Characterization of the cell wall protein Ecm33 family in *Candida glabrata*

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
BMMH	Buffered minimal methanol
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFW	Calcoflour white
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide phosphate
DTT	1,4-Dithiothreitol
ECL	Enhanced chemiluminescent detection
ECWs	Extracted cell walls
EDTA	Ethylene diamine tetraacetic acid
FACS	Fluorescence activated cell sorting
g	Force of gravity
IPTG	Isopropyl beta-D-thiogalactopyranoside
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria-Bertani medium
M	Molar

ml	Milliliter
min	Minute
mg	Milligram
µg	Microgram
µl	Microliter
µm	Micrometer
MM	Minimal medium
MOMP	Mitochondrial outer membrane permeabilisation
mRNA	Messenger RNA
OD	Optical density
p	*P* value
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
sec	Second
SDS	Sodium dodecyl sulphate
SD	Standard deviation
TEMED	N, N, N', N'-tetramethylethylenediamine

tet	Tetracycline
Tm	Annealing temperature
Tris	Tris(hydroxylmethyl)-aminomethan
U	Unit
UV	Ultraviolet
V	Voltage
v	Volume
w	Weight
WT	Wild-type
YPD	Yeast Peptone Dextrose

CHAPTER 1

INTRODUCTION

1.1 Candidiasis

The incidence of fungal infections in humans is increasing worldwide because of the rising numbers of immunocompromised hosts, resulting from AIDS, chemotherapy for cancer patients, and patients receiving immunosuppressive drugs for transplantation and auto-immune diseases. Clinical manifestations of fungal infections may be relatively harmless (e.g. in the case of dermatophytes or *Malassezia* species), but invasive fungal infections (IFIs) can be life-threatening (Giri, S. and Kindo, A. J., 2012; Sifuentes-Osornio, J., Corzo-Leon, D. E. et al., 2012; Mayer, F. L., Wilson, D. et al., 2013).

Candida species, *Aspergillus* species and *Cryptococcus neoformans* are three main organisms causing invasive fungal infections in humans (Liao, Y., Chen, M. et al., 2013). *Candida* species are considered as normal flora which can be found in the oral cavity and the gastrointestinal tract of most healthy humans (Fidel, P. L., Jr., Vazquez, J. A. et al., 1999; Brunke, S. and Hube, B., 2012) and the majority of *Candida* isolates from clinical samples represent asymptomatic colonization (Brunke, S. and Hube, B., 2012). However, when the human body is weakened by prolonged treatment with antibiotics, diabetes, cancer, extreme age, immunosuppression, intravenous catheters or long-term hospitalization, these fungi can cause a broad range of clinical manifestations ranging from local superficial to life-threatening systemic infections, with high morbidity and mortality (Perlroth, J., Choi, B. et al., 2007; Brunke, S. and Hube, B., 2012) (Figure 1). Infections caused by *Candida* species are termed “candidiasis”. More than 90% of candidiasis cases are attributed to only five *Candida* species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. Nevertheless, rare species of *Candida* are isolated from candidiasis patients every year such as *C. guilliermondii*, *C. inconspicua*, *C. rugosa*, and *C. lambica* (Pfaller, M. A., Diekema, D. J. et al., 2010; Giri, S. and Kindo, A. J., 2012). *C. albicans* is the most prevalent species in both systemic and superficial infections

worldwide. The second most prevalent species depends on the site of infection and geography. For example, in the United States, Europe, and Asia-Pacific, *C. glabrata* is considered the second most prevalent species (Marchetti, O., Bille, J. et al., 2004; Borg-von Zepelin, M., Kunz, L. et al., 2007; Pfaller, M. A., Diekema, D. J. et al., 2010), but in Latin America (e.g. Brazil), *C. tropicalis* and *C. parapsilosis* are the most important species after *C. albicans* (Colombo, A. L., Guimaraes, T. et al., 2007).

In contrast to *C. albicans*, *C. glabrata* infections are very difficult to treat, as this organism is often highly resistant to azole antifungals. Amphotericin B and echinocandins are considered as effective drugs to eliminate this pathogenic yeast from the patients, but Amphotericin B has a lot of side effects and echinocandins is still very expensive. *C. parapsilosis* is less susceptible to echinocandins and *C. krusei* is fully resistant to Fluconazole (Table 1). Therefore, infections due to these non-albicans species are causes of increased morbidity and mortality (Marchetti, O., Bille, J. et al., 2004). Consequently, species identification in Candidiasis is essential to enable early and adequate antifungal therapy.

Figure 1. Oral candidiasis in a 2 year old child

The child suffers from acquired immunodeficiency syndrome (AIDS) after infection with human immunodeficiency virus (HIV) by its mother.
(Taken by Tangwattanachuleeporn M., 2011 in Makassar, Indonesia)

Table 1. *In vitro* susceptibilities of antifungal drugs

	C. albicans	*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*C. krusei*	other
Fluconazole	+	+/-	+	+	-	+
Itraconazole	+	+/-	+	+/-	+/-	+
Posaconazole	+	+/-	+	+	+	+
Voriconazole	+	+/-	+	+	+	+
Ketoconazole	+	+	+	+	+	+
Amphotericin B	+	+	+	+	+/-	+
Flucytosine	+/-	+	+/-	+	+/-	+/-
Nystatin	+	+	+	+	+	+
Echinocandins[a]	+	+	+	-	+	+

This data were combinated from (Richter, S. S., Galask, R. P. et al., 2005; Leventakos, K., Lewis, R. E. et al., 2010)

+: in vitro activity, -: no in vitro activity, +/-: modest in vitro activity

[a]: Caspofungin, micafungin, and anidulafungin

1.2 *C. glabrata* genetics and evolution

In the past, *C. glabrata* was classified in the genus of *Torulopsis* and termed *Torulopsis glabrata* because it was thought to be unable to form non-hyphae. Subsequently, when conditions for pseudohypha production in *T. glabrata* were discovered, the organism was reclassified in the genus *Candida* and renamed *C. glabrata* (Fidel, P. L., Jr., Vazquez, J. A. et al., 1999).

C. glabrata belongs to the class Saccharomycetes in the order Saccharomycetales and the family Saccharomycetaceae. The yeast cells of *C. glabrata* are 1 to 4 μm in size and smaller than the *C. albicans* yeast form (4 to 6 μm). *C. glabrata* cannot produce pseudohyphae under normal

condition but it can produce pseudohyphae on solid synthetic low ammonia dextrose nitrogen starvation medium (SLAD) after incubation at 37^{o}C for 2 weeks (Csank, C. and Haynes, K., 2000). Colonies of *C. glabrata* on Sabouraud dextrose agar are smooth, cream colored and glistening. Like *S. cerevisiae*, *C. glabrata* is haploid genome yeast. In contrast, *C. albicans* and several other non-albicans *Candida* species are diploid (Fidel, P. L., Jr., Vazquez, J. A. et al., 1999).

The genome of the reference strain *C. glabrata* CBS138 (originally isolated from human feces) has been sequenced and it shows 13 chromosomes totalling in 12.3 Mb. There are approximately 5,283 coding sequences (CDS), 207 tRNA genes and the genome has a G+C content of 38.8% (http://genolevures.org/cagl.html and (Kaur, R., Domergue, R. et al., 2005)).

The results from phylogenetic analyses show that *C. glabrata* is quite distinct from *C. albicans* and the other pathogenic *Candida* species, but it is closely related to *S. cerevisiae* (Figure 2) (Diezmann, S., Cox, C. J. et al., 2004; Kaur, R., Domergue, R. et al., 2005). *C. glabrata* shares a common ancestor to *S. cerevisiae* and clearly belongs to a clade different from that of other *Candida* species. As a consequence, the CUG codon of *C. glabrata* and *S. cerevisiae* is translated to leucine, but in other Candida species it is translated to Serine (Roetzer, A., Gabaldon, T. et al., 2011). Many genes of *S. cerevisiae* have orthologues in *C. glabrata* and the gene orders are largely conserved between both organisms (Roetzer, A., Gabaldon, T. et al., 2011). However, *C. glabrata* displays a significantly higher degree of gene loss, resulting in a regressive evolution (loss of specific functions) as compared to *S. cerevisiae* (Dujon, B., Sherman, D. et al., 2004). Indeed, *C. glabrata* lacks the genes that are involved in galactose and sucrose assimilation, phosphate, nitrogen, and sulfur metabolism and chemical biosynthesis of nicotinic acid, thiamine and pyridoxine (Kaur, R., Domergue, R. et al., 2005).

The adhesin genes of *C. glabrata* (e.g. Epa protein family) and *S. cerevisiae* (Flo protein family) locate in subtelomeric regions of the chromosomes. Since both adhesin families share some degree of functional homology and the tandem repeat motifs of several Epa and Flo proteins are similar (Roche, 2007). Thus, it is assumed that the *FLO* and *EPA* genes are evolutionarily related but their sequences have diverged beyond recognition. However, Epa adhesin from *C. glabrata* may have occured from adaptation because of the survival within mammalian hosts (Roetzer, A., Gabaldon, T. et al., 2011).

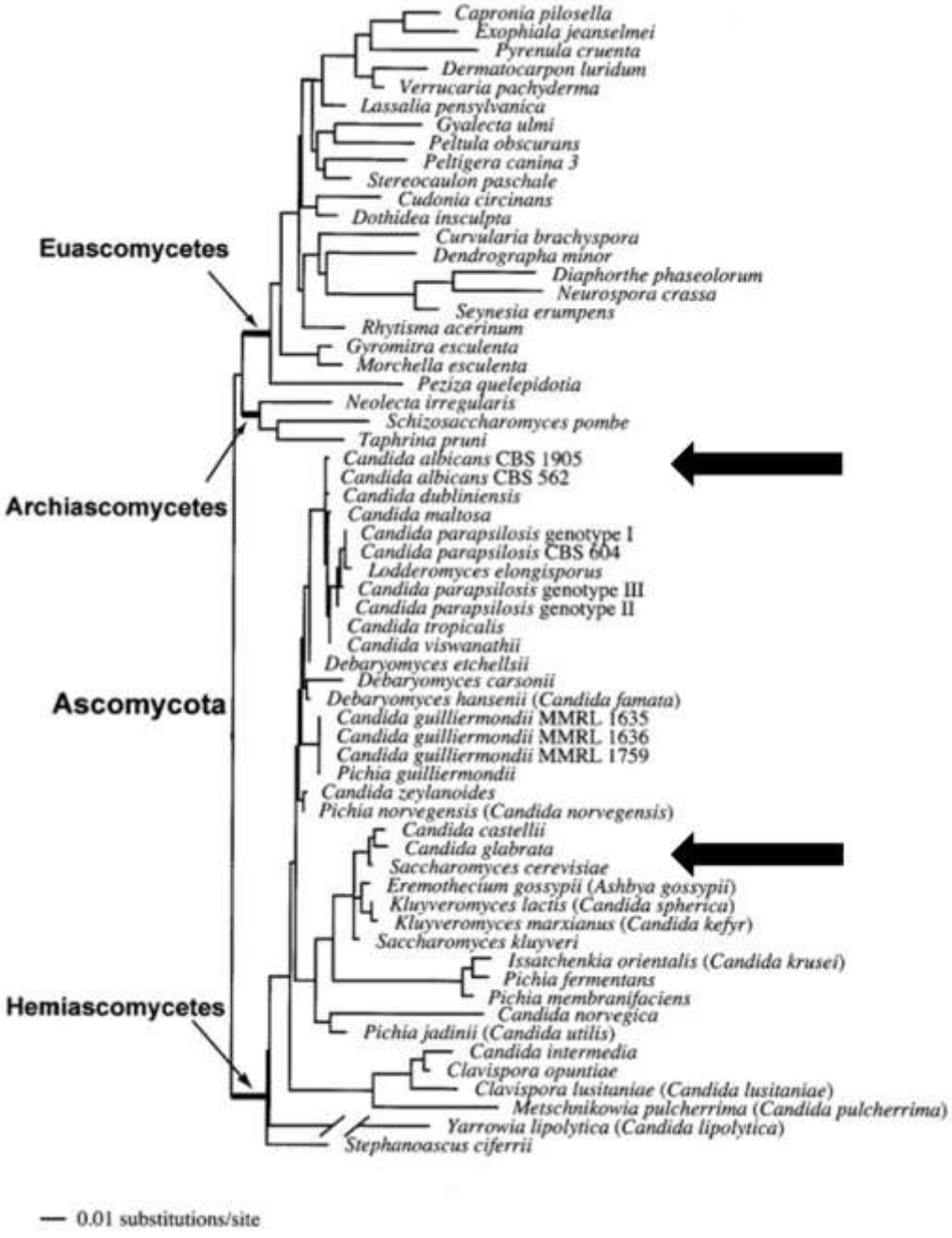

Figure 2. Fungal phylogenetic tree of ascomycetous taxa

Phylogenetic tree based on a combined analysis of the nuclear 18S rDNA and 26 rDNA of ascomycetous taxa. The nodes marking the Euascomycetes, Archiascomycetes, and Hemiascomycetes as well as many terminal branches are supported by homogeneous Bayesian posterior probabilities 95%. Black arrows indicate the origins of the three classes of the phylum Ascomycota. Red arrows indicate *C. albicans* and *C. glabrata* and *S. cerevisiae* (modified from the article of (Diezmann, S., Cox, C. J. et al., 2004)).

Table 2. Comparison of *C. glabrata*, *C. albicans* and *S. cerevisiae*

	C. glabrata	*C. albicans*	*S. cerevisiae*
Number of chromosomes	13 chromosomes	8 chromosomes	16 chromosomes
Length of DNA	12.3 Mb	14.3 Mb	12.1 Mb
Coding genes	5,283	6,202	5,807
Ploidy	Haploid (n)	Diploid (2n)	Diploid (2n)
CUG codon	Leucine	Serine	Leucine
Clade	WGD	CTG	WGD
Virulence	Opportunistic pathogen	Opportunistic pathogen	Non-pathogenic
Major sites of infection	Oral, vaginal, disseminated	Oral, vaginal, disseminated	Non-infectious
Mating genes	Present	Present	Present
Sexual cycle	Unknown	Known	Known
Clonal population structure	Yes	Yes	No
Phenotypic switching	Present	Present	Absent
True hyphae	Absent	Present	Absent
Pseudohyphae	Present	Present	Present
Biofilm formation	Present	Present	Present
Major adhesins	Epa family	Hwp1 and Als family	Flo family
Auxotrophy	Niacin, thiamine, pyridoxine	None	None
Azole resistance	Innate resistance	Susceptible	Susceptible
Mitochondrial function	Petite positive	Petite negative	Petite positive

This table was modified from (Kaur, R., Domergue, R. et al., 2005)

1.3 Pathogenesis and virulence factors of *C. glabrata*

Normally, *C. glabrata* is a commensal microorganism in the human body, but when the host's immune system is compromised, it can also cause severe infections. On mucosal surfaces, *C. glabrata* colonizes by expressing specific adherence-genes such as the *CgEPA* family, *Cg*Silent Information Regulator (*SIR3*), and *Cg*Pyrazinamidase and NiCotinamidase (*PNC1*) enabling it to form biofilms. *C. glabrata* also has an evasive strategy to manage phagocytosis by macrophages. After uptake, macrophages initially induce formation of peroxisomes. These peroxisomes are degraded by *C. glabrata* using the pexophagy pathway, a specific subtype of autophagy. The autophagy related *Cg*AuTophaGy (*ATG*) gene family contributes to the survival of *C. glabrata* during engulfment and this process requires mobilization of resources to survive in phagosome. *C. glabrata* is relative resistant to carbon starvation (Roetzer, A., Gabaldon, T. et al., 2011). Carbon starvation of engulfed *C. glabrata* is associated with the up-regulation of *Cg*IsoCitrate Lyase (*ICL1*) and *Cg*MaLate Synthase (*MLS1*) genes in the glyoxylate cycle (Fukuda, Y., Tsai, H. F. et al., 2013). The oxidative burst response in phagosomes of macrophages is administrated by neutralization of the oxidative reaction and increase the pH in phagosome for the survival of *C. glabrata*. Both reactions contribute to activation of CgYeast AP (*YAP1*), a transcription factor responsive to H_2O_2 and *Cg*SuperOxide Dismutases (*SOD1*). Fequently, *C. glabrata* strains are resistant to antifungal drugs, especially fluconazole, mediated through efflux pumps *CgPDR1* and *CgCDR1* (Ferrari, S., Sanguinetti, M. et al., 2011; Roetzer, A., Gabaldon, T. et al., 2011; Orta-Zavalza, E., Guerrero-Serrano, G. et al., 2013) as shown in Figure 3.

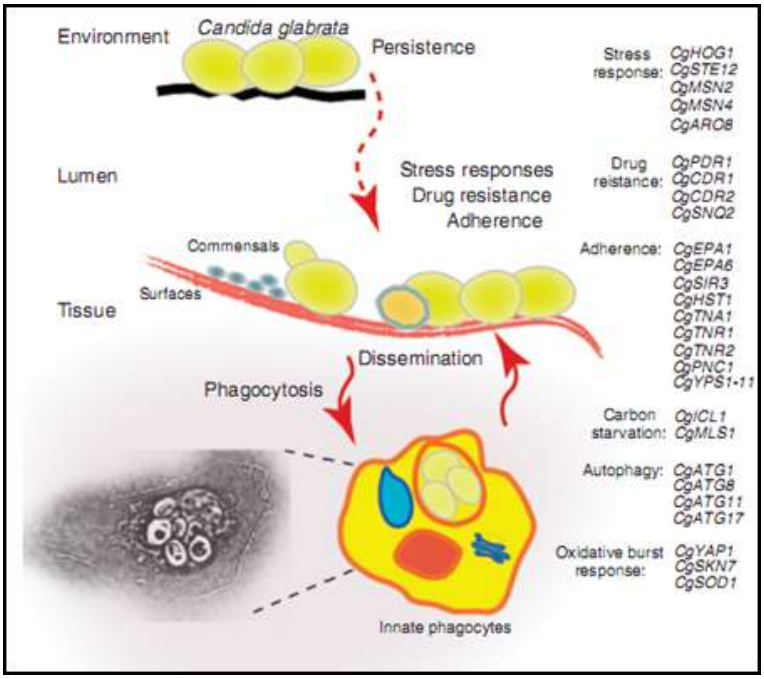

Figure 3. Hypothetical model of *C. glabrata* pathogenesis

C. glabrata is found as normal flora in mucosal areas of humans, such as the gut. In immunocompromised or elderly persons, *C. glabrata* is able to spread into tissues and cause organ failure. The control of phagocytes is necessary for elimination of the invading *C. glabrata*. The right panel shows a list of genes that contribute to each stage of *C. glabrata* infection (Figure taken from (Roetzer, A., Gabaldon, T. et al., 2011)).

1.4 Cell wall structure of *C. glabrata*

1.4.1 The polysaccharide backbone of the *C. glabrata* cell wall

The cell wall of fungi is one of the most important organelle. It determines cell shape, physical strength and limits permeability. Furthermore, it contributes to adherence to host cells or medical materials and to biofilm formation. The cell wall can be considered a "virulence associated factor", since it determines the initial host – fungus interactions leading to mycoses. Moreover, the cell wall triggers the human immune response, especially the innate immunity and it is also connect to antifungal drug resistance (Weig, M., Jansch, L. et al., 2004; de Groot, P. W., Kraneveld, E. A. et al., 2008).

Generally, the fungal cell wall consists of 80-90% polysaccharides, mainly glucans and mannans. In yeast cell wall, the outer layer cell wall is highly enriched with O- and N-linked mannans that are covalently associated with proteins to form glycoproteins, whereas the inner layer contains the skeletal polysaccharides chitin and β-1,3-glucan, which confer strength and cell shape. The outer cell wall proteins are attached to this inner wall framework predominantly by glycosylphosphatidylinositol (GPI) remnants that are linked to the skeleton through a more flexible β-1,6-glucan. The cell wall of molds, e.g. *Aspergillus fumigatus*, it is composed of a unique β-1,3/1,4-glucan skeleton with chitin and galactomannan covalently linked to the nonreducing ends of β-1,3-glucan. The cell wall is mainly coated with GPI proteins, which contain N- and O-glycans (Jin, C., 2012). In comparison, the cell walls of molds have a higher complexity of carbohydrates and chitin components than yeast.

The cell wall of *C. glabrata* contains approximately 54% glucans, 43.8% mannans, 1.2% chitin, and 6.4% proteins (Table 3) and the architecture of the cell wall in *C. glabrata* is shown in Figure 4.

In summary, the cell wall of *C. glabrata* displays a high level of proteins and mannans, but a low level of chitin and glucan, including 1,6-β-glucan and 1,3-β-glucan, when compared with *S. cerevisiae* and *C. albicans* (de Groot, P. W., Kraneveld, E. A. et al., 2008). Therefore, it is assumed that cell wall of *C. glabrata* has more mannoproteins on its outer surface than others yeasts.

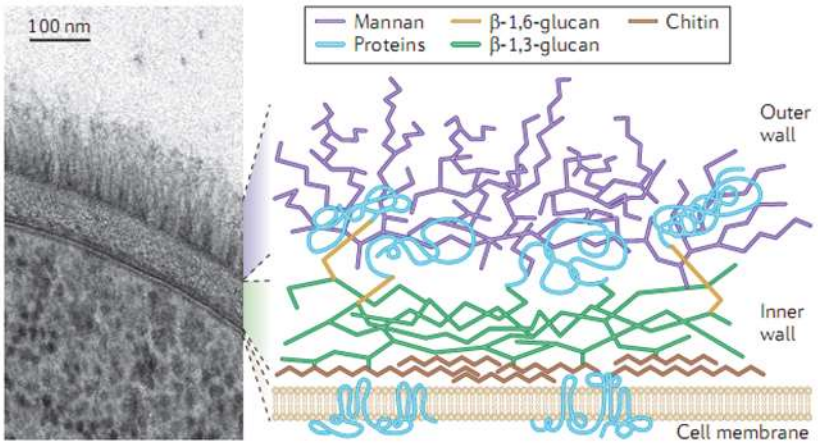

Figure 4. Structure of the yeast cell wall

The outer layer of the cell wall is highly enriched with O- and N-linked mannose polymers (mannans) that are covalently associated with proteins to form glycoproteins, whereas the inner layer contains the skeletal polysaccharides chitin and β-1,3-glucan, which confer strength and cell shape. The outer cell wall proteins are attached to this inner wall framework predominantly by glycosylphosphatidylinositol (GPI) remnants that are linked to the skeleton through a more flexible β-1,6-glucan. Figure was taken from (Gow, N. A., van de Veerdonk, F. L. et al., 2012).

Table 3. Cell wall composition of *C. glabrata* in comparison to *S. cerevisiae* and *C. albicans*

Organisms	Cell wall content						
	Protein (%)	Chitin (%)	Man (%)	Glu (%)	M/G	Alkali-insoluble glucan (%)	
						1,6-β	1,3-β
C. glabrata	6.4 ± 0.1	1.2 ± 0.1	43.8 ± 0.5	54.0 ± 0.2	0.81	4.2 ± 0.1	16.7 ± 1.7
S. cerevisiae	4.0 ± 0.1	1.4 ± 0.2	34.2 ± 1.6	60.3 ± 2.5	0.57	7.1 ± 0.2	26.8 ± 0.9
C. albicans	3.5 ± 0.2	4.2 ± 0.1	26.6 ± 2.3	64.0 ± 4.9	0.42	10.6 ± 0.6	26.2 ± 1.1

Cell wall content values are from exponentially growing cells (in YPD) and are expressed as percentages of freeze-dried cell walls. Man: mannose, Glu: glucose, M/G: ratio of mannose to glucose. Table taken from (de Groot, P. W., Kraneveld, E. A. et al., 2008).

1.4.2 The cell wall proteome of *C. glabrata*

Covalently linked wall protein of *C. glabrata* can be divided 2 groups as (i) GPI cell wall proteins, which can be specifically released with HF-pyridine and (ii) mild alkali-soluble cell wall proteins, which can be released by mild NaOH (Klis, F. M., Brul, S. et al., 2010). The majority of cell wall proteins are linked to the carbohtdrate complex via a covalent bond of a sugar remnant of their GPI anchor and 1,6-β-glucan. The minority of cell wall proteins are attached to 1,3-β-glucan through a linkage which sensitive to mild alkali. The covalently linked wall proteins have a variety of function as (i) carbohydrate-active enzyme such as Crh1, Scw4, and Gas family, (ii) other enzymatic activity such as Plb2, (iii) nonenzymatic cell wall proteins such as Cwp family, Pir family, and Ssr1, and (iv) adhesin such as Epa family, and Awp family (de Groot, P. W., Kraneveld, E. A. et al., 2008).

The most abundant covalently linked wall proteins of *C. glabrata* were identified by LC/MS/MS as Cell Wall Protein (Cwp) 1 family. This proteins are structural cell wall proteins that connected to the cell wall either through the GPI anchor and alkali sensitive linkage (Schmidt, P., 2007; de Groot, P. W., Kraneveld, E. A. et al., 2008).

In silico analysis in *C. glabrata* revealed that there are 106 glycosylphosphatidylinositol-modified (GPI) proteins in the cell wall and 50% of these GPI proteins have features of adhesin-like Cell Wall Proteins (CWPs) (Weig, M., Haynes, K. et al., 2001; Weig, M., Jansch, L. et al., 2004; de Groot, P. W., Kraneveld, E. A. et al., 2008)

One of the noticeable characteristics of *Candida* spp. is their ability to firmly adhere to host surface cells, including the human skin, endothelial, and epithelial mucosal host tissues. Adhesion is considered an important first step of pathogenesis in fungal infections. *Candida* spp. also attach to medical abiotic surfaces such as intravascular and urinary catheters, prosthetic cardiac valves, and denture prostheses (Busscher, H. J., Rinastiti, M. et al., 2010; de Groot, P. W., Bader, O. et al., 2013). Most of known fungal adhesins are large (usally more than 800 amino acids) GPI-modified wall proteins. *In silico* analyses showed that *C. glabrata* has significantly more potential adhesin genes than *C. albicans* and *S. cerevisiae*. Two-thirds of these 66 putative adhesin genes in *C. glabrata* are situated in regions of subtelomeres. In all subtelomeric parts of reference strain CBS138 at least one of putative adhesin gene is present (de Groot, P. W., Kraneveld, E. A. et al., 2008; de Groot, P. W., Bader, O. et al., 2013).

A well-known adhesin family of *C. glabrata* is the *Epa* (epithelial adhesion protein) family with 17 members in reference strain CBS138. The reference strain BG2 has two additional *EPA* genes *EPA4* and *EPA5* (Kaur, R., Domergue, R. et al., 2005; de Groot, P. W., Bader, O. et al., 2013). Additionally, none of the orthologous *EPA* genes in either strain are 100% identical. The *EPA* family is associated with adhesion to human epithelial and endothelial cells, particularly *EPA1*, *EPA6* and *EPA7* (Domergue, R., Castano, I. et al., 2005; Kaur, R., Domergue, R. et al., 2005). Data from Bader et al. revealed that ten progenies of the *C. glabrata* CBS138/ATCC2001 reference strain had a divergent expression of *EPA* family genes, resulting in altered adhesion properties. The low adherence strain CBS138/6 showed significant lower expression of *EPA3*, *EPA6*, *EPA7* and *EPA22* when compared to the other progenies (Bader, O., Schwarz, A. et al., 2012) indicating that the regulation of adhesins in *C. glabrata* is very dynamic. This adaptability may enable individual strains to adjust to different host cell conditions (Kraneveld, E. A., de Soet, J. J. et al., 2011).

The N-terminal part of the Epa proteins contain the binding domains that are similar in sequence to the so-called PA14 domains (anthrax protective antigen). Studies with glycan arrays indicated that PA14 of Epa1, Epa6 and Epa7 bind to oligosaccharides with terminal galactose residues, as they are found in mucin-type *O*-glycans (Zupancic, M. L., Frieman, M. et al., 2008). This finding supports the idea that Epa proteins can directly bind to glycoproteins on the surface of host cell (de Groot, P. W., Bader, O. et al., 2013).

Another important adhesin family in *C. glabrata* is the Pwp family. It consists of seven members that are lectins (de Groot, P. W., Bader, O. et al., 2013). *In vitro* studies showed that Pwp7 plays a role in adherence to human endothelial cells (Desai, C., Mavrianos, J. et al., 2011). Additionlly, six further non-Epa adhesin-like wall proteins were found in the *C. glabrata* cell wall, namely Awp1 to Awp6 (de Groot, P. W., Kraneveld, E. A. et al., 2008; Kraneveld, E. A., de Soet, J. J. et al., 2011). Awp1 is upregulated during biofilm development, and Awp6 was identified by mass spectrometric analysis in cell wall preparations of biofilms. It is assumed that expression of these adhesins depends on many factors which seem to be dependent on the genetic strain background, environmental growth conditions and aggregation (Kraneveld, E. A., de Soet, J. J. et al., 2011; de Groot, P. W., Bader, O. et al., 2013). However, the exact function and protein structure of the Pwp and Awp protein families still needs to be investigated.

1.4.3 Ecm33 protein family

The Ecm33 protein family in *C. glabrata* consists of at least four members as Ecm33 (CAGL0M01826g), Pst1 (CAGL0E04620g), Sps2 (CAGL0H01661g), and Sps22 (CAGL0B00616g). Ecm 33 and Pst1 were found to be very abundant proteins in the cell wall of *C. glabrata* (de Groot, P. W., Kraneveld, E. A. et al., 2008). However, the exact function of the Ecm33 protein family is still unclear. Previous scientific evidences revealed that this family may play a role in cell wall remodeling, cell wall maintenance and cell biogenesis (de Groot, P. W., Ruiz, C. et al., 2001) in the ascomycocetes including *S. cerevisiae* (Pardo, M., Monteoliva, L. et al., 2004), *C. albicans* (Martinez-Lopez, R., Monteoliva, L. et al., 2004; Martinez-Lopez, R., Park, H. et al., 2006) and *A. fumigatus* (Chabane, S., Sarfati, J. et al., 2006).

In *S. cerevisiae*, deletion of *ECM33* (*YBR078w*) resulted in hypersensitivity to cell wall perturbing agents and an increased amount of 1,6-β-glucan-linked proteins secreted to the culture medium. The deletion of *PST1* (*YDR055w*), which is a homologue of *ECM33*, did not show these effects. However, synergistic effects affecting the cell wall were found in the double gene deletion mutant of *ECM33* and *PST1*. The single gene deletion in *ECM33* and the double gene deletion in *ECM33* and *PST1* resulted in a stimulation of phosphorylation in Slt2 protein which is a signaling controller of cell wall integrity (Pardo, M., Monteoliva, L. et al., 2004).

In *C. albicans*, the deletion mutant of *ECM33* (orf19.3010.1) was sensitive to cell wall perturbing agents. It failed to form filaments in Spider medium and its blastospores were larger and more flocculated when compared to the wild-type strain. The cell wall architecture of the *Δecm33* mutant was abnormal because of the thickness of the internal layer of 1,3-β-glucan and chitin. *ECM33* deletion reduced the adherence and invasion capacity to endothelial cells and the FaDu oral epithelial cell line. Moreover, the *Δecm33* mutant showed less virulence in a murine model indicating that the *Ca*Ecm33 protein plays a role in *C. albicans* virulence (Martinez-Lopez, R., Monteoliva, L. et al., 2004; Martinez-Lopez, R., Park, H. et al., 2006; Martinez-Lopez, R., Nombela, C. et al., 2008; Rouabhia, M., Semlali, A. et al., 2012).

In the filamentous fungus *A. fumigatus*, the deletion of *ECM33* results in altered phenotypes as compared to the wild-type strain: (i) rapid conidial germination (ii) a defect in conidial separation, (iii) an increase of conidia size with an increase in the amount of chitin in the cell wall, (iv) conidia were sensitive to the absence of aeration during long-term storage, (v)

resistance to the cell pertubing agents, (vi) conidia were more resistant to killing by phagocytes, whereas the mycelium was more easily killed by neutrophils, and (vii) increased virulence in a mouse model. These results suggest that the *Af*Ecm33 protein contributes in key aspects of cell wall morphogenesis, cell wall integrity and plays a role in *A. fumigatus* virulence (Chabane, S., Sarfati, J. et al., 2006) (Romano, J., Nimrod, G. et al., 2006).

These primliminary findings indicate that the functionanl role of Ecm33 in *S. cerivisiae* is different from the function in *A. fumigatus*. However, so far there is no study that determinated the function of the abundant Ecm33 cell wall protein in the human pathogen *C. glabrata.*

Table 4. The effects of *ECM33* deletion in *S. cerevisiae*, *C. albicans*, and *A. fumigatus*

	Deletion of *ECM33* (Δ*ecm33*)*		
	S. cerevisiae[1]	*C. albicans*[2-4]	*A. fumigatus*[5-6]
1. Shape and size	rounder and larger	rounder and larger	larger
2. Cell perturbing agents	sensitive	sensitive	resistant
3. Cell wall architecture	increased 1,3 and 1,6-β-glucan levels	increased 1,3-β-glucans and chitin levels	increased chitin level
4. Hypha production	N/A	absent[a]	faster
5. Growth rate	ND	2-fold reduced	increased
6. Flocculation	ND	yes	yes
7. Adherence in cell lines	ND	decreased	no significant difference
8. Pathogenicity in mouse	N/A	decreased	increased

*: parental strain in each organism was used to compare a difference of phenotype.
[1]: (Pardo, M., Monteoliva, L. et al., 2004), [2-4]: (Martinez-Lopez, R., Monteoliva, L. et al., 2004; Martinez-Lopez, R., Park, H. et al., 2006; Martinez-Lopez, R., Nombela, C. et al., 2008), and [5-6]: (Chabane, S., Sarfati, J. et al., 2006; Romano, J., Nimrod, G. et al., 2006)
N/A: not applicable, ND: not determined.
[a]: yeast cells were grown in Spider medium for 7 days in 37^{o}C.

1.5 Aims of the study

C. glabrata is the second most frequently observed yeast in clinical specimen. In comparison to *C. albicans* infections, candidiasis caused by *C. glabrata* is more difficult to treat because this species is often resistant to azole antifungals and displays a high degree of adhesive capacities on clinically used catheter materials, facilitating the formation of biofilms.

Proteins of the Ecm33/Pst1 family in *C. glabrata* were experimentally shown to be highly abundant cell wall proteins of this organism. Previous studies indicate that the *ECM33* homologs in *S. cerevisiae*, *C. albicans* and *A. fumigates* are associated with integrity and architecture of the cell wall, adherence capacity, growth rate and with virulence, but the true molecular function of the proteins in these organisms is unknown. Furthermore, phenotypic analysis of *ECM33* deletion mutants in the different species indicates that the functional role of Ecm33 homologs might be different between the organisms. In *C. glabrata* the cell wall is of particular interest, because it holds key functions such as adhesion and detoxification of immune responses (e.g. the oxidative burst of attacking macrophages) during pathogenesis. Therefore, this study focuses on the characterization of the Ecm33 protein family of *C. glabrata*, where the cell wall architecture is significantly different to otherwise closely related fungi. In *C. glabrata*, this family consists of four members: Ecm33, Pst1, Sps2, and Sps22.

The aims of the present study were (i) to generate molecular tools for the study of this gene family in *C. glabrata* such as mutants, revertants, complemented strains, recombinant Ecm33-family proteins, and anti-Ecm33 antibodies, (ii) to characterize the function of Ecm33 and Pst1 of *C. glabrata* in relation to their orthologs in *C. albicans* and *S. cerevisiae* via comparison of the phenotypic differences among the *ECM33* and *PST1* deleted mutants and wild-type in all three organisms, and (iii) to gather data on the biological function of Ecm33 protein in *C. glabrata* by using the generated molecular tools.

CHAPTER 2

MATERIALS AND METHODS

2.1 Disposables

Disposables were purchased from Sarstedt (Nümbrecht, Germany), Greiner bio-one (Frickenhausen, Germany), Corning (Berlin, Germany), and Eppendorf (Hamburg, Germany).

2.2 Chemicals

- General chemicals were purchased from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), and Sigma-Aldrich (Munich, Germany).
- Restriction enzymes were purchased from New England BioLabs Inc (Frankfurt am Main, Germany).
- PCR purification and agarose gel extraction kits were purchased from Qiagen (Hilden, Germany)
- DNA and protein weight standards were purchased from Fermentas (Vilnius, Lithuania)
- Oligonucleotides were purchased from Sigma-Aldrich (Munich, Germany).
- Reagents for Real-time PCR (LightCycler®) were purchased from Roche (Mannheim, Germany)

2.3 Media

All liquid media were sterilized at 121°C for 15 minutes or by filtration (0.22µm pore size) and were kept at 4°C.

LB 1% [w/v] Bacto-tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl, 2% [w/v] agar (for LB agar) in ddH_2O.

For selection LB media, ampicillin (100 µg/ml) or Zeocin^{TM} (25 µg/ml) was added into media after autoclaving.

YPD 1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] dextrose, 2% [w/v] agar (for YPD agar) in ddH_2O.
For selection YPD media, Zeocin^{TM} (200 µg/ml) was added into media after autoclaving.

MM 1.34% [w/v] YNB, 2% [w/v] dextrose, 0.006% [w/v] histidine, 0.008% [w/v] leucine, 0.026% [w/v] tryptophan, 2% [w/v] agar (for MM agar) in ddH_2O.

RPMI 1640 1.04% [w/v] RPMI-1640, 2% [w/v] dextrose, 3.453% [w/v] 3-(N-morpholino)-propanesulphonic acid (MOPS) in ddH_2O.

BMMH 100 mM potassium phosphate pH6, 1.34% [w/v] YNB with ammonium sulfate, 0.00004% [w/v] biotin, 0.5% [v/v] methanol, 0.004% [w/v] histidine in ddH_2O.

2.4 Plasmids

Table 5. Plasmids used in this study

Code	Plasmid	Source
P1	pQE30	Qiagen
P2	pQE30:*CgECM33*	This study
P3	pPICZα	Invitrogen
P4	pPICZα:*CgECM33*	This study
P5	pPICZα:*CgPST1*	This study
P6	pBM51-Leu	Schwarzmüller T., Vienna*
P7	pBM51-Leu:*CgECM33*	This study
P8	pBM51-Leu:*CgPST1*	This study
P9	pBM51-Leu:*CgSPS2*	This study
P10	pBM51-Leu:*CgSPS22*	This study
P11	pBM51-Leu:truncated *CgECM33*	This study
P12	pBM51-Leu:*ScECM33*	This study
P13	pBM51-Leu:*CaECM33*	This study

*: (Schwarzmüller, T., 2009)

2.5 *E. coli* strains

Table 6. *E. coli* strains used in this study

Code	Strains	Plasmid	Gene of interest	Description
1	DH5α	-	-	Empty host
1.1	BL-21	-	-	Empty host
1.2	Rosetta	-	-	Empty host
2	DH5α	pQE30	-	Amplification of pQE30
3	DH5α	pPICZα	-	Amplification of pPICZα
4	DH5α	pPICZα	*CgECM33*	Amplification of pPICZα:*CgECM33*
5	DH5α	pPICZα	*CgPST1*	Amplification of pPICZα:*CgPST1*
6	DH5α	pQE30	*CgECM33*	Amplification of pQE30:*CgECM33*
7	BL-21	pQE30	*CgECM33*	Expression of Ecm33p in *E. coli*
8	Rosetta	pQE30	*CgECM33*	Expression of Ecm33p in *E. coli*
11	DH5a	pBM51-Leu	-	Amplification of pBM51-Leu
15	DH5a	pBM51-Leu	*ECM33*	Amplification of pBM51-Leu:*CgECM33* for revertant construction
17	DH5a	pBM51-Leu	*PST1*	Amplification of pBM51-Leu:*CgPST1* for revertant construction
25	DH5α	pBM51-Leu	*ScECM33*	Amplification of pBM51-Leu:*ScECM33* for complemented strain construction
26	DH5α	pBM51-Leu	*CaECM33*	Amplification of pBM51-Leu:*CaECM33* for complemented strain construction
27	DH5α	pBM51-Leu	truncated *CgECM33*	Amplification of pBM51-Leu:truncated *CgECM33* for complemented strain construction
28	DH5α	pBM51-Leu	*CgSPS2*	Amplification of pBM51-Leu:*CgSPS2* for complemented strain construction
29	DH5α	pBM51-Leu	*CgSPS22*	Amplification of pBM51-Leu:*CgSPS22* for complemented strain construction

-: none

2.6 Yeast strains

Table 7. Yeast strains used in this study

Code	Organisms	Strains	Source and reference	Description
Y15	*P. pastoris*	X33	Invitrogen	Wild-type
Y16	*P. pastoris*	GS115	Invitrogen	*his4*
Y17	*P. pastoris*	KM71H	Invitrogen	*arg4aox1:ARG4*
Y18	*P. pastoris*	GS115:Albumin	Invitrogen	Control for secreated protein
Y19	*P. pastoris*	GS115:LacZ	Invitrogen	Control for intracellular protein
Y20	*P. pastoris*	GS115: 1,6-β-glucanase	Bom IJ., Vlaardingen[1]	1,6-β-glucanase
Y21	*P. pastoris*	GS115:*CgECM33*	This study	Ecm33 protein expression
Y22	*P. pastoris*	GS115:*CgPST1*	This study	Pst1 protein expression
Y23	*P. pastoris*	X33:*CgECM33*	This study	Ecm33 protein expression
Y24	*P. pastoris*	X33:*CgPST1*	This study	Pst1 protein expression
Y25	*S. cerevisiae*	Auxotroph BY4741	Gil C., Madrid[2]	Background wild-type
Y26	*S. cerevisiae*	*Δecm33*	Gil C., Madrid[2]	FBEHO41-01A
Y27	*S. cerevisiae*	*Δecm33*	Gil C., Madrid[2]	BY4741
Y28	*S. cerevisiae*	*Δpst1*	Gil C., Madrid[2]	BY4741
Y29	*S. cerevisiae*	*Δpst1/Δecm33*, YP1-10C	Gil C., Madrid[2]	FBEHO41-01A
Y30	*S. cerevisiae*	*Δpst1/Δecm33*, YP1-1B	Gil C., Madrid[2]	FBEHO41-01A
Y31	*C. albicans*	Clinical strain	NRZ, Göttingen	Pathogen
Y34	*C. glabrata*	CBS138/ATCC2001	Schwarzmüller T., Vienna[3]	Wild-type
Y35	*C. glabrata*	Auxotroph *Δhtl*	Schwarzmüller T., Vienna[3]	Background wild-type
Y36	*C. glabrata*	*Δpst1*	Schwarzmüller T., Vienna	Single deletion mutant
Y37	*C. glabrata*	*Δecm33*	Schwarzmüller T., Vienna	Single deletion mutant
Y40	*C. glabrata*	*Δpst1/Δecm33*	Schwarzmüller T., Vienna	Double deletion mutant

Table 7. Yeast strains used in this study (continued)

Code	Organisms	Strains	Source or reference	Description
Y43	*C. glabrata*	*Δpst1:CgPST1*	This study	Completed revertant
Y45	*C. glabrata*	*Δecm33:CgECM33*	This study	Completed revertant
Y47	*C. glabrata*	*Δpst1/Δecm33:CgPST1*	This study	Incompleted revertant
Y49	*C. glabrata*	*Δpst1/Δecm33:CgECM33*	This study	Incompleted revertant
Y51	*C. albicans*	Auxotroph *Caf2*	Gil C., Madrid[4]	Background wild-type
Y53	*C. albicans*	*Δecm33:Δecm33*	Gil C., Madrid[4]	Homozygous mutant
Y55	*C. albicans*	*Δecm33:Δecm33:ECM33:ECM33*	Gil C., Madrid[4]	Revertant
Y60	*C. glabrata*	*Δecm33:ScECM33*	This study	Complemented strain
Y62	*C. glabrata*	*Δecm33:CaECM33*	This study	Complemented strain
Y64	*C. glabrata*	*Δecm33:CgPST1*	This study	Complemented strain
Y65	*C. glabrata*	*Δecm33:*truncated *CgECM33*	This study	Complemented strain
Y67	*C. glabrata*	*Δecm33:CgSPS2*	This study	Complemented strain
Y68	*C. glabrata*	*Δecm33:CgSPS22*	This study	Complemented strain

[1]: (Bom, I. J., Dielbandhoesing, S. K. et al., 1998)
[2]: (Pardo, M., Monteoliva, L. et al., 2004)
[3]: (Schwarzmüller, T., 2009)
[4]: (Martinez-Lopez, R., Monteoliva, L. et al., 2004).
NRZ: National Reference Center for Systemic Mycoses, Göttingen, Germany

2.7 Oligonucleotides

Table 8. Oligonucleotides used in this study

Code	Name	Sequence	Description
1	F-CG-LM	CCGAATTCCAGAACTCTACATCTGACGATGTTCC	Ecm33p expression in *Pichia pastoris*; EcoRI
2	R-CG-LM	CAGGTACCAGACTTCTTGGTCTTGGTGGAGGA	Ecm33p expression in *Pichia pastoris*; KpnI
3	F-CG-LE	CCGAATTCGCTAACACTACAGTCCCAGATGTT	Pst1p expression in *Pichia pastoris*; EcoRI
7	CG-LE-XbaI	CCTCTAGAAGCACCCTTGGACTTCTGCT	Pst1p expression in *Pichia pastoris*; XbaI
8	For-AOX1	GACTGGTTCCAATTGACAAGC	Checking of the insert fragment size in pPICZα
9	Rev-AOX1	GCAAATGGCATTCTGACATCC	Checking of the insert fragment size in pPICZα
10	alpha-Factor	TACTATTGCCAGCATTGCTGC	Checking of the insert fragment size in pPICZα
11	hk2	CGTCAAGACTGTCAAGGAGGG	Checking of the mutant clones
12	hk3	CATCATCTGCCCAGATGCGAAG	Checking of the mutant clones
13	F-MuE-Cont	AGCGTTTATTTGGTAGAAGGTG	Checking of the mutant clones
14	R-MuE-Cont	CTTCAAGTCCTGAAAGTTGC	Checking of the mutant clones
15	F-MuM-Cont	GATATGATGGATCCCGTGTC	Checking of the mutant clones
16	R-MuM-Cont	TTTTATGGGTTCCATCAGAG	Checking of the mutant clones
17	F-LM-pQE	CCGGATCCCAGAACTCTACATCTGACGATGTTCC	Ecm33p expression in *E.coli*; BamHI
18	R-LM-pQE	CACTGCAGAGACTTCTTGGTCTTGGTGGAGGA	Ecm33p expression in *E.coli*; PstI
19	RT-ECM33-F	CAACACCGTCTCCGGTAACT	Reverse transcription-Real time-PCR
20	RT-ECM33-R	GTCTTGGTGGAGGAAGTGGA	Reverse transcription-Real time-PCR
21	RT-PST1-F	CCGTCTCCTTCGCTAACTTG	Reverse transcription-Real time-PCR
22	RT-PST1-R	AGAAGTTACCGGCCTTGGTT	Reverse transcription-Real time-PCR
23	RT-SPS2-F	CAATTGTTAGAGGCGGGAAA	Reverse transcription-Real time-PCR
24	RT-SPS2-R	TCGCGCTGTTCTCTTGTCTA	Reverse transcription-Real time-PCR
25	RT-SPS22-F	TTCCGGTGGTCTCATGATCT	Reverse transcription-Real time-PCR
26	RT-SPS22-R	CTACGGACTTGCCCCTGATA	Reverse transcription-Real time-PCR
27	RT-ACT1-F	TTCCAGCCTTCTACGTTTCC	Reverse transcription-Real time-PCR
28	RT-ACT1-R	TCTACCAGCAAGGTCGATTC	Reverse transcription-Real time-PCR
29	RT-SLT2-F	AATTTGTGATTTCGGGCTTG	Reverse transcription-Real time-PCR
30	RT-SLT2-R	TCCCCTTGAATAACGGTTTG	Reverse transcription-Real time-PCR

Table 8. Oligonucleotides used in this study (continued)

Code	Name	Sequence	Description
31	1F-Rev-ECM	CCCTCGAGATGAAGGTCACATCATTGGT	Generation of *ECM33* revertant; XhoI
32	2R-Rev-ECM	CAGGATCCTCATAGCAAAGCGACGGCGA	Generation of *ECM33* revertant; BamHI
33	3F-Rev-PST	CCCTCGAGATGCAATTGAATCATCTTTTG	Generation of *PST1* revertant; XhoI
34	4R-Rev-PST	CAGGATCCCTATATTAGAGCCAAAGCAAC	Generation of *PST1* revertant; BamHI
43	M13 F	GTAAAACGACGGCCAGT	For Topo2.1 sequencing
44	M13 R	CAGGAAACAGCTATGAC	For Topo2.1 sequencing
53	F-Seq	GTTTTCCCAGTCACGACGTT	Checking of the inserted fragment in pBM51-Leu
54	R-Seq	CCAAGGGGGTGGTTTAGTTT	Checking of the inserted fragment in pBM51-Leu
57	F_Ca_Xho	CACTCGAGATGCAATTCAAGAACATTCTTGC	Generation of *CaECM33* complemented strain; XhoI
58	R_Ca_Bam	CAGGATCCTTAGAATAAAGCAACACCAACAGC	Generation of *CaECM33* complemented strain; BamHI
61	F_Sc_Xho	CACTCGAGATGCAATTCAAGAACGCTTTGAC	Generation of *ScECM33* complemented strain; XhoI
62	R_Sc_Bam	CAGGATCCTTATAGTAAGGCAACGCCAACAG	Generation of *ScECM33* complemented strain; BamHI
69.2	F-CgSps22_Sal	CCGTCGACATGAAGCTTATTTTTGTTGC	Generation of *CgSPS22* complemented strain; SalI
70	R-CgSps22_Bam	CCGGATCCCTAAAAAAATAGTGCACTCG	Generation of *CgSPS22* complemented strain; BamHI
71.2	F-CgSps2_Xho	CACTCGAGATGCACTGCAAGTCACCAAAC	Generation of *CgSPS2* complemented strain; XhoI
72	R-CgSps2_Bam	CCGGATCCTTAAAACAGTATCAAATACATG	Generation of *CgSPS2* complemented strain; BamHI
75	R_truncatedECM	CAGGATCCAGACTTCTTGGTCTTGGTGGAGGA	Generation of truncated *CgECM33* complemented strain; BamHI
76	RT-R-Ca	GAACGGTGCCACATCTACCT	Reverse transcription-Real time-PCR
77	RT-F-Ca	CTGGAGCAGCACCCTTAGAC	Reverse transcription-Real time-PCR
78	RT-R-Sc	TGCTAACGGTTCATTTGCTG	Reverse transcription-Real time-PCR
79	RT-F-Sc	ATGTTGACGGAACCACCAAT	Reverse transcription-Real time-PCR

2.8 *In silico* analysis

Ecm33, Pst1, Sps2, and Sps22 protein sequences of *C. glabrata* were taken from Genomic Exploration of the Hemiascomycete Yeasts (http://www.genolevures.org) and aligned with MEGA4 program. Signal peptide were predicted by using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Omega sites for GPI anchor attachment were predicted by using the big-PI Predictor server (http://mendel.imp.ac.at/gpi/gpi_server.html).

The following programs were used in this study:

- *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org)
- Candida Genome Database (CGD) (http://www.candidagenome.org)
- Genomic Exploration of the Hemiascomycete Yeasts (http://www.genolevures.org)
- Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov)
- The translation of a nucleotide (DNA/RNA) sequence to a protein sequence (http://web.expasy.org/translate/)
- Protein Molecular Weight Calculator (http://www.sciencegateway.org/tools/proteinmw.htm)
- NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/)
- Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html)
- Primer 3 program for designing PCR primers (http://frodo.wi.mit.edu)
- PlasMapper Version 2.0 for plasmid drawing (http://wishart.biology.ualberta.ca/PlasMapper/index.html)
- SignalP 4.1 server for signal peptide prediction (http://www.cbs.dtu.dk/services/SignalP/)
- big-PI Predictor for GPI Modification Site Prediction (http://mendel.imp.ac.at/gpi/gpi_server.html)

2.9 Generation of knock out strains

The two single mutants of *PST1* (*Δpst1*) and *ECM33* (*Δecm33*) and the *PST1/ECM33* double mutant (*Δpst1*/*Δecm33*) in *C. glabrata* were generated and provided by Tobias Schwarzmüller, Vienna, Austria.

Briefly, fusion PCR was used to generate both single mutants and double mutant. The dominant marker *NAT1* was amplified from plasmid pJK863 (Shen, J., Guo, W. et al., 2005) with primers fp_NAT1-U2 (5'-CGTACGCTGCAGGTCGACagcttgcctcgtccccgccg-3') and rp_NAT1-D2 (5'-CTACGAGACCGACACCGctggatggcggcgttagtatcg-3') to add the two 20 bp adaptor sequences U2 and D2. The PCR product was ligated into pGEM-T vector (Promega, Mannheim, Germany), generating plasmid pTS50.

Fusion PCR was used to generate the deletion cassettes (Wach, A., 1996; Noble, S. M. and Johnson, A. D., 2005) (Figure 5 A). The 500 bp long flanking homology regions were amplified from *C. glabrata* strain ATCC2001 genomic DNA with primer pairs 5'5'/5'3' and 3'5'/3'3' adding the adaptor sequence (U1/D1) of 20 bp and purified by ethanol precipitation. A 50 µl reaction (50 mM KCl, 10 mM Tris-HCl (pH 9.0, 25°C), 0.1% TritionX-100, 1.5 mM MgCl2, 0.2 µM dNTPs, 0.5 µM each primer, 1 unit *Taq*-Polymerase and genomic wild-type DNA from strain ATCC2001) was subjected to cycling (93°C for 5 minutes, 35 cycles 93°C for 30 seconds, 45°C for 30 seconds, 72°C for 90 seconds, finally 10 minutes at 72°C) in a thermocycler machine.

The dominant marker *NAT1* was amplified from plasmid pTS50 in a separated PCR reaction using primers 5M and 3M, adding unique barcode tags and complementary U1 and D1 adaptor sequences (Figure 5 B). The marker fragment was gel-purified over a 0.7% agarose gel. A 50 µl reaction (50 mM KCl, 10 mM Tris-HCl (pH 9.0, 25°C), 0.1% TritionX-100, 1.5 mM MgCl2, 0.2 µM dNTPs, 0.5 µM each primer, 1 unit *Taq*-Polymerase and plasmid TS50) was subjected to cycling (93°C for 3 minutes, 32 cycles 93°C for 30 seconds, 49°C for 30 seconds, 72°C for 2.5 minutes, finally 10 minutes at 72°C) in a thermocycler machine.

The fusion PCR was carried out in a 50 µl volume with the same condition as above (1x Ex*Taq* buffer, 0.2 µM dNTPs, 0.5 µM each primer, 2.5 units Ex*Taq*-Polymerase (TaKaRa, Darmstadt, Germany) and 3 µl marker fragment, and 1.25 µl each flanking homology fragment) and subjected to cycling (93°C for 3 minutes, 35 cycles 93°C for 30 seconds, 45°C for 30

seconds, 72°C for 3 minutes, finally 10 minutes at 72°C.) The final deletion construct was purified by ethanol precipitation (Schwarzmüller, T., 2009).

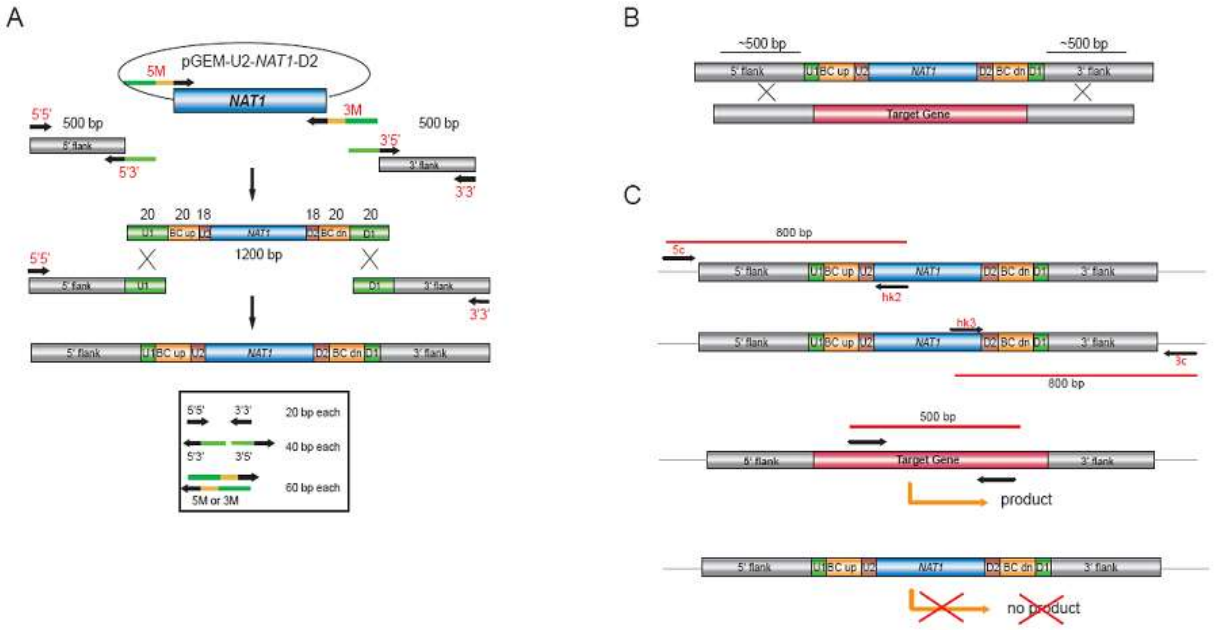

Figure 5. The sequential steps for generation of *C. glabrata* gene deletion cassettes

(A): Fusion PCR using the dominant selectable marker *NAT1* was applied to generate gene deletion cassettes. The 5' and 3' flanking sequences of target genes were integrated in oligonucleotides to amplify the marker fragment and to add overlap sequences. (B): Gene disruption by replacement of the target gene based on homologous recombination. *C. glabrata* strain *Δhtl* was transformed by an electroporation method. (C): Verification of correct integration of the deletion cassette by colony PCR (forward primer outside flanking region and reverse primer inside marker) checking both junctions. Loss-of-gene PCR (internal primer pair) was used to confirm that the coding sequences has not reintegrated in the genome. Reintegration, indicating that the CDS remained in the genome, gave a 500 bp PCR product. Figure taken from (Schwarzmüller, T., 2009).

2.10 Generation of revertants and complemented strains in *C. glabrata*

The set of full revertants (*Δpst1:CgPST1* and *Δecm33:CgECM33*), incompleted revertants (*Δpst1/Δecm33:CgPST1* and *Δpst1/Δecm33:CgECM33*) and complemented strains (*Δecm33*:truncated *CgECM33*, *Δecm33:CgSPS2*, *Δecm33:CgSPS22*, *Δecm33:CgPST1*, *Δecm33:CaECM33* and *Δecm33:ScECM33*) were generated by using the target gene cloned to pBM51-Leu. The expression plasmid contains an autonomously replicating sequence (ARS), a

centromere (CEN), a leucine selectable marker of *C. glabrata* and a *PGK1* (3-phosphoglycerate kinase 1) promotor of *S. cerevisiae* (*ScPGK1*) as shown in Figure 6. This system can restore gene expression in the generated knock out strains (Ma, B., Pan, S. J. et al., 2007; Schwarzmüller, T., 2009).

All of the inserted fragments were amplified with specific primers including adaptors for restriction enzyme sites from CBS138/ATCC2001 genomic DNA. A 50 µl reaction (5 µl of 10x KOD *Taq* buffer, 5 µl of 2 mM dNTPs, 3 µl of 25 $MgCl_2$, 1 µl of 20 µM each primer, 1 µl of KOD *Taq*-Polymerase (1U/µl; Novagen, Darmstadt, Germany), and 2 µl of genomic DNA (50 ng/µl)) was subjected to cycling (95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, XX°C (see annealing temperature in Table 9) for 30 seconds, 70°C for 30 seconds, finally 5 minutes at 70°C in a thermocycler machine. The size of the inserted PCR products are shown in Table 9.

The pBM51-Leu and the inserted fragments were digested with a restriction enzyme. After digestion, the fragments and linearized pBM51-Leu plasmid were purified by using QIAquick Gel Extraction Kit (Qiagen). The ligation between the fragments and linearized pBM51-Leu plasmid were linked by using T4 DNA ligase (Thermo Scientific, MA, USA) as follows: linear vector 50 ng, inserted DNA fragment 250 ng, 2 µl of 10X T4 DNA ligase buffer and 1 µl of T4 DNA ligase (1U/µl). The mixtures were incubated at 25°C for 1 hour and kept in 4°C for overnight. Five µl of the mixture was used for transformation in the competent DH5α *E. coli* using a heat shock transformation protocol (see the detail protocol in 2.10.1 and 2.10.2). The transformant DH5α *E. coli* was selected by using ampicillin drug resistance.

The positive DH5α clones were verified with two approaches as restriction enzyme digestion and DNA sequencing.

Plasmids from pBM51-Leu no. P7-P13 were extracted and used to transform *C. glabrata* by electroporation (see the detail protocol in 2.10.3 and 2.10.4). *C. glabrata* transformats were selected by growth on minimal medium agar without leucine.

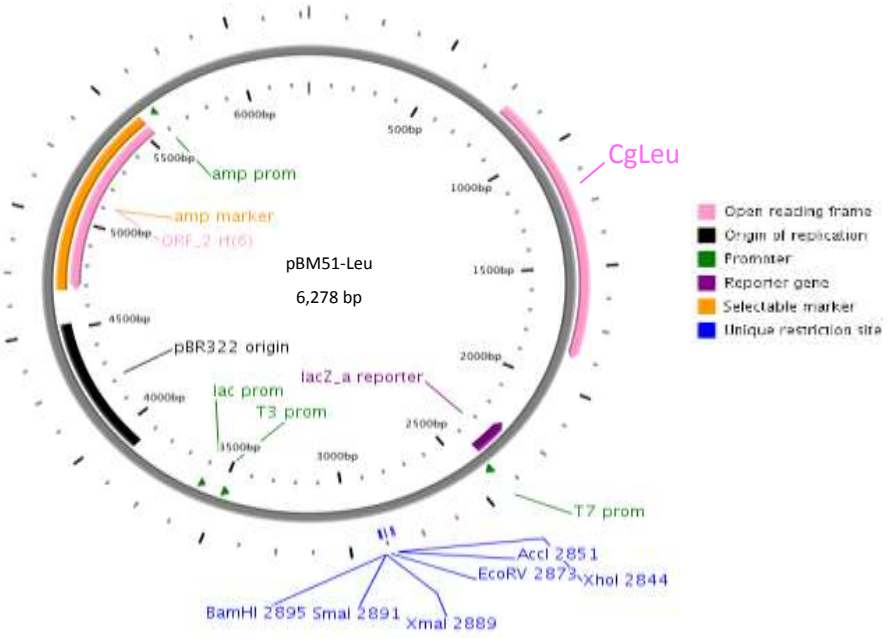

Figure 6. Map of pBM51-Leu

pBM51-Leu map was drawn from online program PlasMapper Version 2.0 (http://wishart.biology.ualberta.ca/PlasMapper/index.html). The pBM51-Leu can be replicated in *E. coli* using pBR322 origin of replication and expressed in *S. cerevisiae* and *C. glabrata* using strong promotor of *S. cerevisiae*. The pBM51-Leu's multiple cloning site contains the following restriction sites: *XhoI*, *AccI*, *EcoRV*, *XmaI*, *SmaI*, and *BamHI*. The marker for selection in *E. coli* is ampicillin resistant and in yeast cell is leucine production.

Table 9. Generation of revertants and complemented strains in *C. glabrata*

Gene	Size (bp)	Primer code	Annealing temp. (^{o}C)	Restriction enzyme
CgECM33	1266	31 and 32	55	*XhoI/BamHI*
CgPST1	1290	33 and 34	55	*XhoI/BamHI*
truncated *CgECM33*	1185	31 and 75	55	*XhoI/BamHI*
CgSPS2	1251	71.2 and 72	58	*XhoI/BamHI*
CgSPS22	1377	69.2 and 70	58	*SalI/BamHI*
CaECM33	1272	57 and 58	60	*XhoI/BamHI*
ScECM33	1290	61 and 62	60	*XhoI/BamHI*

2.10.1 Preparation of competent *E. coli*

A single colony of *E. coli* was grown in 5 ml LB in glass tube at 37°C for overnight with shaking. One ml of overnight culture was inoculated into 100 ml LB in a 250 ml flask and incubated at 37°C for 1.5-3 hours until an OD600 of 0.5. These cells were put on ice for 15 minutes and the cells were collected by centrifugation for 3 minutes at 1,942 x g (3,000 rpm). The cell pellet was treated with cold 0.1 M $CaCl_2$, incubated on ice for 20 minutes and centrifuged to keep the cell pellet. The cell pellet was resuspended with 2 ml cold 0.1 M $CaCl_2$ in 20% glycerol and aliquots of 100 µl competent cells were stored at -80°C (Sambrook, J., Russell, D.W., 2001).

2.10.2 Transformation of *E. coli*

Fifty ng of circular plasmid was added into 100 µl of competent *E.coli* suspension and incubated on ice for 30 minutes. Then a heat shock was applied to the cells for 2 minutes at 42°C in a water bath before the cells were put back on ice. 500 ml of LB was added to the tube and incubated at 37°C for an hour and then plated on LB agar with selective antibiotic drug (Sambrook, J., Russell, D.W., 2001).

2.10.3 Preparation of competent yeast cell

A combination of procedures modified from three protocols was used to prepare the competent *C. glabrata* and *P. pastoris* cells (Wu, S. and Letchworth, G. J., 2004; Lin-Cereghino, J., Wong, W. W. et al., 2005; Gietz, R. D. and Schiestl, R. H., 2007). A single colony of yeast was grown overnight in 5 ml YPD in a glass tube at 30°C with shaking. One ml of an overnight culture was inoculated into 100 ml YPD in 250 ml flask and incubated additionally at 30°C for 3-5 hours until an OD600 of 0.8 – 1.0 was reached. The cells were put on ice for 15 minutes and harvested by centrifugation for 3 minutes at 486 x g (1,500 rpm). The cell pellet was treated with 10 ml cold transformation buffer (7.9 ml of TEDS, 2 ml of 500 mM, and 100 µl of 1M Dithiothreitol (DTT)), incubated on ice for 15 minutes and centrifuged to result in a cell pellet.

The cell pellet was washed with 10 ml cold 1 M Sorbitol and resuspended with 2 ml cold 1 M Sorbitol. Aliquots of 100 µl yeast competent cells were stored at -80°C

2.10.4 Electroporation of yeast

5 µl (50 – 100 ng) of *Pme*I linearized plasmid in case of *P. pastoris* or circular plasmid in case of *C. glabrata* and 100 µl of competent yeast cells was mixed together and loaded into a 1 mm gap electroporation cuvette (PeqLab Biotechnologie GmbH, Erlangen, Germany). The cuvette was incubated on ice for 5 minutes and was electroporated (parameters: charging voltage 1,500 V, resistance 200 Ω and capacitance 25 µF). Immediately after electroporation, one ml cold 1 M sorbitol was added into cuvette and incubated in 30°C for 1 hour, and then plated on minimal media (Lin-Cereghino, J., Wong, W. W. et al., 2005).

2.11 Reverse transcription real-time PCR

The total RNA was isolated using the RNeasy Mini Kit (Qiagen) from cells that were isolated from mid log-phase (OD600 of 0.8 – 1.2). The cells were broken using a mechanical disruption protocol with a Fast Prep 120 machine (RNeasy Mini Handbook page 49 -51) and the RNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, Germany) (Qiagen, 2006; Bader, O., Schwarz, A. et al., 2012)

One µg of total RNA was treated with DNase I (Fermentas) to eliminate genomic DNA and the reaction was stopped by EDTA solution. The pure RNA was reverse transcribed to complementary DNA (cDNA) by using First Strand cDNA Synthesis Kit with the oligo (dT) 18 primer (Fermentas, 2009).

LightCycler® Real-time PCR from Roche system was used for measurement of the level of gene expression. The conditions for a 20 µl reaction were as follows: 4 µl of Master mix, 1 µl of 20 µM each primer, and 2 µl of cDNA (20 ng/µl); 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, 50°C for 10 seconds, 72°C for 10 seconds (Roche, 2007). The value of gene expression was normalized against the two housekeeping genes *ACT1* and *SLT2* in all analyses.

2.12 Growth assay

C. glabrata, *C. albicans* and *S. cerevisiae* cells were grown at 37°C for overnight in a shaking incubator. Cells from the overnight culture were harvested by centrifugastion, washed with ddH_2O and adjusted to a density of 0.5 McFarland ($5X10^6$ cells/ml) in 0.85% NaCl. The adjusted cell suspension was diluted 10-folds (1 ml cell suspension in 9 ml 0.85% NaCl). 100 µl of the diluted cell suspension was added into 96 well plates containing 2-fold concentrated RPMI1640 or YPD. For each strain, growth of three yeast suspensions were monitored at 30°C and 37°C by measuring the OD630 every 3 hours for 36 hours.

2.13 Susceptibility testing to cell perturbing agents

2.13.1 Agar drop dilution assay

Susceptibility testing to cell perturbing agents (Caffeine, Calcofluor white, and Congo red) was performed by agar drop dilution. To prepare the inoculum, yeast cells from agar plates were added to sterile 0.85% NaCl to a density of 0.5 McFarland (5×10^6 cells/ml), diluted 10-fold in sterile 0.85% NaCl. Five µl of the diluted cell suspension was dropped onto agar plate containing cell perturbing agents. The plates were incubated at 30°C for 24 hours in case of *C. glabrata* and 48 hours in case of *C. albicans* and *S. cerevisiae*

2.13.2 Broth micro-dilution assay

Susceptibility testing to cell wall perturbing agents (Calcofluor white, Congo red, Sodium dodecyl sulfate (SDS), Hygromycin B, Brefeldin A, Nikkomycin Z, and Lufenuron) was performed by broth micro-dilution. To prepare the inoculum, cells from agar plates were added to 0.85% NaCl to a density of 0.5 McFarland (5×10^6 cells/ml), diluted 10-fold in 0.85% NaCl and 100 µl of the cell suspension was added to an equal volume of RPMI1640 containing the cell wall perturbing agent. 96 well plates with two-fold serial dilutions of cell wall perturbing agents were incubated at 37°C for 24 hours for *C. glabrata* and for 48 hours for *S. cerevisiae* and *C. albicans*. The cell densities were measured in a spectrophotometer at absorbance 630 (Dynex

Technologies GmbH, Denkendorf, Germany). The minimum inhibitory concentration (MIC) 50 and MIC90 were calculated from the value of absorbance 630 in analogy to the EUCAST methodology (EUCAST, 2008).

2.14 Alcian blue binding assay

Alcian blue is a positively charged, blue colored dye, which binds to negative charges on the cell wall surface. The Alcian blue binding assay was adapted from two previous publications (Odani, T., Shimma, Y. et al., 1996; Hobson, R. P., Munro, C. A. et al., 2004). The overnight culture of yeast cells in YPD were adjusted to an OD600 of 1 in sterile PBS and centrifuged to collect the cells. The cell pellets were suspended in 1 ml of 30 µg/ml Alcian Blue in 0.02 M HCl (pH 3.0), incubated at room temperature for 10 minutes, and pelleted by centrifugation. 100 µl of supernatant was transferred into 96 well plates to measure the absorbance 630 nm by using a spectrophotometer. Alcian blue concentrations were determined by reference to a standard curve. Alcian Blue binding was calculated according to the formula: $x = (u–v) \div n$ where x = Alcian blue binding, u = original Alcian blue concentration (µg/ml), v = final Alcian blue concentration (µg/ml), and n = amount of yeast cells in OD600.

2.15 Flow cytometry measurement

2.15.1 Glucan staining assay

Congo red was used to stain the glucan of the yeast cell wall. Cells from 1 ml of an overnight culture were harvested and washed twice with PBS. Cells were resuspended in 1 ml of 1 M NaOH, boiled at 95°C for 5 minutes, washed again twice with PBS and incubated for 30 minutes at room temperature in 300 µl Congo Red staining solution (200 mg/ml). After washing, stained cells were resuspended in 500 µl of water (Bader, O., Schwarz, A. et al., 2012).

2.15.2 Chitin staining assay

Wheat-germ agglutinin (WGA–Alexafluor 647 conjugate (Invitrogen, OR, USA) was used to stain the chitin of the yeast cell wall. Cells from 1 ml of an overnight culture were

harvested and washed twice with PBS. Cells were resuspended in 1 ml of 1 M HCl, boiled at 95°C for 5 minutes and then washed again twice with PBS. Cells were resuspended in 300 µl WGA staining solution (50 µg/ml), incubated for 30 minutes at room temperature and washed again twice. Stained cells were resuspended in 500 µl water (Bader, O., Schwarz, A. et al., 2012).

After staining, flow cytometric analyses were performed with a FACS Calibur flow cytometer using Cell QuestPro software (BD-Biosciences, NJ, USA). All values were calculated as the mean average fluorescence intensity of 12,000 events in three independent experiments (Bader, O., Schwarz, A. et al., 2012).

2.16 Biofilm properties determination

2.16.1 Hydrophobicity assay

The overnight culture of yeast cells in YPD were washed with ddH_2O and adjusted to OD600 of 1 in sterile water. Two ml of cell suspensions were mixed with 2 ml Octane in glass tube with rubber cover and rigourously vortexed for 30 seconds. After the separation of the two layers, one ml of the bottom aqueous phase was taken to measure the OD600 by using spectrophotometer (de Groot, P. W., Kraneveld, E. A. et al., 2008). Hydrophobicity was calculated as the percentage of cells removed from the aqueous phase.

2.16.2 Polystyrene adhesion assay

Cells were grown in YPD for 24 hours, after that the OD600 was adjusted to 2, and 50 µl of these cell suspensions were transferred into 150 µl fresh YPD medium in polystyrene 96-wells plates (Greiner bio-one). After 24 hours of incubation at 37^{o}C, non-adherent cells were washed out with sterile water and the adherent cells were stained with 0.1% Crystal violet for 5 minutes. Excess Crystal violet was washed away and the cells were lysed with a solution containing 50% ethanol and 1% SDS. The amount of adherent cells was determined by measuring crystal violet released from the lysed cells as the absorbance at 630 nm (Bader, O., Schwarz, A. et al., 2012).

2.16.3 Silicone adhesion assay

1x1 cm. of silicone pieces (AMT Aromando, Duesseldorf, Germany) were incubated for 24 hours at 37^{o}C in 12-wells plates containing 2 ml YPD inoculated at a cell density of 0.08 McFarland with the *C.glabrata* strains. To remove unbound cells by aspiration, silicone pieces were transferred into fresh 12-wells plates containing 1 ml PBS. Bound cells were scraped off, resuspended in 1.2 ml PBS, and quantified by measuring the OD600 (Bader, O., Schwarz, A. et al., 2012).

2.17 Protein expression in *P. pastoris*

Ecm33 and Pst1 protein expression in *Pichia pastoris* was performed using the EasySelectTM Pichia Expression Kit (Invitrogen, CA, USA). Both proteins were expressed without the C-terminal signal peptides and ω-sites of the N-terminal regions.

The inserted fragments of *ECM33* and *PST1* were amplified from *C. glabrata* CBS138/ATCC2001 genomic DNA with specific primers including adaptors for restriction enzyme sites. A 50 µl reaction (5 µl of 10x KOD Taq buffer, 5 µl of 2 mM dNTPs, 3 µl of 25 $MgCl_2$, 1 µl of 20 µM each primer, 1 µl of KOD Taq-Polymerase (1U/µl; Novagen), and 2 µl of genomic DNA (50 ng/ µl)) was subjected to cycling (95°C for 5 minutes, 35 cycles 95°C for 30 seconds, 58°C for 30 seconds, 70°C for 30 seconds, finally 5 minutes at 70°C) in a thermocycler machine. The expected PCR product size of each gene is shown in Table 10. The PCR product of target gene will be inserted at multiple cloning site of pPICZα vector as shown in Figure 7.

The pPICZα and the inserted fragments were digested with restriction enzyme (*EcoRI* and *KpnI* for *ECM33* and *EcoRI* and *XbaI* for *PST1*) and the modified fragments and pPICZα linearlized plasmid purified using the QIAquick Gel Extraction Kit (Qiagen). The ligation of the modified fragments and pBM51 linearlized plasmid were ligated by using T4 DNA ligase (Thermo Scientific) as follows: linear vector 50 ng, inserted DNA 250 ng, 2 µl of 10X T4 DNA ligase buffer and 1 µl of T4 DNA ligase (1U/µl of). The mixtures were incubated at 25°C for 1 hour and kept in 4°C for overnight. A 5 µl of the mixture was used for transformation in the competent DH5α *E. coli* with heat schock transformation protocol (see the detail protocol in

2.10.1 and 2.10.2). The transformant DH5α *E. coli* was selected by using Zeocin^{TM} (Invitrogen, CA, USA) drug resistance.

Positive DH5α clones were verified by restriction enzyme double digestion and DNA sequencing. The pure recombinant pPICZα no. P4 and P5 were extracted and used to transform methylotrophic *P. pastoris* by electroporation (see the detail protocol in 2.10.3 and 2.10.4). The transformant *P. pastoris* was selected by using YPD agar containing Zeocin^{TM}. The recombinant *P. pastoris* can grow in this selective YPD agar because it contains Zeocin resistant gene.

2.17.1 Methanol induced Ecm33 and Pst1 protein expression

ECM33 and *PST1* genes were transformed to the *P. pastoris* strain X33 (wid-type) and GS115 (*his4*). For expression, a representative single colony of each of the four recombinant strains (GS115:*CgECM33*, GS115:*CgPST1*, X33:*CgECM33* and X33:*CgPST1*) was grown in 25 ml BMGH (buffered minimal glycerol histidine) in a 250 ml flask and incubated at 30°C for overnight that these cells will be in log-phase growth. These cells were harvested by centrifugation, resuspended and adjusted to an OD600 of 1 in 100 ml BMMH (buffered minimal methanol histidine) media in 500 ml flask. Then, the cell suspensions were incubated at 30°C in shaking incubator 250 rpm for induction of *ECM33* and *PST1* expression.

One ml of cell suspensions were collected at 24, 48, 72, 96, and 120 hours to determine the time points that express the highest amounts of proteins in the four recombinant strains by using SDS-PAGE (Invitrogen, 2009).

2.17.2 SDS-Polyacrylamide gel electrophoresis

All components of separating gel were mixed and pipetted into assembled gel plates of a BioRad Mini Protean II system. Then, 100 µl of butanol was added onto the top of gel to make a straight top of separating gel and the gel allowed to polymerize. The gel was cleaned by removing butanol and washing with water. All components of collective gel were mixed and pipetted onto the separating gel and that the comb was put into the gel and the gel allowed to polymerize (SDS-PAGE gel preparation is shown in Table 11). SDS-PAGE gels were stored at 4^{o}C in plastic bag until use.

The protein solution was mixed with 5X loading buffer and boiled at 95°C for 10 minutes then loaded onto the SDS-PAGE gel. Coomassie blue was used to stain the proteins in the SDS-PAGE gels.

Table 10. Generation of recombinant *ECM33* and *PST1* plasmids for protein expression

Gene	Size (bp)	Primer code	Annealing temp. (^{o}C)	Restriction enzyme
CgECM33[1]	1,150	1 and 2	58	*Eco*RI/*Kpn*I
CgPST1[1]	1,183	3 and 7	58	*Eco*RI/*Xba*I
CgECM33[2]	1,146	17 and 18	58	*BamH*I/*Pst*I

[1]: protein expression in *P. pastoris*.
[2]: protein expression in *E. coli*.

Table 11. SDS-PAGE gel preparation

	Collective gel	Separating gel		
	5%	10%	12%	15%
Water	1.4 ml	2.95 ml	2.45 ml	1.7 ml
Buffer	630 µl	1.9 ml	1.9 ml	1.9 ml
Acrylamide solution[a]	415 µl	2.5 ml	3 ml	3.75 ml
10% SDS	25 µl	75 µl	75 µl	75 µl
10% APS	25 µl	75 µl	75 µl	75 µl
TEMED	2.5 µl	3 µl	3 µl	3 µl

[a]: 30 % acrylamide and bisacrylamide stock solution at a ratio of 37.5:1

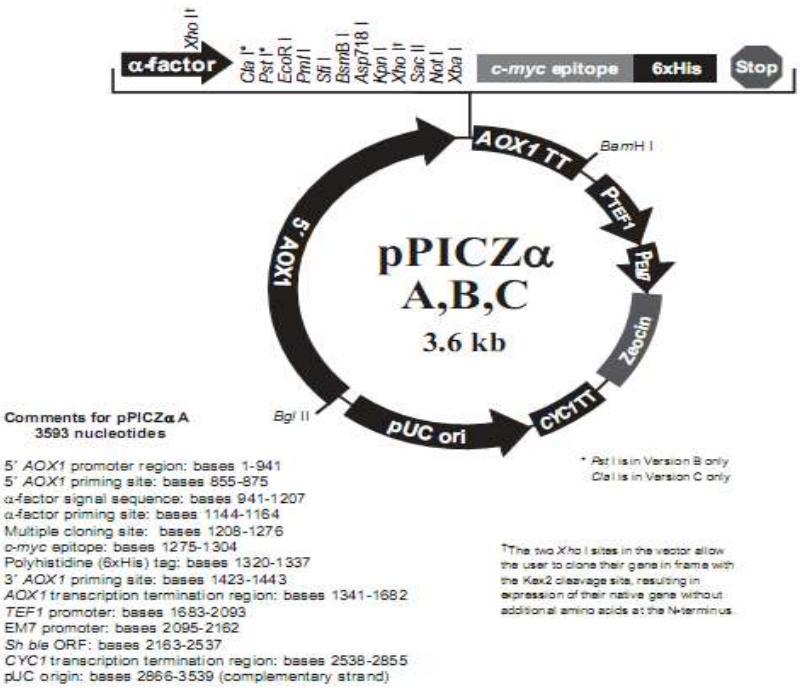

Figure 7. Map of pPICZ*α*

pPICα plasmid is 3.6 kilobases vector that used to express and secrete recombinant proteins in *P. pastoris*. Recombinant protein is expressed as fusions to an N-terminal peptide encoding the *S. cerevisiae* α-factor secretion signal. This plasmid allows high-level, methanol inducible expression of the gene of interest in *P. pastoris*. pPICα plasmid contains the following elements: AOX1 promoter for methanol-induced expression of the gene of interest, α-factor secretion signal for directing secreted recombinant protein expression, Zeocin resistance gene for selection, C-terminal peptide containing the c-myc epitope and a polyhistidine (6xHis) tag for detection and purification. Figure taken from (Invitrogen, 2009).

2.18 Protein deglycosylation

N-glycosidase (Endo-α-N-Acetylgalactosaminidase; New England BioLabs Inc.) and O-glycosidase (PNGase F; New England BioLabs Inc.) were used to be a tool to eliminate N-glycan and O-glycan, respectively. The mixture of substrate and glycosidase enzyme was incubated at 37^{o}C for 2 hours according to the manufacturer's instructions.

2.19 Protein expression in *E. coli* and Anti-Ecm33 antibody production

2.19.1 Cloning of *ECM33* gene in *E. coli*

F-LM-pQE and R-LM-pQE primers (Table 8) were used to amplify the *ECM33* gene of *C. glabrata* without the signal peptide and the GPI-anchoring site. A 50 µl reaction (5 µl of 10x KOD Taq buffer, 5 µl of 2 mM dNTPs, 3 µl of 25 MgCl2, 1 µl of 20 µM each primer, 1 µl of KOD Taq-Polymerase (1U/µl; Novagen) and 2 µl of genomic DNA (50 ng/ µl)) was subjected to cycling (95°C for 5 minutes, 35 cycles of 95°C for 30 second, 58°C for 30 second, 70°C for 30 second, finally 5 minutes at 70°C.) in a thermocycler machine.

The PCR product of *ECM33* (product size 1,146 bp) was cloned into the pQE30 expression vector (Figure 8) by using *BamH*I and *Pst*I restriction enzymes as shown in Table 10. The recombinant plasmid pQE30:*CgECM3*3 was transformed to the *E. coli* strain DH5α for plasmid multiplication and BL-21 for Ecm33 protein expression. Ampicillin resistance was used to select the positive clones (Qiagen, 2003). The detail of the ligation, *E. coli* competent preparation, and transformation were described in 2.10.1 and 2.10.2.

The positive DH5α clones were verified by using restriction enzyme double digestion and DNA sequencing.

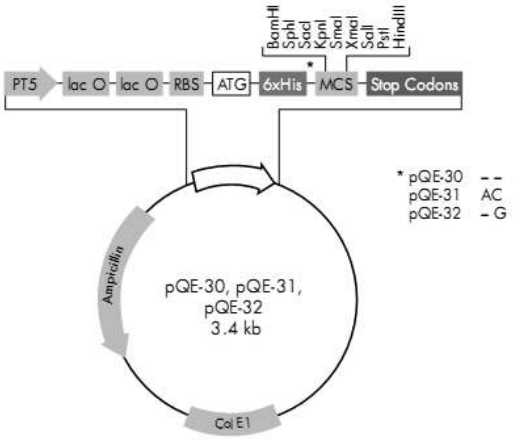

Figure 8. Map of pQE-30

pQE-30 plasmid is 3.4 kilobases vector that used to express recombinant protein in *E. coli*. This vector contains the following elements: the phage T5 promoter transcription–translation system, two lac operator sequences, 6xHis-tag coding sequence, multiple cloning site, β-lactamase gene (*BLA*) conferring resistance to ampicillin, and ColE1 origin of replication (Qiagen, 2003).

2.19.2 *Cg*Ecm33 protein expression in *E. coli*

A single colony of recombinant *E. coli* was grown in 20 ml of LB broth containing 100 µg/ml ampicillin and 25 µg/ml of kanamycin at 37°C for overnight in shaker incubator at 250 rpm. The overnight culture was diluted 50-folds (4 ml in 200 ml LB with antibiotics) and grown at 37°C with vigorous shaking until an OD600 of 0.6-0.8 was reached. IPTG (Roth) was added into the culture medium with a final concentration 1 mM and incubated for 4-5 hours. The Ecm33 expressing cells were harvested by centrifugation at 1,942 x g (3,000 rpm) for 10 minutes and frozen in -80°C (Qiagen, 2003).

2.19.3 Ecm33 protein purification

The pellect from 50 ml culture was mixed with lysis solution for protein expression (5 ml of Bugbuster® (Merck), 0.1 mg of lysozyme powder (Fluka), 5 µl of Benzouase (Novagen), and 220 µl of protease inhibitor cocltail (Roche) and incubated for 40 minutes at room temperature. After incubation, the cloudy solution was centrifuged to collect the pellet and dissolved with 8M urea solution at 40°C for 20 minutes. The protein solution was centrifuged at 1,942 x g (3,000 rpm) to remove the cell debris and the supernatant was kept at -20^{o}C.

His-Tag affinity chromatography was used to purify the Ecm33 protein. Two ml of Ni-NTA resin solution (Qiagen) was added into a column. Columns were equilibrated with 5 CV wash buffer. The lysate was loaded into column and the flow-through was collected in 3 portions of 1 ml. The target protein that bound to Ni-NTA resin was washed with 10 CV wash buffer and the flow-through was collected in 5 portions of 1 ml. The target protein was eluted with wash buffer containing 250 mM imidazole, and collected in 5 portions of 1 ml. All samples were analyzed by using SDS-PAGE (see the detail in 2.17.2).

2.19.4 Protein concentration measurement

BIOQUANT® (Merck) was used to measure the concentration of protein in a solution according to the manufacturer's instructions. 0.05 – 0.5 mg/ml of Bovine serum albumin (BSA) was used to make a calibration curve. The protein solution was mixed with 200 µl of

BIOQUANT®, incubated for 2 minutes at room temperature and measured absorbance at 570 nm.

2.19.5 Western blotting analysis

Protein from SDS-PAGE was transferred onto a PVDF membrane by using wet blotting. After blotting, the PVDF membrane was blocked with 20 ml of 5% (w/v) skim milk for 1 hour and then washed twice with 20 ml of PBST. The PVDF membrane was incubated with the primary antibody at 4°C for overnight and then washed three times with 20 ml of PBST. The secondary antibody was added and incubated at room temperature for 2 hours and the membrane washed three times with 20 ml of PBST. ECL™ Western blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) was used in the detection step and the image was taken with the Fujifilm LAS 4000 system.

2.19.6 Anti-Ecm33 antibody production

The immunisation procedure was performed by Eurogentec, Liège, Belgium. Before start the immunization program, serum of non-immunized rabbits were tested for Ecm33 antibodies to circumvent cross-reactions.

A polyclonal anti-Ecm33 serum was produced with the "anti-protein 87-days polyclonal package" of Eurogentec. Briefly, 100 µg purified *Cg*Ecm33 protein antigen was immunized in a rabbit on day 0, 14, 28, and 56. Serum was taken from the rabbits at day 0 (pre-immune serum) and day 38, 66, and 87 after immunization as shown in Figure 9. The immunized rabbit serum was tested for *Cg*Ecm33 antigen – anti-Ecm33 polyclonal antibody reaction by using immuno-blotting.

Figure 9. The time line of the anti-Ecm33 antibody production

Figure taken from https://secure.eurogentec.com/product/research-anti-protein-87-day-polyclonal-packages.html

2.20 Cell wall protein release assay

An overnight culture of yeast cells in YPD was washed and adjusted to an OD600 of 0.1 in 100 ml of minimum medium (MM). The cell suspension was incubated at 37^{o}C for 24 hours in shaker incubation at 250 rpm. The supernatant was collected by two times of centrifugation at 486 x g (1,500 rpm) for 10 minutes to remove the cells. To concentrate the supernatant, a 15 ml spin column with a pore size of 10 kDa (Sartorius, Göttingen) was used to reduce the volume of supernatant 50-folds. The protein concentration in the supernatant was determined by using BIOQUANT® (see the detail in 2.19.4). Western blot analyses were performed to check for reactivity of released material with rabbit anti-Cwp1 or rabbit anti-1,6-β-glucan.

2.21 Cell wall extraction

Cell wall extraction was performed as described de Groot et al (de Groot, P. W., de Boer, A. D. et al., 2004; de Groot, P. W., Kraneveld, E. A. et al., 2008). Briefly, yeast cells were fully disintegrated with 0.25 to 0.50 mm. glass beads (Emergo BV) in the presence of a protease inhibitor cocktail (Sigma) using a Bio-Savant FastPrep 120 machine (Qbiogene). To remove non-covalently linked proteins and intracellular contaminants, isolated cell walls were washed extensively with 1 M NaCl and twice extracted with 2% SDS, 150 mM NaCl, 100mM Na-EDTA, 100 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.8, for 5 minutes at 100°C. SDS-treated walls were washed with water until no foaming was observed anymore. The extracted cell walls (ECWs) were freeze-dried with lyophilization and stored at 20°C until further use.

2.22 Binding assay

20 µl (20 mg/ml) of extracted cell walls (ECWs) were incubated with 20 µl of ELISA blocking solution for 1 hour at 37^{o}C and washed twice with 1 ml of PBS. The blocked ECWs were incubated with 10 µl (250 µg/ml) of Ecm33 or Pst1 protein for 1 hour at 37^{o}C and washed twice with 1 ml of PBS to remove unbound protein. The pellets were resuspended with 10 µl steriled water and 4 µl of 5X loading buffer. Potential binding between ECWs and recombinant protein was analyzed by western blot analysis.

2.23 Statistical analyses

Three independent experiments were done in all yeast strains. Statistical significance for phenotypic tests was calculated using Student's t-test with Microsoft Excel. p-value < 0.05 were considered to be significant.

CHAPTER 3

RESULTS

3.1 *In silico* analysis of the Ecm33 family

In silico analyses showed that the Ecm33, Pst1, Sps2 and Sps22 proteins of *C. glabrata* consist of a signal peptide, and a short serine/threonine rich region, preceeding an omega site for GPI anchor addition at the C-terminus. Framing the central part of the mature protein four conserved cysteine sites can be found, potentially adding to the protein stability by formation of disulphide bonds as shown in Figure 10 and Figure 11.

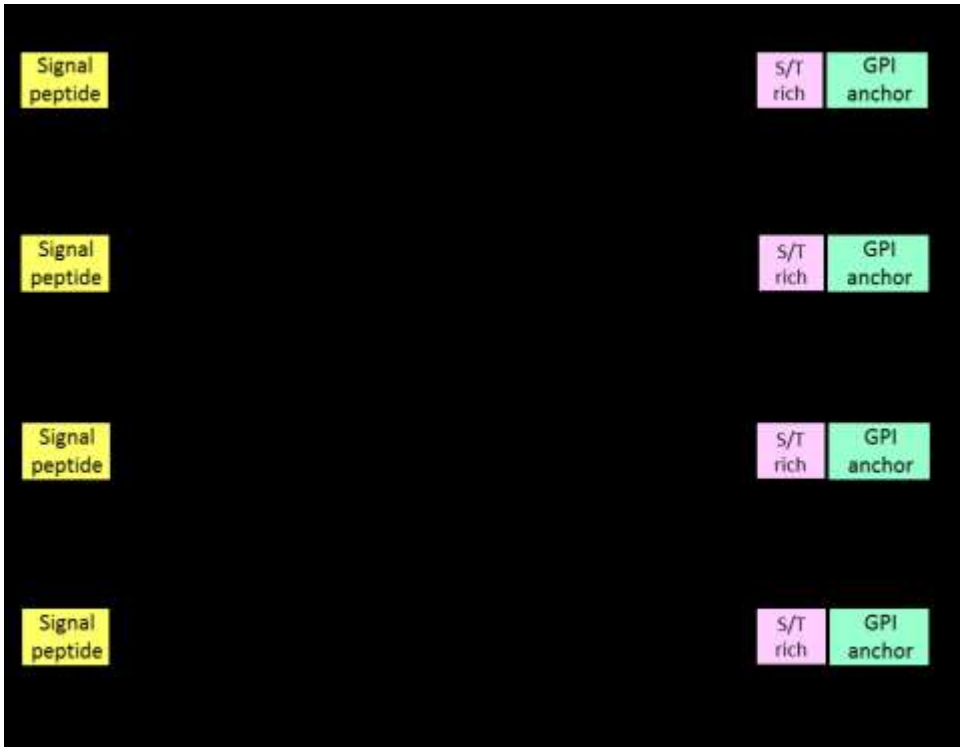

Figure 10. Ecm33 protein family structure of *C. glabrata*

Signal peptides were predicted by using SignalP. Omega sites for GPI anchor attachment were predicted using the big-PI Predictor server. All proteins feature a serine/threonine rich region and four conserved cysteine residues, potentially involved in the formation of disulfide bonds. Residue numbers are given in reference to each individual protein sequence.

Figure 11. Sequence alignment of mature proteins of the Ecm33 families in *C. glabrata*, *C. albicans* and *S. cerevisiae*.

Shaded regions; green: conserved cysteins, red: fully conserved residues, potentially contributing towards function, blue: S/T-rich region. Signal peptides for ER-entry and GPI-modification were removed from the sequences before alignment.

The relatedness of the different proteins of the Ecm33 families of *C. glabrata*, *C. albicans*, *and S. cerevisiae* were analysed by creation of a protein sequence alignment and a phylogenetic tree derived thereof.

Next to the four conserved cysteines already described above for the four proteins from *C. glabrata*, three additional highly conserved regions can be observed in the mature protein sequences: at positions 206-208 an SDT motif, at postions 230-232 an NNN motif and last at positions 375-380 a LK.V.G-motif as shown in Figure 11. Other than that, several conserved proline and glycine residues are found.

Amino acid identity matrices (Table 12 and Table 13) show that *C. glabrata* Ecm33 shares 67% homology with the Ecm33 protein of *S. cerevisiae* and 73% in the Pst1 protein. In contrast, the homology to the respective proteins in *C. albicans* is only 30% and 33%, respectively. Although Sps2 and Sps22 are grouped in the same family, the homology with the Ecm33 protein is only approximately 30% in all three organisms. Phylogenic clustering (Figure 12) and calculation of amino acid identity matrices indicates that the members of the Ecm33 protein family in can be divided into two groups; (i) Ecm33 and Pst1, and (ii) Sps2 and Sps22. An ortholog of Sps22 is not found in *C. albicans*.

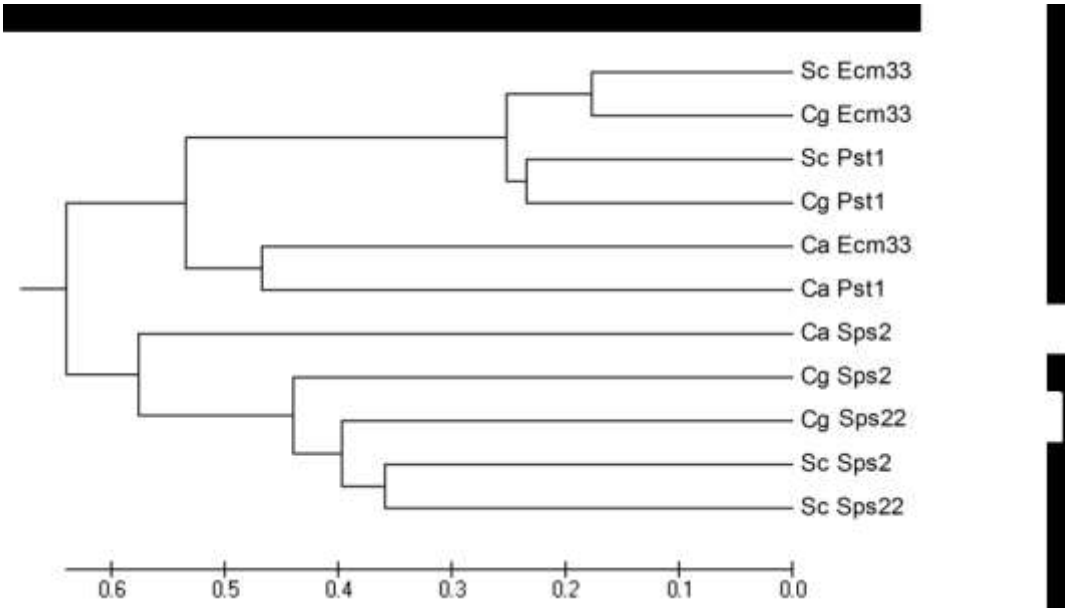

Figure 12. The Ecm33 family phylogeny of *C. glabrata*, *C. albicans* and *S. cerevisiae*.

Phylogenetic clustering of eleven protein sequences of the Ecm33 family in *C. glabrata*, *C. albicans* and *S. cerevisiae* shows separation into two distinct subgroups. An ortholog of the Sps22 protein is not found in the genome of *C. albicans*.

Table 12. Identity matrix of the entire protein in the Ecm33 family of *C. glabrata*, *C. albicans* and *S. cerevisiae*

	Cg-Ecm33	Cg-Pst1	Cg-Sps2	Cg-Sps22	Ca-Ecm33	Ca-Pst1	Ca-Sps2	Sc-Ecm33	Sc-Pst1	Sc-Sps2	Sc-Sps22
Cg-Ecm33	ID	0.62	0.196	0.213	0.308	0.317	0.213	0.67	0.524	0.24	0.216
Cg-Pst1	0.62	ID	0.214	0.213	0.327	0.315	0.203	0.561	0.556	0.235	0.225
Cg-Sps2	0.196	0.214	ID	0.313	0.177	0.213	0.21	0.197	0.2	0.324	0.355
Cg-Sps22	0.213	0.213	0.313	ID	0.201	0.193	0.224	0.2	0.196	0.304	0.347
Ca-Ecm33	0.308	0.327	0.177	0.201	ID	0.366	0.174	0.331	0.31	0.205	0.19
Ca-Pst1	0.317	0.315	0.213	0.193	0.366	ID	0.213	0.315	0.283	0.215	0.205
Ca-Sps2	0.213	0.203	0.21	0.224	0.174	0.213	ID	0.213	0.24	0.237	0.253
Sc-Ecm33	0.67	0.561	0.197	0.2	0.331	0.315	0.213	ID	0.572	0.252	0.238
Sc-Pst1	0.524	0.556	0.2	0.196	0.31	0.283	0.24	0.572	ID	0.233	0.229
Sc-Sps2	0.24	0.235	0.324	0.304	0.205	0.215	0.237	0.252	0.233	ID	0.4
Sc-Sps22	0.216	0.225	0.355	0.347	0.19	0.205	0.253	0.238	0.229	0.4	ID

ID: identity

Table 13. Identity matrix of Ecm33 family effector domains only of *C. glabrata*, *C. albicans* and *S. cerevisiae*

	Cg-Ecm33	Cg-Pst1	Cg-Sps2	Cg-Sps22	Ca-Ecm33	Ca-Pst1	Ca-Sps2	Sc-Ecm33	Sc-Pst1	Sc-Sps2	Sc-Sps22
Cg-Ecm33	ID	0.674	0.278	0.271	0.334	0.351	0.306	0.73	0.613	0.339	0.293
Cg-Pst1	0.674	ID	0.297	0.271	0.362	0.358	0.279	0.616	0.647	0.328	0.303
Cg-Sps2	0.278	0.297	ID	0.371	0.225	0.273	0.303	0.272	0.287	0.445	0.453
Cg-Sps22	0.271	0.271	0.371	ID	0.24	0.243	0.334	0.256	0.265	0.425	0.449
Ca-Ecm33	0.334	0.362	0.225	0.24	ID	0.37	0.231	0.325	0.352	0.268	0.237
Ca-Pst1	0.351	0.358	0.273	0.243	0.37	ID	0.296	0.342	0.333	0.304	0.267
Ca-Sps2	0.306	0.279	0.303	0.334	0.231	0.296	ID	0.293	0.327	0.343	0.366
Sc-Ecm33	0.73	0.616	0.272	0.256	0.325	0.342	0.293	ID	0.622	0.342	0.302
Sc-Pst1	0.613	0.647	0.287	0.265	0.352	0.333	0.327	0.622	ID	0.324	0.305
Sc-Sps2	0.339	0.328	0.445	0.425	0.268	0.304	0.343	0.342	0.324	ID	0.506
Sc-Sps22	0.293	0.303	0.453	0.449	0.237	0.267	0.366	0.302	0.305	0.506	ID

ID: identity

3.2 Confirmation of knock out strains

Single deletion mutants of *PST1* (*Δpst1*) and *ECM33* (*Δecm33*) and a double mutant (*Δpst1/Δecm33*) in *C. glabrata* were provided by Tobias Schwarzmüller (Univerity of Vienna,

Austria). There, the strains were generated by using a fusion PCR to construct a recombinant pTS50 plasmid that was used to transformed the parental strain (wild-type auxotroph *Δhtl*), with selection for *NAT* (nourseothricin-resistance gene) transformants on YPD medium plates (Schwarzmüller, T., 2009).

To comfirm the gene deletion status of the mutants received, the *Δpst1* strain was analysed by using two PCR reactions: the first analysis using primers 13 F-MuE-Cont and 11 hk2 to verify integration of *NAT* gene at the 5' site and the second analysis using the primers 14 R-MuE-Cont and 12 hk3 to confirm integration of the knock out cassette at the 3' end. Similarly, the deletion strain *Δecm33* was tested with primer pair 15 F-MuM-Cont and 11 hk2 for 5' site and primer pair 16 R-MuM-Cont and 12 hk3 for the 3' end. In strain *Δpst1/Δecm33*, the primer pair 13 F-MuE-Cont and 11 hk2 were used to confirm *PST1* gene deletion and primer 16 R-MuM-Cont and 12 hk3 for *ECM33* gene deletion. The PCR product size observed was approximately 800 bp for all reactions as shown in Figure 13 as expected for positive reactions.

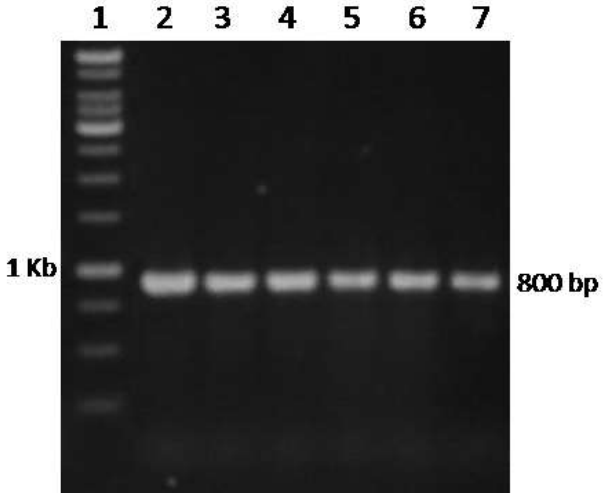

Figure 13. Confirmation of knock out strains by PCR

Lane 1: DNA Marker, lane 2: *Δpst1* DNA with primer no. 13 and 11, lane 3: *Δpst1* DNA with primer no. 14 and 12, lane 4: *Δecm33*DNA with primer no. 15 and 11, lane 5: *Δecm33*DNA with primer no. 16 and 12, lane 6: *Δpst1:Δecm33* DNA with primer no. 13 and 11, and lane 7: *Δpst1:Δecm33* DNA with primer no. 16 and 12

As a further control, reverse transcription real-time PCR was performed to analyze gene expression in the generated mutant strains *Δpst1*, *Δecm33* and *Δpst1/Δecm33* in comparison to the auxotroph parental strain *Δhtl.* In these assays, the housekeeping genes *ACT* and *SLT* were

used for normalization of expression levels. The results confirmed that there was no expression of the deleted genes in the generated knock out strains. Furthermore, no compensatory up-regulation of other family member genes was observed in the gene deletion mutants (Figure 14).

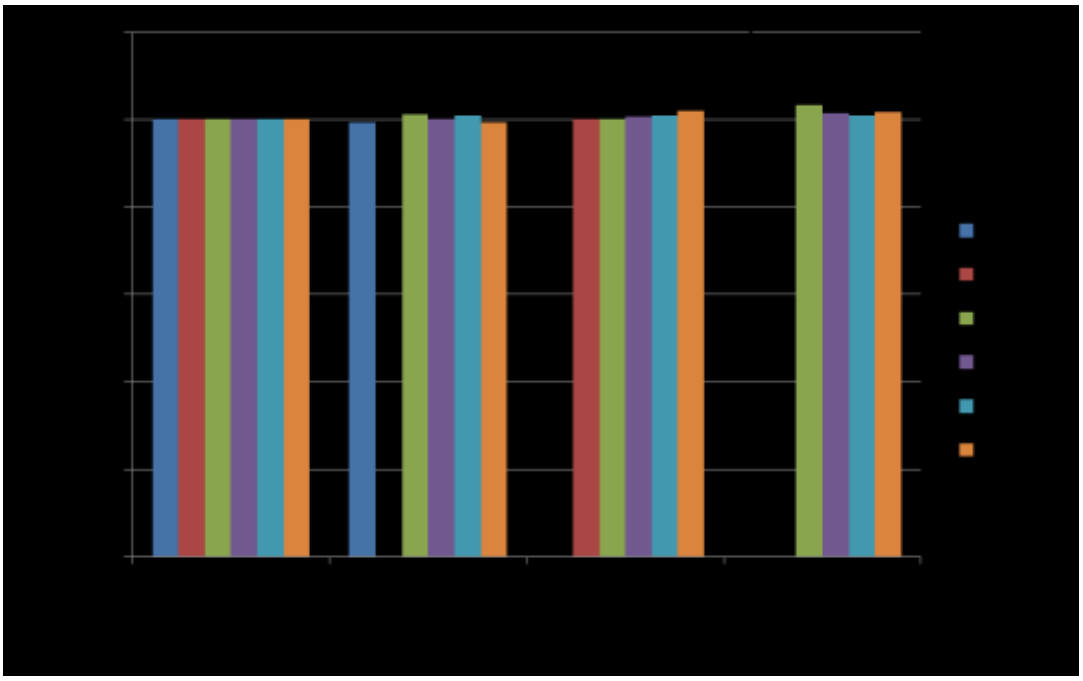

Figure 14. Reverse transcription real-time PCR to analyse the expression levels of the *ECM33* gene family in the generated mutants

Reverse transcription real-time PCR analyses of *ECM33* gene family (average of three independent experiments). The wild-type auxotroph strain *Δhtl* expressed all genes that was tested and the generated mutant strains did not expressed the gene that was deleted. There was no compensatory up-regulation of *SPS2*/*22* or *PST1*/*ECM33* in the mutants. The housekeeping genes *ACT* and *SLT* were used for normalization.

3.3 Generation of revertants and complemented strains

All of the completed revertants (*Δpst1:CgPST1* and *Δecm33:CgECM33*), incompleted revertants *Δpst1/Δecm33:CgPST1* and *Δpst1/Δecm33:CgECM33* and respective complemented strains (*Δecm33*:truncated *CgECM33*, *Δecm33:CgSPS2*, *Δecm33:CgSPS22*, *Δecm33:CgPST1*, *Δecm33:CaECM33* and *Δecm33:ScECM33*) were generated by cloning the respective genes into expression plasmid pBM51 (Figure 6). Briefly, the inserted fragments were amplified with specific primers (Figure 15) and cloned to plasmid pBM51. All recombinant pBM51 candidates

were verified with double restriction enzyme digestion as shown in Figure 16. The confirmed recombinant pBM51 plasmids were transformed into *C. glabrata* by electroporation. To verify the expression of revertant and complemented *C. glabrata*, a reverse transcription real-time PCR were performed for measurement of the level of target gene expression as shown in Figure 18.

All of the revertant strains expressed the inserted gene. The *Δpst1:CgPST1* and *Δpst1/Δecm33:CgPST1* could express *PST1* in the same level as compared to the *Δhtl* auxotrophic wild-type strain. *Δecm33:CgECM33* and *Δpst1/Δecm33:CgECM33* expressed *ECM33*, but approximately 15% and 10% lower as compared to the *Δhtl* auxotrophic strain. As demonstrated before, there was no *PST1* and *ECM33* expression in the respective mutants when the gene was deleted (Figure 17).

In complemented strains, *Δecm33*:truncated *CgECM33*, *Δecm33*:*CaECM33*, and *Δecm33*:*ScECM33* could express *ECM33* at levels around 70%, 90%, and 90% of the *Δhtl* auxotrophic strain, respectively. The strains *Δecm33*:*CgPST1*, *Δecm33*:*CgSPS2*, and *Δecm33*:*CgSPS22* contained two copies of *PST1* and *SPS2* and *SPS22*, respectively. However, there was only a low level of overexpression (~105%) of these genes in these strains, when compared to the *Δhtl* auxotroph and the deletion mutant *Δecm33* (Figure 18).

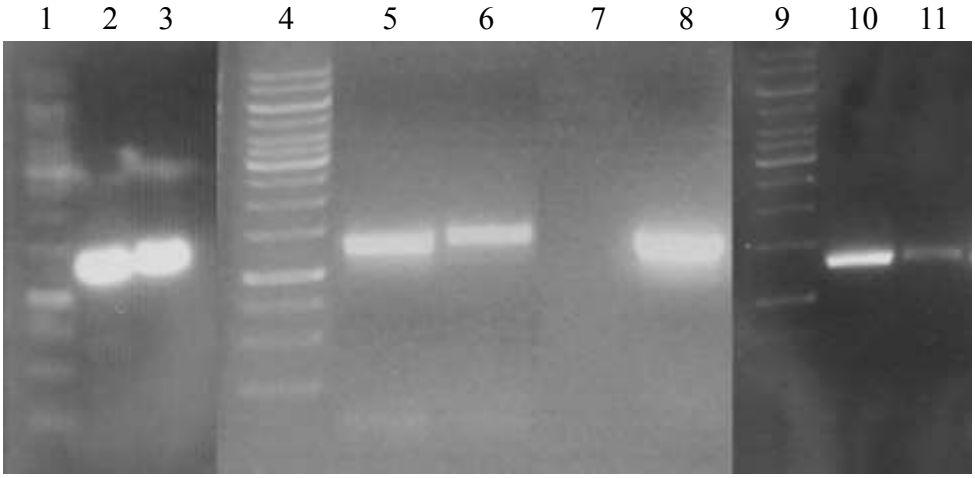

Figure 15. PCR products for recombinant pBM51 construction

PCR products of the interested genes were amplified from genomic DNA in case of *C. glabrata* and cDNA in case of *C. albicans* and *S. cerevisiae* with specific primers (Table 9). All lanes show a the expected specific amplification product. Non-specific PCR products were not observed. Lanes 1,4,9: DNA marker, lane 2: *CgECM33* , lane 3: *CgPST1*, lane 5: *CgSPS22*, lane 6: *CgSPS2*, lane 7: empty, lane 8: truncated *CgECM33*, lane 10: *CaECM33* and lane 11: *ScECM33*

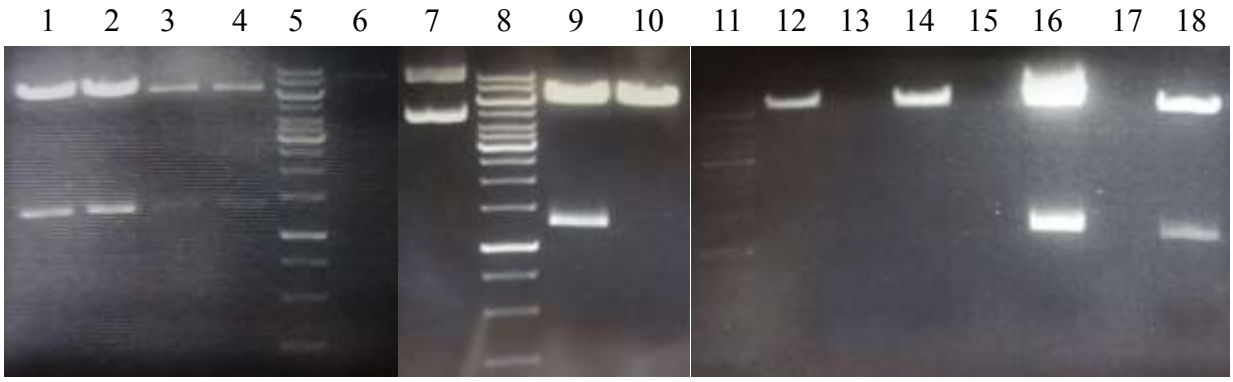

Figure 16. Enzyme digestion for verification of recombinant pBM51 plasmid

The recombinant pBM51 plasmids were extracted from the positive *E. coli* clones. Verification of these recombinant plasmids were performed by using restriction enzyme digestion. All recombinant plasmids containing the inserted genes show two bands on agarose gel. An upper band is pBM51 fragment and a lower band is the inserted gene. Lane 1: pBM51:*CgECM33*, lane 2: pBM51:*CgPST1*, lane 3: pBM51:*CgSPS2*, lane 4: pBM51:*CgSPS22*, lane 5,8,11: DNA marker, lane 6: *BamH*I digested pBM51:*CgECM33*, lane 7: two forms pBM51:truncated *CgECM33* (upper is relaxed form and lower is supercoiled circular), lane 9: pBM51:truncated *CgECM33*, lane 10,12,14: pBM51, lane 13,15,17: empty, lane 16: pBM51:*CaECM33*, and lane 18: pBM51:*ScECM33*

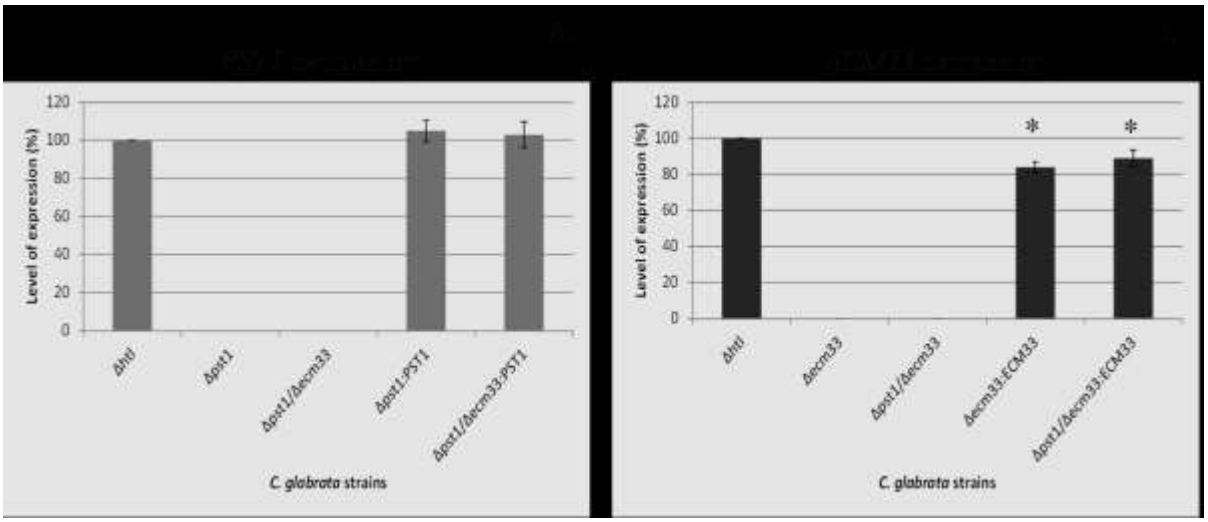

Figure 17. Reverse transcription real-time PCR for the measurement of *PST1* and *ECM33* gene expression in revertant strains

(A.): transcription levels of *PST1*; both *PST1* revertants show *PST1* gene expression as well as the *Δhtl* auxotrophic strains (B.): transcription levels of *ECM33*; both *ECM33* revertants show expression of the *ECM33* genes but not in the same level as the *Δhtl* auxotrophic strains. *: statistically significant differences ($p< 0.05$). Error bars represent the mean value calculated from triplicate experiments (±SD).

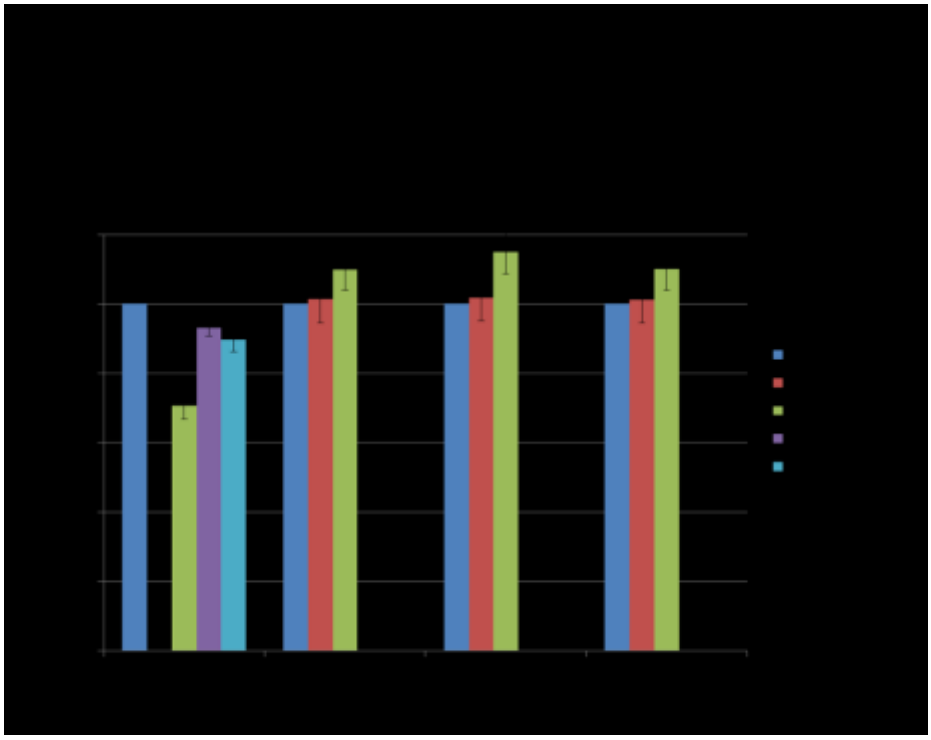

Figure 18. Expression levels of the *ECM33* gene family members in complemented strains

Relative quantification of gene expression of *ECM33* gene family. The complemented strains of the *ECM33* genes from *C. glabrata*, *C. albicans* and *S. cerevisiae* show expression of the introduced genes. Introduction of additional copies of *PST1*, *SPS2*, and *SPS22* in Δ*ecm33* did not lead to significant up-regulation when compared to Δ*ecm33*. The housekeeping genes *ACT* and *SLT* were used for normalization. *: statistically significant differences ($p< 0.05$). Error bars represent the mean value for triplicate experiments (±SD).

3.4 Growth rate determination

To asses whether deletion of any of the genes leads to growth defects and if in such cases the phenotype was recovered in reconstitutet strains, growth rates in YPD and RPMI 1640 media were determined at both 30°C and 37°C. Comparison of growth rates showed that all strains from *C. albicans*, *S. cerevisiae*, *C. glabrata* grew best in YPD at 37°C as shown in Figures 20-22.

The *C. albicans* mutant strain *Δecm33:Δecm33* showed delayed growth as compared to the auxotrophic wild-type strain (Caf2) and its reconstituted control (*Δecm33:Δecm33:ECM33:ECM33*) under all conditions tested as shown in Figure 19.

In *S. cerevisiae*, the growth rate of the auxotrophic wild-type strain BY4741 was similar to the *Δpst1* mutant. Moreover, the growth rate of the deletion mutant *Δecm33* (BY4741 genetic background) was similar to the mutant *Δecm33* (FBEH041 genetic background) but both *Δecm33* strains grew slower when compared with the respective parental auxotrophic wild-type strains and the *Δpst1* mutant. The *Δpst1/Δecm33* strain grew significantly slower than both, the wild-type and the single gene deletion mutants as shown in Figure 20.

In *C. glabrata*, the growth rate of wild-type strain CBS138/ATCC2001 was similar to the auxotrophic wild-type strain *Δhtl* and *Δpst1* mutant. The *Δecm33* mutant grew slower than the wild-type and the *Δpst1* mutant. The *Δpst1/Δecm33* mutant grew significantly slower than the wild-type, *Δpst1*, and *Δecm33* as shown in Figure 21.

The growth rate of the revertants indicated that *Δecm33*:*CgECM33*, *Δpst1/Δecm33:CgPST1*, and *Δpst1/Δecm33:CgECM33* grow slower than the auxotrophic wild-type strain *Δhtl* and the *Δpst1* mutamt, but faster than the *Δecm33* and *Δpst1/Δecm33* as shown in Figure 21. Gene reversion can therefore restore the growth rate from the mutants close to approximate levels of the parental strain, as shown in Figure 21 and Figure 22 A.

Complementations of *CgECM33* with the ortholog from either *S. cerevisiae* or *C. albicans* rescued the growth defect in *C. glabrata* (Figure 23 B). However, addition of a second allele of either *PST1*, *SPS2*, or *SPS22* under the *ECM33* promotor does not rescue the *ecm33* growth defect (Figure 23 A). Neither does introduction of a truncated version of *ECM33* without the GPI anchor attachment site (Figure 23 C).

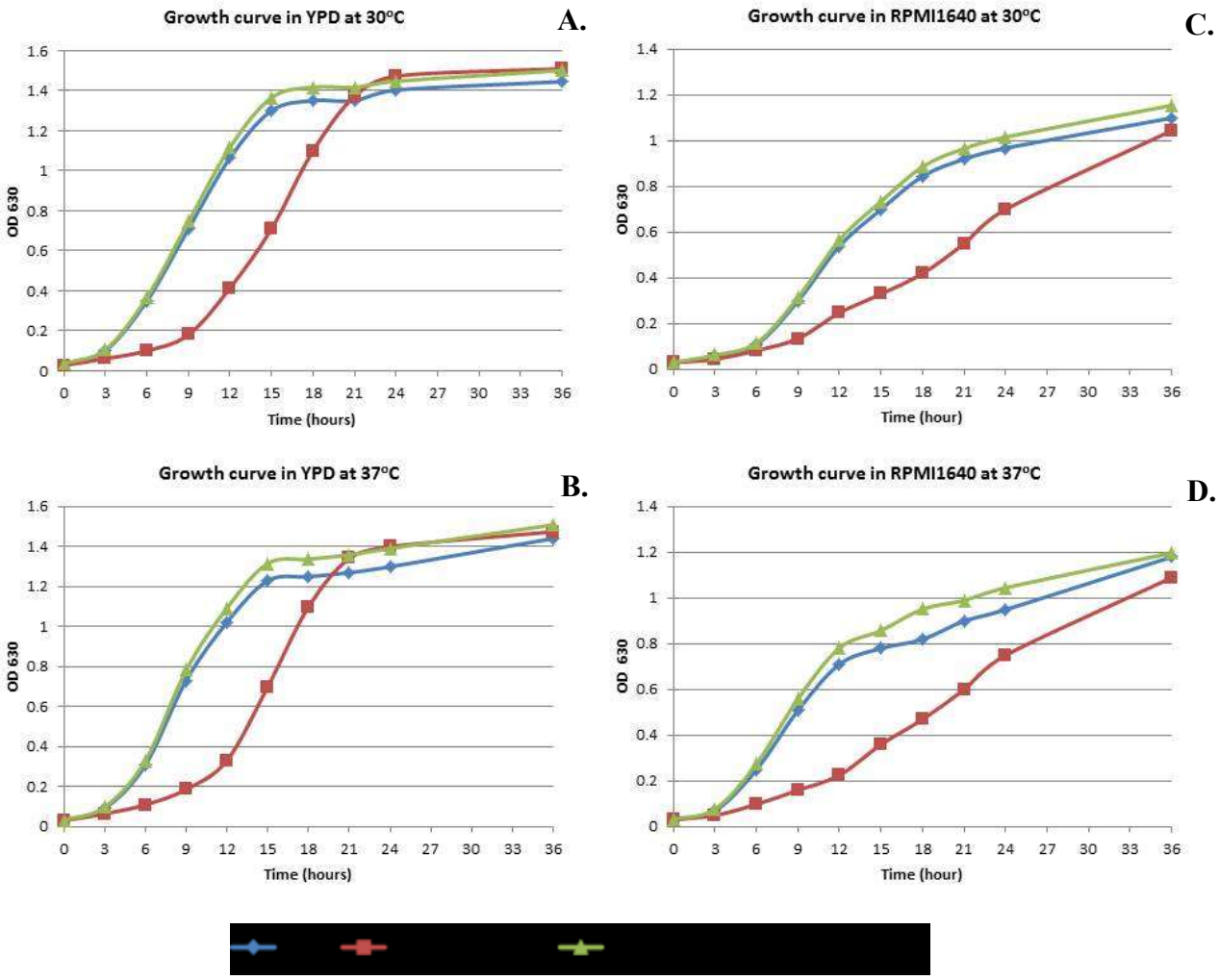

Figure 19. Growth assays of *C. albicans* mutants

(A.): YPD at 30°C, (B.): YPD at 37°C, (C.): RPMI 1640 at 30°C, and (D.): RPMI 1640 at 37°C. The cultures of *C. albicans* cells were grown in 96-well plates monitored for 36 hours and measured an optical density at 630 nm. The Δ*ecm33*:Δ*ecm33* mutant has a slower growth as compared with Caf2 auxotrophic wild-type. The reduced growth rate of the mutant was restored by complementing with *CaECM33*.

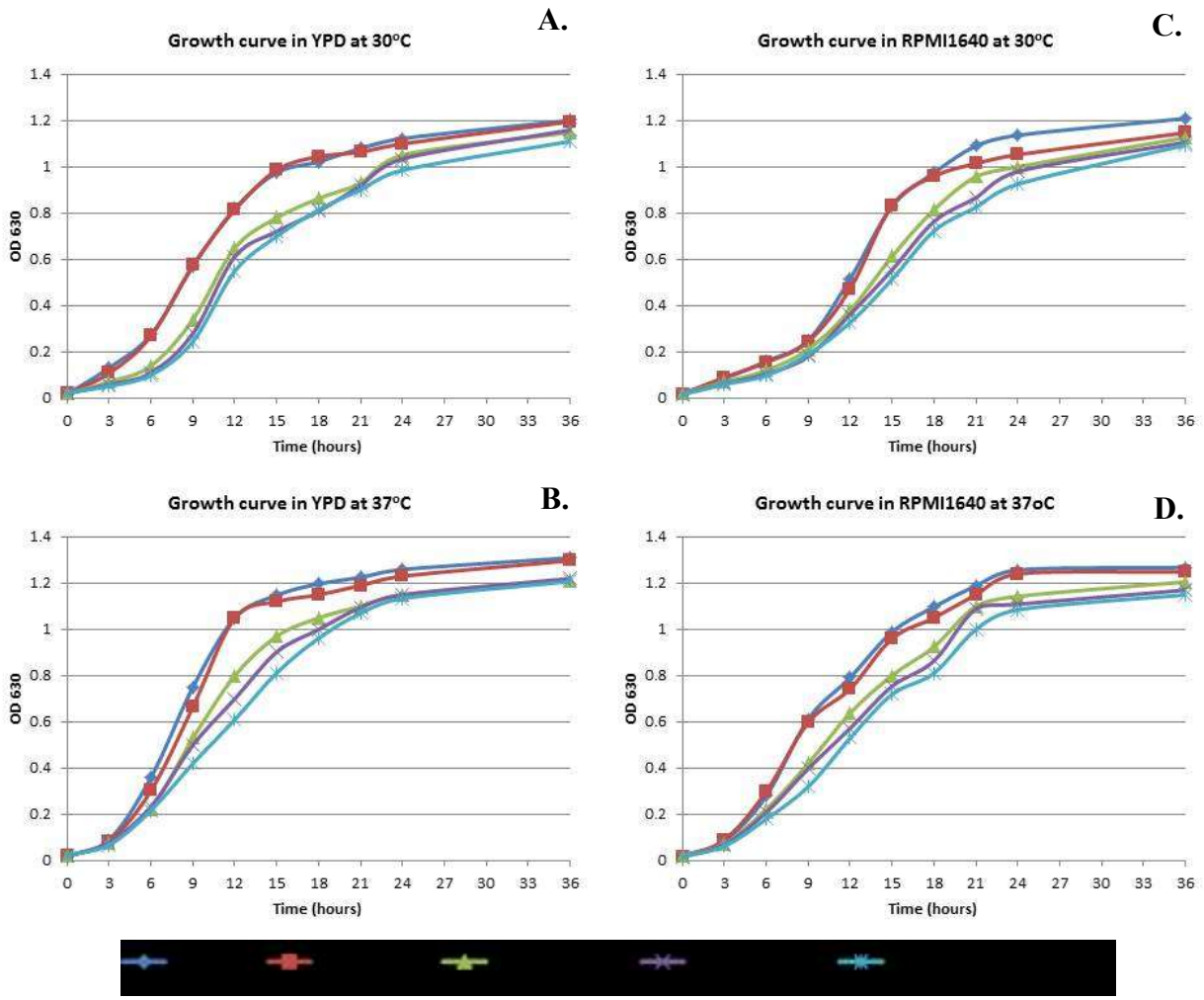

Figure 20. Growth assays of *S. cerevisiae* mutants

(A.): YPD at 30°C, (B.): YPD at 37°C, (C.): RPMI 1640 at 30°C, and (D.): RPMI 1640 at 37°C. The cultures of *S. cerevisiae* cells were grown in 96-well plates, monitored for 36 hours and measured an optical density at 630 nm. The Δ*ecm33* mutant has a slower growth as compared to the BY4741 auxotrophic wild-type and Δ*pst1* mutant. The Δ*pst1*/Δ*ecm33* double mutant has the slowest growth as compared to all other strains. Control strains with the re-introduced genes were not available. Two separate genetic backgrounds were used to construct single and double deletion strains (Pardo, M., Monteoliva, L. et al., 2004).

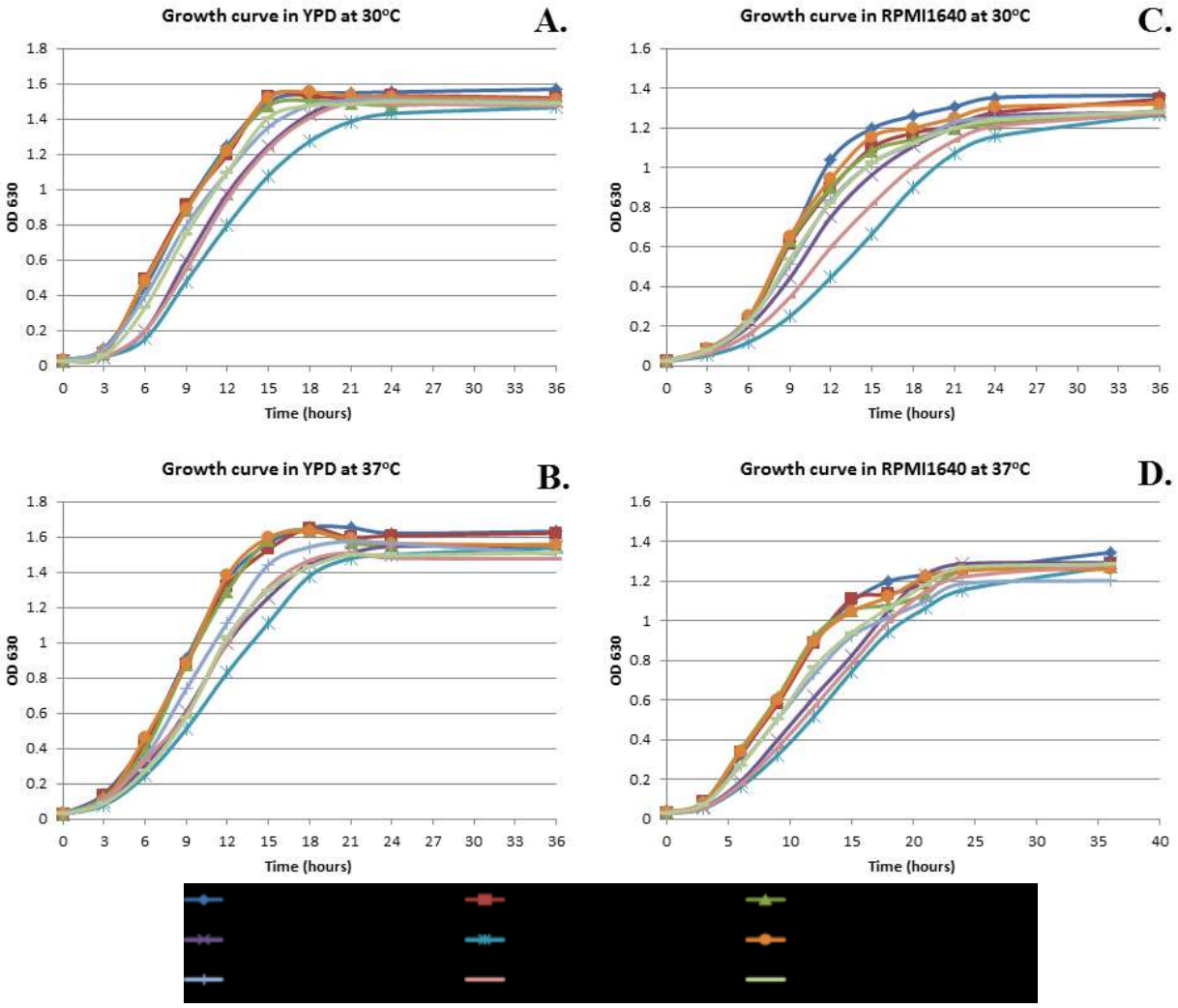

Figure 21. Growth assays of *C. glabrata* mutants

(A.): YPD at 30°C, (B.): YPD at 37°C, (C.): RPMI 1640 at 30°C, and (D.): RPMI 1640 at 37°C. The cultures of *C. glabrata* cells were grown in 96-well plates, monitored for 36 hours and measured an optical density at 630 nm. The Δ*ecm33* mutant has a slower growth as compared to the wild-type and Δ*pst1* mutant. The Δ*pst1*/Δ*ecm33* mutant has the slowest growth as compared to all other strains. The reduced growth rate of the mutant was restored by complementing with *CgECM33*.

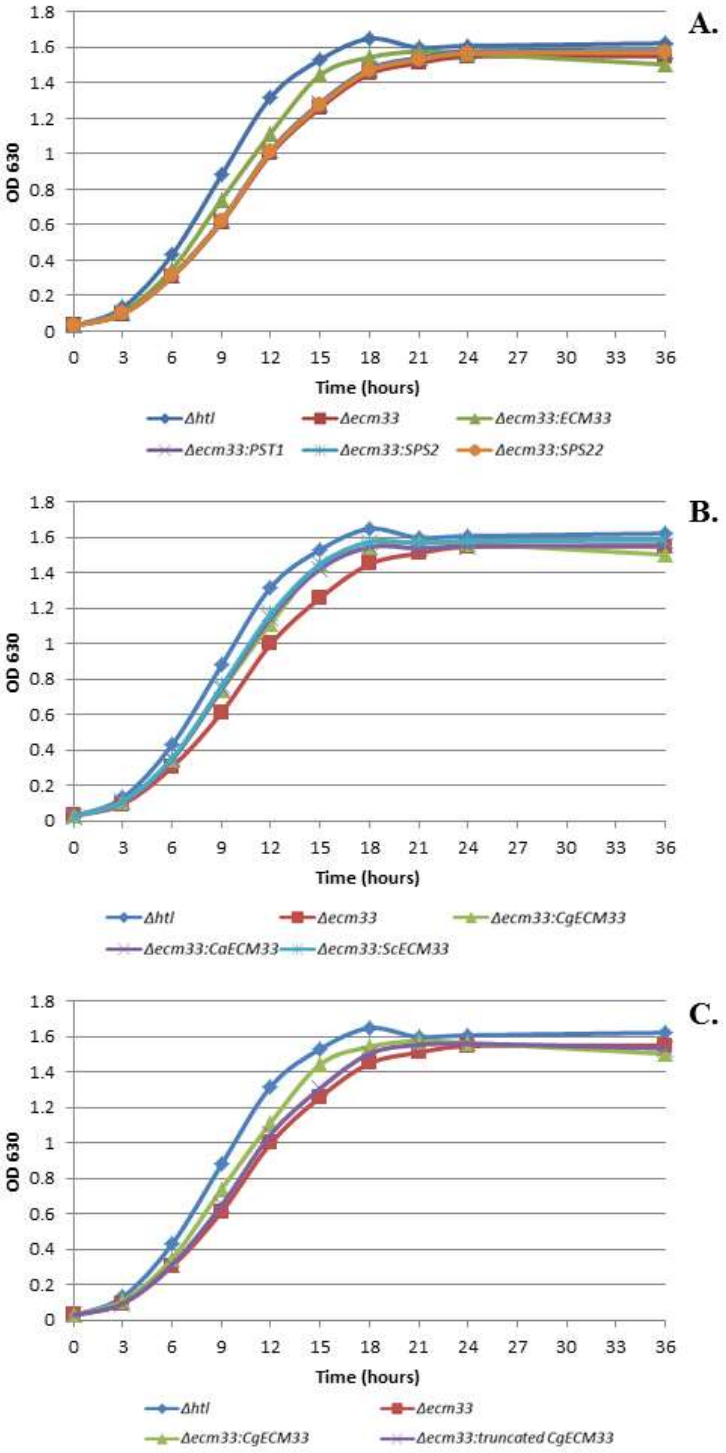

Figure 22. Growth curve of complementation experiments *C. glabrata* in YPD at 37°C

C. glabrata strains were determined in 96-well plates, monitored for 36 hours and measured an optical density at 630 nm. Growth comparisons of (A.): strains complemented with *C. glabrata ECM33*, *PST1*, *SPS2*, and *SPS22*. (B.): complemented strains using *C. albicans* and *S. cerevisiae ECM33*. (C.): complemented strains using truncated *CgECM33*.

3.5 Phenotypic analysis

3.5.1 Measurement of cell wall strength

To measure the effect of the different gene deletions on cell wall strength, growth assays on solid media supplemented with different concentrations of known cell-wall perturbing agents were carried out. As a starting point, previously published concentrations for the respective strains from *C. albicans* (Martinez-Lopez, R., Monteoliva, L. et al., 2004) and *S. cerevisiae* (Pardo, M., Monteoliva, L. et al., 2004) were chosen.

In *C. albicans*, the phenotypic differences between the Δ*ecm33* mutant and the auxotrophic wild-type strain (Caf2) when exposed to 5 mM Caffeine, 50 µg/ml Calcofluor white, and 200 µg/ml Congo red were seen (Figure 23 B).

Similarly, for *S. cerevisiae* the Δ*pst1* mutant had no phenotypic difference compared to the the auxotrophic wild-type strain BY4741, but two independent Δ*ecm33* mutants, showed a significantly decreased growth at concentrations of 10 mM Caffeine, 10 µg/ml Calcofluor white, and 50 µg/ml Congo red. The Δ*pst1*/Δ*ecm33* mutant also displayed a increased susceptibility in these assays (Figure 24 C).

However, there were no phenotypic differences among *C. glabrata* wild-type, auxotrophic wild-type, single mutants and double mutant when tested with 10 mM Caffeine, 500 µg/ml Calcofluor white, and 500 µg/ml Congo red (Figure 23 A). Of note, these concentrations were several fold higher in *C. glabrata* than the concentrations necessary to assay the phenotypes in *C. albicans* and *S. cerevisiae*.

Therefore, additional liquid culture (broth microdilution) assays were performed to confirm these data. The minimum inhibitory concentration (MIC)50- and MIC90-values were used as the indicators for a quantitative evaluation in sensitivity testing.

When the wild-type strains of *C. glabrata, C. albicans* and *S. cerevisiae* were compared in these assays, the CFW and Congo red MIC50- and MIC90-values were significantly higher in *C. glabrata* confirming that this organism is more resistant to cell perturbing agents.

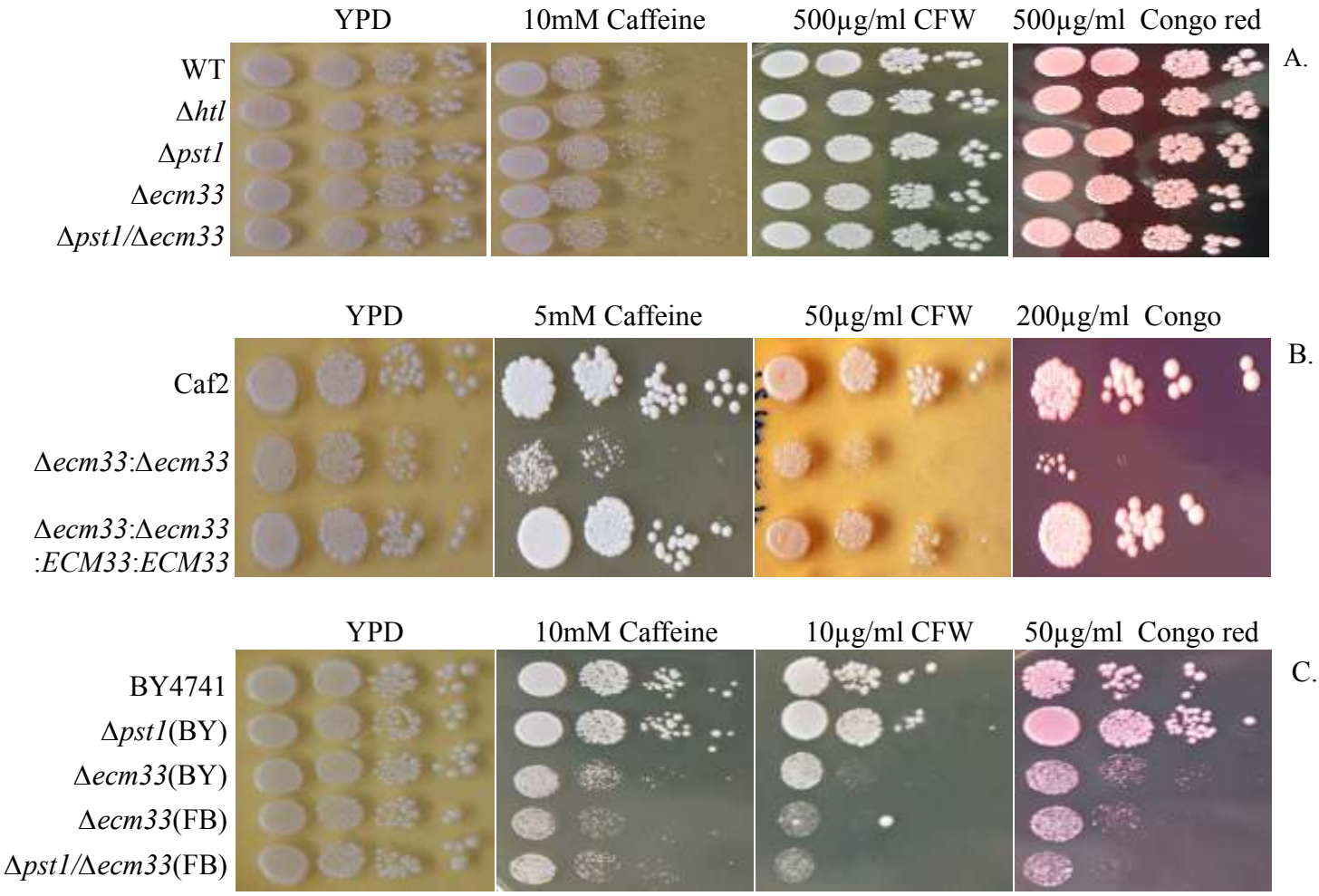

Figure 23. Sensitivity testing of *C. glabrata, C. albicans* and *S. cerevisiae* to cell perturbing agents by using agar drop dilution assay

(A): *C. glabrata*, (B): *C. albicans*, and (C): *S. cerevisiae*. The *ECM33* gene deletion of *C. albicans* and *S. cerevisiae* resulted in the expected susceptibility to cell wall perturbing agents ((Martinez-Lopez, R., Monteoliva, L. et al., 2004; Pardo, M., Monteoliva, L. et al., 2004)); the *Δecm33* mutant of *C. albicans* and the *Δecm33* and *Δpst/Δecm33* mutants of *S. cerevisiae* are susceptible to Caffeine, CFW, and Congo red as compared to wild-type auxotrophic strain. However this effect was not observed in *C. glabrata*.

Using liquid culture assays the *C. glabrata Δecm33* mutant was moderately more sensitive to CFW and Congo red as compared to the wild-type strains. In the double mutant *Δpst1/Δecm33*, these phenotypes were strongly aggravated (Table 14). The broth microdilution assays confirmed that the complemented *C. glabrata* mutants regained their cell wall rigidity. For example, the MIC50 for CFW of the *Δecm33* mutant is 250 µg/ml, but increases to 500 µg/ml in the revertant *Δecm33*:*CgECM33* and reaches the level of the wild-type and auxotrophic strain (Table 14).

In contrast, when *CgPST1*, *CgSPS2* or *CgSPS22* was introduced into the *Δecm33* mutant (*Δecm33*:*CgPST1*, *Δecm33*:*CgSPS2*, and *Δecm33*:*CgSPS22*), the phenotype was not rescued, indicating that these homologs can not take over the function of *ECM33* in *C. glabrata* (Table 14). Furthermore, a truncated version of *CgECM33* without the GPI anchor site was not able to restore the phenotypic defects of the *Δecm33* mutant in *C. glabrata*. In all phenotypic assays, the *Δecm33*:truncated *CgECM33* showed the same MIC50- and MIC90-values as compared to *Δecm33* (Table 14). These phenotypic differences of the revertants (*Δecm33*:truncated *CgECM33* vs *Δecm33*:*CgECM33*) show that the GPI anchor site of the Ecm33 protein is necessary for native Ecm33 function.

Interestingly, the *C. albicans* and *S. cerevisiae* orthologs of *CgECM33* (*CaECM33 and ScECM33*) were able to restore the phenotypes of *CgΔecm33* and the revertants *Δecm33*:*CaECM33* and *Δecm33*:*ScECM33* display similar MIC50- and MIC90-values as the *C. glabrata* wild-type strain.

In *C. albicans*, the mutant strain *Δecm33:Δecm33* was more sensitive to all cell perturbing agents when compared to the auxotrophic wild-type strain (except Lufenuron) and the revertant strains showed the same MIC50- and MIC90-values as the wild-type auxotrophic strain for all tested perturbants (Table 14).

In *S. cerevisiae* neither Brefeldin A, Nikkomycin Z nor Lufenuron did not inhibit the growth of the yeast cells in the tested concentration range. The two strains of *Δecm33* mutants were more sensitive to CFW, Congo red, and Hygromycin B. With all cell perturbing agents, the Δ*pst1* mutant showed similar phenotypes as compared to the wild-type auxotroph (BY4741). However, the *Δpst1/Δecm33* mutant was found to be four-times more sensitive when compared to the wild-type auxotrophic strain or the Δ *pst1* mutant (Table 14).

Table 14. Summary of results obtained by broth microdilution assay

Organisms	Strains/Chemical agents	CFW		Congo red		SDS		Hygromycin		Brefeldin A		Nikkomycin		Lufenuron	
		7.81-1000 μg/ml		3.90-500 μg/ml		0.003125-0.1%		3.125-800 μg/ml		0.234-120 μg/ml		1-128 μg/ml		0.00625-0.4 μg/ml	
		MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
C. glabrata	wild-type	500	NI	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*htl* (auxotroph)	500	NI	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*pst1*	500	NI	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*pst1*/Δ*ecm33*	125	500	250	NI	0.00313	0.00625	50	100	NI	NI	NI	NI	NI	NI
	Δ*pst1:CgPST1*	500	NI	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:CgECM33*	500	1000	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*pst1*/Δ*ecm33:CgPST1*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*pst1*/Δ*ecm33:CgECM33*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:truncated CgECM33*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:CgPST1*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:CgSPS2*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:CgSPS22*	250	1000	500	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:CaECM33*	500	1000	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
	Δ*ecm33:ScECM33*	500	1000	NI	NI	0.00625	0.00625	100	200	NI	NI	NI	NI	NI	NI
C. albicans	*Caf2* (auxotroph)	62.5	125	15.625	31.25	0.00625	0.00625	400	400	3.75	7.5	NI	NI	NI	NI
	Δ*ecm33*:Δ*ecm33*	31.25	62.5	7.81	7.81	0.00625	0.00625	50	100	1.875	3.75	8	NI	NI	NI
	Δ*ecm33*:Δ*ecm33:ECM33:ECM33*	62.5	125	15.625	31.25	0.00625	0.00625	400	400	3.75	7.5	NI	NI	NI	NI
S. cerevisiae	BY4741 (auxotroph)	31.25	62.5	15.625	31.25	0.00625	0.00625	25	50	NI	NI	NI	NI	NI	NI
	Δ*pst1* (BY4741)	31.25	62.5	15.625	31.25	0.00625	0.00625	25	50	NI	NI	NI	NI	NI	NI
	Δ*ecm33* (BY4741)	15.625	31.25	7.81	15.625	0.00625	0.00625	12.5	25	NI	NI	NI	NI	NI	NI
	Δ*ecm33* (FBEHO41-01A)	15.625	31.25	7.81	15.625	0.00625	0.00625	12.5	25	NI	NI	NI	NI	NI	NI
	Δ*pst1*/Δ*ecm33* (FBEHO41-01A)	15.625	15.625	3.9	7.81	0.00313	0.00625	12.5	12.5	NI	NI	NI	NI	NI	NI

MIC50 and MIC90: the minimum inhibitory concentration 50 and 90%, NI: no-inhibition.

3.5.2 Determination of cell surface charge

Alcian blue is a positively charged dye that strongly binds to negative charges on fungal cell walls. Binding of Alcian blue (i.e. the reduction of absorbance in the reaction buffer) was used to quantify the charge state of cell walls. These assays demonstrated that specifically the deletion of *ECM33* led to increased Alcian blue binding in the cell wall of *C. glabrata*, *C. albicans* and *S. cerevisiae*. This effect was observed in *ECM33* deletions (*Δecm33* and *Δpst1/Δecm33* mutants). However, it was not observed in the *Δpst1* mutants (Figure 24). In the complemented strains of *C. glabrata* were not significantly different as compared to wild-type.

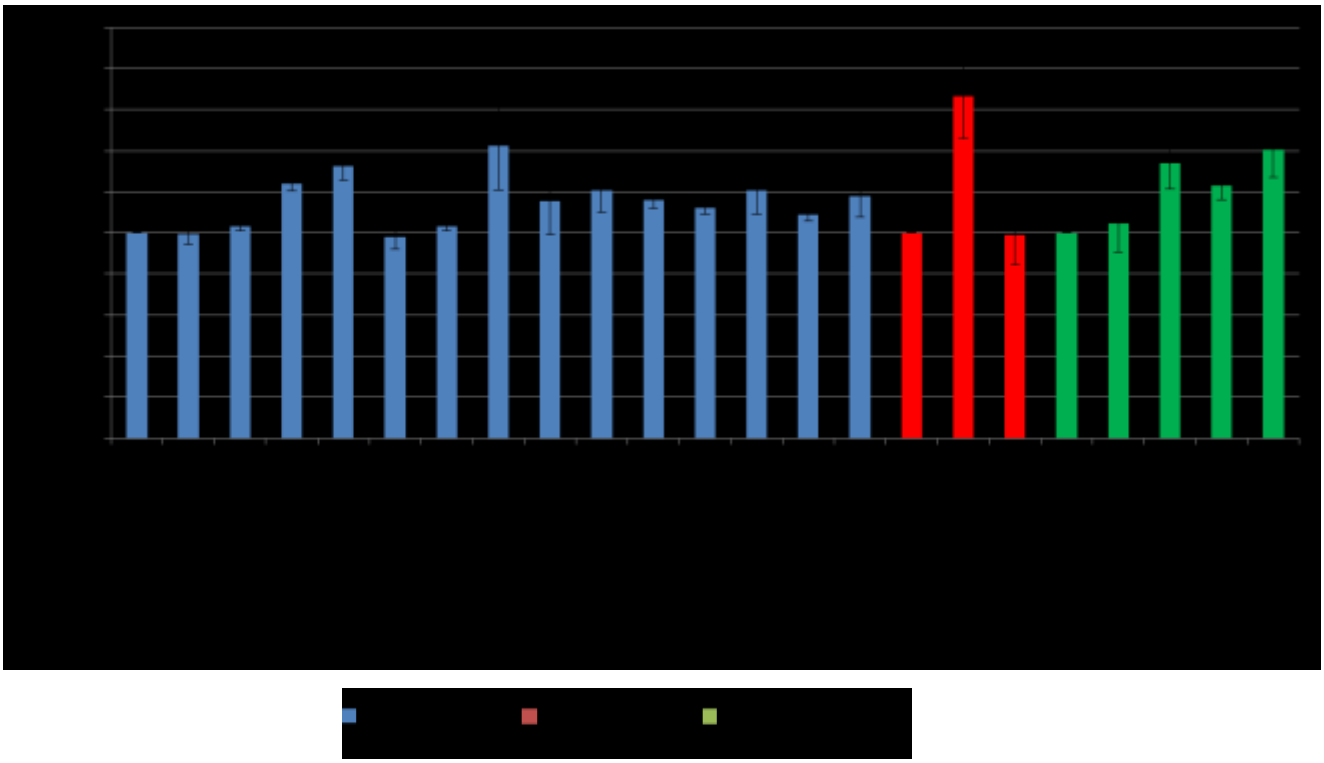

Figure 24. Alcian blue binding of *C. glabrata, C. albicans and S. cerevisiae*

Binding of Alcian blue normalized to the respective wild-type strains (=100%). *: statistic test by student's t-test (significance threshold $p < 0.05$). Error bars represent the mean value calculated from triplicate experiments (±SD).

3.5.3 Determination of cell wall composition

Fluorescence-activated cell sorting (FACS) assays were used to quantify the binding of different substances to the cell wall components glucan and chitin. Alexa-fluor 647-labelled wheat germ agglutinin (WGA) was used to stain chitin and Congo red to stain glucan in the fungal cell wall. Quantification by FACS analysis demonstrated that *ECM33* deleetion (*Δecm33* or *Δpst1/Δecm33*), but not *Δpst1* mutant led to an increased WGA staining in *C. glabrata*, *C. albicans* and *S. cerevisiae* (Figure 25 A).

Overexpression of *CgPST1*, *CgSPS2* or *CgSPS33* in the *Δecm33* mutant strain (*Δecm33:CgPST1, Δecm33:CgSPS2,* and *Δecm33:CgSPS33*) did not compensate the increased WGA binding. However, expression of *ECM33* from *C. albicans* and *S. cerevisiae* rescued this phenotype in the *ECM33* deletion of *C. glabrata* (Figure 25 A).

Consistantly, in all three species examined, no significant differences of the mutants in Congo red binding was observed, implying that the lack of *ECM33* does not affect the total glucan levels of the cells (Figure 25 B).

3.5.4 Measurement of adherence properties

To examine the adherence ability of the yeast cells, adherence assays towards polystyrene and silicone were carried out. In addition, the cell surface hydrophobicity was determined by measuring relocalization of cells from an aqueous to a hydrophobic phase (octane test). These hydrophobicity assays showed that the *C. glabrata Δecm33* mutant was significantly less hydrophobic as compared to the wild-type and auxotrophic strain. In addition, the *C. glabrata Δpst1/Δecm33* mutant was found to be significantly less hydrophobic as compared to the single *Δecm33* mutant. In contrast, the loss of *PST1* alone did not affect the hydrophobicity. There were no significant differences of cell surface hydrophobicity among the gene deletion strains in *C. albicans* and *S. cerevisiae* when compared to wild-type auxotrophic strains (Figure 26 A).

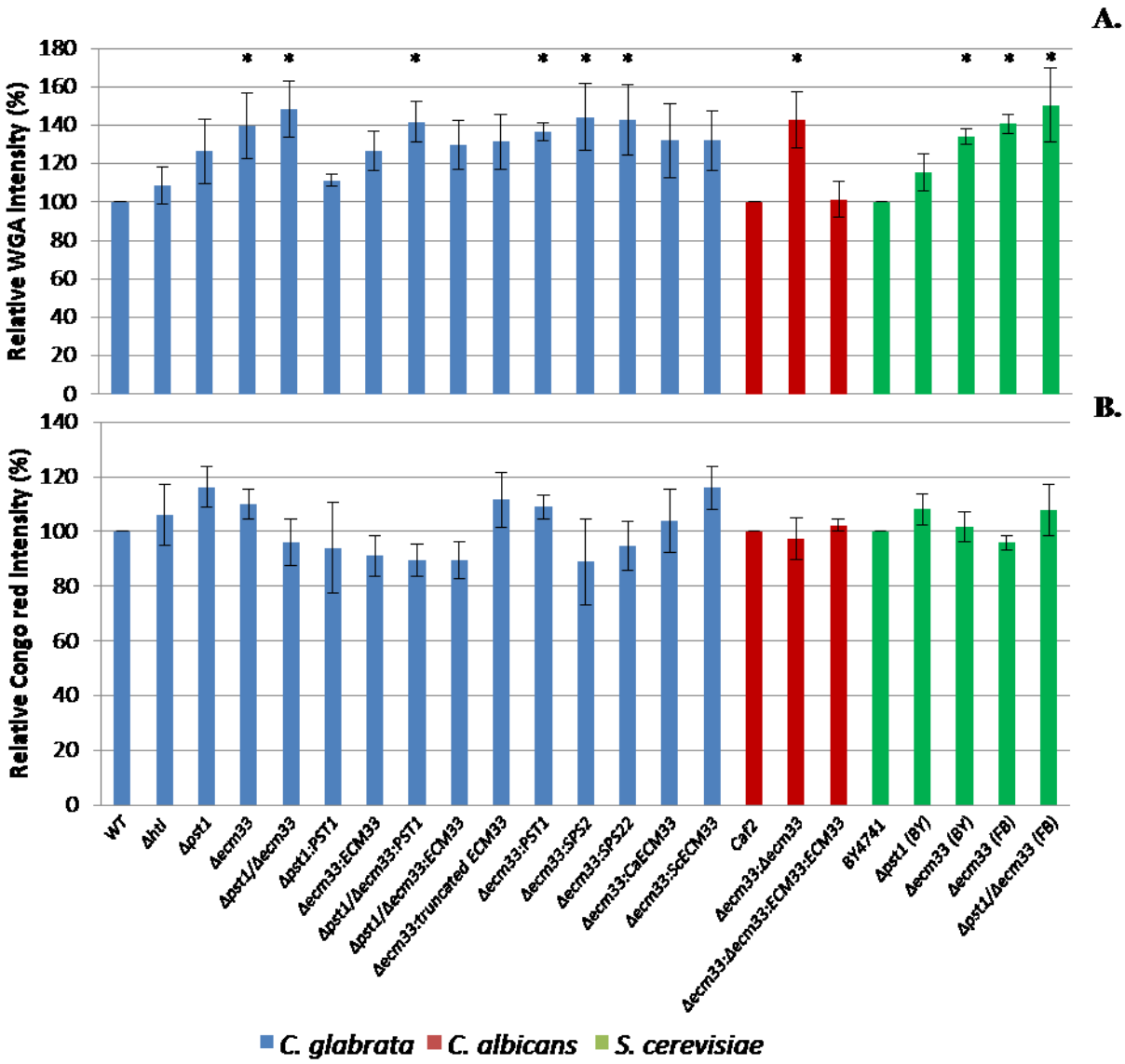

Figure 25. FACS analysis of WGA and Congo red binding in *C. glabrata, C. albicans* and *S. cerevisiae*

Realtive staining of chitin (A: Alexa Fluor® 647 conjugated wheat germ agglutinin) and glucan (B: Congo Red) normalized to the respective wildtype strains (=100%), as measured by FACS. *: statistic test by student's t-test (significance threshold $p < 0.05$). Error bars represent the mean value calculated from triplicate experiments (±SD).

In polystyrene adherence assays, the *C. glabrata Δecm33* and *Δpst1/Δecm33* mutants were significantly less adherent as compared to the wild-type and auxotrophic strains. There were no significant differences between strains of *C. albicans* and *S. cerevisiae*, respectively (Figure 26 B).

In these assays, the results of the hydrophobicity tests show a high correlation to the results of polystyrene tests. For example, the strains showing high hydrophobicity properties also showed, high polystyrene adherence capacities.

The silicone adherence capacity of *C. glabrata* was significantly reduced in the *Δecm33* mutant when compared to the wild-type and auxotrophic strains. Additionally, the double mutant *Δpst1/Δecm33* showed a further significant reduction of about 60% in adherence towards silicone when to the *Δecm33* mutant, which was rescued in the respective reconstituted controls.

Strains of *C. glabrata* heterologously complemented with *CgPST1*, *CgSPS2*, *CgSPS22*, *ScECM33* and *CaECM33* showed no statistically significant differences when compared to the parental *Δecm33* strain.

In *C. albicans*, the *Δecm33* mutant showed a similar reduced silicone adherence capacity. In *S. cerevisiae* this effect was not observed, since the *S. cerevisiae* wild types did not adhere to silicone at all, as shown in Figure 26 C.

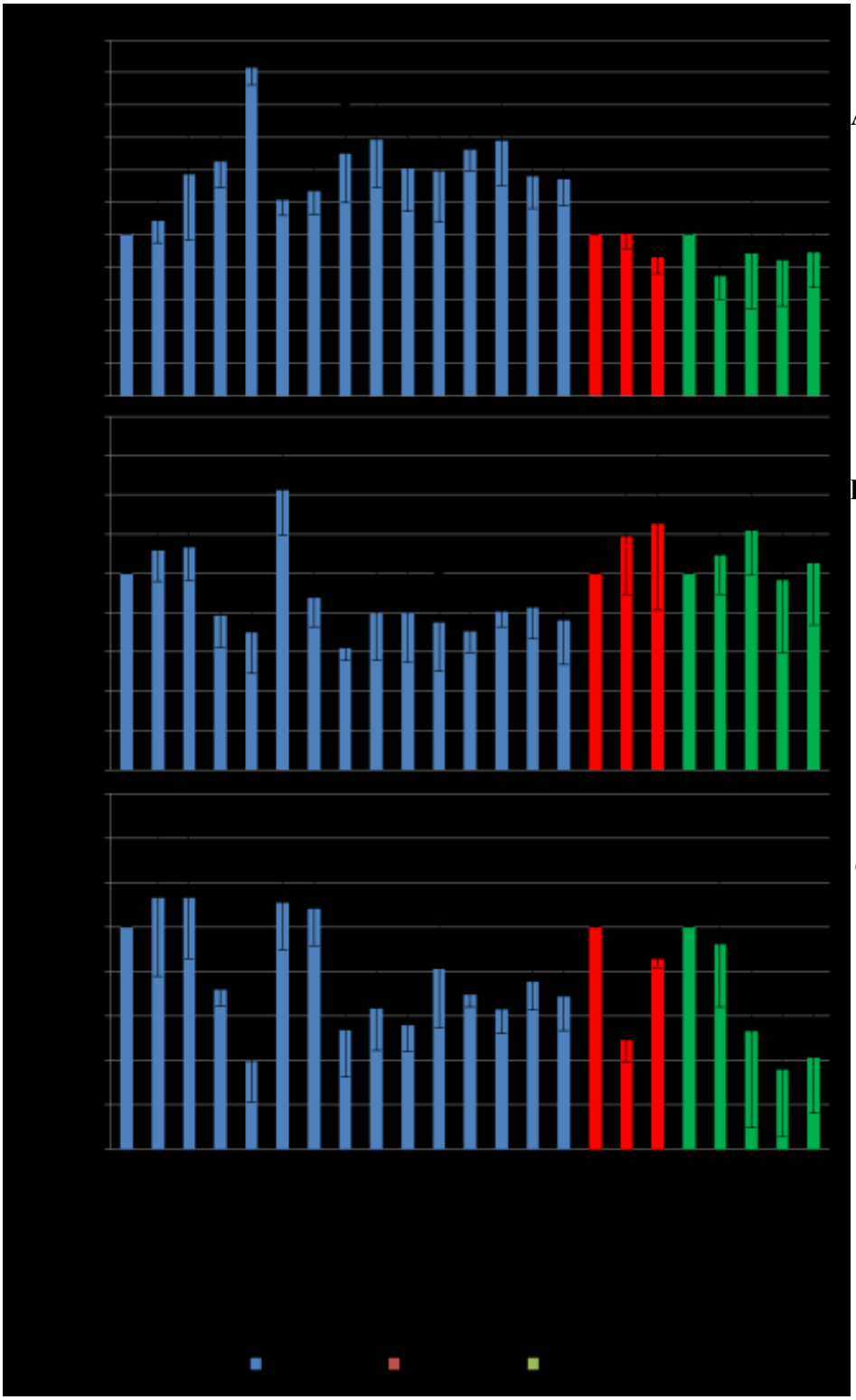

Figure 26. Adherence properties of *C. glabrata, C. albicans and S. cerevisiae*.

Realtive hydrophilicity and adherence proterties normalized to the respective auxotrophic wild-type strains (=100%), (A): hydrophilicity, (B): polystyrene adherence capacity, and (C): silicone adherence capacity. *: statistic test by student's t-test as (significance threshold $p < 0.05$ when compared to auxotrophic wild-type strain). **: significance threshold $p < 0.05$ when compared with *Δecm33*. Error bars represent the mean value calculated from triplicate experiments (±SD).

3.6 *C. glabrata* Ecm33 and Pst1 protein expression in *P. pastoris*

3.6.1 Recombinant pPICZα vector construction

The pPICZα vecor was used for expression of recombinant Ecm33 and Pst1 proteins (EasySelect™ Pichia Expression Kit, Invitrogen). The partial *CgECM33* and *CgPST1* genes without respective signal peptides and ω-sites were amplified and introduced into the pPICZα plasmid. The PCR product size of *CgECM33* and *CgPST1* fragments were 1,150 and 1,183 bp, respectively (Figure 27 A).

The recombinant pPICZα candidates were verified with double restriction enzyme digestion and plasmid DNA sequencing. The recombinant pPICZα:*CgECM33* candidate was digested with *EcoRI* and *KpnI* and the recombinant pPICZα:*CgPST1* candidate was confirmed by *EcoRI* and *XbaI*. The *CgECM33* and *CgPST1* digested fragments have a size of 1,144 and 1,177 bp, respectively (Figure 27 B). Confirmed vector plasmids were transformed into the *P. pastoris* strains X33 and GS115.

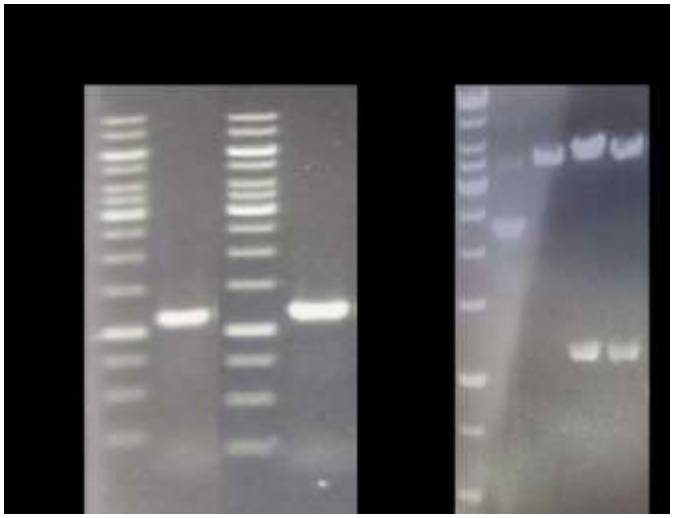

Figure 27. Amplification of *CgECM33* and *CgPST1* genes and verification of both recombinant pPICZα plasmids

A: PCR products of *CgECM33* and *CgPST1* genes. Lane 1,4: DNA marker, lane 2: 1,150 bp of *CgECM33*, and lane 4: 1,183 bp of *CgPST1*.
B: verification of the recombinant pPICZα:*CgECM33* and pPICZα:*CgPST1* plasmids by using double restriction enzyme digestion. Lane 1: DNA marker, lane 2: natural pPICZα (upper band: relaxed form and lower band: supercoiled), lane 3: pPICZα digested with *EcoR*I/*Kpn*I, lane 4: pPICZα:*CgECM33* digested with *EcoR*I/*Kpn*I, and lane 5: pPICZα:*CgPST1* digested with *EcoR*I/*Xba*I

3.6.2 *Cg*Ecm33 and *Cg*Pst1 proteins expression in *P. pastoris*

Resulting from the procedure outlined above, four strains were generated to express recombinant gylcosylated *Cg*Ecm33 and *Cg*Pst1 proteins in *P. pastoris* (GS115:*CgECM33*, GS115:*CgPST1*, X33:*CgECM33*, and X33:*CgPST1*). All strains were tested for recombinant protein expression and subsequently the conditions and time points of protein expression were optimized. The results of SDS-PAGE analyses showed that the *P. pastoris* supernatants from day 3 had the highest concentration of total proteins and that *P. pastoris* GS115 expresses both *Cg*Ecm33 and *Cg*Pst1 proteins to a higher extend as compared to the X33 strains (Figure 28). Based on these results, the GS115:*CgECM33* and GS115:*CgPST1* strains were used to express the proteins for further studies.

In SDS-PAGE analyses, both proteins bands were visible as high molecular weight smears between 130 to 55 kDa (compared to the calculated molecular weight deduced from the amino acid sequence of the recombinant *Cg*Ecm33 and *Cg*Pst1 proteins as 41 and 42 kDa, respectively). To analyse whether the observed higher molecular weight of the recombinant proteins was caused by extensive glycosylation, deglycosylation experiments were performed.

Treatment of the recombinant proteins with N-glycosidase (Endo-α-N-Acetylgalactosaminidase) resulted in a reduced molecular weight from around 100 to 72 kDa to approximately, 55 and 60 kDa, respectively.and a more coherent band in SDS-PAGE gels as shown in Figure 29. However, no reduction in the molecular weight of both proteins was observed when O-glycosidase (PNGase F) was used (Figure 29).

Similarly, double digestion was little different from treatment with N-glycosidase (Figure 29). The results indicated that the recombinant Ecm33 and Pst1 proteins which were produced in *P. pastoris* are heavily N-glycosylated as digestion with N-glycosidase resulted in a significant reduction of the molecular weight.

Recombinant Ecm33 and Pst1 proteins were used subsequently for determination of antibody specificity and cell-wall binding experiments.

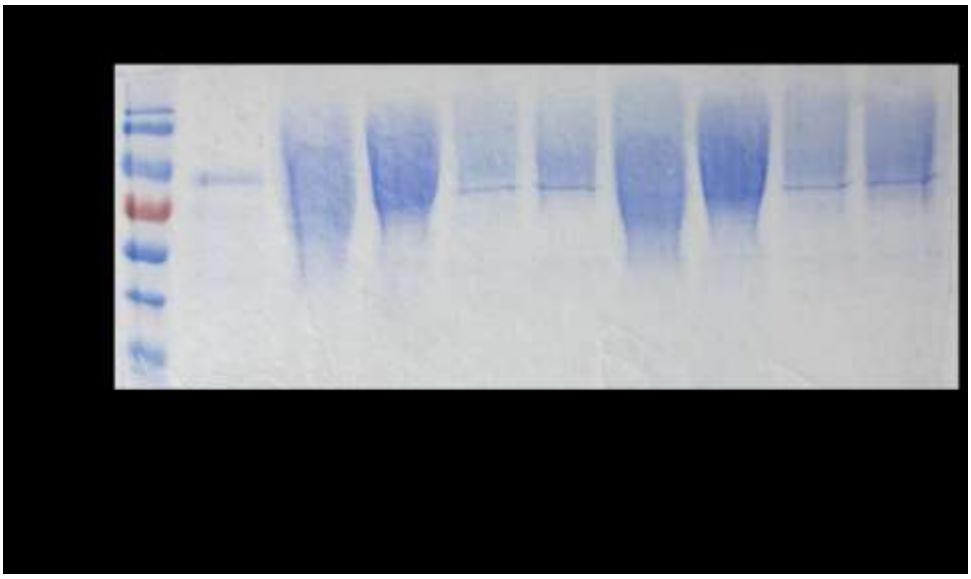

Figure 28. *Cg*Ecm33 and *Cg*Pst1 protein expression in *P. pastoris*

All recombinant *P. pastoris* strains were grown in BMMH media and incubated at 30°C with aeration for 2-3 days. Kex2 expression was used as a positive control of the protein expression (Lesage, G., Tremblay, M. et al., 2001). The GS115:*CgECM33* and GS115:*CgPST1* strains were used to express the proteins for further studies because of the highest protein production. Three days incubation is the best condition time for expression of *Cg*Ecm33 and *Cg*Pst1 proteins.

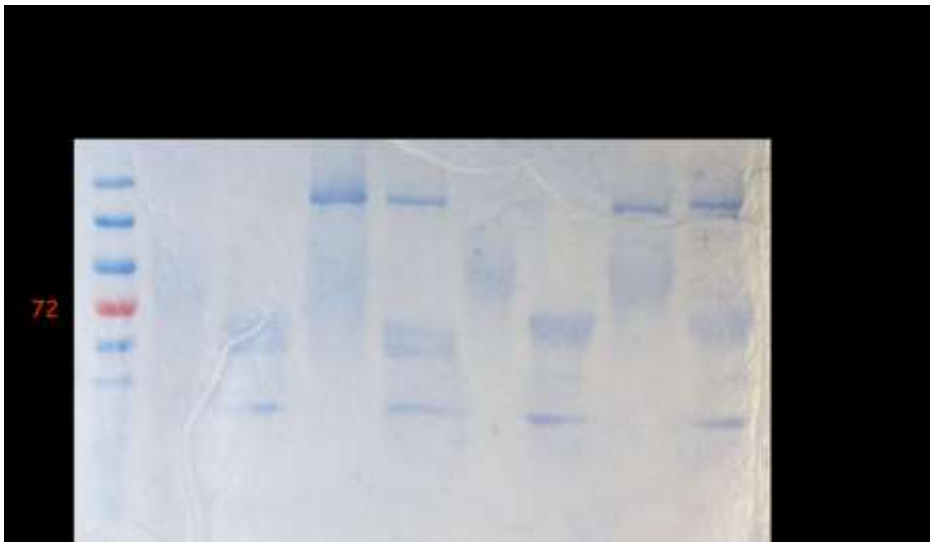

Figure 29. *Cg*Ecm33 and *Cg*Pst1 protein deglycosylation

*Cg*Ecm33 and *Cg*Pst1 recombinant proteins from *P. pastoris* were deglycosylated by using N-glycosidase, O-glycosidase, or by double digestion with N-glycosidae and O-glycosidase. U: untreated, N: treatment with N-glycosidase (molecular weight of 36 kDa), O: treatment with O-glycosidase (molecular weight of 147 kDa), and N/O: double treatment with N-glycosidae and O-glycosidase. *Cg*Ecm33 and *Cg*Pst1 from *P. pastoris* are N-glycosylated but do not appear to be O-glycosylated.

3.7 Anti-Ecm33 antibody production

3.7.1 Generation of plasmids for the expression of recombinant *Cg*Ecm33 protein in *E. coli*

Ecm33 protein expression in *E. coli* was performed using the QIAexpressionist™ system as described by the manufacturerer. The primers F-LM-pQE and R-LM-pQE were used to amplify a 1,150 bp of *ECM33* fragment (Figure 30 A) and cloned to the *BamHI*/*PstI* linearlized pQE30 plasmid. The generated pQE30:*CgECM33* plasmid was confirmed using double restriction enzyme digestion and plasmid DNA sequencing. The recombinant pQE30:*CgECM33* candidate was digested with *BamHI* and *Pst1*. The *CgECM33* digested fragment has a size of 1,144 bp as shown in Figure 30 B. After verification, the pQE30:*CgECM33* plasmid was transformed to the competent *E. coli* strain BL-21.

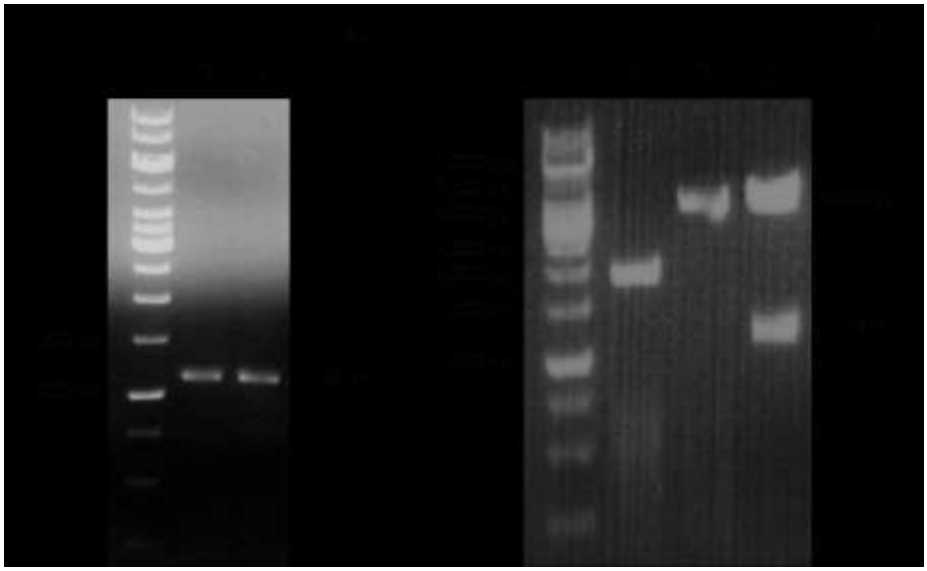

Figure 30. Amplification of the *CgECM33* gene and verification of the generated pQE30:*CgECM33*plasmids

A: PCR products of *CgECM33* gene. Lane 1: DNA marker, lane 2 and 3: 1,150 bp of *CgECM33*. B: verification of the generated pQE30:*CgECM33* plasmids by using double restriction enzyme digestion. Lane 1: DNA marker, lane 2: circular pQE30:*CgECM33* plasmid, lane 3: pQE30 digested with *BamHI*/*PstI*, lane 4: pQE30:*CgECM33* plasmid digested with *BamHI/PstI*.

3.7.2 *Cg*Ecm33 protein expression in *E. coli*

Recombinant *Cg*Ecm33 protein was expressed in *E. coli* for production of anti-Ecm33 polyclonal sera in rabbits. The recombinant *Cg*Ecm33 protein produced in *E. coli* was used for immunization rather than the variant produced in *P. pastoris* because of its lack of glycosylation circumventing production of non-specific antibodies to carbohydrate components. The transformed *E. coli* was induced with 1 mM IPTG for 4-5 hours and the His-Tag affinity chromatography was used to purify the *Cg*Ecm33 protein from cell lysates.

During purification, a band corresponding to recombinant *Cg*Ecm33 protein was not found in the flow through or the washing steps (Figure 31 A). Instead, the recombinant *Cg*Ecm33 was released from the Ni-NTA column in subsequent elution steps 3 and 4 (Figure 31 B).

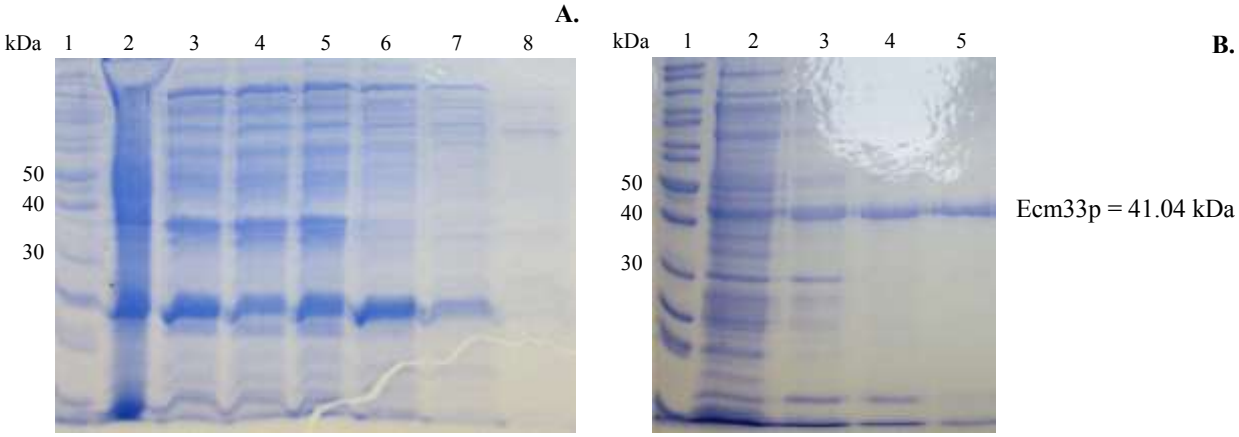

Figure 31. Purification of recombinant *Cg*Ecm33 by His-tag affinity chromatography

A: purification steps; lane 1: protein marker, lane 2: supernatant, lane 3-4: 1st and 2nd flow through, and lane 5-8: 1st to 4th washing. There is no an expected protein band in all steps.
B: elution steps; lane 1: protein marker, lane 2-5:1-4 elution, the expected *Cg*Ecm33 protein band was encountered in all elution steps but the *Cg*Ecm33 in 4th elution was purest.

3.7.3 Anti-Ecm33 antibody production

Production of anti-Ecm33 polyclonal antisera was performed by Eurogentec, Liège, Belgium. The purified recombinant *Cg*Ecm33 protein produced in *E. coli* was immunized in two rabbits (code number 22 and 23) by using 100 µg in each immunization. Serum samples of both animals were analysed before immunization to exclude the preexistence of antibodies cross reacting with *Cg*Ecm33 (data not shown). Serum samples were tested for antibody production at days 0 (pre-immune serum), 38, 66, and 87 after the first immunization by Western blott analysis with the recombinant *Cg*Ecm33 proteins produced in *E. coli* as well as *P. pastoris*. In addition, the recombinant *Cg*Pst1 proteins were used to analyse a cross-reaction. The results demonstrated that the generated polyclonal anti-Ecm33 antibodies can recognize both recombinant Ecm33 proteins from *E. coli* and *P. pastoris* as shown in Figure 32. Moreover, cross reaction of the anti-Ecm33 polyclonal antibody from rabbit no. 23 to the Pst1 protein was observed (Figure 32 B).

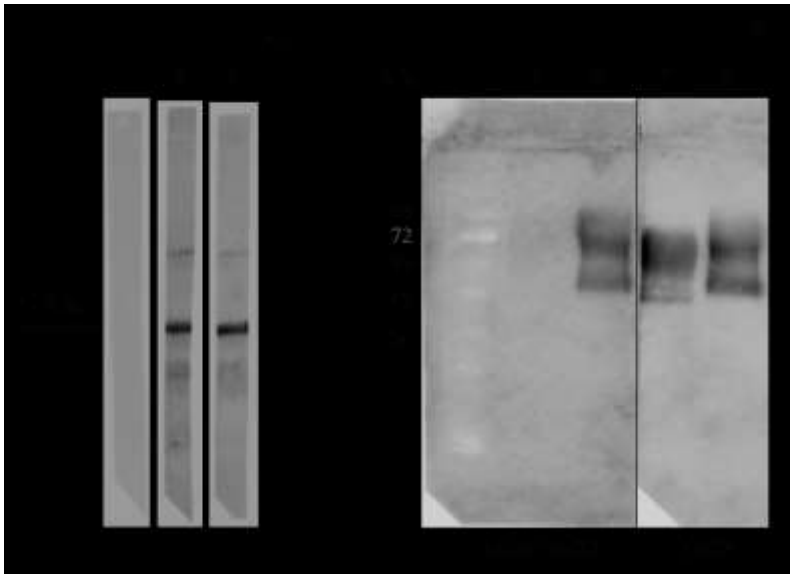

Figure 32. Western blot analyses of the generated anti-*Cg*Ecm33 antisera

A: testing of *Cg*Ecm33 antigen from *E. coli* and anti-CgEcm33 antisera (titer 1:4,000). Lane 1: pre-immune serum, lane 2: serum in 2nd month of rabbit No.22, lane 3: rabbit serum in 2nd month of rabbit No.23. The results demonstrate that the immunized serum in 2nd month of two rabbits can recognize *Cg*Ecm33 antigen purified from *E. coli*.
B: testing of *Cg*Ecm33 *and Cg*Pst1 antigens from *P. pastoris* and anti-*Cg*Ecm33 antisera (titer 1:4,000). Lane 1: protein marker, lane 2: *Cg*Pst1 and rabbit serum No.22, lane 3: *Cg*Ecm33 and rabbit serum No.22, lane 4: *Cg*Pst1 and rabbit serum No. 23, lane 5: *Cg*Ecm33 and rabbit serum No.23. The results demonstrate that serum from rabbit no. 22 can recognize *Cg*Ecm33 only but serum from rabbit no. 23 can recognize both *Cg*Ecm33 and *Cg*Pst1.

3.8 Cell wall protein release assay

To investigate a potential loss of cell wall components in the absence of the Ecm33 protein overnight culture supernatants were collected from *C. glabrata* wild-type and mutant strains and concentrated 50-fold (15 ml spin column pore size 10,000 Da). In the concentrated culture supernatants total protein concentrations were measured and SDS-PAGE and western blot analyses performed to determine a potential release of β-1,6-glucan and/or the abundant glucan-bound structural cell wall protein Cwp1.

Compared to the wild-type, the *C. glabrata* Δ*ecm33* mutant released both, 1,6- β-glucan and Cwp1. The Δ*pst1/*Δ*ecm33* double mutant released Cwp1 and 1,6-β-glucan linked protein to a larger degree than Δ*ecm33* only (Figure 33).

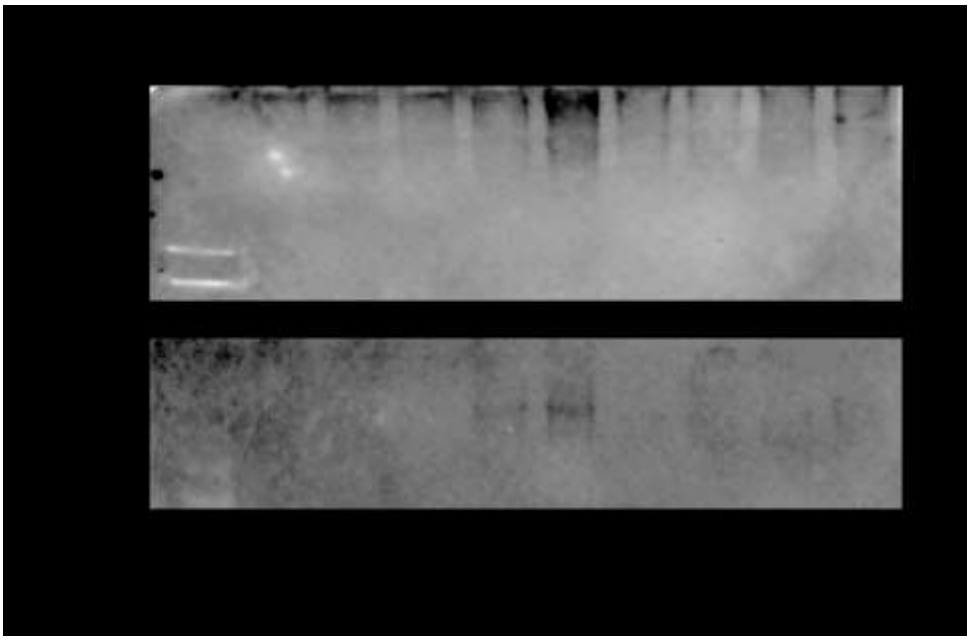

Figure 33. Western blot analyses of culture supernatants

A: Detection of 1,6-β-glucan linked protein in culture supernatants by western blot with monoclonal rabbit anti-β-1,6-glucan antibodies. Note that glucans do not migrate inside the separating gel due to their size, but are rather stacked at the top. B: Detection of Cwp1 protein in culture supernatants by western blot with polyclonal rabbit anti-Cwp1 antibodies.
Lane 1: protein marker, lane 2: concentrated culture supernatant of wild-type, lane 3: Δ*htl* (auxotroph), lane 4: Δ*pst1*, lane 5: Δ*ecm33*, lane 6: Δ*pst1/*Δ*ecm33*, lane 7: Δ*pst1:CgPST1*, lane 8: Δ*ecm33:CgECM33*, lane 9: Δ*pst1/*Δ*ecm33:CgPST1*, lane 10: Δ*pst1/*Δ*ecm33:CgECM33*
[a]: ratio calculated by the formular of (percent of total protein release ÷ percent of growth rate) x 100; growth rate and total protein release was relative value normalized to the auxotrophic wild-type strains.

3.9 Binding assay

As the molecular function of the Ecm33 protein family members is unknown, a binding assay was performed to investigate whether Ecm33 might actively bind to the cell wall.

The results demonstrated that both recombinant Ecm33 and Pst1 proteins bind better to the extracted cell walls (ECWs) of the Δ*ecm33*, *Δecm33:truncated CgECM33* and Δ*pst1*/Δ*ecm33* mutants as compared to wild-type and Δ*pst1* ECWs (Figure 34).

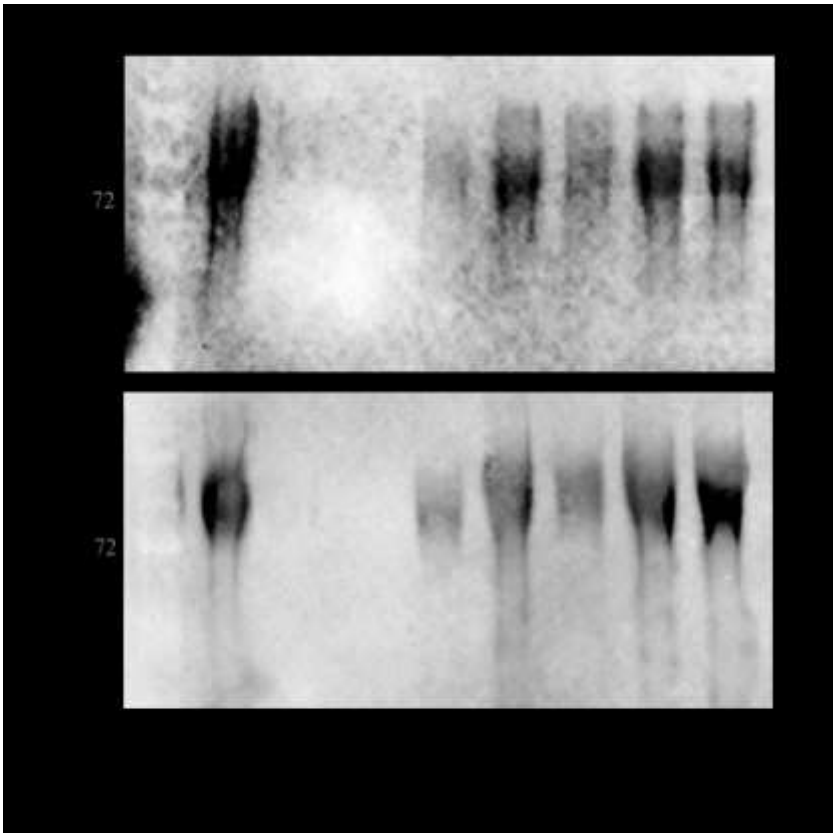

Figure 34. Binding assay

A: Incubation of the extracted cell walls (ECWs) and Pst1 protein, detection with rabbit serum no. 23.
B: Incubation of the extracted cell walls (ECWs) and Ecm33 protein, detection with rabbit serum no. 22.
Lane 1: protein marker, lane 2: Pst1 protein from *P. pastoris* in A. or Ecm33 protein from *P. pastoris* in B., lane 3: wild-type ECWs, lane 4: ELISA blocking solution, lane 5: wild-type ECWs, lane 6: Δ*ecm33* ECWs, lane 7: Δ*pst1* ECWs, lane 8: *Δecm33*:truncated *CgECM33* ECWs, lane 9: Δ*pst1*/Δ*ecm33* ECWs

CHAPTER 4

DISCUSSION

The increased incidence of fungal infections seen over the past decades significantly impacts on human public health (Giri, S. and Kindo, A. J., 2012; Mayer, F. L., Wilson, D. et al., 2013). Among fungal infections, candidiasis is the most frequent and *C. glabrata* is the second most prevalent causative agent of systemic and local candidiasis. *C. glabrata* infection may become difficult to treat because this organism can be highly resistant to anti-mycotic drugs, especially the commonly used substance fluconazole (Borg-von Zepelin, M., Kunz, L. et al., 2007; Pfaller, M. A., Diekema, D. J. et al., 2010). The mortality rate of candidiasis caused from *C. glabrata* infection can be as high as 50% in patients with extended candidemia longer 30 days (Das, I., Nightingale, P. et al., 2011).

For fungal pathogenesis, the fungal cell wall carries key functions. Among proteins localized to the *C. glabrata* cell wall, the a glycosyl-phosphatidylinositol (GPI)-anchored protein Ecm33 is one of the most abundant (de Groot, P. W., Kraneveld, E. A. et al., 2008). Sequence homologs of Ecm33 can be found in many fungi, where full genome sequences are available. Examples are not only budding yeasts, but also fission yeast, dimorphic fungi, dermatophytes, other molds, and plant pathogenic fungi.

Previous studies showed that Ecm33 proteins was associated with cell wall integrity an and biofilm formation, and pathogenicity (Martinez-Lopez, R., Monteoliva, L. et al., 2004; Pardo, M., Monteoliva, L. et al., 2004; Chabane, S., Sarfati, J. et al., 2006; Martinez-Lopez, R., Park, H. et al., 2006; Romano, J., Nimrod, G. et al., 2006; Martinez-Lopez, R., Nombela, C. et al., 2008). Experimental evidence also suggested differences in the biological role of Ecm33 between, for example, yeast and molds. Furthermore, the exact biochemical function of Ecm33 in any fungus remains speculative. Because of the clinical impact of the cell wall and the fact that Ecm33 homologs were identified experimentally in the cell wall proteome as very abundant CWPs in the human pathogen *C. glabrata* (de Groot, P. W., Kraneveld, E. A. et al., 2008), this work was performed to characterize the role of the Ecm33 protein family in this organism and

the results were compared to comprehensive analyses that were carried out with the homologs in *C. albicans* and *S. cerevisiae*.

4.1 *CgECM33* is required for cell fitness, cell wall integrity, and adhesion

The results from agar drop dilution assays using different cell wall perturbing agents demonstrated that the *Δecm33:Δecm33* strain of *C. albicans* and the Δ*ecm33* and Δ*pst1*/Δ*ecm33* strains of *S. cerevisiae* were sensitive to Caffeine, Calcofluor white, and Congo red when compared to the auxotrophic wild-type in each organism. However, there were no phenotypic differences in the Δ*pst1* mutant of *S. cerevisiae* compared to the wild type strain. Our results confirm previous studies which showed that the *Δecm33* mutant in *C. albicans* was susceptible to Calcofluor white, Congo red and Hygromycin B (Martinez-Lopez, R., Monteoliva, L. et al., 2004) and *Δecm33* and *Δpst1/Δecm33* mutants in *S. cerevisiae* had a cell wall weakness, being sensitive to Calcofluor white, Caffeine and Hygromycin B (Pardo, M., Monteoliva, L. et al., 2004). These results indicate that *ECM33* of *C. albicans* and *S. cerevisiae* are required for cell wall integrity.

Surprisingly, all generated mutants in *C. glabrata* did not show the phenotypic differences which were discovered in *C. albicans* and *S. cerevisiae* using agar drop dilution assays. However, we were able to demonstrate that this observation is based on technical limitations of these assays in this organism, as *C. glabrata* shows a high intrinsic resistance towards these substances. Instead, the Δ*ecm33* and Δ*pst1*/Δ*ecm33* mutants in *C. glabrata* show phenotypes when higher concentrations of cell wall perturbing agents are used. Therefore, additional broth micro-dilution assays were developed to explore the generated mutant strains in *C. glabrata*. These assays showed that the Δ*ecm33* mutant in *C. glabrata* was more susceptible to Calcofluor white and Congo red. The Δ*pst1*/Δ*ecm33* double mutant was even more susceptible (approx. 4-times more sensitive when compared to the wild-type) towards Calcofluor white, Congo red, SDS and Hygromycin B. Taken together, these results indicate that *ECM33* in *C. glabrata* is also required for cell wall integrity.

The Δ*ecm33* mutants in *C. albicans*, *S. cerevisiae* and *C. glabrata* had a defect in growth under YPD and RPMI 1640 media at 30^{o}C and 37^{o}C condition. The completed revertant of *C. albicans* and *C. glabrata* showed normal growth as the *Caf2* and *Δhtl* auxotroph strains,

respectively. These data indicate that *ECM33* is an essential protein for growth in the three organisms. Moreover, we found that the Δ*pst1*/Δ*ecm33* double mutant in *C. glabrata* and *S. cerevisiae* had slower growth rates than the *Δecm33* mutant in both media and at both temperatures. These data indicate synergistic effects upon deletion of *ECM33* and *PST1*. However, no significant differences of growth were observed in the *Δpst1* mutant of *S. cerevisiae* and *C. glabrata* when compared to its auxotrophic wild-type. Taken together, Ecm33 is a key protein in the cell wall of *C. glabrata* that determines not only the cell wall strength, but also influences the growth rate of these organisms.

Furthermore, the analyses on the generated *C. glabrata* strains showed that the *Δecm33* mutant displays significantly increased hydrophilic surface properties when compared to the wild-type or auxotrophic wild-type strain. Also, the *Δpst1/Δecm33* double mutant was found to be more hydrophilic as compared to the *Δecm33* single mutant. The results from polystyrene adherence capacity assays correlated with hydrophobicity surface properties of the strains: more hydrophilic (less hydrophobic) isolates showed less polystyrene adherence capacity and consequently, the *C. glabrata* mutants *Δecm33* and *Δpst1/Δecm33* lost polystyrene adherence capacity. In contrast, no phenotypic alterations were observed in terms of hydrophilicity and/or polystyrene adherence capacity in the *C. albicans* and *S. cerevisiae* mutants. However, it should be noted that the parental strains that were used to generate the mutants in *C. albicans* and *S. cerevisiae* do not have any ability to adhere to polystyrene. Therefore, it is difficult to compare and correlate *ECM33* function and the influence on polystyrene adherence in these organisms.

The results of silicone adherence assays demonstrated that the *Δecm33* mutant of *C. glabrata* has a lower adherence capacity to this material as compared to the wild-type strain. The *Δpst1/Δecm33* double mutant is reduced approximately 60% when compared to wild-type and also shows a significant reduction when compared to the *Δecm33* mutant. The *Δecm33* mutant of *C. albicans* is also affected in silicone adherence capacity, but this phenotype was not observed in the *S. cerevisiae* mutants.

The results obtained with *C. glabrata* confirm that *ECM33* affects important adherence capacities of human pathogenic *Candida spp*. and this assumption is further supported by our results with the *C. albicans* mutants and a previous study using a *C. albicans Δecm33* mutant. This study showed that in *C. albicans* the *Δecm33* mutant displays a lower adherence and reduced invasion to endothelial and epithelial cells as compared to auxotrophic wild-type

(Martinez-Lopez, R., Park, H. et al., 2006; Rouabhia, M., Semlali, A. et al., 2012). Therefore, we conclude that beyond cell wall strength and growth, *ECM33* contributes significantly to the adherence capacities of human pathogenic yeasts. As a consequence *ECM33* might be considered a gene associated with virulence in *Candida spp.*

4.2 *ECM33* of *C. glabrata*, *C. albicans*, and *S. cerevisiae* have a similar function but not identical functions.

To explore the function of CgEcm33-family a set of complementation mutants were generated and analyzed. In the *CgΔecm33* strain (i) the homologs of *C. albicans* (*CaECM33*) and *S. cerevisiae* (*ScECM33*), (ii) the *ECM33* homologs of *C. glabrata* (*PST1*, *SPS2* and *SPS22*) and (ii) a truncated version *CgECM33* without the GPI-anchor motif were introduced in order to explore if the phenotypes of the *CgΔecm33* mutant can be rescued. All generated strains were tested for growth, cell wall strength, hydrophobicity and adhesion to polystyrene and silicone. Gene expression analysis confirmed that in both constructed strains, *Δecm33*:*CaECM33* and *Δecm33*:*ScECM33* could be expressed at a levels that compare to approx. 90% of the wild-type *ECM33* gene in *C. glabrata.* Interestingly, the phenotypes of the two complemented strains were very similar to the *C. glabrata* wild-type, indicating that both heterologous homologs can compensate the role of *CgECM33* in terms of cell wall fitness, growth and cell wall composition. However, in the adherence assays and the Alcian blue staining assay, the phenotypes of the *CgΔecm33* mutant could not fully be rescued on all experiments. This might indicate that subtle differences do exist in the multilayered function of the *ECM33* genes that manifest e.g. in the assembly and/or incorporation of cell wall proteins (mediating negative surface charge) including the cell wall adhesins. Another option would be that the Ecm33 protein acts as a adhesion protein itself, but this seems to be not very likely as the *S. cerevisiae Δecm33* mutant expressing *CaECM33* does not adhere to or penetrate epithelial cell whereas the auxotrophic wild type strain of *C. albicans* does (Martinez-Lopez, R., Park, H. et al., 2006).

The next question was whether the homologous genes of the *CgECM33* family can replace the function of *CgECM33*. To answer this question, a second set of overexpression strains were generated in the *CgΔecm33* mutant (*Δecm33*:*CgPST1*, *Δecm33*:*CgSPS2*, and *Δecm33*:*CgSPS22*) and tested as described above. The results of gene expression analyses

showed that there were significantly increased levels of up-regulation in the three overexpression strains, but the expression levels of the integrated genes were found only 20%, 15% and 10%, respectively above the wild-type level. We also excluded that a negative feedback regulation is observed in the *ECM33* gene family of *C. glabrata* and that the gene product from an introduced over-expression plasmid induces the down-regulation of the natural expression of the respective gen homologs. Moreover, the phenotypic results from the three complemented strains displayed very similar phenotypes as the *Δecm33* mutant indicating, that the other three members of this family can not compensate the function of *CgECM33*. These data support the idea that the proteins do not show functional redundancy. However, at this point it cannot be ruled out, that the experimental level of overexpression was too low to induce noticeable compensatory effects or that a few overlapping, but so far unrecognized activities do exist within this family. In agreement with these data in a previous study in *S. cerevisiae* high level expression of *ScPST1* in a *ScΔecm33* mutant (*Δecm33*/Yep352-*PST1* strain) could not compensate or rescue the phenotype of the *Δecm33* strain, when tested with the cell wall perturbing agents Calcofluor white, Congo red and Caffeine. However, a very slight compensation was observed for the hygromycin B phenotype (Pardo, M., Monteoliva, L. et al., 2004).

When the truncated version of Cg*ECM33* without the GPI-anchor motif was reintroduced in the *CgΔEcm33* mutant all the phenotypes observed in the deletion strain could not be rescued. This confirms that the anchor and most likely covalent cell wall incorporation of *ECM33* is necessary for its proper function (see below).

4.3 Ecm33 is associated with alterations in the chemical composition of the cell wall

As outlined above, the results of the Alcian blue staining experiments showed that *ECM33* deletion (*Δecm33* and *Δpst1/Δecm33*) resulted in an increase of negative charges on the cell surface of *C. glabrata* pointing to an altered composition of the cell wall (possibly mediated by macromolecules such as proteins, mannans, glucans, and chitins). In contrast, this effect was not observed when only *PST1* was deleted in *C. glabrata*.

To further investigate these compositional alterations staining of glucan and chitin in the yeast cells walls using wheat-germ agglutinin (WGA–Alexafluor 647 conjugate) and Congo red, respectively were performed and analyzed by using a Flow cytometer. The results of the chitin

staining experiments in the three organisms demonstrated that the *Δecm33* and *Δpst1/Δecm33* mutants, but not the *Δpst1* mutant, had more chitin in cell wall when compared to wild-type auxotrophic strain. Again, when the homologs of the *CgECM33*-family were over expressed in the *C. glabrata Δecm33* mutant (*Δecm33*:*CgPST1, Δecm33*:*CgSPS2,* and *Δecm33*:*CgSPS22*) a reduction of the level of chitin was not observed showing that this alteration in cell wall composition can not be rescued by these *ECM33* family members. In contrast, *ECM33* from *C. albicans* and *S. cerevisiae* could complement this phenotype in *C. glabrata*. The observed increase in the chitin level of the mutant might have different reasons: (i) *ECM33* might be involved in the incorporation of chitin in the cell wall. Consistent with this, a previous report on *C. albicans* showed that *Δchs* (chitin synthase) *2*, *Δchs3*, *Δchs2/Δchs3*, and *Δecm33* mutants could not release melanin pigment to the environment after induction with L-3,4-dihydroxyphenylalanine (L-DOPA) and *N*-acetylglucosamine, while the auxotroph NGY152 could do that. This indicates that a functional *ECM33* gene is required for the production of cell wall chitin components involved in melanin externalization (Walker, C. A., Gomez, B. L. et al., 2010). These analyses were also performed with the generated *C. glabrata* strains, however no melanin externalization, neither in wild-type strains, nor in the mutants was observed in this organism (data not shown). (ii) The increased chitin might also reflect an indirect and compensational effect induced by the cell wall weakening in the *CgΔecm33* mutant. Generally, when fungi face a disruption of cell wall strength, an up-regulation of the cell wall integrity-pathway and subsequently a compensatory increase in chitin production is observed to prevent cell wall collapse (Kapteyn, J. C., Van Egmond, P. et al., 1999). In agreement with our findings, in two previous studies, *S. cerevisiae Δecm33* mutants display an enhanced Calcofluor white fluorescence intensity due to high chitin content on cell wall (de Groot, P. W., Ruiz, C. et al., 2001; Pardo, M., Monteoliva, L. et al., 2004).

The relative quantification of the glucan level in the cell wall exhibited no significant differences between any of the *C. glabrata* strains. Also alterations of the glucan level in cell wall were not observed in the mutants of *C. albicans* and *S. cerevisiae*. These data imply that the lack of *ECM33* does not affect the total glucan level on the cell wall.

However, using Western blot analyses we observed that in *CgΔecm33* mutant released more β-1,6-glucan linked protein to the culture supernatant as compared to wild-type strain and this effect was even more pronounced in the double *Δpst1/Δecm33* mutant. This significant

observation was also made in the *Sc∆ecm33* mutant (de Groot, P. W., Ruiz, C. et al., 2001). In addition, the abundant cell wall protein Cwp1 of *C. glabrata* was detected in the culture supernatant of *∆ecm33* and *∆pst1/∆ecm33* mutants using Western blot analyis. This result is similar to the result of a study by Pardo *et al.* in *∆ecm33* mutant of *S. cereviaise*. This group showed that the *Sc*Cwp1 protein is released to the culture medium in the bakers yeast mutant. In contrast, in a further study by De Groot's group the *Sc*Cwp1 protein was not identified in the culture supernatant of *∆ecm33* mutant of *S. cereviaise*. However, both groups noticed the release of Pir2 cell wall proteins, β-1,6-glucan (Pardo, M., Monteoliva, L. et al., 2004) and also β-1,3-glucan (de Groot, P. W., Ruiz, C. et al., 2001) to the culture supernatant. In summary, the altered cell wall composition and the loss of cell wall components and proteins of the mutants indicate that *ECM33* plays crucial role in the assembly of the cell wall in *C. glabrata* influencing its architecture and function.

4.4 A model for the role of Ecm33 for the fungal cell wall

As shown above, deletion of *ECM33*, but not *PST1*, has a profound effect on the stability and architecture of the cell wall, not only in *C. glabrata*, but also in other fungi such as *C. albicans* and *S. cerevisiae*. The cells are significantly more susceptible to cell (wall) perturbing agents, reflecting a loss of cell wall rigidity. Furthermore, the composition of the cell wall is altered in a way that more chitin is present (potentially representing a compensatory mechanism), and β-1,6-glucan containing material is lost to the environment. Most likely as a consequence of β-1,6-glucan loss, also adhesion is reduced, since adhesins are in turn linked to β-1,6-glucan through GPI-anchors. In total, this leads to reduced fitness, and probably also to reduced virulence, as shown at least for *C. albicans* (Martinez-Lopez, R., Monteoliva, L. et al., 2004; Martinez-Lopez, R., Park, H. et al., 2006; Martinez-Lopez, R., Nombela, C. et al., 2008; Rouabhia, M., Semlali, A. et al., 2012).

These phenotypes can be explained, if we assume that Ecm33 provides a crucial link between β-1,6-glucan and another, as of yet unknown, component of the cell wall (Figure 35).

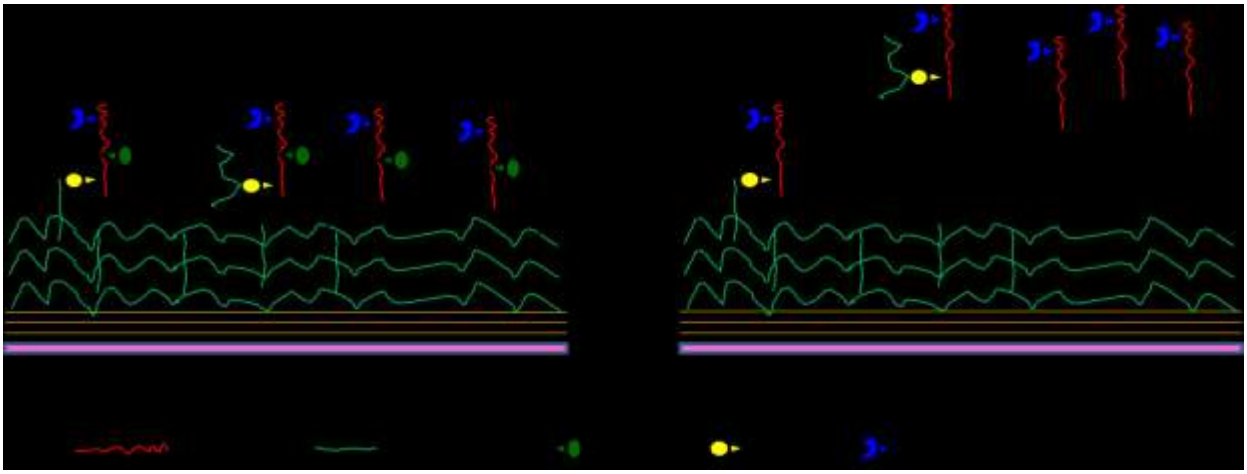

Figure 35. Hypothetical model of Ecm33 contribution to cell wall structure

4.5 Exploring the molecular function of Ecm33

Three possibilities of Ecm33 protein function can be proposed based on our experimental results: Model I: Ecm33 could be a structural protein, where Ecm33 is linked to the cell wall through the GPI anchor and the activity of another protein; Model II: Ecm33 could be a lectin, binding carbohydrate moieties of the cell wall; Model III: Ecm33 itself could be an enzyme, linking 1,6-glucan containing material to the cell wall (Figure 36).

To explore these options, different molecular tools were set up to help deducing data that support any of the model proposed above: active recombinant forms of Ecm33 and Pst1 proteins were made in *P. pastoris*, antisera were raised against recombinant Ecm33 expressed and purified from *E. coli*, and to explore the role of the GPI-anchor, *C. glabrata Δecm33* strain expressing a C-terminally truncated form of Ecm33 (devoid of the GPI anchor) were created.

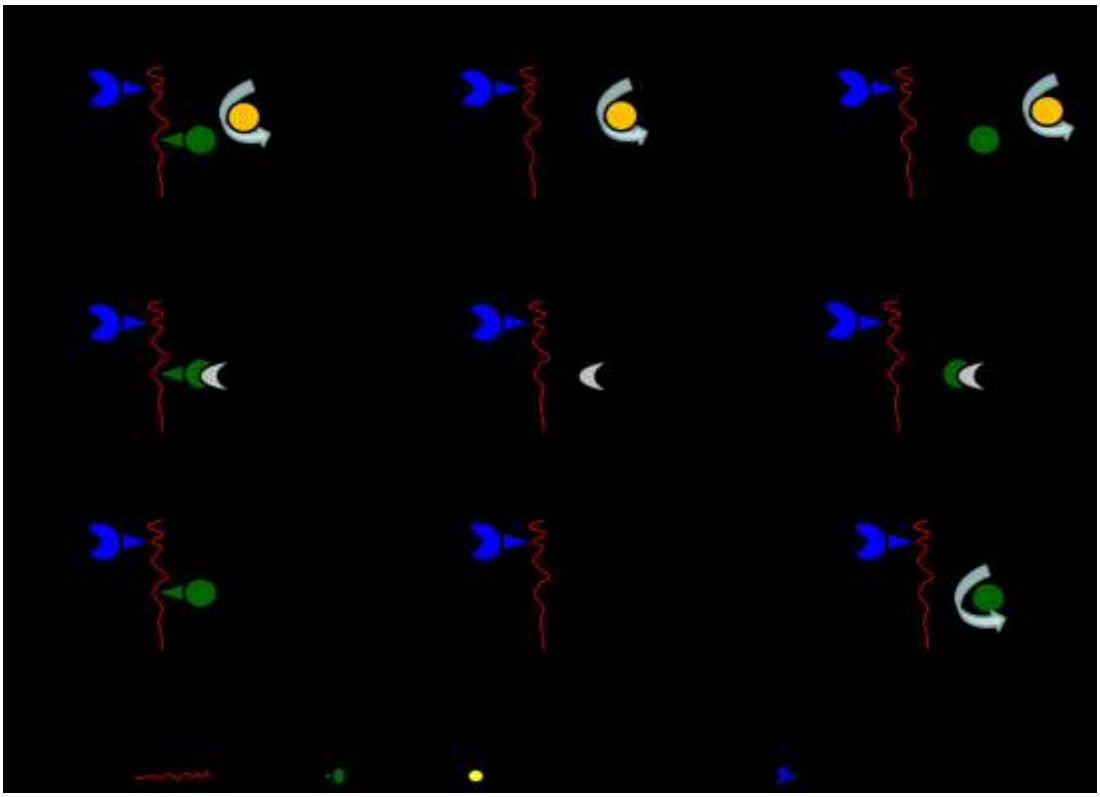

Figure 36. Models of potential Ecm33 functions

If Ecm33 was an enzyme, the GPI anchor should not be needed for full function. We therefore re-investigated all of the phenotypes observed in the *Δecm33* mutant in a strain expressing a truncated, GPI-anchor-less form (Δ*ecm33:*truncated *CgECM33*). As described above, the phenotypic results of Δ*ecm33:*truncated *CgECM33* were similar to *Δecm33* mutant while the strain *Δecm33:CgECM33* was conformable to wild-type, indicating that a truncated form cannot, even partially, complement the loss of *ECM33*. This strongly argues against an enzymatic function of Ecm33. It also implies that the GPI-anchor of Ecm33 protein is a necessary part for its function.

In case that, *ECM33* was a structural component or a lectin, the protein should be found in the cell wall of Δ*ecm33:*truncated *CgECM33* despite the lack of its GPI anchor. In the case of a structural component, Ecm33 should be covalently linked, and not extractable, in the case of a lectin it should bind the cell wall reversibly. To address this, antisera against recombinant Ecm33 produced in *E. coli* were raised. However, although antisera from two animals vividly recognized

the recombinant form of the protein, they failed to recognize any epitope in proteins or cell walls extracted from *C. glabrata.*

Nevertheless, the antisera were able to recognize recombinant Ecm33 produced in *P. pastoris*. One of the antisera also cross-reacted with recombinant Pst1, enabling the study of both proteins. Binding assays of the two recombinant proteins were carried out on extracted cell walls (ECWs) from the different mutant strains. These experiments demonstrated that *Cg*Ecm33 and *Cg*Pst1 protein both bind ECWs from Δ*ecm33*, *Δecm33*:truncated *CgECM33* and Δ*pst1/*Δ*ecm33* better than wild-type and Δ*pst1* ECWs. From this, we can propose that the lack of Ecm33, but not Pst1 protein in the cell wall leaves epitopes which can be actively and reversibly bound by both Ecm33 and Pst1.

In order to identify potential binding partners, initial competition binding experiments with glucose and N-acetylglucosamine (Supplements, chapter 6.4) were carried out. Preliminary results from these experiments suggest a stronger inhibition of Ecm33 binding to the cell wall by glucose than N-acetylglucosamine.

According to our results, it can be summarized that Ecm33 in *C. glabrata* is associated with cell growth, cell wall assembly and integrity, chemical composition of the cell wall and the adherence capacity of the organism. The complementation experiments show that the Ecm33 proteins form *C. albicans*, *S. cerevisiae* and *C. glabrata* have overlapping, but not identical functions, as many phenotypes that were observed in the *CgΔecm33* mutant could be restored by heterologous expression. Interestingly, the adherence phenotype and the altered surface charge could not be complemented by heterologous expression indicating that *Cg*Ecm33 shows subtle functional differences possibly in mediating the proper incorporation of cell wall proteins by acting as a lectin. The results also show that the other members of the Ecm33 protein family in *C. glabrata* (Pst1, Sps2, and Sps22) are functionally not redundant and are not upregulated in response to *ECM33* deletion.

CHAPTER 5

SUMMARY

Candida glabrata is the second most frequent cause of local and systemic human candidiasis and infections with this species are difficult to treat because of frequent azole resistance. *C. glabrata* infections have high mortality rates in immunocompromised patients. During pathogenesis, the cell wall is of particular importance, because it holds key functions such as adhesion and countering of immune defenses.

Next to the structural proteins of the Cwp1-family, Ecm33 and Pst1 have been identified as the most abundant GPI-anchored proteins in the cell wall, but their functions are largely unclear. In *C. albicans* and *S. cerevisiae*, the *ECM33* deletion mutants show partially overlapping phenotypes suggesting defects in cell wall assembly. Therefore, gene disruption and complementation experiments were conducted on these genes in *C. glabrata*.

The results of these experiments show that, similar to *C. albicans* and *S. cerevisiae,* the *C. glabrata* Δ*ecm33* and Δ*pst1/*Δ*ecm33* mutants are susceptible to most cell wall perturbing agents. The mutants have a more negatively charged cell wall surface and release Cwp1 protein and β-1,6-glucan containing material to the environment. Most importantly, as a consequence the mutants lose some adherence capacities, which is of particular interest in terms of *C. glabrata* pathogenicity. With the notable exceptions of the adherence phenotype and the surface charge, the phenotypes are rescued by introduction of *ScECM33* and *CaEMC33*. In contrast, the phenotypes could not be compensated in the Δ*ecm33* mutant of *C. glabrata* by overexpression of the remaining members of the *C. glabrata* ECM33-family (*CgPST1*, *CgSPS2* and *CgSPS22*). However, we can not rule out that this is due to technical limitations of the experimental approaches. From the overall phenotypic data, we can propose that Ecm33 probably is involved in the linkage of β-1,6-glucan to the cell wall. If β-1,6-glucan is lost, so is its carrier function for effector proteins, resulting in the observed phenotypes. The generated phenotypic data also enabled to develope different hypothetical models for the biological function of Ecm33 in *C. glabrata* and the functional analyses performed so far indicate that *Cg*Ecm33 might be a lectin.

These analyses now make it possible to further characterize the biological role of this very abundant cell wall protein, for example using comprehensive binding assays, glycochip experiments and proteomic work on the cell wall and secreted proteomes of the generated mutants. The improved understanding of the cell wall in *C. glabrata* will enable to characterize pathogenicity in this organism and potentially develop new antifungal therapy.

CHAPTER 6

SUPPLEMENTS

6.1 DNA sequence of *CgECM33* for cloning in *E. coli*

DNA sequecnce of *C. glabrata* was taken from online genome database (http://www.genolevures.org) and the restriction enzyme was chosen by checking via NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/).

Online program http://web.expasy.org/translate/ is used to translate DNA sequence to protein sequence. The expected molecular weight of *Cg*Ecm33 protein was calculated from amount of amino acids via http://www.sciencegateway.org/tools/proteinmw.htm. The recombinant CgEcm33 protein expressed in *E. coli* consists of 397 amino acids that this protein has a molecular weight as 41.04 kDa. The *Cg*Ecm33 protein for cloning in *E. coli* is a partial protein that does not have a signal peptide and GPI anchor.

atgAGAGGATCGCATCACCATCACCATCACGGATCC
cagaactctacatctgacgatgttccatctgggtgttctcttggtagcagtgctactgctactgcacagtctgatctggacaagtacagcgggt
gtgagaccattgttggtaacttgaccatcactggtgctcttggctctgctgcgctagccggtgtcaagaagatcgatggttctttgagaatctac
aatgccacctccttgggctcttttgctgctgactccgtcaaggagatcaccggtgctttgaacatgcaggacttgaccatcttgaccactgcgt
ccttcggtgcgctggaagaagtcgacaccatcaacttgatcactctgccagccatctccactttcaacactaacttgcaatccgccaacaaca
tcgtcgtctccgacacctctttggagtccgtcgaaggtttcggctctttgaaagaggtcaacgtcctaaacatcaacaacaacagatacttgaa
ctctttcaagtcctctttggaatccgtgtcaggtgctttgcaattcgcctccaacgccgatgaaaccgccgtgtcctttgacaacttgatctgggc
caacaacatcaccttgagagacgtccaaaacgcctctttcgccaagctggagtccgttaacgcctccctgggtttcatcaacaacaccatctc
cactttgaacttgacccacttgtccaaggtcggtcaatccctatccgttgtctccaacgatgacttgacccagttgtccttcttgaacttgacctcc
gtcggcggtggtctggtcgtcgccaacaacactaacttgaagaccatcgacggtttgaagaacgtccaaactgtcggcggtgccatcgaca
tcaccggtaacttcaccactttggacttgtcctccttgaagtccgtcagaggtggtgccactttcaacaccgtctccggtaacttctcctgctctc
ctttgaagtccctacaaagcaagggtgccatccaaggtgactccttcgtctgcaagaacggtgctacttccacttccatcagcatgtcttccag

atcccgctcttcttccgcctcttcctccgcttccgctactgttaccgccagatccaacgacactgcttccacttcctccaccaagaccaagaagtctCTGCAGCCAAGCTTAATTAGCtga

Annotation; atg and tga: start and stop codon, gray box: 6XHis-tag, gray box: restriction site, black box: primer site, and underline: *ECM33* coding sequence

- Protein sequence of *Cg*Ecm33 expressed in *E.coli*

M R G S H H H H H H G S Q N S T S D D V P S G C S L G S S A T A T A Q S D L D K Y S G C E T I V G N L T I T G A L G S A A L A G V K K I D G S L R I Y N A T S L G S F A A D S V K E I T G A L N M Q D L T I L T T A S F G A L E E V D T I N L I T L P A I S T F N T N L Q S A N N I V V S D T S L E S V E G F G S L K E V N V L N I N N N R Y L N S F K S S L E S V S G A L Q F A S N A D E T A V S F D N L I W A N N I T L R D V Q N A S F A K L E S V N A S L G F I N N T I S T L N L T H L S K V G Q S L S V V S N D D L T Q L S F L N L T S V G G G L V V A N N T N L K T I D G L K N V Q T V G G A I D I T G N F T T L D L S S L K S V R G G A T F N T V S G N F S C S P L K S L Q S K G A I Q G D S F V C K N G A T S T S I S M S S R S R S S S A S S S A S A T V T A R S N D T A S T S S T K T K K S L Q P S L I S

6.2 Process of Ecm33 and Pst1 expressed *P. pastoris* generation

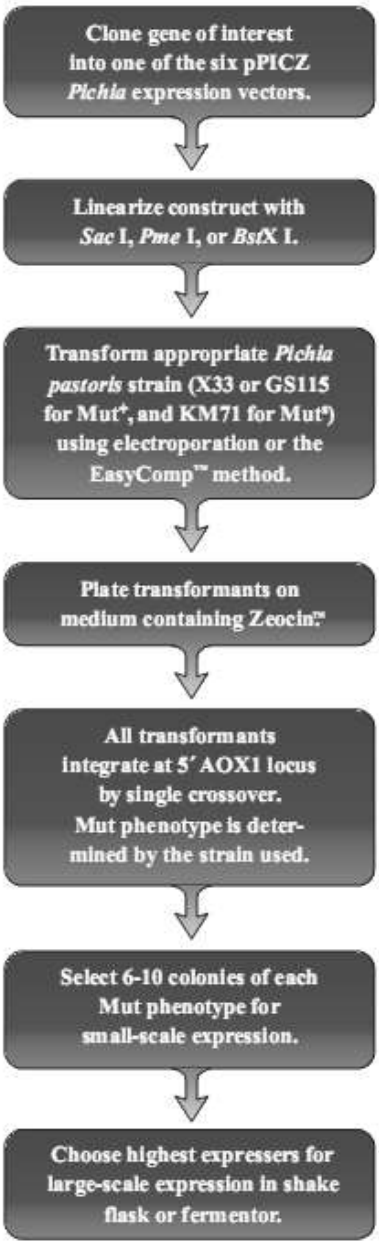

Figure 37. An overview process of Ecm33 and Pst1 expressed *P. pastoris* generation

(Invitrogen, 2009)

6.3 Deglycosylation of *Cg*Ecm33 and *Cg*Pst1 protein and western blot analysis

In experiment 3.6.3 protein deglycosylation, we want to know to exact molecular weight of Ecm33 after deglycosylation. Therefore, we performed the same experiment and used the western blot. The result showed that anti-Ecm33 monoclonal antibody from rabbit can recognize the glycosylation (Figure 38).

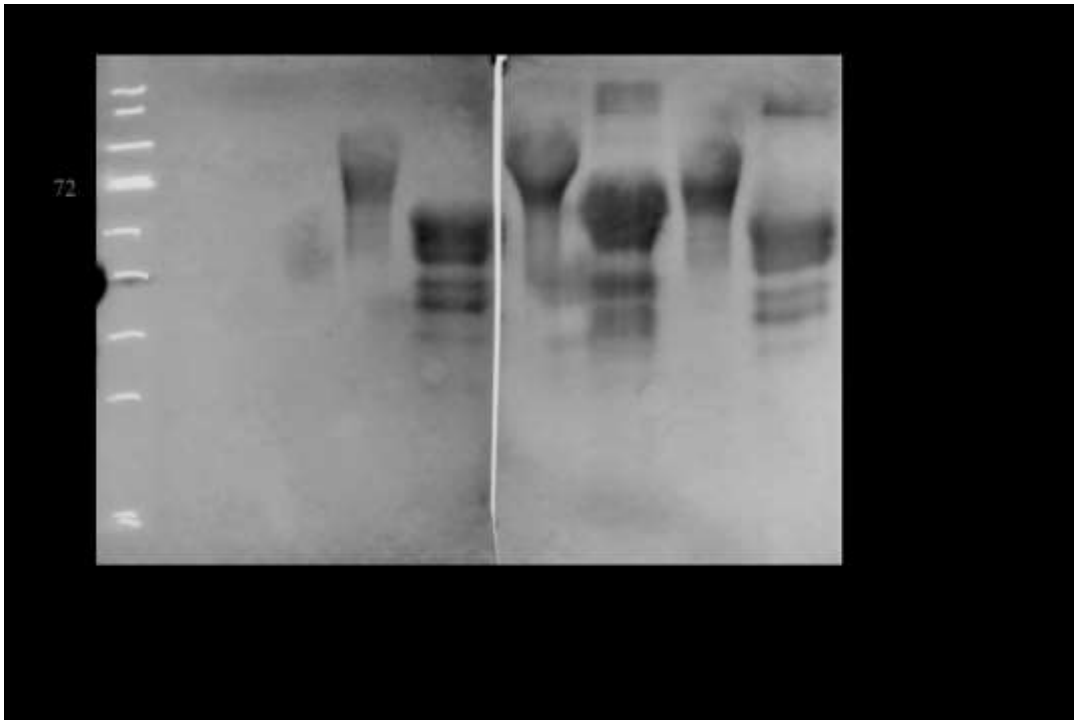

Figure 38. Deglycosylation of *Cg*Ecm33 and *Cg*Pst1 proteins and Western blot analysis

Western blot analysis after for *Cg*Ecm33 and *Cg*Pst1 proteins deglycosylation by double digestion of N-glycosidae and O-glycosidase (N/O: *Cg*Ecm33 or *Cg*Pst1 was incubated with both glycosidase). Lane 1: protein marker, lane 2: *Cg*Pst1 without treatment, lane 3: *Cg*Pst1 with N- and O-glycosidase, lane 4: *Cg*Ecm33 without treatment, lane 5: *Cg*Ecm33 with N- and O-glycosidase, lane 6: *Cg*Pst1 without treatment, lane 7: *Cg*Pst1 with N- and O-glycosidase, lane 8: *Cg*Ecm33 without treatment, and lane 9: *Cg*Ecm33 with N- and O-glycosidase. The serum rabbit no. 22 was tested in lane 2-5 and serum rabbit no. 23 was tested in lane 6-9 with titer 1:4,000.

6.4 The preliminary results of competitive inhibition assay

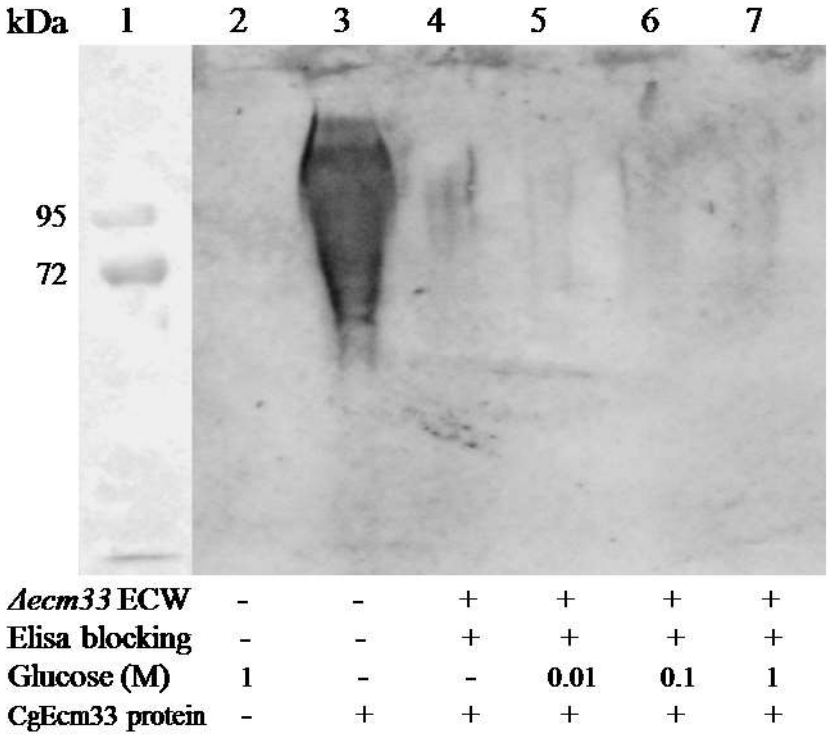

Figure 39. Glucose competitive inhibition assay

20 µl of the extracted cell walls (ECWs; 20 mg/ml) were incubated with ELISA blocking solution, incubated with or without glucose (10 µl of 0.01, 0.1, or 1 M) and then added Ecm33 protein (250 µg/ml) and washed to remove unbound protein. SDS-PAGE electrophoresis and western blot analysis were performed to determine binding complex.
Lane 1: protein marker, lane 2: 1 M of glucose, lane 3: Ecm33 protein from *P. pastoris*, lane 4: co-incubation without glucose competitor, lane 5: co-incubation with 0.01 M glucose competitor, lane 6: co-incubation with 0.1 M glucose competitor, and lane 7: co-incubation with 1 M glucose competitor (lane 4-7 are co-incuabion between *Cg*Ecm33 expressed in *P. pastoris* with Δ*ecm33* ECWs, and glucose was used to prevent binding between *Cg*Ecm33 expressed in *P. pastoris* with Δ*ecm33* ECWs)

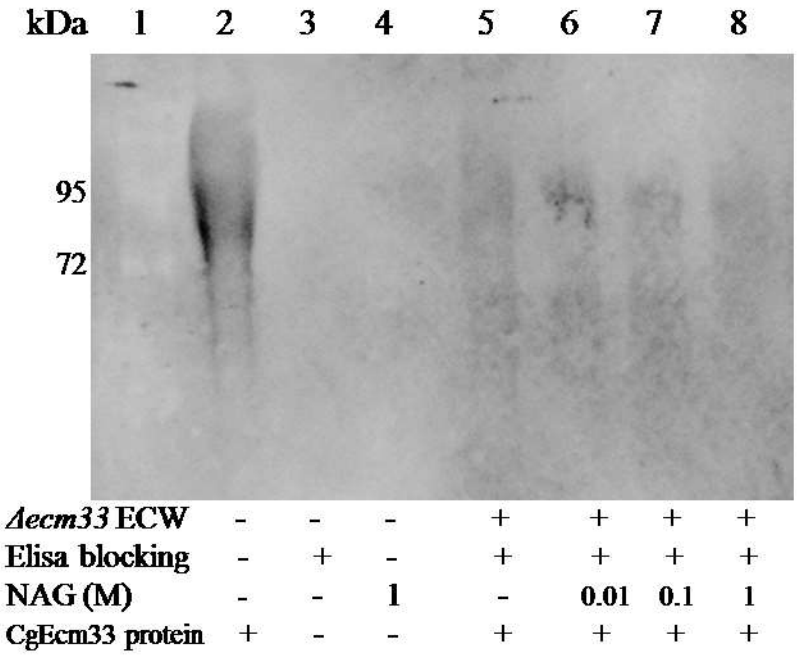

Figure 40. N-acetylglucosamine competitive inhibition assay

20 µl of the extracted cell walls (ECWs; 20 mg/ml) were incubated with ELISA blocking solution, incubated with or without N-acetylglucosamine (NAG; 10 µl of 0.01, 0.1, or 1 M) and then added Ecm33 protein (250 µg/ml) and washed to remove unbound protein. SDS-PAGE electrophoresis and western blot analysis were performed to determine binding complex.
Lane 1: protein marker, lane 2: 1 M of N-acetylglucosamine, lane 3: Ecm33 protein from *P. pastoris*, lane 4: co-incubation without N-acetylglucosamine competitor, lane 5: co-incubation with 0.01 M N-acetylglucosamine competitor, lane 6: co-incubation with 0.1 M N-acetylglucosamine competitor, and lane 7: co-incubation with 1 M N-acetylglucosamine competitor (lane 4-7 are co-incuabion between *Cg*Ecm33 expressed in *P. pastoris* with Δ*ecm33* ECWs, and N-acetylglucosamine was used to prevent binding between *Cg*Ecm33 expressed in *P. pastoris* with Δ*ecm33* ECWs)

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PUBLICATION

During the Ph.D. study, two papers have been published in PLOS ONE and Medical Mycology Journal.

- Bader, O., Schwarz, A., Kraneveld, E., **Tangwattanachuleeporn, M.**, Schmidt, P., Jacobsen, M., Gross, U., De Groot, P., and Weig, M. (2012). Gross karyotypic and phenotypic alterations among different progenies of the *Candida glabrata* CBS138/ATCC2001 reference strain. PLoS ONE 7 (12), e52218.

- **Tangwattanachuleeporn, M.**, Somparn, P., Poolpol, K., Gross, U., Weig, M., and Bader, O. (2013). Prevalence and Antifungal susceptibility of *Cryptococcus neoformans* Isolated from Pigeon Excreta in Chon Buri Province, Eastern Thailand. Med. Mycol. J. Vol. 54.

Other is in process to publish.

- **Tangwattanachuleeporn, M.**, Bader, O., Gross, U., De Groot, P., and Weig, M. (2013). Characterization of the cell wall protein Ecm33 family in *Candida glabrata*. (in preparation).

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