THE DIFFERENTIAL EXPRESSION OF CELL WALL PROTEINS IN THE HUMAN FUNGAL PATHOGEN *CANDIDA ALBICANS*

by

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Abstract

Candida albicans is a prevalent opportunistic fungal pathogen which can cause

serious diseases from deep mucosal infections to fatal systemic infection. The cell wall

of *C. albicans* is the initial point of interaction with the host during infection and it has

an internal polysaccharide layer and an external protein layer. The cell wall proteins are

mainly attached to the external layer through GPI anchors and have various functions

in nutrient capture, virulence and adhesion. Additionally, the cell wall has three copper-

only superoxide dismutases (SOD) SOD4, SOD5 and SOD6 that can protect the yeast

from oxidative damage. The rationale for why *C. albicans* has three unique extracellular

SOD enzymes was unknown. Our hypothesis is that each extracellular SOD is

expressed under unique environmental conditions, allowing the fungus to survive in

many different host niches. To begin to test this hypothesis, we analyzed the expression

of the three SODs in comparison to 42 other cell wall proteins by qRT-PCR under four

stress conditions differing in metal (iron and copper) content, glucose content and cell

morphologies. We found that *SOD5* is upregulated in the hyphal morphogenetic state

together with some hyphal-induced genes involved in cell adhesion and iron uptake

(*RBT5, HWP1, ALS3*, and *HYR1*). *SOD4* is specifically induced during iron starvation with heme-uptake genes *RBT5* and *CSA1*. The expression of *SOD6* is unique and only expressed in yeast-form cells and under iron replete conditions; *SOD6* is co-expressed with the adhesion molecule *RHD3*. The only common pattern with the three *SODs* is their induction by glucose starvation together with cell wall remodeling genes. In copper starvation, the expression of SODs does not change, although we observe for the first time the induction of heme-uptake genes *RBT5*, *CSA1* and *PGA10* with low copper. Overall the three extracellular SODs are induced under very different conditions. Each SOD may be expressed under distinct conditions to protect crucial cell wall proteins from oxidative damage and help *C. albicans* adapt to different environments in the host.

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1. Introduction

1.1 *Candida albicans* as an important fungal pathogen for public health

Candida albicans is a member of the Saccharomycetaceae family of yeast that is a

common commensal microbe of the flora in birds and mammals. It usually colonizes

on mucosal surfaces, such as oral cavity, gastrointestinal tract and urinogenital tract. *C.*

albicans is normally beneficial to the host by preventing other pathogenic fungal

growth and promoting host immune systems (Martins et al., 2014; McCullough et al.,

1996). However, this yeast is also a prevalent opportunistic fungal pathogen and can

cause serious diseases, especially in immunocompromised patients. In the United States,

Candida strains are ranked the fourth most common pathogenic organisms among

nosocomial bloodstream infections (Wisplinghoff et al., 2004). In addition, about 50-

90% of fungal infections are caused by *C. albicans* and the mortality of the patients is

around 30% (McCullough et al., 1996; Nieto et al., 2015). *C. albicans* can cause deep

mucosal infections, such as vaginitis in women and oral-pharyngeal thrush in AIDS

patients (Kim and Sudbery, 2011; Wachtler et al., 2012). The morbidity of systemic

fungal infections is increasing in immunocompromised patients, even in healthy

populations in the last two decades (Eggimann et al., 2003; Martins et al., 2014). In immunocompromised hosts with HIV, cancer, or organ transplantation or other immunosuppressive therapy, *C. albicans* causes severe bloodstream infections first, then infects the internal organs like kidney and liver. This fatal systemic infection has become increasingly prevalent worldwide and causes higher mortality rates in immunocompromised patients (Kim and Sudbery, 2011; Klis et al., 2009; Mavor et al., 2005).

Along with the antifungal-drug use, the antifungal-resistance strains of *C. albicans* has become a major problem around the world (White et al., 1998). There are mainly three clinical anti-fungal therapeutic drugs: polyenes, azoles and echinocandins (Odds et al., 2003). The drug resistance to polyenes is rare but use of this drug is limited due to inherent toxicity. Azoles target fungal sterol biosynthesis and echinocandins target cell wall integrity by inhibiting the synthesis of ß-1,3-glucan. Because azoles and echinocandins target the essential processes of *C. albicans*, the relevant drug resistance evolves rapidly (Cui et al., 2015; Pierce and Lopez-Ribot, 2013). Studies have shown that some *Candida* strains are less sensitive to the azole fluconazole which is a

worldwide therapeutic agent (Sanguinetti et al., 2015). One study shows that the drug-resistance *C. albicans* strains can cause severe oropharyngeal candidiasis in 33% of late-stage AIDS patients (Law et al., 1994). It is clear that better therapies and new antifungal agents are needed to accommodate the increasing rise in antifungal resistance.

C. albicans is a polymorphic yeast that exists in many different morphogenic states, mostly in yeast and filamentous hyphal or pseudo hyphal forms. Yeast cells normally form smooth, white ovoid-shaped colonies. These cells are smaller than hyphal cells and have the ability to disseminate within the bloodstream, and then spread to different host niches. Hyphal cells possess an apical body and septa with simple pores (Klis et al., 2009; Sudbery et al., 2004). It is widely considered that hyphal-form cells are invasive and able to penetrate epithelial cells and evade the phagocytic cells (Jacobsen et al., 2012; Sudbery et al., 2004). The switching between yeast and hyphal morphologies is one of the most necessary factors for the virulence of *C. albicans* (Gil-Bona et al., 2015; Mayer et al., 2013; Saville et al., 2003).

1.2 The cell wall of *Candida albicans*

It is very important for *C. albicans* to interact with the host during the infection.

Some of the most important molecules for host-pathogen interactions are on the surface

of the fungus. The fungal cell wall is the most external structure of *C. albicans*, and it

has several important functions, such as preventing mechanical damage and

maintaining the osmotic strength, as well as activities specific to survival in the host

including adhesion, invasion and nutrient acquisition. Maintaining the integrity and

function of the cell wall is essential for the pathogenic process in *C. albicans* (Gil-Bona

et al., 2015; Klis et al., 2011; McCullough et al., 1996). Therefore, it is important to

understand the functions of the fungal cell wall and cell wall proteins of *C. albicans*.

The cell wall of *C. albicans* is composed of complex polymers of glucose, chitin

and mannoproteins. It has two layers (Fig. 1). The external layer is a protein coat

comprising highly mannosylated glycoproteins and the internal layer is a

polysaccharide layer which consists of branched ß-1,6-glucan, ß-1,3-glucan and a small

number of chitin molecules (Chaffin, 2008; Kapteyn et al., 2000; Klis et al., 2009). This

inner carbohydrate layer of the cell wall provides substantial resistance to intracellular

hydrostatic pressure. Fungal cell wall proteins are mainly found in the external layer

and most are glycosylphosphatidylinositol (GPI) proteins (Fig. 1). The cell wall

proteins are linked by a truncated GPI-anchor to ß-1,6-glucan, which is covalently

linked to ß-1,3-glucan (Kapteyn et al., 2000; Klis et al., 2009).

1.3   The GPI-anchored cell wall proteins of *Candida albicans*

Cell wall proteins have various functions and are important for the virulence of *C.

albicans*. These proteins can protect the cell wall during infection by masking the ß-

glucan layer and preventing detection by the mammalian ß-glucan receptor dectin-1

(Gantner et al., 2005; Reiss et al., 1986; Wheeler and Fink, 2006; Zlotnik et al., 1984).

Some GPI-anchored cell wall proteins have been shown to contribute to coat-forming

and cell wall integrity. Other cell wall proteins are important for adhesion and invasion

of host cells, in capturing nutrients and in stress defense including oxidative stress

(Chaffin, 2008; de Groot et al., 2004; Klis et al., 2009; Klotz et al., 2004; Moreno-Ruiz

et al., 2009; Weissman and Kornitzer, 2004).

A wide number of proteomic studies have been conducted with *C. albicans* to

identify different classes of cell wall proteins. In the various studies that have been

conducted, anywhere from 18 to 48 distinct GPI-anchored proteins have been identified

in the cell wall depending on the technique used to extract the proteins or fungal growth conditions. By searching proteins in the *Candida* Genome Database and making a composite of the various analyses (Gil-Bona et al., 2017; Gil-Bona et al., 2015; Klis et al., 2011; Klis et al., 2009; Sorgo et al., 2010), we identified 45 *C. albicans* proteins most consistently reported as cell wall GPI-anchor polypeptides (Table 1). These various cell wall proteins are the focus of this thesis and can be grouped into specific classes as follows:

RBT5-like family: RBT5-like GPI-anchored proteins include RBT5, PGA10 and CSA1. Studies on RBT5, PGA10 and CSA1 have been showed that they are induced by iron starvation (Chen et al., 2011; Sorgo et al., 2013; Sosinska et al., 2008). These proteins have been proven to be heme receptors and function in iron acquisition. All of them together with plasma membrane PGA7 and secreted protein CSA2, have CFEM domains (common in fungal extracellular membranes) that consist of eight cysteines. Studies have shown that the CFEM proteins play important roles in extracting heme from hemoglobin as well as binding heme and delivering it from host to *C. albicans*. (Kulkarni et al., 2003; Kuznets et al., 2014; Nasser et al., 2016; Weissman and Kornitzer,

2004).

HYR family: This family includes a number of cell wall proteins that are closely related in amino acid sequence to HYR1 (hyphal regulated gene), including HYR3, HYR4, IFF3, IFF4, IFF5, IFF6 and IFF9. The N-terminal regions of all the family members contain predicted exo-α-sialidase domains (Bailey et al., 1996; Klis et al., 2009; Marchler-Bauer et al., 2011; Skrzypek et al., 2010). The function of the members of the HYR gene family is largely unknown.

ALS family: The ALS family has eight members, ALS1, ALS2, ALS3, ALS4, ALS5, ALS6, ALS7, ALS9. ALS proteins have similar three-domain structures and help *C. albicans* bind to various host proteins and peptides (Hoyer, 2001; Klotz et al., 2004). Previous studies have found that ALS1 and ALS3 have functions in adhesion and biofilm formation (Bastidas et al., 2009; Klotz et al., 2004; Nobile et al., 2006). ALS3, interestingly is involved in ferritin binding, and therefore may play an important role in iron acquisition from the host (Almeida et al., 2008).

IHD1-like family: There are six members in this family, IHD1, PGA15, PGA41, PGA42, PGA50, PGA61, and they have conserved seven-cysteine patterns (Skrzypek

et al., 2010). IHD1 is a hyphal-induced protein (Nantel et al., 2002). The functions of this family are unknown.

ECM33-like proteins: This class includes ECM331 and ECM33 that share similar amino acid sequence (de Groot et al., 2004; Mao et al., 2008). ECM33 plays a role in cell wall integrity and stress tolerance (Gil-Bona et al., 2016; Martinez-Lopez et al., 2006). Nothing is known about the function of ECM331.

RHD3-like family: This family includes RHD3, PGA30 and PGA31. Some studies showed that RHD3 is specific to the yeast/budding form of *C. albicans* and may have function in synthesis of cell wall mannan (de Boer et al., 2010). Other research only detected RHD3 in low pH medium, indicating that it is specific to acidic environments of the hosts (Sorgo et al., 2010). The functions of PGA30 and PGA31 are unknown.

PGA59-like proteins: PGA59 and PGA62 have similar three-cysteine containing domains and are believed to be coat-forming proteins which can increase the permeability of the cell wall by disulfide bonding (Moreno-Ruiz et al., 2009).

Carbohydrate metabolism: Some proteins on the cell wall are enzymes involved in carbohydrate metabolism. CRH11 and CRH12 are transglycosylases (Alberti-Segui et

al., 2004; de Groot et al., 2004), and CHT2 is a chitinase (McCreath et al., 1995). BGL2,

PGA4 and PGA5 are ß-1,3-glucanosyltransferases. PGA4 and PGA5 are fungal-

specific proteins which do not have homologues in humans (De Groot et al., 2003).

These proteins may be the potential drug targets for echinocandins. BGL2 is involved

in cell wall biosynthesis and plays roles in the fungal virulence (Sarthy et al., 1997).

UTR2 is a putative glycosidase and has function in cell wall integrity. Its mutants

showed the defect in adhesion and virulence of *C. albicans* (Alberti-Segui et al., 2004).

PHR1 and PHR2 are transglycosidases involved in ß-1,3-glucan modeling. Previous

research shows that PHR1 is induced in high pH and is not involved in vaginal infection

models, while PHR2 is low-pH induced and promotes virulence during vaginal

infection (De Bernardis et al., 1998; Popolo et al., 2017). These cell wall carbohydrate

enzymes may have functions in biofilms formation in the extracellular space (de Groot

et al., 2004; Klis et al., 2009).

SAP family: SAP9 and SAP10 are yapsin-like proteins. They are proposed to have

roles in adhesion and cell wall integrity (Albrecht et al., 2006; Naglik et al., 2003;

Skrzypek et al., 2010). These proteases can cleave cell wall proteins CHT2, YWP1,

ALS2, RHD3, RBT5, ECM33 and PGA4. SAP9 and SAP10 may have the function of

cell wall integrity by their proteolytic cleavage of GPI-anchored cell wall proteins

(Schild et al., 2011).

HWP1-like family: HWP stands for hyphal cell wall protein. HWP1 is hyphal-

associated protein and can help *C. albicans* adhere to the host epithelial surface (Nobile

et al., 2006; Staab et al., 1999). HWP1 has many cysteine residues. RBT1 and PGA8

are similar to HWP1 and studies show that HWP1 and PGA8 are required for adhesion

and biofilm formation (Younes et al., 2011).

Some of the GPI-anchored proteins cannot be classified in specific families, but

they also have some important functions. EAP1 can enhance the adhesion of *C.

albicans* to host epithelial cells (Li and Palecek, 2003), and on the other hand, YWP1

as a yeast-specific cell wall protein reduces the adhesion and promote dispersal of

yeast-form cells (Granger et al., 2005). PGA26 is involved in biofilm formation and

required for virulence (Laforet et al., 2011). SSR1 has similar CFEM domain as RBT5-

family members, but it does not have the function to uptake iron (Sorgo et al., 2013).

PGA6 has been found in hyphal tips but its detailed roles in hyphal formation are

unknown (Elson et al., 2009). The function of PGA45 is unknown.

Lastly, *C. albicans* expresses a unique class of superoxide dismutases SOD4, SOD5 and SOD6 that are extracellular and linked to the cell wall through GPI-anchors. The superoxide dismutases (SODs) are anti-oxidant enzymes that catalyze the disproportionation of superoxide anion to oxygen and hydrogen peroxide and act to protect cells from superoxide toxicity. Studies have shown that SODs are virulence factors during infection and can help *C. albicans* survive under the oxidative damages generated by macrophages and neutrophils (Broxton and Culotta, 2016; de Groot et al., 2004; Fradin et al., 2005; Martchenko et al., 2004). The three extracellular SODs (SOD4, SOD5, SOD6) in *C. albicans* are the members of a novel family of Cu-only SODs that are unique to the fungal kingdom (Gleason et al., 2014; Peterson et al., 2016; Robinett et al., 2017; Schatzman and Culotta, 2018).

It is not clear why *C. albicans* expresses three Cu-only SODs in the cell wall, but this apparent redundancy seems to be a reoccurring theme with cell wall proteins. As described above, virtually every category of cell wall protein consists of multiple members of closely related polypeptides. As one possibility, different members of each

protein family are expressed under distinct conditions in the host (e.g. PHR1 and PHR2

expressed under different pH conditions), and this may be the case for the cell wall Cu-

only SODs. For example, SOD5 has been shown to be induced only under hyphal

conditions, but that is not the case for SOD4 and SOD6 (Heilmann et al., 2011;

Martchenko et al., 2004). The Culotta lab has recently found that this induction of

SOD5 during morphogenesis serves to remove superoxide generated by the yeast itself.

C. albicans expresses a cell surface NADPH oxidase (NOX) enzyme known as FRE8

that produces a burst of extracellular superoxide during morphogenesis to the hyphal

state (Rossi et al., 2017). SOD5 converts FRE8 superoxide to hydrogen peroxide which

is then used to signal hyphal formation through a mechanism that is still unknown

(Rossi et al., 2017).

While SOD5 is hyphal-specific, less is known about SOD4, although some

proteomic studies suggested that SOD4 may be regulated by iron starvation (Klis et al.,

2011; Martchenko et al., 2004; Sorgo et al., 2013; Sosinska et al., 2008). In addition,

nothing is known about SOD6. Considering these three SODs are in the cell wall of *C.*

albicans, they may function to protect certain cell wall components from oxidative

stress during infection, but nothing is known about such processes for now. It is possible

that each of the three SODs is co-expressed with the molecules they protect or regulate

in the cell wall. As one approach, we could analyze the protein composition of the cell

wall under various stress conditions. However, extraction of protein from the cell wall

is extremely difficult and requires harsh conditions (e.g., boiling in acid) followed by

mass spectrometry analysis. Several attempts to define the cell wall proteome have been

made with variable results (Gil-Bona et al., 2017; Gil-Bona et al., 2015; Klis et al., 2011;

Klis et al., 2009; Sorgo et al., 2010). With the limitations in conducting proteomic

studies on cell wall components, we chose a transcriptome approach, where the mRNA

of defined cell wall proteins was analyzed by qRT-PCR. Therefore, the identification

of possible partners for the SODs through gene expression studies is the focus of the

work described here.

The goal of my thesis is to determine how the expression of GPI-anchored cell wall

proteins change under various growth and stress conditions as may be encountered in

the host. Most importantly, I sought to identify genes co-regulated with the cell wall

superoxide dismutases to gain possible insight into the distinct functions of SOD4,

SOD5 and SOD6. In my studies, I focused on four different stress conditions: metal starvation stress (Fe and Cu), glucose starvation stress and stress that may be associated with morphogenesis.

In this study, our hypothesis is that each extracellular SOD is expressed under unique environmental conditions, allowing the fungus to survive in many different host niches. We investigated the gene expression of 45 GPI-anchored cell wall proteins in four different stress conditions. We observed that the three extracellular SODs are induced in different conditions, i.e., *SOD4* is co-regulated with heme-uptake protein genes *RBT5* and *CSA1*. *SOD5* is induced in hyphal cells and co-expressed with iron uptake genes *RBT5*, *ALS3* and adhesion genes *HWP1*, *HYR1*. The expression of *SOD6* is similar to the adhesion molecule *RHD3*. All three *SODs* are induced by glucose starvation with cell wall remodeling genes *PHR1*, *PGA5*, *RHD3* and *PGA30*. In copper-starvation condition, the expressions of three *SODs* are not significantly changed, but the heme-uptake protein genes *RBT5*, *CSA1* and *PGA10* are highly induced when copper is limited. Overall, the expression of cell wall protein genes changes dramatically in response to different stress conditions as may be encountered in the host.

The three copper-only extracellular SODs are expressed differently under distinct conditions with their co-regulated cell wall proteins. They may have the function to protect the crucial cell wall proteins from oxidative damage, which can help *C. albicans* adapt to various environments in the host.

2. Materials and Methods

2.1 Yeast strains and culture conditions

C. albicans strains used in this study include SC5314, and the derivatives CA-IF100 (*arg4Δ/arg4Δ, leu2Δ/leu2Δ::cmLEU2, his1Δ/his1Δ::cdHIS1, URA3/ura3Δ*) and SN152 (*his1Δ/his1Δ, leu2Δ/leu2Δ, arg4Δ/arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434*). The SN152 isogenic *rbt5Δ/Δ*, *csa1Δ/Δ*, *pga10Δ/Δ* and *hyr1Δ/Δ* strains were obtained from the fungal genetic stock center (McCluskey et al., 2010; Noble et al., 2010).

In the studies of hyphal morphology, yeast cells were grown at 30°C in standard enriched medium containing 1% yeast extract, 2% peptone and 2% dextrose overnight for 16 hours to OD_{600} 1-3. Cells were then starved by incubating for 1 hour in water

and morphogenesis induced by incubating in Iscove's Modified Dulbecco's Medium

(IMDM; Quality Biological) at 37°C for 1 hour or 2 hours as described (Rossi et al.,

2017).

For glucose-starvation studies, *C. albicans* strains CA-IF100 were cultured at 30°C

in YP medium containing 1% yeast extract, 2% peptone and 5% glycerol overnight to

log phase. Then these cells were diluted and incubated at 30°C in YP medium with 2%

glucose or 5% glycerol for 2.5 hours as described (Broxton et al., 2018).

In the studies of iron starvation, *C. albicans* strains CA-IF100 were grown in YPD

medium or YPD with 100 µM, 125 µM or 150 µM ferrous iron chelator bathophenan-

throline disulfonic acid (BPS) overnight at 30°C to OD_{600} 1-3.

C. albicans strains CA-IF100 were used in the studies of copper starvation. The

control cultures were grown in SC (synthetic complete) medium containing 0.2% SC,

0.171% yeast nitrogen base, 0.5% ammonium sulfate, 0.2 mg/L $FeCl_3$, 10 µM $CuCl_2$

and 2% glucose. The cells in copper-starvation condition were grown in the same SC

medium with 500 µM of the Cu(I) chelator bathocuproinesulphonate (BCS). All the

cells were cultured overnight at 30°C to log phase.

For copper-uptake studies, cells were first cultured in YPD medium with 500 μM

BCS at 30°C overnight for 16 hours with a starting OD_{600} of 0.005 to stationary phase.

5-10 OD_{600} units of the cells were pelleted, washed once with 1 mL cold TE and then

frozen at -80°C or resuspended in 100 μL Fetal Bovine Serum (FBS; Sigma) at 30°C

for 1 hour. The FBS was thawed in room temperature, then aliquoted into 1.5 mL tubes.

One aliquot (1.5 mL tube) was used only once.

2.2 Biochemical analyses

For RNA analysis, *C. albicans* strains were grown in the cultures as described above.

RNA was obtained from at least 10 OD_{600} cell units by the acid phenol method as

previously described (Li et al., 2015). Then RNA was DNase treated using the

RapidOut DNA Removal Kit (ThermoFisher Scientific). cDNA was prepared from 1

μg of RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (ThermoFisher

Scientific). cDNA was subsequently diluted to 1: 10 and analyzed by qRT-PCR using

iTaq Universal SYBR Green Supermix (Bio-Rad) and values were normalized to *TUB2*.

The fold changes of gene expression were calculated using the ΔCT method. Amplicons

of ~190 bps were obtained using the primers as described in Table 2.

For copper uptake analysis, cell strains were grown in the cultures as described above and 5-10 OD_{600} cell units were washed twice with 1 mL cold TE, once with 1 mL cold MilliQ water. In the final water wash, the densities of cells were measured by OD_{600}. Cells were subsequently pelleted and resuspended in 500 µL of Optima grade nitric acid (Fisher) overnight at 95 °C. Samples were diluted to 2% nitric acid with 4.5 mL MilliQ water the following day and copper content was analyzed by atomic absorption spectroscopy (AAS) performed on an AAnalyst600 graphite furnace atomic absorption spectrometer (PerkinElmer). The copper content was normalized to cell number.

Heat maps shown the grouped data were made by Graphpad Prism 7. Data shown in this study were statistically significantly different for $p \leq 0.05$ using two-tailed paired t-test by Graphpad Prism 7.

3. Results and Discussion

3.1 Cell wall genes induced during hyphal formation

The switching from yeast to hyphae is essential for pathogenesis in *C. albicans*.

There are many ways to induce yeast-to-hyphae transition in laboratory cultures. Previous research has shown that some of the conditions, such as animal serum, induce hyphal formation only transiently. By comparison, inducing hyphal formation by high amino acids with IMDM medium can stably induce hyphae (Heilmann et al., 2011; Rossi et al., 2017). Therefore, we chose to obtain *C. albicans* hyphal cells by growth in IMDM medium.

C. albicans cells can be maintained in the budding yeast form by growth in YPD medium at 30°C. In order to get hyphal cells, the yeast cells were incubated in IMDM medium at 37°C for 1 hour or 2 hours. As in Fig. 2, cells which were incubated in IMDM for 2 hours showed longer germ tubes and clear elongating hyphae compared to cells stimulated with IMDM for 1 hour. To investigate if these conditions were sufficient to induce hyphal-specific genes, we analyzed the expression of *HWP1*, *HYP1*, *ALS3* and *SOD5*, genes previously shown to be induced during morphogenesis to hyphae (Heilmann et al., 2011; Martchenko et al., 2004). As in Fig. 3, gene expression tested by qRT-PCR shows that hyphae-associated genes for cell wall proteins HWP1, HYR1, ALS3 and SOD5 are induced in both 1-hour and 2-hour hyphal conditions, and

the fold changes of the gene expression are similar in both conditions. Based on these experimental results, 2-hour hyphal-induced conditions were chosen to analyze the gene expression patterns for cell wall proteins as measured by qRT-PCR.

The analysis of 45 cell wall protein genes is shown in Fig. 4, organized from the most strongly induced in hyphal conditions (top) to most strongly induced in yeast/budding form (bottom). HWP1 is a known hyphal wall protein and YWP1 is an established yeast wall protein (Granger et al., 2005; Staab et al., 1999). In this experiment, *HWP1* is the most strongly induced in hyphal state (2500 fold) and *YWP1* is mostly highly expressed in yeast cells, validating our approach for examining gene expression changes as a function of morphologic state (Fig. 4). As predicted (Heilmann et al., 2011) *SOD5* is co-induced in hyphal form with some other hyphal-specific genes. *SOD4* does not show significant changes in hyphae versus yeast cells and the expression of *SOD6* is greatly favored in the yeast state.

SOD5 is among the most highly induced cell wall protein gene in the hyphal state, together with genes *HWP1, RBT5, ALS3, IHD1* and *RBT1*. The second tier of hyphal specific genes include *HYR1* and *ECM331* while *PHR1 and PGA30* are marginally

induced by comparison (Fig. 4).

Of the genes most strongly induced during hyphal formation, *HWP1* and *RBT1* are part of the same family of cysteine rich proteins that function in adherence (Nobile et al., 2006; Staab et al., 1999), while the remaining genes (*RBT5*, *ALS3*, *IHD1*, *HYR1* and *ECM331*) are all separate classes of cell wall proteins, each with different functions. IHD1 is a hyphal specific cysteine rich protein of unknown function (Nantel et al., 2002; Skrzypek et al., 2010); HYR1 has predicted sialidase domains but does not show the sialidase activities (Klis et al., 2011; Roggentin et al., 1999), and ECM331 is a member of the ECM family of cell wall proteins of unknown function (de Groot et al., 2004; Mao et al., 2008). Interestingly, two of the cell wall protein genes induced during hyphal transition are involved in iron capture in the host. ALS3 is a ferritin receptor and research has shown that it is important for binding iron during hyphal transition (Almeida et al., 2008). RBT5 is a heme-binding protein, needed for uptake of heme as a nutrient source of iron (Kulkarni et al., 2003; Weissman and Kornitzer, 2004). Curiously however, RBT5 is reported to work together with CSA1 and PGA10 in heme uptake (Kuznets et al., 2014; Nasser et al., 2016), but neither is co-induced with *RBT5*

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during hyphal transition (Fig. 4). It is possible that RBT5 functions alone in heme

uptake in hyphal cells or has another function in hyphae.

As previously mentioned, the three extracellular SODs are expressed at different

morphogenetic states. SOD4 is expressed in both budding and hyphal conditions with

only a small (≈2 fold) preference in hyphae (Fig. 4). It seems that the main function of

SOD4 is not related to different morphologies. Nothing is known about the function of

SOD6 and this study shows that SOD6 is strongly induced in yeast cells over hyphae

(Fig. 4). Interestingly *SOD6* is co-expressed with *PGA8*, an adhesion that is closely

related to the hyphal-specific gene *HWP1* co-expressed with *SOD5* (Fig. 4) (Younes et

al., 2011). The significance of this co-expression of *SOD5* and *SOD6* with closely

related adhesion protein genes awaits future investigations. As described in the

Introduction, SOD5 serves to remove extracellular superoxide generated by the *C.*

albicans fungus during hyphal formation (Rossi et al., 2017). Considering the hyphal-

induced proteins such as RBT5 and ALS3 play important roles in nutrient capture and

virulence in *C. albicans*, SOD5 may be involved in protecting these proteins from the

superoxide generated by the host or *C. albicans* itself. Further studies are required to

understand the functions of these three SODs in different fungal morphologies.

3.2 Cell wall genes induced during glucose starvation

Glucose is a favorable carbon source for *C. albicans*. Since *C. albicans* can colonize in different niches in the host, this yeast has complicated mechanisms of glucose utilization to adapt to low or high glucose environment (Sabina and Brown, 2009). Compared to the medium with 2% glucose, which is usually used to grow *C. albicans in vitro*, glucose can be limiting for *C. albicans* in the host. For example, the concentration of glucose in the bloodstream is around 0.1%, and vaginal environment contains about 0.5% glucose (Barelle et al., 2006; Owen and Katz, 1999). When glucose is absent, *C. albicans* can grow with other carbon sources like glycerol (Childers et al., 2016). During glucose starvation, several studies have shown that some glucose transporters like *HGT17* will be induced, while other glucose transporters like *HGT7* will be repressed (Brown et al., 2006; Broxton et al., 2018). However, very little is known about how cell wall proteins change under glucose-starvation condition.

For these studies, we created a glucose-starvation state by growing cells in the non-fermentable carbon source glycerol as has been previously done (Broxton et al., 2018).

In the experiment of Fig. 5, cells were starved for glucose by growth overnight in glycerol, followed by 2.5-hour growth in either glucose or glycerol. To validate that these conditions were appropriate for monitoring glucose-dependent gene expression, we examined the expression of the glucose transport genes *HGT7* and *HGT17*. As published previously (Broxton et al., 2018), in glycerol condition, *HGT7* is repressed while *HGT17* is induced (Fig. 5).

These same conditions were then used to examine the gene expression profile of the 45 cell wall protein genes. Fig. 6 shows that all three SODs (SOD4, SOD5, SOD6) are induced by glucose starvation, and *SOD5* is the most abundantly induced gene in the glucose-starvation condition with nearly 20-fold induction. The glucose starvation induction of *SOD4* and *SOD6* are about 4 fold. *RBT5* is also significantly induced under glucose starvation with 14 fold. The gene expression changes of *PHR1*, *PGA5*, *RHD3*, *PGA30* and *CHT2* in glucose starvation are 3-10 fold.

In high glucose conditions, *PGA10*, *PGA45* and *ALS9* are significantly induced. PGA10 is a heme receptor and the functions about these glucose-induced genes in glucose regulation are unknown. These glucose-induced genes *PGA10*, *PGA45* and

ALS9 are also mainly in yeast cells versus hyphae, which corresponds to the finding that the concentration of glucose is essential for the pathogenesis and high level of glucose will repress the yeast-to-hyphae transition (Maidan et al., 2005).

Some research has shown that in no or low-glucose conditions, the cell wall mass, architecture and elasticity change dramatically, which influences the stress resistance and virulence of *C. albicans* (Ene et al., 2012; Ene et al., 2015). In response to no-glucose stress, *C. albicans* induces the masking of ß-glucan which contributes to reduction of host immune responses (Ballou et al., 2016; Hopke et al., 2018). Consistent with these effects of low glucose on cell wall sugars, we observed some interesting changes in cell wall carbohydrate metabolism genes. PHR1 is involved in ß-1,3-glucan modeling as described in the *Introduction* (De Bernardis et al., 1998) and we find the gene is highly induced in glycerol condition which correlates with its function in cell wall remodeling. PHR1 may play crucial roles in cell wall integrity and help *C. albicans* adapt to different carbon sources during infection. PGA5 is ß-1,3-glucanosyltransferase and CHT2 is chitinase (De Groot et al., 2003; McCreath et al., 1995). They may also be involved in cell wall remodeling. RHD3 has been shown to involve in the synthesis

of cell wall mannan (de Boer et al., 2010), and PGA30 is in the same family as RHD3. Altogether, glucose starvation leads to induction of several cell wall remodeling genes including PHR1, PGA5, CHT2, RHD3 and PGA30. Although, not directly involved in sugar metabolism, RBT5 is a heme receptor and since respiration requires iron, it may be also involved in glucose regulation. However, as is the case with hyphae-induced genes (Fig. 4), other heme-binding proteins encoding genes *CSA1* and *PGA10* are not induced in glycerol condition, which suggesting that RBT5 may have other important roles in glucose regulation.

Perhaps the most interesting finding with glucose starvation is the induction of all three cell wall SODs. The function of the extracellular SODs (SOD4, SOD5, SOD6) in glucose metabolism is unknown. As one possibility, the SODs may protect cell wall proteins, especially the proteins induced by glucose starvation such as PHR1that are vulnerable to oxidant stress and help *C. albicans* to use different carbon sources other than glucose.

3.3 Cell wall genes induced during iron starvation

Iron is an essential nutrient and plays important roles in biological processes

because of its reduction potential. Host usually limits the availability of iron from the pathogens to prevent the infection, which is termed nutritional immunity (Kehl-Fie and Skaar, 2010). In humans, about 70% of the iron is bound to hemoglobin and the iron in circulation is bound to transferrin (Andrews, 2000). Preliminary studies by Sabrina Schatzman in the lab and other published studies suggest that *SOD4* is induced under iron-starvation condition together with some genes for iron-uptake proteins, such as *RBT5*, *CSA1*, *PGA7* in *C. albicans* (Kulkarni et al., 2003; Kuznets et al., 2014; Nasser et al., 2016; Weissman and Kornitzer, 2004). However, a comparison to all cell wall proteins of *C. albicans* between iron-sufficient and iron-starvation conditions has not been analyzed.

In order to create a condition which is sufficient to induce an iron-starvation response but not inhibits the yeast growth, we first determined a concentration of iron chelator BPS (bathophenanthroline disulfonic acid) in YPD medium, then used this condition to analyze how cell wall protein genes respond when iron is absent. Cells were grown in YPD medium with 100 µM, 125 µM or 150 µM BPS overnight. As seen in Fig. 7A, the growth of cells was not inhibited within 100 µM BPS medium compared

to the medium with 125 µM and 150 µM BPS. In addition, Fig. 7B shows that even though 100 µM BPS is not so toxic to yeast cells as 125 µM and 150 µM BPS, it still allows for good induction of *RBT5*, *CSA1*, *PGA7* and *SOD4*, the genes which have been proved previously to be induced by iron starvation (Chen et al., 2011; Sorgo et al., 2013; Sosinska et al., 2008). Therefore, YPD medium with 100 µM BPS is a good condition to create an iron-starvation environment for *C. albicans*. The following experiment used this condition to analyze the gene expression patterns of the 45 cell wall proteins.

As shown in Fig. 8, the most abundantly induced gene is *RBT5* with nearly a 3000-fold induction. *SOD4* and *CSA1* are also strongly induced by iron starvation with roughly 65 fold and 25 fold respectively. Compared to these genes, *SOD5* is only slightly induced by 4 fold. Opposite to *SOD4* and *SOD5*, the expression of *SOD6* is decreased with iron starvation.

As described in the *Introduction*, much research has shown that RBT5, CSA1 and PGA10 are involved in heme uptake and levels of the proteins in the cell wall are increased when iron is limited (Sorgo et al., 2013; Sosinska et al., 2008). In this study, the level of *RBT5* and *CSA1* mRNA are induced by iron starvation, but the mRNA

expression of *PGA10* does not show an obvious change in the iron-limited medium.

One possibility is that PGA10 is induced on the translational or post-translational level instead of transcriptional level. The extracellular SODs are expressed differently in the iron-starvation condition. SOD4 is co-expressed with the heme-uptake proteins RBT5 and CSA1, which suggest that SOD4 may be involved in heme uptake and may function to protect these heme-binding proteins. The role may be similar to SOD5 under hyphal conditions since *SOD5* is co-expressed with *RBT5* (heme) and *ALS3* (ferritin) receptors for iron uptake (see above Fig. 4). SOD6 seems to play more important roles when iron is replete.

3.4 Cell wall genes induced during copper starvation

Copper is usually potentially toxic to the pathogens (Freinbichler et al., 2011; Halliwell et al., 1985; Macomber and Imlay, 2009). However, on the other hand, it is also an essential transition metal and plays an important role as co-factor in copper-dependent enzymes, such as multi-copper oxidases and superoxide dismutase (SOD) (Cheng et al., 2013; Crichton and Pierre, 2001; Hwang et al., 2002). Previous studies have shown that in the host kidney, copper level rises at first and then decreases in the

later stages of infection with *C. albicans* (Li et al., 2015). *C. albicans* can adapt to this

copper variation in the host by altering the expression of intracellular SOD enzymes

(SOD1, SOD3). SOD1 is a copper/zinc containing cytosolic SOD that is repressed

during copper starvation, while SOD3 is a manganese containing SOD induced with

copper starvation (Besold et al., 2016; Bordo et al., 1994; Culotta et al., 2006; Hwang

et al., 2003; Lamarre et al., 2001; Li et al., 2015; Lindenau et al., 2000). Copper-

starvation conditions can be induced in laboratory cultures by treatment with the copper

chelator BCS (bathocuproinedisulfonic acid), and *C. albicans* responds to this stress by

repressing copper/zinc SOD1 and inducing non-copper SOD3 (Broxton and Culotta,

2016; Li et al., 2015). However, nothing is known about the cell wall proteins including

extracellular SODs (SOD4, SOD5, SOD6) with copper-starvation stress.

In order to create copper starved condition, yeast cells were grown in SC medium

or SC medium with 500 µM BCS. As seen in Fig. 9, in the SC medium with 500 µM

BCS, *SOD3* is induced and *SOD1* is repressed as previously published studies (Broxton

and Culotta, 2016; Li et al., 2015), which validated that these conditions were

appropriate for monitoring the gene expression with copper starvation.

The gene expression of the 45 cell wall proteins was examined in the same conditions and the results were shown in Fig. 10. Unlike the intracellular SODs, the expressions of extracellular copper-only SODs are not significantly changed by copper starvation (Fig. 10). One might expect the copper containing extracellular SODs to be repressed by copper starvation, to spare copper for other copper-requiring enzymes, as has been shown for the repression of intracellular SOD1 with copper starvation (Broxton and Culotta, 2016; Li et al., 2015). However, studies in the Culotta lab have shown that the extracellular copper-only SODs can acquire the metal directly from the extracellular environment (Gleason et al., 2014; Robinett et al., 2017) and thus may not compete with other copper enzymes for intracellular copper. As another possibility, the RNA of these extracellular SODs may be normal in copper-limited conditions, but the enzymatic activities of these SODs are defective because they cannot acquire their copper co-factor. Further research is needed to detect the enzyme activity of SODs in copper-starvation condition.

Although SOD4, SOD5 and SOD6 mRNA does not change with copper starvation, there are a number of other cell wall protein genes induced with low copper. For

example, the heme receptor *RBT5* is the most strongly induced gene with greater that

500-fold induction and the hyphal specific adhesion protein *HWP1* is also strongly

induced with roughly 60 fold. The expressions levels of *CSA1, PGA10, ALS3, ALS9,*

SAP10, IFF5, HYR1, HYR4, and *RBT1* are slightly induced with 2-8 fold. These classes

of cell wall proteins and their possible role in metal homeostasis are discussed as

follows:

RBT5, CSA1 and PGA10 are known as heme receptors that are induced by iron

starvation. My studies show this heme-uptake pathway is also induced by copper

starvation (Fig. 10). There are at least four pathways for iron uptake in *C. albicans*,

including heme uptake, siderophore uptake, ferritin iron uptake and ionic iron uptake,

also known as reductive iron uptake (Bairwa et al., 2017; Heymann et al., 2002; Ismail

et al., 1985; Knight et al., 2005; Knight et al., 2002; Weissman et al., 2008). In *C.*

albicans, multicopper ferroxidases are important in reductive iron-uptake pathway.

These multicopper ferroxidases oxidize ferrous iron to ferric form which then is

transported into the cells. When copper is limited, multicopper ferroxidases have

dysfunctional activities, which disrupts the iron acquisition through the reductive

pathway in *C. albicans* (Bairwa et al., 2017; Cheng et al., 2013; Prohaska, 2011). The induction of the heme-uptake pathway (RBT5, CSA1 and PGA10) may compensate for loss of the copper requiring iron-uptake system. As mentioned above (Fig. 8), *SOD4* has been shown to be induced with heme-uptake system in iron starvation. However, in copper-starvation condition, SOD4 is not co-regulated with heme-uptake proteins. One possibility is that SOD4 cannot maintain its function because it cannot bind enough copper.

Interestingly, uptake of iron from ferritin also requires reductive pathway and in copper starvation, the reductive pathway is defective (Cheng et al., 2013; Dancis 1994; Eck et al., 1999; Prohaska, 2011). ALS3 is a ferritin receptor (Almeida et al., 2008) and the same may be true of the closely related ALS9 protein. Considering that the reductive pathway is defective when copper is limited, ALS3 and ALS9 may be induced in copper-starvation condition to compensate.

HWP1 is a hyphal-specific protein, but in this experiment, *HWP1* is significantly induced in budding cells by copper starvation. *RBT1* is similar to *HWP1* and it is also slightly induced. The rational for inducing *HWP1* and *RBT1* with copper starvation is

not known. As one possibility, copper starvation may trigger yeast-to-hyphae transition, and budding cells in copper-limited condition have the potential tendency to transfer to hyphal cells.

Additional cell wall proteins induced by copper starvation include the HYR-family members IFF5, HYR1 and HYR4. The function of these proteins are not known and it is difficult to predict their connection to copper uptake.

Lastly, SAP10 is a yapsin-like protein in the cell wall (Albrecht et al., 2006) and we find that *SAP10* mRNA is induced by copper starvation. This protease may be induced to somehow increase copper or iron availability. As described below, the source of nutrient copper for *C. albicans* is not known, but it may be one or more globular copper binding proteins in host serum. Proteolytic cleavage of serum copper or iron proteins could increase metal bioavailability for the fungus.

3.5 Cell wall proteins and copper uptake from serum

Much research has described the iron receptors of *C. albicans* and how this yeast uptakes iron from serum (Kuznets et al., 2014; Nasser et al., 2016; Weissman and Kornitzer, 2004). However, nothing is known about copper-uptake receptors of *C.*

albicans when the cells grow in serum. Much of the copper in the serum is bound to globular proteins including ceruloplasmin, albumin and transcuprein (Linder, 2016). Previous study in the Culotta lab has shown that *C. albicans* can use serum as a copper source and accumulate much more copper in the serum compared to YPD medium (Besold et al., 2017). In order to seek the potential copper-uptake receptors that work in the serum, we tested four cell wall proteins which are induced by copper starvation. The heme receptors *RBT5*, *CSA1* and *PGA10* are strongly induced by copper and iron starvation, and *HYR1* is induced by copper starvation. The homozygous deletion mutants to all four strains are currently available and were tested for copper uptake from serum.

The wild type and mutant strains were grown overnight under copper starvation conditions and then incubated for 1 hour with fetal bovine serum as described in *Materials and Methods*. As seen in the Fig. 11, *C. albicans* can significantly uptake copper from the serum, but none of the available mutants for copper-starvation induced cell wall proteins showed a defect in copper uptake. It is possible that a single gene mutant may be not enough to show the significant defect of copper uptake in these

experiments, or we may need to measure more cell wall protein mutants to identify the most crucial protein for copper acquisition from serum.

3.6 A compendium of stress responsive cell wall proteins

In order to compare the gene expression of 45 cell wall proteins in all four stress/growth conditions, the gene expression profiles were analyzed by heat maps, and the proteins are aligned by classification and fold changes of gene expression in four different conditions.

In Fig. 12A, the genes are grouped according to classification of cell wall protein families. Most cell wall proteins fall into families of closely related polypeptides, with the exception of EAP1, YWP1, PGA26, SSR1, PGA6 and PGA45, shown at the bottom of Fig. 12A. Interestingly, there is very little co-expressed of members within any particular family of cell wall protein. For example, the different extracellular SODs are induced in different conditions. With the exception of glucose starvation where all three SODs are induced, the three genes show little co-expression. *SOD4* is most strongly induced with iron starvation and *SOD5* is induced during hyphal formation. *SOD6* seems mainly to be expressed in high-iron and yeast-form conditions. These results are

in line with the possibility that different members of SOD family are expressed in distinct conditions that may be encountered in the host. The same may be true for other family of cell wall proteins where different members are expressed under different stress and environmental conditions.

In Figs, 12B-D, the heat maps are configured according to stress conditions. *RBT5* is interestingly induced in all four conditions, which suggests that it is important for the stress adaption of *C. albicans*. Research has shown that RBT5 is regulated by different transcriptional regulators such as SFU1, TUP1, RFG1 and HOG1(Braun et al., 2000; Enjalbert et al., 2006; Kadosh and Johnson, 2001; Lan et al., 2004; Nobile and Mitchell, 2005). Since RBT5 is a heme receptor, its regulation by the iron repressor SFU1 makes sense. However, the regulation of *RBT5* by the TUP1 and the RFG1 repressor for filamentous growth and by the HOG1 regulator for oxidative and osmotic stress implies that RBT5 may function widely in stress adaptation. RBT5 is involved in hemoglobin utilization with other heme-binding proteins CSA1 and PGA10. However, we find that in glucose-starvation condition and hyphal cells, *CSA1* and *PGA10* are not co-induced with *RBT5*, which suggests that CSA1, PGA10 and RBT5 are not always partners in

response to stress conditions. RBT5 may have other important functions other than heme uptake. Aside from *RBT5*, there are no other cell wall protein genes that are consistently upregulated (or down regulated) under all stress conditions.

Lastly, these heat map analyses allowed us to review cell wall protein genes that are co-regulated with the extracellular SODs in one or more conditions. SOD4 is co-regulated with heme-uptake proteins RBT5 and CSA1 under iron starvation conditions, which suggests that SOD4 may protect these heme-binding proteins from oxidative damage. The expression of *SOD5* is most similar to that of the heme receptor *RBT5* as shown in Fig. 12. Additionally, *SOD5* is induced together with all genes specific to the hyphal state including the ferritin receptor ALS3. Since iron-binding molecules such as ferritin is susceptible to superoxide oxidation (Schatzman and Culotta, 2018), *SOD4* and *SOD5* may play some roles in iron acquisition, although this remains to be determined.

Most curious, *SOD6* seems to be co-expressed with *RHD3* as shown in Fig. 12 (B-F). Both of them are upregulated by glucose starvation and downregulated in iron-limited condition and hyphal state. In low copper medium, they do not show any

apparent change. RHD3 is involved in the synthesis of cell wall mannan (de Boer et al.,

2010), and nothing is known about the function of SOD6. Work in the Culotta lab has

shown that SOD4 and SOD5 are bona fide copper-only SOD enzymes, but attempts to

purify and characterize recombinant SOD6 have failed. SOD6 is a larger molecule and

although it is predicted to bind copper, its role as a SOD enzyme is yet to be determined.

Based on its co-expressed with RHD3, SOD6 may play a role in cell wall remodeling,

although this requires additional studies.

In conclusion, our studies show that the expression of cell wall protein genes change

dramatically in response to different stress conditions. The copper-only extracellular

SODs are expressed differently under distinct conditions and they may function to

protect the crucial cell wall proteins from oxidative damage to help *C. albicans* adapt

to various environments in the host. These comparative expression analyses are just the

starting point for the future studies which are aimed at deciphering the function of cell

wall proteins including the SOD enzymes. Future research are required to continue to

find the cell wall genes that are crucial to acquiring copper in the serum, and search the

function of RBT5 other than iron uptake. Future studies can analyze the expression of

the interested cell wall proteins on protein level, and detect the SOD enzymatic

activities in different stress conditions with their co-expressed proteins.

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Table 1. 45 identified GPI-anchored cell wall proteins in *C. albicans*

Protein	orf number	Family	Functions
SOD4	orf19.2062	SOD	Superoxide dismutase
SOD5	orf19.2060	SOD	Superoxide dismutase
SOD6	orf19.2108	SOD	Superoxide dismutase
RBT5	orf19.5636	RBT5-like	CFEM domain, heme receptor
PGA10	orf19.5674	RBT5-like	CFEM domain, heme receptor
CSA1	orf19.7114	RBT5-like	CFEM domain, heme receptor
HYR1	orf19.4975	HYR-like	Hyphal regulated
HYR4	orf19.3279	HYR-like	Unknown
IFF4	orf19.7472	HYR-like	Unknown
IFF5	orf19.2879	HYR-like	Unknown
ALS3	orf19.1816	ALS-like	Adhesion, biofilm formation, ferritin binding
ALS6	orf19.7414	ALS-like	Unknown
ALS9	orf19.5742	ALS-like	Unknown
IHD1	orf19.5760	IHD1-like	Seven-cysteine pattern, hyphal-induced
PGA15	orf19.2878	IHD1-like	Seven-cysteine pattern
PGA42	orf19.2907	IHD1-like	Seven-cysteine pattern
PGA50	orf19.1824	IHD1-like	Seven-cysteine pattern
PGA61	orf19.5762	IHD1-like	Seven-cysteine pattern
ECM33	orf19.3010.1	ECM33-like	Cell wall integrity, stress tolerance
ECM331	orf19.4255	ECM33-like	Unknown
RHD3	orf19.5305	RHD3-like	Synthesis of cell wall mannan
PGA30	orf19.5303	RHD3-like	Unknown
PGA31	orf19.5302	RHD3-like	Unknown
PGA59	orf19.2767	PGA59-like	Three-cysteine domain, coat-forming
PGA62	orf19.2765	PGA59-like	Three-cysteine domain, coat-forming
CRH11	orf19.2706	carbohydrate enzyme	Transglycosylase
CRH12	orf19.3966	carbohydrate enzyme	Transglycosylase
CHT2	orf19.3895	carbohydrate enzyme	Chitinase
PGA4	orf19.4035	carbohydrate enzyme	ß-1,3-glucanosyltransferase

PGA5	orf19.3693	carbohydrate enzyme	ß-1,3-glucanosyltransferase
BGL2	orf19.4565	carbohydrate enzyme	ß-1,3-glucanosyltransferase, cell wall synthesis
UTR2	orf19.1671	carbohydrate enzyme	Putative glycosidase, cell wall integrity
PHR1	orf19.3829	carbohydrate enzyme	Transglycosidase, ß-1,3-glucan modeling
PHR2	orf19.6081	carbohydrate enzyme	Transglycosidase, ß-1,3-glucan modeling
SAP9	orf19.6928	SAP-like	Yapsin-like, adhesion, cell wall integrity
SAP10	orf19.3839	SAP-like	Yapsin-like, adhesion, cell wall integrity
HWP1	orf19.1321	HWP1-like	Hyphal specific, abundant cysteine residues, adhesion, biofilm formation
RBT1	orf19.1327	HWP1-like	Abundant cysteine residues
PGA8	orf19.3380	HWP1-like	Abundant cysteine residues, adhesion, biofilm formation
EAP1	orf19.1401	Unknown	Adhesion
YWP1	orf19.3618	Unknown	Promoting dispersal
PGA26	orf19.2475	Unknown	Biofilm formation
SSR1	orf19.7030	Unknown	Unknown
PGA6	orf19.4765	Unknown	Unknown
PGA45	orf19.2451	Unknown	Unknown

Table 2. Gene primers used for qRT-PCR

Gene Name			Primer Sequence
TUB2	Forward	orf19.6034	GAGTTGGTGATCAATTCAGTGCTAT
	Reverse		ATGGCGGCATCTTCTAATGGGATTT
SOD4	Forward	orf19.2062	CTTGACGAAGGTGACGATACTGCAA
	Reverse		TTAAAGCAGCAACAACACCGGCAAT
SOD5	Forward	orf19.2060	GCAGATCTTACATTGGCGGTTTATC
	Reverse		CCAAGAGACCATTTACTACTGCTCT
SOD6	Forward	orf19.2108	CTACAGCCCAGAAGAAACTAGG
	Reverse		CCCAATACGAGAGAACCAAAGA
RBT5	Forward	orf19.5636	CTTCCAAGGCTGCTGAAAGT
	Reverse		CGGCAATGACACCACCAATA
PGA10	Forward	orf19.5674	GGTGCTATTGGTGACTGTGT
	Reverse		GCAGATTTGGTGGCTGTTTC
CSA1	Forward	orf19.7114	CTCAACCTGCTGAAACCTCA
	Reverse		CACTTGGGTAGATGGTGTATGG
SSR1	Forward	orf19.7030	CTGCTTCTGCTTCTGCTTCT
	Reverse		AAGCACCAAGACCAACCTTAG
EAP1	Forward	orf19.1401	CTCCAAGGTCAAGCCCATTA
	Reverse		CCCGTAGTTACTGCTGTATGAG
PGA45	Forward	orf19.2451	ATTACCAGCAGCATCACCTAAA
	Reverse		TGACCATCAACTTGTCCATCAG
HYR1	Forward	orf19.4975	ACTGAATCTAAACCTGGTTTC
	Reverse		TACCACCAGTAACAATAGATG
HYR4	Forward	orf19.3279	GCAGGTGATATAGAGCTGACAC
	Reverse		GTAGTGGACGATGACTGACTTG
IFF4	Forward	orf19.7472	GCAACTGCCACAATCACAAG
	Reverse		CAGATCTTCCCAGAGCACTAAC
IFF5	Forward	orf19.2879	TACAGGGTACGAGCCATCTAA
	Reverse		GGTGGTAGTGTATTCTGTTGGT

ALS3	Forward	orf19.1816	TTACTACTTCCACAGCTGCTTC
	Reverse		GGAGCATTACCACCACCATTA
ALS6	Forward	orf19.7414	ACCCATCCCTACTACCACTATT
	Reverse		TCGAGTCAGTGGGATTTGTATAAG
ALS9	Forward	orf19.5742	AGAGAGGTGCCAACTTCAAC
	Reverse		CTAGATGATGGTCCTGGAGTAGA
IHD1	Forward	orf19.5760	GTGTAACTGGTGGCTCTCAA
	Reverse		CATCTGTAGCACCACTACCATC
PGA15	Forward	orf19.2878	CATACTGTGATAAAGCCCTGGA
	Reverse		CCGAATCTTCAGTTGCCTTTG
PGA42	Forward	orf19.2907	AGAGGATGTTTCATTCACAGAGG
	Reverse		CTGAAGTTGTTTGAGCGTTGG
PGA50	Forward	orf19.1824	GACTGCCTAGACTTTGGTGATG
	Reverse		GACGCCAATCCAGAAGTGATAG
PGA61	Forward	orf19.5762	GCAGCAGTACCAGCAGTAATA
	Reverse		ATGGAGCAACTTCTGGTGTAG
ECM33	Forward	orf19.3010.1	AGAGAGTTTCCGGTGGTTTC
	Reverse		CCAGTTTCACTTGAACCAGAAG
ECM331	Forward	orf19.4255	GTCACCATTTCTGGTCCATTTG
	Reverse		GATCCTGATTTGGAGGATGAAGA
PGA30	Forward	orf19.5303	AAGGTGCTGGTGTCAACTAC
	Reverse		ACTTCCATCTTCGGCGATTT
PGA31	Forward	orf19.5302	GGTACCCTTTCCTTTGATGGT
	Reverse		GGAGCAGCAGAAGAAGAAGAA
RHD3	Forward	orf19.5305	TGCCAAATCCGACGATTCA
	Reverse		TAAACGTCACCACCCAAAGT
PGA59	Forward	orf19.2767	GGTTCCGCTTTAGCCACTTA
	Reverse		TAGAAGCAACTGGAGCTTTGG
PGA62	Forward	orf19.2765	TGTCGTCTTATCCGCTGTTG
	Reverse		GTTGACAATGGGCAGTAGGT
YWP1	Forward	orf19.3618	GTACTGCTCTTGAAGGTTCTACTC
	Reverse		CGGCAGAATCACCACTACTT

CRH11	Forward	orf19.2706	CACATCTTCCGCATCATCCA
	Reverse		CGAAGAACTGGTAACGGTAGTC
CRH12	Forward	orf19.3966	GACACATCGGACGACAACAATA
	Reverse		GCTAACCCTTCAGACCCAAATC
UTR2	Forward	orf19.1671	GCTTATTGTTTAGGCGGTTGTG
	Reverse		CCCAACCAGTATAAACCCAGTC
CHT2	Forward	orf19.3895	CAGGAAGCTGACGTTGACAT
	Reverse		CACCAACACCACCACCTAAA
PGA4	Forward	orf19.4035	CGGAGATGTCGTCTGTGTTT
	Reverse		GGACACTTGTTACCACCAGTAG
PGA5	Forward	orf19.3693	CGGATTTATCCGAACCAACAATATC
	Reverse		ATCACGAATAGCGGCTCTAAC
PHR1	Forward	orf19.3829	CGTCCACGTTTGTTCCAAGA
	Reverse		GGCAAGGGATGGGCTTATTT
PHR2	Forward	orf19.6081	CCAGTTTGGTCTGGTGGTATT
	Reverse		TGGTTGGACAAGTGGTTCTG
SAP9	Forward	orf19.6928	GCTCGTCGTACATGTTGTTTG
	Reverse		GTAGAGGTGCCAGATGAAGATG
SAP10	Forward	orf19.3839	TGCAACGTACGATGAAGATGAG
	Reverse		GAATGCCACCACCACCAATA
HWP1	Forward	orf19.1321	TCCTCAACCTGATGTTCCTTG
	Reverse		GGAGTAGTAGCTGGAGTTGTTG
RBT1	Forward	orf19.1327	CTCCATCAACTACCACCGTAAG
	Reverse		GGAGCTGATGATTCTGGAGTAG
PGA8	Forward	orf19.3380	TCGACCACACATTCCATTCC
	Reverse		GAGGTGGCACTTGTAACAGAT
PGA6	Forward	orf19.4765	TAGTGTTGCTCCAGCTGTTC
	Reverse		GTGTGAACACCACTGATGGTA
PGA26	Forward	orf19.2475	TAGTGAACCAACCACAGTAACC
	Reverse		TAGCGACCACACCAACTAAAG
BGL2	Forward	orf19.4565	CCGTCTCCACCATCAAGATTT
	Reverse		ACTGGCACCATCAACTAAGAC
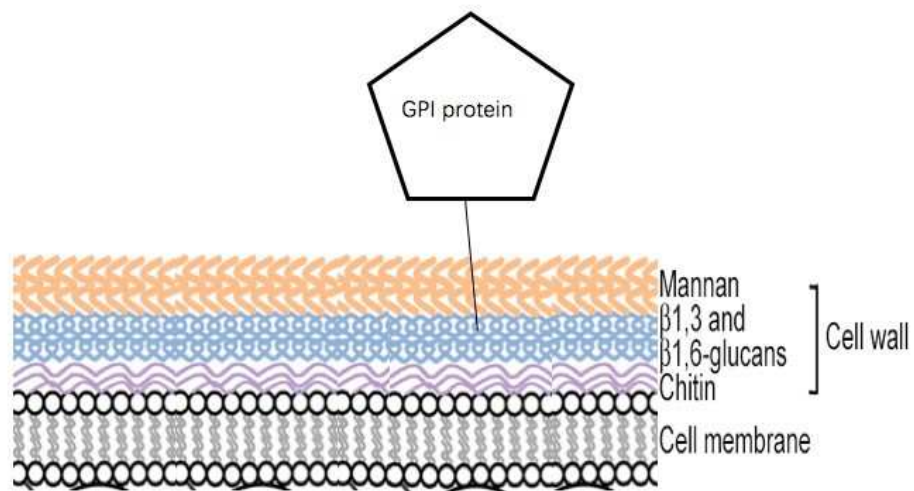
Fig. 1. The cell wall of *C. albicans*

The external layer mainly comprises mannosylated glycoproteins and most of the proteins in the external layer are GPI-anchored proteins. The inner polysaccharide layer consists of ß-1,6 glucans, ß-1,3 glucans and chitin molecules. The GPI-anchored proteins are linked to ß-1,6 glucans, which in turn are linked to ß-1,3 glucans (Chaffin, 2008; Kapteyn et al., 2000; Klis et al., 2009).
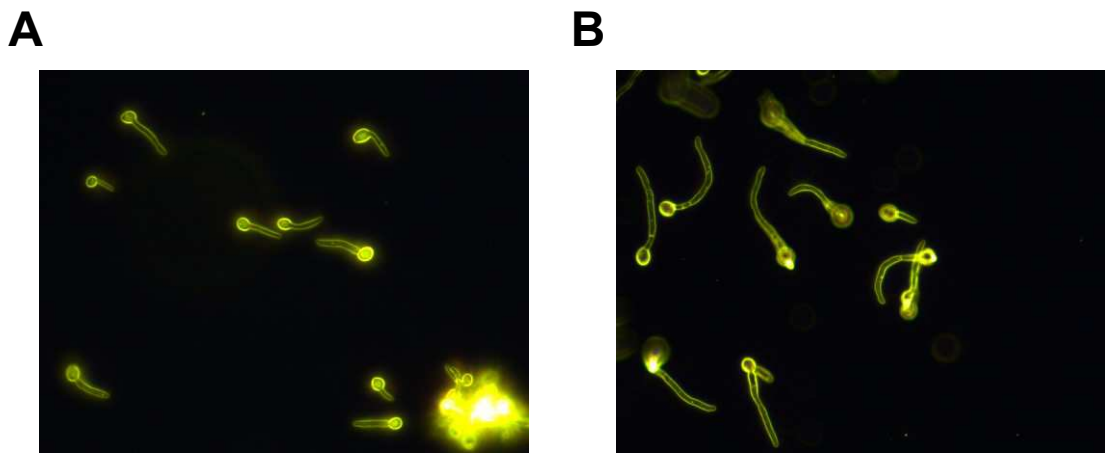
Fig. 2. Hyphal formation of *C. albicans* in IMDM medium

C. albicans strain SC5314 was grown in YPD medium for 16 h at 30°C to log phase, then starved and induced to form hyphae by incubating in IMDM medium at 37°C for 1 h (A) or 2 h (B). The cells were observed by dark-field microscopy. The cells that were cultured for 2 h in IMDM showed distinct elongating hyphae and longer germs tubes compared to 1 h (A).
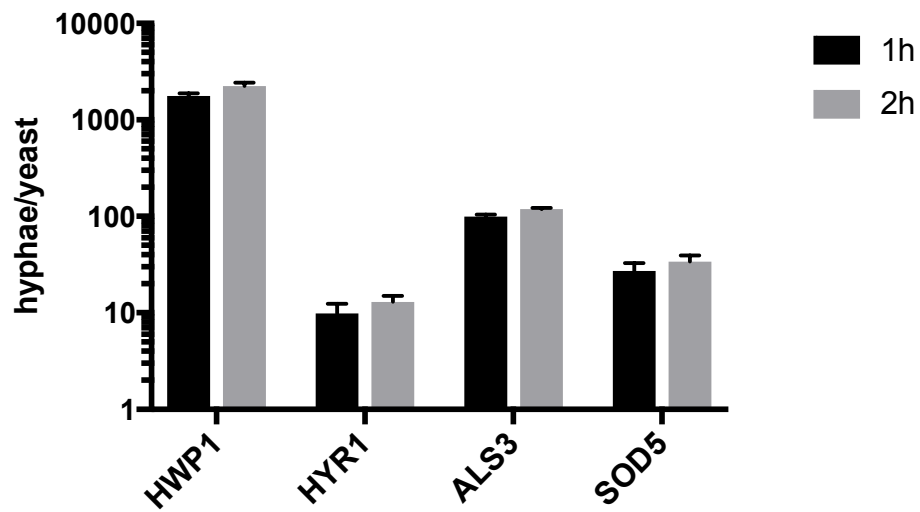
Fig. 3. The expression of *C. albicans* hypha-induced proteins at different time points

The yeast and hyphal cells were grown as in Fig. 2. The expression of *HWP1*, *HYR1*, *ALS3* and *SOD5* was analyzed by qRT-PCR. This figure shows fold changes of the gene expression in 1-hour hyphal culture and 2-hour hyphal culture compared to yeast cells. HWP1, HYR1, ALS3 and SOD5 are hyphal-associated proteins (Heilmann et al., 2011). Results represent the averages of three independent cultures and the error bars are SD.
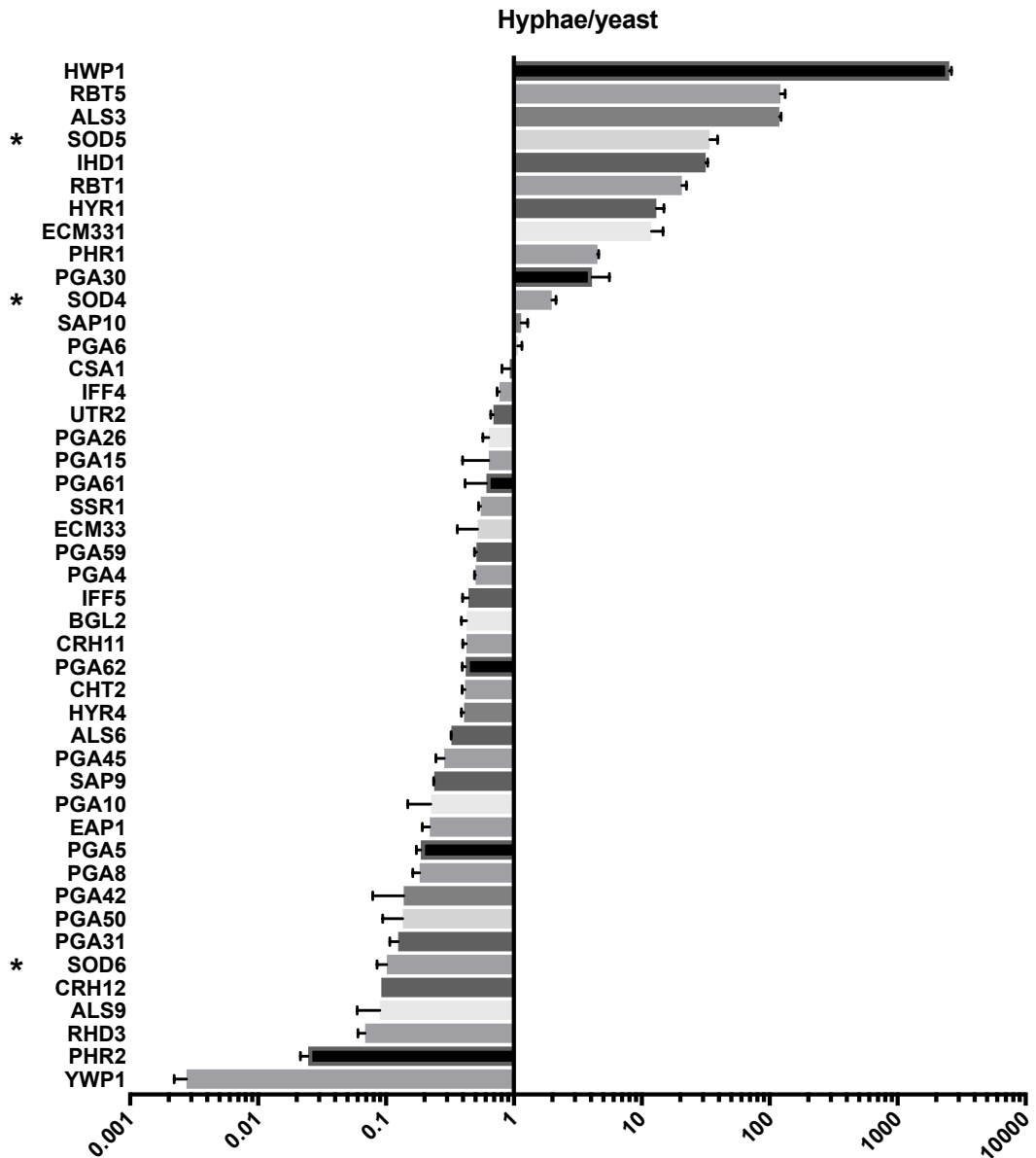
Fig. 4. RNA changes in cell wall protein encoding genes of *C. albicans* in response to hyphal formation

The yeast and hyphal cells were grown as in Fig. 2. The hyphal cells were incubated in IMDM medium for 2 hours. Expression of the indicated genes was quantified by qRT-PCR and normalized to *TUB2*. The results show the fold changes of mRNA in hyphae compared to yeast cells. Values represent the averages of three independent cultures and the error bars are SD.
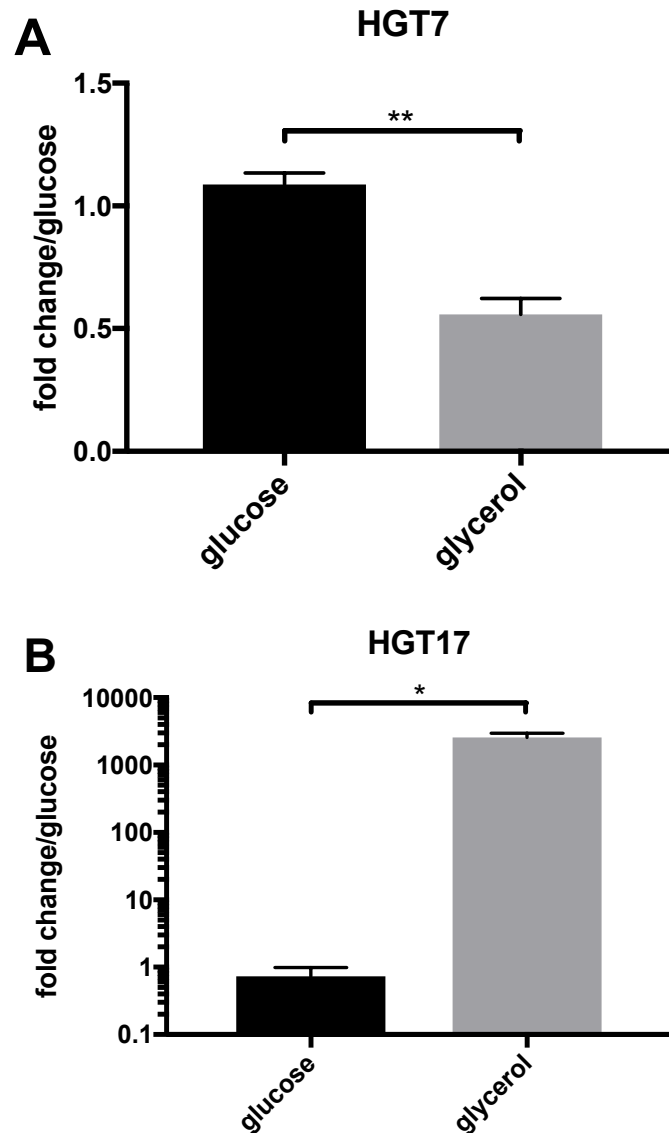
Fig. 5. Expression of glucose transport genes *HGT17* and *HGT7* with glucose starvation

The *C. albicans* strain CA-IF100 was grown in YP + 5% glycerol for 16 h at 30°C to log phase, then diluted and incubated in YP + 2% glucose or YP + 5% glycerol at 30°C for 2.5 h. The expression of *HGT7* (glucose induced) and *HGT17* (glucose repressed) was analyzed by qRT-PCR and compared to *TUB2*. The graphs show the fold changes of gene expression compared to the cells grown in glucose. Results represent the averages of three independent replicates over two experiment trials. The error bars are SE and the significance determined by paired t-test; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. The expression of *HGT7* decreased in glycerol (A), and *HGT17* was significantly induced (B) in glucose-starvation or glycerol conditions.

glucose starvation/control
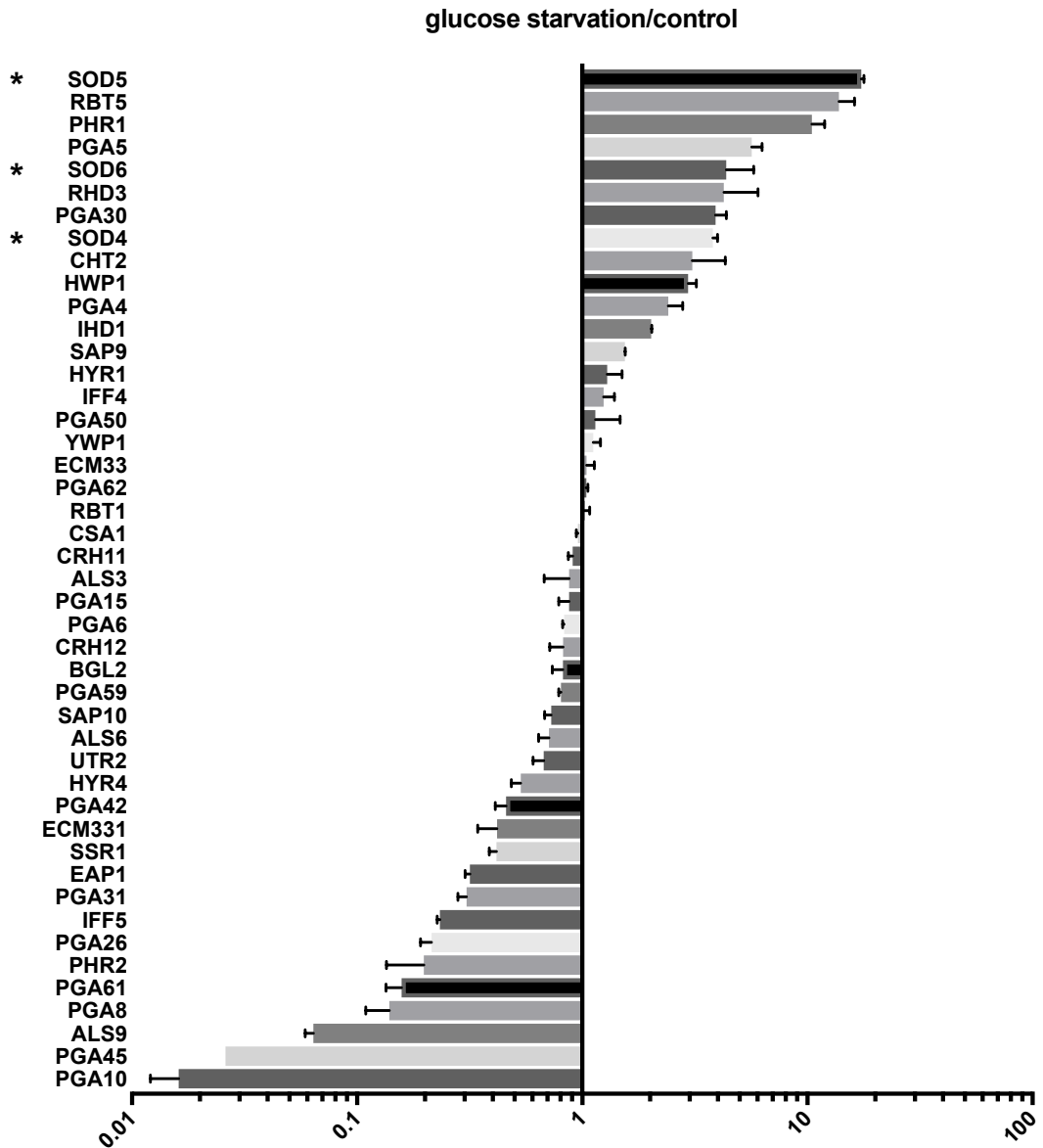
Fig. 6. RNA changes in cell wall protein encoding genes of *C. albicans* in response to glucose starvation

The cells were grown as in Fig. 5. Expression of the indicated genes was quantified by qRT-PCR and normalized to *TUB2*. The results show the fold changes of mRNA in glycerol conditions compared to glucose conditions. Values represent the averages of three independent cultures and the error bars are SD.
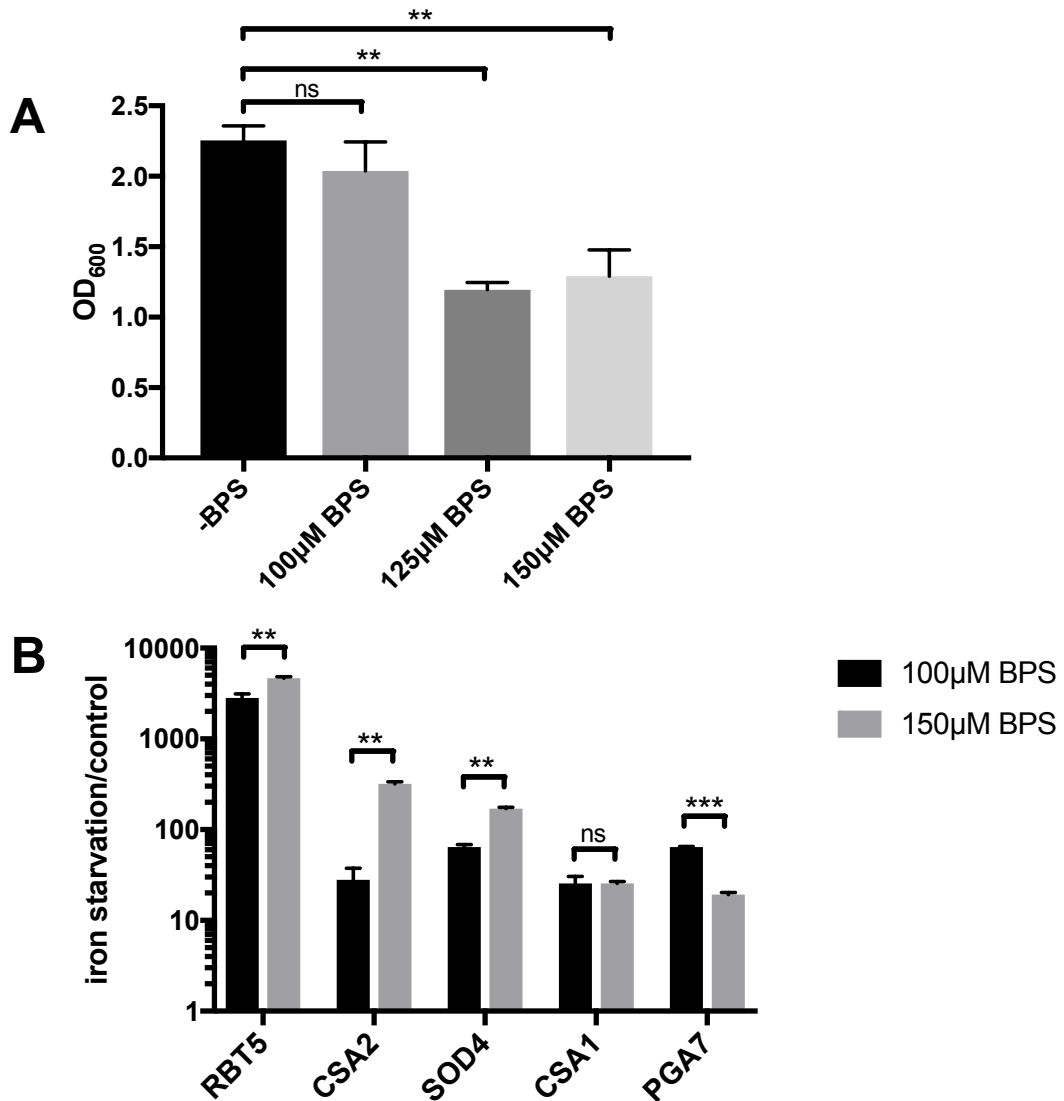
Fig. 7. The effect of the iron chelator BPS on fungal cell growth and expression of known markers of iron starvation

The cultures of *C. albicans* strain SC5314 were grown in YPD medium or YPD with the indicated level of the iron chelator BPS for 16 h at 30°C to log phase. (A) The growth of cells was measured at a wavelength of 600 nm; Results represent the averages of three cultures and the error bars are SD. 125 μM and150 μM BPS are much more toxic to the growth of cells than 100 μM BPS. (B) The expression of known iron regulated genes, *RBT5*, *CSA2*, *SOD4*, *CSA1* and *PGA7* was analyzed by qRT-PCR and compared to *TUB2*. Results represent the averages of three independent replicates and the error bars are SD. The significance determined by paired t-test; $^{***}p = 0.0003$; $^{**}p \leq 0.01$; ns, not significant.
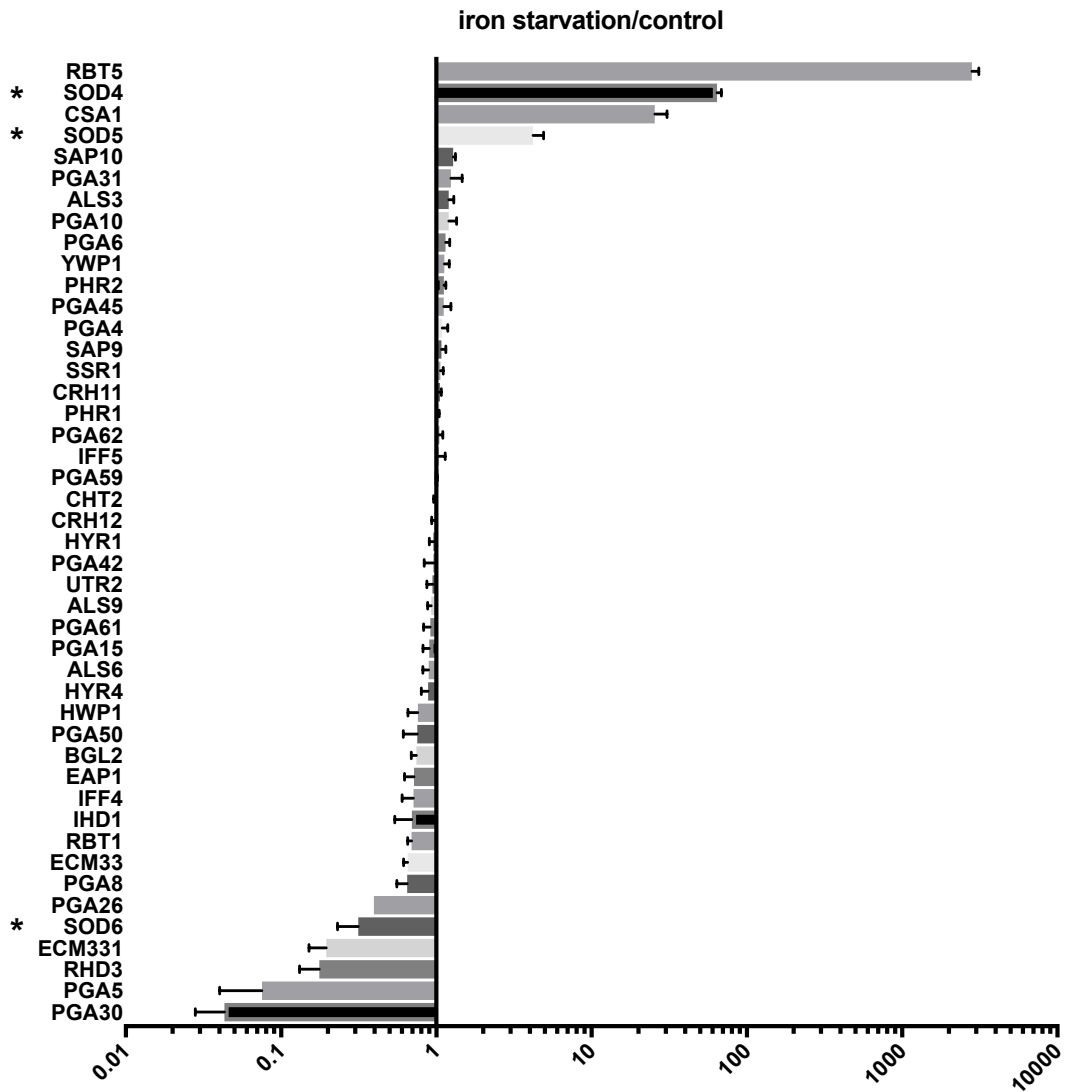
Fig. 8. RNA changes in cell wall protein encoding genes of *C. albicans* in response to iron starvation

The strains were grown in YPD or YPD + 100 μM BPS. Expression of the indicated genes was quantified by qRT-PCR and normalized to *TUB2*. The results show the fold changes of mRNA in 100 μM BPS-treated conditions compared to no BPS conditions. Values represent the averages of three independent cultures and the error bars are SD.
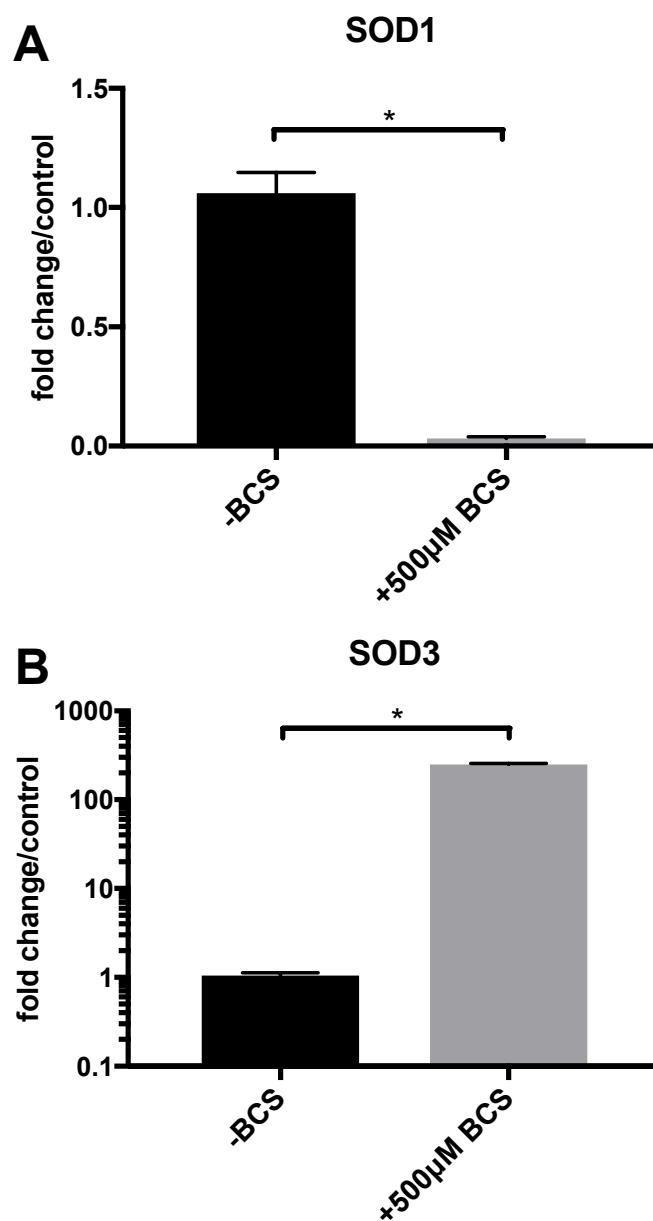
Fig. 9. Expressions of *SOD1* and *SOD3* in copper-starvation condition

The *C. albicans* strain SC5314 was grown in SC medium or SC + 500 μM Cu(I) chelator BCS for 16 hours at 30°C to log phase. Expression of *SOD1* and *SOD3* was analyzed by qRT-PCR and compared to *TUB2*. Results represent the averages of two independent replicates and the error bars are SD. The significance determined by paired t-test; [*]p ≤ 0.05. The graphs show the fold changes of gene expression compared to the cells grown in SC medium without BCS. The expression of *SOD1* decreased in copper-starvation condition (A), and *SOD3* was induced in copper-starvation condition (B).

copper starvation/control
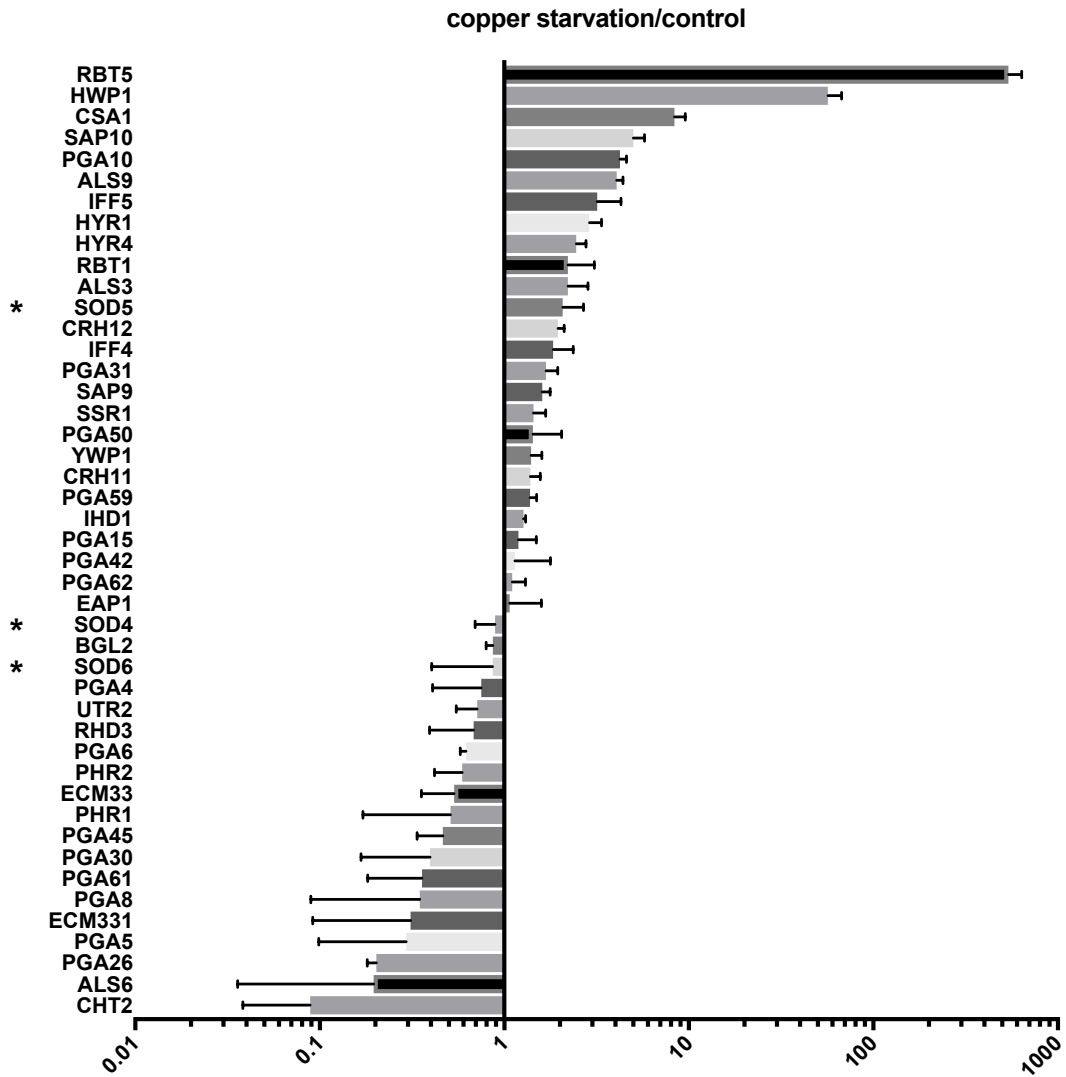
Fig. 10. RNA changes in cell wall protein encoding genes of *C. albicans* in response to copper starvation

The cells were grown as in Fig. 9. Expression of the indicated genes was quantified by qRT-PCR and normalized to *TUB2*. The results show the fold changes of mRNA in 500 μM BCS-treated conditions compared to copper-replete conditions. Values represent the averages of two independent cultures and the error bars are SD.
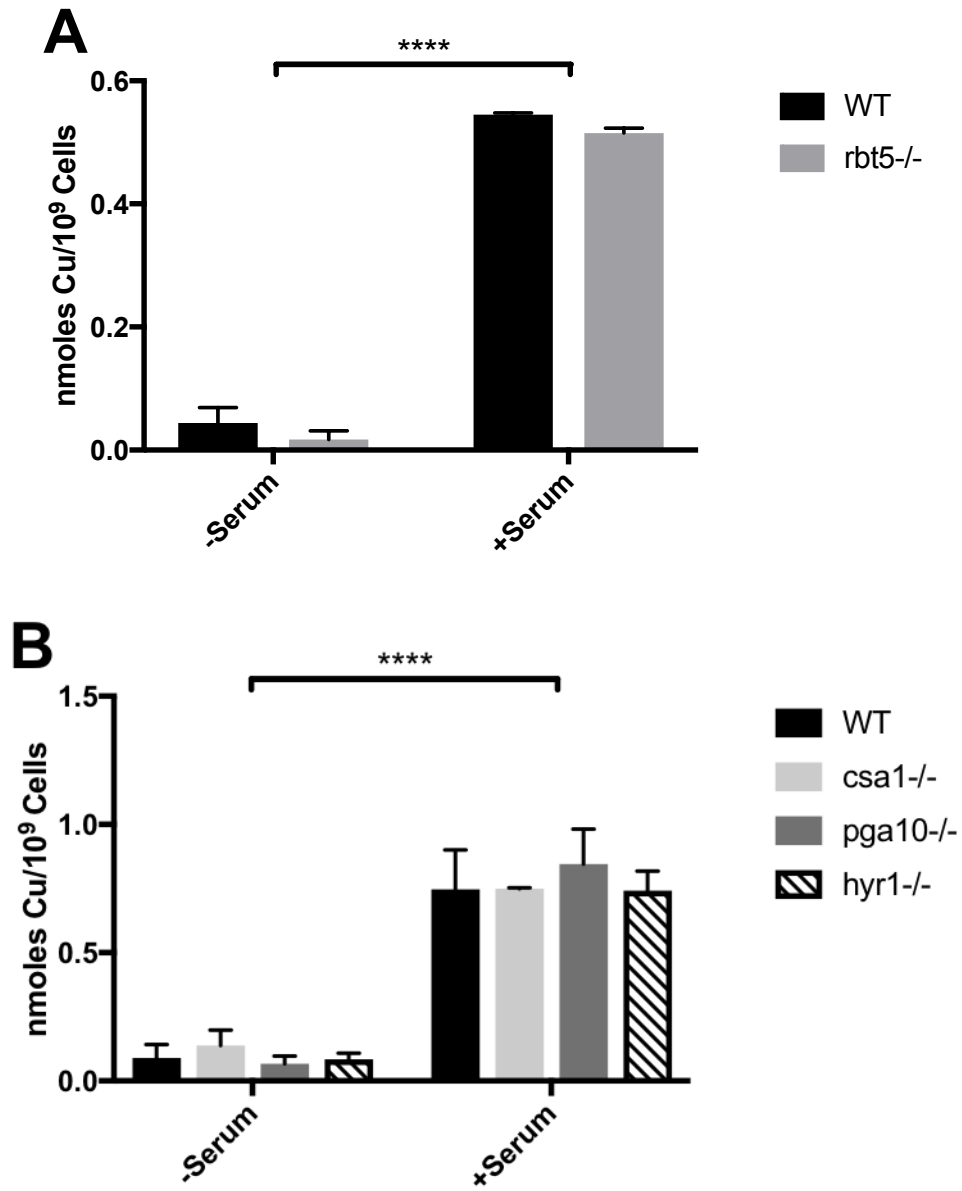
Fig. 11. The effects of select cell wall protein mutants on copper uptake in serum

The designated strains were grown in YPD medium with 500 μM BCS for 16 hours at 30°C to stationary phase. 5 OD_{600} units of cells were washed, pelleted and resuspended in 100 μl serum for 1 hour, or stored in -80°C for 1 hour. The intracellular copper was analyzed by AAS. Results represent the averages of two independent replicates and the error bars are SD. The significance determined by paired t-test; [****]$p < 0.0001$. Strains used: (A) WT SN152 and isogenic *rbt5Δ/Δ* strain; (B) WT SN152 and isogenic *csa1Δ/Δ, pga10Δ/Δ, hyr1Δ/Δ* strains.

Fig. 12. Heat maps of gene expression profiles of cell wall protein encoding genes in four different stress/growth conditions

Shown are fold changes in expression of cell wall protein genes in $\log_{10}$ scale. Data is from Fig. 4, 6, 8, 10. Black stands for baseline value 0. Red shows larger values and green corresponds to the values less than 0. These double gradient heat maps were made by Graphpad Prism 7. (A) The proteins are aligned vertically by classification as described in *Introduction*. (B-F) The proteins are ordered by the fold changes of the gene expression in hyphal form, glucose, iron and copper starvation, respectively.
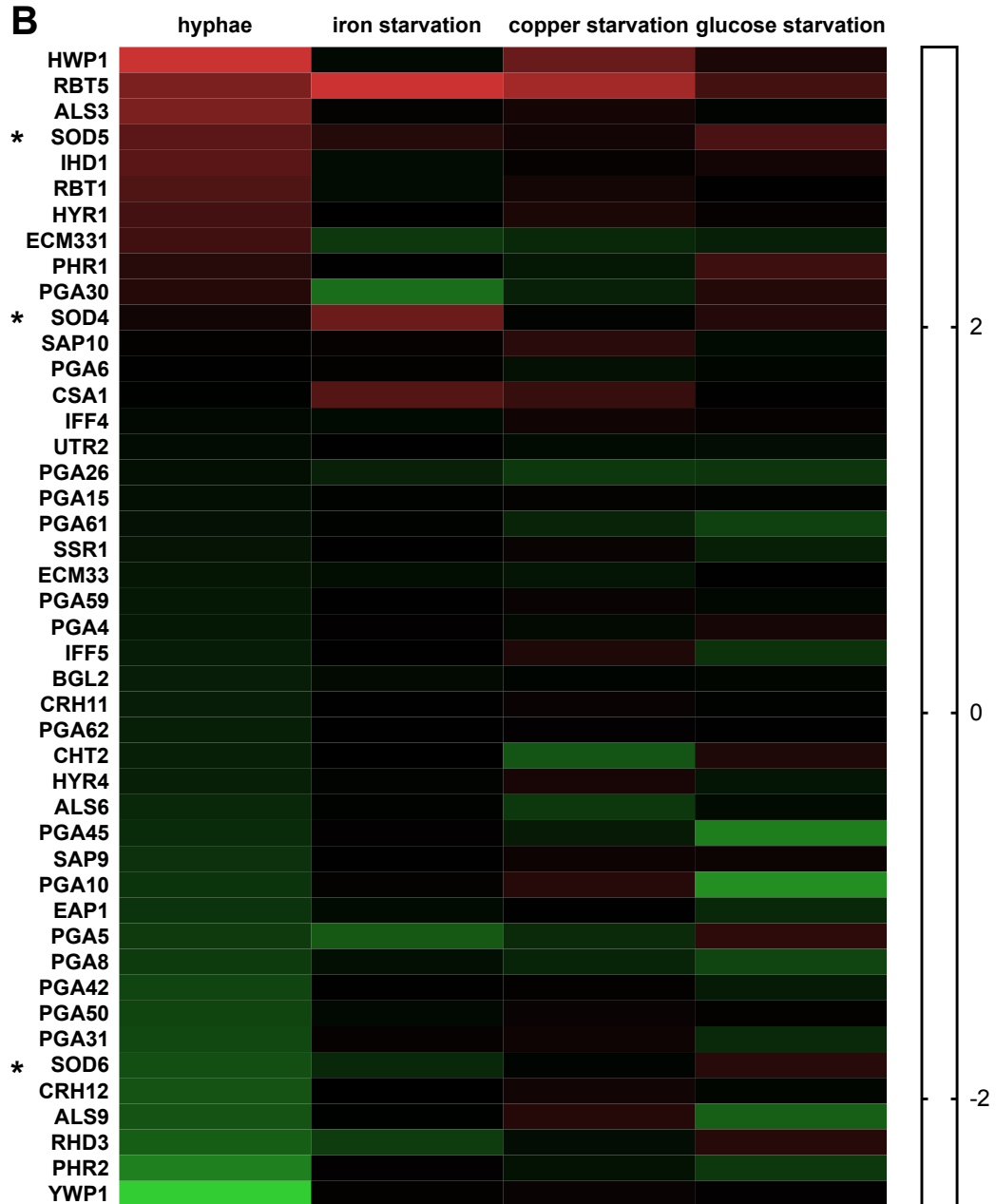
Fig. 12 (Continued). Heat maps of gene expression profiles of cell wall protein encoding genes in four different stress/growth conditions

Shown are fold changes in expression of cell wall protein genes in $\log_{10}$ scale. Data is from Fig. 4, 6, 8, 10. Black stands for baseline value 0. Red shows larger values and green corresponds to the values less than 0. These double gradient heat maps were made by Graphpad Prism 7. (A) The proteins are aligned vertically by classification as described in *Introduction*. (B-F) The proteins are ordered by the fold changes of the gene expression in hyphal form, glucose, iron and copper starvation, respectively.
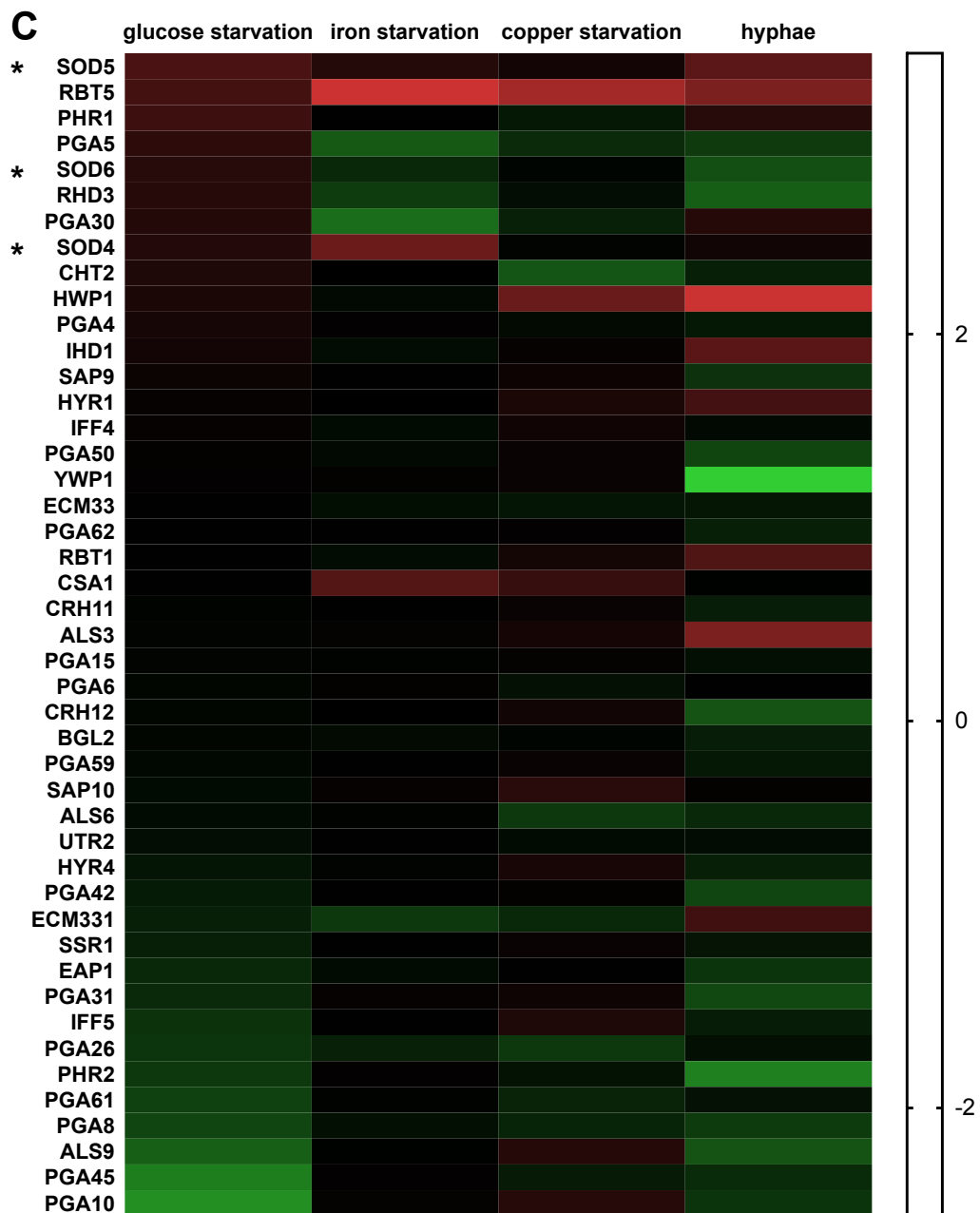
Fig. 12 (Continued). Heat maps of gene expression profiles of cell wall protein encoding genes in four different stress/growth conditions

Shown are fold changes in expression of cell wall protein genes in $\log_{10}$ scale. Data is from Fig. 4, 6, 8, 10. Black stands for baseline value 0. Red shows larger values and green corresponds to the values less than 0. These double gradient heat maps were made by Graphpad Prism 7. (A) The proteins are aligned vertically by classification as described in *Introduction*. (B-F) The proteins are ordered by the fold changes of the gene expression in hyphal form, glucose, iron and copper starvation, respectively.
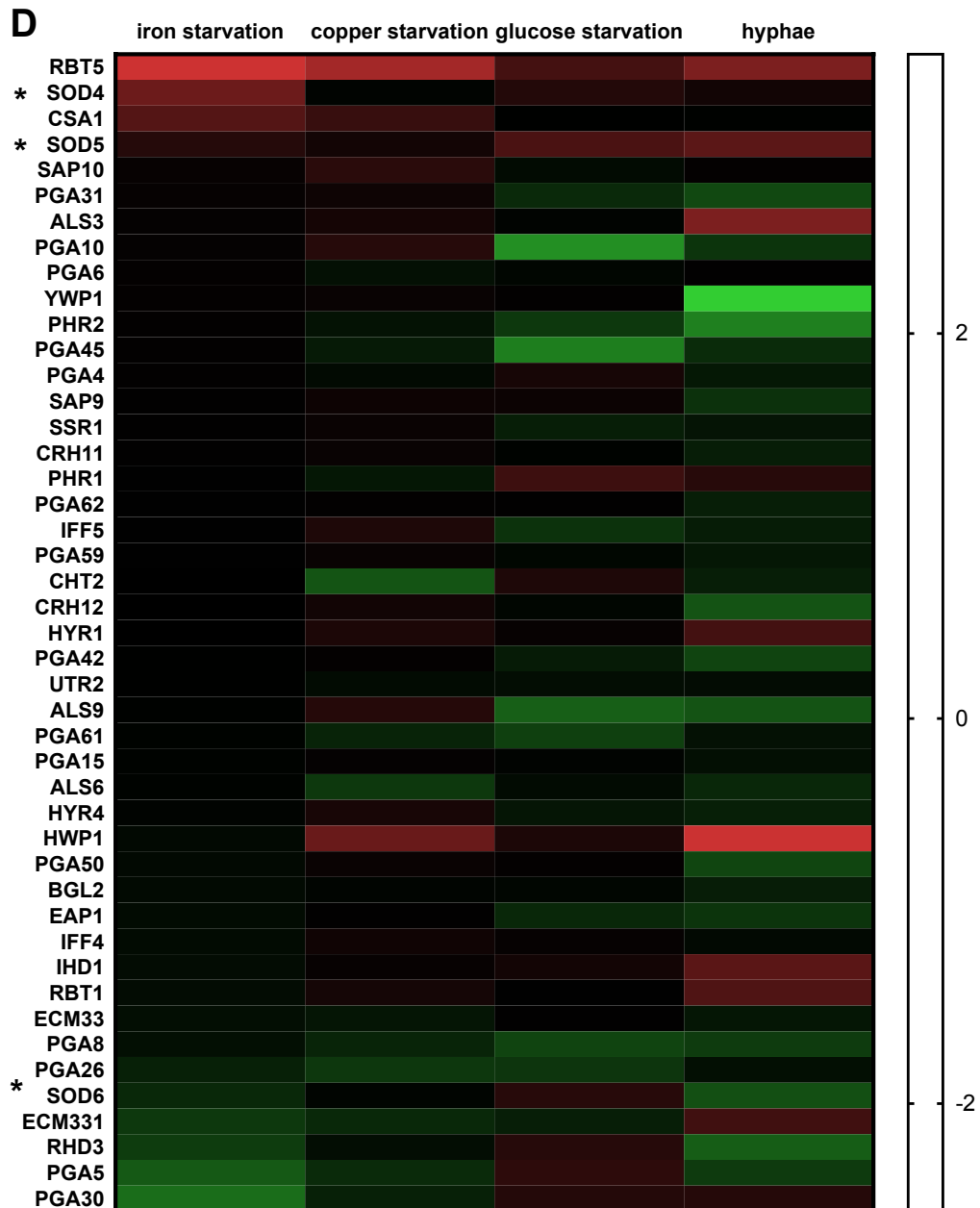
Fig. 12 (Continued). Heat maps of gene expression profiles of cell wall protein encoding genes in four different stress/growth conditions

Shown are fold changes in expression of cell wall protein genes in $\log_{10}$ scale. Data is from Fig. 4, 6, 8, 10. Black stands for baseline value 0. Red shows larger values and green corresponds to the values less than 0. These double gradient heat maps were made by Graphpad Prism 7. (A) The proteins are aligned vertically by classification as described in *Introduction*. (B-F) The proteins are ordered by the fold changes of the gene expression in hyphal form, glucose, iron and copper starvation, respectively.
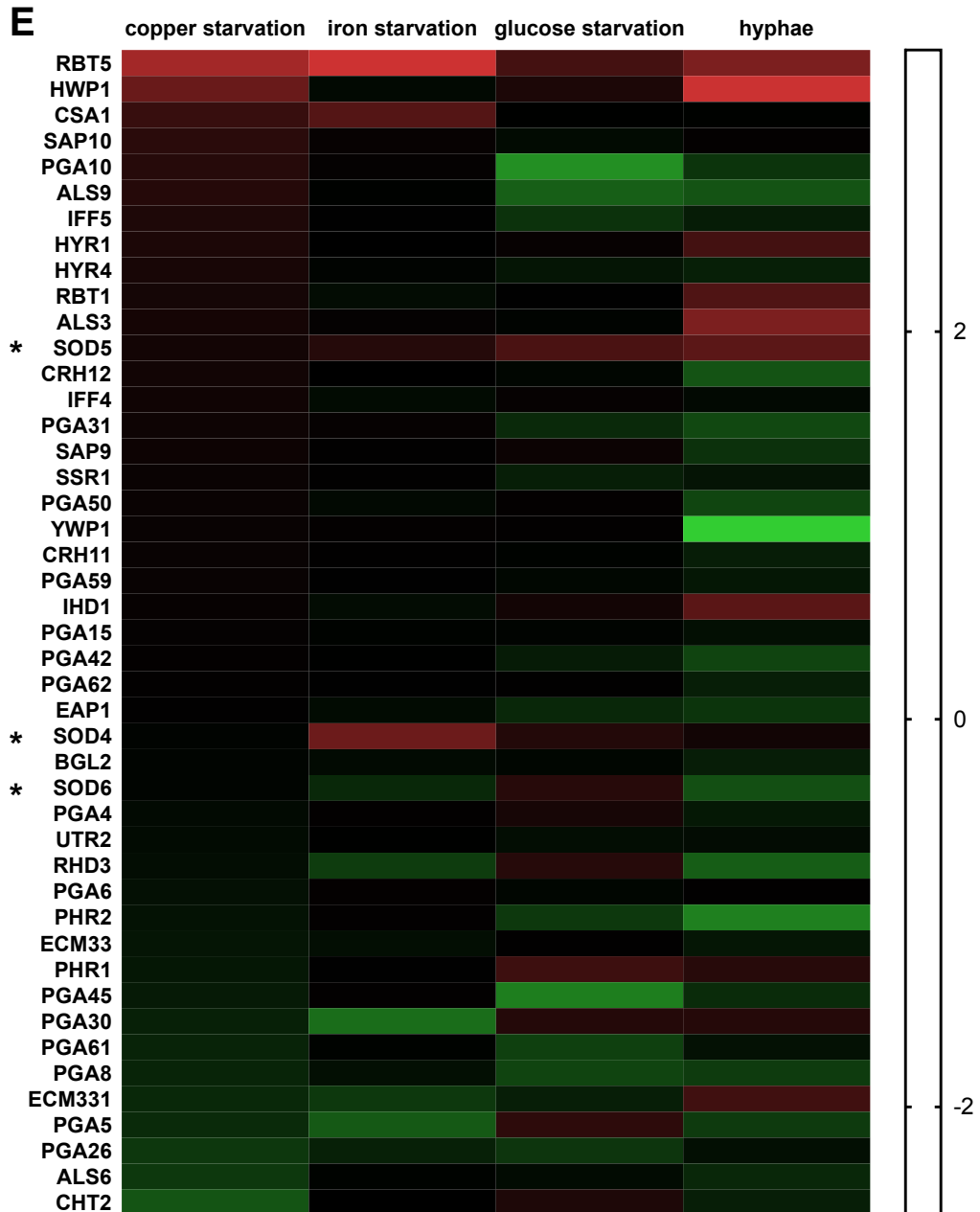
Fig. 12 (Continued). Heat maps of gene expression profiles of cell wall protein encoding genes in four different stress/growth conditions

Shown are fold changes in expression of cell wall protein genes in $\log_{10}$ scale. Data is from Fig. 4, 6, 8, 10. Black stands for baseline value 0. Red shows larger values and green corresponds to the values less than 0. These double gradient heat maps were made by Graphpad Prism 7. (A) The proteins are aligned vertically by classification as described in *Introduction*. (B-F) The proteins are ordered by the fold changes of the gene expression in hyphal form, glucose, iron and copper starvation, respectively.

Curriculum Vitae

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Undergraduate research assistant: Institute of Biochemistry and Biotechnology, Beijing Normal University, June 2014 --- June 2015

Explored the derivatives of GCC which is an immunosuppressant to control the immune response in human body, and researched the properties of the optimal derivative