplication and altered *cis* regulation (Hanikenne *et al.*, 2008). Similarly, in another Brassicaceae, *Noccaea caerulescens*, tandem duplication of *NcHMA4* also contributes to Zn/Cd hyperaccumulation and tolerance (Craciun *et al.*, 2012). Results of these studies indicate the important role of P1B-type ATPase transporters in heavy metal tolerance in plants. On the contrary, very little is known about the function of P1B-type ATPases in fungi especially in basidiomycete fungi.

Referring to the known transporters and pathways in the model species *S. cerevisiae* (see below) and *A. thaliana*, an inventory and comparative analysis of P1B-type ATPases in basidiomycete fungi was performed and is reported in Chapter 2.

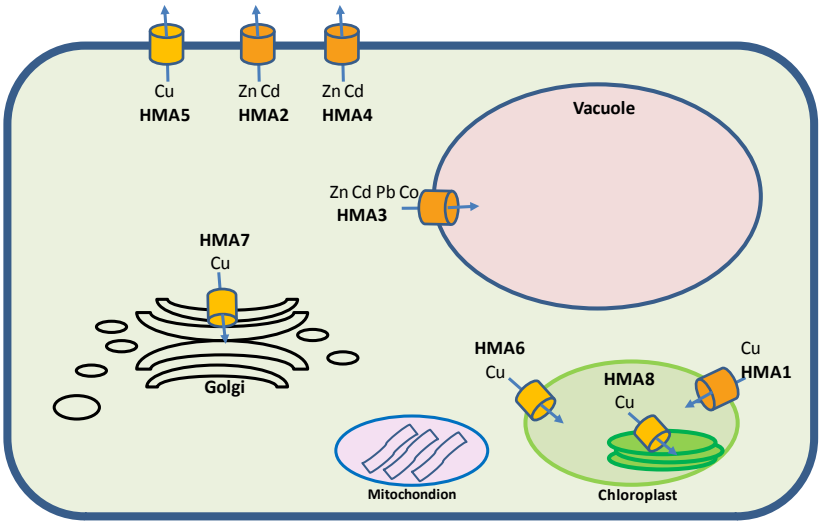


Figure 1.8. Sub-cellular locations and metal specificity of eight P1B-type ATPases (HMA1-8) in the model plant *Arabidopsis thaliana*. AtHMA1 and AtHMA6 located at the chloroplast-envelope import Cu (Boutigny *et al*., 2013). AtHMA2 and AtHMA2 4 function at the plasma membrane and is responsible for Cd and Zn efflux (Eren and Argüello, 2004; Mills *et al.*, 2005). AtHMA3 is responsible for vacuolar storage of Zn, Cd, Pb and Co (Morel *et al.*, 2009); AtHMA5 located at the plasma membrane functions in copper export/detoxification (Andres-Colas, 2006); AtHMA7 transports Cu into the post-Golgi compartment (Hirayama *et al.*, 1999). AtHMA8 located in the thylakoid membrane delivers Cu to Cu-requiring proteins. *Adapted from Williams* and *Mills*, *2005.*

1.5. HEAVY METAL CHELATORS IN CELLS

Despite the relative specificity of the transmembrane metal transporters, both essential and non-essential metals are transported into the cells. Because even the essential metals can be toxic when present in excess, their cellular concentrations are tightly regulated (Ballatori, 2002). It has been shown that total cellular concentrations of zinc or copper are in the millimolar and micromolar range, respectively, cytosolic free zinc and copper ion concentrations is in the femtomolar (10^{-15}) and zeptomolar (10^{-21}) range (far less than one free atom per cell) (Outten and O'Halloran, 2001; Changela *et al.*, 2003; Krämer *et al.*, 2007). This indicates an extraordinary intracellular metal-binding capacity. Metal chelators that buffer cytosolic metal concentrations can be amino acids (cysteine, histidine and proline), peptides (glutathione, phytochelatins), proteins (metallothioneins), polyphenols and phytates, … These chelators have been well studied in plants, especially in the hyperaccumulators (Leitenmaier and Küpper, 2013).

The best documented chelators for copper, cadmium and zinc are **metallothioneins**. Metallothioneins are low molecular weight cysteine-rich proteins that have been found in many species from prokaryotes to humans (Capdevila *et al.*, 2012). Genes coding for metallothioneins have been identified in several fungi. In Chapter 4, the identification and characterization of a novel family of metallothioneins in basidiomycete fungi is reported.

Moreover, specifically for copper, the **copper chaperones** have an essential role in transport and homeostasis. These chaperones bind copper ions and deliver them to particular target proteins where they function as part of the enzymatic activity (see the example with *Saccharomyces cerevisiae* below). Moreover, they transfer copper to specific membrane transporters that evacuate the metal to the extracellular space or into sub-cellular organelles (Harrison *et al.*, 2000).

In addition to proteins, **glutathione** is highly abundant in the cytoplasm and is known to bind both copper and cadmium (Ercal *et al.*, 2001; Bi *et al.*, 2007). In a recent study in human cell lines, it is suggested that glutathione, but not

copper chaperones, is the first intracellular acceptor of copper in the cells (Maryon *et al.*, 2013). The delivery of copper from glutathione to copper-binding proteins has also been suggested previously (Freedman *et al.*, 1989; Ferreira *et al.*, 1993; Banci *et al.*, 2010a). Glutathione is an essential component of cadmium detoxification in various organisms (Howden *et al.*, 1995; Hall, 2002). Especially in plants under heavy metal stress, glutathione plays a key role as an antioxidant metabolite and as a precursor of **phytochelatins**, another important heavy metal scavenger in the cells (Cobbett and Goldsbrough, 2002; Jozefczak *et al.*, 2012). Both glutathione and phytochelatins have been extensively studied in plants, especially in relation to cadmium tolerance (Cobbett and Goldsbrough, 2002). There is less information on the role of these metal chelators in fungi (except in the model yeasts). An increase in glutathione content has been found in the ectomycorrhizal fungus *Paxillus involutus* exposed to cadmium (Courbot *et al.*, 2004). Only few fungi produce phytochelatins in response to cadmium; examples are *Schizosaccharomyces pombe* (Ha *et al.*, 1999), *Candida glabrata* (Mehra *et al.*, 1994) and *Sporobolomyces sp.* (Shine *et al.*, 2015). Phytochelatins are enzymatically synthesized from glutathione by phytochelatin synthase (Grill *et al.*, 1989). In a recent analysis of genome sequences of fungi, it has been shown that phytochelatin synthase homologs are present in several early diverged fungi but not in ascomycetes and basidiomycetes (Shine *et al.*, 2015). These findings indicate that other mechanisms, for example chelation by metallothioneins, might have taken over the role of phytochelatins in metal chelation and detoxification in ascomycetes and basidiomycetes.

1.6. COPPER HOMEOSTASIS IN THE MODEL YEAST *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was the first eukaryotic genome to be completely sequenced. Molecular and biological functions of many genes in *S. cerevisiae* are intensively studied. Accordingly, copper homeostasis in yeast has been studied in great detail. Figure 1.9 shows the copper trafficking in a simplified yeast cell. Following transport via the high affinity copper transporters Ctr1p (Dancis *et al.*, 1994) and Ctr3p (Peña *et al.*, 2000), copper is delivered to various cellular

destinations. A third copper transporter Ctr2p localized on the vacuolar membrane can export stored copper to the cytoplasm (Rees *et al.*, 2004). A number of other well-studied proteins and small molecules participate in copper trafficking in yeast cell. The copper chaperones Atx1p, Cox17p and CCS1 specifically deliver copper to Ccc2p, Sco1p/Cox11p in the mitochondria and to Cu, Zn-superoxide dismutase (Culotta *et al.*, 1997; Pufahl *et al.*, 1997; Heaton *et al.*, 2000).

The *S. cerevisiae* Ccc2p is a P1B-type ATPase transporter that is localized in the post Golgi and delivers copper to enzymes in the secretory pathway (Lowe *et al.*, 2004). Properly functioning Ccc2p is essential for copper incorporation into multicopper oxidases such as ferroxidase Fet3p in the Fet3p/Ftr1p complex that is responsible for high-affinity iron uptake (Taylor *et al.*, 2005). Even though it is not emphasized in *S. cerevisiae*, in some other fungi, orthologs of Ccc2p are crucial for copper incorporation into laccases, a very abundant type of multicopper oxidases in filamentous fungi (Uldschmid *et al.*, 2003; Saitoh *et al.*, 2009).

Copper is mobilized to the mitochondria by Cox17p (Heaton *et al.*, 2000). Two additional mitochondrial inner membrane proteins, Sco1p and Cox11p are implicated in copper ion insertion into cytochrome *c* oxidase (Schulze and Rodel, 1989; Horng *et al.*, 2004). In the cytosol, copper is delivered to the Cu, Zn-superoxide dismutase Sod1p by the CCS1 copper chaperone (Culotta *et al.*, 1997). The Pca1p transporter, a P1B-type ATPase, is localized on the plasma membrane and exports cadmium and can also sequester copper (Adle *et al.*, 2007). It is worth noting that, even though it belongs to the same family as *S. cerevisiae*, the human pathogen yeast *Candida albicans* does not have a Pca1p transporter. Instead, *C. albicans* has a P1B-type ATPase transporter (Crd1p) that is localized on the plasma membrane and is responsible for copper efflux (Riggle and Kumamoto, 2000a); this indicates the presence of different types of copper transporters in fungi.

Two metallothioneins Cup1p and Crs5p are present in yeast and are believed to store and buffer excess copper in vivo (Fogel and Welch, 1982; Culotta *et al.*, 1994). Increased copy number of the *CUP1* gene results in high copper tolerance

(Fogel and Welch, 1982; Adamo *et al.*, 2012). Expression of the *CUP1* gene is regulated by the copper-binding transcription factor Cup2p (synonym Ace1p) which serves as the primary sensor of intracellular Cu^{+} in yeast (Buchman *et al.*, 1989). Both deletion mutants *cup1* and *cup2* are copper sensitive and are often used in the yeast complementation assays to investigate the function of a protein in copper tolerance. In addition to proteins, glutathione is highly abundant in the cytoplasm and is known to bind copper in yeast (Bi *et al.*, 2007).

In *S. cerevisiae*, Ace1p and Mac1p are two well-studied transcription factors involved in copper homeostasis. Ace1p is responsible for the induction of metallothioneins and superoxide dismutase expression in response to stressful levels of copper (Buchman *et al.*, 1990), whereas Mac1p activates a subset of genes under copper-deficient conditions (Jungmann *et al.*, 1993; Labbe *et al.*, 1997).

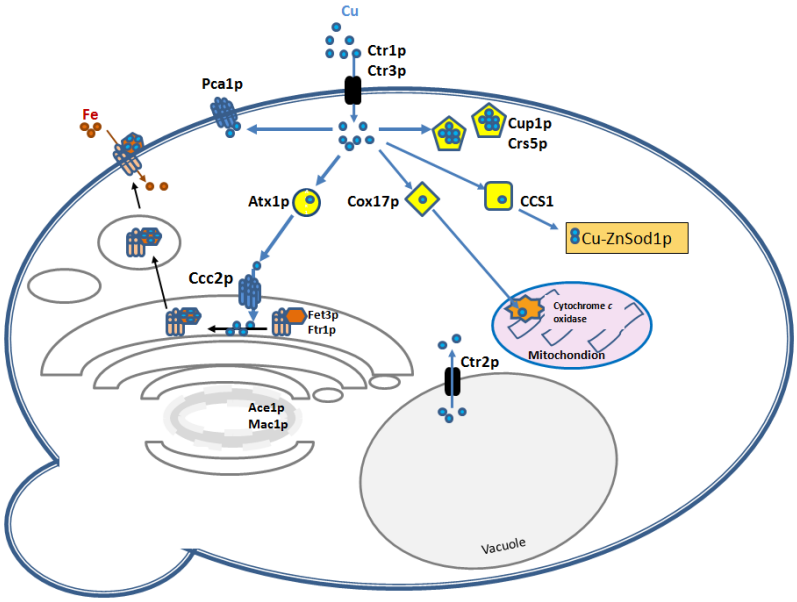


Figure 1.9. Overview of copper trafficking in *S. cerevisiae.* Copper transporters: Ctr1p, Ctr3p and Ctr2p; copper transporting ATPases: Ccc2p and Pca1p; copper chaperones: Atx1p, Cox17p and CCS1; Copper chelators: metallothionein Cup1p and Crs5. Transcription factors involved in copper homeostasis: Ace1p and Mac1p. Fet3p/Ftr1p complex involved in iron uptake is one of the copper-dependent proteins of *S. cerevisiae*

1.7. OBJECTIVES OF THE STUDY

This study aimed to identify and investigate the function of the heavy metal transporting P1B-type ATPases (HMAs) and heavy metal chelators in *S. luteus* with special focus on copper homeostasis.

In this book the followings are reported:

First, an inventory and comparative analysis of different HMAs in basidiomycete fungi (Chapter 2).

Second, functional characterization of the three HMAs in *S. luteus* (Chapter 3).

Third, identification of a novel family of metallothioneins in basidiomycete fungi and characterization of two metallothionein genes of *S. luteus* (Chapter 4).

Chapter 1

CHAPTER 2

Occurrence and diversity of P1B-type ATPases in basidiomycete fungi: identification of putative copper- and zinc-transporting ATPases

2.1. ABSTRACT

The P1B-type ATPases are a subfamily of P-type ATPases that are involved in the transport of heavy metals (Cu, Zn, Cd, Co, Ag and Pb) and the ATPases in this family have also been referred to as HMAs (heavy metal associated). The HMAs are found in bacteria, archaea and eukarya and are suggested to have evolved early in evolution. In fungi, except for those found in the model yeast species, only few HMAs have been identified. We, therefore, conducted a phylogenetic analysis of 345 putative HMA sequences found in 132 genomes of basidiomycetes and of 17 early diverged species of the kingdom Fungi. The HMAs in basidiomycetes subdivide to form three distinct clusters. The three clusters are: CCC2-, CRD1-, and PCA1-type HMAs which have members that are homologous to the *Saccharomyces cerevisiae* Ccc2, Pca1 and the *Candida albicans* Crd1 transporters. So far, only two HMAs belonging to the CCC2-type cluster have been experimentally characterized in basidiomycetes. Most fungal genomes appeared to have at least one HMA of the CCC2-type. Members of the CCC2-type cluster are well conserved and are likely involved in Cu export from the cytosol into the ER-Golgi for secretion. The second cluster –'CRD1-type'- is fungal specific. Members of this cluster are homologous to the ascomycete *C. albicans* Crd1 which is a Cu exporter at the cell plasma membrane. The CRD1-type HMAs are also ubiquitous in basidiomycetes. The third cluster - PCA1-type - has members homologous to the *S. cerevisiae* Pca1 which is a Cd exporter at the plasma membrane. Sequence and phylogenetic analysis identified two sub-groups of PCA1-type HMAs. One sub-group with members that are unique for Polyporales species and have all characteristics of Cu-transporting ATPases. The other sub-group are limited in occurrence and sequence analysis reveals their

characteristics as novel Zn/Co/Cd/Pb transporting HMAs. We propose a further functional characterization of the CRD1- and PCA1-type HMAs, especially the Zn-transporting ATPase HMAs. The occurrence of the three HMA types in basidiomycetes, detailed sequence analysis as well as possible evolutionary relationship with other HMAs in Fungi are discussed.

2.2. INTRODUCTION

Fungi are ubiquitous in the natural environment and play key roles in decomposition, nutrient cycling and transformation of metals. Fungi encounter metals as normal components of the natural environment (Gadd, 2007). Like other organisms, some metals (Cu, Zn, Fe, Mn, …) are essential for fungal metabolism, whereas others have no known biological role (Cd, As, Pb, Hg, Ag, …). Both essential and non-essential metal ions can be toxic when present in excess (Tennstedt *et al.*, 2009). Because of the potential toxicity of metal ions, all living systems possess mechanisms to tightly regulate the distribution of metal ions and to minimize damage under condition of excess metal supply (Eide, 2006). These mechanisms require the participation of different families of metal transporters, chaperones and metal chelators. These include the heavy metal P1B-type ATPases (or CPx-type ATPases), the NRAMPs (natural resistance-associated macrophage proteins), the CDF (cation diffusion facilitator) and the ZIP families (ZRT, IRT-like Protein) (Williams *et al.*, 2000; Hall, 2002).

The P1B-type ATPases are a subfamily of P-type ATPases that pump heavy metal ions (Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, or Cu^{+}) across biological membranes (Williams and Mills, 2005). In plants, P1B-type ATPases are also called HMAs (for heavy metal-associated or heavy metal ATPases). Eight HMA genes in *Arabidopsis thaliana* and nine in rice have been found (Mills *et al.*, 2005; Takahashi *et al.*, 2012). In contrast, only few HMAs have been found in fungi. *Saccharomyces cerevisiae* has two heavy metal ATPases: the Ccc2 localized on post-Golgi membrane transports Cu (Yuan *et al.*, 1997) and the Pca1 protein found on plasma membrane exports Cd and also sequesters Cu via its cysteine-rich N-terminal domain (Adle *et al.*, 2007). *Candida albicans,* the human

pathogenic yeast, possesses Crd1 (for Cu resistance determinant; synonym: Cu resistance protein CaCrp1) that functions as a Cu exporter assuring the yeast's high Cu tolerance (Riggle and Kumamoto, 2000a; Weissman *et al.*, 2000).

Up to present, only few HMAs in other fungi have been identified and all of them are homologous to the *S. cerevisiae* Ccc2. Examples are the Cu-transporting ATPase CtaA of the basidiomycete *Trametes versicolor* (Uldschmid *et al.*, 2003) and the Ccc2 of the human pathogen *Cryptococcus neoformans* (Walton *et al.*, 2005). A few homologs of *S. cerevisiae* Ccc2p have been found in ascomycete fungi such as the CLAP1 of *Colletotrichum lindemuthianum* (Parisot *et al.*, 2002), the Ccc2 of the plant pathogens *Cochliobolus heterostrophus* and *Botrytis cinerea* (Saitoh *et al.*, 2009; Saitoh *et al.*, 2010) and the CtpA of *Aspergillus fumigatus* (Upadhyay *et al.*, 2013). These HMAs are essential for Cu homeostasis, laccases and melanin biosynthesis and are involved in growth, morphogenesis and in the process of infection by these pathogenic fungi.

To our knowledge, except for the characterization of Crd1 (or Crp1) in *C. albicans* by two independent groups (Riggle and Kumamoto, 2000a; Weissman *et al.*, 2000), there is no report on functional characterization of Crd1 orthologs in other fungi. Accordingly, other than Cu, HMAs that transport Zn and other metals are common in bacteria and plants but have not been found in fungi (Saitoh *et al.*, 2009; Blindauer, 2015).

Thanks to the 1000 fungal genome project, more and more genomic and transcriptomic data now become accessible (Grigoriev *et al.*, 2011; Grigoriev *et al.*, 2012; Kohler *et al.*, 2015). The availability of multiple genome sequences provides useful opportunities to compare genomes of closely related fungi; this greatly facilitates accurate annotation, re-annotation, and discovery of new genes.

In the fungal kingdom, the phylum Basidiomycota contains roughly 30,000 species including many important saprotrophs (decay, brown and white rot fungi), beneficial mycorrhizas, as well as destructive plant pathogens (Hibbett, 2006). Understanding how these fungi handle metals is essential for effective control and application of fungi in many aspects of human life.

This study focuses on the identification of P1B-type ATPases in basidiomycete fungi. To understand the diversity and distribution of this protein family, we performed a comprehensive analysis, extracting all HMA-like sequences from current database of basidiomycete fungi, followed by manual curation. Clustering analysis was used to group the sequences, and further analysis of P1B-type ATPase conserved motifs was used to improve this grouping scheme and predict gene functions.

2.3. MATERIALS AND METHODS

2.3.1. Dataset generation

To search for protein sequences of putative HMAs, the protein sequence of the known HMA of *C. albicans* Crd1 was used as query for multiple BLASTp in all basidiomycete genomes available in the MycoCosm portal at the website of the Fungal Genomics Program of the Department of Energy Joint Genome Institute (JGI) (Grigoriev *et al.*, 2012). Five best BLASTp matches for each fungal species were retrieved for further screening. The Crd1 sequence was used as query in order to obtain matches with a long N-terminal sequence where all metal binding motifs GMXCXXC (where X denotes any amino acid residue), CXXC, or CC are expected to be situated. This is important because computer annotated HMAs often lack these motifs when the amino acid methionine (M) in GMXCXXC is mistakenly used as the starting methionine of a protein sequence. Putative HMAs of early diverging species of Fungi were also retrieved for phylogenetic analysis.

All predicted protein sequences were manually checked and when necessary the position of N and C-termini were adjusted. Proteins had to satisfy the following criteria to be accepted for the phylogenetic analysis: (1) contain at least one metal-binding domain at the N-terminus, (2) contain all signature motifs of heavy metal ATPases: the TGEX conserved motif of the phosphatase domain, the CPX motif of the translocation domain, the DKTGT of the ATP binding domain.

Transmembrane domains were predicted using TMHMM Server v.2.0 (http://www.cbs.dtu.dk).

The bacterial Cu-transporting ATPase (copA), the yeast *C. albicans* CaCcc2 and CaCrd1 and *S. cerevisiae* ScCcc2, ScPca1 were retrieved from the National Center for Biotechnology Information website (NCBI) and the *Saccharomyces* genome database (www.yeastgenome.org).

2.3.2. Phylogenetic analysis

Protein sequence alignment and neighbour-joining phylogenetic analysis with Jukes-Cantor distance measure using 1000 bootstrap replicates were performed with the CLC main workbench 7.0.2 (http://www.clcbio.com). First, all retrieved protein sequences were aligned and a phylogenetic tree was constructed. The bacterial copA was used as an out-group. Second, sequences belonging to each of the three groups of HMAs were aligned and phylogenetic trees were constructed separately. Yeast HMAs representing each group were used as an out-group.

The current taxonomic tree of basidiomycete fungi was obtained from the JGI MycoCosm portal and re-drawn so that only the species that were used for the analysis are shown.

2.4. RESULTS

The goal of our survey is to retrieve all types of HMAs from sequenced genomes of basidiomycete fungi (last accessed in March 2015). After eliminating apparent truncated and disrupted sequences, we analyzed 345 sequences found in genomes of 132 basidiomycetes and 17 early diverging species (taxonomic position of the early diverging fungi are indicated in Chapter 1, Figure 1.3). All sequences have the structural characteristics of P1B-type ATPases: contain 8 transmembrane domains, DKTGT, CPC, TGEX motifs and at least one metal-

binding motif (MBD) GMXCXXC. Results of the phylogenetic analysis show that basidiomycete fungi had three main types of HMA proteins: CCC2-, CRD1- and PCA1-type, whose names are adopted from the yeast homologs (Figure 2.1). Table 2.1 shows the types of HMA proteins found in each fungal species. For some species, two HMAs of the same type were found. In the phylogenetic trees and in Table 2.1, species and order names of the three subphyla of Basidiomycota are indicated. Subphylum Pucciniomycotina, Ustilaginomycotina and five large orders of subphylum Agaricomycotina are indicated in different colours; other orders are indicated in black. In order to understand the divergence of the three types, putative HMAs of the early diverging lineages of the kingdom Fungi were included in the analysis and indicated in orange.

Figure 2.2 shows the types and number of copies of HMAs found in each species rearranged on the current taxonomic tree of basidiomycete fungi. The results indicate that CCC2-type HMAs (violet diamond) are present in all species analyzed and two HMAs were found in the genome of *Plicaturopsis crispa*, *Rhizoctonia solani* and *Dioszegia cryoxerica* (Figure 2.2). The CRD1-type HMAs (open square) were found in most basidiomycete genomes and several genomes have more than one copy. The PCA1-type HMAs were found in all species of order Polyporales (open circle) and in some species in other orders within the subphyla Agaricomycotina and Pucciniomycotina (red triangle). Most early diverged species used in this analysis also possess CCC2-type HMAs that are clustered with the CCC2-type HMAs of the basidiomycetes (Figure 2.1). In addition to that, early diverged species possess a second HMA that cluster separately from the CRD1- and PCA1-types.

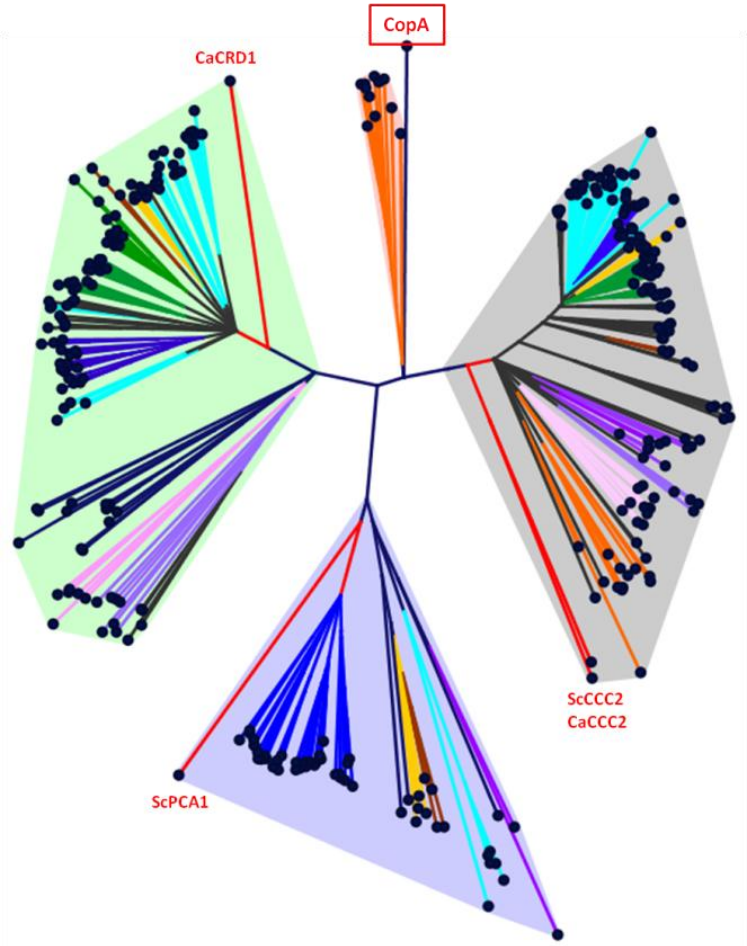


Figure 2.1. Neighbour-joining tree showing three main types of HMA proteins present in genomes of 132 basidiomycetes and 17 early diverging species of the kingdom Fungi.

Species belong to the subphyla Pucciniomycotina and Ustilaginomycotina are indicated in violet and pink. Subphylum Agaricomycotina contains many species; species belong to order Agaricales, Polyporales, Boletales, Russulales, and Auriculariales are indicated in light-blue, green, blue, yellow and brown. All other orders of Agaricomycotina are indicated in black. The bacterial P1B-type ATPase (copA), the yeast *C. albicans* CaCcc2 and CaCrd1 and *S. cerevisiae* ScCcc2, ScPca1 are indicated in red. P1B-type ATPases of early diverging species are indicated in orange . Early diverging specific HMAs are indicated in pink background. CCC2-, CRD1-, PCA1-type clusters are indicated in gray, pale green and violet background, respectively.

Table 2.1. Occurrence and diversity of HMAs in basidiomycete and in the early diverged fungi genomes

Species belong to the subphyla Pucciniomycotina and Ustilaginomycotina are indicated in violet and pink. Subphylum Agaricomycotina contains many species; species belong to order Agaricales, Polyporales, Boletales, Russulales, and Auriculariales are indicated in light-blue, green, blue, yellow and brown background, respectively. All other orders of Agaricomycotina are indicated in white background. Early diverging species are indicated in orange. The number of HMAs of each types are indicated by the number of letter x. The (x) in brackets indicates putative zinc-transporting ATPases. The x indicates HMAs of the early diverging fungi that are not clustered with CCC2-, CRD1-, nor PCA1- HMAs but with CopA (in Figure 1, the cluster with pink background).

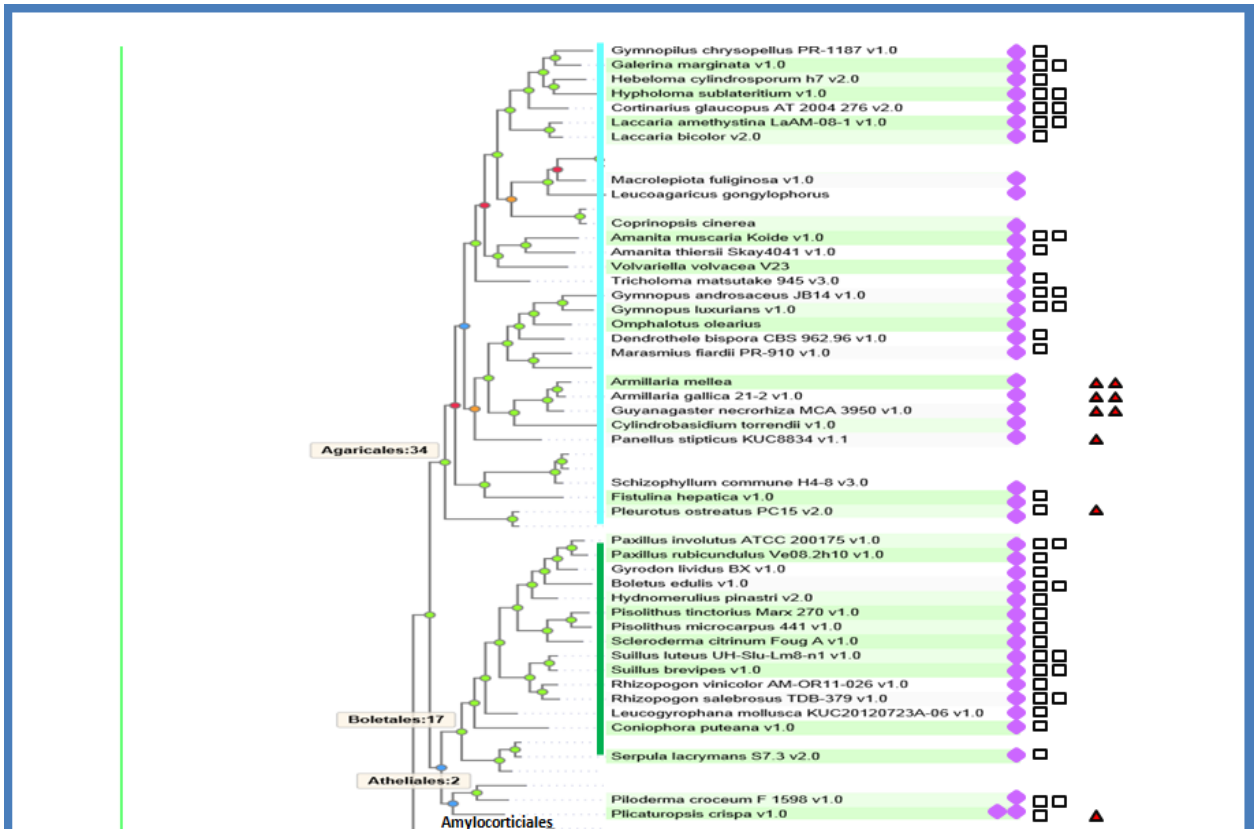
	Sub-phylum	Order	Species	Life style	HMA type			Name abbreviation
					CCC2	CRD1	PCA1	
			Basidiomycetes					
	Agaricomycotina							
1		Agaricales	Amanita muscaria Koide v1.0	Ectomycorrhizal	x	xx		Amamu1|
2		Agaricales	Amanita thiersii Skay4041 v1.0	Litter decomposer	x	x		Amath1|
3		Agaricales	Armillaria gallica 21-2 v1.0	White rot	x		(xx)	Armga1|
4		Agaricales	Armillaria mellea	White rot	x		(xx)	Armme1_1|
5		Agaricales	Coprinopsis cinerea	Litter decomposer	x			Copci1|
6		Agaricales	Cortinarius glaucopus AT 2004 276 v2.0	Ectomycorrhizal	x	xx		Corgl3|
7		Agaricales	Cylindrobasidium torrendii v1.0	White rot	x			Cylto1|
8		Agaricales	Dendrothele bispora CBS 962.96 v1.0	Unknown	x	x		Denbi1|
9		Agaricales	Fistulina hepatica v1.0	Brown rot	x	x		Fishe1|
10		Agaricales	Galerina marginata v1.0	White rot	x	xx		Galma1|
11		Agaricales	Guyanagaster necrorhiza MCA 3950 v1.0	White rot	x		(xx)	Guyne1|
12		Agaricales	Gymnopilus chrysopellus PR-1187 v1.0	Unknown	x	x		Gymch1|
13		Agaricales	Gymnopus androsaceus JB14 v1.0	White rot	x	xx		Gyman1|
14		Agaricales	Gymnopus luxurians v1.0	Litter decomposer	x	xx		Gymlu1|
15		Agaricales	Hebeloma cylindrosporum h7 v2.0	Ectomycorrhizal	x	x		Hebcy2|
16		Agaricales	Hypholoma sublateritium v1.0	White rot	x	xx		Hypsu1|
17		Agaricales	Laccaria amethystina LaAM-08-1 v1.0	Ectomycorrhizal	x	xx		Lacam1|
18		Agaricales	Laccaria bicolor v2.0	Ectomycorrhizal	x	x		Lacbi2|

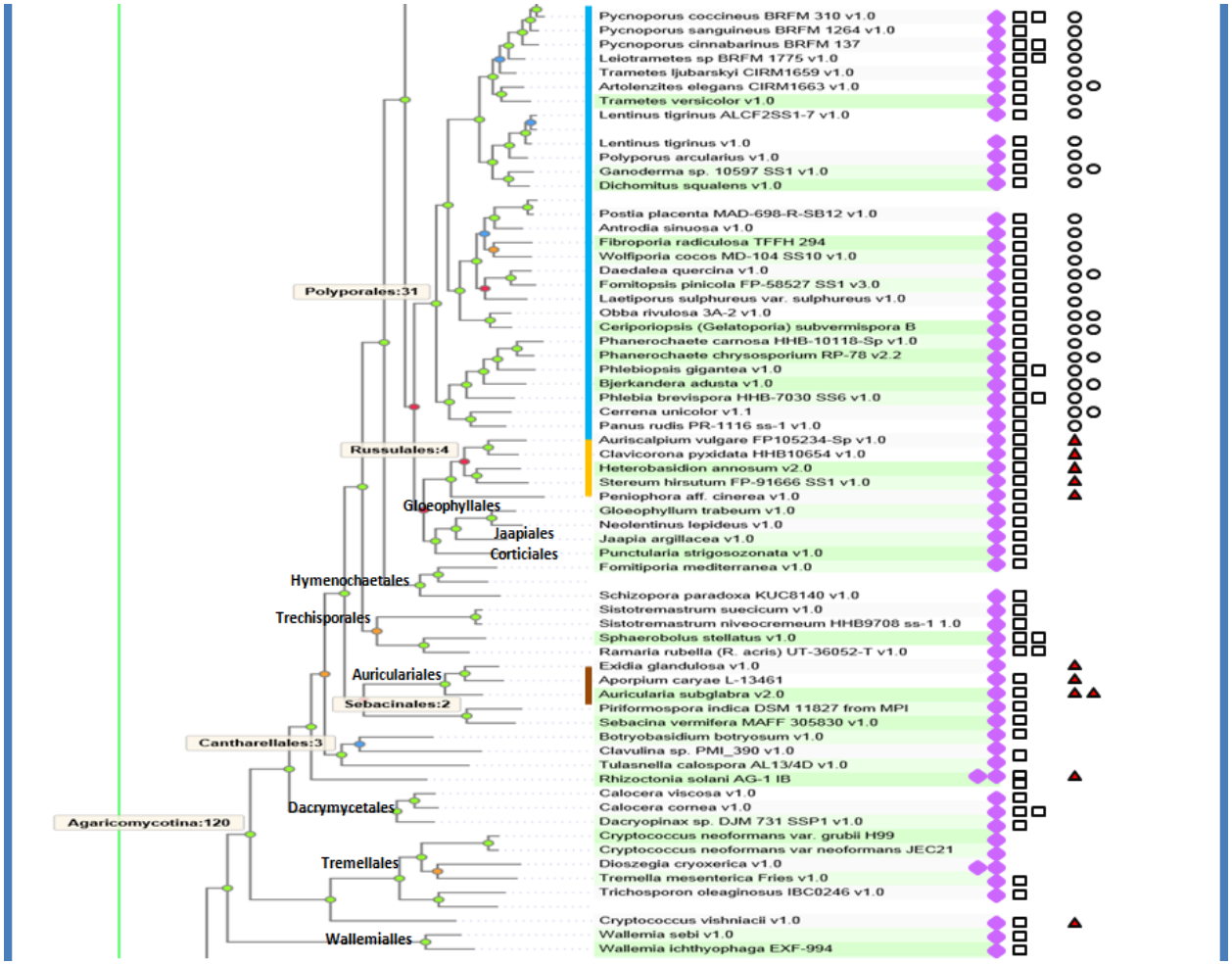
19	Agaricales	Leucoagaricus gongylophorus	White rot	x			Leugo1_1|
20	Agaricales	Macrolepiota fuliginosa v1.0	Litter decomposer	x			Macfu1|
21	Agaricales	Marasmius fiardii PR-910 v1.0	Litter decomposer	x	x		Marfi1|
22	Agaricales	Omphalotus olearius	White rot	x			Ompol1|
23	Agaricales	Panellus stipticus KUC8834 v1.1	White rot	x		(x)	Panst_KUC8834
24	Agaricales	Pleurotus ostreatus PC15 v2.0	White rot	x	x	(x)	PleosPC15_2|
25	Agaricales	Schizophyllum commune H4-8 v3.0	White rot	x	x		Schco3|
26	Agaricales	Tricholoma matsutake 945 v3.0	Ectomycorrhizal	x	x		Trima3|
27	Agaricales	Volvariella volvacea V23	White rot	x			Volvo1|
28	Amylocorticiales	Plicaturopsis crispa v1.0	White rot	xx	x	(x)	Plicr1|
29	Atheliales	Fibulorhizoctonia sp. CBS 109695 v1.0	Unknown	x	x		Fibsp1|
30	Atheliales	Piloderma croceum F 1598 v1.0	Ectomycorrhizal	x	xx		Pilcr1|
31	Auriculariales	Auricularia subglabra v2.0 (delicata)	White rot	x	x	(xx)	Aurde3_1|
32	Auriculariales	Elmerina (Aporpium) caryae v1.0	White rot	x	x	(x)	Elmca1|
33	Auriculariales	Exidia glandulosa v1.0	White-rot	x		(x)	Exigl1|
34	Boletales	Boletus edulis v1.0	Ectomycorrhizal	x	xx		Boled1|
35	Boletales	Coniophora puteana v1.0	Brown rot	x	x		Conpu1|
36	Boletales	Gyrodon lividus BX v1.0	Ectomycorrhizal	x	x		Gyrli1|
37	Boletales	Hydnomerulius pinastri v2.0	Brown rot	x			Hydpi2|
38	Boletales	Leucogyrophana mollusca KUC20120723A-06 v1.0	Unknown	x	x		Leumo1|
39	Boletales	Paxillus involutus ATCC 200175 v1.0	Ectomycorrhizal	x	xx		Paxin1|
40	Boletales	Paxillus rubicundulus Ve08.2h10 v1.0	Ectomycorrhizal	x	x		Paxru1|
41	Boletales	Pisolithus microcarpus 441 v1.0	Ectomycorrhizal	x	x		Pismi1|
42	Boletales	Pisolithus tinctorius Marx 270 v1.0	Ectomycorrhizal	x	x		Pisti1|
43	Boletales	Rhizopogon salebrosus TDB-379 v1.0	Ectomycorrhizal	x	xx		Rhisa1|
44	Boletales	Rhizopogon vinicolor AM-OR11-026 v1.0	Ectomycorrhizal	x	x		Rhivi1
45	Boletales	Scleroderma citrinum Foug A v1.0	Ectomycorrhizal	x	x		Sclci1|
46	Boletales	Serpula lacrymans S7.3 v2.0	Brown rot	x	x		SerlaS7_3_2|
47	Boletales	Suillus brevipes v1.0	Ectomycorrhizal	x	xx		Suibr1|
48	Boletales	Suillus luteus UH-Slu-Lm8-n1 v1.0	Ectomycorrhizal	x	xx		Suilu1|
49	Cantharellales	Botryobasidium botryosum v1.0	Brown rot	x	x		Botbo1|
50	Cantharellales	Rhizoctonia solani AG-1 IB	Pathogen	xx	x	(x)	Rhiso1|
51	Cantharellales	Tulasnella calospora AL13/4D v1.0	Orchid mycorrhizal	x			Tulca1|
52	Corticiales	Punctularia strigosozonata v1.0	White rot	x	x		Punst1|
53	Dacrymycetales	Calocera cornea v1.0	Brown rot	x	xx		Calco1|
54	Dacrymycetales	Calocera viscosa v1.0	Brown rot	x	x		Calvi1|
55	Dacrymycetales	Dacryopinax sp. DJM 731 SSP1 v1.0	Brown rot	x	x		Dacsp1|
56	Filobasidiales	Cryptococcus vishniacii v1.0	Unknown	x	x	(x)	Cryvi1|
57	Geastrales	Sphaerobolus stellatus v1.0	White rot	x	xx		Sphst1|

58	Gloeophyllales	Gloeophyllum trabeum v1.0	Brown rot	x	x		Glotr1_1|
59	Gloeophyllales	Neolentinus lepideus v1.0	Brown rot	x	x		Neole1|
60	Gomphales	Ramaria rubella(R.acris) UT-36052-T v1.0	Unknown	x	xx		Ramac1|
61	Hymenochaetales	Fomitiporia mediterranea v1.0	White rot	x	x		Fomme1|
62	Hymenochaetales	Schizopora paradoxa KUC8140 v1.0	White rot	x	x		Schpa1|
63	Jaapiales	Jaapia argillacea v1.0	White rot	x	x		Jaaar1|
64	Polyporales	Antrodia sinuosa v1.0	Brown rot	x	x	x	Antsi1|
65	Polyporales	Artolenzites elegans CIRM1663 v1.0	White rot	x	x	xx	Artel1|
66	Polyporales	Bjerkandera adusta v1.0	White rot	x	x	xx	Bjead1_1|
67	Polyporales	Ceriporiopsis (Gelatoporia) subvermispora B	White rot	x	x	xx	Cersu1|
68	Polyporales	Cerrena unicolor v1.1	White rot	x	x	xx	Cerun2|
69	Polyporales	Daedalea quercina v1.0	Brown rot	x	x	xx	Daequ1|
70	Polyporales	Dichomitus squalens v1.0	White rot	x	x	x	Dicsq1|
71	Polyporales	Fibroporia radiculosa TFFH 294	Brown rot	x	x	x	Fibra1|
72	Polyporales	Fomitopsis pinicola FP-58527 SS1 v3.0	Brown rot	x	x	x	Fompi3|
73	Polyporales	Ganoderma sp. 10597 SS1 v1.0	White rot	x	x	xx	Gansp1|
74	Polyporales	Laetiporus sulphureus var. sulphureus v1.0	Brown rot	x	x	x	Laesu1|
75	Polyporales	Leiotrametes sp BRFM 1775 v1.0	White rot	x	xx	x	Leisp1|
76	Polyporales	Lentinus tigrinus ALCF2SS1-7 v1.0	White rot	x	x	x	Lenti7_1|
77	Polyporales	Obba rivulosa 3A-2 v1.0	White rot	x	x	xx	Obbri1|
78	Polyporales	Panus rudis PR-1116 ss-1 v1.0	White rot	x	x	x	Panru1|
79	Polyporales	Phanerochaete carnosa HHB-10118-Sp v1.0	White rot	x	x	x	Phaca1|
80	Polyporales	Phanerochaete chrysosporium RP-78 v2.2	White rot	x	x	xx	Phchr2|
81	Polyporales	Phlebia brevispora HHB-7030 SS6 v1.0	White rot	x	xx	x	Phlbr1|
82	Polyporales	Phlebiopsis gigantea v1.0	White rot	x	x	x	Phlgi1|
83	Polyporales	Polyporus arcularius v1.0	White rot	x	x	x	Polar1|
84	Polyporales	Postia placenta MAD-698-R-SB12 v1.0	Brown rot	x	x	x	Pospl1|
85	Polyporales	Pycnoporus cinnabarinus BRFM 137	White rot	x	xx	x	Pycci1|
86	Polyporales	Pycnoporus coccineus BRFM 310 v1.0	White rot	x	xx	x	Pycco1|
87	Polyporales	Pycnoporus sanguineus BRFM 1264 v1.0	White rot	x	x	x	Pycsa1|
88	Polyporales	Trametes ljubarskyi CIRM1659 v1.0	White rot	x	x	x	Tralj1|
89	Polyporales	Trametes versicolor v1.0	White rot	x	x	x	Trave1|
90	Polyporales	Wolfiporia cocos MD-104 SS10 v1.0	Brown-rot	x	x	x	Wolco1|
91	Russulales	Auriscalpium vulgare FP105234-Sp v1.0	Unknown	x	x	(x)	Aurvu1|
92	Russulales	Clavicorona pyxidata HHB10654 v1.0	White rot	x	x	(x)	Clapy1|
93	Russulales	Heterobasidion annosum v2.0	White rot	x	x	(x)	Hetan2|
94	Russulales	Rickenella mellea v1.0 (Peniophora aff. cinerea)	White rot	x	x	(x)	Ricme1|
95	Russulales	Stereum hirsutum FP-91666 SS1 v1.0	White rot	x	x	(x)	Stehi1|

96	Sebacinales	Sebacina vermifera MAFF 305830 v1.0	Orchid symbiont	x	x		Sebve1|
97	Sebacinales	Piriformospora indica DSM 11827 from MPI	Endophyte	x	x		Pirin1|
98	Trechisporales	Sistotremastrum niveocremeum HHB9708 ss-1 1.0	White rot	x	x		Sisni1|
99	Trechisporales	Sistotremastrum suecicum v1.0	White rot	x	x		Sissu1|
100	Tremellales	Cryptococcus neoformans var neoformans JEC21	Pathogen	x			Cryne_JEC21_1|
101	Tremellales	Cryptococcus neoformans var. grubii H99	Pathogen	x			Cryne_H99_1|
102	Tremellales	Dioszegia cryoxerica v1.0	Unknown	xx			Diocr1|
103	Tremellales	Tremella mesenterica Fries v1.0	White rot	x	x		Treme1|
104	Tremellales	Trichosporon oleaginosus IBC0246 v1.0	White rot	x	x		Triol1|
105	Wallemiales	Wallemia ichthyophaga EXF-994	Saprophyte	x	x		Walic1|
106	Wallemiales	Wallemia sebi v1.0	Saprophyte	x	x		Walse1|
	Ustilaginomycotina						
107	Entylomatales	Tilletiopsis washingtonensis MCA 4186 v1.0	Pathogen	x	x		Tilwa1|
108	Exobasidiales	Exobasidium vaccinii MPITM v1.0	Pathogen	x	x		Exova1|
109	Exobasidiales	Meira miltonrushii MCA 3882 v1.0	Endophyte	x			Meimi1|
110	Georgefisheriales	Tilletiaria anomala UBC 951 v1.0	Unknown	x			Tilan2|
111	Microbotryales	Sporisorium reilianum SRZ2	Pathogen	x	x		Spore1|
112	Microstromatales	Jaminaea sp. MCA 5214 v1.0	Endophyte	x	x		Jamsp1|
113	Ustilaginales	Testicularia cyperi MCA 3645 v1.0	Pathogen	x			Tescy1|
114	Ustilaginales	Ustilago maydis	Pathogen	x	x		Ustma1|
115	Ustilaginomycotina	Pseudozyma antarctica T-34	Unknown	x	x		Psean1_1|
116	Ustilaginomycotina	Pseudozyma hubeiensis SY62	Pathogen	x			Psehu1|
117	Ustilaginomycotina	Ustilaginomycotina sp. SA 807 v1.0	Endophyte	x	x		Ustsp1|
	Pucciniomycotina						
118	Agaricostilbales	Agaricostilbum hyphaenes ATCC MYA-4628 v1.0	Parasite	x			Agahy1|
119	Atractiellales	Atractiellales sp. v2.0	Endophyte	x	x		Atrsp2|
120	Classiculales	Naiadella fluitans ATCC 64713 v1.0	Parasite	x			Naifl1|
121	Pucciniomycotina	Erythrobasidium hasegawianum ATCC 9536 v1.0	Unknown	xx	x	(x)	Eryha1|
122	Pucciniomycotina	Rhodotorula minuta MCA 4210 v1.0	Unknown	x	x		Rhomi1|
123	Heterogastridiales	Heterogastridium pycnidioideum ATCC MYA-4631 v1.0	Parasite	x	x	(x)	Hetpy1|
124	Mixiales	Mixia osmundae IAM 14324 v1.0	Pathogen	x	x		Mixos1|
125	Pucciniales	Melampsora laricis-populina v1.0	Pathogen	x			Mellp1|
126	Pucciniales	Puccinia graminis	Pathogen	x			Pucgr1|
127	Pucciniomycotina	Rhodotorula sp. JG-1b	Unknown	x			Rhosp1|
128	Pucciniomycotina	Sporobolomyces linderae CBS 7893 v1.0	Unknown	x	x		Spoli1|
129	Pucciniomycotina	Sporobolomyces roseus v1.0	Unknown	x			Sporo1|
130	Tritirachiales	Tritirachium sp. CBS 265.96 v1.0	Unknown	x	x	(x)	Trisp1|
131	Uredinalles	Cronartium quercuum f. sp. fusiforme G11 v1.0	Pathogen	x			Croqu1|
132	Uredinalles	Puccinia striiformis f. sp. tritici PST-130	Pathogen	x	x		Pucst1|

	Sub-phylum	Order	Species	Life style	HMA type			Name abbreviation
					CCC2	CRD1	PCA1	
			Early diverged divisions					
	Glomeromycota							
133			Rhizophagus irregularis DAOM 181602 v1.0		xx		X	Gloin1
	Mucoromycotina							
134			Backusella circina FSU 941 v1.0				X	Bacci1
135			Hesseltinella vesiculosa NRRL3301 v1.0				X	Hesve1
136			Lichtheimia hyalospora v1.0		x		X	Lichy1
137			Mucor circinelloides CBS277.49 v2.0		xx		X	Mucci2
138			Phycomyces blakesleeanus NRRL1555 v2.0		x		X	Phybl2
139			Rhizopus microsporus var. chinensis CCTCC M201021		x		X	Rhich1
140			Rhizopus microsporus var. microsporus ATCC52813 v1.0		x		X	Rhimi1_1
141			Rhizopus oryzae 99-880 from Broad		x		X	Rhior3
142			Umbelopsis ramanniana AG # v1.0		x		X	Umbra1
	Entomophthoromycotina							
143			Conidiobolus thromboides FSU 785 v1.0		x			Conth1
	Blastocladiomycotina							
144			Catenaria anguillulae PL171 v1.0		x		X	Catan1
	Kickxellomycotina							
145			Coemansia reversa NRRL 1564 v1.0		x		X	Coere1
146			Martensiomyces pterosporus CBS 209.56 v1.0		x		X	Marpt1
147			Ramicandelaber brevisporus CBS 109374 v1.0		x		X	Rambr1
	Chytridiomycotina							
148			Batrachochytrium dendrobatidis JAM81 v1.0		x		X	Batde5
	Cryptomycota							
149			Rozella allomycis CSF55		x			Rozal1


Gymnopilus chrysopellus PR-1187 v1.0
Galerina marginata v1.0
Hebeloma cylindrosporum h7 v2.0
Hypholoma sublateritium v1.0
Cortinarius glaucopus AT 2004 276 v2.0
Laccaria amethystina LaAM-08-1 v1.0
Laccaria bicolor v2.0
Macrolepiota fuliginosa v1.0
Leucoagaricus gongylophorus
Coprinopsis cinerea
Amanita muscaria Koide v1.0
Amanita thiersii Skay4041 v1.0
Volvariella volvacea V23
Tricholoma matsutake 945 v3.0
Gymnopus androsaceus JB14 v1.0
Gymnopus luxurians v1.0
Omphalotus olearius
Dendrothele bispora CBS 962.96 v1.0
Marasmius fiardii PR-910 v1.0
Armillaria mellea
Armillaria gallica 21-2 v1.0
Guyanagaster necrorhiza MCA 3950 v1.0
Cylindrobasidium torrendii v1.0
Panellus stipticus KUC8834 v1.1
Agaricales:34
Schizophyllum commune H4-8 v3.0
Fistulina hepatica v1.0
Pleurotus ostreatus PC15 v2.0
Paxillus involutus ATCC 200175 v1.0
Paxillus rubicundulus Ve08.2h10 v1.0
Gyrodon lividus BX v1.0
Boletus edulis v1.0
Hydnomerulius pinastri v2.0
Pisolithus tinctorius Marx 270 v1.0
Pisolithus microcarpus 441 v1.0
Scleroderma citrinum Foug A v1.0
Suillus luteus UH-Slu-Lm8-n1 v1.0
Suillus brevipes v1.0
Rhizopogon vinicolor AM-OR11-026 v1.0
Rhizopogon salebrosus TDB-379 v1.0
Leucogyrophana mollusca KUC20120723A-06 v1.0
Coniophora puteana v1.0
Boletales:17
Serpula lacrymans S7.3 v2.0
Atheliales:2
Piloderma croceum F 1598 v1.0
Plicaturopsis crispa v1.0
Amylocorticiales


Pycnoporus coccineus BRFM 310 v1.0
Pycnoporus sanguineus BRFM 1264 v1.0
Pycnoporus cinnabarinus BRFM 137
Leiotrametes sp BRFM 1775 v1.0
Trametes ljubarskyi CIRM1659 v1.0
Artolenzites elegans CIRM1663 v1.0
Trametes versicolor v1.0
Lentinus tigrinus ALCF2SS1-7 v1.0
Lentinus tigrinus v1.0
Polyporus arcularius v1.0
Ganoderma sp. 10597 SS1 v1.0
Dichomitus squalens v1.0
Postia placenta MAD-698-R-SB12 v1.0
Antrodia sinuosa v1.0
Fibroporia radiculosa TFFH 294
Wolfiporia cocos MD-104 SS10 v1.0
Daedalea quercina v1.0
Fomitopsis pinicola FP-58527 SS1 v3.0
Laetiporus sulphureus var. sulphureus v1.0
Obba rivulosa 3A-2 v1.0
Ceriporiopsis (Gelatoporia) subvermispora B
Phanerochaete carnosa HHB-10118-Sp v1.0
Phanerochaete chrysosporium RP-78 v2.2
Phlebiopsis gigantea v1.0
Bjerkandera adusta v1.0
Phlebia brevispora HHB-7030 SS6 v1.0
Cerrena unicolor v1.1
Panus rudis PR-1116 ss-1 v1.0
Auriscalpium vulgare FP105234-Sp v1.0
Clavicorona pyxidata HHB10654 v1.0
Heterobasidion annosum v2.0
Stereum hirsutum FP-91666 SS1 v1.0
Peniophora aff. cinerea v1.0
Gloeophyllum trabeum v1.0
Neolentinus lepideus v1.0
Jaapia argillacea v1.0
Punctularia strigosozonata v1.0
Fomitiporia mediterranea v1.0
Schizopora paradoxa KUC8140 v1.0
Sistotremastrum suecicum v1.0
Sistotremastrum niveocremeum HHB9708 ss-1 1.0
Sphaerobolus stellatus v1.0
Ramaria rubella (R. acris) UT-36052-T v1.0
Exidia glandulosa v1.0
Aporpium caryae L-13461
Auricularia subglabra v2.0
Piriformospora indica DSM 11827 from MPI
Sebacina vermifera MAFF 305830 v1.0
Botryobasidium botryosum v1.0
Clavulina sp. PMI_390 v1.0
Tulasnella calospora AL13/4D v1.0
Rhizoctonia solani AG-1 IB
Calocera viscosa v1.0
Calocera cornea v1.0
Dacryopinax sp. DJM 731 SSP1 v1.0
Cryptococcus neoformans var. grubii H99
Cryptococcus neoformans var neoformans JEC21
Dioszegia cryoxerica v1.0
Tremella mesenterica Fries v1.0
Trichosporon oleaginosus IBC0246 v1.0
Cryptococcus vishniacii v1.0
Wallemia sebi v1.0
Wallemia ichthyophaga EXF-994
Polyporales:31
Russulales:4
Gloeophyllales
Jaapiales
Corticiales
Hymenochaetales
Trechisporales
Auriculariales
Sebacinales:2
Cantharellales:3
Dacrymycetales
Agaricomycotina:120
Tremellales
Wallemiales

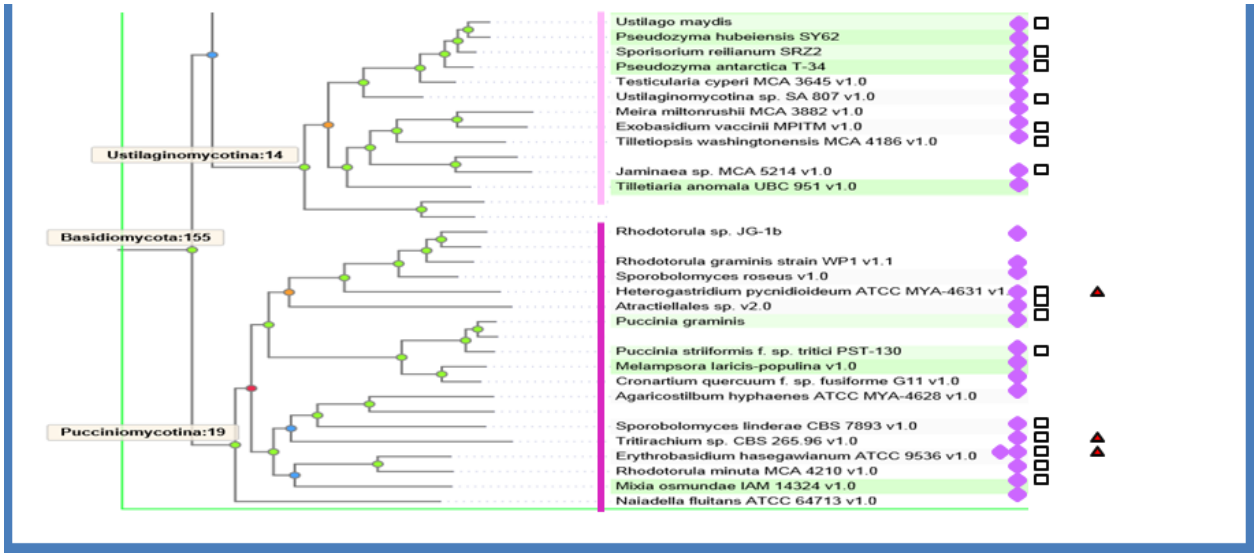


Figure 2.2. Diversity and abundance of HMA proteins in basidiomycete fungi.

The current taxonomic tree of basidiomycete fungi was re-drawn from MycoCosm web portal of the Fungal Genomics Program of the Department of Energy Joint Genome Institute (JGI); green and gray background colours are JGI's illustration and not related to the content of this report. Numbers of putative HMAs in each species are indicated in different symbols: CCC2- and CRD1-type HMAs are indicated in violet diamond and open square; PCA1-type HMAs are indicated in open circle (sup-group I) and red triangle (sub-group II).

2.4.1. The CCC2-type HMAs

The CCC2-type HMAs were present in all species of basidiomycetes. In total 135 sequences of the CCC2-type HMAs were aligned and a phylogenetic tree was constructed (Figure 2.3). CCC2-type HMAs of species from the same order appeared together in the same cluster. Protein sequences of the CCC2-type are well conserved. The signature motifs of P1B-type ATPase (CPCX(6)P, DKTGT, HP and GDGINDXP) were perfectly conserved. Most sequences contained two metal-binding GMTCXXC motifs. There were only few exceptions: CCC2-type HMA of *Hypholoma sublateritium* (Agaricales/ Agaricomycotina) had three GMTCXXC and the species in the order Tremellales and Filobasidiales had only one. We found putative Golgi signal motifs MDVLVM or a derivative MDVLXX in all CCC2-type sequences at the N-terminus of the third transmembrane domain.

Interestingly, the number of metal-binding motifs detected in CCC2-type HMAs of basidiomycetes are reduced when compared to the early diverging species. HMAs of basidiomycetes contain one to three metal-binding motifs whereas HMAs of early diverged species contain four or five. For illustration, Figure 2.4 shows the N-terminal sequence of the CCC2-type HMA of the arbuscular mycorrhizal Glomeromycotina species: *Rhizophagus irregularis*. This HMA contains five metal-binding motifs in which four are embedded in highly homologous fragments obviously resulting from duplications. The coding sequence of this HMA contains a short sequence repeat. This (CAT)n repeat was identified via RepeatMasker server (Smit *et al.*, 1996) based on curated repeat libraries. If a frameshift mutation occurs, the (CAT)n repeat will be translated to give a stretch of histidine (H) or isoleucine (I) (Figure 2.4). Histidine-rich stretches are often found in Zn-transporting P1B-type ATPases in bacteria and plants (Blindauer, 2015).

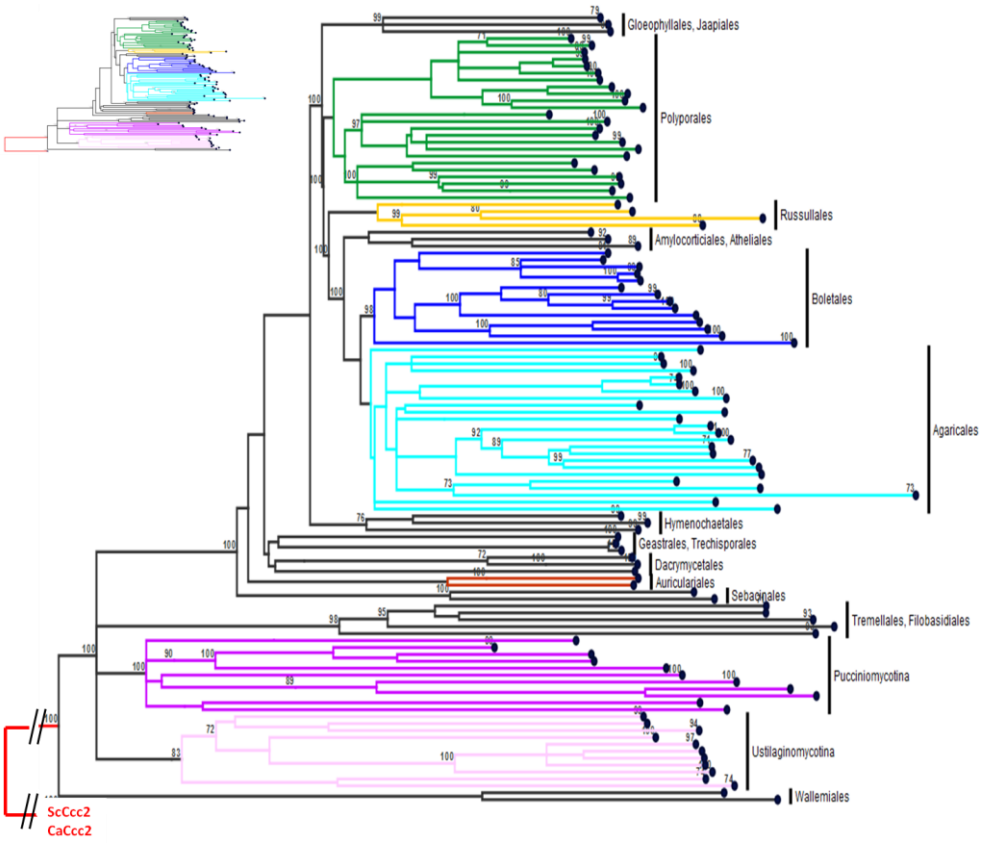


Figure 2.3. Phylogenetic relation of CCC2-type HMAs found in different taxonomic orders of basidiomycete fungi. HMAs are clustered on branches on the basis of the similarity of their amino acid sequences. Protein sequences were aligned and a neighbour-joining tree was constructed using the CLC Main Workbench software. Bootstrap values were calculated from 1000 replicates but only values greater than 70 % are shown. Species belong to the subphyla Pucciniomycotina and Ustilaginomycotina are indicated in violet and pink. Subphylum Agaricomycotina contains many species; species belong to order Agaricales, Polyporales, Boletales, Russulales, and Auriculariales are indicated in light-blue, green, blue, yellow and brown, respectively. All other orders of Agaricomycotina are indicated in black. *Saccharomyces cerevisiae* ScCCC2 and CaCCC2 were used as an out-group and indicated in red. Miniature shows the same tree with the original branch lengths.

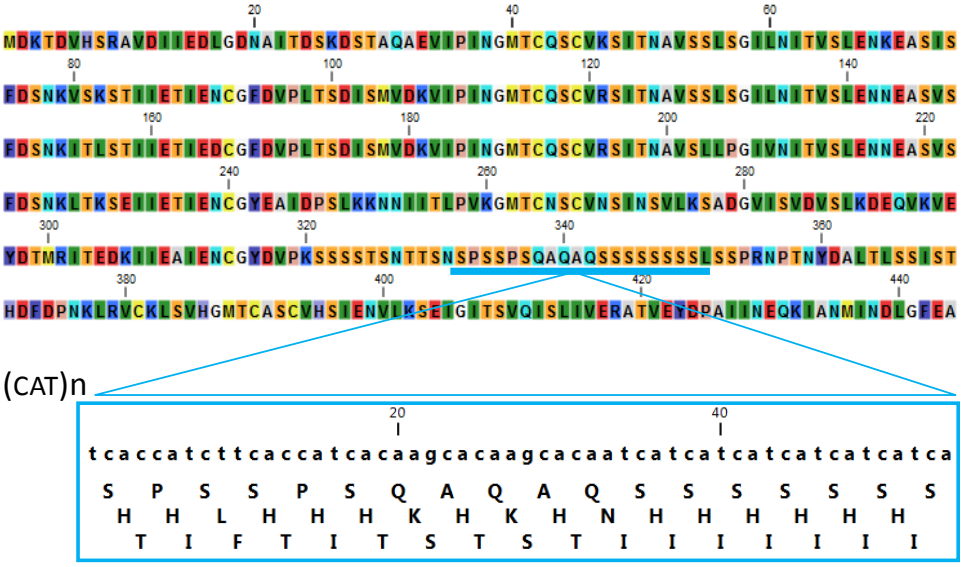


Figure 2.4. The presence of five metal-binding motifs GMTCXXC and a short sequence repeat ((CAT)n) in the N-terminal of the CCC2-type HMA (Jgi|Gloin|218161|) of *Rhizophagus irregularis* (Glomeromycotina), an early diverging species in the kingdom Fungi.

This HMA contains five metal-binding motifs in which four are embedded in highly homologous fragments obviously resulting from duplications. Coding sequence of this HMA contains a short sequence repeat (CAT)n. Assuming that a frameshift mutation occurs, the (CAT)n repeat will be translated into a stretch of histidine (H) or isoleucine (I).

2.4.2. The CRD1-type HMAs

The first apparent characteristic of CRD1-type that differs with CCC2-type HMAs is that most of them have a longer N-terminal domain. The N-terminal domain of CRD1-type HMAs often contains additional (up to three) CXXC motifs located before the first metal-binding GMXCXXC motif. Second, CRD1-type HMAs have from one to five (in *Erythrobasidium hasegawianum/* Pucciniomycotina) GMXCXXC motifs; among these motifs, the last one does not contain a Threonine (T) but Phenylalanine (F), Tyrosine (Y) or Histidine (H). The CRD1-type HMAs are less conserved because of the diversity in the numbers and combinations of the CXXC and GMXCXXC motifs. Especially CRD1-type HMAs among species in the Agaricales order were substantially different from each other (Figure 2.5).

Our dataset of CRD1-type HMAs contains 125 sequences collected from 105 basidiomycete genomes out of the total 132 genomes analyzed. The survey indicates that CRD1-type HMAs are present in most (approximately 80 %) species analyzed (Figure 2.2, Figure 2.5 and Table 2.1). Many genomes have more than one CRD1-type HMA. In fact, in the genome of the ectomycorrhizal fungi *Amanita muscaria*, *S. luteus*, *S. brevipes* and *Paxillus involutus,* CRD1-type HMAs are encoded by two genes that are syntenically arranged and are likely the result of a recent gene duplication event (Chapter 3, Figure 3.3).

The CRD1-type HMAs in basidiomycetes sub-divide into two clusters (Figure 2.5). The larger cluster consists of all Agaricomycetes. The putative Golgi signal motif MDVLXX was not found in HMAs in this cluster, instead, all HMAs contain a MNLLXS, which is highly similar to that of *C. albicans* Crd1 (MNLLMS)-- a Cu exporter localized on the plasma membrane of the yeast. On the other hand, HMAs in the other cluster do not contain the MNLLXS but several different sequences MD-(L/T/S)-LV-(A/S/T); some of those are more similar to the putative Golgi signal motifs of the *S. cerevisiae* and *C. albicans* Ccc2: MDTLVC or MDVLVC, respectively.

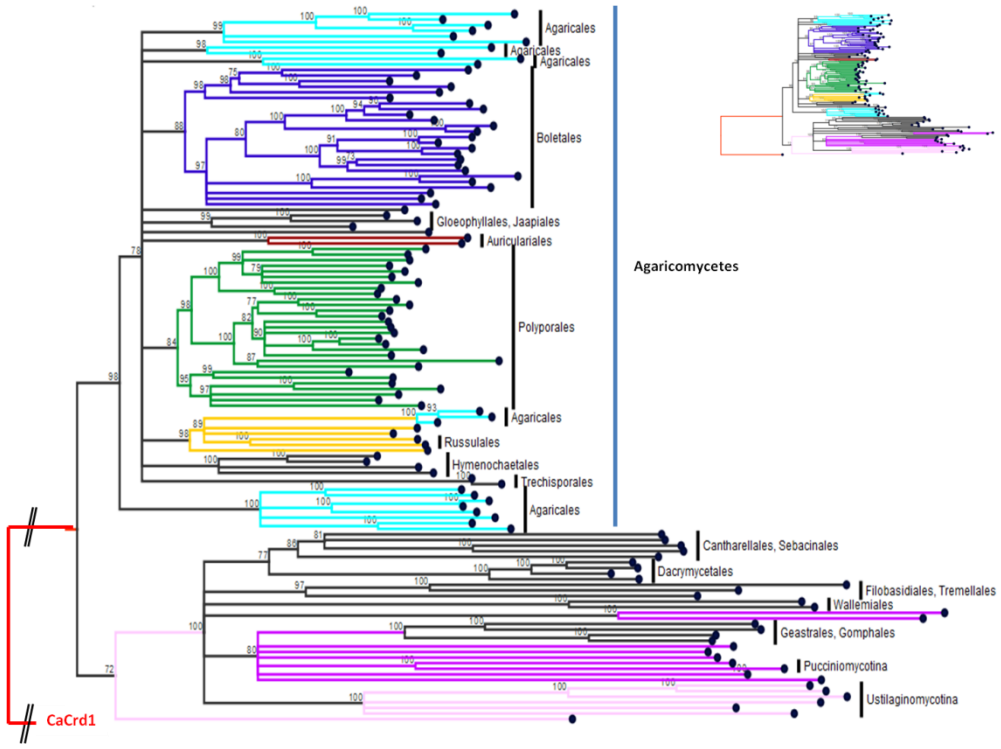


Figure 2.5. Phylogenetic relation of CRD1-type HMAs found in different taxonomic orders of basidiomycete fungi. HMAs are clustered on branches on the basis of the similarity of their amino acid sequences. Protein sequences were aligned and a neighbour-joining tree was constructed using the CLC Main Workbench software. Bootstrap values were calculated from 1000 replicates but only values greater than 70 % are shown. Species belong to the subphyla Pucciniomycotina and Ustilaginomycotina are indicated in violet and pink. Subphylum Agaricomycotina contains many species; species belong to order Agaricales, Polyporales, Boletales, Russulales and Auriculariales are indicated in light-blue, green, blue, yellow, and brown, respectively. All other orders of Agaricomycotina are indicated in black. *Candida albicans* CaCrd1 was used as an out-group and indicated in red. Miniature shows the same tree with the original branch lengths.

2.4.3. The PCA1-type HMAs

The PCA1-type HMAs were found in approximately 35 % of genomes analyzed hence form the smallest group of basidiomycete HMAs (Figure 2.1). Our dataset of PCA1-type HMAs contains 58 sequences collected from 27 Polyporales species and 19 species belonging to other orders. PCA1-type HMAs are present in all species of the Polyporales order and in some other orders of subphylum Agaricomycotina as well as in three species belonging to subphylum Pucciniomycotina (Figure 2.2, Figure 2.6 and Table 2.1). PCA1-type HMAs are present in the genomes of many wood-rotting but not in any of the ectomycorrhizal fungi analyzed in this study (Table 2.1). The main characteristic of the PCA1-type that differentiates it from other HMA types is the cysteine-rich N-terminal domain that contains multiple (up to 16) CC motifs. Besides, several single cysteine residues and occasionally one or two CXC and CCC motifs were found just before the metal binding motif GMXCXXC.

Proteins belonging to this group form two small sub-groups (I and II) (Figure 2.6). Sub-group I comprised of proteins from merely Polyporales species. All proteins of sub-group I had a typical GMTCXXC, whereas proteins of the sub-group II contained a distinctive GMDCXXC motif, in which 'D' stands for aspartic acid (Figure 2.7). Proteins belonging to sub-group II were found in only a few species scattered over six different orders of the subphylum Agaricomycotina and in three species of Pucciniomycotina. Another characteristic of the sub-group II is the presence of a histidine-rich region (as HHHH or HX stretches) in the N-terminal domains of HMAs found in species of Auriculariales, Russulales, Amylocorticiales, and Cantharellales. These histidine-rich regions locate at the area where a short sequence repeat was detected in *R. irregularis* (Figure 2.4). Long histidine-rich stretches are often found in Zn-transporting P1B-type ATPases in plants and bacteria (Blindauer, 2015). The PCA1-type of *Exidia glandulosa* (Auriculariales) contains an additional HX stretch (Figure 2.7). *Tritirachium sp.* (Pucciniomycotina) PCA1-type HMA also contains a long HX stretch in the corresponding region. These histidine-rich regions were not detected in PCA1-type HMAs in other genomes.

The PCA1-type HMAs do not have similar signal motifs with either the CCC2-type or the CRD1-type HMAs in the corresponding region.

Figure 2.8 summarises the sequence features of different types of P1B-type ATPases found in basidiomycete fungi. Sequences were retrieved from three basidiomycete genomes: the CCC2- and CRD1-type HMAs of *S. luteus* (Boletales/ Agaricomycotina); the PCA1-type (I) of *T. versicolor* (Polyporales/ Agaricomycotina); the PCA1-type (II) of *S. hirsutum* (Russulales/ Agaricomycotina). In all sequences, eight transmembrane segments (TM) were predicted. Metal binding motifs CXXC, GMXCXXC and CC are indicated within the N-terminal domain of the proteins.

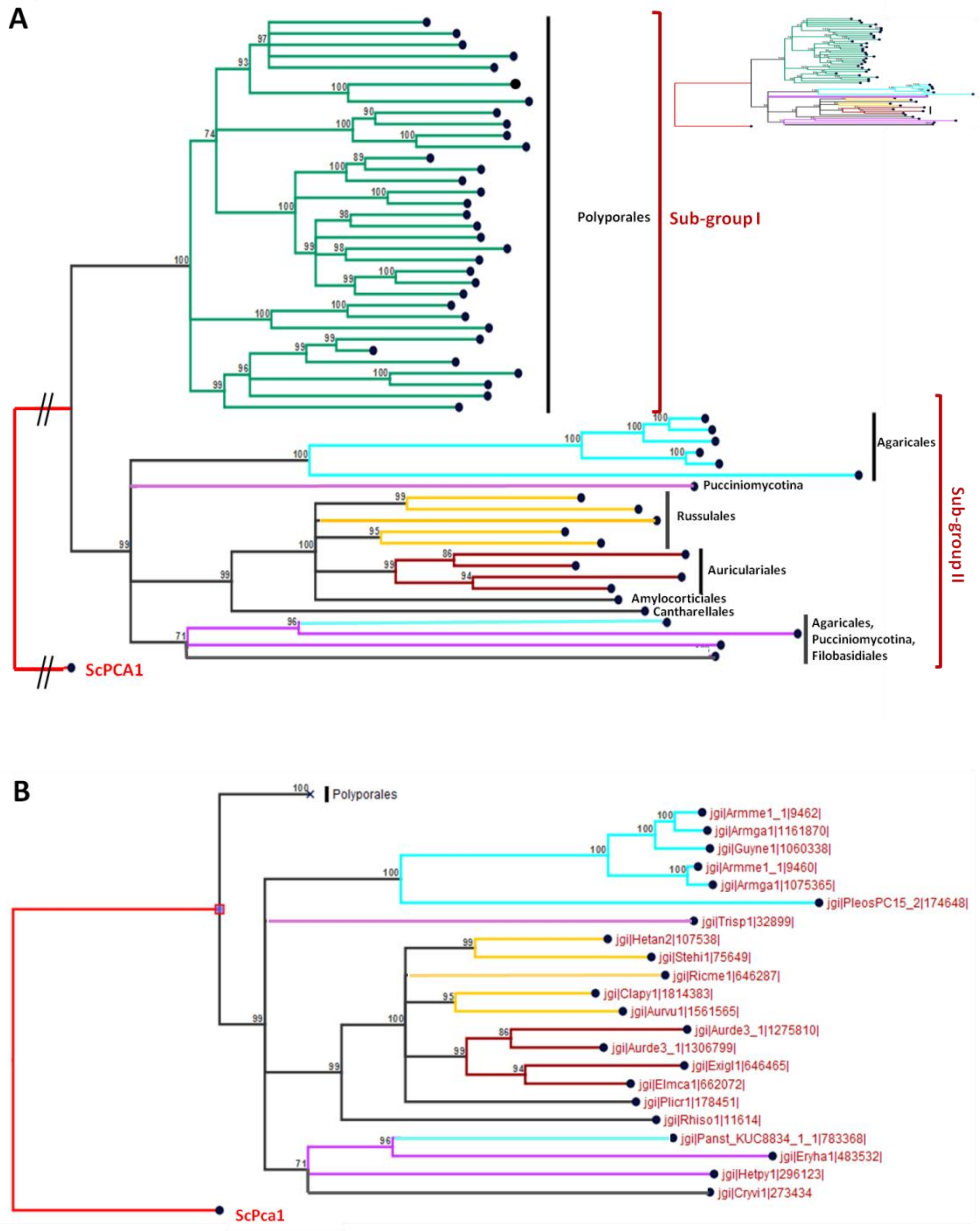


Figure 2.6.

(A) Phylogenetic relation of PCA1-type HMAs found in different taxonomic orders of basidiomycete fungi. HMAs are clustered on branches on the basis of the similarity of their amino acid sequences. Protein sequences were aligned and a neighbour-joining tree was constructed using the CLC Main Workbench software. Bootstrap values were calculated

from 1000 replicates but only values greater than 70 % were shown. Species belong to the subphylum Pucciniomycotina are indicated in violet. Subphylum Agaricomycotina contains many species; species belong to order Agaricales, Polyporales, Russulales and Auriculariales are indicated in light-blue, green, blue, yellow, and brown, respectively. All other orders of Agaricomycotina are indicated in black. *Saccharomyces cerevisiae* ScPca1 was used as an out-group and indicated in red. Miniature shows the same tree with the original branch lengths.

(B) Sub-group II - the putative zinc-transporting P1B-type ATPases. Species names and JGI protein IDs are provided.

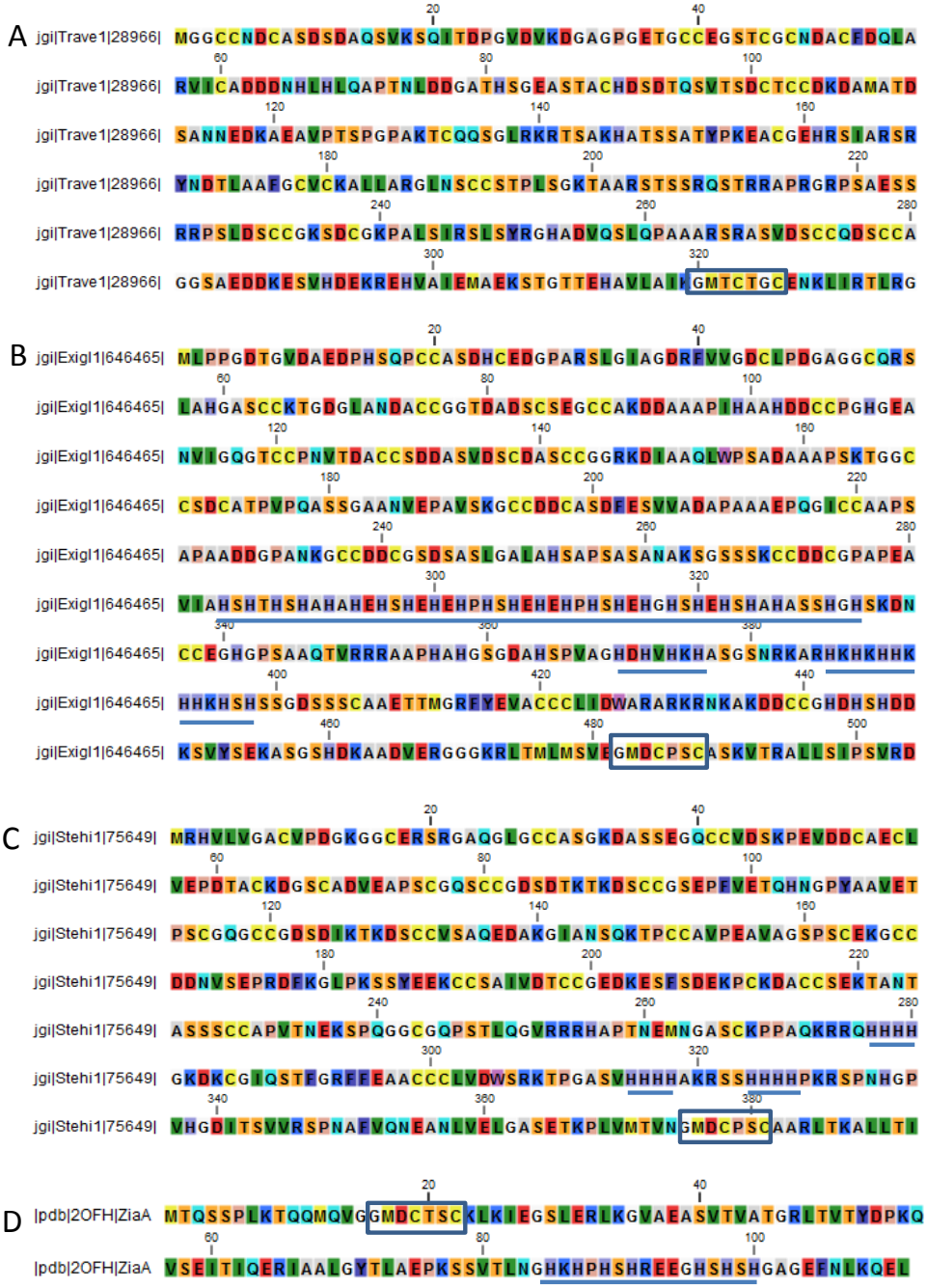


Figure 2.7. Some examples of N-terminal metal binding domains of PCA1-type HMAs in basidiomycete fungi and of ZiaA - a Zn-transporting ATPase of the cyanobacteria *Synechocystis* PCC 6803. The N-terminal sequences of (A) *Trametes versicolor*

(Polyporales), (B) *Stereum hirsutum* (Russulales), (C) *Exidia glandulosa* (Auriculariales) showing (1) the Cysteine-rich regions of PCA1-type HMAs; (2) the presence of a GMTCXXC motif (A, boxed) in a HMA protein belonging to sub-group I of a Polyporales species; (3) the presence of Histidine-rich stretches (underlined) and a GMDCXXC motif (B, C, boxed) in HMA proteins belonging to sub-group II. The N-terminal sequence of ZiaA, a zinc-transporting P1B-type ATPase of the cyanobacteria *Synechocystis* PCC 6803, is included for comparison.

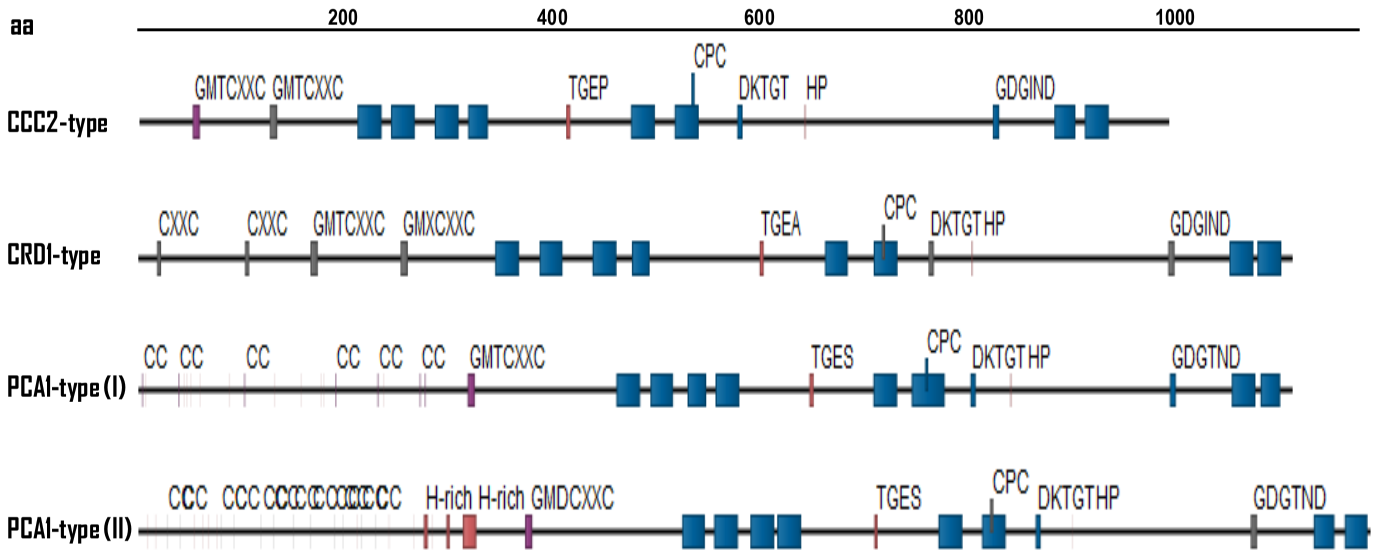


Figure 2.8. The sequence features of three main types of P1B-type ATPases found in basidiomycete fungi. The protein features were drawn to scale from four representatives. The sequences were retrieved from three basidiomycete genomes: the CCC2- and CRD1-type HMAs of *Suillus luteus* (Boletales/ Agaricomycotina), JGI20904, JGI797637; the PCA1-type (I) of *Trametes versicolor* (Polyporales/ Agaricomycotina), JGI28966; the PCA1-type (II) of *Stereum hirsutum* (Russulales/ Agaricomycotina) JGI75649. Metal binding motifs CXXC, GMXCXXC and CC are indicated within the N-terminal domain of the proteins. Multiple cysteine residues in N-terminal domain of PCA1-type HMAs are indicated on the sequence but not labelled for simplicity. Conserved P1B-type ATPase motifs are indicated: TGEX, CPC, DKTGT, HP and GDGXND. Putative transmembrane domains (blue rectangle) were predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.5. DISCUSSION

In this chapter we present a phylogenetic analysis of HMAs in basidiomycete fungi and discuss the characteristics, evolution/ divergence of the three main groups of HMAs. Our data are consistent with previously published phylogenetic analysis which was reported by Saitoh *et al.*(2009). The major differences are that our analysis is expanded to include many more sequences including putative HMAs of early diverging species and a detailed analysis of differences in each type of HMAs within basidiomycetes. Based on the analysis we identified a novel group (PCA1-type, sub-group II) of HMAs that are most likely Zn-transporting ATPases.

2.5.1. The CCC2

First of all, CCC2-type HMAs were found in all basidiomycetes and in the early diverged fungi. Sequences in this group are homologous to *S. cerevisiae* and *C. albicans* Ccc2. In *S. cerevisiae* and *C. albicans*, Ccc2 transporter is localized on post-Golgi membranes (Yuan *et al.*, 1997; Weissman *et al.*, 2002b). The Ccc2 transporter receives Cu from chaperones and transports it into the lumen of the Golgi where Cu is then inserted into Cu-dependent ferroxidase protein that is essential for high affinity Fe import. In basidiomycetes, two CCC2-type proteins were experimentally characterized: the CtaA of the Polyporales *T. versicolor* (Uldschmid *et al.*, 2003) and the Ccc2 of the human pathogen *C. neoformans* (Walton *et al.*, 2005). These proteins are not only involved in Cu and Fe homeostasis but also in laccase production in *T. versicolor* and melanin production and virulence in *C. neoformans*. The presence of CCC2-type HMAs in all basidiomycetes and in the early diverged species indicate that CCC2-types proteins have important roles in fungi. However, deletion of the *CCC2* gene causes several deficiencies but is not lethal in neither yeasts (Fu *et al.*, 1995b; Weissman *et al.*, 2002b) nor *C. neoformans* (Walton *et al.*, 2005).

2.5.2. The CRD1

The CRD1-type group contains the proteins homologous to the ascomycete *C. albicans* Crd1 transporter. This transporter is localized on the plasma membrane

and functions as a Cu-efflux pumps assuring *C. albicans* high Cu tolerance (Riggle and Kumamoto, 2000a; Weissman *et al.*, 2000). Deletion of the *CRD1* gene also increases the fungus sensitivity to Cd and Ag (Riggle and Kumamoto, 2000a). Thus, we assume that CRD1 orthologs in basidiomycetes are also involved in metal homeostasis and detoxification. However, except for Crd1, there is no further information on this type of HMAs in other fungi. Results from our survey indicate that this group of HMAs are ubiquitous in basidiomycetes; some fungi have more than one copy. Therefore, it will be interesting to investigate the function of CRD1-type HMAs in other fungi.

It has been presumed that *C. albicans* Crd1 evolved from an existing Ccc2 during the time *C. albicans* diverged from *S. cerevisiae* as a result of adaptation to very different environments -- an animal body versus rotting fruit (Weissman *et al.*, 2000). Here we show that the presence of CRD-type HMAs is not limited to the ascomycete *C. albicans* but much more ubiquitous in basidiomycetes. The CRD1-type HMA, therefore, must have existed in the ancestor of the Dikarya before the split of the ascomycetes and basidiomycetes.

2.5.3. The PCA1 (I)

The last group of HMAs consists of the proteins most homologous to the *S. cerevisiae* Pca1. This transporter is found on the plasma membrane; it exports Cd and also sequesters Cu via its cysteine-rich N-terminal domain (Adle *et al.*, 2007). It has been proposed that CRD1- and PCA1-types are fungal specific HMAs (Saitoh *et al.*, 2009). Sub-group I of PCA1-type HMAs were found only in Polyporales species which are typical wood degraders. These sequences showed high similarity with the *S. cerevisiae* PCA1: contains a metal-binding GMTCXXC motif together with multiple CC motifs in the N-terminal domain. All Polyporales species have one or two HMAs of this type.

2.5.4. The PCA1 (II): novel Zn-transporting ATPase

Proteins of sub-group II, on the other hand, are found scattered in different genomes and clearly differ from sub-group I in the N-terminal metal binding domain. When examining the sequences of the proteins we found several characteristics similar to HMAs from bacteria and plants. Firstly, GMDCXXC motif was found in sequences in sub-group II of PCA1-type HMAs. This GMDCXXC is a

Zn binding motif of several Zn-transporting HMAs found in bacteria. Examples are the *E. coli* ZntA that exports Zn (and divalent metals) from the cytoplasm to the periplasm (Rensing *et al.*, 1997; Hou and Mitra, 2003) and ZiaA that transports Zn out of the cyanobacterium *Synechocystis* PCC 6803 (Banci *et al.*, 2010b). In cyanobacterium *Synechocystis* PCC 6803, swapping the Cu binding motif GMRCAAC of the Cu transporter PacS with the Zn binding motif GMDCTSC of the Zn transporter ZiaA enhances the Zn affinity of the metal binding motif by up to two orders of magnitude and the aspartic acid residue (D) in the GMDCXXC motif is proposed to stabilize Zn binding together with the two cysteines in the binding motif (Badarau *et al.*, 2013). Secondly, a histidine-rich region in the vicinity of the metal-binding domain is involved in Zn binding in Zn-transporting ATPase (ZiaA) in the cyanobacterium *Synechocystis PCC 6803* (Banci *et al.*, 2010b)(cited in Figure 2.7). Moreover, both a histidine-rich region and a CC motif are found in P1B-type transporters such as *A. thaliana* HMA2 (Eren and Argüello, 2004), HMA4 (Mills *et al.*, 2003) and Bxa1 cyanobacterium *Oscillatoria brevis* (Tong *et al.*, 2002) that assure multiple heavy metal tolerance in these organisms. The histidine-rich region and CC motifs in *A. thaliana* HMA2 and HMA4 are present in the C-terminus of the protein sequences instead of the N-terminus as found in the HMAs of basidiomycetes and the cyanobacteria ZiaA and Bxa1 mentioned above. Collectively, our results indicate that PCA1-type HMAs in sub-group II are most likely Zn (and divalent metals)-transporting ATPases. Protein IDs of the sub-group II HMAs are provided in Figure 2.6B. Zinc-transporting ATPases have not yet been reported in the fungal kingdom.

It has been proposed that the presence of Zn (and divalent metals Cd/Co/Pb) P1B-type ATPases in plants evolved together with the formation of the plastids, and is the result of horizontal gene transfer events from the prokaryotic endosymbiont (Cobbett *et al.*, 2003). Our results indicate that non-photosynthetic fungi do have putative Zn-(and divalent ions) transporting P1B-type ATPases. The data also show that only a minority of basidiomycetes possess the gene (22 sequences in 19 species among 132 basidiomycete species analyzed).

Investigating the sequence of the CCC2-type HMA of the early diverged mycorrhizal fungus *R. irregularis* we found a short sequence repeat located close to the last metal-binding motif GMTCXXC in the metal-binding domain of the

protein (Figure 2.4). This sequence repeat can be frameshift translated into a stretch of histidine residues similar to and at the exact location of the histidine-rich region found in putative Zn- transporting ATPases of species of Auriculariales, Russulales, Amylocorticiales, and Cantharellales (Figure 2.7). We hypothesize that among myriad changes in the ancestor HMA gene sequence to gain a new protein, insertion/deletion of such a short sequence repeat might be the origin/absence of the histidine-rich sequence of Zn-transporting ATPases.

2.5.5. Occurrence and function

The presence of P1B-type ATPases – transmembrane proteins - in simple forms of life such as bacteria can be linked directly to metal export and detoxification. In more complex eukaryotes, some of the P1B-type ATPases might have diversified to perform other functions such as export metals into different cell organelles (such as vacuoles, ER-Golgi, mitochondria) to supply metals to metal-dependent enzymes, or export metals out of the cytoplasm for detoxification, storage in the vacuoles, … On the other hand, parasitic organisms might have adopted extremely specialized characteristics and depend mostly on the host's supply of metals (nutrients) therefore may lack certain transporters.

In this study, we showed that there are three different types of HMAs present in basidiomycete fungi. The CCC2-type HMAs are the most ubiquitous. Functional characterization of two CCC2-type HMAs in basidiomycete *T. versicolor* (Uldschmid *et al.*, 2003) and *C. neoformans* (Walton *et al.*, 2005) confirm their roles in Cu and Fe homeostasis, in laccase production (in *T. versicolor*) and melanin production and virulence (in *C. neoformans*). Subcellular localization of the transporters were not investigated in these studies. Instead, the CtaA of *T. versicolor* is assumed to localize in the Golgi network based on its similarity in sequence and function with the *S. cerevisiae* Ccc2p. Function and localization of CRD1- and PCA1- type HMAs in basidiomycetes have not yet been investigated. The yeast ortholog *C. albicans* Crd1 is localized on the plasma membrane (Weissman *et al.*, 2000), whereas plasma membrane localization of the *S. cerevisiae* Pca1 is only induced/ promoted upon Cd exposure (Adle *et al.*, 2007). Based on sequence analysis, we found a putative Golgi signal motif MDVLVM or a derivative (MDVLXX) in all CCC2-type sequences. Similar motifs have been

found in CCC2-type HMA of two ascomycete fungi: *C. heterostrophus* and *A. fumigatus* (Saitoh *et al.*, 2009; Upadhyay *et al.*, 2013).

The roles of CCC2-type homologs in Cu-regulated production of either laccase, melanin or both have been emphasized in several studies in basidiomycete (Uldschmid *et al.*, 2003; Walton *et al.*, 2005) and ascomycete fungi (Saitoh *et al.*, 2010; Upadhyay *et al.*, 2013). Laccases are multicopper oxidases that are produced by many ascomycetes and basidiomycetes and that are particularly abundant in the white-rot fungi responsible for degradation of lignin related compounds (Baldrian, 2006). It is likely that other types of HMAs take part in Cu homeostasis and, therefore, also are involved in production of laccases and melanin. In this study, we found that the order Polyporales, which contains mostly white-rot fungi (Table 2.1), is particularly rich in HMAs and has the 'Polyporales-specific' PCA1-type. A CCC2-type HMA and a Cu chaperone of the polypore *T. versicolor* are involved in laccase production (Uldschmid *et al.*, 2003). Whether other HMAs compete with this CCC2-type in Cu supply for laccases or collaborate in the production, and how these HMAs are regulated is an interesting subject for future studies.

2.5.6. Divergence and localization

The current data are not yet sufficient to reconstruct the diversification of the three types of HMAs. It was, however, interesting to observe that most of the early diverging species have a CCC2-type HMA together with a specific type of HMA, in which the latter is more similar to the bacteria Cu-transporting ATPase CopA (Figure 2.1). We hypothesize that from a common ancestor the CCC2-type was diverged before CRD1- and PCA1-type HMAs. The CCC2-type HMA is essential for fungi and therefore conserved through the course of evolution. The other two types of HMAs likely diverged later and perform different functions from the CCC2-type and from each other.

It is conceivable that proteins must be localized at their appropriate sub-cellular compartment to perform their function. As discussed earlier, the CCC2-type HMAs likely localize in the Golgi network. Their sequences contain a putative Golgi signal motif MDVLVM or a derivative (MDVLXX). In the sequences of CRD1-type HMAs of Agaricomycetes (Figure 2.5, the larger cluster) these motifs were not found. Together with the fact that they are orthologs of Crd1 and carry a

similar (putative) signal motif with Crd1, we predict that the CRD1-type HMAs of Agaricomycetes localize on the plasma membranes. The rest of CRD1-type and the PCA1-type HMAs contain several different sequence motifs at the corresponding region. Some of them showed similarity with the Golgi signal motifs of the yeast Ccc2. We have applied several bioinformatics tools such as ProtComp, LocSigDB, PSORTb (for prokaryotes) to predict their sub-cellular localization. However, the results were not consistent. Experimental verification is therefore needed.

2.5.7. Conclusions and remarks

We applied bioinformatic approaches using computational predictions, homology and motif analysis to determine P1B-type ATPases' diversity and predict their functions in basidiomycete fungi. We show that the CCC2- and CRD1-type HMAs are ubiquitous in Basidiomycetes whereas the PCA1-type HMAs are less common. In addition to that, we identified putative Zn-transporting ATPases that have been thought only to exist in bacteria and photosynthetic plants.

There is a possibility that some HMAs or copies were not found in certain species in this study due to sequence quality and our elimination criteria. Besides, we cannot exclude that some genes are in fact pseudogenes which are not functional in certain species. Experimental characterization of putative HMAs in the species of interest is therefore needed. This may include detailed analysis of the promoter sequence, experimentally manipulation of the corresponding genes in model species (such as *S. cerevisiae*) and determine gene/protein expression and regulation in different metal conditions, … Moreover, it is important to find out the exact localization of the HMA transporters in the cell. In addition to that it will be also informative to compare closely related species that have or lack a certain type of HMA to gain a deeper insight into their functions/collaborations.

Results of our analysis indicate that comparative analysis of protein sequences from species at different evolutionary distances is a powerful method for annotating genes and highlighting unique species-specific sequences. The identification of the novel PCA1-type HMAs of Polyporales and the putative Zn-transporting ATPases reported here is an example. We plan to further investigate the diversification, gene expansion/loss of HMAs throughout fungal evolution.

CHAPTER 3

Three heavy metal ATPases having a role in Cu homeostasis and Cd detoxification in the ectomycorrhizal fungus *Suillus luteus*

3.1. ABSTRACT

The ectomycorrhizal basidiomycete *Suillus luteus* harbours three genes coding for P1B-type ATPases involved in Cu and Cd trafficking. *SlHMA1* encodes for a structural and functional homolog of the *Saccharomyces cerevisiae* Cu transporter Ccc2p (*CCC2* gene). When expressed in *S. cerevisiae* cells, *SlHMA1* complements the *ccc2* mutant deficient in high affinity iron uptake. Expression of *SlHMA1* also rescues the Cu sensitive phenotype of the *cup2* mutant. GFP fusion SlHma1 seems to localize on the endoplasmic reticulum/ Golgi membranes in yeast. In *S. luteus*, the expression of the *SlHMA1* gene and its potential interacting partners (*ATX1*- and *FET3*-like) is induced by Cu. The *SlHMA2* and *SlHMA3* are paralogs that show similarity with the Cu exporter coding gene *CRD1* of the pathogenic yeast *Candida albicans*. Heterologous expression of full length *SlHMA2* and *SlHMA3* in *S. cerevisiae* does not result in functional Cu transport proteins. A natural mutation of *SlHMA2* causing a frameshift and truncated protein in the *S. luteus* isolate LM8n1 is discovered. When expressed in yeast cells, the truncated *SlHMA2** and *SlHMA3** rescue both Cu and Cd sensitive mutants. In *S. luteus*, transcription of both genes is greatly induced by Cu and Cd exposure. Promoter analysis of all three ATPases reveals several binding sites for metal-responsive transcription factors.

3.2. INTRODUCTION

The ectomycorrhizal (ECM) fungus *Suillus luteus*, a basidiomycete, is an important root symbiont of young pine trees. Because its host plants are commonly grown in harsh environments and at various metal polluted sites, and because of the existence of metal tolerant ecotypes, this fungus is a good model species to investigate metal tolerance and homeostasis mechanisms. Previous studies confirmed that metal pollution has triggered adaptive Zn, Cd and Cu tolerance in *S. luteus* and that metal tolerant isolates can alleviate metal toxicity in their host plants (Adriaensen *et al.*, 2005; Colpaert, 2008). In comparison to plants, there is very little information on the molecular and cellular mechanisms underlying metal tolerance in ECM fungi in general. With the recent release of the full genome sequence of *S. luteus* (Kohler et al., 2015), identification of genes involved in micronutrient homeostasis and metal tolerance mechanisms is greatly facilitated. This study focuses on identification and functional analysis of P1B-type ATPases involved in Cu and Cd homeostasis and Cd detoxification in this fungus.

P-type ATPases are membrane proteins found in plasma membranes of bacteria and eukaryotes as well as in membranes of particular eukaryotic organelles. P-type ATPases function to transport a variety of different compounds, including ions, phospholipids and small organic compounds, using ATP hydrolysis for energy. The P1B-type ATPases are a subfamily of P-type ATPases that pump metal ions across biological membranes (Williams and Mills, 2005). There are only two P1B-type ATPases in humans (ATP7A and ATP7B), and they are well characterized because mutations in these genes result in an imbalance of Cu distribution in the human body -- the Menkes and Wilson diseases. These ATPases are normally localized to the trans-Golgi network in cultured cells but they are relocated to the plasma membrane in the presence of elevated extracellular Cu in order to export excess Cu from the cells (Goodyer *et al.*, 1999; La Fontaine and Mercer, 2007). In plants, the P1B-type ATPases – known as heavy metal ATPases (HMAs) – are very diverse in terms of tissue distribution, sub-cellular localization and metal specificity, and HMAs play an important role in the translocation and detoxification of different heavy metals. So far, eight

HMA genes in *Arabidopsis thaliana* and nine in rice have been found (Mills *et al.*, 2005; Takahashi *et al.*, 2012). In fungi, *Saccharomyces cerevisiae* yeast has two heavy metal ATPases: the Ccc2p localized on post-Golgi membranes transports Cu (Yuan *et al.*, 1997) and the Pca1p protein found on plasma membranes exports Cd and also sequesters Cu via its cysteine-rich N-terminal domain (Adle *et al.*, 2007). The human pathogenic yeast *Candida albicans* possesses an important P1B-type ATPase (Crd1) that functions as a Cu exporter assuring the yeast's high Cu tolerance (Weissman *et al.*, 2000). Phylogenetically closer to *S. luteus*, in the filamentous basidiomycete *Trametes versicolor*, a P1B-type ATPase transporting Cu has been identified and functionally characterized (Uldschmid *et al.*, 2003). In the current study, three genes coding for two different types of heavy metal transporting P1B-type ATPases in *S. luteus* were identified. We cloned the three genes and performed functional analysis. A yeast expression system was used to investigate the function and sub-cellular localization of the corresponding proteins. The expression of the genes in *S. luteus* exposed to different metals was analyzed using quantitative real-time PCR. Moreover, we searched for potential binding sites for different metal-responsive transcription factors in the promoter regions of the genes. Possible roles of these genes in homeostasis and detoxification of Cu and Cd are discussed.

3.3. MATERIALS AND METHODS

3.3.1. Identification of *S. luteus* HMAs

Protein sequences of functionally characterized HMA transporters were retrieved from the National Center for Biotechnology Information database (NCBI). These include CopA and PacS of *Escherichia coli* and the cyanobacteria *Synechocystis sp PCC6803*; CCC2 and CLAP1 of the plant-pathogenic ascomycetes *Cochliobolus heterostrophus* and *Cochliobolus lindemuthianum*; CtaA of the basidiomycete *T. versicolor*; Ccc2 and Pca1 of *S. cerevisiae* and Crd1 of *C. albicans*. To identify genes encoding HMA proteins in *S. luteus,* BLASTp was performed at the website of the Functional Genomics Program of the Department of Energy Joint Genome

Institute (JGI) (Grigoriev *et al.*, 2012). Protein sequence of CtaA Cu transporter of the basidiomycete *T. versicolor* (Uldschmid *et al.*, 2003) was used as query. Sequence alignment and phylogenetic analysis were performed with the CLC main workbench 7.0.2 (http://www.clcbio.com). Transmembrane domains were predicted using TMHMM Server v.2.0 (http://www.cbs.dtu.dk). The physical map of HMA gene clusters of *S. luteus* and related species were re-drawn from the genome browser of each species in the JGI website.

3.3.2. Cloning of *SlHMAs*

Monokaryons of the ectomycorrhizal basidiomycete *S. luteus* named LS5n1, LS5n32 and LM8n1 (in short for UH-Slu-Lm8-n1, the monokaryon used for JGI *S. luteus* genome database) obtained from basidiospores released by sporocarps collected from heavy metal polluted site in Lommel, Belgium were used. This site is heavily contaminated with zinc, cadmium, lead, … due to the pyrometallurgical activities during the period between 1904 and 1974. The cDNA libraries were made using the SMARTer PCR cDNA synthesis kit (Clonetech, US) following the manufacturer instructions. Specific primers were designed to amplify full-length coding sequences with or without stop codon (for GFP fusion construct; small letters) of *SlHMA1* (F: GCCCCTCGCGGCTATCTGAAGCATGTC; R: tcaAACACTATCCGACATTTCCATCGGG), *SlHMA2* (F: ATCGCACCTCCGAGTCATGA-GCACCTC; R: tcaAAGCTGTATCTTCGGCTCCCTGTACAA), *SlHMA3* (F: CCGTTCC-CCCACCCTCTAATCACAC; R: ttaGAGCTGTATCTTTGGCTCTTTGTACATATTGAG). For cloning of truncated *SlHMA2* and *SlHMA3*, the reverse primers were tcaGACGTCTGGTAGGAGCCTGTGCA and tcaTGATAATGGTGACGTCGGGCTGGAG, respectively. 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 3000 base pairs 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 pAG306GAL-ccdB and pAG306GAL-ccdB-EGFP (Alberti *et al.*, 2007) for functional complementation tests or for protein localization by GFP fluorescence. 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. Inserts were sequenced in both directions to assure correct fusion.

The yeast strains used for heterologous expression of *SlHMAs* were BY4741 (MATa; his3Δ1; leu2Δ; met15Δ0; ura3Δ0), Δzrc1 (BY4741; MATa; his3Δ1; leu2Δ; met15Δ0; ura3Δ0; YMR243c::kanMX4), Δycf1 (BY4741; MATa; his3Δ1; leu2Δ; met15Δ0; ura3Δ0; YDR135c::kanMX4), Δccc2p (BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR270w::kanMX4), Δcup2 (BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL166w::kanMX4) obtained from Euroscarf (EUROSCARF, Frankfurt, Germany, 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 galactose, 0.77 g/L CSM-URA, 2 % agar in case of solid medium, pH 5.8). Plates were incubated at 30 °C for five days before imaging.

The yeast drop assay was used to test whether expression of *S. luteus* genes can complement yeast loss-of-function mutations. A single 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 ferrozine or metals as indicated. Ferrozine (a ferrous iron chelator) was added to create iron-limiting condition. The yeast mutant lacking a Cu-transporting P1B-type ATPase (Δccc2p) is incapable of high-affinity iron uptake and cannot grow under this condition. The drop test was repeated at least three times using different yeast colonies.

3.3.3. Localization of SlHMA1::GFP fusion by confocal imaging

Transformed yeast cells were grown in SD medium with 2 % galactose to induce expression of the SlHMA::GFP fusion. Cells were grown to an OD_{600nm} of between 1-1.5, vacuolar membranes were selectively stained with the red fluorescence

probe FM4-64 (Molecular Probes, Invitrogen) following (Vida and Emr, 1995). Stained yeast cells were observed at 20 °C with a Zeiss LSM 510 META confocal imaging system (Carl Zeiss Mikroskopie, Jena, Germany). For EGFP fluorescence analysis we used the 488 nm excitation line of the laser and a BP 500-550 nm emission filter. For FM4-64 fluorescence analysis we used the 488 nm excitation line of the laser and a LP 560 nm emission filter. Image processing was carried out with ImageJ software (NIH, Bethesda, MD, USA).

3.3.4. Cultivation of *S. luteus* for the gene expression assay

Five monokaryons of *S. luteus* LS5n1, LS5n32, LS5n14, LS5n42 and LM8n1 were used. Fungal inocula of 0.5 x 0.5 cm were grown for eight days on cellophane-covered solid modified Fries medium (28 mM glucose, 5.4 mM ammonium tartrate, 1.5 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 5 µM $CuSO_4 \cdot 5H_2O$, 20 µM $ZnSO_4 \cdot 7H_2O$, 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) (Fries, 1978).

One gram of mycelium was 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. Every two days, half of the medium was replaced with fresh medium to obtain regular growth and uniform fungal spheres. Fungal spheres of about 0.3-0.5 cm diameter were used for the experiments. Fresh mycelial spheres (approximately 100 mg fresh weight) were transferred to Petri dishes containing 10 ml of Fries medium and were further grown for 24 h with shaking (120 rpm). Metals were added as sulfates ($CuSO_4.5H_2O$ and $3CdSO_4.8H_2O$) to the sphere cultures to obtain the following final concentrations: 0, 10, 20, 40 µM Cd or 0, 100, 500 µM Cu. The cultures were placed at 23 °C on a shaker incubator (70 rpm). After six hours, the spheres were collected in four replicates, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

3.3.5. Gene expression analysis with RT-qPCR

Total RNA was extracted using the RNeasy Plant Kit (Qiagen, France), according to the manufacturer's instructions. RNA concentration and purity was evaluated

spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, Wilmington, DE, USA). DNase treatment with the TURBO DNA-free™ Kit (Ambion, Life Technologies, Paisley, UK) was performed to eliminate possible genomic DNA contamination. For each sample, one µg of RNA 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 10-fold dilution of the cDNA was prepared in 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM Na_2-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, 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). All primer sequences are provided in the *Supplementary information,* Table S1 with GenBank accession or JGI protein ID number.

Reference genes were selected out of a set of ten candidate genes that show stable expression under different metal treatment conditions (Muller *et al.*, 2007; Ruytinx *et al.*, 2011) together with putative homologs of the classical reference genes actin and tubulin. Gene expression was calculated according to the $2^{-\Delta Cq}$ method, relative to the sample with the highest expression in all isolates. Obtained values were used in geNorm program (Vandesompele *et al.*, 2002) to find the most stable reference genes for the Cu and Cd treatment separately. According to the geNorm, four genes were needed to normalize the Cd treated samples (*AM085168, JGI805314, JGI810455, GR975621*), and three

genes (*AM085168, JGI805314, JGI810455*) were needed to normalize the Cu treated samples. Relative expression was expressed in fold changes: metal-treated sample set relatively to the non-treated control.

Mean values of at least three biological repeats were used to plot figures, and error bars represent the standard error of the means. The datasets were analyzed via the linear model procedure in R (R Development Core Team, 2013). Both normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) were tested. Data were analyzed using the one-way ANOVA procedure and Dunnett's test was used to compare different treatments with a control.

3.3.6. Promoter analysis

A qualitative and quantitative analysis of the transcription factor (TF) binding sites present in the promoter sequences of the three *SlHMAs* of isolate LM8n1 was made. We retrieved the upstream DNA sequences, approximately 1000 bp from the start codon of the three *SlHMAs*. We used the JASPAR core database (Portales-Casamar *et al.*, 2010) to scan the promoter sequences for binding sites of common, well-defined transcription factors. In a second step, we manually searched for metal-responsive elements (MREs) using known TF-binding sequences as queries. In which, the consensus sequence of metal regulatory elements (denoted here as yMRE) was HTHNNGCTGD (D = A, G or T; H = A, C or T; N = any nucleotides); the copper sensing element (CuSE) was DDDHGCTGD; and the MTF1-binding (MTF1-BD) metal response element was TGCRCNC (R = A or G).

3.4. RESULTS

3.4.1. Identification and characterization of *S. luteus* HMAs

Our BLASTp search in the genome database of *S. luteus* revealed three predicted Cu-transporting P1B-type ATPase protein sequences (JGI protein ID 20904, 1915649, 797637). These sequences are named 'heavy metal ATPase'

SlHma1, 2 and 3. The respective genes are *SlHMA1*, *2* and *3*. By comparing to the previously characterized orthologs we concluded that SlHma1 belongs to the CCC2-type group of HMAs and the SlHma2 and 3 belong to the CRD1-type group of HMAs. The JGI gene model of *SlHMA1* was correct but that of *SlHMA2* and *3* needed corrections at the N and C termini. We then re-sequenced the *HMA2* and *3* genes in several isolates of *S. luteus* and found that the incorrect prediction was caused by an unprocessed intron in the DNA sequence of *SlHMA2*, and because the monokaryon that was sequenced for the genome database (UH-Slu-Lm8-n1 or LM8n1 for short) itself carries a frameshift deletion of 11 bp (position 1048-1058) (*Supplementary information*, Figure S3.1). The dikaryon 'parent' strain of LM8n1 is a heterozygote and carries both a 'correct' and a 'frameshift' allele of *SlHMA2*. This frameshift deletion was not present in other isolates used in this study. The corrected sequences of SlHma1, 2 and 3 are shown in Figure 3.1A and 3.1B. All SlHmas contain eight transmembrane domains, two GMXCXXC (X stands for any amino acid) metal-binding motifs, a highly conserved DKTGT motif in the phosphorylation domain, a GDGIND motif in the ATP-binding domain and a CPC motif in the translocation domain. The SlHma2 and 3 have two additional metal-binding motifs CXXC in the N-termini that differentiate them from SlHma1. The latter motif is characteristic for the second cluster (CRD1-type) in the phylogenetic tree (Figure 3.2).

The phylogenetic tree was constructed using the *S. cerevisiae* Pca1 (synonym: Cad2) protein sequence as an out-group. The Pca1 is a Cd-transporting P1B-type ATPase responsible for Cd tolerance in yeast (Shiraishi *et al.*, 2000; Adle *et al.*, 2007). Orthologs of Pca1 are found in plants and several ascomycetes (Saitoh *et al.*, 2009). We did not find any orthologs of Pca1 in *S. luteus* nor in other sequenced Boletales (see also Chapter 2). Representatives of known HMAs in bacteria (CopA and PacS) and in fungi were included in the phylogenetic tree. SlHma1 groups closely, with high bootstrap values, to all CCC2-type proteins from the basidiomycete *T. versicolor*, the four ascomycetes fungi (*S. cerevisiae, C. albicans*, *C. heterostrophus* and *C. lindemuthianum*), and to the two bacterial CCC2-type HMAs. Whereas, the SlHma2 and 3 proteins cluster with the yeast *C. albicans* Crd1.

DNA sequencing of the three HMAs showed that all three genes were interrupted by short introns of approximately 50 bp. *SlHMA1* is located on scaffold 8; the gene has four exons and codes for 986 aa. *SlHMA2* and *3* are located on scaffold 4, have 12 exons and code for 1119 and 1112 aa, respectively. The latter two genes shared high similarity (Figure 3.1B) and are probably the result of a gene duplication event. To find out if the gene duplication also exists in other species, we searched for the HMAs in four other basidiomycete genomes (*Suillus brevipes, Piloderma croceum, Paxillus involutus and Amanita muscaria*) and the results are shown in Figure 3.3. Except for *A. muscaria* that belongs to the order Agaricales, the other four species belong to the Boletales. It is obvious that *S. brevipes,* the most closely related species to *S. luteus* with a sequenced genome, carries a highly similar cluster of genes with two putative HMA genes of the CRD1-type. The gene duplication was also found in other Boletales (*P. croceum* and *P. involutus*) and Agaricales (*A. muscaria*).

A

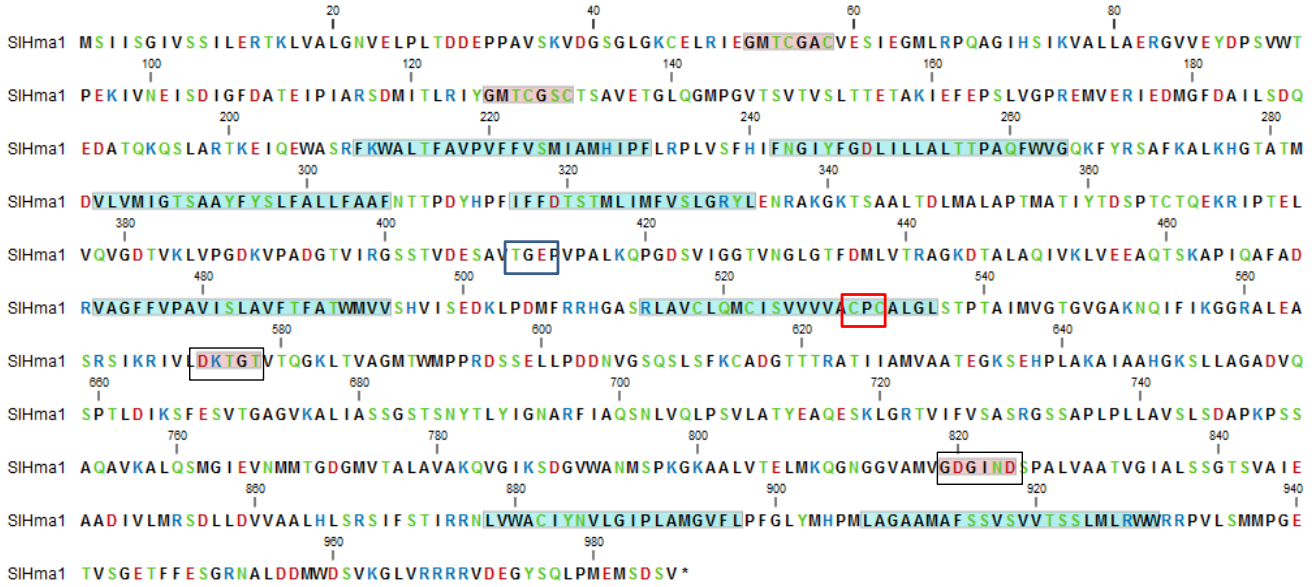
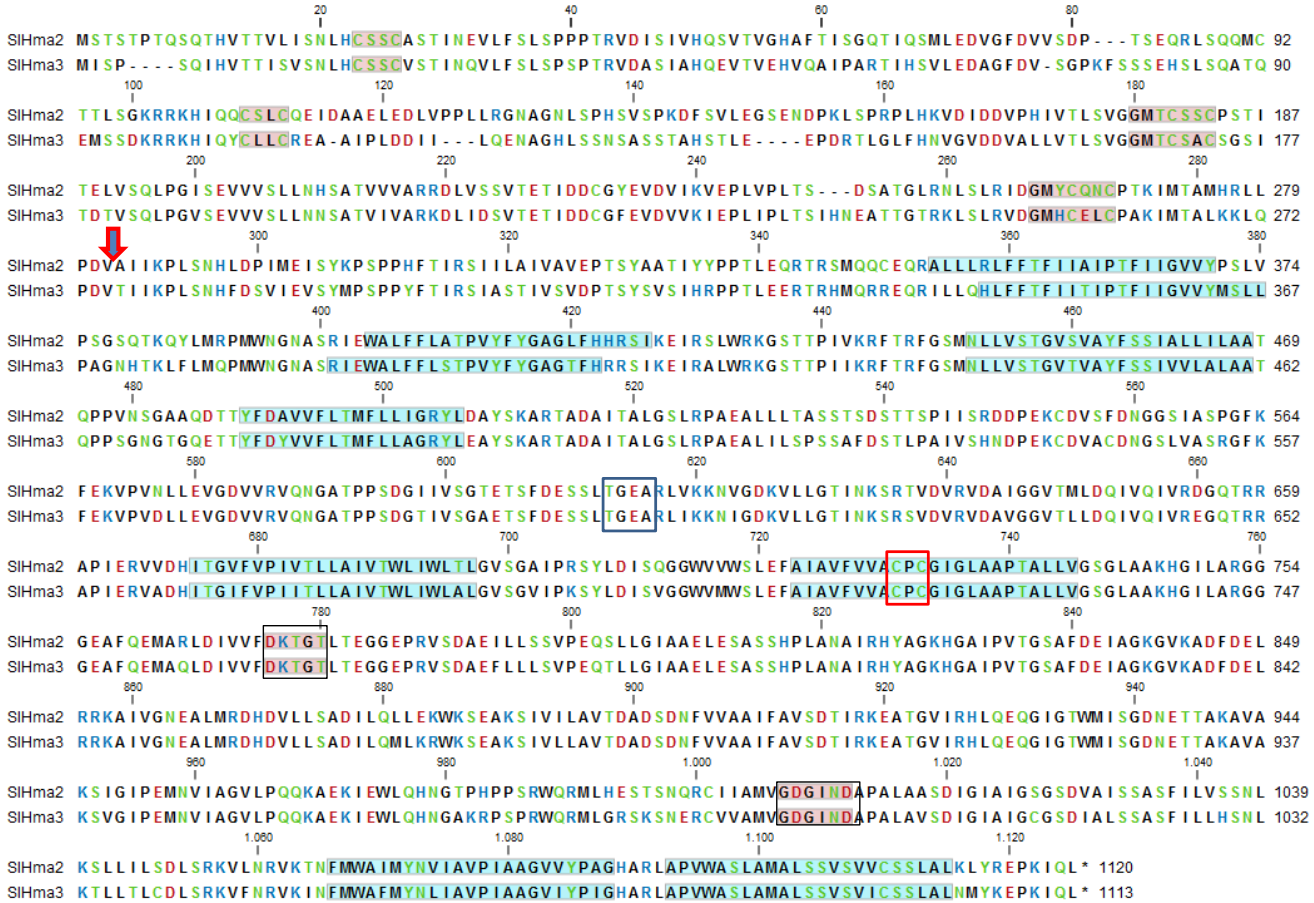


Figure 3.1. Amino-acid sequence and putative functional domains of the SlHma1 (A), SlHma2 and SlHma3 (B, *next page*)

The amino-acid sequence deduced from the DNA sequence data of SlHma1 (A), SlHma2 and 3 share high similarity and are presented together (B). The predicted functional domains and the P1B-type ATPase highly conserved motifs are indicated as follows: an N-terminal region with 2 (SlHma1) or 4 (SlHma2 and 3) cytosolic CXXC (pink shaded) metal binding domains (MBD), 8 putative transmembrane domains (TM, light blue). Connecting TM4 and TM5 is the phosphatase domain with the conserved motifs (blue box) TGEP in SlHma1 and TGEA in SlHma2 and 3), TM6 is predicted to be part of the translocation domain and has the CPC motif (red box). Between TM6 and TM7 is the phosphorylation and ATP binding domains with the highly conserved sequences (shaded, black box) DKTGT and GDGIND respectively. The red arrow in (B) indicates the end of the truncated SlHma2* and 3* proteins.

B


SlHma2 MSTSTPTQSQTHVTTVLISNLHCSSCASTINEVLFSLSPPPTRVDISIVHQSVTVGHAFTISGQTIQSMLEDVGFDVVSDP---TSEQRLSQQMC 92
SlHma3 MISP----SQIHVTTISVSNLHCSSCVSTINQVLFSLSPSPTRVDASIAHQEVTVEHVQAIPARTIHSVLEDAGFDV-SGPKFSSSEHSLSQATQ 90
SlHma2 TTLSGKRRKHIQQCSLCQEIDAAELEDLVPPLLRGNAGNLSPHSVSPKDFSVLEGSENDPKLSPRPLHKVDIDDVPHIVTLSVGGMTCSSCPSTI 187
SlHma3 EMSSDKRRKHIQYCLLCREA-AIPLDDII---LQENAGHLSSNSASSTAHSTLE----EPDRTLGLFHNVGVDDVALLVTLSVGGMTCSACSGSI 177
SlHma2 TELVSQLPGISEVVVSLLNHSATVVVARRDLVSSVTETIDDCGYEVDVIKVEPLVPLTS---DSATGLRNLSLRIDGMYCQNCPTKIMTAMHRLL 279
SlHma3 TDTVSQLPGVSEVVVSLLNNSATVIVARKDLIDSVTETIDDCGFEVDVVKIEPLIPLTSIHNEATTGTRKLSLRVDGMHCELCPAKIMTALKKLQ 272
SlHma2 PDVAIIKPLSNHLDPIMEISYKPSPPHFTIRSIILAIVAVEPTSYAATIYYPPTLEQRTRSMQQCEQRALLLRLFFTFIIAIPTFIIGVVYPSLV 374
SlHma3 PDVTIIKPLSNHFDSVIEVSYMPSPPYFTIRSIASTIVSVDPTSYSVSIHRPPTLEERTRHMQRREQRILLQHLFFTFIITIPTFIIGVVYMSLL 367
SlHma2 PSGSQTKQYLMRPMWVNGNASRIEWALFFLATPVYFYGAGLFHHRSIKEIRSLWRKGSTTPIVKRFTRFGSMNLLVSTGVSVAYFSSIALLILAAT 469
SlHma3 PAGNHTKLFLMQPMWVNGNASRIEWALFFLSTPVYFYGAGTFHRRSIKEIRALWRKGSTTPIIKRFTRFGSMNLLVSTGVTVAYFSSIVVLALAAT 462
SlHma2 QPPVNSGAAQDTTYFDAVVFLTMFLLIGRYLDAYSKARTADAITALGSLRPAEALLLTASSTSDSTTSPIISRDDPEKCDVSFDNGGSIASPGFK 564
SlHma3 QPPSGNGTGQETTYFDYVVFLTMFLLAGRYLEAYSKARTADAITALGSLRPAEALILSPSSAFDSTLPAIVSHNDPEKCDVACDNGSLVASRGFK 557
SlHma2 FEKVPVNLLEVGDVVRVQNGATPPSDGIIVSGTETSFDESSLTGEARLVKKNVGDKVLLGTINKSRTVDVRVDAIGGVTMLDQIVQIVRDGQTRR 659
SlHma3 FEKVPVDLLEVGDVVRVQNGATPPSDGTIVSGAETSFDESSLTGEARLIKKNIGDKVLLGTINKSRSVDVRVDAVGGVTLLDQIVQIVREGQTRR 652
SlHma2 APIERVVDHITGVFVPIVTLLAIVTWLIWLTLGVSGAIPRSYLDISQGGWVVWSLEFAIAVFVVACPCGIGLAAPTALLVGSGLAAKHGILARGG 754
SlHma3 APIERVADHITGIFVPIITLLAIVTWLIWLALGVSGVIPKSYLDISVGGWVMWSLEFAIAVFVVACPCGIGLAAPTALLVGSGLAAKHGILARGG 747
SlHma2 GEAFQEMARLDIVVFDKTGTLTEGGEPRVSDAEILLSSVPEQSLLGIAAELESASSHPLANAIRHYAGKHGAIPVTGSAFDEIAGKGVKADFDEL 849
SlHma3 GEAFQEMAQLDIVVFDKTGTLTEGGEPRVSDAEFLLLSVPEQTLLGIAAELESASSHPLANAIRHYAGKHGAIPVTGSAFDEIAGKGVKADFDEL 842
SlHma2 RRKAIVGNEALMRDHDVLLSADILQLLEKWKSEAKSIVILAVTDADSDNFVVAAIFAVSDTIRKEATGVIRHLQEQGIGTWMISGDNETTAKAVA 944
SlHma3 RRKAIVGNEALMRDHDVLLSADILQMLKRWKSEAKSIVLLAVTDADSDNFVVAAIFAVSDTIRKEATGVIRHLQEQGIGTWMISGDNETTAKAVA 937
SlHma2 KSIGIPEMNVIAGVLPQQKAEKIEWLQHNGTPHPPSRWQRMLHESTSNQRCIIAMVGDGINDAPALAASDIGIAIGSGSDVAISSASFILVSSNL 1039
SlHma3 KSVGIPEMNVIAGVLPQQKAEKIEWLQHNGAKRPSPRWQRMLGRSKSNERCVVAMVGDGINDAPALAVSDIGIAIGCGSDIALSSASFILLHSNL 1032
SlHma2 KSLLILSDLSRKVLNRVKTNFMWAIMYNVIAVPIAAGVVYPAGHARLAPVWASLAMALSSVSVVCSSLALKLYREPKIQL* 1120
SlHma3 KTLLTLCDLSRKVFNRVKINFMWAFMYNLIAVPIAAGVIYPIGHARLAPVWASLAMALSSVSVICSSLALNMYKEPKIQL* 1113

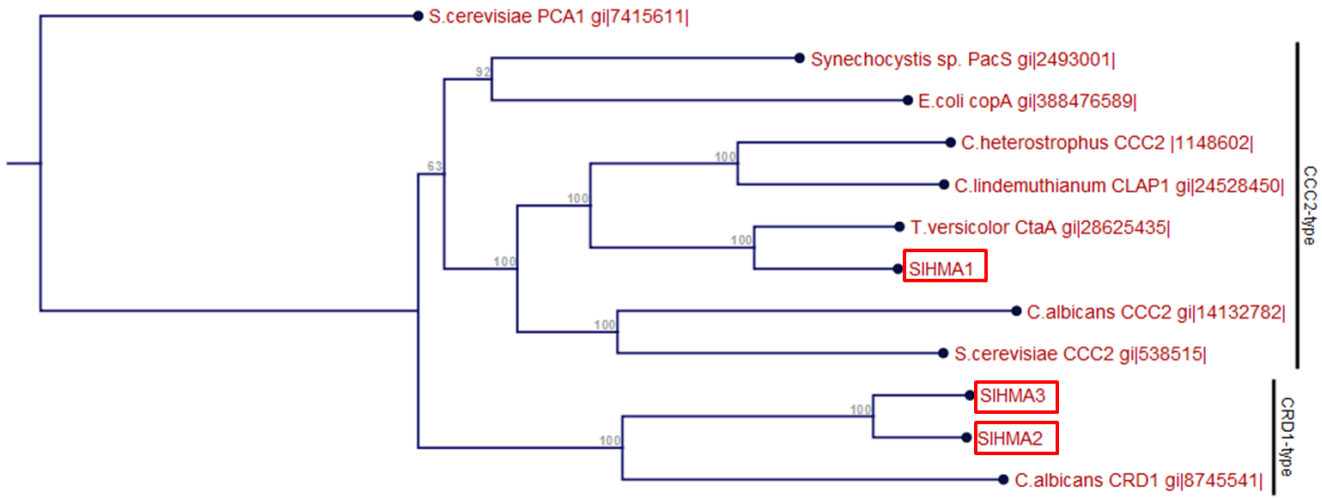


Figure 3.2. Phylogenetic analysis of SlHmas and functionally characterized P1B-type ATPases.

Protein sequences of known P1B-type ATPases were retrieved from NCBI and GI numbers are indicated. Sequence alignment and phylogenetic analysis were performed with the CLC main workbench 7.0.2 (www.clcbio.com). Bootstrap values were calculated from 1000 replications and presented as percentages.

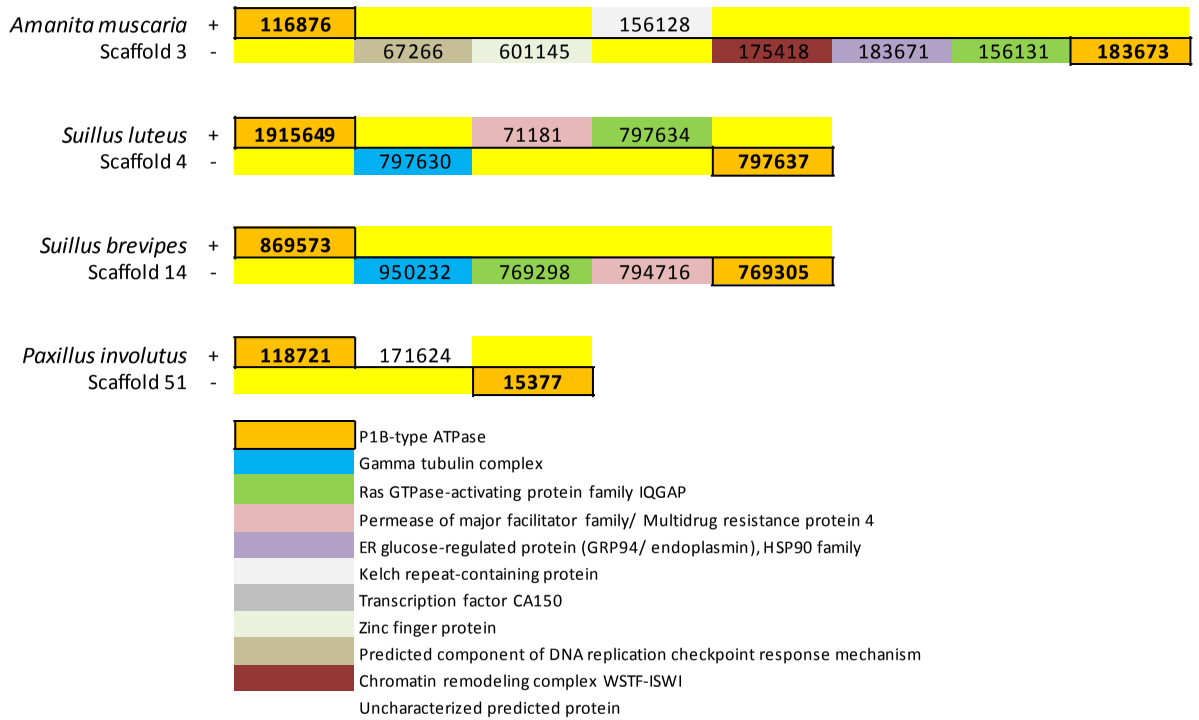


Figure 3.3. Physical map of the *S. luteus* and related species HMA gene clusters. Numbers are JGI predicted protein ID. Protein ID 1915649 and 797637 are SlHma2 and SlHma3, respectively. Homologous genes are shown in identical colours.

Table 3.1. Yeast strains used in this study

Strain	Genotype	Function of the deleted gene	Phenotype	Accession number *
BY4741	MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0		Wild-type	Y00000
Δccc2	BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR270w::kanMX4	Cytosolic copper metallochaperone that transports copper to the secretory vesicle copper transporter Ccc2p for eventual insertion into Fet3p, which is a multicopper oxidase required for high-affinity iron uptake	Deficient in Fe uptake	Y03629
Δcup2	BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL166w::kanMX4	Copper-binding transcription factor; activates transcription of the metallothionein genes CUP1-1 and CUP1-2 in response to elevated copper concentrations	Cu sensitive	Y04533
Δzrc1	BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR243c::kanMX4	Vacuolar membrane zinc transporter, transports zinc from the cytosol into the vacuole for storage; also has a role in resistance to zinc shock resulting from a sudden influx of zinc into the cytoplasm	Zn sensitive	Y00829
Δycf1	BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ 0; YDR135c::kanMX4	Vacuolar glutathione S-conjugate transporter of the ATP-binding cassette family, has a role in detoxifying metals such as cadmium, mercury, and arsenide	Cd sensitive	Y04069

(*) Accession number of the collection of deletion strains of the European *Saccharomyces cerevisiae* archive for functional analysis (EUROSCARF)

3.4.2. Yeast functional complementation

By comparing the HMA sequences of different *S. luteus* isolates (monokaryons or dikaryons) we found several types of sequence variations; some of these mutations resulted in an altered amino acid, deletion/insertion of an amino acid, or a truncated protein (in the case of *SlHMA2* mentioned above). We suspect that these variations might cause variations in the function of the recombinant proteins. Therefore, gene constructs were made using cDNA libraries of at least two different monokaryons. The coding sequences were cloned into the yeast expression vector pAG306GAL-ccdB (Addgene, 14139). The *SlHMAs* genes were then under the control of a GAL1-inducible promoter (Alberti *et al.*, 2007). Gene constructs were transformed into the chosen yeast mutants and a wild type (Table 3.1); at least three transformants were tested in the drop assays.

In *S. cerevisiae*, Ccc2p is a Cu-transporting P1B-type ATPase, located in the membrane of the late or post Golgi compartment (Yuan *et al.*, 1997). Ccc2p receives Cu from the chaperone Atx1 and transfers the ions into the lumen of the Golgi where Cu is then inserted into Cu-dependent enzymes such as the Fe oxidase Fet3p. Fet3p then functions with the high-affinity Fe transporter Ftr1p to import Fe (Stearman *et al.*, 1996). Thus, the *ccc2* mutant lacks Fet3p activity and is incapable of high-affinity Fe uptake. This mutant cannot survive on Fe-limited medium.

The gene constructs pAG306GAL-*SlHMA1, -SlHMA2,* and *-SlHMA3* were transformed into a *ccc2* mutant. Serial dilutions of transformants were made on Ferrozine-containing media to create an Fe-limiting condition. Glucose in the medium was replaced by galactose to induce expression of the *HMAs*. The results showed that only *SlHMA1* expression could restore viability of the *ccc2* mutant cells (Figure 3.4).

To find out if *SlHMAs* had a role in metal resistance as their homologs from other species, we transformed the gene constructs into yeast mutants that were sensitive to Cu (*cup2*), Cd (*ycf1*), or Zn (*zrc1*). The Cu-sensitive phenotype of *cup2* was fully complemented by *SlHMA1* on medium with up to a tested concentration of 75 µM Cu, but not or only partially by *SlHMA2* and *3* (Figure 3.4B). Expression of neither of the genes could restore growth of *ycf1* or *zrc1* on

Cd or Zn containing media; on the contrary, all three *SlHMAs* made the *ycf1* mutant even more sensitive to Cd (Figure 3.4C).

Intrigued by the unexpected results with *SlHMA2* and *3*, and the natural frameshift mutation of *SlHMA2* of the LM8n1 isolate, we cloned and expressed the sequence prior to the frameshift deletion of *SlHMA2* and the corresponding part of *SlHMA3* (denoted as truncated *SlHMA2** and *3**). The resulting portions of SlHma2 and 3 proteins were of 282 and 278 aa in length, starting from the N-termini and covered the metal-binding domains of the two proteins. Expression of the truncated genes rescued both *cup2* and *ycf1* yeast mutants on Cu and Cd containing media (Figure 3.4B, C).

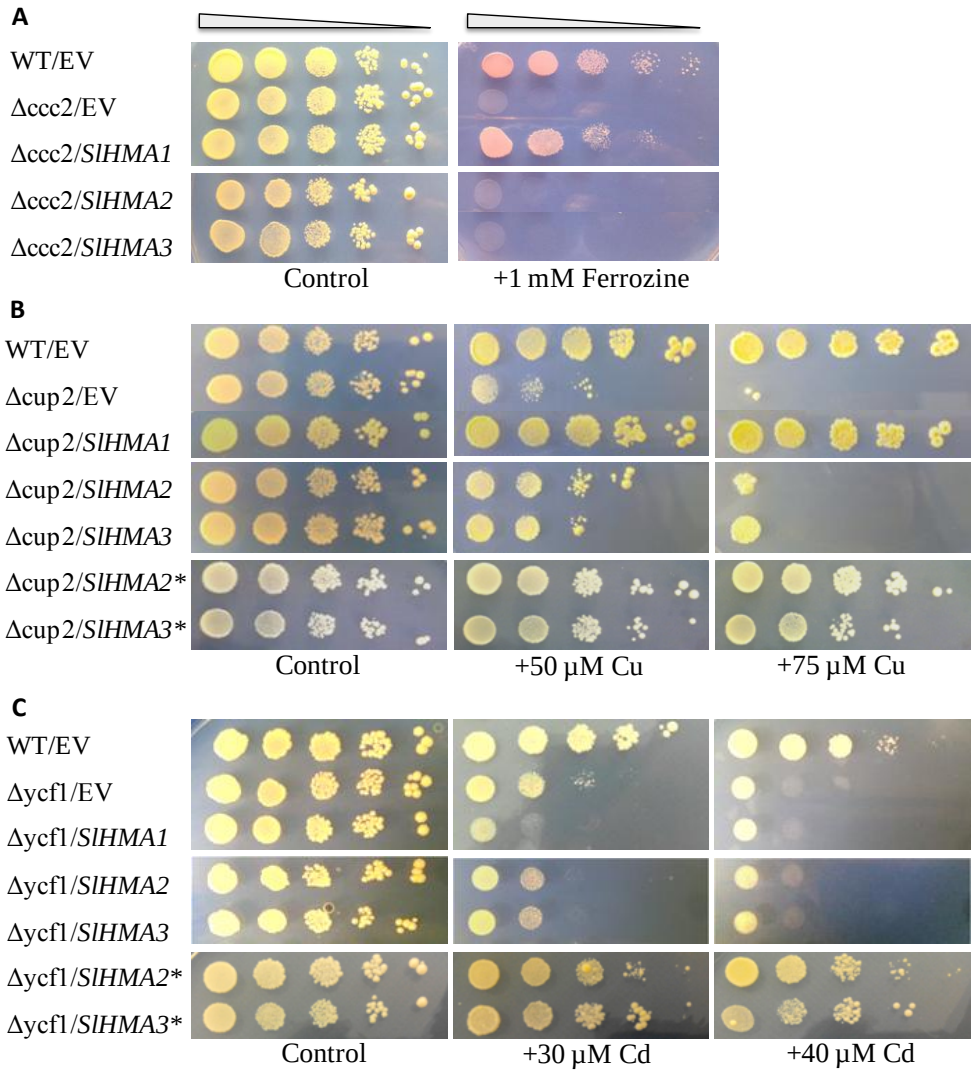


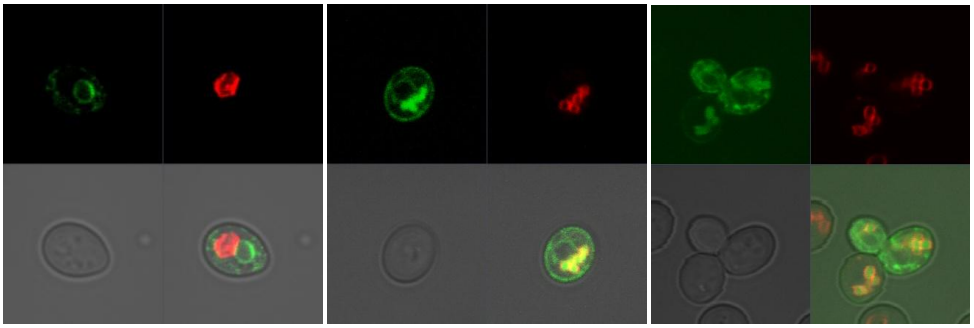
Figure 3.4. Complementation of *S. cerevisiae* mutants on selective media.

Yeast mutant strains were transformed with the empty vector (EV) pAG306GAL, with vectors containing full length *SlHMA1*, *2* or *3*, or truncated genes (*SlHMA2** and *3**). 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 Fe chelator (ferrozine, A) or metals (B,C) as indicated. Plates were incubated for five days at 30 °C before imaging.

3.4.3. Localization of SlHma proteins in yeast

Considering that all SlHma proteins were predicted to contain eight transmembrane domains, and that the yeast homologues of SlHmas are localized on the post-Golgi (Ccc2p) or the plasma membrane (Crd1), it is important to identify the location of the SlHma proteins. The SlHma1 protein could complement the *ccc2* yeast mutant and could rescue the *cup2* mutant from high Cu toxicity; this indicates that the SlHma1 protein functions as a Cu efflux transporter that removes Cu from the cytosol, either by extruding Cu out of the cell or by transporting Cu into intracellular compartments as its yeast homolog does. We, therefore, constructed a SlHMA::EGFP fusion to determine the intracellular localization of heterologously expressed proteins in yeast.

For SlHma1, EGFP fluorescence was found forming a ring-like pattern, typical of endoplasmic reticulum localization in yeast. EGFP was not present on the vacuolar membrane since the vacuolar membrane was clearly stained with FM4-64 (red) and no overlap between the green fluorescence and FM4-64 signal was observed (Figure 3.5A). In some cells, EGFP fluorescence was also detected sporadically on the periphery of the cells. For SlHma2 and 3, a different localization of the EGFP signal was observed (Figure 3.5B and C). Apart from the ring-like pattern as seen with SlHma1, the fluorescence of SlHma2 and 3 was also found on the periphery of the cells ranging from sporadical to a relatively smooth line (Figure 3.5B) resembling plasmalemma localization. Sometimes, cytoplasmic aggregation, and vacuolar accumulation of the fusion EGFP of SlHma2 and 3 were observed.



A B C

Figure 3.5. Confocal images of SlHma1::EGFP (A), SlHma2::EGFP (B) and SlHma3:EGFP (C) fusion in yeast.

Cells were grown overnight in SD medium with galactose to induce gene expression. Four different images from the same cells are shown: GFP fluorescence, bright field, red fluorescence of FM4-64 stained vacuoles and the overlay image. In (A) SlHma1::EGFP fusion expresses a ring-like pattern, typical of ER-Golgi without overlap with the FM4-64 signal of the stained vacuole. In (B) and (C), not only the ring-like pattern is present but fusion GFP is found on plasma membranes and as aggregates inside the vacuoles.

3.4.4. Expression of *SlHMAs* in *S. luteus* exposed to high Cu and Cd

Complementation of *SlHMA1* and truncated *SlHMA2* and *3* in yeast indicated that the three genes might be involved in Cu and Cd resistance in *S. luteus*. We therefore conducted quantitative real-time PCR (RT-qPCR) experiments to verify changes in the expression of *SlHMAs* in response to exogenous Cu and Cd. Both monokaryons and dikaryons of *S. luteus* can easily grow on solid agar plates and in a liquid medium. For these experiments liquid cultures were preferred. Nutrient availability and metal exposure is more homogenous for individual cells and harvest of mycelia is more convenient in time dependent experiments. Five monokaryons were selected, in which four were monokaryons isolated from a single 'parent' sporocarp and LM8n1, the monokaryon that was sequenced for the *S. luteus* genome database. Isolates LS5n1 and LS5n32 are tolerant to Cd, and the other three LS5n14, LS5n42, LM8n1 are sensitive. There is no clear difference in their tolerance to Cu. Pictures of these isolates on Cd and Cu containing media are provided in *Supplementary information*, Picture S3.1.

All five isolates were exposed to increased concentrations of Cu and Cd for six hours. A six hour exposure to both 100 and 500 µM Cu induced a three- to four-fold increase in expression of *SlHMA1* in all isolates. Cd exposure did not induce significant changes in *SlHMA1* expression in none of the isolates (Figure 3.6). Both Cu and Cd exerted a great effect on the expression of *SlHMA2* and *SlHMA3*. Cu exposure strongly induced expression of *SlHMA2* and *SlHMA3*, up to 30 and 16 times, respectively. Exposure to Cd induced *SlHMA2* and *SlHMA3* to a lesser extent, up to four times with the highest Cd concentration (40 µM). Cu and Cd treatments also significantly induced expression of *SlHMA2* that carried the frameshift deletion in isolate LM8n1.

Because the *SlHMAs* responded to Cu and Cd treatments, we decided to investigate the expression of genes that code for proteins that potentially interact with the *SlHMAs,* particularly *SlHMA1* which showed Ccc2p-like characteristics. As described for the yeast *S. cerevisiae*, Cu is captured by metallo-chaperone Atx1 before being delivered to the Cu transporter Ccc2p for eventual incorporation into Fet3p/Ftr1 complex. Cu loaded Fet3/Ftr1 then travels

to the plasma-membrane to take part in Fe uptake. Deletion of *FTR1* and/or *FET3* also leads to Cu sensitivity in yeast (Shi *et al.*, 2003; Stoj *et al.*, 2007).

We used as query the known cognate proteins of the yeast Ccc2p to search for their homologs in the *S. luteus* genome. We identified putative genes encoding for two Cu chaperones *ATX(*9264) and *COX*(799011), a ferric reductase *FRE*(319406), a ferroxidase *FET3*(805776), and an iron permease *FTR1*(805774). Names for these genes were adopted from the yeast homologs and the protein IDs from the *S. luteus* genome database were provided in brackets. It is worth noting that *COX*(799011) is an homolog of the yeast Cox17, a chaperone that delivers Cu to the mitochondrion for assembly of the cytochrome *c* oxidase (Heaton *et al.*, 2000). *Saccharomyces cerevisiae* has a third chaperone that delivers Cu to superoxide dismutase (Culotta *et al.*, 1997) but we could not find any homologs in *S. luteus*. Since neither of the chaperones has been studied in detail, we analyzed the expression of both chaperones in Cu treated *S. luteus*. As expected, Cu treatment induced expression of *ATX*(9264) and *FET3*(805776) in all isolates (Figure 3.6), and *COX*(799011) in four out of five isolates (data not shown). In contrast *FRE*(319406) and *FTR1*(805774) had similar gene expression patterns but did not alter significantly with Cu exposure (data not shown).

3.4.5. Promoter analysis of *SlHMAs*

We subsequently searched for transcription factor (TF) binding sites present in the promoter sequences of the *SlHMAs* of LM8n1. We used the JASPAR core database (Portales-Casamar *et al.*, 2010) to scan the promoter sequences for binding sites of common, well-defined transcription factors. In a second step, we manually searched for metal-responsive elements (MREs) using known TF binding sequences as queries. In the 2014 release, the JASPAR core database for fungi contained a set of 177 yeast *S. cerevisiae* TF binding profiles. Scanning the *SlHMAs* promoters we found that promoters of all three *SlHMAs* contained binding sites for MSN2, MSN4, SKN7, SKO1, STB5, TFs related to osmotic and oxidative stress responses. We do not yet know if *S. luteus* has homologs of these TFs. The scan also predicted several binding sites for TF Mac1p in all three promoters. In *S. cerevisiae,* Mac1p activates a set of genes involved in import of

Cu under Cu-deficient conditions (Jungmann *et al.*, 1993; Labbe *et al.*, 1997). Manually searching for metal regulatory elements (denoted here as yMRE) using the consensus sequence HTHNNGCTGD (D = A,G or T; H = A,C or T; N = any nucleotides), binding sites for TF Ace1p of *S. cerevisiae* (Buchman *et al.*, 1990), and for TF Amt1 of the *Candida glabrata* (Zhou and Thiele, 1993) we found one yMRE in *SlHMA1* and three in the *SlHMA2* promoter. Position of the predicted TF binding sites is provided in the *Supplementary information*, Figure S3.2. Using as query another motif that is highly similar with the yMRE: DDDHGCTGD denoted as Cu sensing element (CuSE) and binding site for TF Cuf1 of *S. pombe* (Beaudoin *et al.*, 2003) we could also detect two CuSEs in *SlHMA1* and three in the *SlHMA2* promoter.

In the *S. luteus* genome we found two candidate homologs of the TFs Ace1, Amt1, Mac1 and Cuf1 which have protein IDs: 31562 and 809277. We performed gene expression analysis of these two candidate genes and found that expression of 31562 was strongly induced by Cu in three out of five tested isolates, and was induced by Cd treatments in all isolates (Figure 3.7) whereas 809277 did not show any induction.

Finally, in the promoter of all *SlHMAs* (two in *SlHMA1* and one in *SlHMA2* and *3*) the consensus sequence TGCRCNC (Thiele, 1992) corresponding to another type of MREs found in higher eukaryotes and mammals was detected. Transcription factor MTF1 (metal response element-binding TF-1) that specifically binds to this MRE in human, mouse and the fruit fly *Drosophila* is well documented (Palmiter, 1994; Wang *et al.*, 2004b; Günther *et al.*, 2012). One homolog of MTF1 is present in the *S. luteus* genome: 798587. We performed gene expression analysis of this putative MTF1 gene and found that its expression was not significantly altered by Cu but was induced by Cd treatments in all isolates (Figure 3.7).

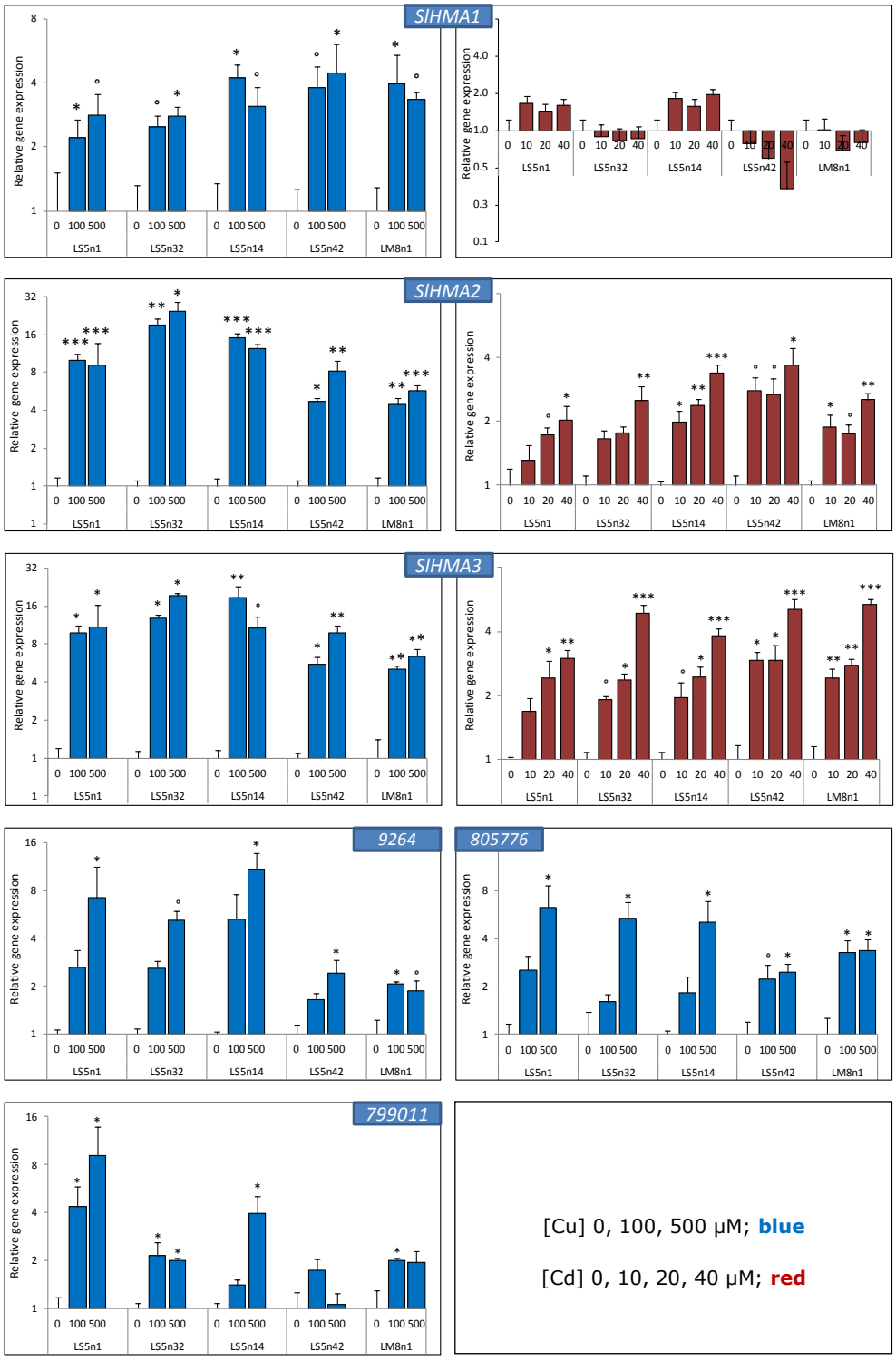

SlHMA1
SlHMA2
SlHMA3
9264
805776
799011
Relative gene expression
0 100 500
0 10 20 40
LS5n1
LS5n32
LS5n14
LS5n42
LM8n1
[Cu] 0, 100, 500 µM; blue
[Cd] 0, 10, 20, 40 µM; red

Figure 3.6. Relative expression of *SlHMA1, 2* and *3* and potential interacting copper chaperone *ATX(9264)* and ferroxidase *FET(805776)* in *S. luteus* exposed to high Cu and Cd. Five *S. luteus* isolates were grown in liquid cultures for eight days. Treatments were started by adding the metals to the medium (Cu: 0, 100, 500 µM, presented in blue or Cd: 0, 10, 20, 40 µM, presented in red) and incubated for six hours. Gene expression was measured by RT-qPCR and presented as fold changes (metal-treated relative to non-treated control). Data are given as mean ± SE of four biological replicates. Statistics were performed for each isolate separately. Relative expression fold changes are represented on a $\log_2$ scale y axis. Dunnett's comparison was performed to test for significant difference of each treatment to the non-treated control; (°), (*), (**), and (***) indicate significant differences at $p<0.1$, $p<0.05$, $p<0.01$, $p<0.001$, respectively.

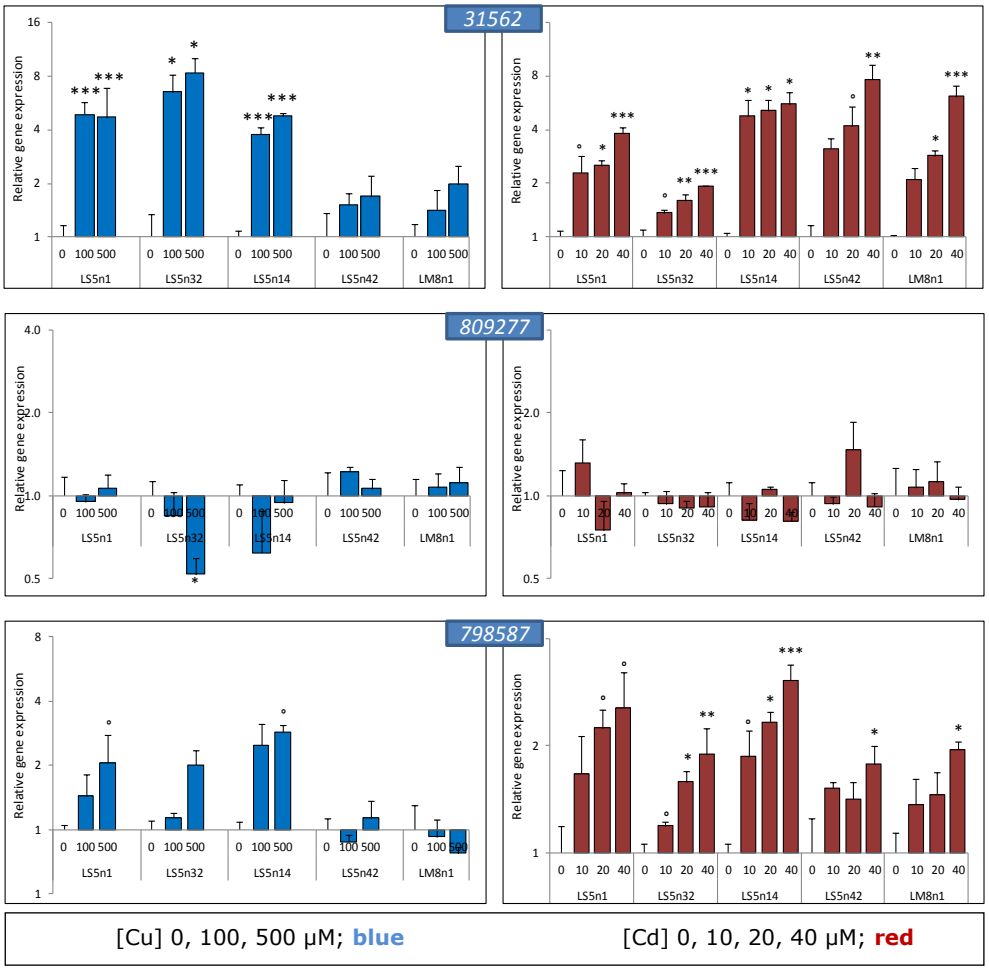


Figure 3.7. Relative expression of *SlHMAs'* potential transcription factors in *S. luteus* exposed to high Cu and Cd. Five *S. luteus* isolates were grown in liquid cultures for eight days. Treatments were started by adding the metals to the medium (Cu: 0, 100, 500 µM, presented in blue or Cd: 0, 10, 20, 40 µM, presented in red) and incubated for six hours. Gene expression was measured by RT-qPCR and presented as fold changes (metal-treated relative to non-treated control). Data are given as mean ± SE of four biological replicates. Statistics were performed for each isolate separately. Relative expression fold changes are represented on a $\log_2$ scale y axis. Dunnett's comparison was performed to test for significant difference of each treatment to the non-treated control; (°), (*), (**), and (***) indicate significant differences at $p<0.1$, $p<0.05$, $p<0.01$, $p<0.001$, respectively.

3.5. DISCUSSION

In the present study, we identified three transporter genes (*SlHMA1, 2* and *3*) involved in Cu and Cd homeostasis and detoxification in *S. luteus*. The *SlHMA1* could be well characterized. The protein sequence deduced from the *SlHMA1* gene sequence reveals a high similarity of SlHma1 with the Ccc2-type Cu transporting ATPase that is found in members from all kingdoms (Fu *et al.*, 1995a; Yuan *et al.*, 1995; Rensing *et al.*, 2000; Rensing and Mitra, 2007). The data obtained from the heterologous expression in different yeast mutants confirm that SlHma1 specifically transports Cu but not Cd nor Zn. The heterologous expression of GFP-tagged SlHma1 shows that SlHma1 seems to localize in the ER-Golgi complex. This is expected from the high homology of SlHma1 with Ccc2p. Via successful complementation of a *ccc2* mutant, we could also confirm that SlHma1 transports Cu following a similar route as its yeast homolog. Since SlHma1 fully complemented the *ccc2* mutant, it probably could interact with the yeast Cu chaperone Atx1 to obtain Cu and to transfer Cu to the yeast Fet3/Ftr1 complex which fulfils its role in Fe acquisition by the cells. Our results enlarge the list of successful yeast complementation tests in which the two human genes ATP7A (Payne and Gitlin, 1998) and ATP7B (Hung *et al.*, 1997) can complement deletion of *CCC2*, as well as *C. albicans CCC2* (Weissman *et al.*, 2002a), homologs found in the basidiomycete fungus *T. versicolor* (Uldschmid *et al.*, 2003), and a few plants (Hirayama *et al.*, 1999; Southron *et al.*, 2004).

Even though Ccc2 homologs and their interacting proteins are highly conserved in various organisms from bacteria, yeast to human, gene expression seems to be regulated slightly differently. For example, transcriptional expression of *CCC2* of the yeasts *S. cerevisiae* and *C. albicans* are not influenced by Cu but induced in low Fe conditions (Yamaguchi-Iwai *et al.*, 1996; Weissman *et al.*, 2002a). We showed, on the other hand, that the *SlHMA1* gene expression was induced by Cu exposure in the different isolates of *S. luteus*. Similarly, transcriptional regulation of the Cu chaperone *ATX1* in yeast is not influenced by Cu (Lin *et al.*, 1997), whereas the *ATX* homolog of *T. versicolor* is strongly regulated by Cu

availability (Uldschmid *et al.*, 2002). In our study, we showed that expression of *SlHMA1* as well as its candidate chaperone *ATX*(9264) and ferroxidase *FET*(805776) were influenced by Cu conditions.

CCC2-type transporters are important for the biosynthesis of Cu containing enzymes such as amine oxidase, tyrosinase, and multicopper oxidases including ferroxidase and laccases. Deletion of *CCC2*-type genes results in loss of laccase activities in some fungi (Parisot *et al.*, 2002; Saitoh *et al.*, 2009). Compared to yeasts, filamentous fungi have a larger set of Cu containing enzymes that can be a target of CCC2-type transporter proteins (Saitoh *et al.*, 2009). In the ectomycorrhizal fungus *Laccaria bicolor*, 11 genes coding for multicopper oxidases including nine laccases and two ferroxidases have been identified (Courty *et al.*, 2009). Browsing the *S. luteus* genome we could find at least 10 candidate genes for multicopper oxidases including the *FET*(805776) that was included in our gene expression analysis. Homolog of *FET*(805776) in the yeast *S. cerevisiae* is Fet3p. This ferroxidase acquires Cu from Ccc2p in the post-Golgi and forms a complex with Ftr1p to target for the cell surface to play a role in importing iron (Taylor *et al.*, 2005). *FET3* and *FTR1* expression is induced under conditions of low iron or high Cu. Mutant *fet3* is sensitive to Cu (Shi *et al.*, 2003). Induction of *FET*(805776) in Cu treated *S. luteus* is in accordance with the role of Fet3p in the yeast model. In addition to Fet3-like proteins, Cu could be incorporated into other multicopper oxidase such as laccases. Most laccases are extracellular enzymes (Baldrian, 2006) and Cu is a strong laccase inducer in a number of fungal species in the laboratory and in industry (Giardina *et al.*, 1999; Palmieri *et al.*, 2000; Piscitelli *et al.*, 2011; Tinoco *et al.*, 2011; Janusz *et al.*, 2013). It is likely that in *S. luteus,* together with *FET*(805776) ferroxidase, secretion of laccases also takes part in removing Cu from the cytoplasm.

Regarding *SlHMA2* and *3,* heterologously expressed full length of *SlHMA2* and *3* in yeast did not result in phenotype complementation. Whereas, expression of the truncated N-terminal metal binding domains of SlHma2 and SlHma3 complemented the Cu- and Cd-sensitive phenotypes of yeast mutants. Although the Ccc2-type transporter is well conserved from bacteria to human, the Crd1-type proteins are far less conserved (Saitoh *et al.*, 2009). The yeast *S. cerevisiae* itself does not have a homolog of Crd1-type but a paralog: Pca1,

which functions mainly as a Cd exporter at the plasma membrane and which is to some extent involved in Cu and iron homeostasis (Adle *et al.*, 2007). To our knowledge, except for the characterization of Crd1 in *C. albicans* (Riggle and Kumamoto, 2000a; Weissman *et al.*, 2000), there is no other report on heterologous expression and characterization of a Crd1-type protein in other fungi.

Failure to express the full SlHma2 and 3 might be caused by various reasons. Above all, heterologous expression of proteins in general and membrane proteins in particular is challenging. Topology of some proteins can be modified when they are produced in heterologous systems and the proper folding and localization of proteins with a large molecular weight and high number of transmembrane fragments are generally less successful (Bernaudat *et al.*, 2011). It will be more advisable to express the SlHma2 and *3* in other systems such as in *C. albicans* or, preferably in a closely related basidiomycete.

Expression of SlHma2 and 3 metal binding domains could rescue yeast mutants on high Cu and Cd concentrations and that expression of the genes *in vivo* was strongly induced by Cu and Cd indicate that SlHma2 and 3 might be involved in Cu homeostasis and Cd detoxification in *S. luteus*. In *C. albicans*, expression of *CRD1* gene is also induced more by Cu than Cd and *crd1* null mutant is found to be sensitive to both metals (Riggle and Kumamoto, 2000a). As long as localization of SlHma2 and 3 remains unknown we cannot answer if SlHma2 and 3 function as Cu and Cd exporters as observed with their Crd1 ortholog. However, it is clear that SlHma2 and SlHma3 do not function as SlHma1 in the Ccc2p-like pathway. We could also show that the three genes responded differently to Cu and Cd. Expression patterns of *SlHMA2* and *3* were markedly different from *SlHMA1* expression. *SlHMA2* and *3* expression was induced approximately two times more than that of *SlHMA1* in all tested Cu conditions and *S. luteus* isolates, and the expression of both genes was induced in response to Cd treatments whereas *SlHMA1* was not. Moreover, the level of induction of *ATX1*(9264) and *FET*(805776) corresponded only to the level of induction of *SlHMA1*. There were two putative copper chaperones *ATX1*(9264) and *COX*(799011) in the *S. luteus* genome and both genes were induced by exposure to Cu but not by Cd. We wondered whether other factors interact to

deliver Cu and Cd to SlHma2 and 3 or the two truncated proteins can chelate Cu and especially Cd by themselves. In fact, SlHma2 and 3 possess each four CXXC motifs that are known to participate in binding of Cu and Cd in a number of chaperones and metal transporters. Examples are the *S. cerevisiae* Atx1, human Atox1, *A. thaliana* Cdl19 and various bacteria P1B-type ATPases (Suzuki *et al.*, 2002; Narindrasorasak *et al.*, 2004; Banci *et al.*, 2006). Complementation of the N-terminal portion of SlHma2 and 3 in the Cu and Cd sensitive yeasts partially proved that the proteins could chelate the metals and protect the cells. It will be interesting to express and purify the truncated SlHma2 and 3 and determine their ability to bind Cu, Cd and other metals in vitro, as well as investigate their interactions with potential chaperone(s). To our knowledge, there is no evidence on the role of Crd1-type transporters in metal storage or transferring Cu to Cu-containing proteins but this possibility should not be neglected.

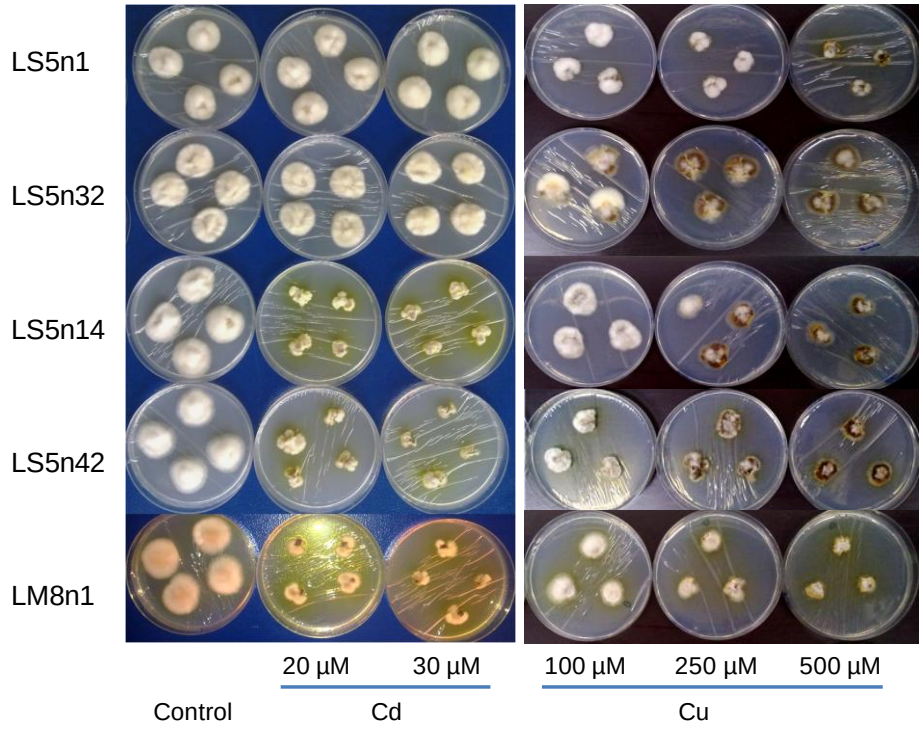
The fact that *S. luteus* and some other basidiomycetes maintain two homologs of SlHma2 and 3 indicates that the genes have some physiological significance in these fungi. It is possible that malfunctioning of one gene can be compensated by the other. It is also possible, though not likely, in the case of *S. luteus* because of the high similarity in sequence and gene expression patterns, that the two proteins may evolve differently, locate on different membranes and acquire different functions.

The *S. luteus* genome allows us to systematically track the network of metal transporters and regulators that determine the fate of essential and non-essential metals in this fungus. Our search for transcription factor binding sites on the promoters of the three *SlHMAs* revealed several possibilities for gene regulation. In general, expression of all three *SlHMAs* might be influenced by various factors because their promoters contain elements that are known to be involved in metal, osmotic and oxidative stress responses. The presence of metal-responsive elements in all *SlHMAs'* promoters is particularly convincing. Referring to the yeast model, in *S. cerevisiae*, Cu ions regulate expression of a number of genes through TF Ace1p and Mac1p. Ace1p is responsible for the induction of metallothioneins and superoxide dismutase expression in response to stressful levels of Cu, whereas Mac1p activates a subset of genes under Cu-deficient conditions (Gross *et al.*, 2000). The situation is different in the fission

yeast *S. pombe;* TF Cuf1 of *S. pombe* binds DNA in the Ace1-like TF binding site but functions in the manner of Mac1p (Beaudoin and Labbé, 2001). In the pathogenic yeast *Cryptococcus neoformans,* TF Cuf1 is involved both in Cu acquisition and Cu detoxification (Jiang *et al.*, 2011). The presence of binding sites for these TFs in *SlHMAs'* promoters is indicative of the genes regulation. From the analysis we could find one potential TF: sequence ID 31562 that increased transcription under both Cd and Cu treatments in a pattern similar to *SlHMA2* and *SlHMA3*. We predict that *SlHMA2* and *3* might be direct targets of this ACE1-like transcription factor, similar to that of the yeast system (Gross *et al.*, 2000). Another potential TF is the MTF1 homolog (798587) that has binding sites in all *SlHMAs'* promoters and its expression was up-regulated under Cd exposure.

All together, the present study represents the first steps into the understanding of the role of heavy metal ATPases in Cu and Cd homeostasis in *S. luteus*. SlHma1 is a structural and functional homolog of the yeast *S. cerevisiae* Ccc2p that transports Cu into the ER-Golgi to be incorporated into Cu containing enzymes and is important for Cu resistance. SlHma2 and 3 are paralogs that share sequence similarity with the Cu exporter Crd1 of *C. albicans*. Even though we were not successful in expressing functional full length of SlHma2 and 3 proteins in the yeast *S. cerevisiae,* we could show that overexpression of the proteins' metal binding domain could rescue Cu and Cd sensitive yeast mutants. Transcriptional responses of the genes and their putative transcription factors to excess Cu and Cd strongly suggest the involvement of these genes in the metal homeostasis and detoxification in *S. luteus*.

3.6. SUPPLEMENTARY INFORMATION



Picture S3.1. Growth of five monokaryons of *S. luteus* on Cd and Cu containing medium.

Fungal inocula of 0.5 x 0.5 cm were grown on cellophane-covered solid Fries medium containing metals as indicated. Fungal cultures were placed at 23 °C. Images were taken after eight days.

Table S3.1. Primers used in the study

	Usage	Name*	Sequence 5'-3'	Amplicon size (bp)	Primer efficiency**
1	Cloning	SlHMA1F	GCCCCTCGCGGCTATCTGAAGCATGTC		
2	Cloning	SlHMA1R	tcaAACACTATCCGACATTTCCATCGGG		
3	Cloning	SlHMA2F	ATCGCACCTCCGAGTCATGAGCACCTC		
4	Cloning	SlHMA2R	tcaAAGCTGTATCTTCGGCTCCCTGTACAA		
5	Cloning	SlHMA3F	CCGTTCCCCCACCCTCTAATCACAC		
6	Cloning	SlHMA3R	ttaGAGCTGTATCTTTGGCTCTTTGTACATATTGAG		
7	Cloning truncated gene	trHMA1R	tcaTCTTGGCTGTCTCGGTGGTGAGTGAG		
8	Cloning truncated gene	trHMA2R	tcaGACGTCTGGTAGGAGCCTGTGCA		
9	Cloning truncated gene	trHMA3R	tcaTGATAATGGTGACGTCGGGCTGGAG		
10	qPCR	qHMA1F	CATCGTTCTCATGCGTTCTG	102	2.057 ± 0.079
11	qPCR	qHMA1R	ATACACGCCCAGACGAGATT		
12	qPCR	qHMA2F	ACCCCACATCCACCTTCAC	102	1.979 ± 0.015
13	qPCR	qHMA2R	GGGTGCATCATTGATACCG		
14	qPCR	qHMA3F	GTTGTGTCGTTGCAATGGTC	103	2.068 ± 0.083
15	qPCR	qHMA3R	GATAATGCGATGTCGCTTCC		
16	qPCR	qATX9264F	CGCCGAGCACAACTATCAAC	82	1.933 ± 0.068
17	qPCR	qATX9264R	CGCCTTGGTCAGTACACGA		
18	qPCR	qCOX799011F	TGCATGCGAGGACTTGGATT	93	1.909 ± 0.060
19	qPCR	qCOX799011R	CGCGGTGGGCTGATTATGAA		

20	qPCR	qFET805776F	CCTCTTGCTGCATTCTCCAAA	106	1.935 ± 0.048
21	qPCR	qFET805776R	GTTCAAGAGGGCAGGTTGCT		
22	qPCR	q31562F	AGCACCTGCAGCTGAATCAT	101	2.085 ± 0.097
23	qPCR	q31562R	AGCTTTGGGTCGTTGGACAT		
24	qPCR	q809277F	TCAGACAGGTCGCACTTAGC	106	1.986 ± 0.013
25	qPCR	q809277R	CGCGGTCTTCCGTTCTGATA		
26	qPCR	qMTF798587F	CACAAGCGGACACACAATGG	133	2.031 ± 0.066
27	qPCR	qMTF798587R	GACAAATTCGACGACTCAGCG		
28	qPCR	qFRE319406F	CTAGCCCTTGCATTTGTCGG	80	2.069 ± 0.053
29	qPCR	qFRE319406R	GGCTCGGTAATATCTCGGCG		
30	qPCR	qFTR805774F	AAGGTGTTGTCTTCGTCGGA	99	2.086 ± 0.068
31	qPCR	qFTR805774R	AACCGCAGATAAGACCGCAA		
32	qPCR reference	JGI810455F	GTTCTGGGAGGTGGTTTCTGATG	110	1.926 ± 0.080
33	qPCR reference	JGI810455R	GCCGATTTCATTGTAGTAAACCGA		
34	qPCR reference	GR975621F	CCGGTTTCAGTAATACAGAGTCCT	96	2.077 ± 0.056
35	qPCR reference	GR975621R	GGTCTGTTTACCTTACTTTATTATGTCCACC		
36	qPCR reference	AM085168F	GGGCGTTTGACGAAGCTCATC	98	2.045 ± 0.014
37	qPCR reference	AM085168R	CGATCTCGAGGAGCTGTGTTCCA		
38	qPCR reference	JGI805314F	TGATGAACGAGGGAGTCGTG	113	2.008 ± 0.103
39	qPCR reference	JGI805314R	GGAATGGTCCAGAGATCGCA		

* JGI protein identification number, GenBank accession number (GR, AM; deposited by Ruytinx *et al.* 2011) and names of *S. cerevisiae* orthologs are included when applicable. ** Primer efficiency (e ± standard deviation) was calculated on a standard curve generated using a twofold dilution series of a mixed cDNA sample over at least five dilution points (e = 10^(−1/slope))

Figure S3.1. Sequence of *SlHMA2* showing position of the 11 bp (977-987) frameshift deletion on exon 3 of the isolate LM8n1. The deletion results in a difference in the deduced amino acid sequence (boxed). Full gene and coding sequence of *SlHMA2* of the five *S. luteus* isolates were amplified by PCR from genomic DNA and cDNA before cloning into pCR4TOPO TA cloning vector (Life technologies, Paisley, UK) for sequencing.

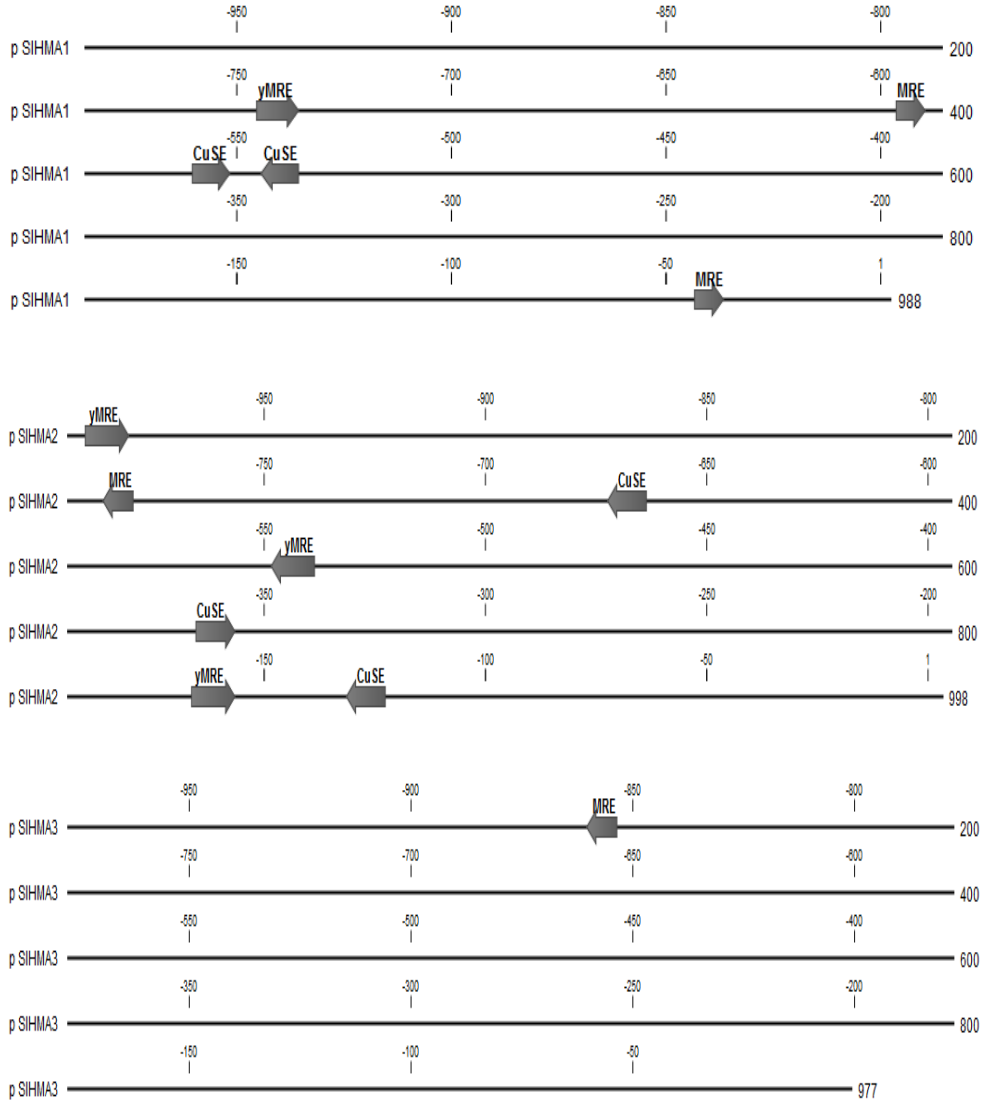


Figure S3.2. Position of the predicted metal response elements on promoter region of the three *SlHMAs.*

- yMRE sequence: HTHNNGCTGD (D = A,G or T; H = A,C or T; N = any nucleotides); binding site for *S. cerevisiae* Ace1p, Mac1p and *Candida glabrata* Amt1
- CuSE sequence: DDDHGCTGD; binding site for *S. pombe* Cuf1
- MRE sequence: TGCRCNC (R = A or G); binding site for higher eukaryotes (humans, mouse, fly *Drosophila melanogaster)* MTF1

CHAPTER 4

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

4.1. ABSTRACT

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 genes that code for potential heavy metal chelators in *S. luteus.* Two metallothionein coding genes, *SlMTa* and *SlMTb,* were identified. The genes encode two deduced peptides of 67 and 65 aa residues in which 16 and 15, respectively, are cysteine residues arranged in the typical CXC and CXXC motifs of metallothionein-like proteins. When expressed in *Saccharomyces cerevisiae* cells, both *SlMTa* and *SlMTb* can rescue the *cup2* copper sensitive mutant. *In vivo*, transcription of both *SlMTa* and *SlMTb* are induced by copper but not cadmium nor zinc. Promoter analysis and comparison of promoter sequences of the novel MT genes of two *Suillus* species (*S. luteus and S. brevipes*) revealed several copper-sensing and metal-response elements that are conserved across both species and are most likely binding sites for metal responsive transcription factors. These results indicate that SlMta and SlMtb function as copper metallothioneins. Moreover, sequence search revealed that homologs of *S. luteus* MTs (52 protein sequences) are present in 48 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. These 48 fungi harbouring one or both MT genes have different life styles and ecological functions. The roles of the novel MTs in copper tolerance in basidiomycete fungi

and their possible application are discussed. Results of this study will assist in the identification of orthologs of *SlMTa* and *SlMTb*, as well as other types of fungal MTs.

4.2. INTRODUCTION

Metallothioneins (MT) are small proteins (molecular weight 4 - 8 kDa) that bind heavy metals, such as zinc, copper, cadmium, silver etc. 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). Metallothioneins are present in a vast range of taxonomic groups, ranging from prokaryotes to humans (Capdevila *et al.*, 2012). 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 Kagi (Binz and Kägi, 1999). Alternatively, based on their metal-binding abilities, MTs can be classified into different metal groups such as the Zn-thioneins and Cu-thioneins (Palacios *et al.*, 2011). 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 and 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 Hg; (3) carrier of essential metals to apo-metalloproteins; (4) free radical scavenging and protection against oxidative damage (Capdevila *et al.*, 2012). In plants, the existence of four MT families is known. Their metal specificity, production, and regulation in a variety of tissues are well studied. Plant MTs are not only constitutively expressed, but the production of different types of plant MTs is also stimulated by a myriad of endogenous and exogenous agents in both a temporally and spatially regulated manner (Leszczyszyn *et al.*, 2013). Accordingly, beside metal homeostasis and detoxification, plant MTs have been

linked to a variety of biotic and abiotic stresses (Zhu *et al.*, 2009), but also in 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 sporadically characterized in yeasts, a few other ascomycetes (*Neurospora crassa*, *Candida albicans*, *Candida glabrata, Yarrowia lipolytica, …*) and only a handful of basidiomycetes (*Paxillus involutus*, *Laccaria bicolor*, *Hebeloma sp., Russula atropurpurea, Cryptoccocus neoformans, …*). Among those identified, most are copper-binding MTs, although silver (Osobová *et al.*, 2011) and zinc (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), responses to oxidative stress (Reddy *et al.*, 2014), in fungal-plant symbiosis interactions (Lanfranco *et al.*, 2002; Bergero *et al.*, 2007) as well as in fungal virulence in the case of pathogens (Tucker *et al.*, 2004; Ding *et al.*, 2013).

Thanks to the 1000 fungal genomes project (Grigoriev *et al.*, 2011), a myriad 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 (in which about 20,000 belong to the subphylum Agaricomycotina) including many important saprotrophs, and mycorrhizal fungi (Hibbett, 2006). The ectomycorrhizal (ECM) basidiomycete *Suillus luteus* is a very 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 been investigated. 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.

4.3. MATERIALS AND METHODS

4.3.1. The fungus

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 KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 5 µM $CuSO_4 \cdot 5H_2O$, 20 µM $ZnSO_4 \cdot 7H_2O$, 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).

4.3.2. Identification of *SlMTa* and *SlMTb*

To identify genes encoding for MTs in *S. luteus,* BLASTp was performed at the JGI genome portal. Protein sequences of known MTs of different organisms were used as queries (*Supplementary information*). Sequence alignment was performed with the CLC main workbench 7.0.2 (http://www.clcbio.com).

4.3.3. Cloning of *SlMTa* and *SlMTb* genes

A cDNA library was made using the SMARTer PCR cDNA synthesis kit (Clonetech, US) following the manufacturer's instructions. Specific primers were designed to amplify full-length coding sequences of *SlMTa* (F: ACAAAAACCATAATGGCGAC CTGCAG; R: TCACTTTGACTCGCAGGTACATGCTAGA), *SlMTb* (F: GCGCTCTGCAT-CAACATGGCTAAAGAC; 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 functional complementation of *SlMTa* and *SlMTb* were BY4741 (MATa; his3Δ1; leu2Δ; met15Δ0; ura3Δ0), Δzrc1 (BY4741; MATa; his3Δ1; leu2Δ; met15Δ0; ura3Δ0; YMR243c::kanMX4), Δycf1 (BY4741; MATa; his3Δ1; leu2Δ; met15Δ0; ura3Δ0; YDR135c::kanMX4), Δyap1 (BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YML007w::kanMX4) and Δcup2 (BY4741; MATa; 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 galactose, 0.77 g/L CSM-URA, 2 % agar in case of solid medium, pH 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.

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

Suillus luteus inocula of 0.5 x 0.5 cm 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 Fries medium and were grown further for 24 h with shaking (120 rpm). Metals were added as sulfates ($CuSO_4.5H_2O$, $CdSO_4.3/8H_2O$ and $ZnSO_4.7H_2O$) 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. RNA concentration and purity was evaluated spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, Wilmington, DE, USA). DNase treatment with the TURBO DNA-free™ Kit (Ambion, Life Technologies, Paisley, UK) was performed to eliminate possible genomic DNA contamination. 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 Na_2-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. Reference genes and 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).

All primer sequences are provided in the *Supplementary information* Table S4.2 with JGI protein ID or GenBank accession number deposited previously (Ruytinx *et al.*, 2011). Gene expression was calculated according to the $2^{-\Delta Cq}$ method, relative to the sample with the highest expression and expression of genes of interest was normalized to three reference genes (*Supplementary information,* Table S4.2). The reference genes were selected (out of ten candidates) based on their high expression stability determined by geNorm (Vandesompele *et al.*, 2002). Relative expression was expressed in fold changes for each time point: metal-treated sample relative to the non-treated control set to 1.

Mean values of four biological repeats were calculated, and error bars represent the standard error of the means. The datasets were analyzed via the linear model procedure in R (R development core team, 2013). Both normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) were tested. Data were analyzed using the one-way ANOVA procedure and Dunnett's test was used to compare different treatments with a control.

4.3.5. Promoter analysis of *SlMTa* and *SlMTb* genes

Upstream DNA sequences, approximately 1500 bp from the start codon, of *MTa* and *MTb* of *S. luteus* and *S. brevipes* were retrieved from JGI genome database. Genomic DNA sequence with promoter regions and coding sequences were aligned using CLC main work-bench 7.0.2 software (http://www.clcbio.com). Putative transcription factor binding sites were searched manually using their consensus sequences as follow: ARE (TGACNNNGC), STRE (CCCCT), CuSE

(DDDHGCTGD), CuSE* (DDHGCTGD), yMRE (HTHNNGCTGD), MTF1-BD (TGCRCNC), in which D = A,G or T; H = A,C or T; N = any nucleotides; R = A or G.

4.4. RESULTS

4.4.1. Identification of *SlMTa* and *SlMTb*

A number of known MT protein sequences from literature were used as queries for BLASTp in the *S. luteus* genome database. In most cases the BLASTp (expect value E = 10^{-5}, scoring matrix BLOSUM62, applied to filtered model proteins) returned no hits. BLASTp using as a query the longest metallothionein Cmt2 (183 aa) of the human fungal pathogen *Cryptococcus neoformans* H99 (Ding *et al.*, 2011) gave one positive hit with a 40 % identity of an hypothetical protein 802625. By increasing the expected value (to E = 10^{-4}) and re-BLASTp in the *S. luteus* genome using 802625 as a query we found its paralog: 84059. None of the protein sequences were annotated. Both 84059 and 802625 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. A comparison to the query sequence (Cmt2) and to all known 15 families of metallothioneins we found that *S. luteus* Mts had a unique sequence particularly in the arrangement of cysteine residues (Figure 4.1 and *Supplementary information*).

4.4.2. Homologs of SlMta and SlMtb in the Basidiomycota fungi

Expanding our BLAST searches to all sequenced basidiomycetes in the JGI database (last access in April 2015; 152 genomes were available) we found a number of potential homologs of SlMta and SlMtb. Figure 4.1A shows 52 sequences that are most likely homologs of SlMta and SlMtb obtained from fungi

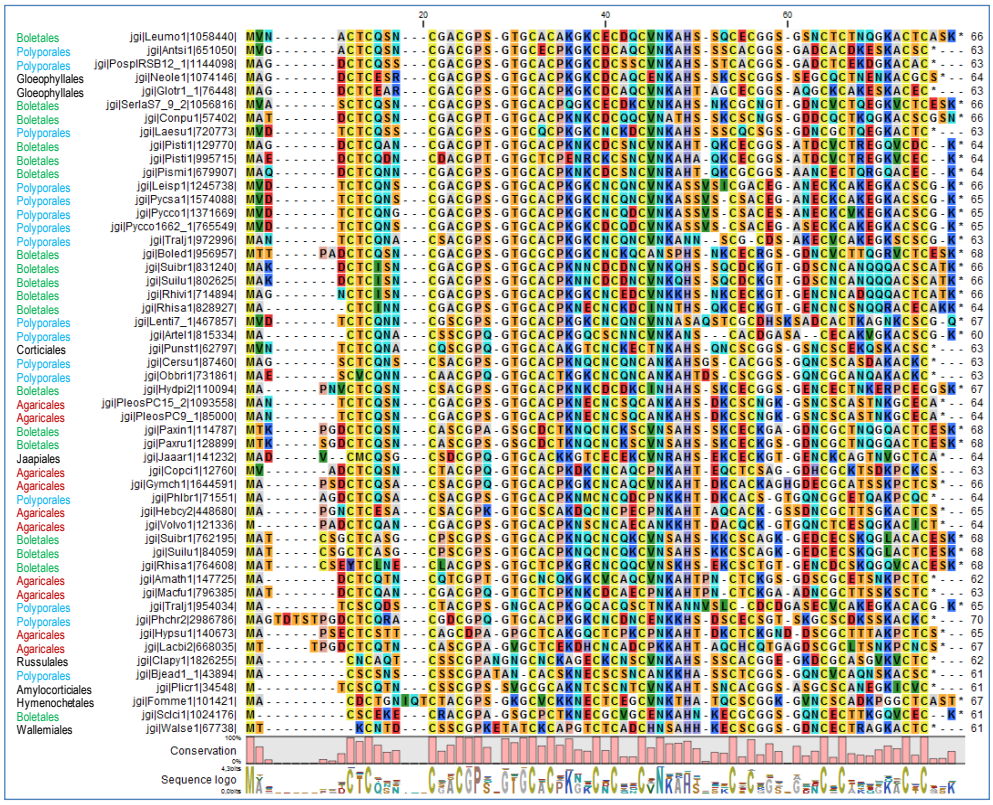
from ten different orders of the subphylum Agaricomycotina. A list of fungal species is provided in the *Supplementary information*, Table S4.1. Sequence alignment of 18 putative MTs of *S. luteus* and other Boletales species was performed separately (Figure 4.1B) to show that these MTs are intensively conserved among species in the same 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 their Mtb's are completely identical (Figure 4.1B).

Both sequence alignments show that the putative MTs, except for some exceptions, share common features (1) The sequence does not contain any CC or CCC motifs, (2) does not have long spacers typical for plants MTs, (3) is of approximately 60-70 aa and contains 15-16 cysteine residues, (4) one histidine but no other aromatic amino acid is present. Seven cysteine-rich boxes can be distinguished and are indicated in Figure 4.1B. The abundance and arrangement of cysteine residues of these novel MTs are different from known MT families as described (Binz and Kägi, 1999) and reviewed recently (Capdevila *et al.*, 2012).

A few variations exist in the cysteine-rich motifs among species listed in Figure 4.1: four Polyporales species (*Leiotrametes sp*., *Pycnoporus cinnabarinus*, *Pycnoporus sangguineus*, *Trametes ljubarskyi*) have CXXC instead of CXC in cysteine-rich box V (Figure 4.1 A). Three Suilloid species (*S. luteus, S. brevipes, and Rhizopogon salebrosus*) have an additional cysteine residue in the cysteine-rich box I (Figure 4.1B), whereas *Wallemia sebi* (Wallemiomycetes, Basidiomycotina) lacks one cysteine. Other than these 52 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) (data not shown). 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 were longer and homologous to the *Cryptococcus neoformans* Cmt2 were found in the genomes of *Dioszegia cryoxerica* and *Tremella mesenterica* (order Tremellales, Agaricomycotina), *Rhodotorula sp.* (Pucciniomycotina), and *Sporisorium reilianum* (Ustilaginomycotina).

Chapter 4

A



B

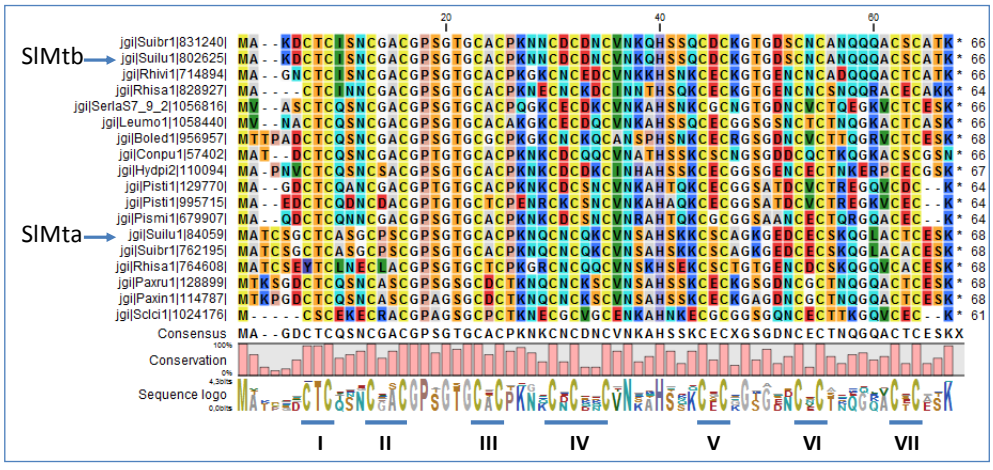


Figure 4.1. Sequence alignment of (A) 52 putative MTs found in basidiomycete fungi and (B) 18 putative MTs found in 14 species belonging to the order Boletales. Sequences were retrieved from the JGI genome database and JGI protein IDs are provided. The three largest orders Agaricales, Boletales, Polyporales 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).

4.4.3. 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* mutants. The coding sequences of SlMta and SlMtb were cloned into the yeast expression vector pAG426GAL-ccdB (Addgene, 14155). The *SlMTa* and *SlMTb* genes were then under the control of a GAL1-inducible promoter (Alberti *et al.*, 2007). Gene constructs were transformed into Cu (*cup2*), Cd (*ycf1* and *yap1*), or Zn (*zrc1*) sensitive yeast mutants and a wild type. Results of the drop assay showed that the Cu sensitive phenotype was complemented by *SlMTa* and *SlMTb* (Figure 4.2A). Overexpression of *SlMTa* slightly improved growth of the cadmium sensitive mutants *ycf1* and *yap1* (Figure 4.2B and C). Overexpression of neither of the MT genes could restore the growth of the zinc sensitive mutant (Figure 4.2D).

4.4.4. 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 the fungus was exposed to Cu, Cd or Zn (Figure 4.3). Results show that transcription of *SlMTa* was induced by Cu after three hours and transcription of both genes was induced after six hours exposure. Exposure to high concentration of Cd and Zn did not induce significant changes in expression of *SlMTa* and *SlMTb*.

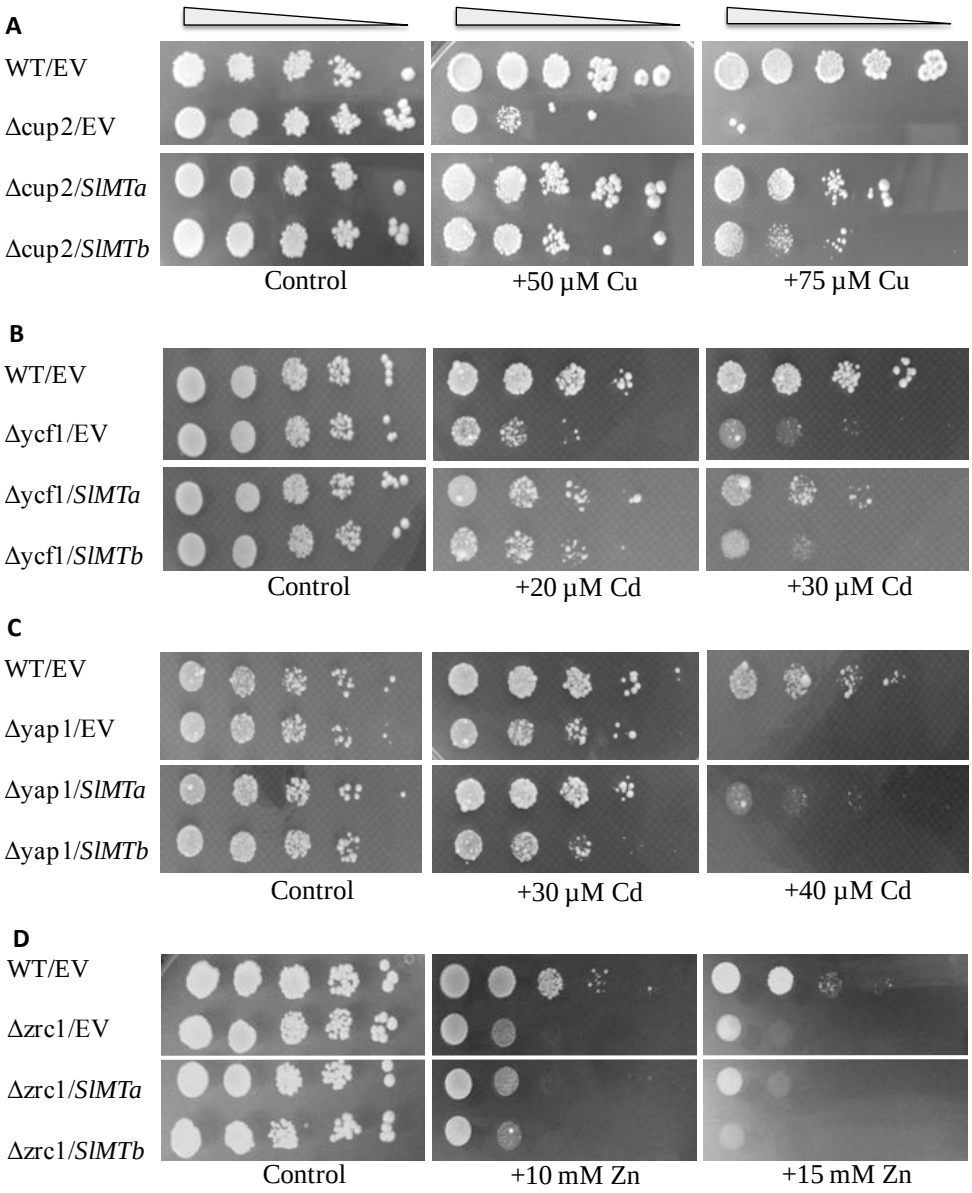


Figure 4.2. Functional complementation of *S. cerevisiae* mutants on selective media. Yeast mutant strains were transformed with the empty vector (EV) pAG426GAL or with vector containing coding sequence of SlMTa and SlMTb. Wild-type strain BY4741 (WT) was transformed with EV as a control. Yeast cultures 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.

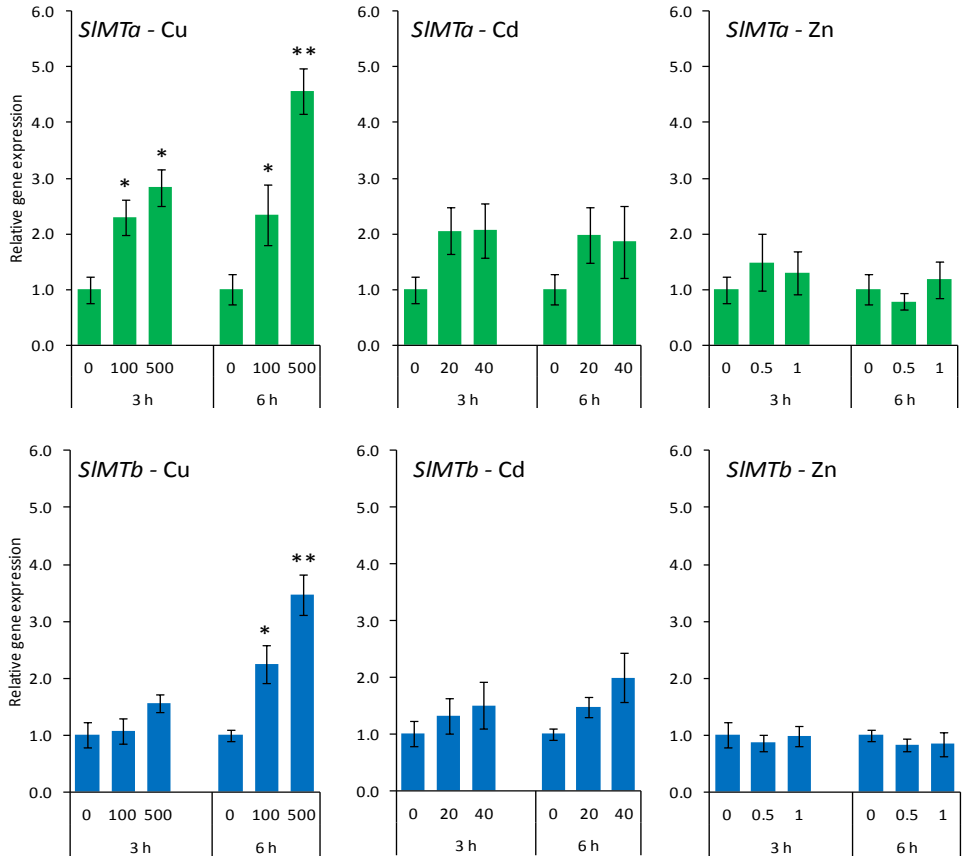
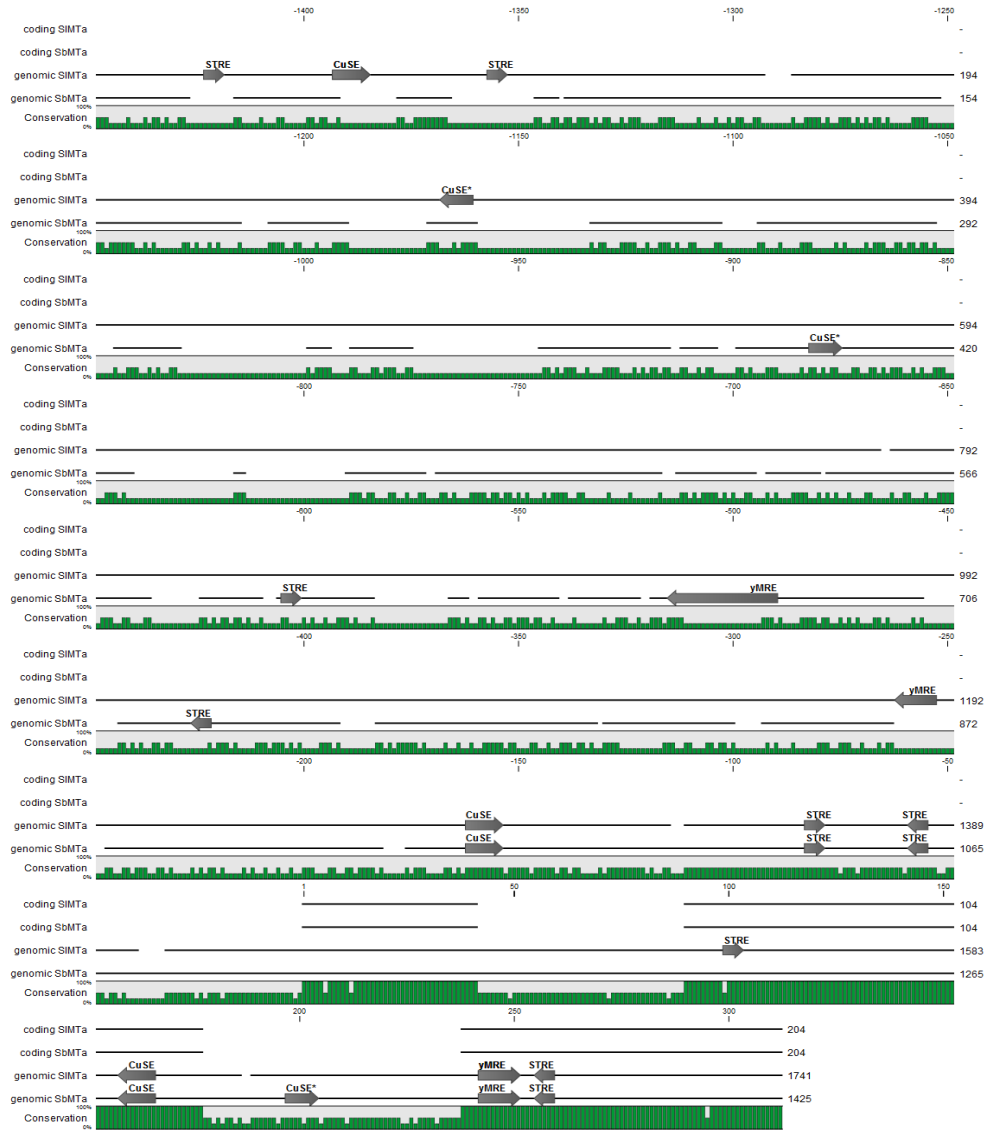


Figure 4.3. Relative expression of *SlMTa and SlMTb* in a *S. luteus* monokaryon (isolate UH-Slu-Lm8-n1) exposed to high Cu, Cd or Zn. The monokaryon was grown in liquid culture. Treatments were started by adding the metals to the medium (Cu: 0, 100, 500 µM, Cd: 0, 20, 40 µM, or Zn: 0, 0.5, 1 mM) and incubated for three and six hours. Gene expression was measured by RT-qPCR and presented as fold changes (metal-treated sample relative to non-treated control). Data are given as means ± SE of four biological replicates. Statistics was 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; (*) and (**) indicate significant difference at $p<0.05$ and $p<0.01$, respectively.

4.4.5. Promoter analysis of *SlMTa* and *SlMTb*

A promoter analysis was performed on two Suillus species (*S. luteus* and *S. brevipes*) which have identical and nearly identical peptide sequences of Mta and Mtb (Figure 4.1B). We conducted an *in silico* examination of promoter sequences of the genes and at the same time compared promoter sequences between these two species. Comparing the coding sequences of *MTa* of the two species we found three synonymous and one non-synonymous single nucleotide polymorphisms (SNP) (Figure 4.4A). All four SNPs in MTb genes (Figure 4.4B) were synonymous and did not alter the peptide sequence. Introns and promoter regions of both genes were less conserved. The promoter region of *MTa* differed substantially whereas *MTb* shared high similarity between both species. Despite the difference in the sequences, promoter regions of *MTa* of both species contained similar responsive elements including the general stress responsive element (STRE), an antioxidant response element (ARE), metal responsive (yMRE - "y" stands for "yeast" in this paper) and copper sensing elements (CuSE) (Figure 4.4A). The promoter region of *MTb* of both species was similar in sequence and contained another type of metal responsive element (MTF1-BD), 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 in both species (Figure 4.4B).

A


coding SlMTa
coding SbMTa
genomic SlMTa
genomic SbMTa
Conservation
STRE
CuSE
CuSE*
yMRE
194
154
394
292
594
420
792
566
992
706
1192
872
1389
1065
104
104
1583
1265
204
204
1741
1425

B

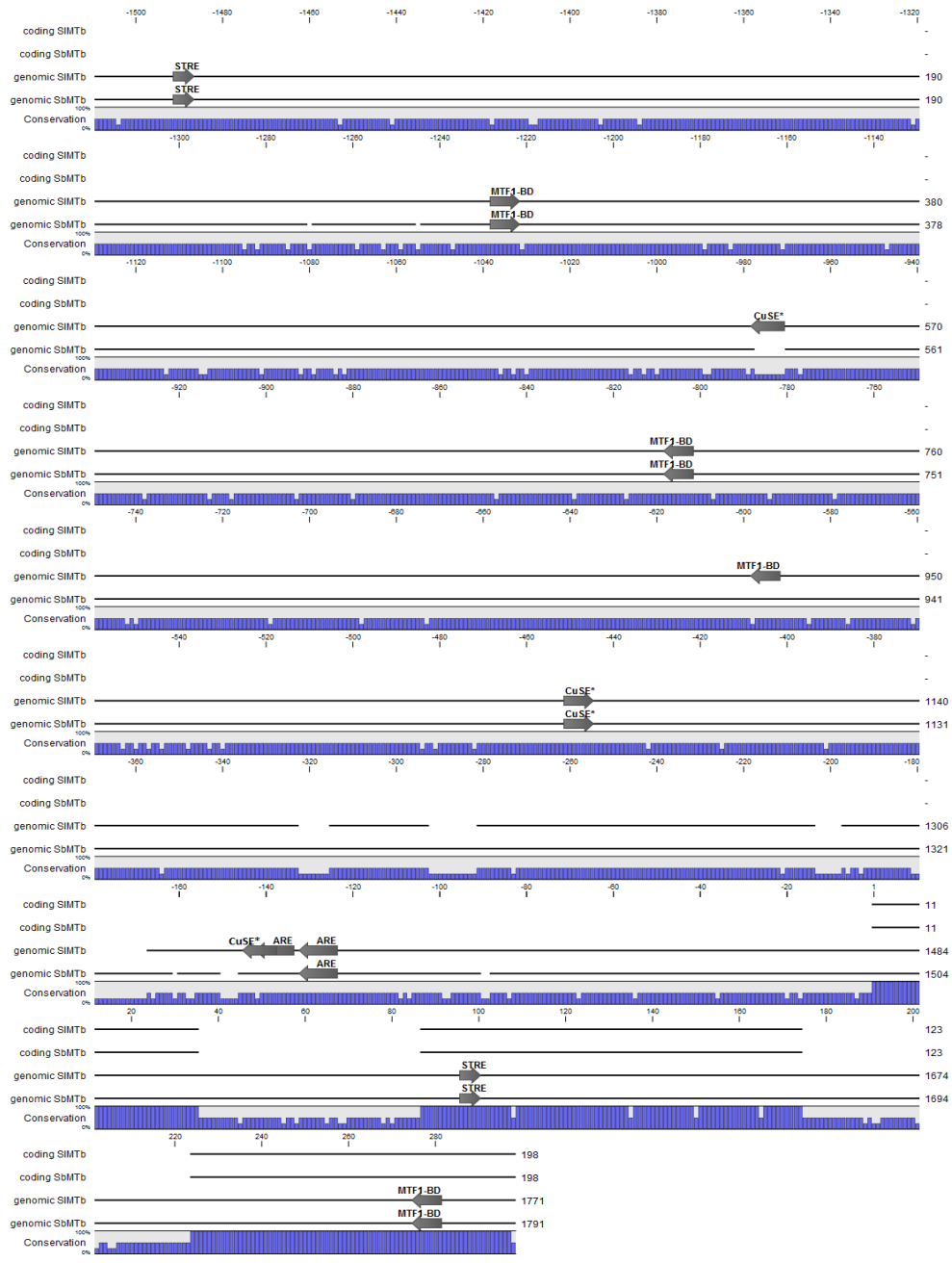


Figure 4.4. Sequence alignment of coding and genomic sequences of *MTa* (A) and *MTb* (B) genes from two Suillus species: *S. luteus* (*SlMTa, b*) and *S. brevipes* (*SbMTa, b*). Sequences were retrieved from the JGI genome database. All sequences are numbered

from the translation start codon. Promoter regions of approximately 1500 bp upstream of the translation start codon are included. Predicted TF binding sites are indicated: ARE (TGACNNNGC), STRE (CCCCT), CuSE (DDDHGCTGD), CuSE* (DDHGCTGD), yMRE (HTHNNGCTGD), MTF1-BD (TGCRCNC), in which D = A, G or T; H = A, C or T; N = any nucleotides; R = A or G. Coding sequences are provided showing that both *SlMTa* and *SlMTb* have three exons. Bar graph shows sequence conservation.

4.5. 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 genes were characterized and their sequence structure, ability to complement the Cu sensitivity of the *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) and are reviewed more recently (Capdevila *et al.*, 2012) showing the high heterogeneity in length and primary structure of the MT sequences. In addition to the six fungal MT families (families 8 to 13) in this classification, several functionally studied fungal MTs are not (yet) classified. Identification 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's found in fungi are Cmt1 (122 aa) and Cmt2 (183 aa) of a basidiomycete: *Cryptococcus neoformans* (Ding *et al.*, 2011) (*Supplementary information*). 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 sequence (60 - 70 aa) and the distribution of cysteine residues are also different from Cmt1 and Cmt2 that were used initially as queries in our search.

In fact, in fungi, a number of metallothionein coding genes have been characterized *(Supplementary information).* Examples include *CUP1* and *CRS5* from the model yeast species *S. cerevisiae* (Fogel and Welch, 1982; Culotta *et al*., 1994), *CRD2* in *C. albicans* (Riggle and Kumamoto, 2000b), *CuMT* in *Neurospora crassa* (Munger *et al.*, 1987), Pimt1 in *Paxillus involutus,* a Boletales fungus (Bellion *et al.*, 2007), as well as MTs in a few Agaricales fungi: *Hebeloma*

cylindrosporum HcMT1 and *HcMT2* (Ramesh *et al.*, 2009), *H. mesophaeum HmMTs* (Sacky *et al.*, 2014), *Laccaria bicolor LbMT1* and *LbMT2* (Reddy *et al.*, 2014). In addition, the Cmt1 and Cmt2 of the pathogenic fungus *C. neoformans* were identified and characterized in many details (Ding *et al.*, 2011; Ding *et al.*, 2013). Homologs of these families have not been found in *S. luteus*. It is interesting to note that at least one homolog of SlMta/ SlMtb is present in the genome of *Amanita sp., H. cylindrosporum*, *P. involutus* and *L. bicolor* (Figure 4.1B), ECM fungi in which several other MTs are already discovered. Accordingly, apart from SlMta and SlMtb, an additional MT-like sequence was also found in *S. luteus* (sequence: MINNNTEQCNCTTSCSCKVGSCDCKK). 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 the novel Mta and Mtb 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 Mta/Mtb 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 the novel MT also plays a role in metal ion homeostasis and osmotic stress tolerance of Wallemia.

In Figures 4.1A and 4.1B, comparison of the primary structure of the MT homologs revealed that these proteins are very well conserved. The coding regions of the MT genes were also strongly homologous, whereas, the non-coding sequences were more divergent. This was illustrated by the comparison of the genes from two closely related Suillus species, *S. luteus* and *S. brevipes* (Figure 4.4A and 4.4B). It is also worth mentioning that most of the MT genes analyzed in this report contain three exons and the second exon consists of exactly 88 bp in all species.

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 *cup2* transformants expressing *SlMTa* grew better than *SlMTb* on all Cu concentrations tested. Transformants expressing *SlMTa* also slightly improved growth of Cd sensitive *ycf1* and *yap1* mutants but not completely restored their growth on high Cd concentrations. These results suggest that there exist differences in the metal-binding abilities of SlMta and SlMtb proteins. We are currently investigating the possibility to use purified recombinant SlMta and SlMtb to identify metal binding properties of these proteins.

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). Regulation of MT genes is often associated with particular transcription factors and well-documented TFs are the Ace1 (also known as Cup2) in *S. cerevisiae* (Buchman *et al.*, 1989), Cuf1 in *Schizosaccharomyces pombe* (Beaudoin *et al.*, 2003), Cuf1 homolog in *C. neoformans* (Ding *et al.*, 2011) and MTF1 in human, mouse and the fly *Drosophila* (Heuchel *et al.*, 1994; Palmiter, 1994; Wang *et al.*, 2004b; Günther *et al.*, 2012). Accordingly, promoters of most MT genes contain conserved DNA sequence motifs (metal responsive elements) that are binding sites for these TFs. The corresponding metal responsive elements of Ace1, Cuf1 and MTF1 are the yeast metal responsive element (yMRE), copper sensing element (CuSE), and MTF1-binding (MTF1-BD, also called MRE in several studies), respectively. In addition, the *S. cerevisiae* MTs are also regulated by stress responsive TFs such as Msn2p and Msn4p that bind to the general stress responsive element (STRE) (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). Several mammalian MTs are regulated by specific TFs and reactive oxygen species through the antioxidant response element (ARE) that is present in their promoter (Andrews, 2000; Nerland, 2007).

In the basidiomycete *C. neoformans*, 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 other TFs in regulation of MT genes in basidiomycete fungi. However, potential TF binding sites (MRE, CuSE, STRE and MTF1-BD) have been found in the promoter regions of several basidiomycete MT genes (Ramesh *et al.*, 2009; Ding *et al.*, 2011; Eastwood *et al.*, 2011b). Likewise, in this study, promoter analysis of *SlMTa* and *SlMTb* shows the presence (co-existence) of different putative response elements (Figure 4.4); this also indicates the complexity of *SlMTa* and *SlMTb* regulation. First of all, we found that alignment of promoter sequences of homologs from *S. luteus* and *S. brevipes* provided additional confirmation on the possible role of the responsive elements. Elements that are evolutionarily conserved (across species) are most likely essential for the regulation of the genes. Secondly, transcription of both MT 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. Thirdly, we found that despite the similarity in protein sequences, *SlMTa* and *SlMTb* might be regulated differently. Here we could only show that *SlMTa* responded to Cu exposure earlier than *SlMTb* (Figure 4.3). Possibly regulation of the expression of the two MT genes might be more differential in other conditions (for example, metal exposure time and doses, growth condition and developmental stages of the fungi, …). Based on the presence of responsive elements in their promoters, *SlMTa* might be regulated by Ace1-like and *SlMTb* by MTF1-like transcription factors. At least one homolog of Ace1 (also Cuf1) and MTF1 were found in the *S. luteus* genome (data not shown). Their role in regulation of MT genes is likely very complicated and remains to be elucidated. In fact, in *S. cerevisiae*, the TF Ace1 activates the MT genes Cup1 and CRS5 (Buchman *et al.*, 1989; Culotta *et al.*, 1994), resulting in protection of cells against Cu toxicity. In *S. pombe*, Cuf1 plays a dual role: at high Cu exposure it induces the expression of Cu detoxification genes and under conditions of Cu scarcity it activates Cu transporter gene expression (Beaudoin and Labbé, 2001). 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). On the other hand, 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 these two Polyporales species. It is likely that *SlMTa*/*SlMTb* homologs of *P. chrysoporium* and *C. subvermispora* are also targets of these TFs. It is interesting in subsequent experiments to conduct loss-of-function and gain-of-function experiments to obtain more direct evidence on the physiological roles of these MTs. We have not yet a stable protocol for gene manipulation in *S. luteus*. However, gene transformation methods are available for other basidiomycete fungi such as *H. cylindrosporum* (Marmeisse *et al.*, 1992) and *Suillus bovinus* (Hanif *et al.*, 2002) and they may be used as an alternative.

The subphylum Agaricomycotina contains about one-third of the described basidiomycete species and accommodates a diverse array of fungi, in size, life style (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) among 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 Cu and heavy metals in general will help to develop new technologies for the control and efficient use of these fungi in the future.

4.6. SUPPLEMENTARY INFORMATION

Table S4.1. 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)

	Abreviation	Name
1	jgi|Amamu1|	Amanita muscaria Koide v1.0
2	jgi|Amath1|	Amanita thiersii Skay4041 v1.0
3	jgi|Antsi1|	Antrodia sinuosa v1.0
4	jgi|Artel1|	Artolenzites elegans CIRM1663 v1.0
5	jgi|Bjead1_1|	Bjerkandera adusta v1.0
6	jgi|Boled1|	Boletus edulis v1.0
7	jgi|Cersu1|	Ceriporiopsis (Gelatoporia) subvermispora B
8	jgi|Clapy1|	Clavicorona pyxidata HHB10654 v1.0
9	jgi|Conpu1|	Coniophora puteana v1.0
10	jgi|Copci1|	Coprinopsis cinerea
11	jgi|Fomme1|	Fomitiporia mediterranea v1.0
12	jgi|Glotr1_1|	Gloeophyllum trabeum v1.0
13	jgi|Gymch1|	Gymnopilus chrysopellus PR-1187 v1.0
14	jgi|Hebcy1|	Hebeloma cylindrosporum h7 v2.0
15	jgi|Hydpi1|	Hydnomerulius pinastri v2.0
16	jgi|Hypsu1|	Hypholoma sublateritium v1.0
17	jgi|Jaaar1|	Jaapia argillacea v1.0
18	jgi|Lacbi2|	Laccaria bicolor v2.0
19	jgi|Laesu1|	Laetiporus sulphureus var. sulphureus v1.0
20	jgi|Leisp1|	Leiotrametes sp BRFM 1775 v1.0
21	jgi|Lenti7_1|	Lentinus tigrinus ALCF2SS1-7 v1.0
22	jgi|Leumo1|	Leucogyrophana mollusca KUC20120723A-06 v1.0

23	jgi|Macfu1|	Macrolepiota fuliginosa v1.0
24	jgi|Neole1|	Neolentinus lepideus v1.0
25	jgi|Obbri1|	Obba rivulosa 3A-2 v1.0
26	jgi|Paxin1|	Paxillus involutus ATCC 200175 v1.0
27	jgi|Paxru1|	Paxillus rubicundulus Ve08.2h10 v1.0
28	jgi|Phchr2|	Phanerochaete chrysosporium v2.0
29	jgi|Phbr1|	Phlebia brevispora HHB-7030 SS6 v1.0
30	jgi|Pismi|	Pisolithus microcarpus 441 v1.0
31	jgi|Pisti1|	Pisolithus tinctorius Marx 270 v1.0
32	jgi|PleosPC15_2|	Pleurotus ostreatus PC15 v2.0
33	jgi|PleosPC9_1|	Pleurotus ostreatus PC9 v1.0
34	jgi|Plicr1|	Plicaturopsis crispa v1.0
35	jgi|PosplRSB12_1|	Postia placenta MAD-698-R-SB12 v1.0
36	jgi|Punst1|	Punctularia strigosozonata v1.0
37	jgi|Pycco1|	Pycnoporus coccineus BRFM 310 v1.0
38	jgi|Pycco1662_1|	Pycnoporus coccineus BRFM 310 v1.0
39	jgi|Pycsa1|	Pycnoporus sanguineus BRFM 1264 v1.0
40	jgi|Rhisa1|	Rhizopogon salebrosus TDB-379 v1.0
41	jgi|Rhivi1|	Rhizopogon vinicolor AM-OR11-026 v1.0
42	jgi|Sclci1|	Scleroderma citrinum Foug A v1.0
43	jgi|SerlaS7_9_2|	Serpula lacrymans S7.9 v2.0
44	jgi|Suibr1|	Suillus brevipes v1.0
45	jgi|Suilu1|	Suillus luteus UH-Slu-Lm8-n1 v1.0
46	jgi|Traj1|	Trametes ljubarskyi CIRM1659 v1.0
47	jgi|Volvo1|	Volvariella volvacea V23
48	jgi|Walse1|	Wallemia sebi v1.0

Table S4.2. Primers used in the study

	Usage	Name	Sequence 5'-3'	Amplicon size (bp)	Primer efficiency*
1	Cloning	SlMTaF	ACAAAAACCATAATGGCGACCTGCAG		
2	Cloning	SlMTaR	TCACTTTGACTCGCAGGTACATGCTAGA		
3	Cloning	SlMTbF	GCGCTCTGCATCAACATGGCTAAAGAC		
4	Cloning	SlMTbR	CTACTTCGTTGCGCAACTGCACGCCTGC		
5	qPCR	qMTaF	TGTGTCAACAGCGCACATTC	125	1.865 ± 0.097
6	qPCR	qMTaR	GCACCCATTCATCATCACTTT		
7	qPCR	qMTbF	CTGTGCCAATCAACAGCAGG	91	1.915 ± 0.106
8	qPCR	qMTbR	TGGATACTCGACGGCATGTG		
9	qPCR reference	JGI810455F	GTTCTGGGAGGTGGTTTCTGATG	110	1.926 ± 0.080
10	qPCR reference	JGI810455R	GCCGATTTCATTGTAGTAAACCGA		
11	qPCR reference	GR975621F	CCGGTTTCAGTAATACAGAGTCCT	96	2.077 ± 0.056
12	qPCR reference	GR975621R	GGTCTGTTTACCTTACTTTATTATGTCCACC		
13	qPCR reference	AM085168F	GGGCGTTTGACGAAGCTCATC	98	2.045 ± 0.014
14	qPCR reference	AM085168R	CGATCTCGAGGAGCTGTGTTCCA		

* Primer efficiency (e ± standard deviation) was calculated on a standard curve generated using a twofold dilution series of a mixed cDNA sample over at least five dilution points (e = 10^(−1/slope))

Protein sequences of (**A**) some examples of members of the five fungal MT families (family 8-13) proposed in the classification of Binz and Kägi (1999) and (**B**) un-classified fungal MTs discussed in the text.

(A)

Family 8

Neurospora crassa MT (*Ascomycete*)

MGD**C**G**C**SGASS**C**N**C**GSG**C**S**C**SN**C**GSK **26 aa**

Family 9

Candida glabrata MT1 (*Ascomycete*)

MAND**C**K**C**PNG**C**S**C**PN**C**ANGG**C**Q**C**GDK**C**E**C**KKQS**C**HG**C**GEQ**C**K**C**GSHGSS**C**HGS**C**G**C**GDK**C**E**C**K **63 aa**

Family 10

Candida glabrata MT2 (*Ascomycete*)

MPEQVN**C**QYD**C**H**C**SN**C**A**C**ENT**C**N**CC**AKPA**C**A**C**TNSASNE**C**S**C**QT**C**K**C**QT**C**K**C** **52 aa**

Family 11

Yarrowia lipolytica MT3 (*Ascomycete*)

MEFTTAMLGASLISTTSTQSKHNLVNN**CCC**SSSTSESSMPAS**C**A**C**TK**C**G**C**KT**C**K**C** **55 aa**

Family 12

Saccharomyces cerevisiae Cup1 (*Ascomycete*)

MFSELINFQNEGHE**C**Q**C**Q**C**GS**C**KNNEQ**C**QKS**C**S**C**PTG**C**NSDDK**C**P**C**GNKSEETKKS**CC**SGK **61 aa**

Family 13

Sacharomyces cerevisiae CRS5 (*Ascomycete*)

MTVKI**C**D**C**EGE**CC**KDS**C**H**C**GST**C**LPS**C**SGGEK**C**K**C**DHSTGSPQ**C**KS**C**GEK**C**K**C**ETT**C**T**C**EKSK**C**N**C**EK**C** **69 aa**

(B)

Suillus luteus (Basidiomycete)

SlMta MAT**C**SG**C**T**C**ASG**C**PS**C**GPSGTG**C**A**C**PKNQ**C**N**C**QK**C**VNSAHSKK**C**S**C**AGKGED**C**E**C**SKQGLA**C**T**C**ESK **67 aa**

SlMtb MAKD**C**T**C**ISN**C**GA**C**GPSGTG**C**A**C**PKNN**C**D**C**DN**C**VNKQHSSQ**C**D**C**KGTGDS**C**N**C**ANQQQA**C**S**C**ATK **65 aa**

Cryptococcus neoformans (Basidiomycete)

Cmt1 MA**C**N**C**PLQKNTQ**CC**STSEAQDK**C**T**C**QKGN**C**E**C**KA**C**PNSTKTSESGGKAST**C**N**C**GGSGEA**C**T**C**PPGQ**C**A**C**DK**C**

PKKAKSVST**C**G**C**GGSGAA**C**S**C**PPGK**C**A**C**DN**C**PKQAQEKVSS**C**A**C**SGSGAA **122 aa**

Cmt2 MAFNPNPEKTTS**CC**STSKAQDK**C**T**C**PKGK**C**E**C**ET**C**PKSTKTPGSGP**C**N**C**GVKEKVST**C**G**C**NGSGAA**C**T**C**

PPGQ**C**A**C**DS**C**PRKAKSVST**C**G**C**GGSAAA**C**S**C**PPGK**C**A**C**DS**C**PKQAQEKVSS**C**A**C**NGSGAA**C**T**C**PPGK**C**S**C**SG**C**

PAQAKENPADQPTT**C**G**C**QGVFVA**C**T**C**PPGQ**C**A**C**DG**C**PAKAK **183 aa**

Agaricus bisporus (Basidiomycete)

ABMET1 MPATT**C**ASK**C**GEA**C**A**C**ANN**C**Q**CC**SNNEVPKNQH**C**GMSS**C**G**C**GDS**C**G**C**KPDE**C**K**C** **54 aa**

ABMET2 MPATM**C**TFK**C**GEA**C**A**C**TNN**C**Q**C**RKDNNNSTVPENHGH**C**GIDG**C**N**C**GDA**C**A**C**TREG**C**K**C**H **59 aa**

Laccaria bicolor (Basidiomycete)

LbMT1 MISNTSAFANAA**C**GDHSS**C**G**C**AQD**C**S**C**AS**C**G**C**KCASG **37 aa**

LbMT2 MLFNTLTPISRASSTG**CCC**TS**C**K**C**TS**C**T**C**GTAPVNEAG**C**GSTT**C**N**C**TN**C**A**C**KPEE**C**K**C** **58 aa**

Paxillus involutus Pimt1 *(Basidiomycete)*

Pimt1 MNTITSVPVNFNN**C**GSNS**C**G**C**GSS**C**A**C**KPGE**C**K**C** **34 aa**

Hebeloma mesophaeum (Basidiomycete)

HmMT1 MQFTSTLVNQA**C**GSAN**C**S**C**DSS**C**T**C**SSGS**C**HAPVNQA**C**GSSS**C**N**C**NSS**C**G**C**DSNN**C**N**C**S **59 aa**

HmMT2 MQIVQNTLVSRTRTPD**C**T**C**GT**C**E**C**APT**C**T**C**AAPVNQSG**C**GSSS**C**T**C**TS**C**A**C**KPGE**C**K**C** **58 aa**

HmMT3 MQIVQKSSE**C**T**C**DP**C**E**C**GAN**C**T**C**AAPVNQSSG**C**GSSS**C**T**C**TS**C**A**C**KPGE**C**K**C** **52 aa**

Hebeloma cylindrosporum (Basidiomycete)

HcMT1 MQFTSILVNQA**C**GSDN**C**Q**C**DAA**C**T**C**SSGS**C**HAPVNRA**C**GSSD**C**N**C**NSS**C**G**C**ESNN**C**N**C**N **59 aa**

HcMT2 MQIVQNSLVSQSSG**C**T**C**TS**C**K**C**GSN**C**T**C**GAPVNQSSG**C**GSSS**C**T**C**TS**C**T**C**KAGE**C**K**C** **57 aa**

Podospora anserina (Ascomycete)

PaMt1 MGGS**C**N**C**SGSASS**C**S**C**GSD**C**S**C**SG**C**K **26 aa**

Gigaspora margarita (Glomeromycete)

GmarMT1 M**C**QN**C**K**C**GSA**C**Q**C**GTN**C**T**C**PKDYTTSSTTQQQQQSTDTQKKTAS**C**GVST**C**R**C**GEV**C**K**C**TKGN**C**K**C** **65 aa**

CHAPTER 5

General conclusions and future perspectives

The present study represents the first steps into the understanding of the role of the heavy metal transporters and metal chelators in copper homeostasis and, to a certain extent, cadmium detoxification in the ectomycorrhizal fungus *Suillus luteus*.

First, this study provides an **inventory and comparative analysis** of the P1B-type ATPases in basidiomycete fungi (Chapter 2). We determined the diversity and abundance of this family of heavy metal transporters in 132 basidiomycete genomes. We showed that **HMAs are ubiquitously present** in most basidiomycetes. Our data indicate that there are three main types of HMA transporters; in which two (the CRD1- and PCA1-types) are specific for fungi that have not been documented. We showed that the CRD1-type candidates in *S. luteus* are likely involved in **copper and cadmium** homeostasis and detoxification (Chapter 3). Our data indicate, albeit in an indirect manner, that several **PCA1-type HMAs are likely zinc-transporting ATPases** fulfilling a function previously unknown in fungi. We identified the Polyporales-specific PCA1-type HMAs and propose to further investigate their roles in metals, especially copper, homeostasis. More important, the most evolutionary **conserved HMA is the CCC2-type**; the representative **SlHma1** in *S. luteus* is confirmed experimentally as a copper exporter localized in the ER-Golgi similar to its *S. cerevisiae* ortholog that is essential in the secretory pathway (Figure 5.1).

Second, we discovered a novel, highly **conserved family of metallothioneins** that is present in *S. luteus* and a number of basidiomycete fungi (Chapter 4). Two *S. luteus* metallothionein genes of this family were functionally characterized; they show typical copper-metallothionein characteristics.

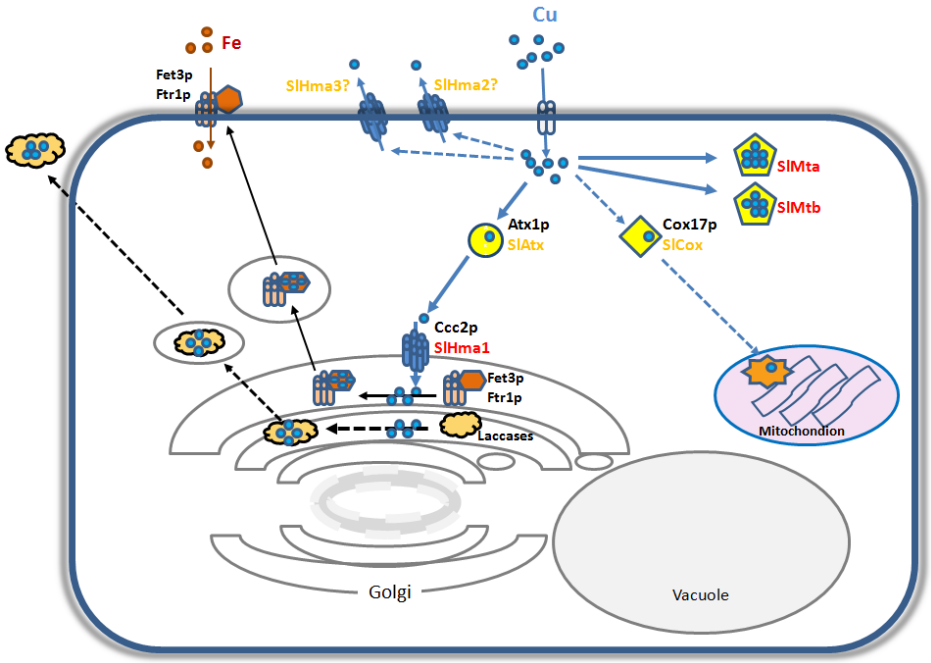


Figure 5.1. Scheme of factors involved in copper homeostasis in a *S. luteus* cell. A HMA transporter (SlHma1) and two metallothioneins (SlMta and SlMtb) whose functions were confirmed experimentally are indicated in red. Copper chaperones and transporters that likely function as indicated but that required additional work are illustrated in orange. Dotted arrows indicate possible pathways that were proposed and discussed in the text. Names of homologous proteins in *S. cerevisiae* are indicated in black.

There are, however, some unresolved questions in our work. In Chapter 2, we would like to identify the diversification of HMAs in the taxonomic phylogeny and to discuss the occurrence of HMA gene gain and loss. This may be done with the protein tree and taxonomic tree reconciliation analysis. In Chapter 3, the localization of SlHma2 and SlHma3 could not be verified. It might be better in future to test different expression times, other expression vectors or eventually other expression systems than *S. cerevisiae* to verify the localization of these transporters.

Concerning the metallothioneins (Chapter 4), the ubiquitous and highly conserved features of the novel MT family indicate that they are important for

basic cellular functions in basidiomycete fungi. It will be interesting in subsequent experiments to conduct loss-of-function and gain-of-function experiments to obtain more convincing evidence on the physiological roles of these MTs. This can be done in other basidiomycete fungi that have an established transformation protocol. Besides, it will be interesting to study the metal binding characteristics of the proteins.

Results of this study open several possibilities for future research. An area that I would have liked to add to the research is the regulation of the transporters and chelators. As discussed in Chapter 3 and 4, several transcription factors binding sites in the promoter region of HMAs and metallothionein genes were found. In Chapter 3, I proposed several potential metal responsive transcription factors for further studies. Functional characterization of these transcription factors will extend our understanding of homeostasis and detoxification mechanisms of not only copper and cadmium but possibly some other metals. A transcription factor may function alone or together with other proteins and may be involved in regulation of different genes. Therefore, it will be interesting to find out if expression of other metal-related genes, for example, laccases – the prominent multicopper oxidases that have crucial roles in lignin degradation, morphogenesis, pathogenesis, stress defence, … in fungi - are regulated by the same transcription factors with the copper transporters and metallothioneins.

In addition to that, the putative copper chaperones identified in Chapter 3 are likely involved in the function of HMA transporters; this can be verified by further characterization of the genes. The present study indicates the link between copper and iron transport via the function of the CCC2-type HMAs (SlHma1 in *S. luteus*) and between copper and cadmium via the metal binding ability of the CRD1-type HMAs (SlHma2 and SlHma3 in *S. luteus*).

Moreover, we call attention to further investigate the involvement of different types of P1B-type ATPases in metal (Cu, Cd and Zn) homeostasis in basidiomycete fungi. The novel family of metallothioneins reported here can also be a target in further research on metal homeostasis and tolerance of basidiomycete fungi.

Chapter 5

REFERENCES

Adamo, G.M., Lotti, M., Tamás, M.J., Brocca, S., 2012. Amplification of the *CUP1* gene is associated with evolution of copper tolerance in *Saccharomyces cerevisiae*. Microbiology 158, 2325-2335.

Adle, D.J., Sinani, D., Kim, H., Lee, J., 2007. A cadmium-transporting P1B-type ATPase in yeast *Saccharomyces cerevisiae*. Journal of Biological Chemistry 282, 947-955.

Adriaensen, K., Vrålstad, T., Noben, J.-P., Vangronsveld, J., Colpaert, J.V., 2005. Copper-adapted *Suillus luteus*, a symbiotic solution for pines colonizing Cu mine spoils. Applied and Environmental Microbiology 71, 7279-7284.

Alberti, S., Gitler, A.D., Lindquist, S., 2007. A suite of Gateway cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. Yeast 24, 913-919.

Álvarez, J.M., Canessa, P., Mancilla, R.A., Polanco, R., Santibáñez, P.A., Vicuña, R., 2009. Expression of genes encoding laccase and manganese-dependent peroxidase in the fungus *Ceriporiopsis subvermispora* is mediated by an ACE1-like copper-fist transcription factor. Fungal Genetics and Biology 46, 104-111.

Andres-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker, J.R., Puig, S., Penarrubia, L., 2006. The *Arabidopsis* heavy metal P-type ATPase *HMA5* interacts with metallochaperones and functions in copper detoxification of roots. Plant Journal 45, 225-236.

Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochemical Pharmacology 59, 95-104.

Axelsen, K.B., Palmgren, M.G., 1998. Evolution of substrate specificities in the P-type ATPase superfamily. J Molecular Evolution 46, 84-101.

Badarau, A., Baslé, A., Firbank, S.J., Dennison, C., 2013. Investigating the role of zinc and copper binding motifs of trafficking sites in the cyanobacterium *Synechocystis* PCC 6803. Biochemistry 52, 6816-6823.

Baldrian, P., 2003. Interactions of heavy metals with white-rot fungi. Enzyme and Microbial Technology 32, 78-91.

Baldrian, P., 2006. Fungal laccases – occurrence and properties. FEMS Microbiology Reviews 30, 215-242.

Ballatori, N., 2002. Transport of toxic metals by molecular mimicry. Environmental Health Perspectives 5, 689-694.

Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., Zovo, K., Palumaa, P., 2010a. Affinity gradients drive copper to cellular destinations. Nature 465, 645-648.

Banci, L., Bertini, I., Ciofi-Baffoni, S., Poggi, L., Vanarotti, M., Tottey, S., Waldron, K.J., Robinson, N.J., 2010b. NMR structural analysis of the soluble domain of ZiaA-ATPase and the basis of selective interactions with copper metallochaperone Atx1. Journal of Biological Inorganic Chemistry 15, 87-98.

Banci, L., Bertini, I., Ciofi-Baffoni, S., Su, X.C., Miras, R., Bal, N., Mintz, E., Catty, P., Shokes, J.E., Scott, R.A., 2006. Structural basis for metal binding specificity: the N-terminal cadmium binding domain of the P1-type ATPase CadA. Journal of Molecular Biology 356, 638-650.

Beaudoin, J., Labbé, S., 2001. The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. Journal of Biological Chemistry 276, 15472-15480.

Beaudoin, J., Mercier, A., Langlois, R., Labbe, S., 2003. The *Schizosaccharomyces pombe* Cuf1 is composed of functional modules from two distinct classes of copper metalloregulatory transcription factors. Journal of Biological Chemistry 278, 14565-14577.

Bellion, M., Courbot, M., Jacob, C., Blaudez, D., Chalot, M., 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiology Letters 254, 173-181.

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

Bergero, R., Lanfranco, L., Ghignone, S., Bonfante, P., 2007. Enhanced activity of the GmarMT1 promoter from the mycorrhizal fungus *Gigaspora margarita* at limited carbon supply. Fungal Genetics and Biology 44, 877-885.

Bernaudat, F., Frelet-Barrand, A., Pochon, N., Dementin, S., Hivin, P., Boutigny, S., *et al*., 2011. Heterologous expression of membrane proteins: choosing the appropriate host. PLoS ONE 6, e29191.

Bi, W.X., Kong, F., Hu, X.Y., Cui, X., 2007. Role of glutathione in detoxification of copper and cadmium by yeast cells having different abilities to express cup1 protein. Toxicology Mechanisms & Methods 17, 371-378.

Binder, B.M., Rodriguez, F.I., Bleecker, A.B., 2010. The copper transporter RAN1 is essential for biogenesis of ethylene receptors in *Arabidopsis*. Journal of Biological Chemistry 285, 37263-37270.

Binz, P.-A., Kägi, J.R., 1999. Metallothionein: Molecular evolution and classification. In: Klaassen, C. (Ed.), Metallothionein IV. Birkhäuser Basel, pp. 7-13.

Blindauer, C.A., 2015. Advances in the molecular understanding of biological zinc transport. Chemical Communications 51, 4544-4563.

Boutigny, S., Sautron, E., Finazzi, G., Rivasseau, C., Frelet-Barrand, A., Pilon, M., Rolland, N., Seigneurin-Berny, D., 2014. HMA1 and PAA1, two chloroplast-envelope PIB-ATPases, play distinct roles in chloroplast copper homeostasis. Journal of Experimental Botany 65, 1529-1540.

Branco, S., Gladieux, P., Ellison, C.E., Kuo, A., LaButti, K., Lipzen, A., Grigoriev, I.V., *et al*., 2015. Genetic isolation between two recently diverged populations of a symbiotic fungus. Molecular Ecology 24, 2747-2758.

Buchman, C., Skroch, P., Dixon, W., Tullius, T.D., Karin, M., 1990. A single amino acid change in *CUP2* alters its mode of DNA binding. Molecular and Cellular Biology 10, 4778-4787.

Buchman, C., Skroch, P., Welch, J., Fogel, S., Karin, M., 1989. The *CUP2* gene product, regulator of yeast metallothionein expression, is a copper-activated DNA-binding protein. Molecular and Cellular Biology 9, 4091-4095.

Canessa, P., Álvarez, J.M., Polanco, R., Bull, P., Vicuña, R., 2008. The copper-dependent ACE1 transcription factor activates the transcription of the mco1 gene from the basidiomycete *Phanerochaete chrysosporium*. Microbiology 154, 491-499.

Capdevila, M., Bofill, R., Palacios, Ò., Atrian, S., 2012. State-of-the-art of metallothioneins at the beginning of the 21st century. Coordination Chemistry Reviews 256, 46-62.

Casalino, E., Sblano, C., Landriscina, C., 1997. Enzyme activity alteration by cadmium administration to rats: the possibility of iron involvement in lipid peroxidation. Archives of Biochemistry & Biophysics 346, 171-179.

Changela, A., Chen, K., Xue, Y., Holschen, J., Outten, C.E., O'Halloran, T.V., Mondragon, A., 2003. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science 301, 1383-1387.

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486.

Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annual Review of Plant Biology 53, 159-182.

Cobbett, C.S., Hussain, D., Haydon, M.J., 2003. Structural and functional relationships between type 1B heavy metal-transporting P-type ATPases in *Arabidopsis*. New Phytologist 159, 315-321.

Colpaert, J.V., 2008. Heavy metal pollution and genetic adaptations in ectomycorrhizal fungi. In: Avery S.V., Stratford M., and West P.V. (Eds), Stress in yeasts and filamentous fungi. Elsevier, Academic Press, London, pp. 157-173.

Colpaert, J.V., Adriaensen, K., Muller, L.A.H., Lambaerts, M., Faes, C., Carleer, R., Vangronsveld, J., 2005. Element profiles and growth in Zn-sensitive and Zn-resistant Suilloid fungi. Mycorrhiza 15, 628-634.

Colpaert, J.V., Muller, L.A.H., Lambaerts, M., Adriaensen, K., Vangronsveld, J., 2004. Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi. New Phytologist 162, 549-559.

Colpaert, J.V., Vandenkoornhuyse, P., Adriaensen, K., Vangronsveld, J., 2000. Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. New Phytologist 147, 367-379.

Colpaert, J.V., Wevers, J.H.L., Krznaric, E., Adriaensen, K., 2011. How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Annals of Forest Science. 68, 17-24.

Courbot, M., Diez, L., Ruotolo, R., Chalot, M., Leroy, P., 2004. Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Applied and Environmental Microbiology 70, 7413-7417.

Courty, P.E., Hoegger, P.J., Kilaru, S., Kohler, A., Buee, M., Garbaye, J., Martin, F., Kues, U., 2009. Phylogenetic analysis, genomic organization, and expression analysis of multi-copper oxidases in the ectomycorrhizal basidiomycete *Laccaria bicolor.* New Phytologist 182, 736-750.

Craciun, A.R., Meyer, C.L., Chen, J., Roosens, N., De Groodt, R., Hilson, P., Verbruggen, N., 2012. Variation in HMA4 gene copy number and expression among *Noccaea caerulescens* populations presenting different levels of Cd tolerance and accumulation. Journal of Experimental Botany 63, 4179-4189.

Culotta, V.C., Howard, W.R., Liu, X.F., 1994. CRS5 encodes a metallothionein-like protein in *Saccharomyces cerevisiae.* Journal of Biological Chemistry 269, 25295-25302.

Culotta, V.C., Klomp, L.W.J., Strain, J., Casareno, R.L.B., Krems, B., Gitlin, J.D., 1997. The copper chaperone for superoxide dismutase. Journal of Biological Chemistry 272, 23469-23472.

Dancis, A., Haile, D., Yuan, D., Klausner, R., 1994. The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. Journal of Biological Chemistry 269, 25660 - 25667.

De Smet, H., Blust, R., Moens, L., 2001. Cadmium-binding to transferrin in the plasma of the common carp *Cyprinus carpio*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 128, 45-53.

Ding, C., Festa, Richard A., Chen, Y.-L., Espart, A., Palacios, Ò., Espín, J., Capdevila, M., Atrian, S., Heitman, J., Thiele, Dennis J., 2013. *Cryptococcus neoformans* copper detoxification machinery is critical for fungal virulence. Cell Host & Microbe 13, 265-276.

Ding, C., Yin, J., Tovar, E.M., Fitzpatrick, D.A., Higgins, D.G., Thiele, D.J., 2011. The copper regulon of the human fungal pathogen *Cryptococcus neoformans* H99. Molecular Microbiology 81, 1560-1576.

Dorta, D.J., Leite, S., DeMarco, K.C., Prado, I.M., Rodrigues, T., Mingatto, F.E., Uyemura, S.A., Santos, A.C., Curti, C., 2003. A proposed sequence of events for cadmium-induced mitochondrial impairment. Journal of Inorganic Biochemtry 97, 251-257.

Eastwood, D., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., *et al*., 2011a. The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science 333, 762 - 765.

Eastwood, D.C., Bains, N.K., Henderson, J., Burton, K.S., 2011b. Genome organization and transcription response to harvest of two metallothionein-like genes in *Agaricus bisporus* fruiting bodies. Journal of Microbiology & Biotechnology 21, 455-463.

Eide, D.J., 2006. Zinc transporters and the cellular trafficking of zinc. Biochimica et Biophysica Acta 7, 711-722.

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Current Topics in Medicinal Chemistry 1, 529-539.

Eren, E., Argüello, J.M., 2004. *Arabidopsis* HMA2, a divalent heavy metal-transporting P(IB)-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. Plant Physiology 136, 3712-3723.

Ferreira, A.M., Ciriolo, M.R., Marcocci, L., Rotilio, G., 1993. Copper(I) transfer into metallothionein mediated by glutathione. Biochemical Journal 292, 673-676.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R., Henrissat, B., *et al.*, 2012. The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336, 1715 - 1719.

Fogel, S., Welch, J.W., 1982. Tandem gene amplification mediates copper resistance in yeast. Proceedings of the National Academy of Sciences 79, 5342-5346.

Foster, A.W., Robinson, N.J., 2011. Promiscuity and preferences of metallothioneins: the cell rules. BMC Biology 9, 25-25.

Frank, B., 2005. On the nutritional dependence of certain trees on root symbiosis with belowground fungi (*an English translation of A.B. Frank's classic paper of 1885*). Mycorrhiza 15, 267-275.

Freedman, J.H., Ciriolo, M.R., Peisach, J., 1989. The role of glutathione in copper metabolism and toxicity. Journal of Biological Chemistry 264, 5598-5605.

Fridovich, I., 1995. Superoxide radicals and superoxide dismutase. Annual Review of Biochemistry 5, 321-324.

Fries, N., 1978. Basidiospore germination in some mycorrhiza-forming hymenomycetes. Transactions of the British Mycological Society 70, 319-324.

Fu, D., Beeler, T.J., Dunn, T.M., 1995. Sequence, mapping and disruption of CCC2, a gene that cross-complements the $Ca2^{+}$-sensitive phenotype of csg1 mutants and encodes a P-type ATPase belonging to the $Cu2^{+}$-ATPase subfamily. Yeast 11, 283-292.

Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycological Research 111(1) 3-49.

Georgatsou, E., Mavrogiannis, L.A., Fragiadakis, G.S., Alexandraki, D., 1997. The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. Journal of Biological Chemistry 272, 13786-13792.

Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G., Sannia, G., 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus1*. Biochemical Journal 341, 655-663.

Gietz, R.D., Schiestl, R.H., 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nature Protocols 2, 31-34.

Goodyer, I.D., Jones, E.E., Monaco, A.P., Francis, M.J., 1999. Characterization of the Menkes protein copper-binding domains and their role in copper-induced protein relocalization. Human Molecular Genetics 8, 1473-1478.

Green Iii, F., Clausen, C.A., 2003. Copper tolerance of brown-rot fungi: time course of oxalic acid production. International Biodeterioration & Biodegradation 51, 145-149.

Grigoriev, Cullen, D., Goodwin, S., Hibbett, D., Jeffries, T., Kubicek, C., Kuske, C., Magnuson, J., Martin, F., Spatafora, J., Tsang, A., Baker, S., 2011. Fueling the future with fungal genomics. Mycology 2, 192 - 209.

Grigoriev, I.V., Nordberg, H., Shabalov, I., Aerts, A., Cantor, M., Goodstein, D., Kuo, A., *et al*., 2012. The genome portal of the Department of Energy Joint Genome Institute. Nucleic Acids Research 40, 22.

Grill, E., Loffler, S., Winnacker, E.L., Zenk, M.H., 1989. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proceedings of the National Academy of Sciences 86, 6838-6842.

Gross, C., Kelleher, M., Iyer, V.R., Brown, P.O., Winge, D.R., 2000. Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. Journal of Biological Chemistry 275, 32310-32316.

Guillen, Y., Navias, D., Machuca, A., 2009. Tolerance to wood preservatives by copper-tolerant wood-rot fungi native to south-central Chile. Biodegradation 20, 135-142.

Günther, V., Lindert, U., Schaffner, W., 2012. The taste of heavy metals: Gene regulation by MTF-1. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1823, 1416-1425.

Ha, S.B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B., Cobbett, C.S., 1999. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. The Plant Cell 11, 1153-1164.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. Journal of Experimental Botany 53, 1-11.

Halliwell, B., Gutteridge, J.M.C., 1985. The importance of free radicals and catalytic metal ions in human diseases. Molecular Aspects of Medicine 8, 89-193.

Hanif, M., Pardo, A., Gorfer, M., Raudaskoski, M., 2002. T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. Current Genetics 41, 183-188.

Hanikenne, M., Talke, I.N., Haydon, M.J., Lanz, C., Nolte, A., Motte, P., Kroymann, J., Weigel, D., Kramer, U., 2008. Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature 453, 391-395.

Harrison, M.D., Jones, C.E., Solioz, M., Dameron, C.T., 2000. Intracellular copper routing: the role of copper chaperones. Trends in Biochemical Sciences 25, 29-32.

Hartwig, A., 2001. Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxidants & Redox Signaling 3, 625-634.

Hastrup, A.C.S., Green Iii, F., Clausen, C.A., Jensen, B., 2005. Tolerance of *Serpula lacrymans* to copper-based wood preservatives. International Biodeterioration & Biodegradation 56, 173-177.

Heaton, D., Nittis, T., Srinivasan, C., Winge, D.R., 2000. Mutational analysis of the mitochondrial copper metallochaperone Cox17. Journal of Biological Chemistry 275, 37582-37587.

Hegelund, J.N., Schiller, M., Kichey, T., Hansen, T.H., Pedas, P., Husted, S., Schjoerring, J.K., 2012. Barley metallothioneins: MT3 and MT4 are localized in the grain aleurone layer and show differential zinc binding. Plant Physiology 159, 1125-1137.

Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Aguet, M., Schaffner, W., 1994. The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. The EMBO Journal 13, 2870-2875.

Heyno, E., Klose, C., Krieger-Liszkay, A., 2008. Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. New Phytologist 179, 687-699.

Hibbett, D.S., 2006. A phylogenetic overview of the Agaricomycotina. Mycologia 98, 917-925.

Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A., Ecker, J.R., 1999. RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. Cell 97, 383-393.

Horng, Y., Cobine, P., Maxfield, A., Carr, H., Winge, D., 2004. Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome *C* oxidase. Journal of Biological Chemistry 279, 35334 - 35340.

Hou, Z., Mitra, B., 2003. The metal specificity and selectivity of ZntA from *Escherichia coli* using the acylphosphate intermediate. Journal of Biological Chemistry 278, 28455-28461.

Howden, R., Andersen, C.R., Goldsbrough, P.B., Cobbett, C.S., 1995. A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiology 107, 1067-1073.

Hung, I.H., Suzuki, M., Yamaguchi, Y., Yuan, D.S., Klausner, R.D., Gitlin, J.D., 1997. Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. Journal of Biological Chemistry 272, 21461-21466.

Janusz, G., Kucharzyk, K.H., Pawlik, A., Staszczak, M., Paszczynski, A.J., 2013. Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation. Enzyme and Microbial Technology 52, 1-12.

Jiang, N., Liu, X., Yang, J., Li, Z., Pan, J., Zhu, X., 2011. Regulation of copper homeostasis by Cuf1 associates with its subcellular localization in the pathogenic yeast *Cryptococcus neoformans* H99. FEMS Yeast Research 11, 440-448.

Jozefczak, M., Remans, T., Vangronsveld, J., Cuypers, A., 2012. Glutathione is a key player in metal-induced oxidative stress defenses. International Journal of Molecular Sciences 13, 3145-3175.

Jungmann, J., Reins, H.A., Lee, J., Romeo, A., Hassett, R., Kosman, D., Jentsch, S., 1993. MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. EMBO Journal 12, 5051-5056.

Kägi, J.H.R., 1991. [69] Overview of metallothionein. In: James F. Riordan, B.L.V. (Ed.), Methods in Enzymology. Academic Press, pp. 613-626.

Kägi, J.H.R., Schaeffer, A., 1988. Biochemistry of metallothionein. Biochemistry 27, 8509-8515.

Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annual Reviews Pharmacology Toxicology 39, 267-294.

Kohler, A., Kuo, A., Nagy, L.G., Morin, E., Barry, K.W., Buscot, F., Canback, B., Choi, C., *et al.*, 2015. Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nature Genetics 47, 410-415.

Krämer, U., Talke, I.N., Hanikenne, M., 2007. Transition metal transport. FEBS Letters 581, 2263-2272.

Krznaric, E., Verbruggen, N., Wevers, J.H.L., Carleer, R., Vangronsveld, J., Colpaert, J.V., 2009. Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd. Environmental Pollution 157, 1581-1588.

Krznaric, E., Wevers, J.H.L., Cloquet, C., Vangronsveld, J., Vanhaecke, F., Colpaert, J.V., 2010. Zn pollution counteracts Cd toxicity in metal-tolerant ectomycorrhizal fungi and their host plant, *Pinus sylvestris*. Environmental Microbiology 12, 2133-2141.

Kues, U., Ruhl, M., 2011. Multiple multi-copper oxidase gene families in basidiomycetes - what for? Current Genomics 12, 72-94.

La Fontaine, S., Mercer, J.F.B., 2007. Trafficking of the copper-ATPases, ATP7A and ATP7B: Role in copper homeostasis. Archives of Biochemistry and Biophysics 463, 149-167.

Labbe, S., Pena, M.M., Fernandes, A.R., Thiele, D.J., 1999. A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. Journal of Biological Chemistry 274, 36252-36260.

Labbe, S., Zhu, Z., Thiele, D.J., 1997. Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. Journal of Biological Chemistry 272, 15951-15958.

Laity, J.H., Lee,B.M., Wright, P.E., 2001. Zinc finger proteins: new insights into structural and functional diversity. Current Opinion in Structual Biology 11, 39-46.

Landeweert, R., Hoffland, E., Finlay, R.D., Kuyper, T.W., van Breemen, N., 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology & Evolution 16, 248-254.

Lanfranco, L., Bolchi, A., Ros, E.C., Ottonello, S., Bonfante, P., 2002. Differential expression of a metallothionein gene during the presymbiotic versus the

symbiotic phase of an arbuscular mycorrhizal fungus. Plant Physiology. 130, 58-67.

Leitenmaier, B., Küpper, H., 2013. Compartmentation and complexation of metals in hyperaccumulator plants. Frontiers in Plant Science 4, 374.

Leonhardt, T., Sacky, J., Simek, P., Santrucek, J., Kotrba, P., 2014. Metallothionein-like peptides involved in sequestration of Zn in the Zn-accumulating ectomycorrhizal fungus *Russula atropurpurea*. Metallomics 6, 1693-1701.

Leszczyszyn, O.I., Imam, H.T., Blindauer, C.A., 2013. Diversity and distribution of plant metallothioneins: a review of structure, properties and functions. Metallomics 5, 1146-1169.

Li, Z., Szczypka, M., Lu, Y., Thiele, D., Rea, P., 1996. The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. Journal of Biological Chemistry 271, 6509-6517.

Lin, S.-J., Pufahl, R.A., Dancis, A., O'Halloran, T.V., Culotta, V.C., 1997. A role for the *Saccharomyces cerevisiae* ATX1 gene in copper trafficking and iron transport. Journal of Biological Chemistry 272, 9215-9220.

Lowe, J., Vieyra, A., Catty, P., Guillain, F., Mintz, E., Cuillel, M., 2004. A mutational study in the transmembrane domain of Ccc2p, the yeast Cu(I)-ATPase, shows different roles for each Cys-Pro-Cys cysteine. Journal of Biological Chemistry 279, 25986-25994.

MacDiarmid, C.W., Milanick, M.A., Eide, D.J, 2002. Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. Journal of Biological Chemistry 277, 39187-39194.

Macomber, L., Imlay, J.A., 2009. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proc Natl Acad Sci U S A 106, 8344-8349.

Margoshes, M., Vallee, B.L., 1957. A cadmium protein from equine kidney cortex Journal of the American Chemical Society 79, 4813-4814.

Marmeisse, R., Gay, G., Debaud, J.C., Casselton, L.A., 1992. Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. Curr Genet 22, 41-45.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., Estruch, F., 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO Journal 15, 2227-2235.

Maryon, E.B., Molloy, S.A., Kaplan, J.H., 2013. Cellular glutathione plays a key role in copper uptake mediated by human copper transporter 1. American Journal of Physiology Cell Physiology 304, 20.

Mehra, R.K., Mulchandani, P., Hunter, T.C., 1994. Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. Biochemical and Biophysical Research Communications 200, 1193-1200.

Mills, R.F., Francini, A., Ferreira da Rocha, P.S.C., Baccarini, P.J., Aylett, M., Krijger, G.C., Williams, L.E., 2005. The plant P1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. FEBS Letters 579, 783-791.

Mills, R.F., Krijger, G.C., Baccarini, P.J., Hall, J.L., Williams, L.E., 2003. Functional expression of AtHMA4, a P1B-type ATPase of the Zn/Co/Cd/Pb subclass. The Plant Journal 35, 164-176.

Mohanta, T.K., Bae, H., 2015. The diversity of fungal genome. Biological Procedures Online 17, 1-9.

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., Richaud, P., 2009. AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. Plant Physiology. 149, 894-904.

Muller, L.A., Craciun, A.R., Ruytinx, J., Lambaerts, M., Verbruggen, N., Vangronsveld, J., Colpaert, J.V., 2007. Gene expression profiling of a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate exposed to increased external zinc concentrations. Mycorrhiza 17, 571-580.

Munger, K., Germann, U.A., Lerch, K., 1987. The *Neurospora crassa* metallothionein gene. Regulation of expression and chromosomal location. Journal of Biological Chemistry 262, 7363-7367.

Narindrasorasak, S., Zhang, X., Roberts, E.A., Sarkar, B., 2004. Comparative analysis of metal binding characteristics of copper chaperone proteins, Atx1 and ATOX1. Bioinorganic Chemistry and Application. 2(1-2):105-23.

Nerland, D.E., 2007. The antioxidant/electrophile response element motif. Drug Metabolism Reviews 39, 235-248.

Op De Beeck, M., Ruytinx, J., Smits, M.M., Vangronsveld, J., Colpaert, J.V., Rineau, F., 2015. Belowground fungal communities in pioneer Scots pine stands growing on heavy metal polluted and non-polluted soils. Soil Biology and Biochemistry 86, 58-66.

Osobová, M., Urban, V., Jedelský, P.L., Borovička, J., Gryndler, M., Ruml, T., Kotrba, P., 2011. Three metallothionein isoforms and sequestration of intracellular silver in the hyperaccumulator *Amanita strobiliformis*. New Phytologist 190, 916-926.

Outten, C.E., O'Halloran, T.V., 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292, 2488-2492.

Padamsee, M., Kumar, T.K., Riley, R., Binder, M., Boyd, A., Calvo, A.M., Furukawa, K., Hesse, C., *et al*., 2012. The genome of the xerotolerant mold

Wallemia sebi reveals adaptations to osmotic stress and suggests cryptic sexual reproduction. Fungal Genetics & Biology 49, 217-226.

Palacios, Ò., Atrian, S., Capdevila, M., 2011. Zn- and Cu-thioneins: a functional classification for metallothioneins? JBIC Journal of Biological Inorganic Chemistry 16, 991-1009.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., Sannia, G., 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus.* Appl Environ Microbiol 66, 920-924.

Palmiter, R.D., 1994. Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proceedings of the National Academy of Sciences 91, 1219-1223.

Parisot, D., Dufresne, M., Veneault, C., Laugé, R., Langin, T., 2002. clap1, a gene encoding a copper-transporting ATPase involved in the process of infection by the phytopathogenic fungus *Colletotrichum lindemuthianum.* Molecular Genetics & Genomics 268, 139-151.

Payne, A.S., Gitlin, J.D., 1998. Functional expression of the menkes disease protein reveals common biochemical mechanisms among the copper-transporting P-type ATPases. Journal of Biological Chemistry 273, 3765-3770.

Peña, M.M.O., Puig, S., Thiele, D.J., 2000. Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. Journal of Biological Chemistry 275, 33244-33251.

Peterson, R.L., Massicotte, H.B., Melville L.H., 2004. Mycorrhiza: anatomy and cell biology. NRC Research press and CABI publishing, Ottawa and Wallingford. p 7-42.

Piscitelli, A., Giardina, P., Lettera, V., Pezzella, C., Sannia, G., Faraco, V., 2011. Induction and transcriptional regulation of Laccases in Fungi. Current Genomics 12, 104-112.

Polanco, R., Canessa, P., Rivas, A., Larrondo, L.F., Lobos, S., Vicuna, R., 2006. Cloning and functional characterization of the gene encoding the transcription factor Ace1 in the basidiomycete *Phanerochaete chrysosporium.* Biological Research 39, 641-648.

Porcheron, G., Garénaux, A., Proulx, J., Sabri, M., Dozois, C.M., 2013. Iron, copper, zinc, and manganese transport and regulation in pathogenic Enterobacteria: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. Frontiers in Cellular and Infection Microbiology 3, 90.

Portales-Casamar, E., Thongjuea, S., Kwon, A.T., Arenillas, D., Zhao, X., Valen, E., Yusuf, D., Lenhard, B., Wasserman, W.W., Sandelin, A., 2010. JASPAR

2010: the greatly expanded open-access database of transcription factor binding profiles. Nucleic Acids Research 38, D105-D110.

Pufahl, R.A., Singer, C.P., Peariso, K.L., Lin, S.J., Schmidt, P.J., Fahrni, C.J., Culotta, V.C., Penner-Hahn, J.E., O'Halloran, T.V., 1997. Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science 278, 853-856.

R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org. R Foundation for Statistical Computing, Vienna, Austria.

Rademacher, C., Masepohl, B., 2012. Copper-responsive gene regulation in bacteria. Microbiology 158, 2451-2464.

Ramesh, G., Podila, G.K., Gay, G., Marmeisse, R., Reddy, M.S., 2009. Different patterns of regulation for the copper and cadmium metallothioneins of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Applied and Environmental Microbiology 75, 2266-2274.

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

Rees, E.M., Lee, J., Thiele, D.J., 2004. Mobilization of intracellular copper stores by the Ctr2 vacuolar copper transporter. Journal of Biological Chemistry 279, 54221-54229.

Rensing, C., Fan, B., Sharma, R., Mitra, B., Rosen, B.P., 2000. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. Proceedings of the National Academy of Sciences 97, 652-656.

Rensing, C., Mitra, B., 2007. Zinc, cadmium, and lead resistance and homeostasis. In: Nies, D., Silver, S. (Eds.), Molecular Microbiology of Heavy Metals. Springer Berlin / Heidelberg, pp. 321-341.

Rensing, C., Mitra, B., Rosen, B.P., 1997. The zntA gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. Proceedings of the National Academy of Sciences 94, 14326-14331.

Riggle, P.J., Kumamoto, C.A., 2000a. Role of a *Candida albicans* P1-Type ATPase in resistance to copper and silver ion toxicity. Journal of Bacteriology 182, 4899-4905.

Rousseau, J.V.D., Sylvia, D.M., Fox, A.J., 1994. Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. New Phytologist 128, 639-644.

Rutherford, J.C., Bird, A.J., 2004. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. Eukaryotic Cell 3, 1-13.

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., *et al*., 2013. The role of metallothionein in oxidative stress. International Journal of Molecular Sciences 14, 6044-6066.

Ruytinx, J., 2013a. Mechanisms of metal homeostasis and detoxification in metal sensitive and tolerant isolates of *Suillus luteus,* an ectomycorrhizal fungus. Doctoral dissertation, University Hasselt, Diepenbeek.

Ruytinx, J., Craciun, A., Verstraelen, K., Vangronsveld, J., Colpaert, J., Verbruggen, N., 2011. Transcriptome analysis by cDNA-AFLP of *Suillus luteus* Cd-tolerant and Cd-sensitive isolates. Mycorrhiza 21, 145-154.

Ruytinx, J., Nguyen, H., Van Hees, M., Op De Beeck, M., Vangronsveld, J., Carleer, R., Colpaert, J.V., Adriaensen, K., 2013b. Zinc export results in adaptive zinc tolerance in the ectomycorrhizal basidiomycete *Suillus bovinus*. Metallomics 5, 1225-1233.

Sacky, J., Leonhardt, T., Borovicka, J., Gryndler, M., Briksi, A., Kotrba, P., 2014. Intracellular sequestration of zinc, cadmium and silver in *Hebeloma mesophaeum* and characterization of its metallothionein genes. Fungal Genetics & Biology 67, 3-14.

Saitoh, Y., Izumitsu, K., Morita, A., Tanaka, C., 2010. A copper-transporting ATPase BcCCC2 is necessary for pathogenicity of *Botrytis cinerea*. Molecular Genetics and Genomics 284, 33-43.

Saitoh, Y., Izumitsu, K., Tanaka, C., 2009. Phylogenetic analysis of heavy-metal ATPases in fungi and characterization of the copper-transporting ATPase of *Cochliobolus heterostrophus.* Mycological Research 113, 737-745.

Sautron, E., Mayerhofer, H., Giustini, C., Pro, D., Crouzy, S., Ravaud, S., *et al*., 2015. HMA6 and HMA8 are two chloroplast Cu^{+}-ATPases with different enzymatic properties. Bioscience Report 20, 20.

Schmitt, A.P., McEntee, K., 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences 93, 5777-5782.

Schulze, M., Rodel, G., 1989. Accumulation of the cytochrome c oxidase subunits I and II in yeast requires a mitochondrial membrane-associated protein, encoded by the nuclear SCO1 gene. Molecular and General Genetics 216, 37-43.

Shi, X., Stoj, C., Romeo, A., Kosman, D.J., Zhu, Z., 2003. Fre1p Cu2+ reduction and Fet3p Cu1+ oxidation modulate copper toxicity in *Saccharomyces cerevisiae*. Journal of Biological Chemistry 278, 50309-50315.

Shine, A.M., Shakya, V.P., Idnurm, A., 2015. Phytochelatin synthase is required for tolerating metal toxicity in a basidiomycete yeast and is a conserved factor involved in metal homeostasis in fungi. Fungal Biology & Biotechnology 28, 3.

Shiraishi, E., Inouhe, M., Joho, M., Tohoyama, H., 2000. The cadmium-resistant gene, CAD2, which is a mutated putative copper-transporter gene (PCA1), controls the intracellular cadmium-level in the yeast *S. cerevisiae.* Current Genetics 37, 79-86.

Silva, R.F., Lupatini, M., Trindade, L., Antoniolli, Z.I., Steffen, R.B., Andreazza, R., 2013. Copper resistance of different ectomycorrhizal fungi such as *Pisolithus microcarpus*, *Pisolithus sp.*, *Scleroderma sp.* and *Suillus sp.* Brazilian Journal of Microbiology 44, 613-621.

Simm, C., Lahner, B., Salt, D., LeFurgey, A., Ingram, P., Yandell, B., Eide, D.J., 2007. Saccharomyces cerevisiae vacuole in zinc storage and intracellular zinc distribution. Eukaryotic Cell 6, 1166-1177.

Smit, A.F., Hubley, R., Green, P., 1996. RepeatMasker Open-3.0.

Socha, A.L., Guerinot, M.L., 2014. Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants. Frontiers in Plant Science 5, 106.

Southron, J.L., Basu, U., Taylor, G.J., 2004. Complementation of *Saccharomyces cerevisiae* ccc2 mutant by a putative P1B-ATPase from *Brassica napus* supports a copper-transporting function. FEBS Letters 566, 218-222.

Speisky, H., Gomez, M., Carrasco-Pozo, C., Pastene, E., Lopez-Alarcon, C., Olea-Azar, C., 2008. Cu(I)-glutathione complex: a potential source of superoxide radicals generation. Bioorganic & Medicinal Chemistry 16, 6568-6574.

Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D., Dancis, A., 1996. A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science 271, 1552-1557.

Stoj, C.S., Augustine, A.J., Solomon, E.I., Kosman, D.J., 2007. Structure-function analysis of the cuprous oxidase activity in Fet3p from *Saccharomyces cerevisiae*. Journal of Biological Chemistry 282, 7862-7868.

Suzuki, N., Yamaguchi, Y., Koizumi, N., Sano, H., 2002. Functional characterization of a heavy metal binding protein CdI19 from *Arabidopsis*. The Plant Journal 32, 165-173.

Takahashi, R., Bashir, K., Ishimaru, Y., Nishizawa, N.K., Nakanishi, H., 2012. The role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice. Plant Signaling & Behavior 7, 1605-1607.

Taylor, A.B., Stoj, C.S., Ziegler, L., Kosman, D.J., Hart, P.J., 2005. The copper-iron connection in biology: structure of the metallo-oxidase Fet3p. Proceedings of the National Academy of Sciences 102, 15459-15464.

Tennstedt, P., Peisker, D., Böttcher, C., Trampczynska, A., Clemens, S., 2009. Phytochelatin synthesis is essential for the detoxification of excess zinc and contributes significantly to the accumulation of zinc. Plant Physiology. 149, 938-948.

Thiele, D.J., 1992. Metal-regulated transcription in eukaryotes. Nucleic Acids Research 20, 1183-1191.

Thurston, C.F., 1994. The structure and function of fungal laccases. Microbiology 140, 19-26.

Tinoco, R., Acevedo, A., Galindo, E., Serrano-Carreon, L., 2011. Increasing *Pleurotus ostreatus* laccase production by culture medium optimization and copper/lignin synergistic induction. Journal of Industrial Microbiology & Biotechnology 38, 531-540.

Tong, L., Nakashima, S., Shibasaka, M., Katsuhara, M., Kasamo, K., 2002. A novel Histidine-rich CPx-ATPase from the filamentous cyanobacterium *Oscillatoria brevis* related to multiple-heavy-metal cotolerance. Journal of Bacteriology 184, 5027-5035.

Tucker, S.L., Thornton, C.R., Tasker, K., Jacob, C., Giles, G., Egan, M., Talbot, N.J., 2004. A fungal metallothionein is required for pathogenicity of *Magnaporthe grisea*. The Plant Cell 16, 1575-1588.

Uldschmid, A., Dombi, R., Marbach, K., 2003. Identification and functional expression of ctaA, a P-type ATPase gene involved in copper trafficking in *Trametes versicolor.* Microbiology 149, 2039-2048.

Uldschmid, A., Engel, M., Dombi, R., Marbach, K., 2002. Identification and functional expression of tahA, a filamentous fungal gene involved in copper trafficking to the secretory pathway in *Trametes versicolor.* Microbiology 148, 4049-4058.

Upadhyay, S., Torres, G., Lin, X., 2013. Laccases involved in 1,8-dihydroxynaphthalene melanin biosynthesis in *Aspergillus fumigatus* are regulated by developmental factors and copper homeostasis. Eukaryotic Cell 12, 1641-1652.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7), research0034.1–0034.11.

Vangrondsveld, J., Colpaert, J.V., Van Tichelen, K.K. 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environmental pollution 94, 131-140.

Vida, T.A., Emr, S.D., 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. Journal of Cell Biology. 128, 779-792.

Waldron, K.J., Robinson, N.J., 2009. How do bacterial cells ensure that metalloproteins get the correct metal? Nature Reviews Microbiology 7, 25-35.

Walton, F.J., Idnurm, A., Heitman, J., 2005. Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans.* Molecular Microbiology 57, 1381-1396.

Wang, B., Qiu, Y.L., 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16, 299-363.

Wang, Y., Fang, J., Leonard, S.S., Rao, K.M., 2004a. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radical Biology & Medicine 36, 1434-1443.

Wang, Y., Lorenzi, I., Georgiev, O., Schaffner, W., 2004b. Metal-responsive transcription factor-1 (MTF-1) selects different types of metal response elements at low vs. high zinc concentration. Biological Chemistry 385, 623-632.

Weissman, Z., Berdicevsky, I., Cavari, B.-Z., Kornitzer, D., 2000. The high copper tolerance of *Candida albicans* is mediated by a P-type ATPase. Proceedings of the National Academy of Sciences 97, 3520-3525.

Weissman, Z., Shemer, R., Kornitzer, D., 2002a. Deletion of the copper transporter CaCCC2 reveals two distinct pathways for iron acquisition in *Candida albicans.* Molecular Microbiology 44, 1551-1560.

Williams, L.E., Mills, R.F., 2005. P1B-ATPases – an ancient family of transition metal pumps with diverse functions in plants. Trends in Plant Science 10, 491-502.

Williams, L.E., Pittman, J.K., Hall, J.L., 2000. Emerging mechanisms for heavy metal transport in plants. Biochimica et Biophysica Acta - Biomembranes 1465, 104-126.

Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., Klausner, R.D., 1996. Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. The EMBO Journal 15, 3377-3384.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13, 1471-2105.

Yuan, D., Dancis, A., Klausner, R., 1997. Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. Journal of Biological Chemistry 272, 25787 - 25793.

Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., Klausner, R.D., 1995. The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proceedings of the National Academy of Sciences 92, 2632-2636.

Zhou, P., Thiele, D.J., 1993. Rapid transcriptional autoregulation of a yeast metalloregulatory transcription factor is essential for high-level copper detoxification. Genes & Development 7, 1824-1835.

Zhu, W., Zhao, D.X., Miao, Q., Xue, T.T., Li, X.Z., Zheng, C.C., 2009. *Arabidopsis thaliana* metallothionein, AtMT2a, mediates ROS balance during oxidative stress. Journal of Plant Biology 52, 585-592.