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FACULTEIT INDUSTRIËLE INGENIEURSWETENSCHAPPEN
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Masterproef

Functional characterization of heavy metal transporters of the mycorrhizal fungus *Suillus luteus*

Promotor :
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Proefschrift ingediend tot het behalen van de graad van master in de industriële wetenschappen: biochemie

Gezamenlijke opleiding Universiteit Hasselt en KU Leuven

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universiteit hasselt | KU LEUVEN

Foreword

This master thesis is the result of my internship project in the specialization Master in Biochemistry at KHLim. The practical proceedings are implemented during the second semester of the academic year 2013 - 2014, in the Centrum voor Milieukunde (CMK).

I would like to take this opportunity to thank everyone who supported the realization of this thesis and the supervision during the implementation of this assignment. I express my sincere gratitude to my promotor, Drs. Hoai Nguyen and my intern promoter, Ing. Liesbet Pauls, for their valuable directions and patient guidance. Besides, I am grateful to the CMK team, and also to everyone who helped and encouraged me in any way. Also, I would like to recommend CMK as an ideal and supportive work environment for achieving an internship and for integrating in professional life.

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Abstract (English)

Since the Industrial Revolution, human activities have led to an enormous rise of heavy metal concentrations in certain areas. Heavy metals are toxic for living organisms, even in minor concentrations. Some organisms, however, developed specific survival strategies. Some of these strategies are mediated by the Ycf1 and Yor1 ATP binding cassette (ABC)-transporters. In the yeast *Saccharomyces cerevisiae*, the Ycf1 transporter is involved in vacuolar sequestration, the Yor1 in efflux of heavy metal ions.

The research topics in this master thesis are the identification of homologues of these two transporters in the ectomycorrhizal fungus *Suillus luteus*, and the investigation if these homologues also have a role in heavy metal tolerance, more specifically in cadmium tolerance. By means of a phylogenetic analysis, with the aid of bioinformatic tools like NCBI, JGI and MEGA6, the evolutionary relationship was shown. The *Ycf1* gene was isolated from *S. luteus* and introduced into a *Ycf1* deletion yeast strain. This strain was subjected to a complementation test by drop assay, wherein it was cultivated on media containing a gradient of cadmium concentrations.

The phylogenetic analysis has shown that *S. luteus* and all Boletales fungi in this study have one homologue of Ycf1 and at least one homologue of Yor1. The complementation test indicated that the expression of *SlYcf11* might rescue the yeast mutants in media with high cadmium concentrations, but further detailed analysis is required to confirm the function of the *SlYcf1* gene.

Abstract (Nederlands)

Sinds de Industriële Revolutie zijn door menselijke activiteiten grote hoeveelheden aan zware metalen terechtgekomen in bepaalde gebieden. Deze zware metalen zijn zelfs in kleine hoeveelheden toxisch voor levende organismen. Een aantal organismen ontwikkelden specifieke strategieën om in deze condities te overleven. Enkele van deze mechanismen worden, bijvoorbeeld in de gist *Saccharomyces cerevisiae*, gemedieerd door de ATP binding cassette (ABC)-transporters Ycf1 en Yor1. De Ycf1 transporter geeft de mogelijkheid tot sequestratie van zware metalen in de vacuole en de Yor1 transporter tot efflux van zware metaalionen.

In deze masterproef worden de homologen van deze twee transporters geïdentificeerd in de ectomycorrhiza schimmel *Suillus luteus*. Daarnaast wordt onderzocht of deze homologen een rol spelen in de zware metaaltolerantie, meer bepaald de cadmiumtolerantie. Door middel van een fylogenetische analyse werd de evolutionaire verwantschap aangetoond, met behulp van bioinformatica tools zoals NCBI, JGI en MEGA6. Het *Ycf1* gen werd geïsoleerd uit *S. luteus* en ondergebracht in een giststam met een*Ycf1* deletie . Deze stam werd onderworpen aan een complementation test via drop assay, waarbij het werd uitgeplaat op voedingsbodems met verschillende concentraties aan cadmium.

De fylogenetische analyse toonde aan dat *S. luteus*, net als alle andere schimmels behorende tot de Boletales, één Ycf1 homoloog heeft en minstens één Yor1 homoloog. De complementation test gaf een indicatie dat de expressie van het *SlYcf11* gen de mutant giststammen in staat stelt te overleven in media die hogere concentraties aan cadmium bevatten, maar verdergaand onderzoek is vereist om de precieze functie van het *SlYcf11* gen te bepalen.

Introduction

Research topic

Since the beginning of the Industrial Revolution, human activities have led to an enormous rise of metal concentrations in certain areas. Some metals, for instance cadmium (Cd) and lead (Pb), can be extremely toxic for the human body and for the environment. However, some of these metals, e.g. zinc (Zn) and copper (Cu), are essential in biological functions, in appropriate quantities. Toxic metals are often referred to as 'heavy metals".

This study focuses on such a metal-contaminated area in the Northern Campine region in Belgium. Large quantities of zinc, cadmium and lead have spread over this region due to zinc processing industry, in particular pyrometallurgical industry. This contamination has induced specific adaptations in organisms inhabiting these areas. In their struggle for life these organisms have developed several mechanisms in order to survive (e.g. extrusion of heavy metal ions, sequestration in the cell vacuoles). A fine example of such an organism is the Basidiomycete ectomycorrhizal fungus *S. luteus*, which belongs to the order of the Boletales. *S. luteus* is commonly found in coniferous forests, where it lives in symbiosis with the widely spread pine tree *Pinus sylvestris.* Several studies have already investigated *S. luteus'* ability to adapt and thrive on these polluted sandy soils. Even more, its complete genome is already sequenced and free available online. For these two reasons, *S. luteus* is an interesting research organism.

The Centrum voor milieukunde (CMK), situated in the University Hasselt at Diepenbeek, researches this fungus' specific pathways for survival in metal stress conditions in physiological and molecular scales such as gene expression and regulation. Regarding the future, CMK's perspective is to obtain knowledge that might be used for new methods such as bioremediation, either by immobilizing or by extracting excessive heavy metal ions out of contaminated soils.

Research question and objective

The ATP Binding Cassette transporters are a group of transmembrane proteins. Under normal conditions, they ensure the active transport of diverse compounds like amino acids, peptides, proteins, and lipids across cellular membranes. Several studies also report their role in the transportation of heavy metal ions [1,2].

With the complete genome sequence of *S. luteus* available, we will first identify some potential metal transporters (Ycf1- and Yor1-like proteins) of the ABC transporter family. The second objective of this master thesis is to help reveal the precise function of these potential *Ycf1* and *Yor1* homologues, and, if possible, to study their regulation.

To obtain this knowledge, firstly the sequence of the potential transporters is compared with homologous sequences from closely related species. The function of those reference proteins is already described to a greater extent. Also, in order to test the exact influence of heavy metal exposure, the genes will be isolated from the mycorrhizal fungus *Suillus luteus*. With the aid of vector plasmids their expression will be induced in the yeast *Saccharomyces cerevisiae*. This recombinant strain will be exposed to various levels of heavy metal concentrations, together with a mutant without the gene, in order to compare.

1 Theoretical background

1.1 Heavy metals

The term "heavy metals" mostly refers to a range of chemical elements, categorized in the lower periods of the periodic table. In fact, this definition remains inadequate, because "heavy metals" is also commonly used for certain elements that exhibit toxic properties. Of course there is a causal link: the position of the element in the period table is related to chemical properties of compounds that include the element [3].

Most heavy metals occur naturally in the soil. But human activities like mining, agriculture and landfill of domestic wastes have led to an enormous rise of concentrations, up to a level that can be harmful for humans and for the environment. On the other hand, it has been shown that a number of heavy metals are essential micronutrients that function in cell metabolism, including cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn) [3]. Co and Zn for instance are part of the active site in certain plant and animal enzymes. Only when present in excess they become toxic [4]. As for other elements such as cadmium (Cd), lead (Pb) and mercury (Hg) [5], they are considered strictly toxic. They have no known role in biological functions.

Heavy metals exert toxicity in various ways. They bind to proteins and disrupt the protein structure or block functional groups (e.g. sulfhydryl groups [6]), causing inactivation or downregulation of enzymes and transport systems [5]. Also (hydroxyl)radicals are formed which lead to oxidative stress. The primary effects lead to alteration of the composition and fluidity of the membrane lipids, disruption of electron transport, and therefore of processes that depend on these, like photosynthesis and mitochondrial respiration [6]. Even genotoxic effect can occur, damage to genetic material, which can lead to mutations.

As for the visible effects of heavy metals: inhibition of plant growth will be observed (Fig. 1), and also color changes, an impaired water balance, closing of the stomata, and, at very high concentrations, necrosis (Fig. 2).

Certain toxic components are biodegradable. This is not the case for heavy metals, the only ways to dispose of them are immobilization [5] and removal.

Fig. 1: Toxic effect of Cu on rice (*Oryza sativa*). The plant on the left is a control, the plant on the right was treated with 0,5 mg/l of Cu [7]

Fig. 2: Necrosis in older mustard leaves after treatment with 100 mg Cd/kg soil [8]

1.2 Heavy metal detoxification and tolerance

Driven by natural selection, certain species responded to rather extreme conditions by developing coping mechanisms, in order to maintain and to thrive. Therefore certain species develop highly resistant proteins. A striking example is the taq polymerase of *Thermus aquaticus*, the bacterium that is able to survive in hot springs.

Development of more resistant proteins is not obvious in stress conditions due to the presence of heavy metals. Instead, coping strategies (Fig. 3) are focused on avoiding the build-up of toxic metal concentrations in vulnerable locations in the cell.

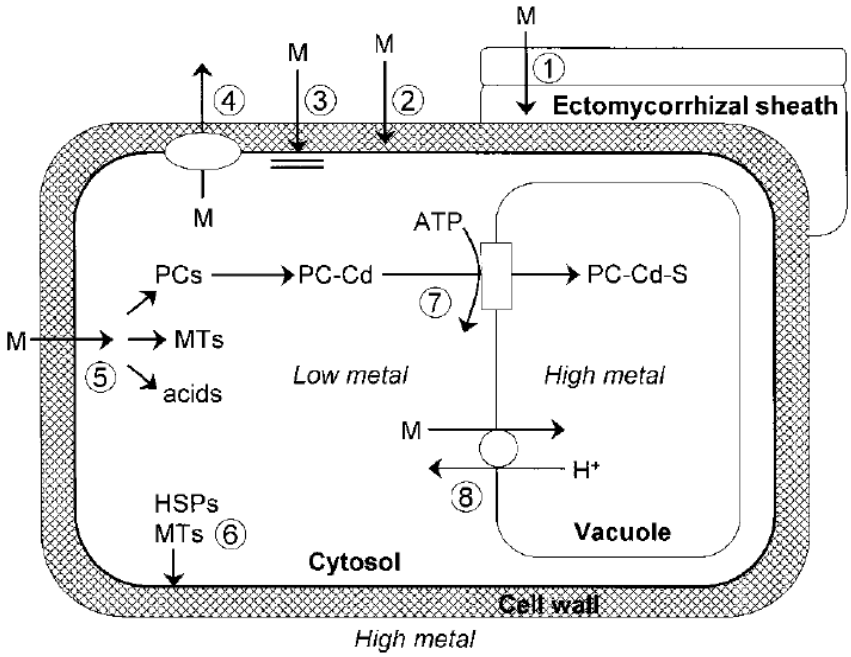

Fig. 3: Potential cellular mechanisms available for metal detoxification and tolerance [6]. A plant cell is depicted here, but most mechanisms are similar in other organisms. 1: symbiosis; 2: ligation to cell walls and root exudates; 3: reduced uptake; 4: active efflux; 5: intracellular ligation; 6: restoration and protection of plasma membranes and proteins; 7: transport into vacuole; 8: accumulation in vacuole

1.2.1 Symbiosis between organisms

Although it is not always considered in general reviews of plant metal tolerance mechanisms, some plants are able to engage in a symbiotic relationship with certain micro-organisms. Mycorrhizal networks, symbiotic cooperation between specific plants and fungi, demonstrate this. The fungus acts as an interphase between plant and soil, so the plant takes benefit of the fungus' resistance mechanisms [6].

1.2.2 Ligation to cell walls and root exudates

Cell walls are complex three-dimensional structures, consisting of proteins, lipids, polysaccharides and large organic macromolecules such as chitosan and glucans [6]. A number of these function as metal chelating agent, transforming the metal into a non-toxic form, thus prohibiting its entrance into the cell and thus decreasing the bioavailability. This ligation of specific components to cell walls is called biosorption [9]. Adsorption to the cell wall however has merely limited capacity. Mycorrhizal fungi frequently carry out biosorption to handle the presence of heavy metals.

A similar mechanism is the adsorption of metals by root exudates. Buckwheat roots, for example, exude oxalic acid in order to transform aluminium ions into non-toxic Al-oxalate [6].

1.2.3 Reduced uptake of heavy metal ions

The plasma membrane acts as a barrier between the interior of the cell and the exterior. This barrier reduces the uptake of heavy metals into the cell. This can be observed in *Holcus lanatus*, among others. Arsenic finds its way towards the inner cell, via the same mechanism that phosphates use. In case of excessive arsenic concentrations, this pathway will be blocked, resulting in reduced levels of both arsenic and phosphate intake [6].

1.2.4 Disposal of the heavy metal ions by means of efflux

Active efflux transporters function as pumps, in order to transport heavy metal ions in the cytosol back to the exterior. In many cases these transporters belong to the ATPases or cation/H^+ antiporters. Such efflux systems are capable of handling Cu, Cd, Zn, Co and Ni ions. This mechanism is most observed in bacteria [6].

1.2.5 Intracellular ligation of heavy metal ions

Within the cell, high-affinity ligands perform chelations. As a result, the heavy metals are stored as complexes, in a non-toxic form. These ligands can be organic acids, amino acids, sulfides [10], phytochelatins (PCs) and metallothioneins (MTs) [11].

Phytochelatins and metallothioneins are cysteine-rich, heavy metal-binding protein molecules. Both groups are primarily identified in plants. Phytochelatins possess a "general structure (Ɣ-Glu Cys)n-Gly " wherein n values from 2 up to 11. In plants, phytochelatins are considered responsable for Cd tolerance (Fig.), but eventually they also increase tolerance for other heavy metals. Metallothioneins are much larger, and are involved in Cu, Zn and Cd binding. Their exact function is not quite clear. They serve primarily as chelating agents, but it is also possible that metallothioneins help restore cell membranes and protect the cell against oxidative stress. [6].

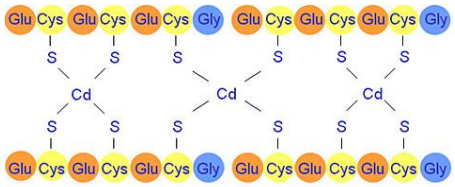

Fig. 4: Cadmium phytochelatin complex [12]

1.2.6 Restoration of damaged plasma membranes and proteins

In order to reduce or avoid damage, proteins and plasma membranes need to be protected and repaired as much as possible. This is where heat shock proteins (HSPs) en metallothioneins (MT) (see above) play a role. It is known that growth at higher than preferable temperatures increases the expression of heat shock proteins. This also happens in other kinds of stress condition. Heat shock proteins not only act as molecular chaperones in protein folding and assembly, they also have a protecting and healing effect on proteins [6].

1.2.7 Vacuolar sequestration

Another way of effectively removing heavy metal ions out of the cytosol, apart from efflux into the exterior, is transportation and storing into a vacuole. This mechanism is primarily known to enhance Zn and Cd tolerance. Free metal ions as well as complex metal ions can be sequestered in the vacuole [6].

1.3 Mycorrhizal fungus

A mycorrhiza is the symbiotic association between specific soil fungi and vascular plants. The basic requirement for this symbiosis is the exchange of nutrients. Mycorrhizal fungi form an interface between plant roots and soil, in fact they are part of the root system [5]. Fungal hyphae are smaller in diameter than roots or root hairs, so they can penetrate and exploit a larger volume of soil. A considerably larger surface area means more efficient absorption of water and mineral nutrients. The fungus provides these nutrients to the vascular plant in exchange for photosynthesis products such as glucose and sucrose. The fungus itself is not capable of photosynthesis, because the fungus' cells do not contain chlorophyll. Mycorrhizal fungi even extract phosphate ions from soils with a high pH, so the plant also takes benefit. [13]. Even more, the fungal symbiotic partner is also equipped to provide protection for the vascular plant. It shields the plant from pathogenic organisms or unfavorable soil conditions such as raised heavy metal concentrations and extreme pH values [13].

Different major categories of mycorrhizal associations occur: ectomycorrhizae and endomycorrhizae (Fig. 5). In ectomycorrhizae, the hyphae form a mantle around the root, the hyphal sheath, and only penetrate the cortex, never the cells within the root. In endomycorrhizae the hyphae penetrate the cell wall and the cell membrane and reach the cytoplasm. Endomycorrhizae are further classified into five groups: arbuscular, ericoid, arbutoid, monotropoid, and orchid mycorrhizae, based on their symbiotic partner's family and the relationship between fungus and plant.

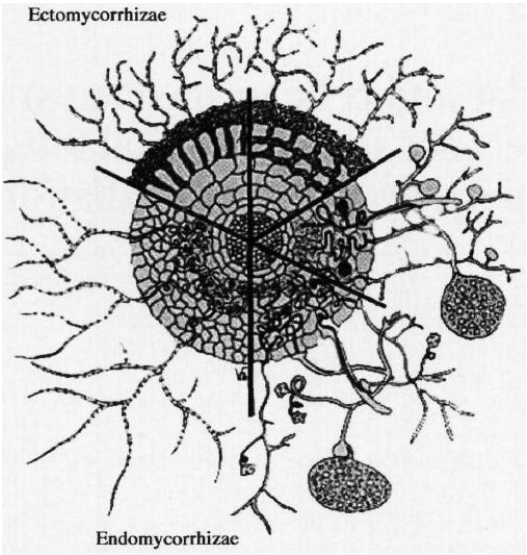

Fig. 5: Different types of mycorrhizae [5]

1.3.1 Mycorrhizal fungus *Suillus luteus*

S. luteus is a mycorrhizal fungus, of the ectomycorrhiza type. *S. luteus*, also called "slippery jack" is categorized in the kingdom of the fungi, phylum Basidiomycota, class Agaricomycetes , order Boletales [14]. *S. Luteus* is very common throughout Europe, Asia and North-America, always in or near pine woods. The species is a pioneer, rapidly spreading throughout disturbed soils. It even thrives in the heavy metal contaminated soils of northern Limburg, distinctively in newly grown pine stands (Pinus sylvestris), where the litter layer is still relatively thin [13]. In the late summer, the fruiting body forms above ground level, and gives an edible mushroom (Fig. 6).

Fig. 6: Fruiting body of *S. luteus* [15]

The reproduction of *S. Luteus* proceeds in the same manner as with all other Basidiomycota, via sexual and asexual reproduction. Asexual reproduction takes place by budding or by spore formation. Starting the process of budding, a bulge will form in the cell membrane. The nucleus divides, and one of the nuclei migrates into the bulge. Then, the bulge will be constricted, resulting in a new cell. Budding can take place in any cell. Asexual spore formation takes place in the so-called primary or haploid mycelium, which is monokaryotic and possesses one, haploid nucleus (n). This sporogenesis only occurs in specialized cells, named conidiophores. Mitosis precedes the formation of a cellular structure that includes one of the nuclei, which is detached as a spore.

In Fig. 7, the sexual reproduction cycle of the Basidiomycota is schematically illustrated. Sexual reproduction is the result of the fusing of two haploid mycelia to produce the dikaryotic or secundary mycelium (n+n). In this appearance, the fungus engages in a symbiotic relationship with a vascular host plant. The fungus retrieves sugars from this cooperation, which provides in the energy that is necessary for shaping the fruiting body. In the case of Basidiomycota it is named basidiocarp, this is the mushroom's typical cap and stipe.

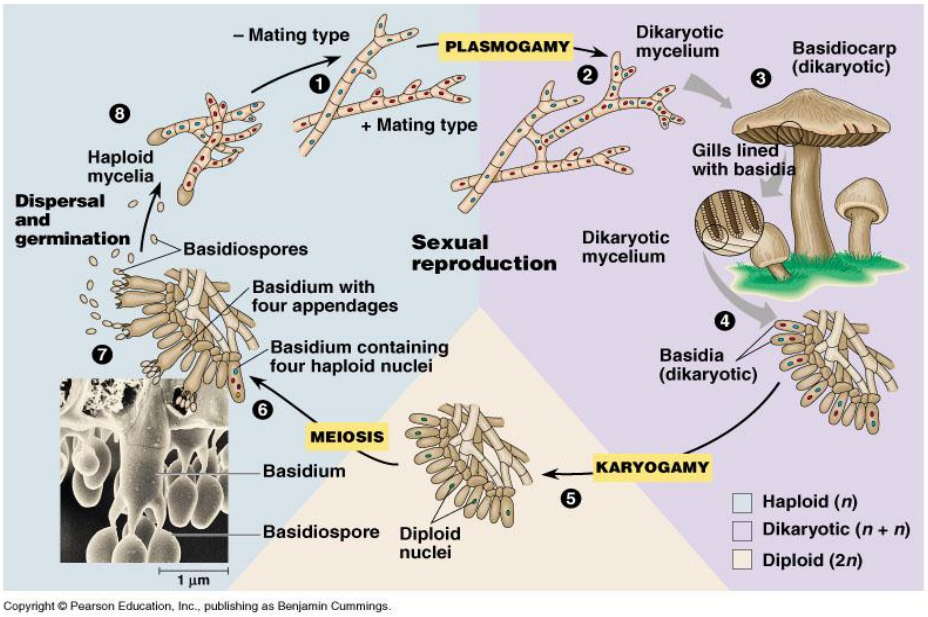

Fig. 7: Sexual reproduction cycle of the Basidiomycota [16]

Inside the pores, in the underside of the cap, basidia are formed (Fig. 8). These are cells wherein two haploid nuclei fuse to produce one diploid nucleus (2n). Subsequently the nucleus is divided in four haploid nuclei by meiosis. These are constricted and detached in order to form basidiospores, leaving the basidia behind, which is now without nucleus. Wind or animals eventually convey these spores to a favourable underground, where they can form a new primary mycelium.

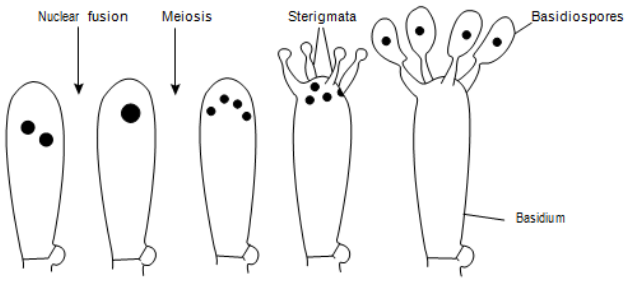

Fig. 8: Formation of basidiospores in the basidia [17]

1.4 ABC-transporters

ATP Binding Cassette (ABC)–transporters belong to the transmembrane proteins, one of the largest known protein families [1]. They are present in all living cells, prokaryotic cells as well as eukaryotic cells. ABC proteins are mainly, but not exclusively, responsible for active transport of diverse substrates like amino acids, peptides, proteins and lipids across biological membranes. Some ABC proteins also have other functions, for instance as ion channels or receptors and are involved in mRNA translation and ribosome biogenesis. An organism does not function properly without ABC transporters. Defective human ABC genes affect the functioning of transport channels, involving cystic fibrosis, adrenoleukodystrophy, various cholesterol disorders, … [2].

The typical structure of an ABC transporter consists of two nucleotide-binding domains (NBD's) and two transmembrane domains (TMD's), although variations occur (Fig. 9). The NBD's perform the ATP hydrolysis that provides the energy required for protein activity.

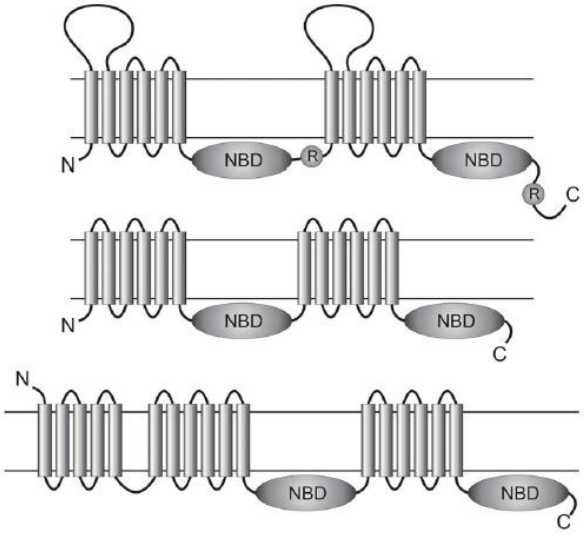

Fig. 9: Different ABC protein subclasses. From the top down: ABC-A, ABC-B, ABC-C [1]

The Human Genome Organization (HUGO) has classified all ABC proteins, encountered in eukaryotes, into 8 subfamilies, ranging from ABC-A to ABC-H, according to their structure and the amino acid sequence of their NBD's. Later on, prokaryotic ABC transporters are grouped into a ninth subfamily, the ABC-I proteins [1,18].

1.4.1 Subclass ABC-C transporters: Ycf1 and Yor1

Most of the ABC-C subfamily proteins are transporters that mediate the efflux of xenobiotic compounds [2] . This ABC-C subfamily is further separated into seven groups, among them group VI including the Yor1-like transporters and group VII including the Ycf1-like transporters.

Yeast cadmium factor (Ycf1) is characterized in 1994 in the yeast *Saccharomyces cerevisiae*, based on its ability to confer cadmium resistance [19]. Ycf1, located in the vacuolar membrane (Fig 10), is a GS-X transporter. It transports glutathione-conjugated components, certain pigments, and some heavy metals like Cd, Pb, Sb, Hg and As.

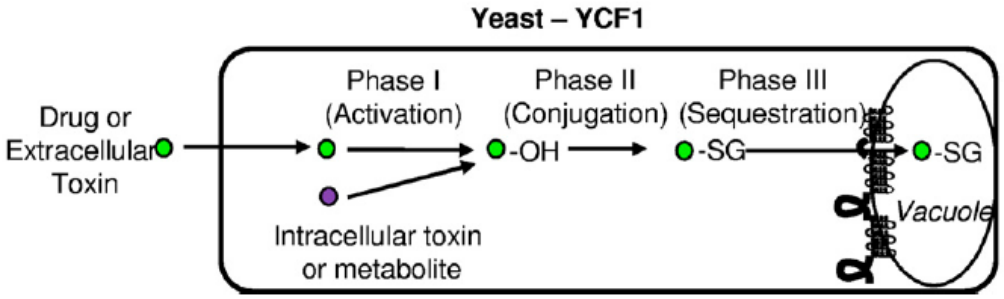

Fig. 10: Cellular detoxification mechanism: phase I: activation leads to increased chemical activity; phase II: enzymatic conjugation of glutathione to the toxin; phase III: sequestration of the conjugated toxin in the vacuole, mediated by Ycf1 [2]

Later on, so-called Ycf1 homologues have been identified in several other species. Most of the fungi have only one Ycf1 homologue except for *Suillus pombe* and *Puccinia graminis*, they contain two Ycf1 homologues [1].

The Yor1 transporter is the only member of the ABC subfamily that is known to be located in the plasma membrane [2]. Yor1 is considered a multispecific organic anion transporter, for it mediates the export of components like phospholipids, reveromycin A and oligomycin. Oligomycin is an antibiotic that inhibits ATP synthase and therefore oxidative phosphorylation. Yor1 features the ability of transporting oligomycin out of the cell, hence the name yeast oligomycin resistence (yor1) transporter [17]. Irregularities in the *Yor1* gene cause increased sensitivity for certain drugs and xenobiotics in organisms.

Just like Ycf1, Yor1 functions as a GS-X transporter, urging Cd-$(GS)_2$ complexes into the exterior of the cell. Therefore Yor1 is also implicated in Cd tolerance. Yor1 homologues have been found in other organisms. *Sphenodon punctatus* for example possesses five Yor1 homologues and *Batrachochytrium dendrobatidis* even has 11 homologues [1].

Two model organisms have been studied in order to understand ABC transporters: the yeasts *Saccharomyces cerevisiae* and less extensively *Schizosaccharomyces pombe*. *S. cerevisiae* is the first eukaryote of which the genome is completely sequenced. For *S. cerevisiae*, 22 proteins are identified as ABC transporters and they have been fully characterized and localized (Fig. 11) [2]. A full characterization of the genome is only partly implemented for other organisms.

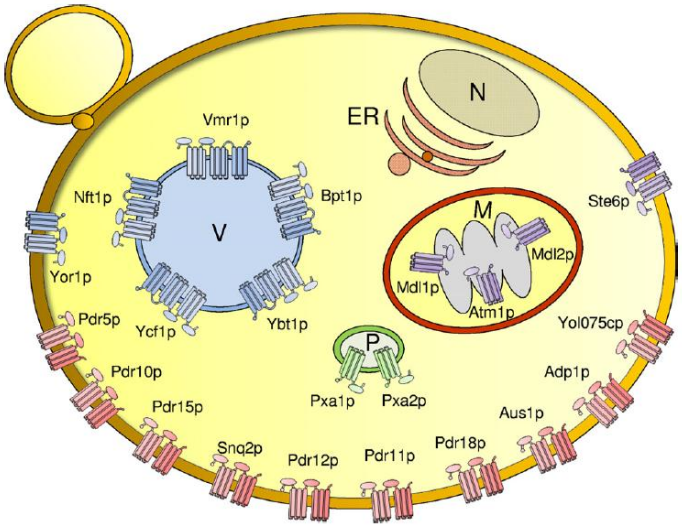

Fig. 11: Location of the ABC transporters in a cell of *S. cerevisiae* [2]

2 Material and methods

2.1 Phylogenetic analysis of the ATP binding cassette proteins in the Phylum Basidiomycota

To determine potential functions of a protein, its sequence is compared with the known sequences of other species. The higher the similarity, the more probable that the proteins exert the same function. The degree of resemblance is shown and interpreted by constructing a phylogenetic tree. In addition, the conserved domains of the proteins are compared.

2.1.1. Drafting the phylogenetic tree

The reference peptide sequences of Ycf1 and Yor1 in *S. cerevisiae* were retrieved from the NCBI databases [20].

Protein sequences of fungal species were retrieved from the Joint Genome Institute database [21]. Firstly the CLUSTER function of the JGI database was used. Any hits in species not belonging to the Basidiomycota phylum were omitted. A number of newly sequenced species were not included in the CLUSTER and not yet annotated. These sequences were found by searching specifically for Ycf1 and Yor1 with BLASTP in the database of *S. luteus* closely related species. Subsequently all data were checked manually for complete sequences and comparable domains. Duplicate sequences were removed.

MEGA6 was used for drawing a phylogenetic tree. Having imported the data, the sequences were aligned via the ClustalW algorithm. The tree itself was constructed via the Neighbor-Joining algorithm.

To estimate the reliability of the phylogenetic tree, the option “bootstrap testing” was used while drawing the tree. Phylogenetic bootstrapping is a standard technique for inferring confidence values on phylogenetic trees [22]. This technique is based on reconstructing many trees from minor variations of the input data, trees called bootstrap replicates [23].

2.1.2 Determination of highly conserved domains

Highly conserved regions are sequences or domains that have remained very similar in different species during the course of evolution. Their association with molecular functions such as binding or catalysis explains this [24]. For these functions are vital and alterations are likely to be lethal. So there is a natural selection pressure that kept these domains unchanged in different species. The more resemblance of the conserved domains in two proteins, the higher the level of confidence that their functions will match. To identify the conserved domains in a sequence, the Conserved Domain Database (CDD) was used.

2.2 Functional test of *S. luteus* Ycf1-like protein (SlYcf1)

2.2.1 Cloning of *SlYcf1* gene

1. Isolation of the *Ycf1* gene by PCR

In a previous study in the laboratory, the cDNA library of *S. luteus* was made from LM8n1 and LS5n32 monokaryons and then the full coding sequence of *SlYcf1* gene was isolated by means of PCR.

The *SlYcf1* genes from LM8n1 and LS5n32 were then cloned into a Gateway entry vector pCR8/GW/TOPO (Invitrogen, Life technologies) (Annex D), using the pCR8/GW/TOPO TA Cloning Kit (Invitrogen, Life technologies) [25]. These entry vectors were used as materials for this study. The Gateway technology was selected, with the intention of sub-cloning the gene into different expression vectors.

2. Sub-cloning *SlYcf1* into yeast expression vectors

The LR recombination reaction was performed to transfer the *SlYcf1* genes from the pCR8/GW/TOPO construct into the gateway destination yeast expression vectors pAG426GAL-ccdB from Addgene and pYES-DEST52 from Life Technologies (annex E and F).

The LR reactions contained 1-7 µl entry vector, 1 µl destination vector and an amount of TE buffer pH 8.0 so that 8 µl reaction mix was obtained. 2 µl of LR clonase II enzyme mix was added to each sample and incubated for one hour at 25 °C. Afterwards 1 µl Proteinase K 2 µg/µl was added to terminate the reaction. This was performed according to the standard protocol of the pCR8/GW/TOPO TA Cloning Kit (Invitrogen, Life technologies) [25].

3. Transformation into *E. coli*

After the LR reaction, the expression construct was transformed into *E. coli* according to the One Shot Chemical Transformation Protocol of the pCR8/GW/TOPO TA Cloning Kit [25].

Briefly, 3 µl of the LR reaction was added to one tube of One Shot® competent *E.coli* and incubated on ice for 30 min. After a heat-shock of 30 seconds at 42 °C, the tubes were transferred back to ice and 250 µl S.O.C. medium was added. Next, the tubes were incubated, shaking at 37°C for 1 hour. After that, the bacteria were spread on LB-medium (Annex A, Table 2) with ampicillin (100 µg/ml) for positive selection [25]. After incubating overnight at 37 °C, one positive colony was picked to let grow further in five milliliter of liquid LB medium with 100 µg/ml ampicillin in order to obtain enough plasmids for yeast transformation.

4. Plasmid Extraction

The extraction of the vector constructs was carried out by means of the GeneJET Plasmid Miniprep Kit of Thermo Scientific [26]. The kit utilizes spin column technology.

The overnight cultures of *E. coli* in liquid LB medium containing the expression constructs was centrifuged at 3000 g for 10 min in order to harvest the cells. Each sample was resuspended in 250 µl Resuspension Buffer. The cells were lysed with 250 µl Lysis Solution and then neutralized with 350 µl Neutralization Solution. The water phase, containing the suspended plasmid DNA, was separated by centrifugation 12000 g for 60 s and transferred to a spin column. Other cell residues were washed off with 500 µl Wash Solution and centrifuged at 12000 g for 60 s. The plasmid DNA itself was eluted with 50 µl water [26].

5. Yeast transformation

The mutant yeast strain used for the expression of *SlYcf1* was BY4741 (MAT a; his3Δ1; leu2Δ 0; met15Δ 0; ura3Δ 0; YDR135c:kanMX4), obtained from Euroscraf [27]. For the study of the Ycf1 homologue of *S. luteus* in the yeast *S. cerevisiae*, it is not possible to use a wild type *S. cerevisia* strain, since *S. cerevisiae* possesses a Ycf1 homologue itself. Therefore a mutant strain with a *Ycf1* gene knockout is selected as host cell.

The expression construct was transferred into the yeast *S. cerevisiae* according to the lithium acetate transformation protocol described by Gietz & Woods [28].

From a yeast overnight culture on 30 °C, on solid Yeast Peptone Dextrose (YPD) medium (Annex A, Table 3), the content of an inoculating loop was transferred to 10 ml of liquid 2xYPD medium and then incubated at 30 °C on a shaker. After 4 hours 2 ml of the culture was taken and centrifuged at 12000 g for 30 seconds to pellet the cells. The transformation mix was added to the pellet: 48 µl Polyethylene glycol (PEG) 3500 50% w/v, 7.2 µl lithium acetate (LiAc) 1.0 M, 10 µl boiled single strand carrier DNA 2 mg/ml and 6.8 µl Plasmid DNA (1 µg). This mix was incubated at 42 °C for 60 min. Afterwards, the mix was centrifuged and the supernatans discarded. The pellet was resuspended in 150 µl sterile water, spread on synthetic dextrose medium without uracil (SD -Ura) and incubated at 30 °C.

2.2.2 Yeast functional complementation test

The influence of the genes on heavy metal tolerance was observed by means of drop assay. This was established by plating the different dilutions of the recombinant yeast strains in spots (Fig.) and on culture media which contained a range of heavy metal concentrations. In case the genes are involved in heavy metal tolerance, yeast cells containing the construct should be able to survive higher concentrations.

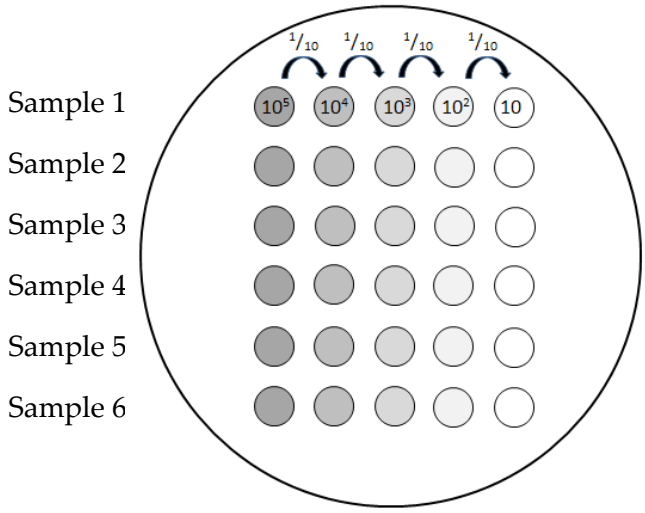

Fig. 12: Schematic representation drop assay

While preparing a drop assay, it is necessary to plate out a wild type and a mutant yeast strain which contain the vector without the gene of interest, apart from the mutant yeast strain that contains the expression construct with the gene of interest.

One colony of each yeast strain was incubated overnight in SD liquid medium without uracil. The OD at 600 nm was measured with a spectrophotometer. Each culture was adjusted to OD = 0.100 by adding more culture or more sterile water, then a 1/10 dilution series was made and 10 µl of each dilution was dropped on the SD plates containing galactose (annex A, table 4) instead of glucose (annex A, table 5) and with concentrations of cadmium of 0, 10, 20, 30, 40 µM.

3 Results

3.1 Phylogenetic analysis of ABC binding cassette proteins in the Phylum Basidiomycota

3.1.1. Phylogenetic tree of Ycf1 and Yor1

From the resulting phylogenetic tree, a simplified version was made to obtain a more comprehendible view (Annex B). Only hits in species belonging to the same order as *S. luteus*, the Boletales, were retained. Also the controls of *S. pombe* and *S. cerevisiae* were edited.

ABC-transporters of *S. luteus* are marked in blue, those of yeast in red. The homologues of Ycf1 and Yor1 form the upper half of the phylogenetic tree. The lower half is likely to be formed by the proteins that belong to other subfamilies of the ABC-C transporters. For verification, a number of transporters belonging to these other ABC-C subgroups have been identified: Ybt1, ABC1 and ABC4.

Group I of the ABC-C proteins are the Ybt1- like proteins [1]. De peptide sequence of Ybt1 of *S. cerevisiae* was retrieved in the NCBI databases and with BLASTP the Ybt1 homologue was found in *S. luteus*. This homologue was already included in the tree, Prot. Id. 80892, Locus 217. The proteins ABC1 and ABC4 of *S. pombe* belong to the ABC-C group IV. They also were used for searching the homologues in *S. luteus*, Prot. Id. 798765, Locus 2703.

From the phylogenetic tree, also a close-up of the Ycf1 and Yor1 part was edited (Annex C). In this tree, also hits in species which do not belong to the order of the Boletales are included. The same indications are used, only the Boletales are marked in green, and species belonging to other orders in black. The Boletales are more closely related, so they group together in the phylogenetic tree. An overview was made of the number of Ycf and Yor1 homologues in each species (Table 1).

Table 1: Number of Ycf1 and Yor1 homologues. The species belonging to the Boletales are marked in gray

Species	Ycf1	Yor1
Saccharomyces cerevisiae	1	1
Schizosaccharomices pombe	2	0
Boletus edulis	1	1
Coniophora puteana	1	1
Hydnomerulius pinastri	1	1
Leucogyrophana mollusca	1	1
Paxillus involutus	1	2
Paxillus rubicundulus	1	2
Pisolithus microcarpus	1	2
Pisolithus tinctorius Marx	1	6
Scleroderma citrinum	1	4
Serpula lacrymans	1	1
Suillus brevipes	1	1
Suillus luteus	1	2
Amanita muscaria Koide	1	2
Hebeloma cylindrosporum	1	5
Laccaria amethystina	1	2
Laccaria bicolor	1	2
Piloderma croceum	1	1
Sebacina vermifera	1	3
Tulasnella calospora	1	2

3.1.2 Determination of highly conserved domains

For the study of the conserved domains, the sequences of *Ycf1* and *Yor1* from *S. crevisiae* and their homologues found in *S. luteus* were entered in the Conserved Domain Database. In addition, in order to compare the conserved domains that were noted in the sequences, an alignment for Ycf1 (Fig. 13) and Yor1 (Fig. 14) was defined with the aid of CLC Sequence Viewer [30]. In Ycf1 and in Yor1 the same conserved domains were noted: 1. Walker A/P loop, 2. Q - loop, 3. ABC transporter signature motif, 4. Walker B, 5. D- loop, 6. H-loop/switch region [24].

The regions Walker A, Walker B, Q-loop, D-loop and H-loop form the nucleotide (ATP) binding site of the ABC protein. Between the sequences, slight variations are visible in the conserved domains. Variation is possible and tolerated as long as the function of the region is preserved [30].

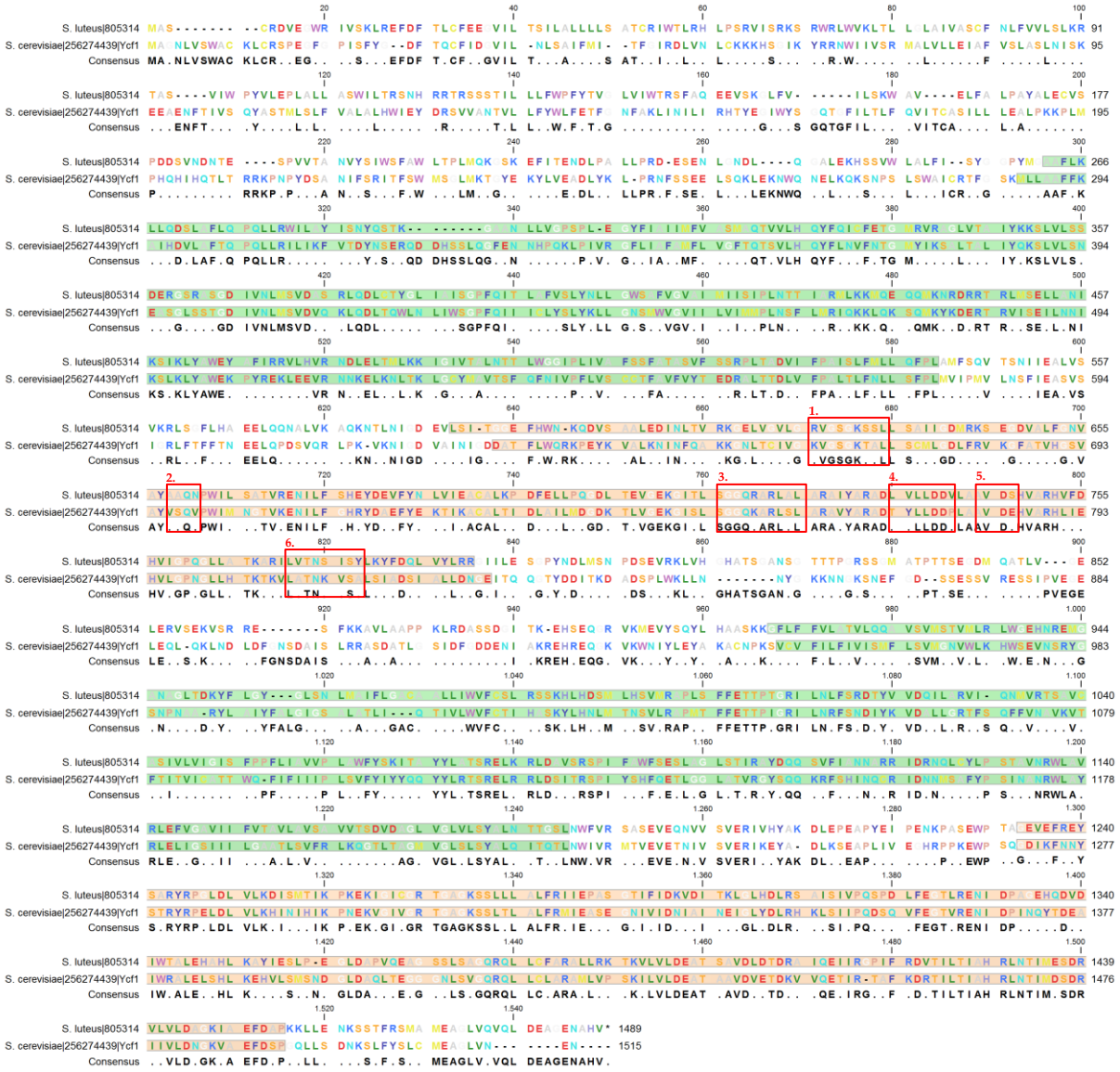

Fig. 13: Alignment *of S. cerevisiae* Ycf1 and the Ycf1-like homologue in *S. luteus*. Residues are Rasmol coloured. In the sequences, the background of transmembrane regions are marked in green, the background of ATP-binding cassette domains in orange. Highly conserved domains are framed in red [29]

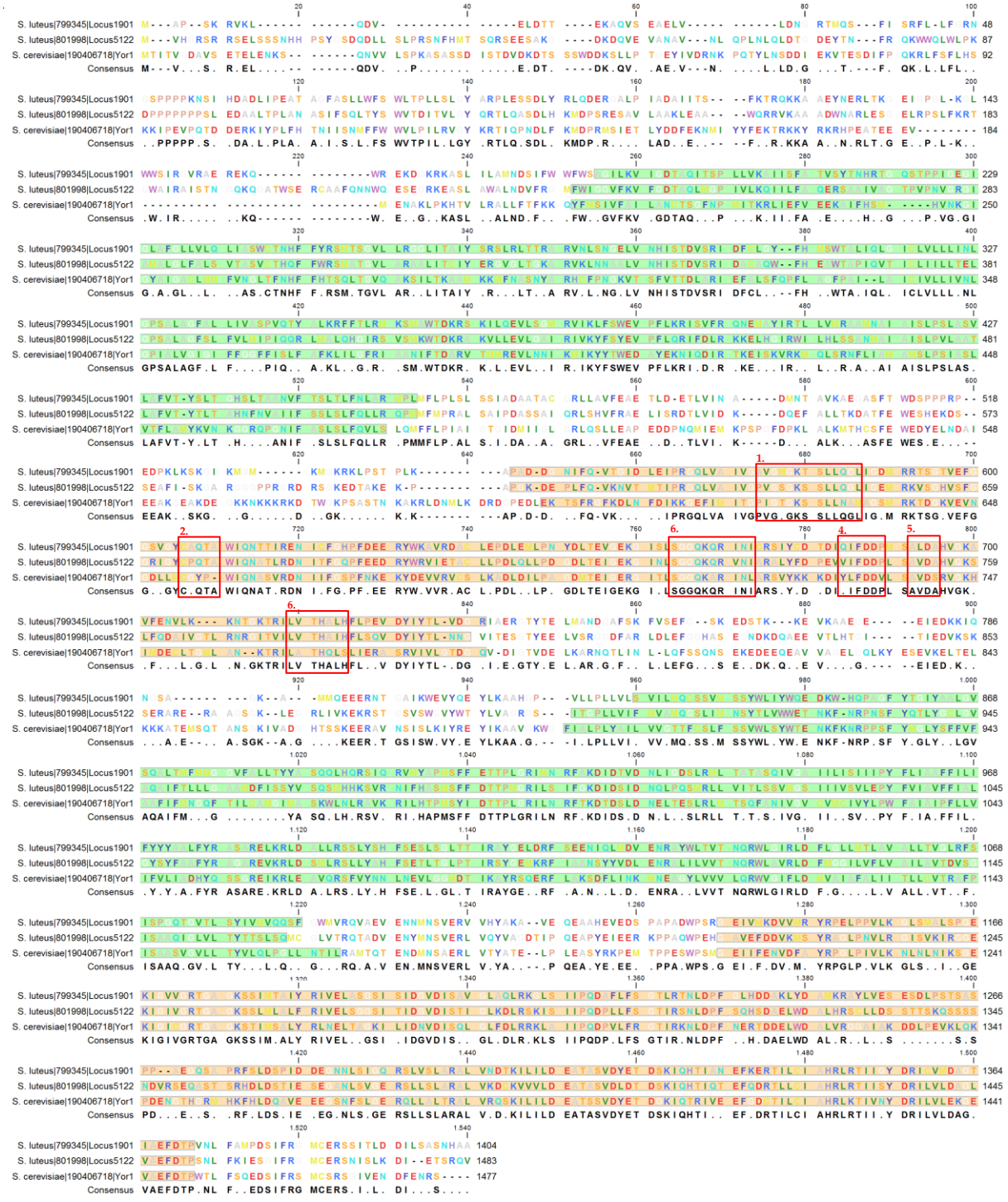

Fig. 24: Alignment between Yor1 of *S. cerevisiae* and the Yor1-like homologues in *S. luteus*. Residues are Rasmol coloured. In the sequences, the background of transmembrane regions are marked in green, the background of ATP-binding cassette domains in orange. Highly conserved domains are framed in red [29]

3.1.3 Discussion

Results of the phylogenetic analysis demonstrate that all species, with exclusion of *S. pombe*, possess one homologue of Ycf1 protein exactly. For Yor1, *S. pombe* had no homologue. Kovalchuk and Driessen report the multiple loss of ABC-A transporter genes in fungi [1]. It is possible that this is also the case here with its Yor1 homologue, which belongs to the ABC-C transporters. In contrast, most species possess several homologues of the Yor1 protein. As much as six homologues are counted in *P. tinctorius*. It probably concerns gene duplicates, or otherwise alternative splicing. These numbers of homologues correspond to the reports of Kovalchuk and Driessen [1]. *S. luteus* appeared to possess one Ycf homologue and two Yor1 homologues, *S. brevipes* one Ycf homologue and one Yor1 homologue.

The study of the conserved domains has shown that there are small variations between the sequences but also that the conserved domains are located in the same places. These variations are possible as long as the function of the region is preserved [30]. Only in the first ATP-binding cassette domain, conserved domains are found. Normally, conserved domains should also exist in the second ATP-binding cassette domain [31]. The Conserved Domain Database (CDD) based its search on certified protein data [24]. For the second ATP-binding cassette domain, possibly there is no matching matrix for the program to rely on.

The proteins SlYcf1 and SlYor1 showed high similarity to their homologues in *S. cerevisiae*. For *S. cerevisiae*, it is proven that the Ycf1 transporter is involved in the vacuolar sequestration of heavy metal ions [19] and the Yor1 transporter in efflux of heavy metal ions [2]. Probably, SlYcf1 and SlYor1 of *S. luteus* have the same function.

3.2 Functional test of *S. luteus* Ycf1-like protein (SlYcf1)

The cloning of the *SlYcf1* genes from the *S. luteus* strains LM8n1 and LS5n32 into the entry vector pCR8/GW/TOPO and afterwards in the expression vector pAG426GAL-ccdB had already been performed into the yeast mutant in a previous study in the lab. LM8n1 is a sensitive monokaryon, LS5n32 is a Cd tolerant monokaryon.

For the first drop assay (Fig. 15) of the yeast strain containing the pAG426GAL-ccdB *SlYcf1* constructs, SD -ura plates containing galactose and cadmium concentrations of 0, 10, 20, 30 and 40 µM were used. As control, SD -ura plates containing glucose with the same cadmium concentrations were utilized. On the plates, also a wild type *S. cerevisiae* strain (WT pAG) and the mutant yeast strain from Euroscraf (M pAG) was added. Both strains contain the expression vector, but no *SlYcf1* gene.

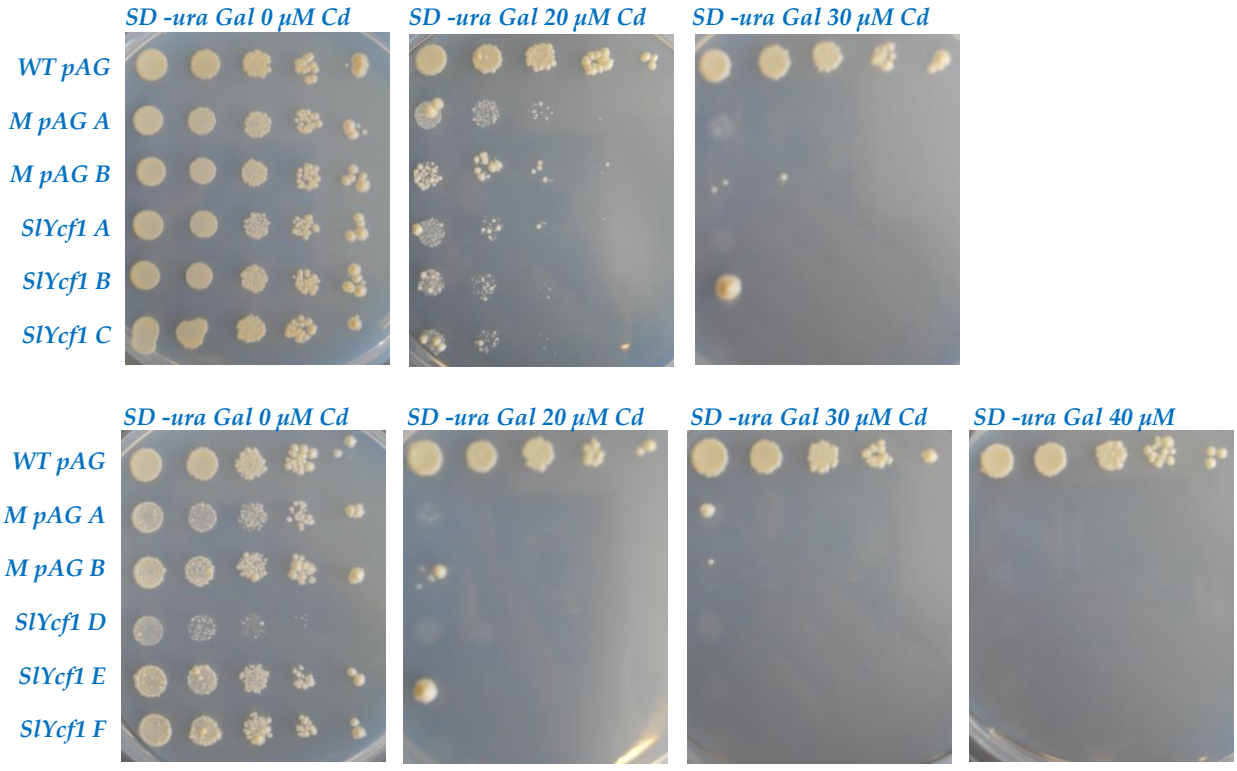

Fig. 35: Drop assay of Ycf1 pAG. Only the SD -ura containing galactose are shown

The plate SD -ura 40 µM Cd from the first series was contaminated with a white fluffy fungus, and is not shown. On the SD -ura plates containing cadmium, only a small quantity of cells were able to survive. Even on the plate with the lowest concentration of cadmium, 20 µM, the number of colonies was limited. On the plates with 30 µM Cd, only the mutant controls have shown any growth, in the lowest dilutions of culture (the first spot in the row of each sample). The larger colony of SlYcf1 B might have been contaminated.

For the functional test of pYES-DEST52 *SlYcf1*, a first drop assay was performed in order to estimate to which extent the yeast strain was rescued. Because the drop assay with pAG426GAL-ccdB *SlYcf1* only showed growth at the lowest cadmium concentrations on plates containing galactose, only concentrations of 0, 10, 20 and 30 µM Cd were used. On the plates with glucose, no reduced growth was observed, herefore also an SD -ura medium containing glucose with 50 µM Cd was used.

For this drop assay (Fig. 16), only one of pYES-DEST52 *SlYcf1* strains was used in duplicate.

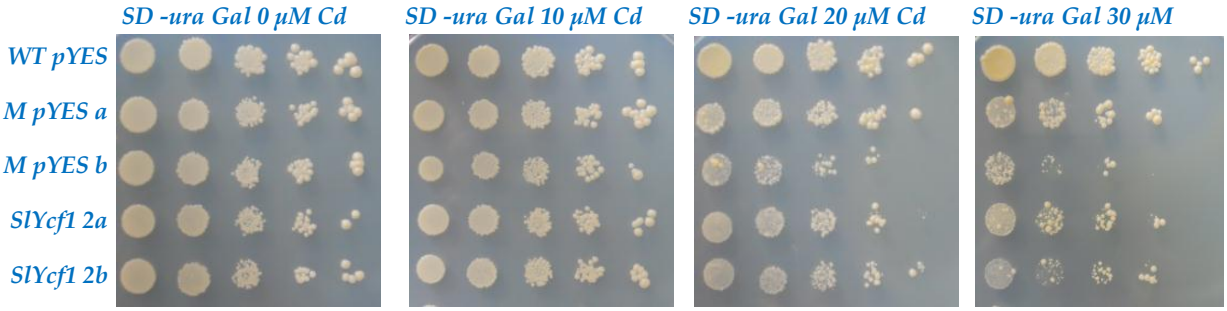

Fig. 46: First drop assay of pYES-DEST52 *SlYcf1*. Only the SD -ura containing galactose are shown

On the first drop assay with pYES-DEST52 *SlYcf1*, the strains were able to survive at higher cadmium concentrations. Reduced growth is observed upward from 20 µM Cd, in the highest dilutions of culture. On 30 µM cadmium, the highest dilution of culture has no growth. One of the mutant strains, M pYES a, did not show growth either on the fourth dilution of culture. On the SD -ura plates containing glucose, there was now reduced growth visible, only one mutant strain and one strain with the gene has grown on the first dilution of culture.

For a second drop assay with *S. cerevisiae* pYES-DEST52 *SlYcf1*, cadmium concentrations of 0, 10, 20, 30 and 50 µM Cd were used. Four different strains containing the pYES-DEST52 *SlYcf1*were plated out in duplicate.

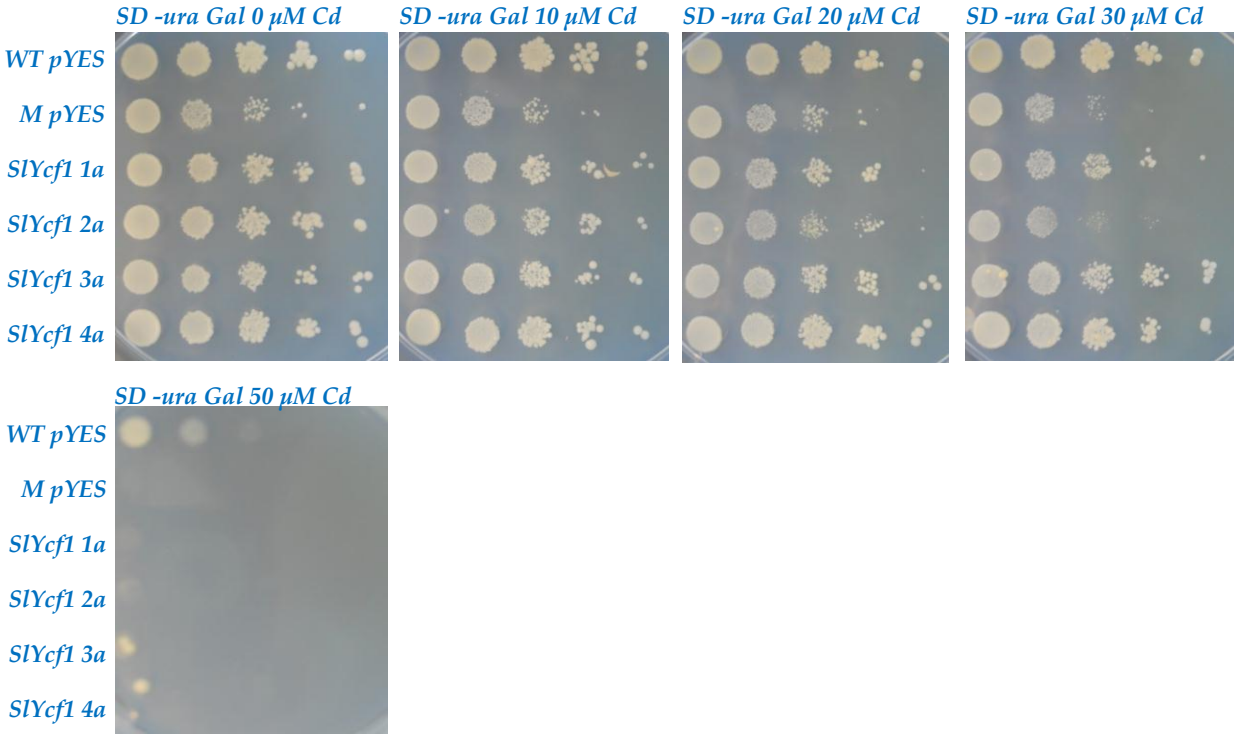

Fig. 57: One of the duplicate sets of the second drop assay of pYES-DEST52 *SlYcf1*

The strain *SlYcf1 2* showed markedly less growth than the other strains containing pYES-DEST52 *SlYcf1*. On 0, 10 and 20 µM Cd, strain *SlYcf1 2* grew equally but on 30 µM Cd, in the third and fourth dilution of culture there were fewer and smaller colonies present, and no growth on the fifth dilution of culture. In contrary, the other strains did grow up to the fifth dilution. For the strains SlYcf1 3 and SlYcf1 4, some colonies were even visible on the first dilution of 50 µM Cd. The mutant control strain showed less growth than the strains with the *SlYcf1* gene.

3.2.1 Discussion

The projected growth pattern in the drop assay would predict identical results for the different strains in plates without cadmium, while, in cadmium containing plates, growth of mutant controls would be prohibited in comparison with the strains with the *SlYcf1* gene. The wild type *S. cerevisiae* still contains its own *Ycf1* gene and should also be able to survive at higher cadmium concentrations.

On the drop assay with the expression vector pAG426GAL-ccdB, the strain *SlYcf1 D* is not growing equally to the other strains on the plates without cadmium. Probably a mistake was made while preparing the dilution. For the recombinant strains, most of them were able to grow on 20 µM Cd, *SlYcf1 A* and *SlYcf1B* even up to the third dilution of culture. *SlYcf1 D* and *SlYcf1F* are not rescued by the *SlYcf1* gene. On the plates containing cadmium, it was expected that the strains with the *SlYcf1* gene would be able to survive a higher cadmium concentration. However, the mutant strains grow even better. It is possible that the cells contain a low vector copy number, or that something went wrong in the sequence while cloning the *SlYcf1* gene [25], resulting in a disturbed functioning. Another explanation could be that a recombinant protein is toxic [32,33] for the cell, or that the host cell makes use of other folding proteins [34].

On the plates containing glucose, there was no significant difference between the plates with cadmium or without cadmium. It is difficult to make a comparison between the plates containing glucose and the plates containing galactose. The favoured carbon source for *S. cerevisiae* is glucose. Galactose is only used in the absence of glucose and requires induction. [35,36]

The pYES-DEST52 *SlYcf1* expression construct yields better results than the pAG426GAL-ccdB expression construct. The strains were capable of growing on higher concentrations of cadmium. The recombinant strains SlYcf1 1, SlYcf1 3 and SlYcf14 were possibly rescued by the *SlYcf1* gene. This cannot be stated for certain, taking into account that the mutant control shows slightly reduced growth also in the plates with 0 and 10 µM Cd, so for these strains further research is demanded. The SlYcf1 2 strains exhibit reduced survival at 20 and 30 µM cadmium and appear not to be rescued, but there is a chance that the pYES-DEST52 *SlYcf1* expression construct is present to a lesser extent in these strains.

4 Conclusion

Several ABC-C transporter subgroups are distinctly visible in the phylogenetic tree; ABC-C group VI, the Yor1 homologues, and ABC-C group VII, the Ycf1 homologues, among others. In *S. luteus*, one homologue of the Ycf1 protein and two homologues of the Yor1 protein were identified. These homologues are probably also involved in respectively heavy metal tolerance by vacuolar sequestration and efflux of heavy metal ions.

This presumption is supported by the research on conserved domains and the complementation test. Although minor variations occur in the conserved sequences, the same conserved domains are recognized by CDD, and they are localized in the same places in the sequences. The complementation test indicated that the *SlYcf1* gene might rescue the yeast mutants with the *Ycf1* gene knock-out, but further analysis of these strains is required.

The next stage in this research could be the repeating of the complementation test for the found Yor1 homologues. In addition, the *SlYcf1* gen could be transformed from the entry vector into another expression vector that provides the construct with a GFP tag in order to find the location of the proteins in the cell. The performing of these stages would form a suitable research project for a following master thesis.

This research lines up with other studies aimed on acquiring insight in heavy metal tolerance mechanisms. The Ycf1 and Yor1 proteins in the yeast *S. cerevisiae* are already known with certainty to have a mediating role. In this thesis, for the first time a start was made on the functional characterization of the Ycf1 and Yor1 homologues in another organism, *S. luteus*. In case evidence is obtained that these ABC transporters in *S. luteus* also are involved in heavy metal tolerance, it would be interesting to investigate the ABC genes and their regulation. Furthermore it will be possible to select/breed *S. luteus* with higher heavy metal tolerance to inoculate on pine trees specially for growing in polluted areas. The cloning and functional complementation techniques in the present investigation can also be applied to search for ABC transporters in other fungi or organisms.

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Annexes

List of annexes

Annex A: Culture media

Table 2: Composition Luria-Bertani (LB) broth medium

Component	Quantity
Tryptone	10 g/l
Yeast extract	5 g/l
Sodium Chloride (NaCl)	10 g/l
Agar	10 g/l
pH	7

Table 3 Composition YPD medium

Component	Quantity
Bactoyeast	10 g/l
Bactopeptone	20 g/l
D-glucose	20 g/l
Bactoagar	20 g/l
pH	5.6-5.8

Table 4: Composition SD -ura Gal medium

Component	Quantity
Yeast nitrogen base	1.7 g/l
$(NH_4)_2SO_4$	5 g/l
D-Galactose	20 g/l
SD -ura drop out mix	0.77 g/l
Agar	20 g/l
pH	5.6-5.8

Table 5: Composition SD -ura Glu medium

Component	quantity
Yeast nitrogen base	1.7 g/l
$(NH_4)_2SO_4$	5 g/l
D-Glucose	20 g/l
SD -ura drop out mix	0.77 g/l
Agar	20 g/l
pH	5.6-5.8

Annex B: Phylogenetic tree of ABC-C transporters

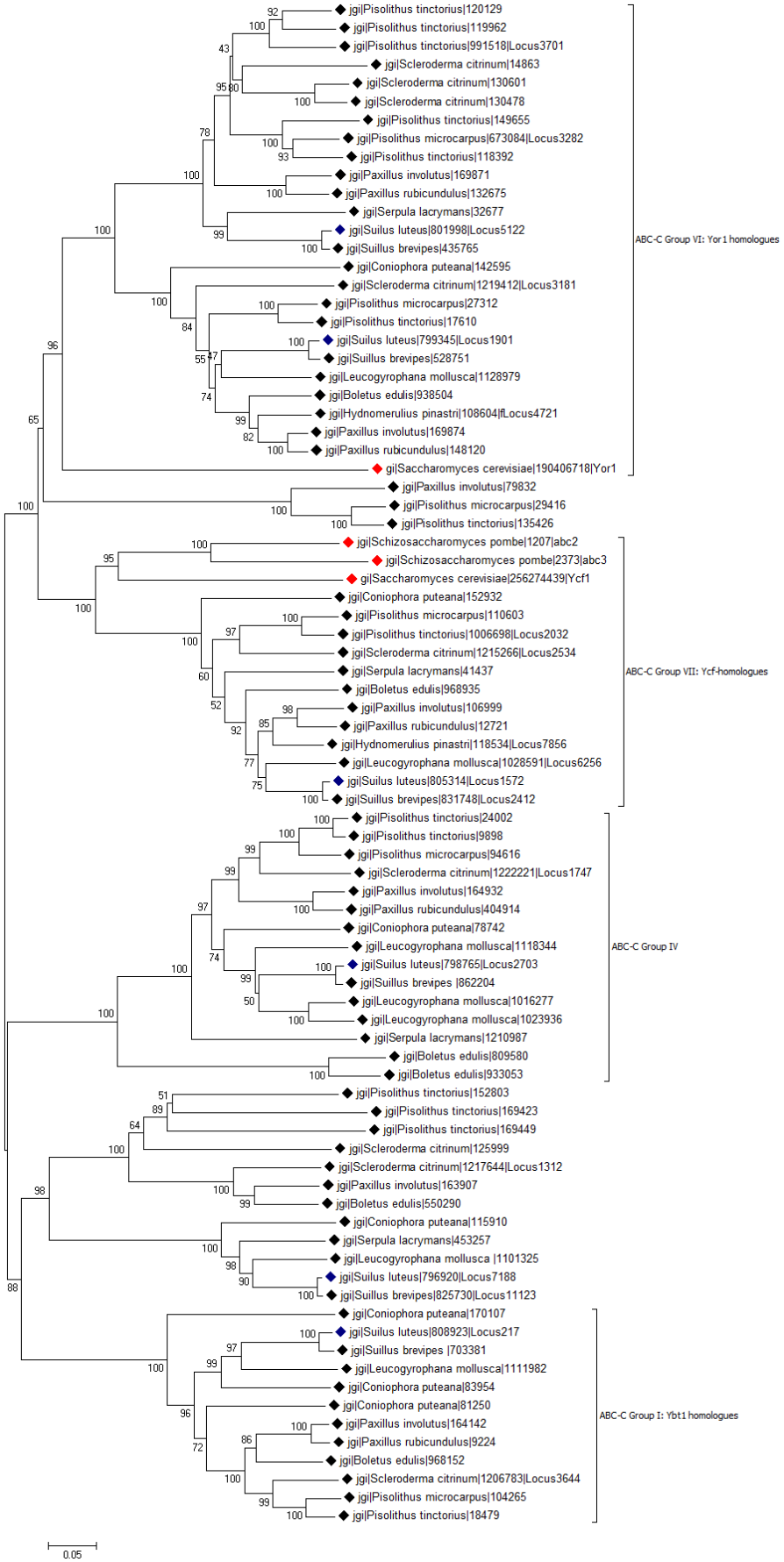

Fig. 18: Phylogenetic tree of ABC-C transporters, only Boletales by MEGA6

Annex C: Phylogenetic tree Ycf- and Yor Homologues

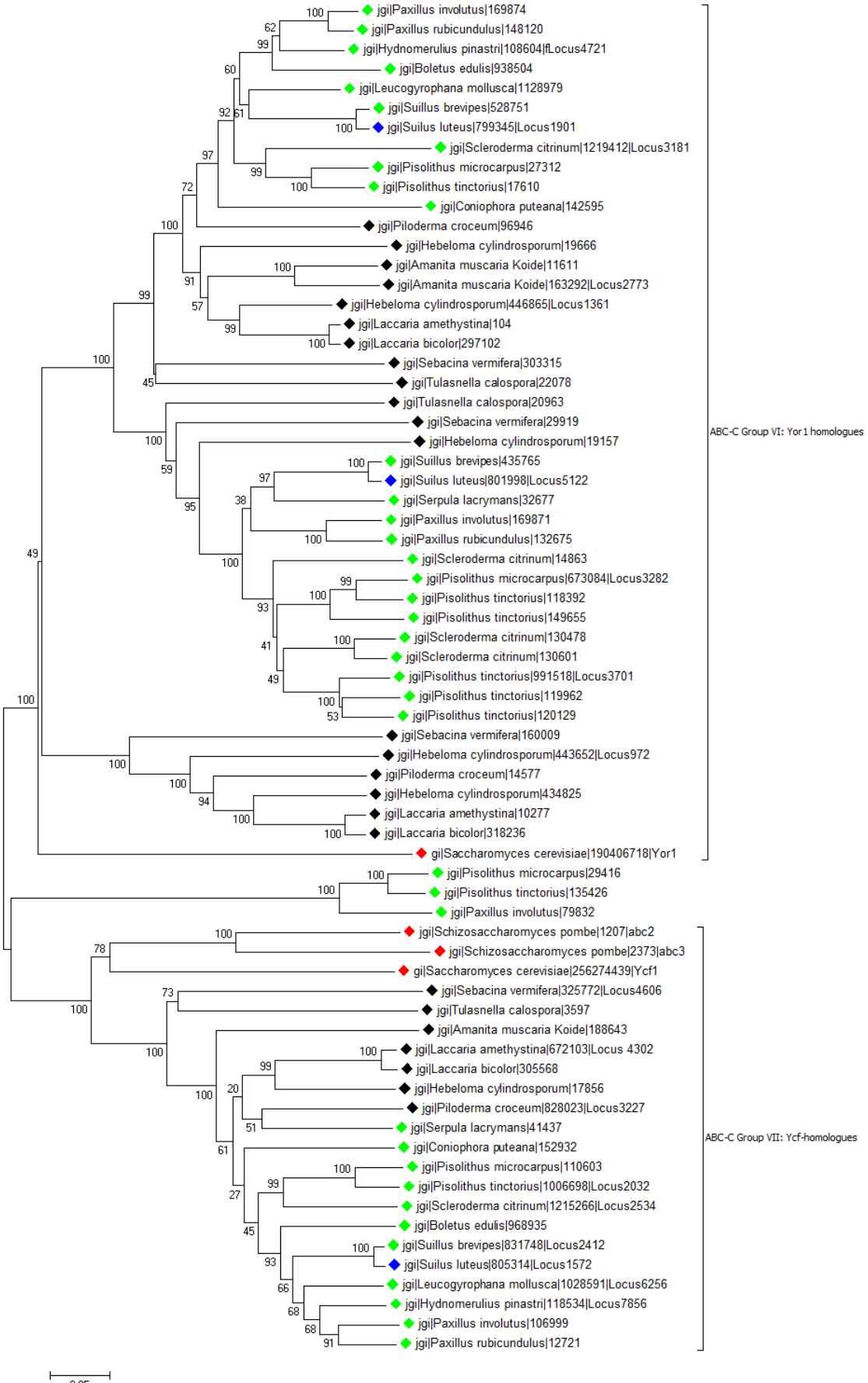

Fig. 69: Phylogenetic tree of all Yor1 and Ycf1 homologues by MEGA6

Annex D: Entry vector pCR®8/GW/TOPO®TA

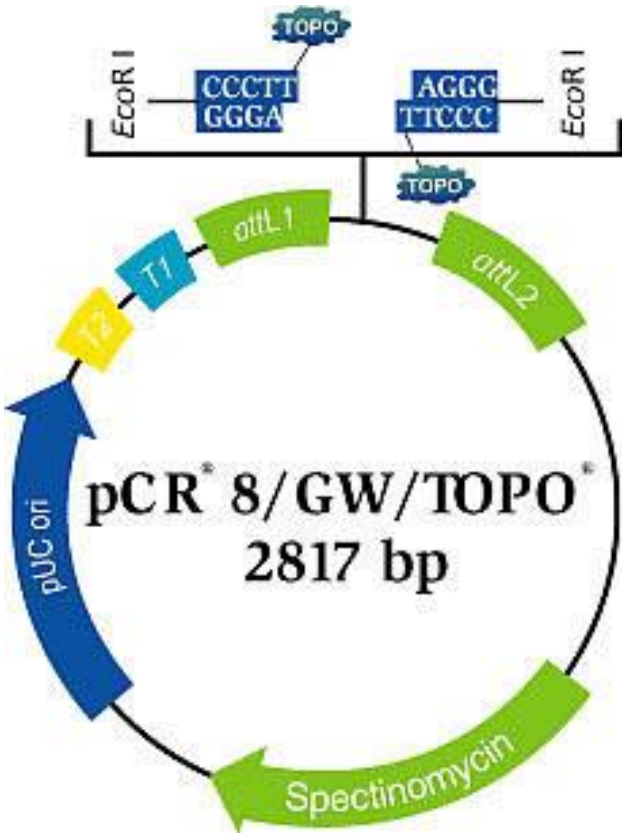

Fig.20: Entry vector pCR®8/GW/TOPO®TA of Invitrogen [37]

Annex E: Yeast Expression Vector Gateway® pAG426GAL-ccdB

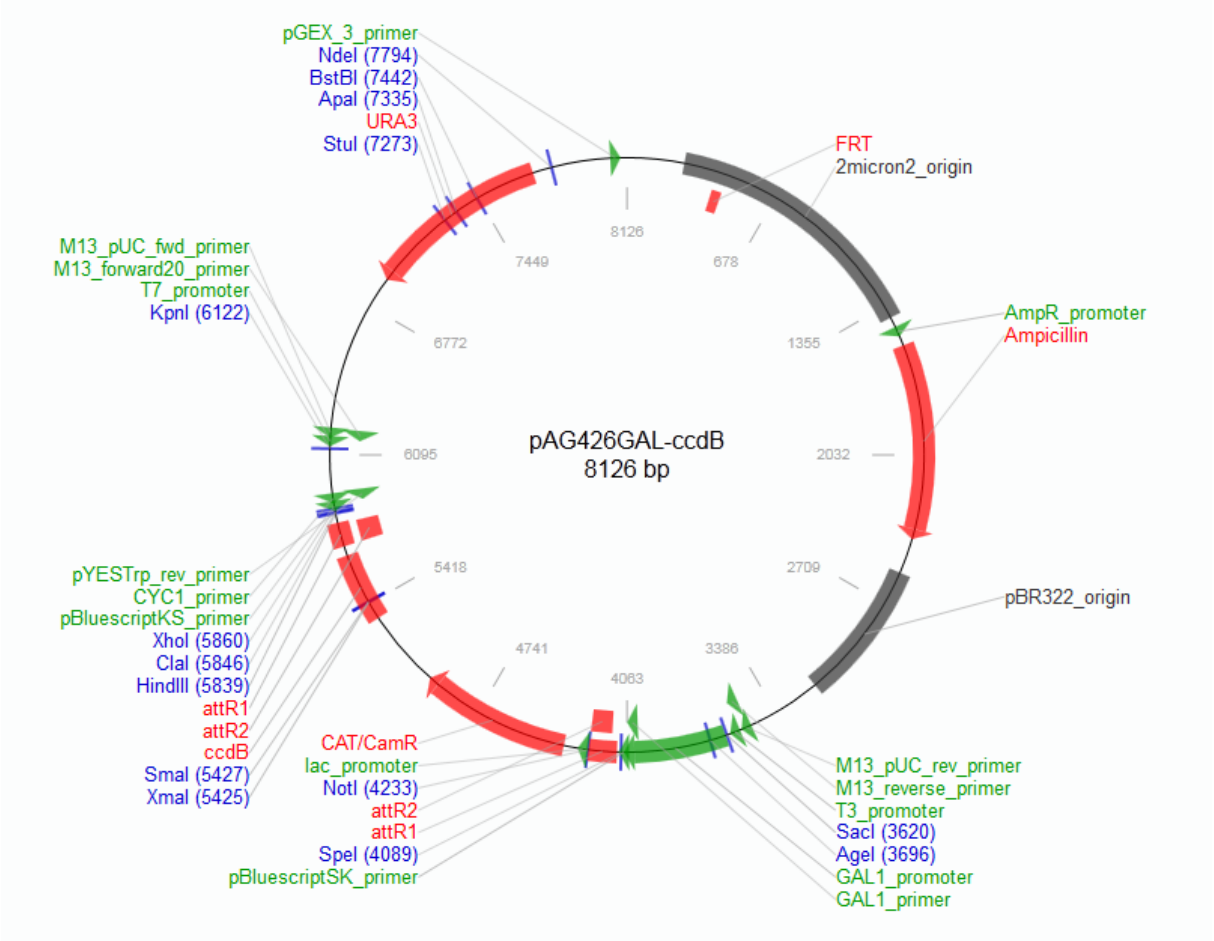

Fig. 21: Gateway® pAG426GAL-ccdB Vector [38]

Annex F: Yeast Expression Vector Gateway® pYES-DEST52

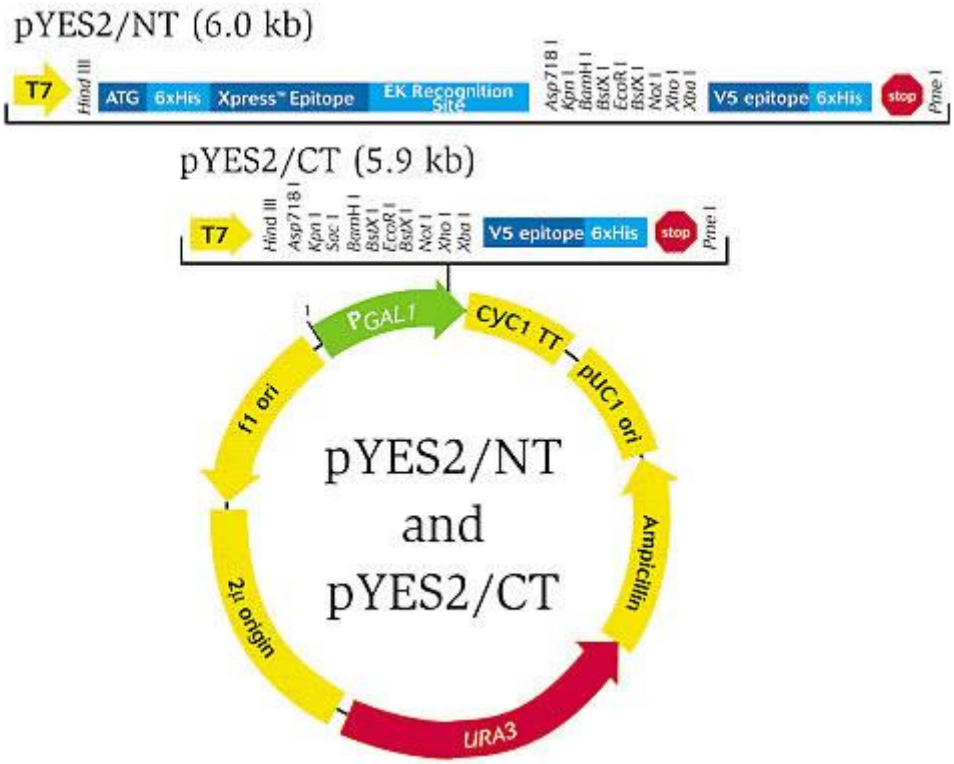

Fig. 22: Gateway® pYES-DEST52 Vector [39]

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