

**Characterisation of the cell wall protein Pga29p in the human
pathogenic fungus *Candida albicans***

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Tag der mündliche Prüfung:

To my parents
Jan and Marga de Boer

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Abstract

Candida albicans is an opportunistic fungal pathogen that is responsible for most of the *Candida* infections in humans. It can cause superficial infections on the mucosa, nails or skin and in severely immunocompromised individuals it is able to disseminate via the bloodstream and infect deep tissues, which can lead to life-threatening situations. In order to improve the treatment against *C. albicans* infections, a thorough understanding of the pathogenesis on a molecular level is needed. The last two decades, the cell wall of *C. albicans* has been an interesting object of research for investigators around the world, mainly because this structure is not present in mammalian cells and therefore it is a potential drug target. Moreover, the upper layer of the cell wall consists of mannoproteins that play an important role in the infection process of *C. albicans*.

In this study we characterised Pga29p (Predicted GPI-anchored protein 29), a small glycosylphosphatidylinositol modified cell wall protein (GPI-CWP) that is abundantly present in the yeast cell wall of *C. albicans*. In a previous study, Pga29p was among a small group of 14 cell wall proteins (CWPs) that could be identified with liquid chromatography-tandem mass spectrometry (LC/MS/MS) (De Groot et al., 2004). Putative homologs of Pga29p are mainly found in pathogenic *Candida* species, but their functional roles during human infection are completely unknown so far.

For characterisation of Pga29p, we raised polyclonal antiserum against recombinant Pga29p, which was used for studying the cell wall incorporation of the protein. Immunoblot analysis of different groups of purified cell wall protein (CWP) fractions, showed that Pga29p is linked to the β -1,3-glucan framework via β -1,6-glucan.

As the yeast-to-hyphae transition is of major importance for *C. albicans* to establish an infection, we monitored the transcriptional and translational levels of *PGA29* during hyphal development. In addition, Pga29p levels were determined in a deletion mutant that is locked up in the yeast form and lacks the transcription factors Efg1 and Cph1, both of which are known to be key-regulators of hyphae formation. During the yeast-to-hyphae transition *PGA29* gene products were strongly downregulated. In agreement with this, Pga29p levels were upregulated in the *EFG1/CPH1* deletion mutant compared with the wild type strain SC5314. Taken together, these data show that Pga29p is an abundant, yeast-specific protein that is covalently embedded into the cell wall of *C. albicans*.

To elucidate its function, the level of Pga29p incorporation in the cell wall was systematically determined under different growth conditions and cell (wall) stresses. Only

yeasts that were exposed to the cell wall perturbant Calcofluor White (CFW) incorporated higher levels of Pga29p in their cell wall, which might indicate that *C. albicans* attempts to stabilise its perturbed cell wall by upregulating Pga29p levels under this specific situation. In order to study the function of Pga29p in more detail, $\Delta pga29/\Delta pga29$ deletion mutants were generated by using a PCR-based transformation method. Possible structural alterations in the cell wall of this deletion strain were examined by transmission electron microscopy and by quantitative determination of the different cell wall components. These experiments showed that *PGA29* deletion had no direct effect on the thickness of the cell wall and the levels of chitin and protein. However, the mannose/glucose ratio in $\Delta pga29/\Delta pga29$ cell walls was significantly reduced, indicating the absence of Pga29p results in a reduced mannose level in the cell wall. In order to determine whether *PGA29* deletion caused phenotypical changes in *C. albicans* that could be related to decreased cell wall integrity, we performed assays in which growth, sensitivity to cell wall stress and antimycotics were tested. We could show that the $\Delta pga29/\Delta pga29$ strain did not exhibit significant differences compared to the control strains. Further investigation was conducted to exclude the possibility that the function of Pga29p was compensated by its family members, Pga30p and Pga31p, or other CWPs. Northern analysis showed that *PGA30* and *PGA31* were not upregulated in the $\Delta pga29/\Delta pga29$ strain, indicating that the *PGA29* gene is not redundant. This result was confirmed on a translational level, by analysing the full cell wall proteome of the $\Delta pga29/\Delta pga29$ strain making use of a “cell wall shaving” LC/MS/MS-technique. These comprehensive proteomic analyses further showed that an upregulation of other CWPs is not taking place in the mutant, suggesting that Pga29p does not play a significant role in maintaining the cell wall integrity in *C. albicans* but might have another function for the yeast-form of this human pathogen.

In order to study a possible role of Pga29p in the establishment of *C. albicans* infection, the $\Delta pga29/\Delta pga29$ strain was tested in two virulence models: a mouse model of systemic infection and the reconstituted human epithelium (RHE) oral model. Both experiments showed that *PGA29* deletion results in a reduction of virulence. The conditions in these virulence models promote the formation of hyphae, a growth form in which *PGA29* is downregulated. These data suggest that Pga29p is involved in the initial stages of the infection, where *C. albicans* faces the host in the yeast-morphology. Experiments that tested the adherence capacity and ability to form hyphae of the $\Delta pga29/\Delta pga29$ strain showed that the reduction of virulence was not dependent on these two virulence characteristics. As cell wall mannans are known as important pathogen associated molecular patterns (PAMPs), further experiments

were conducted to investigate whether the reduced mannan in the *Δpga29/Δpga29* strain could be responsible for altered host cell interactions which may lead to the observed reduced virulence. O-mannosylation of Pga29p by protein-O-mannosyltransferase 1 (Pmt1p) was proven by analysis of CWP extracts from *C. albicans* mutants that are deficient in O-glycosylation. RHE infected with the *Δpga29/Δpga29* strain showed a reduced expression of proinflammatory cytokines. This diminished immune response could be related with the decrease of mannan in the cell wall or the reduced tissue damage that was caused by *Δpga29/Δpga29* strain. However, upon stimulation with *Δpga29/Δpga29* mutants, murine primary dendritic cells (mDCs) and human polymorphonuclear leukocytes (PMNs) did not show altered cytokine or oxidative burst levels, respectively. These results indicate that the reduced virulence may not be related to an altered pathogen host cross-talk via the mannans. We propose that Pga29p is involved in the initial stage of infection, prior to hyphae development, which designates this protein as the first yeast-specific CWP of *C. albicans* that directly plays a role in virulence.

1. Introduction

The opportunistic fungus *Candida albicans* is a major cause of human fungal infections. These infections may be confined superficially to the mucosa, but in immunocompromised hosts they can develop systemically and ultimately lead to life-threatening situations. Although antifungal therapies are available and recent epidemiological studies reveal a stable or a slightly decreasing trend of systemic infections, *C. albicans* is still a serious medical problem. It is therefore of great importance to unravel the molecular mechanisms that are involved in the infection process of this pathogen, thereby revealing particular virulence factors which might be putative targets for antifungal drugs development. In the last decade, intensive research has led to a significant increase of our knowledge about *C. albicans*. Despite this fact, unfortunately, powerful therapeutic strategies to combat systemic candidosis did not arise yet. Nevertheless, researchers around the world are confident in finding a solution and are steadfastly continuing their exploration.

In this introduction we will shortly discuss the taxonomy, growth, morphology, genetics and medical relevance of *C. albicans*. Furthermore we describe the cell wall with particular emphasis on the role of glycosylphosphatidylinositol modified proteins (GPI-proteins) in virulence.

1.1 *C. albicans*: classification, growth, morphology and genetics

1.1.1 Classification

The classification of fungi is primarily based on characteristics of spores and fruiting bodies that are produced during sexual reproduction. However, due to the analyses of PCR-amplified ribosomal RNA genes in the early 1990s (White et al., 1990), the fungal taxonomy has been undergoing a molecular revolution.

Candida species are classified as yeasts and are subdivided in the phylum of the *Ascomycota*. The genus *Candida* comprises more than a 150 species, of which only a minority is medically significant. For many years, the absence of any sexual form was considered as the main characteristic that was shared between the *Candida* species (Odds, 1988). However, it has been shown that *C. albicans* is able to mate *in-vitro* and *in-vivo* (Hull et al., 2000)

1.1.2 Growth

C. albicans is capable to adapt itself to a wide range of environmental conditions, for example *in vivo* it can grow on the relatively acidic vaginal mucosa (pH 4.5) and in the blood (pH 7.4), whereas *in vitro* it is able to grow in the pH range between 2.5 and 10 and at temperatures ranging from 5°C to 46°C (Odds, 1988; Hubbard et al., 1986). Optimum growth of *C. albicans* is observed under aerobic conditions, but it is able to grow under anaerobic conditions (Eklund and Jarmund, 1983) elevated concentrations of CO₂ (Iralu, 1971; Webster and Odds, 1987). The maximal achieved doubling time is just under one hour (Odds, 1988).

1.1.3 Morphology

Already in the 19th century it was reported that *C. albicans* is able to grow as yeast, pseudohyphae, true hyphae and chlamydo spores (Audrey, 1887). A yeast cell (also known as blastospore or blastoconidium) is the unicellular form of *C. albicans*. Cell division of yeast cells is regulated by a process called 'budding'. In this process a parent cell (mother cell) produces near one of its poles a new cellular out-growth which is designated as the 'bud' or 'daughter cell'. During its development, the bud increases in size and at a certain time point mitosis occurs followed by the generation of septum around the bud-neck, which leads ultimately to cell separation (Odds, 1988).

True hyphae are microscopic tubular structures that contain cells that are separated by septa. A hypha originates from a single elongated yeast cell, known as a 'germ tube', that continuously grows by apical extension. From hyphae new buds may develop that bud off laterally just behind the septa (Odds, 1988). Several factors induce hyphal growth such as temperature (37°C), a neutral pH value, CO₂ concentration, nutrient deprivation and serum (Odds, 1988).

Although pseudohyphae resemble true hyphae, they appear to be an intermediate form between yeast cells and hyphae. The development of pseudohyphae emerges from a yeast cell or hypha by a process that is very similar to the typical blastospore budding. However, in this case each generation of buds remains attached to each other and the cells form an elongated shape (Odds, 1988; reviewed by Sudbery et al., 2004).

Chlamydo spores are spherical, thick-walled cells, that can be three to four times larger than yeast cells. *C. albicans* and *C. dubliniensis* are the only species that are able to produce these structures of which the biological function remains to be elucidated (reviewed by Staib and Morschhäuser, 2007).

1.1.4 Genetics

C. albicans is considered to be the most important fungal species that causes infections in humans and therefore it was one of the first eukaryotic pathogens selected for genome sequencing. Due to its widespread and increasing use in molecular analyses, the genome of the clinical isolate SC5314 was sequenced by the Stanford Genome Technology Center in 1998. The complete and annotated sequence was published in 2004 by Jones et al. (2004) who assembled the diploid genome. The release of the genome sequence facilitated the molecular manipulation of *C. albicans* and it enabled to establish transcript and proteomic profiling projects, which resulted in a considerable increase in the amount of biological data.

Two main differences distinguish *C. albicans* from other yeast species, such as the bakers yeast *Saccharomyces cerevisiae*, which frequently was used as model for *C. albicans*. For instance, *C. albicans* does not follow the universal codon usage, because it translates the CUG codon in a serine instead of a leucine residue (Santos et al., 1993). This unusual codon usage has to be taken into account for recombinant expression of *C. albicans* proteins. Moreover, *C. albicans* is a diploid organism; consequently two rounds of allele disruption have to be performed in order to delete a single gene. During the last decade, several systems have been developed for gene deletion (Alani et al., 1987; Gow et al., 1994; Wilson et al., 1999; Morschhäuser et al., 1999; Wilson et al., 2000; Reuss et al., 2004; Morschhäuser et al., 2005). Most studies that describe gene functions use auxotrophic strains in their gene disruption methods. These strains are impaired in producing a certain compound (mostly an amino acid), due to deletion of genes encoding enzymes that are active in the respective metabolic pathways. The auxotrophy is complemented with a gene disruption cassette that consists of the respective auxotrophic marker and sequences sharing homology with the gene of interest at the 5' and 3' ends. After gene disruption the auxotrophy is restored, enabling selection of transformants on minimal medium. One potential problem that could arise by using this method is that the ectopically inserted auxotrophic marker is not properly expressed from the integration locus, which has been shown in case of the *URA3* marker gene. Changing the location of *URA3* results in an altered Ura3p activity (orotidine 5'-monophosphate carboxylase activity) (Lay et al., 1998; Cheng et al., 2003; Brand et al., 2004) and influences the phenotypes of *C. albicans* mutants in virulence models (Lay et al, 1998; Bain et al., 2001; Sundstrom et al, 2002; Cheng et al., 2003; reviewed by Staab and Sundstrom, 2003). However, directing the *URA3* marker to the high-expression *RPS10* locus (*RPS1*) can avoid these problems (Brand et al., 2004). Another technique that has been established by Reuss and Morschhäuser (2004), circumvents all problems with auxotrophy by utilizing a disruption

cassette containing an antibiotic resistance marker that can be excised out of the genome. In this way, wild type strains can be used and the same disruption cassette can be utilized multiple times in order to delete the second allele or other genes. This method has been successfully applied in several studies; however, gene disruption in auxotrophic strains is still preferred by many scientists.

1.2 Medical relevance

1.2.1 *C. albicans* infections and predisposing conditions

C. albicans and other *Candida* species are part of the normal flora of the skin, mucous membranes and gastrointestinal tract. During or soon after birth the mucosal surfaces of most human beings are colonized and the risk of an endogenous *Candida* infection is therefore always present. An infection with *Candida* is referred to the term ‘candidiasis’ or ‘candidosis’. All forms of visible infections that are located at the surface, such as skin, nail, oral and vaginal infections, are defined as ‘superficial candidosis’. One of the most well-known *Candida* infection that occurs superficially is oral candidosis (also known as oral thrush) which causes lesions on the tongue, lips, gums or palate. All other infections that are located in the respiratory, urinary tract or organs could be classified as ‘systemic or invasive candidosis’ (proposed by Odds, 1988). Systemic candidosis can occur in a single organ, but the fungus can disseminate via the blood stream and subsequently infect other organs (Odds, 1988). Another form of infection is the development of biofilms on implanted or indwelling devices (for example catheters or prosthetic heart valves), which has become an increasing clinical problem, particularly because of the resistance of biofilms against a wide range of antifungal drugs (reviewed by Douglas, 2002).

The most important factor that contributed to the increase of candidosis over the last two decades is the growing number of immunocompromised individuals, which can be divided in groups who suffer from mucosal or cutaneous barrier disruption, neutrophil dysfunctioning, defects in cell-mediated immunity, metabolic disorders or have an extreme young or old age (reviewed by Segal et al., 2006). Additionally, other risk factors for infections are the increase use of broad-spectrum antibiotics, cytotoxic chemotherapies, and transplantation (reviewed by Nucci and Marr, 2005, Pfaller and Diekema, 2004).

1.2.2 Epidemiology of invasive candidosis

Although the list of opportunistic fungi causing serious life-threatening infections is steadily increasing (reviewed by Walsh et al., 2004), *Candida* species are evidently the number one

cause of systemic mycoses worldwide (reviewed by Pfaller and Diekema, 2007). More than 17 different *Candida* species have been reported to be agents of invasive candidosis in humans (reviewed by Hazen, 1995; Pfaller and Diekema, 2004). This number will continue to grow as laboratories are forced to provide identification to the species level, as a facility in optimizing therapy for candidal infections (reviewed by Rex and Pfaller, 2002; Pappas et al., 2004; Pfaller and Diekema, 2004; Spellberg et al., 2006). The five most common species *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* are ascribed to more than 90% of invasive *Candida* infections. Up to 70% are related to *C. albicans*, making it by far the most frequently isolated species from *Candida* bloodstream infections (Pfaller and Diekema, 2007).

Candida species are the fourth leading cause of nosocomial bloodstream infection in the United States (Pfaller, 1998; Wisplinghoff et al., 2004). The absolute number for *Candida* bloodstream infections in the US varies approximately between 10,500 to 42,000 per year (Pfaller and Diekema, 2007). These infections are associated with a high mortality rate that ranges from 46% to 75%, reflecting the severity of this illness (Diekema and Pfaller, 2004; Pfaller and Diekema, 2007). As a result of high mortality rates and prolonged length of hospital stays, *Candida* infections also have their impact on health care costs, which is estimated at one billion dollar per year in the US alone (Rentz et al., 1998).

1.2.3 Medication

Several antifungal agents are used to treat systemic fungal infections. The three major groups of drugs are the polyenes, azoles and the echinocandins.

The first described polyene with specific antifungal activity that could be used against candidosis is nystatin (Hazen and Brown, 1950), but the most well known antifungal polyene is amphotericin B, which has been used for many years as a standard drug to treat life-threatening *Candida* infections (Odds, 1988). It binds ergosterol, the main sterol in fungal membranes, causing membrane perturbation which results in leakage of cellular contents. The antifungal selectivity of amphotericin B is relatively low and therefore it is toxic to mammalian cells, often causing nephrotoxicity (reviewed by Odds et al., 2003).

Azoles are the largest class of drugs that are used in antifungal therapies. They can be classified in two groups, the imidazoles and triazoles. Similar to the polyenes, the azoles are causing disorganisation of the fungal membrane by targeting the fungal cytochrome P-450, which inhibits the 14 α -demethylation of lanosterol in the ergosterol biosynthetic pathway (Vanden Bossche et al., 1995). Depletion of ergosterol causes an accumulation of several

14 α -methyl-sterols thereby altering permeability and fluidity of the fungal membrane with secondary consequences for membrane-bound enzymes (Marichal et al., 1985)

Echinocandins are fungal secondary metabolites that consist of a cyclic hexapeptide core with a lipid side-chain responsible for the antifungal activity. They form a relative new class of antifungal agents, that target a complex of enzymes which are responsible for synthesizing the polysaccharide β -1,3-glucan for the fungal cell wall. The component to which echinocandins bind is the Fks1p protein, however, whether this protein is the catalytic subunit involved in glucan synthesis and whether the binding site on Fks1p is internal or external to the cell membrane remains to be clarified (Odds et al., 2003). In the late 1990s, the echinocandin-class compounds anidulafungin, caspofungin and micafungin were tested for clinical development (Vanden Bossche, 2002). Several studies recently showed that these three compounds are relatively safe and effective for the treatment of *Candida* infections (reviewed by Cappelletty and Eiselstein-McKittrick, 2007).

1.2.4 Resistance to antifungal agents

The resistance of *Candida* species to antifungal agents is a notorious fact that needs to be encountered from different angles, such as combination therapies with different drugs and revealing the mechanisms behind resistance. Although clinicians are not in exigencies, *Candida* infections should not be trivialized and should be acknowledged as a significant medical problem. Therefore the identification of new potential drug targets is essential for maintaining the mortality rates as low as possible.

1.2.5 Fungal vaccines

The persistent need for novel approaches to cure *Candida* infections eventuated in a growing medical interest for fungal vaccine development. The principle of vaccination depends on stimulation of the host immune system by using antigens of the pathogen. Since most of the fungal infections occur in patients with a deficient immune system, the task of finding a proper vaccine is difficult and will be the new challenge for the academic community in the coming years (reviewed by Cassone, 2008).

Theoretically, antibody production against pathogenic fungi can be induced by vaccination in at-risk patients before they become immunocompromised. IgG antibodies might remain in the body with a protective titre for several weeks to months (Cassone, 2008). Data show, that vaccination before immunosuppression could work for many fungal infections (Gigliotti et al., 2003, Zheng et al, 2005). However, vaccines, that exclusively induce protective responses of

shorter persistence (antifungal T cells, pro-inflammatory cytokines and activation of macrophages or neutrophils), seem less suitable for this purpose.

Passive vaccination with monoclonal recombinant antibodies have shown to be a promising tool to confer efficient protection against fungal infection (Beninati et al., 2000; Matthews et al., 2003, De Bernardis et al., 2006; Selvakumar et al., 2006). As β -glucan is present in most of the human fungal pathogens, the anti- β -glucan antibodies are probably one of the best option for therapeutic use (Bromuro et al., 2002; Masuoka, 2004; Torosantucci et al., 2005; Cassone and Torosantucci, 2006). Nevertheless, antibody-based immunotherapy should carefully be considered with respect to antibody specificity, affinity and isotype. For example, antibodies with identical specificity but of different isotypes may result in a non-protective or even a disease-enhancing effect in patients (Casadevall et al., 2004, Beenhouwer et al., 2007).

1.3 Virulence factors of *C. albicans*

The ability of *C. albicans* to switch from a yeast to a hyphal form is considered as one of the most important virulence factors that cause infection in the host. Hyphae are able to penetrate tissue cells of the host, thereby causing severe damage. As gene expression of other virulence factors are co-regulated with hyphal morphogenesis, it has been difficult to assess the degree of contribution of hyphae in virulence (reviewed by Kumamoto and Vinces, 2005). However, the *hgc1* deletion mutant generated by Zheng et al. (2004) showed the importance of hyphae in the infection process. Disruption of *HGC1* (G1 cyclin gene) results in an impaired hyphal development, but does not effect the expression of the hypha-associated genes *HWPI*, *HYR1* and *ECE1*. This indicates that *HGC1* encodes not a regulator of hyphal gene expression, but a component of the hyphal formation machinery. The *hgc1* deletion mutants cause decreased lethality in mice, supporting the fact that hyphae are indeed an important virulence factor. Hyphal morphogenesis and co-regulation of virulence factors is regulated by a number of transcription factors, of which *TUP1*, *NRG1*, *RFG1*, *EFG1* and *CPH1* are the most well characterised (Braun and Johnson, 1997; Murad et al., 2001; Braun et al., 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Stoldt et al., 1997; Liu et al., 1994). Deletion mutants lacking *TUP1*, *NRG1* and *RFG1* show filamentous growth under non-filamentous growth conditions, suggesting these transcription factors act as repressors of hyphal growth (Braun and Johnson, 1997; Murad et al., 2001; Braun et al., 2001; Kadosh and Johnson, 2001). In contrast, deletion of *EFG1* or *CPH1* results in defective hypha formation, indicating that they are positive regulators (Lo et al., 1997). A double null *efg1/cph1* mutant is unable to cause mortality in mice (Lo et al., 1997), showing that *EFG1* and *CPH1* play an important

role in virulence. The disability to generate proper hyphae is probably not the only cause for the avirulent phenotype of this strain. Several studies revealed that Efg1 regulates a large number of genes, including several that are associated with virulence (Lane et al., 2001; Nantel et al., 2002; Sohn et al., 2003; Doedt et al., 2004; H Marcus et al., 2004). Virulence factors that are controlled by Efg1 are for example the secreted aspartic proteases (SAPs), which may degrade host proteins in the extracellular matrix (Naglik et al., 2003), and GPI-modified adhesins such as Als1p, Als3p and Hwp1 that play a role in adhesion to host tissue (Hoyer et al., 1998; Sharkey et al., 1999; Fu et al., 2002). These adhesins allow *C. albicans* cells to be tightly attached to host tissue cells, thereby facilitating the hyphae to invade tissue by mechanical force (Kumamoto and Vines, 2005). Interestingly, deletion mutants that constitutively grow in filaments show a reduced virulence as well (reviewed by Gow et al., 2002), indicating that hyphae are not the only growth form that is important for virulence. The ability of *C. albicans* to switch from the yeast to the hyphal form and vice-versa is probably necessary to infect the host. As described above (in **1.1.3 Morphology**) this transition is induced under certain conditions, however, *C. albicans* is also able to spontaneously switch back and forth between two different phenotypes. This process is called ‘phenotypic switching’, which is a shift between a yeast form that grows as smooth and white colonies on agar media (white phase) and an elongated, rod-like form that grows as flattened grey colonies (opaque phase) (Rikkerink et al., 1988; Rustchenko et al., 1994; Slutsky et al., 1985, 1987; reviewed by Soll et al., 1993; Soll, 1997). This change can influence several characteristics such as physiological characteristics, adhesion to host cells, virulence and the immune response of the host (Soll, 1997).

1.4 The cell wall of *C. albicans*

The cell wall of the bakers yeast *Saccharomyces cerevisiae* has been extensively studied over the years which has led to a substantial amount of information about its molecular organisation (reviewed by Orlean 1997; Lipke and Ovalle, 1998; Cabib et al., 2001; Klis et al., 2002, 2006 Lesage and Bussey, 2006) Due to this, it has been designated as a standard model for cell walls of other ascomycetous yeasts, mycelial species and even for basidiomycetous fungi (reviewed by De Groot et al., 2005).

Four major functions for the yeast cell wall have been described: (1) The stabilisation of osmotic conditions within the cell; the cell wall resists the osmotic pressure from the cell, which results in a restricted water influx that prevents disturbance of the internal reaction conditions and a rupture of the plasma membrane. (2) Protection against mechanical stress;

the sturdiness and high elasticity of the cell wall offers the cell protection against mechanical stress. (3) Cell shape maintenance; the cell wall is needed for morphogenesis, for example in *C. albicans* the cell wall contributes to the shape of yeast cells, pseudohyphae, true hyphae and chlamydospores. (4) The cell wall as a scaffold for proteins; the polysaccharides in the cell wall can be considered as a supporting framework for the heavily N- and O-glycosylated proteins that can for example reduce the permeability of the cell wall, thereby protecting the skeletal network of polysaccharides against foreign macromolecules (Klis et al., 2006).

1.4.1 Molecular organisation of the cell wall

Electron microscopic data showed that *S. cerevisiae* and *C. albicans* have a bi-layered cell wall, consisting of an electron-dense fibrillar outer layer and an underlying transparent inner layer (Chaffin et al., 1998; Tokunaga et al., 1986; Baba et al., 1989; Hagen et al., 2004; Osumi, 1998). Pioneer studies on the cell wall of *S. cerevisiae* revealed that the fibrils can be removed by protease, whereas the inner layer is resistant against proteolytic activity and can be only digested by β -glucanases (Kopecka et al., 1974; Zlotnik et al., 1984). These data showed that cell wall of *S. cerevisiae* contains a protein-rich upper layer covering a polysaccharide inner layer. Similar data have been obtained with other ascomycetous yeasts and mycelial fungi (reviewed by De Groot et al., 2005). Tokunaga et al. (1990) showed for *C. albicans* that the outer fibrillar layer could attach with the membrane of human buccal epithelial cells, indicating the presence of adhesion proteins in the cell wall.

Further extensive biochemical analysis showed that the cell wall of *S. cerevisiae* consists of an elastic three-dimensional β -1,3-glucan network, which is kept together through hydrogen bonds. To this framework covalently linked proteins and chitin are attached. Cell wall studies later proved that the cell wall of *C. albicans* has a similar structure (Figure 1) (Klis et al., 2001).

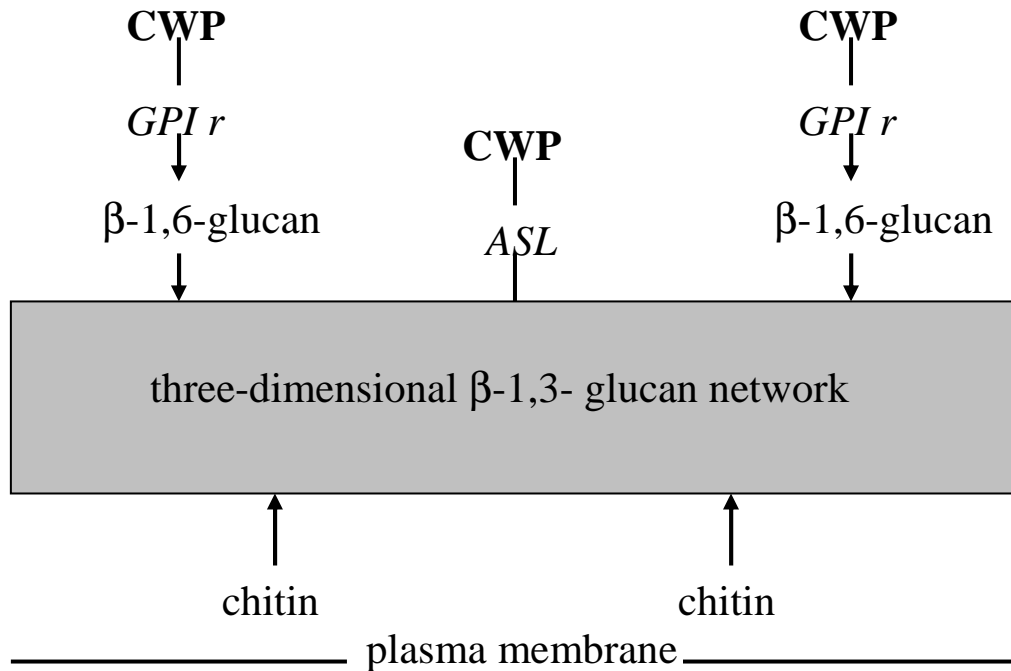


Figure 1. Molecular organisation of the cell wall of *C. albicans*.

The covalently linked cell wall proteins (CWPs) can be classified in two groups: the GPI-CWPs and the CWPs with an alkali sensitive linkage (ASL), which include the Pir proteins. GPI-CWPs form the majority of the covalently linked proteins. The *GPI r* is a lipidless remnant of the GPI-anchor. The arrows represent glycosidic linkages and point from the reducing end of a macro molecule to a non-reducing end of an acceptor polysaccharide. For the sake of clarity non-covalently linked proteins are not shown (Adapted from Klis et al., 2006).

1.4.2 Non-conventional cell wall-associated proteins

Several studies showed the association of glycolytic enzymes and other abundant cytosolic proteins with the cell wall (Chaffin et al., 1998; Delgado et al., 2003; Edwards et al., 1999, Motshwene et al., 2003; Urban et al., 2003). These proteins can be easily released by reducing agents, like mercaptoethanol, which explains the fact that they are not identified in cell walls washed with a hot solution of SDS and β -mercaptoethanol (De Groot et al., 2004; Yin et al., 2005)

The absence of glycosylation and an N-terminal signalling peptide indicates that these proteins might be transported to the cell surface by a non-conventional export pathway. However, other possibilities that could explain their presence in the wall have to be considered (reviewed by De Groot et al., 2005). For example, aging or damaged cells could release cytosolic proteins that are –due to their relatively high isoelectric point- able to bind to

the large number of negatively charged phosphate groups, which are present as phosphodiester bridges in N- and O- carbohydrate side chains in the cell wall.

1.4.3 Covalently linked cell wall proteins and glycosylation

Covalently linked cell wall proteins (CWPs) are mostly heavily glycosylated with polysaccharides consisting of mannose molecules (mannan) that are either N-linked or O-linked. N-linked glycosylation is initiated by α -glucosidases I and II and α -1,2-mannosidase in the endoplasmatic reticulum (ER) generating the oligosaccharide $\text{Man}_8\text{GlcNac}_2$ to which an outer chain consisting of up to 150 mannose residues is attached in the Golgi (reviewed by Cutler, 2001). O-linked glycosylation is performed by protein-O-mannosyltransferases (Pmt proteins) that are located in the ER where they catalyse the addition of mannose to serine or threonine residues (reviewed by Lengler et al., 2008). In the Golgi, the chain is further extended up to five mannose residues by the α -1,2-mannosyltransferases Mnt1p and Mnt2p (reviewed by Ernst and Prill, 2001).

Due to its outer location, mannans are readily recognised by the host immune system and therefore they are considered as the most important pathogen-associated molecular patterns (PAMPs) of *C. albicans*. The pattern recognition receptors (PRRs) that are involved in recognising mannan structures are the mannose receptor (MR), Toll-like receptor 4 (TLR4), Toll like receptor 2 (TLR2), DC-SIGN and the dectin 2 receptor (reviewed by Netea et al., 2008). After recognising mannan, the receptors induce several signalling cascades that primarily induce the expression of T helper 1 (TH1)-type cytokines, which confer protection against disseminated *C. albicans* infections (Netea et al., 2006, 2008; Mencacci et al., 1994). Although less exposed to the outside than mannans, cell wall structures such as glucan and chitin are likely to play a role in the recognition of *C. albicans* (Netea et al., 2008).

Types of covalently linked cell wall proteins

The covalently linked CWPs are divided in two groups, the GPI-proteins and the Proteins with internal repeats (Pir proteins) or alkali-sensitive linkage (ASL) cell wall proteins (Figure 1).

1.5 Alkali-sensitive linkage proteins

The PIR CWPs (protein with internal repeats) of *S. cerevisiae* were the first proteins found to be linked through a mild-alkali-sensitive linkage to the β -1,3-glucan network (Kandasamy et al., 2000, Kapteyn et al., 1999, Kapteyn et al., 2000, Mrsa et al., 1997 and Toh-e et al., 1993).

Later their presence has been confirmed in the cell wall of *C. albicans* and other ascomycetous yeasts (Frieman et al., 2002, Jaafar and Zueco, 2004, Kandasamy et al., 2000, Kapteyn et al., 2000 and Weig et al., 2004). At the N-terminal part PIR proteins consist of a pro-peptide that is cleaved off in Golgi apparatus by the serine proteinase Kex2p. Furthermore they contain in the central part repeats that are rich in the amino acid glutamine. The C-terminal region contains the so-called four-cysteine domain, in which four cysteines with fixed spacing are conserved.

During the last years, experimental evidence has accumulated that PIR proteins play a major role in cell wall strengthening. In *S. cerevisiae* a strong upregulation of PIR genes was observed during cell wall stress (Boorsma et al., 2004, Garcia et al., 2004, Kapteyn et al., 2000 and Lagorce et al., 2003). Deletion mutants of PIR genes in *S. cerevisiae* and *C. albicans* show a defective growth phenotype and are sensitive to compounds that affect cell wall assembly (Mrsa and Tanner, 1999; Martinez et al., 2004). All PIR proteins are probably linked to β -1,3-glucan via their repetitive sequences, which is shown by the release of Pir4p in *S. cerevisiae* from the cell wall after deleting its single repetitive sequence (Castillo et al., 2003). Recently, it has been shown that Pir4p is attached to the cell wall via a glutamine residue at position 74 within this repetitive sequence (QIGDGQ₇₄VQ; Ecker et al., 2006). The authors proposed that between the γ -carboxyl of a glutamate (originated from the glutamine 74 residue) and a sugar hydroxyl an extremely alkali sensitive ester-linkage might be formed. As other PIR members in *S. cerevisiae* and *C. albicans* contain more repetitive sequences, multiple linkages can be formed that interconnect several β -1,3-glucan chains, which enhances cell wall stability.

It has been recently reported that cells walls of *S. cerevisiae* and *C. albicans* contain proteins that do not show homology to PIR proteins, but also are covalently linked to the cell wall with an alkali-sensitive bond (De Groot et al., 2004; Yin et al., 2005), for example, the endo- β -1,3-glucanase Scw1p of *C. albicans*.

1.6 Glycosylphosphatidylinositol modified (GPI) proteins

GPI-proteins are present in lower as well as in higher eukaryotic organisms (Eisenhaber et al., 2001). In *C. albicans* and other fungi they are linked to the plasma membrane or covalently incorporated in the cell wall, in which they perform various functions that range from cell wall assembly to virulence (De Groot et al., 2005; Richard and Plaine, 2007). Nearly all GPI-proteins share common regions in their amino acid sequence: a N-terminal hydrophobic signal sequence which is needed for secretion, a sequence that is rich in serine or

threonine residues and a C-terminal hydrophobic domain which is cleaved off and replaced with a GPI-anchor, a lipid that resides in the endoplasmatic reticulum. It should be noted that not all GPI-proteins contain the serine/threonine rich domain (Richard and Plaine, 2007). The length C-terminal hydrophobic domain normally varies between 15 or 30 amino acids, which (Udenfriend and Kodukula, 1995) contains a cleavage site (the ω -site) to which the GPI-anchor is attached. The ω -site could be an asparagine, serine, glycine, alanine, aspartic acid or cysteine residue followed by two amino acids with short side chains. This ω -region is followed by a spacer region of 4 to 24 amino acids followed by an hydrophobic tail of approximately 10 amino acids (De Groot et al., 2003).

In the last decade, large-scale comparative-genomics studies of fungal GPI-proteins have been made possible due to the availability of fungal genome sequences. The prediction whether proteins contain a GPI-anchor addition is based on characteristics described above. However, the lack of a strict definition for these characteristics, leads often to an identification of a different numbers of GPI-proteins.

Five prediction studies on *C. albicans* GPI-proteins have been described so far (Sundstrom, 2002; De Groot et al., 2003; Eisenhaber et al., 2004; Garcera et al., 2003; Richard and Plaine 2007). Sundstrom (2002) predicted 152 ORFs to be coding for GPI-anchored proteins, however, these data were obtained from the incomplete genome assembly 6. De Groot et al., Eisenhaber et al. and Garcera et al. used assembly 19 and predicted 102, 104 and 169 GPI-anchored proteins, respectively. In the study of Richard and Plaine (2007) the former three data sets were combined which resulted in a list of 115 GPI-anchored proteins. Strikingly, the number of putative GPI-proteins identified in *C. albicans* is considerably higher than those identified in *S. cerevisiae*, which could be explained by the different growth forms of *C. albicans* or its virulence traits.

Several studies showed that some GPI-proteins in *S. cerevisiae* and *C. albicans* undergo further processing and are transported from the plasma membrane to the cell wall (Caro et al., 1997). In *S. cerevisiae* it has been shown that GPI-cell wall proteins (GPI-CWPs) share common sequence motifs. Two different signal sequences for cell surface localization in *S. cerevisiae* have been suggested: (1) the specific amino acid residues valine, isoleucine, or leucine at the site ω -4 or ω -5 upstream of the GPI-attachment site (the ω -site) with tyrosine or asparagine at the site ω -2 acting as a positive signal for cell wall localization and (2) the dibasic residues, arginine and lysine, for instance, in the region upstream of the ω -site for plasma membrane localization (Vossen et al., 1997; Hamada et al., 1999). Richard and Plaine (2007) applied these signal sequence algorithms to their predicted list of 115 GPI-anchored

proteins of *C. albicans* and additionally to a previously published list of 58 GPI-proteins of *S. cerevisiae* (Caro et al., 1997). This analysis showed that the percentages of cell wall and membrane-bound proteins are similar for *S. cerevisiae* and *C. albicans*, however, in case of *C. albicans*, these predictions are not necessarily in accordance with subsequent experimental data. For example, Dfg5p contains several dibasic amino acids upstream of the putative ω -site, which predicts its localisation in the plasma membrane. However, it is reported that Dfg5p is localized in the plasma membrane as well as in the cell wall (Spreghini et al., 2003), which indicates that this algorithm might be less suitable for predicting the localisation of GPI-proteins in *C. albicans* or that additional signals might be present.

The approximately 100 predicted GPI-proteins from *C. albicans* can be classified in four groups: (1) the proteins with putative functions related to cell wall biogenesis or remodeling, (2) the proteins with putative functions related to cell-cell adhesion and interactions, (3) the proteins with enzymatic activity and (4) the proteins with unknown functions. The latter comprises the largest group, representing about two-thirds of the total number predicted GPI-anchored proteins. Due to their putative localisation at the cell surface several of these proteins might be involved e.g in mediating adhesion of *C. albicans* to host cells, which would designate them as an important class of virulence factors. Additionally, revealing their unique function might explain the highly adaptive behaviour of *C. albicans* in the host, with respect to the immune response.

It is believed that GPI-proteins in eukaryotic cells are mainly located in the plasma membrane and possibly many of them are retained or alternatively are directed to other cell organelles. It has been shown that GPI-proteins are present in lipid rafts, which are specialized regions of the membrane bearing higher levels of cholesterol and sphingolipids. However, among 29 identified proteins in lipid rafts of *C. albicans*, only two are GPI-anchored (Insenser et al., 2006), which is rather a small number compared with rafts of higher eukaryotes (Sharom and Lehto, 2002). This low variety of GPI-anchored proteins is also found in lipid rafts of *Cryptococcus neoformans* (Siafakas et al., 2006), indicating that this phenomena could be particularly related with fungi.

GPI-CWPs have also been identified directly from isolated cell walls from *C. albicans* exponential-phase yeast cells (De Groot et al., 2004). By using liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis, De Groot et al. (2004) identified 14 cell wall proteins, of which 12 are predicted to be GPI-anchored. In view of the approximate 100 computational predicted GPI-anchored proteins of which 30 to 50% are predicted to be linked to the cell wall, this number is surprisingly low. Although some proteins might not be

detected because their glycosylated tryptic peptides fall out of the range of the MS-analysis, the authors suggested that these 12 identified GPI-proteins represent almost all the covalently linked proteins in the cell wall of exponential-phase *C. albicans* yeast cells. This view is supported by several facts: (1) The method that was used is sensitive enough to identify a protein even when 500 molecules per cell wall are present. (2) The number of identified proteins did not increase when 100 times more cell walls were used. (3) With SDS-PAGE only a limited number of proteins could be visualised, which was also previously shown for cell wall proteins of *S. cerevisiae* (Cappellaro et al., 1998; Mrsa et al., 1997, 1999). (4) In the different morphologies of *C. albicans* only a particular set of genes that encode GPI-proteins is expressed, for example Hwp1p, Hyr1p, and Als3p are specific for the hyphal form (Bailey et al., 1996; Hoyer et al., 1998; Staab et al., 1999), whereas Pga24p, Pir1p, and Cht2p are yeast specific and repressed during hyphal growth (Nantel et al., 2002 ; Sohn et al., 2003). The proteins that were identified by De Groot and co-workers originated from cells that were cultured at one particular temperature (30°C) and in one type of medium; different types of medium and temperatures or other host driven conditions could induce an up-regulation of other GPI-proteins.

1.6.1 The role of GPI-proteins in the virulence of *C. albicans*

As described above (in **1.3 Virulence factors of *C. albicans***) several extracellular GPI-adhesins are allowing *C. albicans* cells to adhere to host tissue, suggesting these proteins are associated with virulence. However, in most cases, it is difficult to determine whether GPI-proteins are directly or indirectly involved in the infection process. Apart from systems biology, transcription analysis and proteomics, the phenotypical analysis of gene deletion mutants is one of the classical and most reliable methods to get insight in the function of a protein. Richard and Plaine (2007) reported in a review that a total 28 deletion mutants exist for genes encoding GPI-anchored proteins in *C. albicans*, of which 15 have been tested in virulence assays. Within this group, 3 mutants were as virulent as their reference strain and 13 mutants showed a reduction in virulence, which implicates the role of GPI-proteins in the virulence of *C. albicans*. It should be noted that most of these GPI-proteins are not directly involved in the infection process, but their absence in the deletion mutants leads to secondary effects that contribute to a reduced virulence. For the majority of the GPI-proteins the function is not known, however, at the time of writing several laboratories around the world are engaged in generating mutants and testing their phenotypes in virulence models. In the

section below, we briefly describe a number of *C. albicans* GPI-proteins of which the respective deletion mutants are reported to be attenuated in virulence.

Phr1p, Phr2p, Crh11p, Utr2p and Ecm33p are proteins that are involved in the assembly and modelling of the cell wall (Saporito-Irwin et al., 1995; Ghannoum et al., 1995; Mühlischlegel and Fonzi, 1997; Pardini et al., 2006; Martinez-Lopez et al., 2004, 2006). These GPI-CWPs are necessary for proper cell separation and deletion of the encoding genes mostly has an effect on the ability of generating hyphae, which is crucial for virulence. Moreover, these mutants have a modified cell wall structure or composition, which might alter recognition by the host immune system and could lead to a reduced virulence.

One important characteristic that distinguishes *C. albicans* from other yeasts is that it is able to tightly adhere with host tissue (Borg-Von Zepelin and Wagner, 1995). Adhesion of *C. albicans* cells to host cells is the initial step in the infection process, which is mainly mediated by the GPI-CWPs of the Als (Agglutinin like sequences) protein family and Hwp1p (Hyphal wall protein 1). The first *ALS* gene was described in 1995 as a homologue of a sexual agglutinin in *S. cerevisiae* (Hoyer et al., 1995). The Als family comprises 8 members (Hoyer, 2001), of which each exhibits the typical features of a GPI-protein with in addition a central domain that consists of 36-amino acid tandem repeats (Hoyer, 2001; Zhao et al., 2003). Interestingly, there is a high variation in the number of tandem repeats and the C-terminally located VASES (valine, alanine, serine, glutamic acid and serine) repeats between alleles of the same *ALS* gene or in sequences of the same *ALS* gene among different strains. For example, in 66 different *C. albicans* strains 60 different forms of *ALS7* were found, generating 49 different genotypes (Zhang et al., 2003). Additionally, an analysis of the genotype data revealed that more tandem repeats are present in the genome of the strains that were more pathogenic. Not only the repeats vary in *ALS* genes, in a study by Zhao et al. (2003) it was shown for the *ALS9* gene that its two alleles vary from each other in the 5' part of the ORF. The repeats in the genome could favour genetic reshuffling of domains, resulting in CWPs with new functions (reviewed by Verstrepen et al., 2004). It has been shown for *S. cerevisiae* that the variation in intragenic repeats, which mostly occurs in CWPs, alters the phenotypes in different strains (Verstrepen et al., 2005). It is not unlikely that this is also the case for *C. albicans*, which provides an escape mechanism for the fungus against the host immune response. On protein level the structural diversity within the Als family was also confirmed and these variations were likely to be responsible for the functional diversity within the family (Sheppard et al., 2004)

Deletion mutants have been generated for *ALS1* (Fu et al., 2002), *ALS3* (Zhao et al. 2004), *ALS4* (Zhao et al., 2005), *ALS7* (Nobile et al., 2003)) and an attempt to disrupt *ALS2* has been made, however the gene seems to be essential to *C. albicans* viability (Zhao et al., 2005). The *als1* and *als3* null mutants show a reduced adherence and virulence in different kinds of models (Fu et al., 2002; Zhao et al., 2004). The *als4* mutant has a reduced ability to adhere but its virulence is comparable to that of the wild type (Zhao et al., 2005), which shows the redundancy of functions within the *ALS* family. It should be noted that these phenotypes might depend on the background strain that is used, as the sequences of *ALS* genes greatly vary among different strains. Moreover, a single reference strain can evolve differently from laboratory to laboratory, creating an even more complicated scenario to interpret data from phenotypical analyses.

The reduced virulence and adherence exhibited by the *ALS* deletion mutants suggests the Als protein family is involved in cell-cell adherence. Other studies confirm this assumption, for example, Als5 expression in *S. cerevisiae* results in a higher binding capacity to variety of substrates, such as extra-cellular matrix-coated beads, human buccal epithelial cells and various peptide sequences (Gaur et al., 1999, 2002; Klotz et al., 2004). This binding activity seems not to be solely localised to the N-terminal immunoglobulin like region, but also depends on the tandem repeats domain (Rauceo et al., 2006).

Als3p has shown to be important for adhesion and plays a crucial role in biofilm development (Nobile et al., 2006; Nobile and Mitchell, 2005; Zhao et al., 2003). Recently, this protein was discovered as the first fungal invasin that mimics host cell cadherins and induces endocytosis of *C. albicans* by binding to N-cadherin on endothelial cells and E-cadherin on oral epithelial cells (Phan et al., 2007).

Another GPI-CWP that plays a role in adhesion is Hwp1p, which is exclusively expressed in germ tubes and true hyphae (Staab et al., 1999, 2003b). This protein is known as an unconventional adhesin that shares sequence similarities with mammalian small proline-rich proteins, thereby mimicking human transglutaminase substrates. Due to this, covalent bonds are established that enable stable attachments between the germ tubes of *C. albicans* and the host cells (Staab et al., 1999).

An important virulence factor of *C. albicans* is its extracellular proteolytic activity, which is produced by 10 members of the Sap family. In contrast to the secreted Sap1p-Sap6p, which degrade host proteins and cause tissue damage (Naglik et al., 2003), the GPI-anchored Sap9p and Sap10p seem to target fungal cell surface proteins. Deletion of *SAP9* and *SAP10* causes misprocessing of *C. albicans* CWPs, resulting in sensitivity against compounds that target the

cell wall and an attenuated virulence in a model of oral candidosis. Furthermore, the *sap10* null mutant exhibits a reduced adhesion to buccal epithelial cells, whereas deletion of *SAP9* resulted in an increased adhesion (Albrecht et al., 2006).

Another protein family with enzymatic activity is the phospholipase B family (Plb), that consists of 5 members of which Plb3p, Plb4p and Plb5p are predicted to be GPI-anchored. *PLB5* expression is increased in hyphal growth conditions and deletion of the gene results in a reduced organ colonisation in a mice model. During infection, secreted and cell surface linked Plbs are likely to come in close contact with host tissue cells and therefore they could be involved in the degradation of host cell membranes (Theiss et al., 2006).

In order to proliferate during a systemic infection *C. albicans* needs to acquire several nutrients, including iron. Iron acquisition plays a key role in the infection process and therefore it is a major virulence factor. The two highly homologous GPI-proteins Rbt5p and Rbt51p (Pga10p) are upregulated during iron starvation and deletion of the *RBT5* gene leads to a reduced ability of *C. albicans* to use haemin and haemoglobin as iron sources, which suggests Rbt5p is involved in iron uptake (Weismann and Kornitzer, 2004).

While residing in the host, *C. albicans* can encounter an oxidative burst from phagocytes, which consists of several reactive oxygen species that react with cellular components. This leads to inactivation of enzymes, to disruption of membranes, to mutations, and ultimately to cell death (Halliwell and Gutteridge, 1990 and 1999). However, *C. albicans* protects itself with superoxide dismutases (SOD) that detoxify these reactive oxygen species. Mutants lacking the GPI-protein Sod5p (Pga3p) are more sensitive to hydrogen peroxide and exhibit a reduction in virulence in mice (Martchenko et al., 2004).

The function of the hyphal regulated GPI-proteins Hyr1p and Rbt1p remains unclear, as the respective deletion mutants do not show phenotypical differences compared with the wild type (Bailey et al., 1996; Braun et al., 2000).

1.7 The Predicted GPI-anchored protein 29 (Pga29p)

The *PGA29* gene was initially discovered in a transcription analysis, in which 6333 open reading frames were investigated for differential gene expression between the yeast and hyphal form of *C. albicans* (Nantel et al., 2002). *PGA29* was identified as one of the genes being most strongly downregulated by *C. albicans* during its transition from yeast to hyphae and consequently it was designated as 'Repressed during Hyphae Development 3' (*RHD3*; Nantel et al., 2002). Nantel and co-workers noted that this gene encodes a putative membrane protein which is rich in serine residues. This was confirmed one year later by De Groot et al.

(2003) who identified Pga29p in a genome-wide analysis to predict GPI-proteins, which indicated the protein is localized at the cell surface and is linked to the plasma membrane and/or cell wall of *C. albicans*. With the aid of mass spectrometry, the presence of Pga29p in isolated cell walls of exponential-phase yeast cells was proven (De Groot et al., 2004). The study revealed detailed information about the N-terminal signal cleavage peptidase site for Pga29p and SDS-PAGE showed that the protein is abundantly present in yeast cell walls (De Groot et al., 2004). Pga29p is small in size (the unprocessed protein consists of 204 amino acids) and does not contain conserved domains in its amino acid sequence. De Groot and co-workers (2004) reported that it might be needed to maintain the rigidity of the cell wall, which is supported by the fact that *PGA29* is among the genes that are highly expressed in protoplasts that regenerate their cell wall (Castillo et al., 2006). In the literature Pga29p has not been classified as a putative virulence factor of *C. albicans*, probably because its expression is low in iron depleted (Lan et al., 2004) as well as in hyphal stimulating conditions (Nantel et al., 2002), both of which are known to particularly induce the expression of genes encoding virulence factors.

1.8 Aim of the study

Although our knowledge about the pathogenicity of *C. albicans* on molecular level has been considerably increasing over the last decade, the exact strategy by which this fungal pathogen infects humans remains unclear. In order to successfully proliferate in the host, *C. albicans* needs an ample of genes that are either directly or indirectly related with virulence. Several of these genes encode GPI-proteins that reside in the plasma membrane and/or cell wall and are therefore exposed to the extracellular environment, allowing them to contact host tissue during infection. The function of approximately two-thirds of the predicted GPI-proteins is still unknown. Though transcript profiling studies give researchers hints, speculating about their putative function is often difficult because of the lack of functional homologs in other fungal species. It is believed that a number of these GPI-proteins are involved in virulence and therefore their characterisation will answer a part of the remaining questions concerning the pathogenesis of *C. albicans*.

In this study we characterised Pga29p, an abundant GPI-CWP that belongs to the group of GPI-proteins with unknown function. We generated anti-Pga29p polyclonal antibodies with which we studied the type of linkage through which Pga29p is connected to the cell wall and the Pga29p incorporation in the cell wall of *C. albicans* cells that had grown in different conditions. Further analysis was carried out by testing double deletion mutants in several

phenotypic assays, including virulence models. Furthermore, we attempted to obtain information about the role of this protein in the host immune response.

2. Methods

2.1 Chemicals

Chemicals were purchased from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sigma Aldrich (München, Germany). Media to culture *C. albicans* were purchased from Qbiogene and Difco (Merck) and cell culture media were purchased from Biochrom (Berlin, Germany).

2.2 Kits, plasmids and enzymes

Enzymes and kits were purchased from Invitrogen (Paisley, UK), New England Biolabs (Ipswich, MA, USA), Novagen (Darmstadt, Germany), Promega (Madison, WI, USA), Q-biogene (Irvine, CA, USA), Qiagen (Hilden Germany), Roche Molecular Diagnostics (Pleasanton, CA, USA) and Stratagene (Cedar Creek, TX, USA).

Table 1. Plasmids

Plasmids used in this study	Source
pSTblue-1	Novagen, Darmstadt
pQE30	Qiagen, Hilden
pQE30- <i>PGA29</i>	This study
pRS-ARG4 Δ SpeI	Wilson et al. (1999)
pGEM-HIS1	Wilson et al. (1999)
CIp10	Murad et al. (2000)
CIp10- <i>PGA29</i>	This study

Table 2. Enzymes

Enzymes used in this study	Source
Restriction enzymes	New England Biolabs
Taq DNA polymerase	Roche
KOD hot start DNA polymerase	Novagen
Reverse transcriptase	Qiagen, Invitrogen
Shrimp alkaline phosphatase	Roche
DNA Quick ligase	New England Biolabs
Quantazym <i>ylg</i> TM (recombinant β -1,3-glucanase)	Q-biogene
Recombinant β -1,6-glucanase	Bom et al., 1998

2.3 Table 3. Media used in this study

Media	Components
LB *	5g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl
YPD *	10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose
YNB *	6.7 g/l yeast nitrogen base (without amino acids, with (NH ₄) ₂ SO ₄), 20 g/l glucose
Sabouraud *	Purchased from Oxid
Limited iron medium (LIM, filter sterilised) *	1.7 g/l yeast nitrogen base without copper and iron ions, 0.79 g/l complete supplement mixture, 20g/l glucose, 5 g/l (NH ₄) ₂ SO ₄ , 0.25 mM CuSO ₄ and supplemented with 100 mM of bathophenanthroline disulphonic acid (Sigma Chemical Co.).
Modified Lee (filter sterilised)	35.7 g/l Hepes, 0.07 g/l arginine, 0.2 µM ZnSO ₄ ×7H ₂ O, 1 µM FeCl ₃ , 0.2 g/l MgCl ₂ , 0.25 µM CuSO ₄ , 0.147 g/l CaCl ₂ , pH 4.5 or pH 6.7
SB (N-acetylglucosamine, filter sterilised)	3.35 g/l of YNB without amino acids, with ammonium sulphate, 4.5 g/l NaCl. With 1.1513 g/l L-Proline (10mM) or 0.553 g/l N-acetylglucosamine (2.5 mM)
Rice-Tween-agar *	25 g/l rice, 1% (v/v) Tween 80
Spider *	10 g/l Nutrient Broth, 10 g/l mannitol, 2 g/l K ₂ HPO ₄ .
mDC	High glucose DMEM with glutamine supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 5-10% X-conditioned medium as a source of GM-CSF.
D-MEM	For adhesion assay: DMEM supplemented with 1 mM sodium pyruvate and 1% gentamycin.
RPMI	RPMI-1640
Modified RPMI	RPMI-1640 w glutamine w/o bicarbonate, 25 mM HEPES, 0.02% Bovine Serum Albumin (BSA), 20 mM Glucose, 10 mM NaOH, 20 µg/ml uridine.

*(In case of solid media: 2% agar, for spider medium 1.35%)

2.4 **Table 4. *C. albicans* strains used in this study**

Strain	Genotype	Parental strain	Reference
SC5314	Wild type	--	Gillum et al. (1984)
BWP17	<i>Δura3::λimm434/Δura3::λimm434</i> <i>his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i>	SC5314	Wilson et al. (1999)
<i>Δpga2/Δpga29</i>	<i>Δpga29/Δpga29</i> , <i>pga29::HIS1/pga29::ARG4</i> , <i>rp10::URA3</i>	BWP17	This study
<i>Δpga29/Δpga29/PGA29</i>	<i>Δpga29/Δpga29</i> , <i>pga29::HIS1/pga29::ARG4</i> , <i>rp10::PGA29-URA3</i>	BWP17	This study
<i>Δpmt1/Δpmt1</i>	<i>Δpmt1::hisG /Δpmt1::hisG</i> <i>Δura3::λimm434/URA3</i>	CAI4 (Fonzi and Irwin, 1993)	Prill et al. (2005)
<i>Δpmt2/PMT2</i>	<i>Δpmt2::hisG/PMT2 Δura3::λimm434/URA3</i>	CAI4	Prill et al. (2005)
<i>Δpmt4/Δpmt4</i>	<i>Δpmt4::hisG/Δpmt4::hisG Δura3::λimm434/URA3</i>	CAI4	Prill et al. (2005)
<i>Δpmt5/Δpmt5</i>	<i>Δpmt5::hisG/Δpmt5::hisG</i> <i>Δura3::λimm434/URA3</i>	CAI4	Prill et al. (2005)
<i>Δpmt6/Δpmt6</i>	<i>Δpmt6::hisG/Δpmt6::hisG</i> <i>Δura3::λimm434/URA3</i>	CAI4	Prill et al. (2005)
<i>Δefg1/Δefg1</i>	<i>Δura3::λimm434/Δura3::λimm434</i> <i>efg1::hisG/efg1::hisG-URA3-hisG</i>	CAI4	Lo et al. (1997)
<i>Δefg1/Δefg1</i> <i>Δcph1/Δcph1</i>	<i>cph1::hisG/cph1::hisG</i> <i>efg1::hisG/Δefg1::hisG-URA3-hisG</i>	CAI4	Lo et al. (1997)

Table 5. *E. coli* strains used in this study

Strain	Genotype	Source
DH5 α	<i>F' end A1 hsd R17 (r_k⁻, m_k⁻) sup E44 thi-1 l rec A1 gyr A96 rel A1 Δ(arg F⁻ lac ZYA) U 169 y80 Δ lacZΔM15</i>	Qiagen, Hilden, Germany
M15 with plasmid pREP4	<i>F' proA⁺B⁺ lacI^q Δ(lacZ)M15 zzf::Tn10(Tet^R)/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5</i>	Qiagen, Hilden, Germany
NovaBlue Singles™	<i>endA1 hsdR17 (r_k⁻ m_k⁺) supE44 thi-1 recA1 gyrA96 relA1 lac F'[proA⁺B⁺ lacI^qZ Δ M15::Tn10] (Tet^R)</i>	Novagen, Darmstadt, Germany
XL1-Blue supercompetent cells	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]</i>	Stratagene, La Jolla, CA

2.5 Primers that were used in this study

Table 6. Primers used for recombinant expression of *PGA29* in *E. coli*

Primer name	Primer sequence	Localisation (in bp)
ExCaPGA31-F	CGGCGGATCCACCATCTCATCCATTCAATTATTTGCC	44-72
ExCaPGA31-R	GCGCAAGCTTACCTTCAAAGTTGGAAACAGAGGC	523-555
PGA29-pm-F	CCGTTAGACAATTGTTCACTTTGGGTGGTG	227-256
PGA29-pm-R	CACCACCCAAAGTGAACAATTGTCTAACGG	227-256
PGA29-pm2-F	CCGTTAGACAATCGTTCACCTTTGGGTGGTG	227-256
PGA29-pm2-R	CACCACCCAAAGTGAACGATTGTCTAACGG	227-256

Table 7. Primers used to generate digoxigenin PCR probes (Northern blot experiments)

Primer name	Primer sequence	Localisation (in bp)
Act1F-RT	ATGAAGCCCAATCCAAAAGAGGTA	122-145
Act1R-RT	GAAATCCAAAGCAACGTAACACAA	601-624
Pga29F-RT	TCTGCTCTTGCTACCATCTCATCC	34-57
Pga29R-RT	AGCAACAACAGCAGCCATACCA	579-600
PGA30F-RT	CAAAACATACTGAAGCTCCAATA	491-514
PGA30R-RT	AATAGCAGCAGCAGCAAGAG	800-819
PGA31F-RT	TAAAGGTGGTGATGGTGCTATTCC	597-620
PGA31R-RT	ACCACCAGTGAGGGCGTTAG	995-1014

Table 8. Primers used for PCR based transformation (Capital letters represent the sequence of the untranslated region of *PGA29*)

Primer name	Primer sequence
PGA31pGEM-F	TTAGTTCATTATAACAAAAAACAATTA GTTATACCCATCAAATCAAACgttttcccagtcacgacgtt
PGA31pGEM-R	TAGAATTTAAAAGTCCGCAACGGATACATA ATTAGTCTTGCTCTTGCTTTAtgtggaattgtgagcggata

Table 9. Primers used to generate the *PGA29* PCR product for complementation, to verify mutants with PCR and to generate digoxigenin PCR probes (Southern blot)

Primer name	Primer sequence	Localisation (in bp)
Kompl-PGA29-F	gtcgacACAAATGGGTTTCGGCTTCTT	-898--878
Kompl-PGA29-R	acgcgtATTCTGACCGTTGTTGCCACTT	+464--486
PGA29-F	ATAGTGGTGGTGGAGGAGAACAA	-827--804
HIS-R	GGACGATTATATTGACCATTTCTG	605-628
HIS-F	GAGTGGTGCCGATATACAGTTTAG	87-110
PGA29-R	CCAACCTTTCGCTGAGACCA	+322--341
ARG4-R	CCACAAAAGAAAAACCCTGACTCA	874-897
ARG4-F	CCATTCTTGTCTGATTTGTTCTAA	217-240
RP10-1F	TTTTAGGGGCAATCAGCAACATA	-861--838
Clp10.2-R	TGGAAAGCGGGCAGTGAG	3175-3192
PGA29-best- <i>knk</i> -F	GAAGTGGCAACAACGGTCAGA	+364 --385
URA3-F	CCTCACCAGTAGCACAACGAT	47-67
RP10-3R	AATTTCTGACTTTCTGTATCTA	+372--395
URA3-R	TGTCATAATAAGCCAATCAAATCC	592-615
ARG4-S-F	TGAGTCAGGGTTTTTCTTTTGTGG	874-897
ARG4-S-R	TTAGAACAAATCAGACAAGAATGG	217-240

Table 10. RT-PCR primers used in infected RHE experiment

Primer name	Primer sequence
GAPDH-F	GCACCACCAACTGCTTAGCACC
GAPDH-R	GTCTGAGTGTGGCAGGGACTC
GM-CSF-F	GTGGCCTGCAGCATCTCTGCAC
GM-CSF-R	CCTGGACTGGCTCCCAGCAGTC
TNF- α -F	GGGACCTCTCTCTAATCAGCCCTCTGG
TNF- α -R	GACGGCGATGCGGCTGATGG
IL-1 α -F	CACTCCATGAAGGCTGCATGG
IL-1 α -R	ACCCAGTAGTCTTGCTTTGTGG
IL-1 β -F	CGATCACTGAACTGCACGCTCCG
IL-1 β -R	GGTGAAGTCAGTTATATCCTGGCCG
IL-6-F	CACAGACAGCCACTCACCTC
IL-6-R	TTTCTCCGTGACCGTCTTTT
IL-8-F	GCAGCTCTGTGTGAAGGTGCAG
IL-8-R	GCATCTGGCAACCCTACAACAG

Table 11. RT-PCR Primers used in Candida- mDC interaction assay

Primer name	Primer sequence
ACT1-F	GGTCATCACTATTGGCAACG
ACT-R	TCCATACCCAAGAAGGAAGG
IFN- β -F	TCAGAATGAGTGGTGGTTGC
IFN- β -R	GACCTTTCAAATGCAGTAGATTCA
IFN- γ -F	TCAAGTGGCATAGATGTGGAAGAA
IFN- γ -R	TGGCTCTGCAGGATTTTCATG
IL-10-F	GGTTGCCAAGCCTTATCGGA
IL-10-R	ACCTGCTCCACTGCCTTGCT
IL-12-F	GGAAGCACGGCAGCAGAATA
IL-12-R	AACTTGAGGGAGAAGTAGGAATGG
TNF- α -F	CAAAATTTCGAGTGACAAGCCTG
TNF- α -R	GAGATCCATGCCGTTGGC

2.6 Basic Local Alignment Search Tool (BLAST)

To find homologs of Pga29p, the Pga29p protein sequence was used to search against the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), the FGI *Candida* database (http://www.broad.mit.edu/annotation/genome/candida_group/Blast.html) and the *Candida dubliniensis* data base (<http://www.genedb.org/genedb/cdubliniensis/>).

2.7 Anti-Pga29p polyclonal antibodies

2.7.1 Cloning of *PGA29* in the pQE30 expression vector

The coding sequence of *PGA29* extending from codon 15 (at the signal cleavage site) to codon 182 (before the predicted GPI-anchoring site) was amplified with primers ExCaPGA31-F and ExCaPGA31-R (Table 8). The amplicon was cloned as a *Bam*HI-*Hind*III PCR fragment in the pQE30 vector, thereby generating pQE30-*PGA29*. In this way the ORF included of a polyhistidine tag, which was necessary for Ni-purification. *C. albicans* does not follow the universal codon usage, for it translates the leucine-specific CTG codon in a serine. Because the CTG codon at position 193 is translated as leucine in *E. coli*, we introduced two point mutations, using the primers PGA29-pm-F and PGA29-pm-R (Table 6) for the first point mutation and the primers PGA29-pm2-F and PGA29-pm2-R (Table 6) for the second point mutation. Using the QuickChange II Site-directed mutagenesis kit (Stratagene). The point mutations were confirmed by restriction analysis with enzyme *Mfe*I and by sequencing. The pQE30-*PGA29* vector was transformed in the *E. coli* strain M15 and recombinant Pga29p was isolated and purified under denatured conditions (8 M urea).

2.7.2 *PGA29* overexpression and purification

An overnight culture of *E. coli* M15 (with pREP4) with the pQE30-*PGA29* vector was 50 times diluted in 1 liter LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The culture was incubated at 37°C with vigorous shaking until an optical density at 600 nm (OD₆₀₀) of 0.6 was reached. *PGA29* expression was induced by adding IPTG to a final concentration of 1 mM and the culture was further incubated for 5 hours. Cells were harvested by centrifugation at 4000×g for 20 min and were frozen with liquid nitrogen. The cell pellet was thawed on ice and cells were lysed by resuspending them in buffer B (5 ml per gram wet weight), which contains 100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 8.0. The cells were gently shaken for 1 hr at room temperature and subsequently the lysate was centrifuged at 10,000×g for 30 min in order to pellet the cellular debris. The supernatant was collected in 50 ml tubes and 1 ml of 50% Ni-NTA resin slurry was added to 4 ml lysate. This mixture was gently shaken for 1 hr at room temperature and subsequently loaded in empty columns. After the flow-through was collected the Ni-NTA resin was two times washed with 4 ml buffer C, which contains 100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 6.3. Recombinant Pga29p was eluted from the Ni-NTA resin by adding 4 times 0.5 ml buffer D (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 5.9) followed by addition of 4 times 0.5 ml buffer E (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 4.5).

2.7.3 Generation of anti-Pga29p polyclonal antibodies.

Recombinant Pga29p was dialysed 3 times against phosphate-buffered saline (PBS) in order to decrease the urea concentration. The immunisation procedure was performed by a commercial company (Eurogentec, Liège, Belgium). Two New Zealand White rabbits were immunised with the 200 µg protein on day 0, 14, 28 and 50. On day 65 the rabbits were sacrificed and serum was collected. The serum was tested for anti-Pga29p activity by using immuno-blotting with recombinant Pga29p and HF-pyridine cell wall extract as antigens.

2.8 Cell wall extraction, digestion and immuno blot analysis

2.8.1 Cell wall isolation

For cell wall isolation we used a method that was developed in the laboratory of Frans Klis from the university of Amsterdam (De Groot et al., 2004). An overnight culture was diluted up to an OD₆₀₀ of 0.1 in 300 ml YPD medium. The cells were harvested in the mid-exponential growth phase (OD₆₀₀ = 2.0) with centrifugation for 10 min at 4000×g at 4°C. The cells were one time washed with 200 ml of distilled water and centrifuged for 10 min at 4000×g at 4°C. Then the cell were washed in 200 ml of 10 mM Tris-HCl, pH 7.5 and centrifuged for 10 min at 4000×g at 4°C. In order to weaken the cells, cell pellets were frozen in liquid nitrogen and subsequently thawed. The cell pellets were resuspended in 10 mM Tris-HCl, pH 7.5 and divided over 6 screw-cap tubes. The tubes were centrifuged for 1 min at 9000×g. After the supernatant was discarded, the cell pellets were resuspended in 200 µl 10 mM Tris-HCl, pH 7.5. Cold glass beads with a diameter of approximately 500 nm (Sartorius, Göttingen, Germany) were added to the cells and the excess of dry beads was discarded. In total 30 µl protease inhibitor was added to the cells (5 µl per tube). The tubes were cooled down on ice and then transferred into the Fast Prep machine (Fastprep FP120, Bio101, Savant). To break the cells the samples were shaken for 20 sec at speed 6 and were subsequently transferred on ice. After 5 min cooling on ice, the tubes were shaken again for 20 sec at speed 6. The samples were transferred on ice and 1 M of ice-cold NaCl was added to inhibit protease activity. The content of the tubes was collected in a 50 ml tube and the supernatant was transferred in a second 50 ml tube. The remaining glass beads in the first tube were six times washed by shaking vigorously with 5 ml of 1 M NaCl. After every washing step the supernatant was collected in the second 50 ml tube. This tube was centrifuged for 10 min at 4000×g and the cell wall pellet was one time washed with 40 ml of 1 M NaCl and two times with 40 ml of distilled water. The cell wall pellets were resuspended in 5 ml of distilled water and was divided over 5 eppendorf tubes of 2 ml. The tubes were centrifuged for 5 min

at 20,000×g. The pellets were resuspended in 1 ml SDS extraction buffer of pH 7.8 which contains 50 mM Tris-HCl, 100 mM EDTA, 2% SDS and 100 mM β -mercapto-ethanol. The samples were boiled in a hot block for 5 min and subsequently cooled down at room temperature for 5 min. The samples were centrifuged for 5 min at 20,000×g. Resuspending and boiling the samples in SDS extraction buffer was two times repeated. Then the samples were three times thoroughly washed in 1.5 ml distilled water. The cell wall pellets were frozen in liquid nitrogen and freeze-dried overnight in a freeze dryer (Edwards). After freeze-drying the dry-weight of the cell wall pellets was determined.

2.8.2 Cell wall digestions with enzymes and chemicals

For each cell wall digestion we routinely used 4 mg of dry-weight cell wall. In case of double treatments (β -1,3-glucanase and subsequently β -1,6-glucanase or β -1,6-glucanase and subsequently NaOH) 8 mg cell wall was used.

For β -1,3-glucanase digestion, cell walls were resuspended in 200 μ l 50 mM Tris-HCl, pH 7.4, 0.7 μ l β -mercapto-ethanol and 6 μ l Quantazyme. Cell walls were incubated at 37°C and were shaken in an Eppendorf shaker at 850 rpm. In case of a second treatment with β -1,6-glucanase, the supernatant was dialysed (Spectrum laboratories, Canada, 3500 kDa MWCO) overnight in 4 l of distilled water and subsequently freeze-dried.

For β -1,6-glucanase digestion, cell walls were resuspended in 200 μ l Na₂HPO₄ at pH 5.5, 2 μ l protease inhibitor, 2,5 μ l β -1,6-glucanase. Cell walls were incubated at 37°C and were shaken in an Eppendorf shaker at 850 rpm. Prior to a second treatment with NaOH, cell walls were two times boiled in SDS extraction buffer and three times washed in distilled water followed by freeze-drying overnight.

Besides β -1,6-glucanase digestion a second method was used to liberate GPI-proteins from the cell wall. Cell walls were resuspended in 300 μ l hydrogen fluoride pyridine (HF-pyridine, Fluka) and were incubated for three hrs on ice. The samples were gently mixed every 30 min. In order to quench the HF-pyridine, 300 μ l of distilled water was added to the samples. The samples were further diluted with 1.4 ml of distilled water and were transferred to a dialysis tube. Dialysis was done overnight against distilled water. In order to dialyse efficiently, the water was changed several times until the samples obtained an appropriate pH value. Samples were collected in 14 ml tubes (Greiner) and centrifuged at 4000×g. The supernatant was used for immuno-blot analysis.

For mild alkali treatment cell walls were resuspended in 91 μl of ice-cold 30 mM NaOH solution and were incubated at 4°C overnight. The samples were neutralized by adding 109 μl of ice-cold 30 mM acetic acid.

2.8.3 Immuno-blot analysis

Three times Laemmli sample buffer pH 8.9 was added to β -1,3-glucanase, β -1,6-glucanase and NaOH cell wall protein extracts and were heated at 100°C for 5 minutes. After cooling down at room temperature, samples were centrifuged at 20,000 \times g in order to pellet the cell walls. For SDS-PAGE analysis 30 μl of the supernatant was loaded per lane, which is equal to the amount of protein released from 0.4 mg dry-weight cell walls. The proteins were separated on 11% polyacrylamide gels with Laemmli buffer and were transferred overnight to polyvinylidene difluoride membranes.

To enhance specificity of Pga29p detection, membranes were treated with 50 mM periodic acid, 100 mM sodium acetate at room temperature for 30 min with shaking. This treatment was not performed for membranes that would be treated with Concanavalin A (Con A) antibodies. The membranes were washed in PBS for 10 min and were subsequently blocked for 1 hour with a solution 5% skim milk powder in PBS pH 7.2 (for Con A antibody treatment: blocking was performed in 3 % (w/v) BSA, 5 mM CaCl_2 and 2.5 mM MnCl_2 in PBS pH 6.8). Membranes were incubated for 1-2 hours with anti-Pga29p polyclonal antibodies (1000 times diluted) or with Con A antibodies in blocking buffer, were subsequently washed three times in PBS and were incubated for 2 hours with Goat-Anti-Rabbit-Peroxidase antibodies in blocking buffer (no secondary antibody is needed for membranes treated with Con A antibodies, for these are labelled with peroxidase).

Membranes were washed for 4 times in PBS and were incubated for 1 min with ECL solutions (GE Healthcare, Buckinghamshire, UK). High performance chemiluminescence films (GE Healthcare, Buckinghamshire, UK) were exposed for 2, 5 and 10 min. Differences in expression between the samples were determined with the program ScanPack 3.0 (Grus and Nuske, Aachen, Germany).

2.9 *PGA29* expression in hyphae

2.9.1 RNA isolation from *C. albicans*

An overnight culture was diluted up to an OD₆₀₀ of 0.1 in 20 ml YPD medium. The cells were harvested at an OD₆₀₀ of 1 or further incubated at 37°C with 10% fetal calf serum (FCS) for 15 min, 30 min or 45 min. The samples were centrifuged for 5 min at 4000×g at 4°C.

The cell pellets were resuspended in 1 ml DEPC-water and transferred to 2 ml eppendorf tubes. The samples were centrifuged for 1 min at 20,000×g and the pellets were resuspended in 600 µl TES, which contains 10 mM Tris-HCl pH 7.5, 10 mM EDTA and 0.5% SDS. To this 600 µl acidic phenol were added followed by 20 sec of vortexing. The samples were incubated for 60 min at 65°C and were vortexed every 15 min for 10 sec. Then the tubes were incubated for exactly 10 sec in a mixture of ethanol and dry-ice and were subsequently centrifuged for 10 min at 20,000×g. Five hundred µl of the water phase were collected in new tubes and 500 µl acidic phenol were added. The samples were vortexed for 10 sec and incubated for 10 sec in a mixture of ethanol and dry-ice. Then the samples were centrifuged for 10 min at 20,000×g and subsequently 400 µl of the water phase were collected in a new tube. To this 400 µl neutral phenol/chloroform/isoamylalcohol (25:24:1) were added, followed by 10 sec of vortexing. The samples were centrifuged for 10 min at 20,000×g and 300 µl of the water phase were collected in a new tube. RNA was precipitated with 30 µl of 3 M sodium acetate pH 5.2 and 750 µl 100% ethanol. The samples were gently mixed and were incubated for 5 min in a mixture of ethanol and dry-ice. To collect the RNA the samples were centrifuged for 10 min at 20,000×g and were subsequently washed in 500 µl of 70% ethanol. The RNA pellets were air-dried and dissolved in 50 µl of DEPC-water. The concentration of the RNA was measured spectrophotometrically at 260 nm.

2.9.2 Northern blot analysis

For Northern blot analysis we used 20 µg RNA per sample, which was dissolved in 20 µl DEPC-water. To the samples 5× RNA sample buffer was added, which contained 1×MOPS buffer, 5×blue juice loading dye, 5.7% formaldehyde and 30.8% formamide. Then the samples were incubated at 65°C for 10 min and were cooled on ice for 5 min. The samples were loaded on a 1.2% agarose gel (150 ml), which contained 1×MOPS buffer and 2.7 ml of 37% formaldehyde. Electrophoresis was performed at 65 V in 1×MOPS buffer. Prior to blotting, the gels were two times washed for 20 min in 250 ml DEPC-water in order to remove the formaldehyde. RNA was transferred for 16 hrs on a nylon-membrane (Hybond–

N+, GE Healthcare) with a 20×SSC solution. After transferring the RNA, the membranes were washed in 6×SSC and UV-treated with 700 kJ for 30 sec (Hoefer UVC500 UV Crosslinker, MA, USA) in order to cross-link the RNA to the membranes. The membranes were incubated for 1 to 2 hrs at 42°C in prehybridisation solution, which contained 50% formamide, 6×SSC, 5×Denhardt's reagent, 0.5% SDS and 100 µg hering sperm DNA (5 min boiled prior to adding). Five to 10 µl of the digoxigenin PCR probes (*PGA29*, *PGA30*, *PGA31* and *ACT1*; for primer sequences see Table 7.) were diluted in 100 µl water and boiled for 5 min followed by the addition of 100 µl of formamide. The probes were incubated on ice for 5 min and were subsequently added to the prehybridisation solution. After an overnight incubation at 42°C, the membranes were washed two times for 20 min at 42°C in 200 ml washing buffer containing 2×SSC and 0.5% SDS. Then the membranes were washed two times for 30 min at 65°C in a washing buffer containing 0.1×SSC and 0.5% SDS. The membranes were equilibrated for 1 min in washing buffer which contained 0.1 M maleic acid, 0.15 M sodium chloride and 0.2% Tween pH 7.5. The membranes were incubated for 1 to 2 hrs with a blocking buffer which was produced as follows: 5 g of milk powder was dissolved in 20 ml distilled water. Then 1 M of sodium hydroxide solution was added and the mixture was heated till a light brownish color appeared. The solution was cooled down and 5 ml of 1 M maleic acid was added, resulting in a final concentration of 50 mM maleic acid. Under constant stirring the solution was titrated with 1 M HCl to a pH of 8.0. To remove precipitated caseine, the solution was centrifuged at 4000×g and the supernatant was collected. After blocking, the membranes were incubated with blocking buffer and 10 µl of anti-digoxigenin-POD Fab fragments (Roche) per 10 ml blocking buffer was added. After 1 hr of incubation, the membranes were two times washed with washing buffer which contained 0.1 M maleic acid, 0.15 M sodium chloride and 0.2% Tween pH 7.5. Then the membranes were briefly washed in one time PBS, pH 7.2 and were incubated for 1 min with ECL solutions (GE Healthcare). Exposure times to high performance chemiluminescence films (GE Healthcare, Buckinghamshire, UK) ranged from 20 min to 16 hrs.

2.10 *PGA29* deletion

2.10.1 Generation of gene disruption cassettes

To knock-out both alleles of *PGA29*, gene disruption cassettes were generated by PCR using the primers *PGA31pGEM-F* and *PGA31pGEM-R* (Table 8). The plasmids *pGEM-HIS1* and *pRS-ARG4ΔSpeI* (Wilson et al., 1999) were used as a template. As selectable markers we

used *ARG4* and *HIS1* with flanking regions of 50 bp that share homology with the untranslated regions of *PGA29*. To complement the *URA3* auxotrophy in the double deletion mutants, we used the CIp10 (Murad et al., 2000), which integrates in the *RPS10* locus. For complementation of *PGA29*, a *SalI-MluI* *PGA29* PCR fragment of 1900 bp was amplified with primers Kompl-PGA29-F and Kompl-PGA29-R (Table 9.) and was subsequently digested and cloned in the CIp10 plasmid, generating CIp10-*PGA29*. The *PGA29* fragment consisted of 898 bp promoter region and 486 bp terminator region. For transformation CIp10 and CIp10-*PGA29* were linearized with the restriction enzyme *StuI*.

2.10.2 Transformation of *C. albicans*

For the most part, we used the transformation protocol from Walther and Wendland (2003). From an overnight culture, the BWP17 strain was inoculated in 50 ml YPD medium supplemented with 80 mg/ml uridine at an OD₆₀₀ of 0.2. Cells were incubated at 30°C at 200 rpm. After reaching an OD₆₀₀ of 1.0, cells were collected by centrifugation with 4000×g at 4°C and were one time washed in 30 ml sterile water. Subsequently cells were washed with 30 ml TEL, which contains 10 mM Tris-HCl, 1 mM EDTA and 100 mM lithium acetate, pH 7.5. The cell pellet was resuspended in 1.5ml TEL. The transformation mixture contained 100 µl cells, 100 µg (10 µl) Yeast Maker Carrier DNA (Clontech), 600µl 40% Polyethylene glycol 4000 in TEL and 10-15 µg knock-out cassette dissolved in 25-100 µl water. After vortexing, the transformation mixture was incubated overnight at 30°C. The next day heat shock was performed at 44°C for 15 min. Cells were pelleted with 800×g and were subsequently resuspended in 200µl TE, which contains 10 mM Tris-HCl and 1 mM EDTA, pH 7.5. Cells were plated out on YNB+2% glucose agar medium, which was supplemented with 20 µg/ml arginine, 20 µg/ml histidine and 40 µg/ml uridine as required and were incubated at 30°C. After 2 to 4 days colonies were subcultured for genomic DNA isolation.

2.10.3 DNA isolation from *C. albicans*

To harvest cells, a 10 ml overnight YPD culture was centrifuged in a 50 ml tube for 5 min at 4000×g. The cell pellet was washed with 1 ml of 1 M sorbitol and the cells were divided over two 2 ml eppendorf tubes. The samples were centrifuged for 5 min at 20,000×g. Then the cells were resuspended in 1 ml zymolyase solution, which contains 0.64 M sorbitol, 30 mM potassium phosphate pH 7.5, 6.4 mM DTT and 0.25 mg/ml zymolase 20 T (Seikagaku Corporation, Tokyo, Japan). The samples were incubated at 37°C for 1.5-2 hrs and were

gently mixed every 20 min. Then the samples were centrifuged for 1 min at 20,000×g and the pellets were subsequently washed with 1 ml of 1 M sorbitol. After 5 min centrifugation for 5 min, the samples were resuspended in 600 µl of 50 mM EDTA/0.2 % SDS solution followed by addition of 600 µl of phenol/chloroform. The samples were vortexed for 20 sec and were subsequently centrifuged for 10 min at 20,000×g. Five hundred µl of the water phase were collected and transferred to a new tube. The phenol/chloroform extraction was repeated and 400 µl of the water phase was collected. Then 400 µl of chloroform were added to the samples and after vortexing for 10 sec the samples were centrifuged for 10 min at 20,000×g. Three hundred µl of the water phase was collected and transferred to a new tube. For precipitation, 30 µl of 3 M sodium acetate pH 5.2 solution and 660 µl of 100% ethanol were added to the samples. The samples were gently mixed and were incubated for overnight at -20°C. The next day samples were centrifuged for 10 min at 20,000×g and were subsequently washed with 500 µl of 70% ethanol. The DNA pellets were air-dried and were subsequently dissolved in 50 µl water. To remove the RNA, 1 µl of 10 mg/ml RNase A was added to the samples. After mixing, the samples were stored overnight at 4°C. In order to estimate the DNA concentration, 1 µl of each sample was checked visually on a 1% agarose gel.

2.10.4 Southern blotting

Eight µg digested genomic DNA per sample and 12.5 µl of the digoxigenin DNA molecular weight marker were loaded on a 0.8% agarose gel. Electrophoresis was performed overnight at 20 V. In order to depurinate the DNA, the gel was washed in 0.25 M HCl solution. Then the gel was washed two times for 20 min in denaturing solution, which contains 1.5 M NaCl and 0.5 M NaOH, followed by two times washing for 20 min in neutralisation solution which contains 1.5 M NaCl and 0.5 M Tris-HCl pH 8.0. DNA was transferred for 16 hrs on a nylon-membrane (Hybond-N+, GE Healthcare) with a 20×SSC solution. After transferring the DNA, the membranes were washed in 6×SSC and UV-treated with 700 kJ for 30 sec (Hoefer UVC500 UV Crosslinker, MA, USA) in order to cross-link the DNA to the membranes. The membranes were incubated for 2 hrs at 37°C in prehybridisation solution (see Northern blotting). Five to 10 µl of the digoxigenin PCR probes (*ARG4*, *HIS1* and *URA3*; for primers see Table 9.) were diluted in 100 µl water and boiled for 5 min followed by the addition of 100 µl of formamide. The probes were incubated on ice for 5 min and were subsequently added to the prehybridisation solution. After an overnight incubation at 37°C, the membranes were washed two times for 20 min at 37°C in 200 ml washing buffer containing 2×SSC and

0.5% SDS. Then the membranes were washed two times for 30 min at 65°C in a washing buffer containing 0.1×SSC and 0.5% SDS. The membranes were equilibrated in PBS pH 7.2 for 1 min at room temperature followed by 1 hr blocking in 5% milk powder, 0.1% Tween and PBS pH 7.4. Then 10 µl anti-digoxigenin-POD Fab fragments (Roche) per 10 ml blocking buffer were added. After 2 hrs of incubation, the membranes were washed three times for 10 min with PBS and 0.1% Tween pH 7.2. Then the membranes were incubated with ECL solutions and high performance chemiluminescence films (GE Healthcare, Buckinghamshire, UK) were exposed for 7-60 min.

2.11 Cell wall composition analysis

2.11.1 Electron microscopy

Cells were harvested at 2000 rpm using a Stat Spin Microprep 2 table top centrifuge. The pellet was resuspended with 10% Dextran. A drop of the paste was placed on a flat embedding specimen holder (Leica Microsystems, Wetzlar, D-35578 Germany) and frozen in a Leica EM PACT high pressure freezer, and Automatic Freeze Substitution Unit (AFS). The samples were substituted at -90°C in a solution containing anhydrous acetone, 0.1% tannic acid for 24 hrs and in anhydrous acetone, 2% OsO₄ for additional 8 hrs. After a further incubation over 20 hrs at -20°C samples were warmed up to +4°C and subsequently washed with anhydrous acetone. The samples were embedded at room temperature in Agar 100 (Epon 812 equivalent) at 60°C over 24 hrs. Images were taken in a Philips CM120 electron microscope (Philips Inc.) using a TemCam 224A slow scan CCD camera (TVIPS, Gauting, Germany).

2.11.2 Chitin determination

For each strain 4 mg of dry weight cell walls was resuspended in 100 µl 1 M NaOH solution and were subsequently boiled for 10 min in order to extract proteins. After cooling at room temperature, 1 M of HCl was added followed by centrifugation for 5 min at 20,000×g. The supernatant was collected for protein determination. The cell wall pellets were hydrolysed in 1 ml of 6 M HCl at 100°C for 17 hrs. A standard curve was generated with different concentrations of D(+)-glucosamine. The HCl was evaporated by air stream at 100°C. The samples were resuspended in 1 ml distilled water and were centrifuged for 10 min at 20,000×g. The supernatants were transferred to new eppendorf tubes. To 0.1 ml sample (supernatant) 0.1 ml Solution A was added, which contains 1.5 M Na₂CO₃ solution and 4%

acetylacetone. The samples were incubated at 100°C for 20 min, subsequently cooled down at room temperature and 0.7 ml of 96% ethanol was added followed by the addition of 0.1 ml solution B, which contains 1.6 g of *p*-dimethyl-aminobenzaldehyde, 30 ml of concentrated HCl and 30 ml of 96% ethanol. After incubation of 1 hr at room temperature, the OD was measured at 520 nm.

2.11.3 Protein determination

The protein concentration of cell walls was determined with the bicinchoninic acid protein assay (BCA assay, Pierce Biotechnology). To 10 µl of the supernatant (see Chitin determination) 1ml of BCA solution was added. A standard curve was generated with different concentrations of BSA (0-10 µg). The samples were 30 min incubated at 37°C and were measured spectrophotometrically at 563 nm.

2.11.4 Determination of the mannose/glucose ratio

In order to determine the glucose/mannose ratio of the cell walls, the polysaccharides in cell wall preparations were hydrolyzed by using H₂SO₄ (sulphuric acid) (Dallies et al. 1998). For each strain 3 independent cell wall samples were used. Briefly, 4 mg of dry weight cell walls were incubated with 100 µl 72% (v/v) H₂SO₄ for 3 hrs at room temperature. Samples were then diluted with 575 µl distilled water to obtain a 2 M H₂SO₄ solution, and incubated for 4 hrs at 100°C. Samples were analyzed by HPLC (LKB) on a REZEX organic acid analysis column (Phenomenex, Torrance, CA) at 40°C with 7.2 mM sulphuric acid as eluent, using a RI 1530 refractive index detector (Jasco, Great Dunmow, UK). The chromatograms were analyzed using AZUR chromatography software and compared with chromatograms of known amounts of mannose and glucose.

2.11.5 Cell wall proteome of the *Δpga29/Δpga29* mutants

2.11.5.1 Sample preparation for MS analysis

Samples for in-solution digestions were desalted and rebuffered with 50 mM NH₄HCO₃ by using 0.5-kDa-cutoff centrifugal filters (Millipore). In-solution digestion with trypsin (sequencing grade; Roche Diagnostics, Indianapolis, Ind.) was carried out overnight at 37°C using a CWP/trypsin ratio of 50:1.

2.11.5.1 MS analysis

After digestion, peptides were desalted and collected on Omix C₁₈ pipette tips (Varian), washed with 0.1% formic acid, and eluted with 60% acetonitrile–0.1% formic acid. Proteolytic digests derived from 30 µg of freeze-dried cell walls were fractionated on a 150-mm by 75-µm (length×inner diameter) reversed-phase capillary column (PepMap C₁₈; Dionex) using an Ultimate nano-LC system (Dionex). The peptides were separated over a period of 30 min with a two-step linear gradient of 0 to 50% acetonitrile plus 0.1% formic acid, and the outflow at 300 nl/min was directly electrosprayed into the Q-ToF1 (Waters) operating in data-dependent MS and MS/MS mode. The Masslynx software (Waters) was instructed to select ions ranging from m/z 350 to 1,500 with a charge state of at least 2+ above a base peak intensity (BPI) ion count threshold of 40 for collision-induced fragmentation using argon as the collision gas. The resulting MS/MS spectra were processed with the Biolynx tool of the Masslynx software program, which generated a peak list (.pkl file) with all precursor and product ions calculated to the corresponding MH⁺ charge state. Each LC/MS/MS run was repeated at least twice, thereby excluding abundant ions from previous runs. For protein identification, the resulting spectra and amino acid sequences were analyzed using MASCOT software and compared with *in silico* digests of the CandidaDB protein database (<http://genolist.pasteur.fr/CandidaDB/>). This database was created by the European Union-funded consortium Galar Fungail by performing independent annotation of sequence data (contig assembly 19) obtained from the Stanford Genome Technology Center (<http://www-sequence.stanford.edu/group/candida>).

2.12 Phenotypic analysis

2.12.1 Growth determination at different pH values

To determine the growth rate we cultured our strains in liquid YPD medium with 1% glucose at different pH values. Medium was buffered with 10 mM CH₃COONa (for pH 4.0 and 5.0), 10 mM NaH₂PO₄ (for pH 6.0 and 7.0) and 10 mM Tris-HCl (for pH 8.0 and pH 9.0). Overnight cultures were diluted with water until a turbidity of 0.5 McFarland units was reached. Cells were diluted 100 times and subsequently 100 µl cells were mixed with 100 µl of 2 times concentrated medium. The cells were cultured in 96-wells plates at 30°C and OD₆₀₀ was measured at 15 min time intervals.

2.12.2 Growth rate determination with microdilution spot assay

An overnight YNB culture was diluted up to an OD₆₀₀ of 0.2 and incubated at 30°C, 200 rpm. After reaching an OD₆₀₀ of 1.0, the cells were diluted to a cell density of 8.0×10⁵ in 50 mM Tris-HCl, pH 7.5. This cell suspension was serially diluted using 1/5 dilution steps. In this way, six different dilutions were made and of each 3 µl was spotted on the surface of the YPD or YNB agar medium that was supplemented with 0-75µg/ml Calcofluor White (CFW) or 0.01-0.05% sodium dodecyl sulphate (SDS).

2.12.3 β-1,3-glucanase sensitivity assay

To determine the sensitivity to β-1,3-glucanase of the mutants we used the recombinant β-1,3-glucanase Quantazym. From an overnight YNB culture, fresh cultures were inoculated to a starting OD₆₀₀ of 0.1 and were subsequently grown at 30°C till an OD₆₀₀ of 2.0 was reached. Then the cultures were collected by centrifugation, washed one time in 50 mM Tris-HCl, pH 7.5 and resuspended in the same solution. The cells were diluted to an OD₆₀₀ of 1.0 in 50 ml of Tris-HCl, pH 7.5 and β-mercaptoethanol was added to a final concentration of 40 mM. After an incubation of 1 hour at room temperature, 5 U/ml Quantazym was added and the samples were incubated in a water bath at 37°C, 150 rpm. The decrease of the OD₆₀₀ was measured over a time period of 1 hour. The experiment was repeated two times and from the average values a curve was generated

2.12.4 Determination of antimycotic susceptibility using E-test

The E-test (Inverness, Cologne) was used to assess the susceptibility to Fluconazole, Intraconazole, Voriconazole, Caspofungin, 5-Fluocytosin and Amphotericin B. Five colonies from Sabouraud agar were collected and resuspended in water. The cells were diluted to 0.5 McFarland units and were streaked out on RPMI agar medium supplemented with 0.05% methylene blue. E-test strips were laid on top of the agar medium and the plates were incubated for 24 hrs at 35°C. Growth inhibition was determined visually according to the suggestions of the manufacturer.

2.12.5 Yeast-to-hyphae transition

The ability of the mutants to generate hyphae was tested in several media. Serum induced hyphae formation was tested in liquid or solid YPD medium. For liquid medium, yeast cells were grown to the mid-log phase (OD₆₀₀ = 0.5) at 30°C in YPD medium. The cells were then

harvested by centrifugation and two times washed in PBS. The cells were resuspended in pre-warmed YPD medium + 10% FCS, pH 7.0 (unbuffered) and incubated at 30°C and 37°C for 5 hours. Every 30 min the cells were observed with light microscopy in order to check germ tube development. For hyphae formation on solid YPD medium with 10% FCS, a liquid overnight culture of yeast cells was two times washed in PBS. To determine the number of cells per ml, cells were counted in a Neubauer counting chamber. The cell suspension was diluted accordingly and 200 cells per Petri dish were plated out. The cells were incubated at 30°C and 37°C for 7 days.

For testing pH-regulated hyphal formation we used modified Lee's medium (Buffo et al., 1984). Briefly, cells were plated out on YNB plates and incubated overnight at 30°C. The next day single colonies were collected and were incubated in modified Lee's medium, pH 4.5 at 25°C for 24 hours. To induce hyphae, 0.5 ml of this culture was diluted in 25 ml of modified Lee's medium, pH 6.7. Over a time period of 5 hours, every 30 min hyphal formation was checked with light microscopy.

For N-acetylglucosamine induced hyphal formation an overnight YPD culture was harvested by centrifugation and two times washed with PBS. Cells were resuspended at an $OD_{600} = 1.0$ in 25 ml pre-warmed SB medium with 10 mM proline. After 1 hour incubation at 30°C, cells were inoculated at an OD_{600} of 0.1 in 25 ml pre-warmed SB medium with 2.5 mM N-acetylglucosamine. The cells were incubated at 37°C for 5 hours and every 30 min hyphal formation was checked with light microscopy.

Nutrient-deprived hyphal induction was performed with Spider medium with mannitol as a sole carbon source (Liu et al., 1994). An overnight YPD culture was two times washed in PBS. To determine the number of cells per ml, cells were counted in a Neubauer counting chamber. The cell suspension was diluted accordingly and 200 cells were plated out per Petri dish. The cells were incubated for 7 days at 30°C or 37°C.

Quorum sensing-based induction of hyphae was carried out in YPD, pH 7.5 buffered with a correct proportion of Na_2HPO_4 and NaH_2PO_4 to a 100 mM final concentration. Cells were grown either at 30°C or 37°C to the stationary phase. Hyphal growth was triggered by diluting these cultures to an OD_{600} of 0.5 in fresh pre-warmed buffered YPD (Enjalbert and Whiteway, 2005). The cells were observed every 30 min over a time period of 5 hours.

Chlamydospore formation was tested on Rice-Tween agar. From YNB agar colonies were collected with a loop and were streaked out on Rice-Tween agar. In order to reduce the

oxygen concentration, a coverslip was placed on the cells and were incubated at 25°C for 5 days.

2.12.6 Hyphae-to-yeast-transition

Hyphal growth was induced in 10 ml YPD + 10% FCS as described above. After 3.5 hours of hyphal growth, hyphae were collected by centrifugation and three times washed in 1×PBS. In order to stimulate blastoconidia growth, hyphae were resuspended in 20 ml YPD and incubated at 30°C. Blastoconidia formation was observed every 30 min over a time period of 7 hours.

2.12.7 Adhesion assays

Three different adhesion assays were used in this study. In the first assay the colony forming units were counted after incubating the *C. albicans* cells with plastic or Caco-2 cells (Dieterich et al, 2002). Caco-2 cells were grown in D-MEM medium supplemented with 10% FCS in 24-well polystyrene plates. The cells were incubated at 37°C in a 5% CO₂, 95% humidity atmosphere and grown to a confluent stage. *C. albicans* strains were grown at 30°C to the mid-log phase or stationary phase and were diluted in 250 µl D-MEM medium supplemented with 10% FCS without the addition of antibiotics to a concentration of 400 cells/ml. The cells were distributed in the 24-well plates and incubated at 37°C, 5% CO₂ for 15, 30, 45, 60 and 120 min. Non-adherent cells were collected by vigorously shaking of the plates followed by aspirating the media. The media with non-adherent cells was plated out on YPD agar. In addition each well was washed with PBS and this was also plated out. Adherent cells were scraped off using a cell scraper (LPS life sciences) and 300 µl PBS. The cells were plated out on YPD agar and incubated at 30°C overnight. The next day colony forming units were counted.

In the second assay, adhesion of *C. albicans* yeast cells was determined on tissue culture-treated and non-treated polystyrene Petri dishes. Cells were grown in YPD at 30°C or 37°C for 24 hours and were diluted in YPD or PBS, pH 7.4 to an OD₆₀₀ of 2.0. Cells were spotted in 25 µl drops on the plates and were incubated for 3, 4, 6 and 24 hrs in a humidified chamber at 30°C or 37°C. The drops were carefully aspirated and on the adherent cells 25 µl of 0.1% crystal violet solution was spotted. After an incubation of 30 min at room temperature, cells were washed with water or a 1 M NaCl solution. The adhesion of cells was visually determined.

For the hyphal adhesion assay, cells were grown in YPD at 30°C or 37°C for 24 hours and were diluted in modified RPMI to a concentration of 100 or 200 cells/ml. The cells were distributed in tissue culture treated 24-well plates (1 ml per well) and were incubated at 37°C in a humidified chamber. Cells were incubated for 3 days and adhesion was examined at intermediate times by gently shaking the plates. After shaking, the proportion of adherent and non-adherent colonies was assessed.

2.13 Phenotypic analysis in infection models

2.13.1 Mouse model for systemic candidosis

For infection studies, parental and mutant strains were grown at 30°C in YED agar plates (1% Difco Yeast extract, 2% glucose, and 2% agar). After 24 hrs, cells were harvested, washed twice with PBS and diluted to the desired density in the same buffer for injection into the lateral tail vein of the mice at a volume of 0.5 ml.

Both male and female BALB/c mice, ranging in age from 12 to 16 weeks and with a weight of about 18 g, were used in the virulence studies. All animal studies were carried out in cooperation with the Animal Facilities of the School of Medicine at the Universidad Complutense of Madrid.

2.13.2 Reconstituted Human Epithelial model based on oral candidosis

Well-established models of reconstituted human oral epithelium (oral RHE), based on the buccal epithelial carcinoma cell line TR146, were supplied by SkinEthic Laboratories, Nice, France (Rupniak et al., 1985). Strains were cultured over 3 days in order to obtain synchronized yeast populations. Strains were streaked out on Sabouraud agar medium and were incubated for 24 hours at 37°C. Colonies were collected with a loop and were resuspended in 5 ml 0.9% NaCl solution. The cells were washed three times in 0.9% NaCl solution and were resuspended in 5 ml water. The cell density was determined with a Neubauer counting chamber and the suspensions were diluted to 2×10^6 cells/ml. The cells were collected by centrifugation and were resuspended in 10 ml YPD medium. Cultures were grown in 15 ml Falcon tubes for 16 hours at 25°C, 200 rpm. The next day the cells were 3 times washed as described above and the suspensions were diluted to 4×10^6 cells/ml. The cells were collected by centrifugation and resuspended in 10 ml YPD. The cultures were grown in 15 ml Falcon tubes for 24 hours at 37°C, 200 rpm. Cells were 3 times washed in 1×PBS and the cell suspensions were adjusted to 2.0×10^6 cells in 50 µl 1×PBS. Oral RHE

samples were incubated with or without 2×10^6 cells in 50 μ l PBS for 24 h at 37°C with 5% CO₂ at 100% humidity. All experiments were performed in the absence of antibiotics and antimycotics.

2.13.3 Light microscopy

Light microscopical studies were performed as previously described using paraffin-embedded oral RHE specimens (Schaller et al. 2002, 2004). Briefly, a part of each specimen was fixed, postfixed and embedded in glycide ether, and cut using an ultramicrotome (Ultracut). Semi-thin sections (1 μ m) were studied with a light microscope after staining with 1 % toluidine blue and 1 % pyronine G (Merck). The histological changes of the mucosa were evaluated on the basis of 50 sections from five different sites for each infected epithelium.

2.13.4 Epithelial cell damage assay

In all experiments, the release of LDH from epithelial cells into the surrounding medium at 24 hours was used as a measure of epithelial cell damage. LDH activity was analysed spectrophotometrically by measuring the reduced nicotinamide adenine dinucleotide disappearance rate at 340 nm as a main wavelength during the LDH-catalysed conversion of pyruvate to lactate according to Wróblewski and John (1955). The determination of the LDH activity was performed by using the Cytotoxicity Detection Kit (Roche).

2.13.5 RNA isolation and quantitative RT-PCR

Total RNA was isolated from oral RHE samples using the NucleoSpin RNA II-kit (Macherey-Nagel, Düren, Germany) including DNA digestion. For assessing RNA concentration and purity, UV spectroscopy was used (Bio Photometer, Eppendorf). cDNA was synthesized using the Superscript III first-strand synthesis supermix for qRT-PCR (Invitrogen) according to the manufacturer's instructions. Real-time PCR reactions were performed on a Light Cycler 480 instrument with LightCycler 480 SYBR Green I Master (Roche Molecular Diagnostics) in a total reaction volume of 10 μ l. Primer pairs that were used for amplification are listed in table 10. Data on the expression level were obtained in the form of crossing point (CP) values based on the 'second derivative maximum' method as computed by the LightCycler 480 software (Roche Molecular Diagnostics). Both standard and experimental samples were performed in triplicate and the average Cp reading used. Fold difference in gene expression was normalized to the housekeeping genes YWHAZ and G6PD, since detailed preliminary investigations using 10 different housekeeping genes (ALDOA, B2M, G6PD, GAPDH,

HMBS, POLR2A, SDHA, TBP, UBC and YWHAZ) indicated that these genes showed the most constant level of expression.

2.13.6 Determination of cytokine release

Culture medium was collected after 24 hrs of infection and assayed for GM-CSF, IL-6, IL-8, and TNF- α , using commercially available ELISA kits (DuoSet; R&D Systems, Minneapolis, MN, USA).

2.14 Interaction assays with immune cells

2.14.1 Primary culture myeloid dendritic cells (mDCs)

This protocol is based on method described previously by Inaba et al. (1992). The bone marrow isolation was performed as described above for mMPs. Black six mice (6-8 weeks old) were dissected, bones were rinsed with 70% ethanol and PBS and subsequently kept on ice in ice-cold PBS. At the knee joint, the femur and tibia were separated and the bone marrow was flushed by using a 20 ml syringe with a 27GX374 needle with DMEM with 10% FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. The bone marrow cell suspension was centrifuged at 700 \times g for 7 min. The pellet was resuspended in 1 ml of red cell lysis buffer, pH 7.2, which contained 10 mM KHCO₃, 150 mM NH₄Cl and 0.1 mM EDTA. Lysis was immediately stopped with 1 ml of mDC medium. Cells were collected by centrifugation at 700 \times g for 7 min and the pellet was resuspended in 5 ml mDC medium. The cell density was determined and the suspension was distributed in a 24 well plate with 1.0 \times 10⁶ cells per well in 1 ml. Cells were incubated at 37°C in a 5% CO₂, 95% humidity atmosphere and grown for 4 days. Then 0.5 ml was removed from each well and 1 ml of fresh mDC medium was added. After 3 days, non-adherent mDC aggregates were collected by flushing the medium against the well wall with a 1 ml Pipet set at 800 μ l, in order to limit the amount of adherent cells to be taken up. All cells were pooled and replated to a desired cell density.

2.14.2 In vitro interaction of mDCs with *C. albicans* strains.

Overnight YPD cultures of the *C. albicans* strains were diluted in 25 ml YPD medium to an OD₆₀₀ of 0.2-0.3 and were incubated at 30°C until OD₆₀₀ of 1.0 was reached. The cells were collected by centrifugation at 1200 \times g for 5 min and were washed one time in PBS. After centrifugation the cells were resuspended in 1 ml of PBS and the cell density was determined by using a CASY cell counter (Innovatis, Bielefeld, Germany). The fungal cells were diluted

in pre-warmed high-glucose DMEM medium without phenol red so that that the fungal-mammalian cell interaction was performed at a ratio of 2 fungal cells per mDC (multiplicity of infection ration, MOI).

One day prior to the interaction assay, the mDCs were plated at a cell density of $1.0-1.25 \times 10^5$ cells/cm² in a volume of cell culture medium of 0.2-0.4 ml/cm² and were incubated at 37°C in a 5% CO₂, 95% humidity atmosphere.

The media from the mDC culture dishes were aspirated and were replaced with high-glucose DMEM without phenol red with or without *C. albicans*. The *Candida*- mDCs interaction was performed at a 2:1 MOI in a 2 ml volume in 6 cm dishes. The *Candida* cell suspension was simply added to the mDC media in a 200 µl volume. The dishes were incubated at 37°C in a 5% CO₂, 95% humidity atmosphere for a 20 min 'infection pulse'. After the infection pulse cells were either collected or further incubated for an additional 90 min time period.

2.14.3 RNA isolation from mDCs and quantitative RT-PCR

The dishes were placed on ice and the media was removed, cell layers were collected by scrapping in 175 µl RNA lysis buffer (Promega). The samples were centrifuged at 11,000×g for 8 min and the supernatants were collected. RNA extraction was performed with the SV Total Isolation System (promega), according to the instructions of the manufacturer. Samples were eluted in 50 µl RNase-free sterile water. The RNA concentration was measured at an OD₂₆₀ and the quality of the samples was checked on a 1% agarose urea-TBE gel.

Reverse transcription was performed with 1 µg total RNA. Real-time PCR reactions were performed on a Light Cycler 480 instrument with LightCycler 480 SYBR Green I Master (Roche Molecular Diagnostics). Primers that were used for amplification are listed in table 11.

2.14.4 Determination of oxidative burst in PMNs upon interaction with *C. albicans*.

To determine the oxidative burst of human PMNs upon interaction with our *C. albicans* strains, we used a method which is based on the conversion of Dichlorfluorescein Diacetat (DCFH-DA) to the green fluourescent Dichlorfluorescein (DCF) by oxygen radicals (Radsak et al., 2004). The *Candida* – PMN interaction was performed at a MOI of 1:1 or 5:1 in 96-well plates with 25 µM DCF-DA in 100 µl RPMI 1640 supplemented with 5% FCS. As a positive control we incubated the PMNs with 50 ng/ml phorbol 12-myristate 13-acetate (PMA). The relative fluorescence units (RFU) were measured every 5 minutes in an ELISA-reader (GENios, Tecan, Crailsheim) at 37°C for 145 min. Experiments were repeated four times.

3. Results

Pga29p was identified in a genome-wide screen for putative GPI-proteins in *C. albicans* (De Groot et al., 2003). The extracellular presence of the protein was confirmed with mass spectrometry, revealing that it resides in the cell wall via a covalent linkage (De Groot et al., 2004). Interestingly, cell wall protein extracts analysed with Coomassie stained SDS-PAGE gels showed that Pga29p is abundantly present in the cell wall of yeast cells (De Groot et al., 2004), which is reflected in the MS identification of a large number of peptides from cell walls treated with trypsin. The Pga29p family comprises the three members Pga29p, Pga30p and Pga31p which are small GPI-CWPs that are covalently linked in the cell wall (De Groot et al., 2003, 2004; Castillo et al., 2008). Pga29p shares 50% and 45% sequence identity with Pga30p and Pga31p, respectively (Castillo et al., 2008) and the encoding genes are located at chromosome 4 within a range of 7467 base pairs. In order to obtain information about the distribution of Pga29p homologs in other fungal species, we performed BLAST searches with the Pga29p protein sequence against the NCBI protein database, the Fungal Genome Initiative *Candida* database at the Broad institute and the *Candida dubliniensis* database. In order to obtain a selective group of proteins a stringent E-value cut off of 1.0×10^{-4} was used. We found homologs in other human pathogenic *Ascomycetes*: *C. dubliniensis*, *C. guilliermondii*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *Lodderomyces elongisporus* and in *Debaryomyces hansenii* and *Pichia stipitis* (Table 16, Appendix). For none of these identified homologs functional characteristics do exist so far. Additionally, these BLAST results show that homologs of Pga29p are widespread among well-known virulent *Candida* species

3.1 Antibody generation against Pga29p

In order to study Pga29p in detail, we generated polyclonal antibodies against this protein. This was achieved by cloning *PGA29*, adapting the sequence for the altered codon usage by performing targeted point mutations and producing recombinant Pga29p in *E. coli*. The purified protein was subsequently used for rabbit immunisation. Briefly, we cloned a truncated form of the *PGA29* open reading frame that extended from codon 15 to codon 182, thereby omitting the hydrophobic N-terminal signal and GPI-anchor motif sequence. *C. albicans* is a *Candida* species that translates the leucine CUG codon in serine. Consequently, we introduced two point mutations in the CTG codon at position 193 of *PGA29*, generating a TCG codon which is translated in *E. coli* as a serine. We overexpressed the truncated *PGA29* in *E. coli* and verified the protein production with SDS-PAGE analysis (not shown).

Recombinant Pga29p was bearing a 6×His-tag, which facilitated the purification with Ni-NTA resin (Figure 1A). The purified recombinant Pga29p was used for rabbit immunisation (described in the methods section) and the presence of anti-Pga29p antibodies in the collected rabbit serum samples were tested with immuno-blot analysis. The strong signal on the membrane showed that the antibodies against recombinant Pga29p were successfully generated (Figure 1B). In order to investigate whether these antibodies specifically recognize endogenous Pga29p, we first treated isolated cell walls of the *C. albicans* wild type SC5314 with the chemical HF-pyridine, which specifically cleaves phosphodiester bonds, through which GPI-CWPs are linked to β -1,6-glucan chains (De Groot et al., 2004). This HF-pyridine cell wall extract was separated by SDS-PAGE and transferred to a PVDF membrane, which was treated with our anti-Pga29p serum. We observed a specific signal (Figure 1C), that was of identical size as the Pga29p band in the Coomassie brilliant blue stained SDS-gel from De Groot et al. (2004) (Figure 1D), thereby confirming that the serum contained polyclonal antibodies that specifically recognise Pga29p.

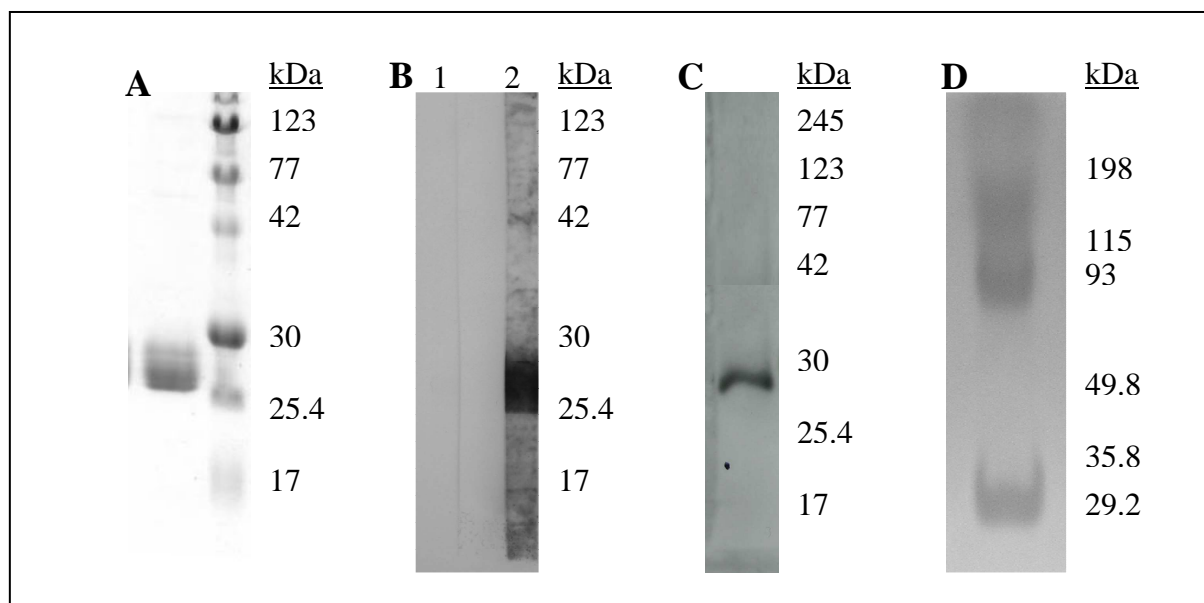


Figure 1. Generation of anti-Pga29p polyclonal antibodies.

(A) SDS-PAGE analysis (11%, Coomassie brilliant blue stained) of purified recombinant Pga29p. (B) Immuno-blot analysis with rabbit anti-Pga29p serum and recombinant Pga29p as an antigen. Lane 1) Rabbit serum before immunisation. Lane 2) Rabbit serum after immunisation. (C) HF-pyridine cell wall extract was separated with SDS-PAGE analysis (11%) and analysed with Immuno-blot analysis by using anti-Pga29p serum. (D) SDS-PAGE analysis (Coomassie brilliant blue stained gradient gel 2-20%) of HF-pyridine cell wall extract, from which the 29.2 kDa band was identified as Pga29p (De Groot et al., 2004). The numbers at the right of the figures indicate the sizes of standard molecular mass markers.

3.2 Pga29p incorporation into the cell wall

Different treatments can be used to release CWPs from purified cell walls such as HF-pyridine or β -1,6-glucanase to release GPI-proteins, β -1,3-glucanase to release GPI- and proteins with an alkali sensitive linkage and 30 mM NaOH to exclusively release proteins with an alkali sensitive linkage (Klis et al., 2001; Figure 2A). In 2004, De Groot and co-workers mass spectrometrically identified Pga29p in purified cell walls of *C. albicans* indicating that the protein is covalently attached

To unequivocally verify how Pga29p is incorporated into the carbohydrate backbone of the cell wall, we treated yeast cell walls with β -1,3-glucanase or β -1,6-glucanase and analysed the supernatants of these samples with immuno-blot assays. Digesting cell walls with β -1,6-glucanase released Pga29p (Figure 2B, lane 1), whereas digesting cell walls with β -1,3-glucanase released a high molecular weight smear (lane 3). The β -1,3-glucanase extract was dialysed and subsequently used for a second treatment with β -1,6-glucanase, which shifted the signal to 30 kDa (lane 4) indicating the release of Pga29p from a bigger complex: in the high molecular smear in lane 3 is Pga29p attached to large fragments of β -1,3-glucan. Digesting these fragments with β -1,6-glucanase resulted in the release of Pga29p (lane 4), which proved that this protein is covalently linked to β -1,3-glucan via β -1,6-glucan chains. The cell wall protein Cwp1p in *S. cerevisiae* and *C. glabrata* is either single linked via β -1,6-glucan or double linked via β -1,6-glucan and through an alkali labile linkage (Kapteyn et al., 2001; Weig et al., unpublished data). In order to show whether this is also the case with Pga29p we additionally treated (i) β -1,6-glucanase digested cell walls (lane 2) and (ii) untreated cell walls (lane 5) with 30 mM NaOH to unravel a possible second linkage which is alkali labile. However, no signal was detected; indicating that Pga29p no additional linkage to the cell wall via an alkali labile bond.

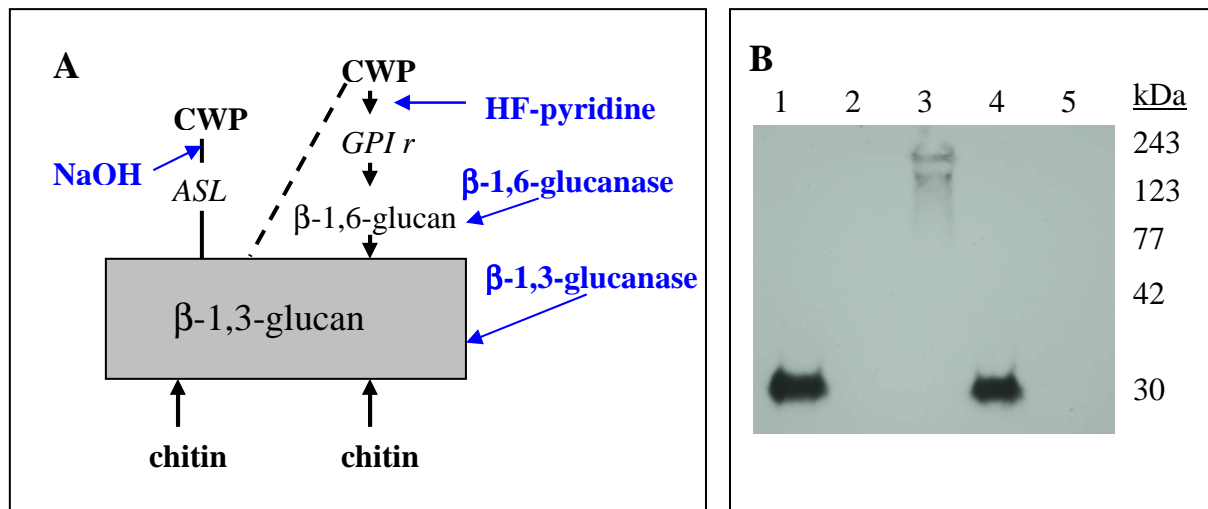


Figure 2. Covalent incorporation of Pga29p in the cell wall of *C. albicans*.

(A) Covalently linked cell wall proteins (CWP) can be liberated from the cell wall with different chemicals and enzymes, which are shown in blue. The black arrows indicate glycosidic linkages and point from the reducing end of a macro molecule to a non-reducing end of an acceptor polysaccharide. The dashed line indicates the double linkage by which some GPI-CWPs are linked. *ASL* and *GPI r* indicate the alkali sensitive linkage and the GPI remnant, respectively.

(B) Cell wall proteins were separated by SDS-PAGE (11%) and analysed by immuno-blotting using anti-Pga29p polyclonal antibodies. Lane 1) β -1,6-glucanase extract. Lane 2) NaOH extract of cell walls previously treated with β -1,6-glucanase. Lane 3) β -1,3-glucanase extract. Lane 4) β -1,3-glucanase extract treated with β -1,6-glucanase. 5) NaOH extract. Sizes of standard molecular mass markers are shown at the right.

3.3 Pga29p is a yeast-specific protein

3.3.1 *PGA29* expression during the yeast-to-hyphal transition

The CWP incorporation drastically changes upon the morphological transition of *C. albicans* from the yeast to the hyphal form, an important process for virulence (Sohn et al., 2003; Ebanks et al., 2006; Castillo et al., 2008). Furthermore, the expression of several genes that encode GPI-proteins is related with particular growth forms, for example *HWPI* is exclusively expressed in the hyphal form (Staab et al., 1999). Yeast wall protein 1 (Ywp1, Pga24p) is an example of a GPI-protein that specifically related to the yeast form of *C. albicans* (Nantel et al., 2002; Sohn et al., 2003; Granger et al., 2005). We showed previously that Pga29p is abundantly present in the yeast form of *C. albicans* (De Groot et al., 2004). To examine whether the protein level changes during the transition from yeast to hyphae, cell walls were isolated from *C. albicans* cells that were induced to develop hyphae by stimulation with 10% serum and a temperature shift from 30°C to 37°C. After two hours of hyphal induction the Pga29p level in the cell wall clearly decreased, but remained detectable even

after 4 hours of hyphal growth (Figure 3A). Additionally, Northern blot analysis showed that *PGA29* transcript levels rapidly decreased within 45 minutes during the yeast-to-hyphae transition (Figure 3B). These data show that Pga29p is a yeast-specific protein, though the protein remains in the cell wall for several hours after hyphal induction.

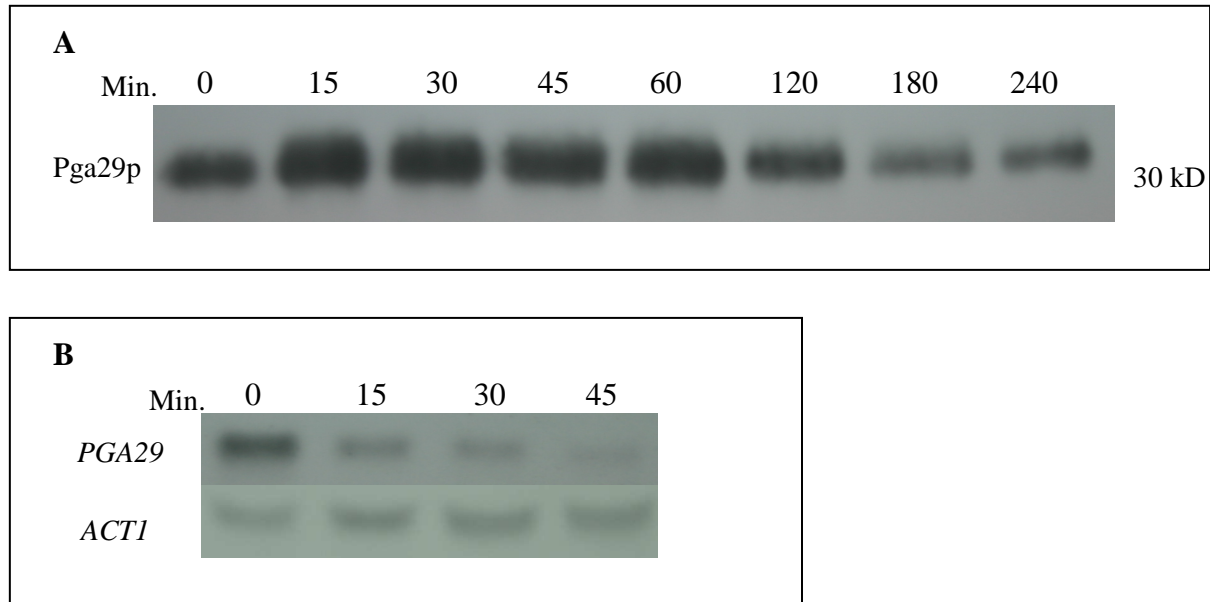


Figure 3. *PGA29* expression in yeast-to-hyphal transition.

Immuno-blot (A) and Northern blot (B) analyses were used to study the expression of *PGA29* during the transition from yeast to hyphae. (A) In the immuno-blot analysis, Pga29p was detected with the generated anti-Pga29p polyclonal antibodies. Each lane was loaded with β -1,6-glucanase extract derived from 0.4 mg dry-weight cell walls. (B) In the Northern blot analysis, *PGA29* transcript was detected with a digoxigenin probe. As a loading control a probe against *ACT1* transcript was used. The numbers indicate the time in minutes after hyphal induction.

3.3.2 *PGA29* expression in deletion mutants that are impaired in hyphal development

EFG1 and *CPH1* encode transcription factors that regulate the transition from yeast to hyphae in *C. albicans*. Nantel et al. (2002) showed an increased expression of *PGA29* in $\Delta efg1/\Delta efg1$ (2.5 times) and $\Delta efg1/\Delta efg1 \Delta cph1/\Delta cph1$ (3.9 times) mutants. Both strains are impaired in their ability for hyphal development. In order to investigate whether Pga29p is highly incorporated in the cell wall of these mutants, we analysed β -1,6-glucanase CWP extracts with immuno-blot analysis (Figure 4). This revealed that the cell wall of the $\Delta efg1/\Delta efg1$ mutant (lane 1) contained 1.17 times and $\Delta efg1/\Delta efg1 \Delta cph1/\Delta cph1$ mutant (lane 2) contains 2.28 times more Pga29p than the wild type (lane 3), which supports the idea that Pga29p is a yeast-specific protein and that Efg1p and Cph1p negatively regulate its expression.

To determine whether these transcription factors might directly regulate *PGA29* expression we searched by *in silico* analysis for their potential binding sites in the 2 kb region upstream

of the start codon of *PGA29*. Efg1p was shown to bind to the consensus sequences CANNTG (known as E-box) (Leng et al., 2001). The exact binding site for Cph1p is not known, however for our studies we used the consensus sequence TGAAACA, which is a specific binding site for the *S. cerevisiae* transcription factor Ste12p, a homologue of Cph1p. We found in total 8 E-box sequences (Figure 5) upstream of the *PGA29*-ORF which suggests that Efg1p might directly regulate *PGA29* via these binding sites. Ste12 binding sites were not identified with the preliminary approach described above. However, it should be stressed that this approach is based on information derived from bakers yeast and therefore this result does not reliably exclude Cph1 as a direct regulator of *PGA29*.

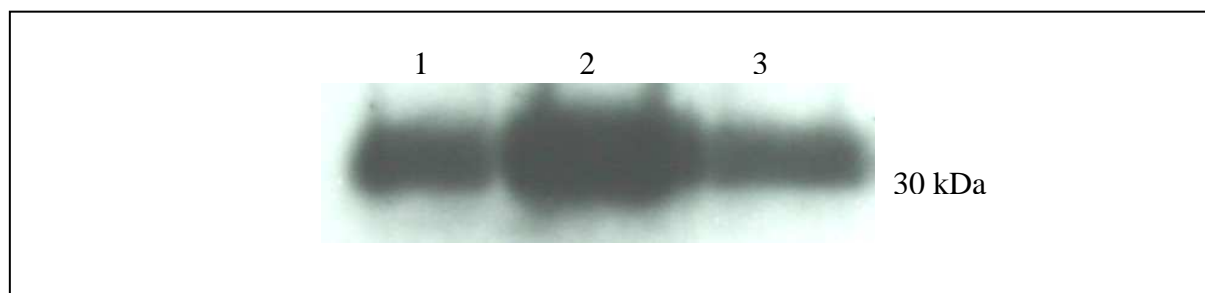


Figure 4. Pga29p incorporation in cell walls of *C. albicans* $\Delta efg/\Delta efg1$ and $\Delta efg1/\Delta efg1$ $\Delta cph1/\Delta cph1$ mutants.

β -1,6-glucanase cell wall protein extracts of the mutants and of the control strain were separated by SDS-PAGE (11%) and analysed by immuno-blotting using anti-Pga29p polyclonal antibodies. Lane 1) $\Delta efg1/\Delta efg1$. Lane 2) $\Delta efg1/\Delta efg1$ $\Delta cph1/\Delta cph1$. Lane 3) SC5314.

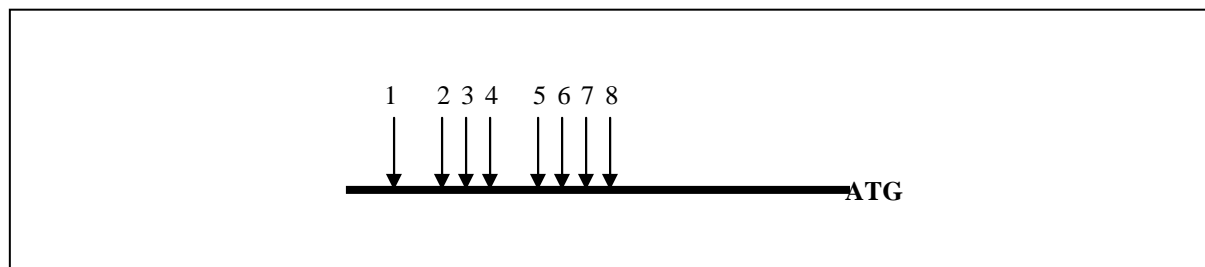


Figure 5. E-boxes in the *PGA29* promoter sequence.

We searched for E-boxes (CANNTG) 2000 base pairs upstream of the start codon of *PGA29*. In total 8 E-boxes were found, which were mainly located in the first 1000 base pairs of the promoter region. E-box #1) 177. #2) 411. #3) 501. #4) 613. #5) 840. #6) 968. #7) 1006. #8) 1104 (in base pairs)

3.4 Functional analysis of Pga29p

3.4.1 Pga29p upregulation under cell wall stress

In order to elucidate the function of Pga29p, we sought for conditions in which Pga29p is upregulated. Pga29p is a small protein without any conserved domains, therefore it seems possible that this GPI-protein may have a structural role for maintaining cell wall integrity in *C. albicans*. To investigate this, we analysed β -1,6-glucanase protein extracts from yeast cell walls which were isolated from cells that underwent cell wall stress during growth. The level of incorporated Pga29p increased 2.2 times in cells that were grown in YPD supplemented with 25 μ g/ml CFW, a compound which induces cell wall perturbation due to its binding to chitin in the cell wall (Figure 6, lane 2). However, Pga29p upregulation was not observed during growth in the presence of SDS (Figure 6, lane 3), a detergent that penetrates the plasma membrane and consequently disorganises its structure. Our data suggest that Pga29p upregulation is more related with perturbation of the cell wall and

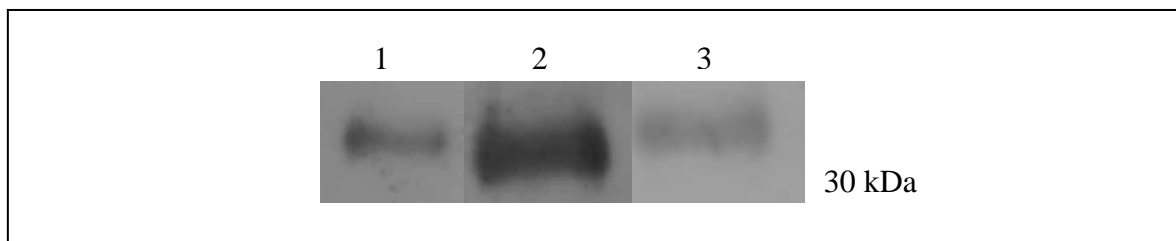


Figure 6. Pga29p incorporation under CFW and SDS stress.

C. albicans wild type SC5314 cells were grown under 25 μ g CFW or 0.01% SDS and were harvested when an OD₆₀₀ of 2.0 was reached. Isolated cell walls were treated with β -1,6-glucanase and the extracts were analysed by immuno-blotting using anti-Pga29p polyclonal antibodies. Lane 1) Wild type SC5314 grown in YPD medium at 30°C. Lane 2) Wild type SC5314 grown under CFW-stress. Lane 3) Wild type SC5314 grown with 0.01% SDS

We also searched for growth conditions that induce downregulation of Pga29p. As mRNA levels of *PGA29* decrease during the yeast-to-hyphal transition, we selected two factors that play a role in hyphal development. A neutral pH and a temperature of 37°C are two major factors for hyphal development. Downregulation was not observed when cells were grown at a pH 7.5, whereas a strong downregulation was observed under high temperatures of 37°C and 42°C (Figure 7).

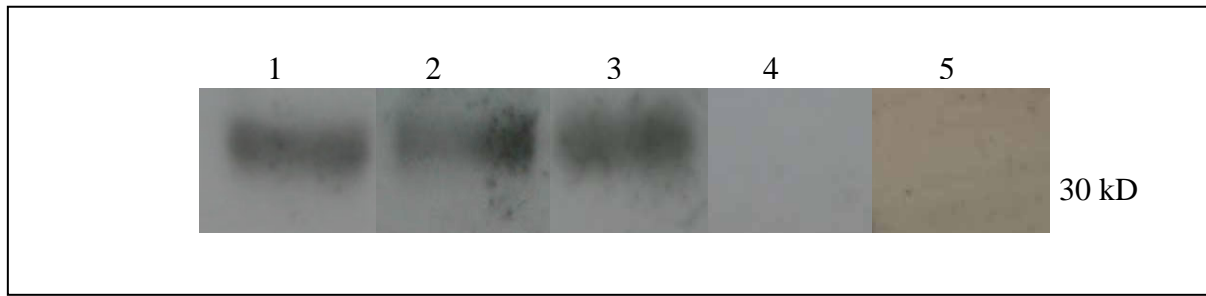


Figure 7. Pga29p incorporation under different pH values and temperatures.

C. albicans wild type SC5314 cells were grown at different pH values and temperatures. After OD₆₀₀ of 2.0 was reached, cells were harvested and cell walls were isolated. Cells walls were treated with β -1,6-glucanase and extracts were analysed by immuno-blotting using anti-Pga29p polyclonal antibodies. Lane 1) pH 4.0. Lane 2) pH 5.75. Lane 3) pH 7.5. Lane 4) 37°C. Lane 5) 42°C.

3.5 Generation of *PGA29* deletion mutants

To more deeply explore the function of Pga29p, we introduced double allele deletions in the triple auxotrophic strain BWP17 (Wilson et al., 1999), by using PCR products as disruption cassettes that consist of a selectable marker (*HIS1* or *ARG4*) and at both ends 50 base pairs that share homology to the *PGA29* untranslated genome sequence. To complement the *URA3* auxotrophy in the Δ *pga29*/ Δ *pga29* mutants, we directed the linearized CIp10 vector to the *RPS10* locus, which facilitates appropriate expression of *URA3* (Brand et al., 2004). This locus was also used to generate the revertants (Δ *pga29*/ Δ *pga29*/*PGA29*) with the linearized CIp10-*PGA29* vector.

3.5.1 Verification of *PGA29* deletion

The first allele of *PGA29* was disrupted with a *HIS1* knock-out cassette (Figure 8), of which the integration at the *PGA29* locus was confirmed by two different PCRs. For correct integration at the 5 prime region a PCR was performed with the primers PGA29-F (binding position: -827- -804) and HIS-R (binding position: 605-628) that resulted in a product of 1799 base pairs (Figure 9A, lane 2). For correct integration at the 3 prime region a PCR was performed with the primers HIS-F (binding position: 87-110) and PGA29-R (binding position: +322 - +341) that resulted in a 2433 bp product (Figure 9A, lane 1). Sequencing of both PCR products confirmed correct integration of the *HIS1* knock-out cassette. Southern blot analysis was performed in order to verify the single integration of the *HIS1* knock-out cassette in the genome. Genomic DNA of mutants was digested with the restriction enzyme *EcoRI*, which cuts up- and downstream of the knock-out cassette at the positions -854 and +2122, respectively (Figure 8). The expected fragment size of 4890 base pairs was confirmed

by using a probe against *HIS1*, which was generated with primers HIS-F and HIS-R (Figure 9B).

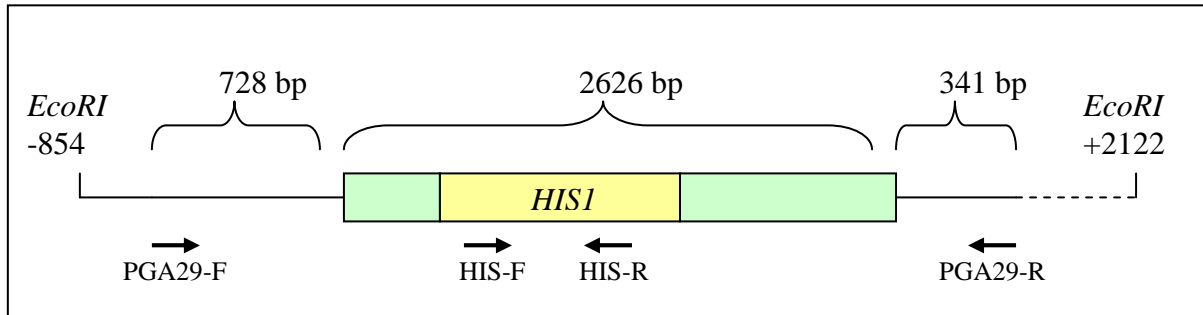


Figure 8. Verification of the integration of the *HIS1* knock-out cassette with PCR and Southern blot analysis. Schematic representation of *PGA29* deletion with the *HIS1* knock-out cassette. The yellow region represents the *HIS1* ORF. The green regions represent *HIS1* promoter, *HIS1* terminator and plasmid (pGEM-*HIS1*) sequences. The arrows indicate the primer binding positions. *EcoRI* cuts in the *PGA29* untranslated regions at positions -854 and +2122. Sizes of the knock-out cassette and untranslated regions are indicated in base pairs (bp).

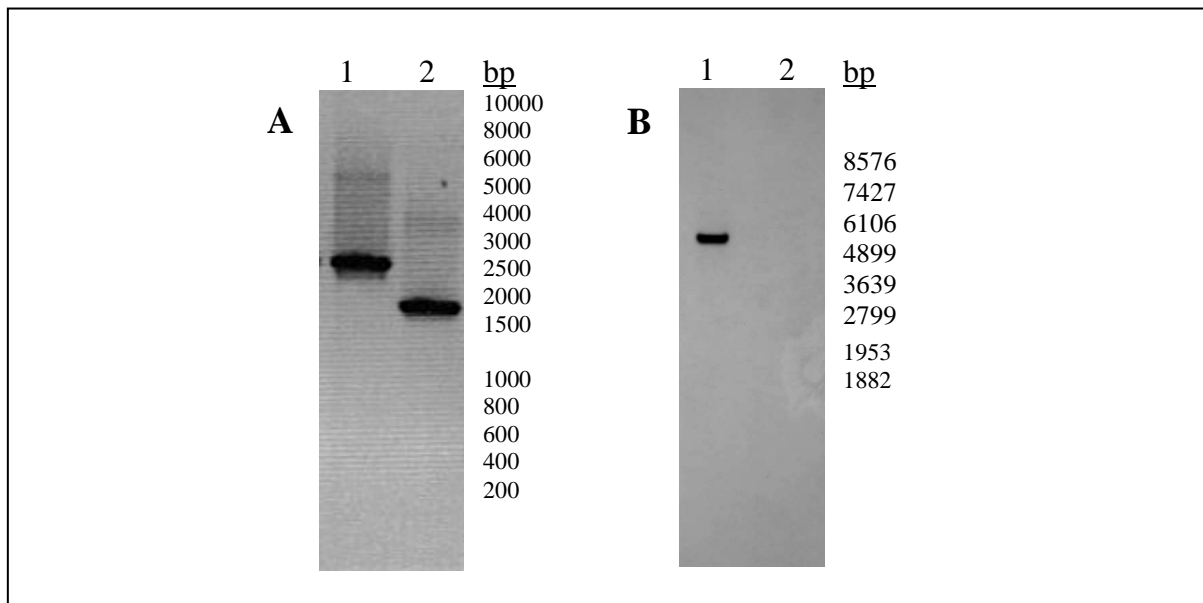


Figure 9A and B. Verification of the integration of the *HIS1* knock-out cassette with PCR and Southern blot analysis. (A) Two different PCRs were performed which resulted in products that included the genome and *HIS1* cassette sequences. The PCR products were visualised by agarose gel electrophoresis. Lane 1) PCR of 3 prime region. Lane 2) PCR of 5 prime region. (B) Detection of the 4890 bp *EcoRI* fragment with Southern blot analysis. *EcoRI* digestion of genomic DNA from the mutant (lane1) and from the parental strain BWP17 (negative control) (lane2). The numbers at the right indicate the size of standard markers in base pairs.

The second allele was disrupted with the *ARG4* knock-out cassette (Figure 10), which integrated in the anti-sense direction of the *PGA29* locus. Correct integration was confirmed by amplifying the 5 prime region with the primers PGA29-F (binding position: -827- -804)

and ARG4-R (binding position: 874-897), which resulted in a product of 1453 base pairs (Figure 11A, lane 1). Additionally, the 3' prime region was amplified with the primers ARG4-F (binding position: 217-240) and PGA29-R (binding position: +322 - +341), which resulted in a fragment of 1119 base pairs (Figure 11A, lane 2). Both PCR products were sequenced and confirmed correct integration of the *ARG4* knock-out cassette. For Southern analysis, genomic DNA of the mutants was digested with the restriction enzyme *MfeI*, which cuts up- and downstream of the *ARG4* knock-out cassette at positions -1253 and +3325 (Figure 10). This *MfeI* fragment of 6738 base pairs was detected by using a probe against *ARG4* (Figure 11B, lane 1), which was generated with the primers ARG4-S-F and ARG4-S-R. An additional signal was observed of 2942 base pairs (Figure 11B, lane 2), which is due to the binding of the *ARG4* probe with remaining sequences of the *ARG4* locus. This was confirmed by using *MfeI* digested genomic DNA from the parental strain BWP17 as control.

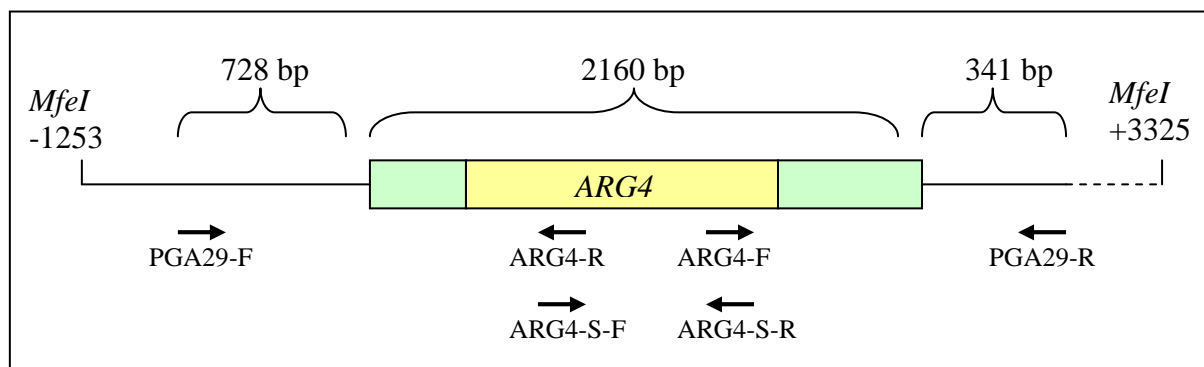


Figure 10. Verification of the integration of the *ARG4* knock-out cassette with PCR and Southern blot analysis. Schematic representation of *PGA29* deletion with the *ARG4* knock-out cassette. The yellow region represents the *ARG4* ORF. The green regions represent *ARG4* promoter, *ARG4* terminator and plasmid (pRS-*ARG4*Δ*SpeI*) sequences. The arrows indicate the primer binding positions. *MfeI* cuts in the *PGA29* untranslated regions at positions -1253 and +3325. Sizes of the knock-out cassette and untranslated regions are indicated in base pairs (bp).

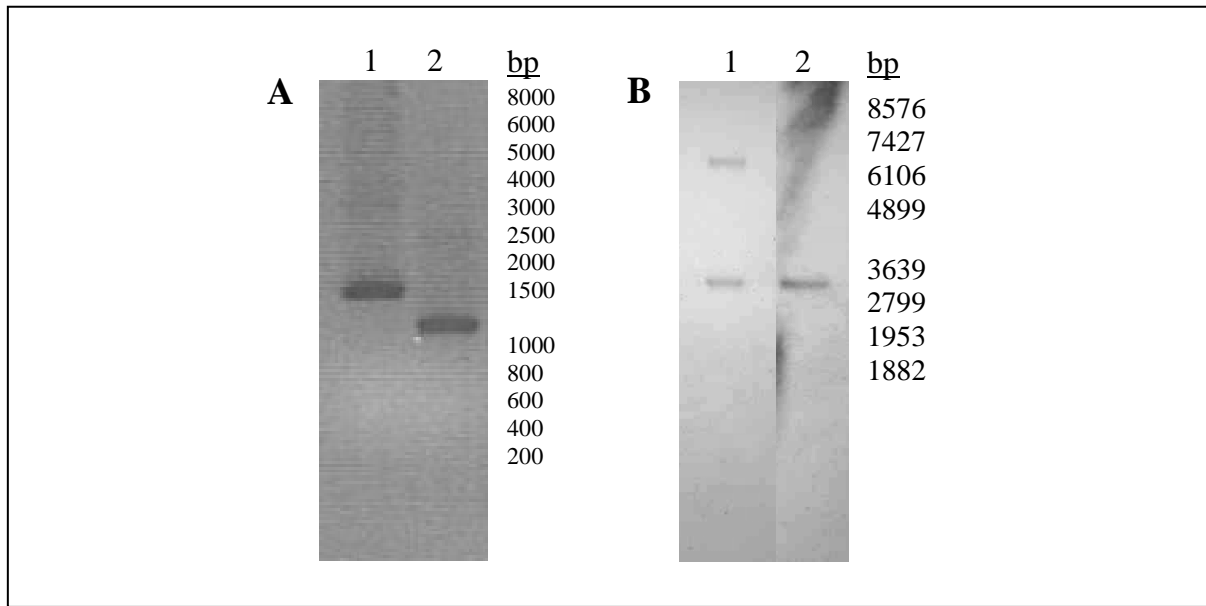


Figure 11A and B. Verification of the integration of the ARG4 knock-out cassette with PCR and Southern blot analysis. (A) Two different PCRs were performed which resulted in products that included the genome and ARG4 cassette sequences. The PCR products were visualised by agarose gel electrophoresis. Lane 1) PCR of 5 prime region. Lane 2) PCR of 3 prime region. (B) Detection of the 6711 bp *MfeI* fragment with Southern blot analysis. *MfeI* digestion of genomic DNA from the mutant (lane 1) and from parental BWP17 strain (lane2). The ARG4 probe hybridized with remaining sequences in the original ARG4 locus, which resulted in an extra signal with a size of 2942 bp. The numbers at the right indicate the size of standard markers in base pairs.

3.5.2 Verification of URA3 and PGA29 complementation

The integration of the CIp10 plasmid in the *RPS10* locus was verified by two different PCRs and Southern analysis (Figure 12). The 5 and 3 prime regions were amplified with the primers RP10-1F (binding position: -861- -838) and CIp10.2-R (binding position in CIp10: 3175-3192) and URA3-F (binding position: 47-67) and RP10-3R (binding position: +372 - +395), which resulted in the fragments of 1971 and 1767 base pairs (Figure 13A), respectively. Both PCR products were sequenced and this confirmed correct integration of the CIp10 plasmid. For Southern blot analysis, genomic DNA of the mutants was digested with the restriction enzyme *EcoRI*, which cuts in the cassette and further downstream in the genome sequence at positions 133 of the *URA3* ORF and +2130 of the RP10 untranslated region (Figure 12). The expected fragment *EcoRI* of 3422 base pairs was detected by using a probe against *URA3* (Figure 13B), which was generated with the primers URA3-F (binding position: 47-67) and URA3-R (binding position: 592-615).

The integration of the CIp10-*PGA29* plasmid in the *RP10* locus was confirmed by two different PCRs and Southern analysis (Figure 14). For correct integration at the 5 prime region a PCR was performed with the primers RP101F (binding position: -861- -838) and *PGA29*-best-*knk*-F (binding position: +364 - +385) which resulted in a 1746 bp product

(Figure 11B). For correct integration at the 3 prime region the PCR was performed as previously described for Clp10 integration (Figure 15A). Sequencing of both PCR products confirmed correct integration of the Clp10-*PGA29* plasmid. Southern blot analysis was performed in order to verify the single integration of the Clp10-*PGA29* plasmid (Figure 15B). This was performed as previously described for Clp10 integration.

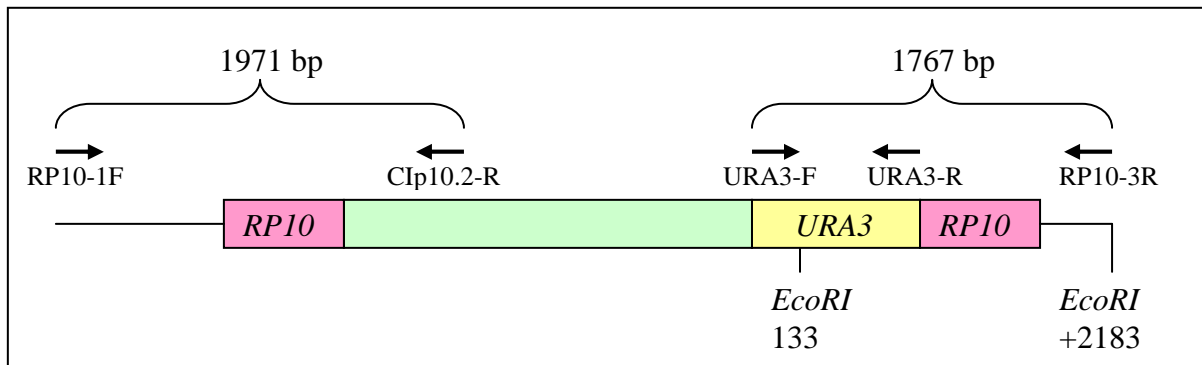


Figure 12. Verification of the integration of Clp10 with PCR and Southern blot analysis. Schematic representation of the *URA3* complementation in the $\Delta pga29/\Delta pga29$ strain with the linearised Clp10. The yellow region represents the *URA3* ORF. The green regions represent plasmid sequences of Clp10. The red regions represent the *RP10* loci. One extra *RP10* locus was generated with Clp10 integration. The arrows indicate the primer binding positions. *EcoRI* cuts in the *URA3* ORF at position 133 and in the *RP10* untranslated region (3 prime) at position +2122. Sizes of the knock-out cassette and untranslated regions are indicated in base pairs (bp).

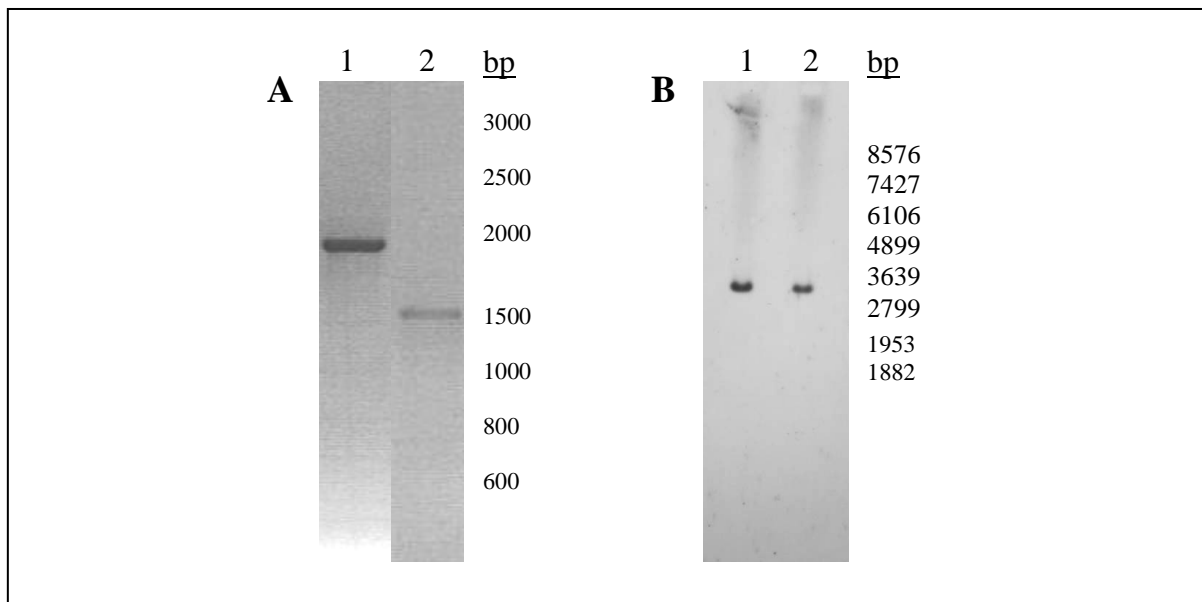


Figure 13A and B. Verification of the integration of Clp10 with PCR and Southern blot analysis. (A) Two different PCRs were performed which resulted in products that included the genome and Clp10 sequences. The PCR products were visualised by agarose gel electrophoresis. Lane 1) PCR of 5 prime region. Lane 2) PCR of 3 prime region. (B) Detection of the 3422 bp *EcoRI* fragment with Southern blot analysis with *URA3* probe. Lane 1 and 2) *EcoRI* digestion of genomic DNA of two transformants. The numbers at the right indicate the size of standard markers in base pairs.

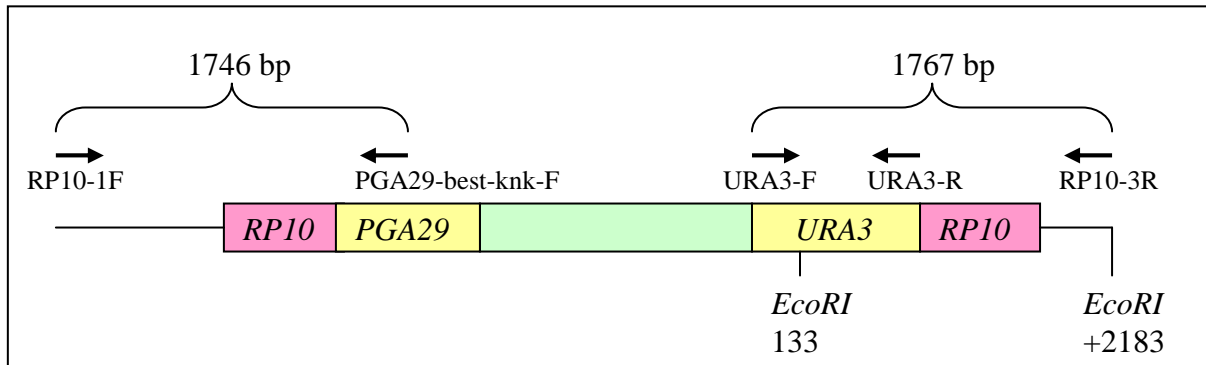


Figure 14. Verification of the integration of Clp10-*PGA29* with PCR and Southern blot analysis. Schematic representation of the *PGA29* and *URA3* complementation in the $\Delta pga29/\Delta pga29$ strain with the linearised Clp10-*PGA29*. The yellow region represents the *PGA29* gene and *URA3* ORF. The green regions represent plasmid sequences of Clp10. The red regions represent the *RP10* loci. One extra *RP10* locus was generated with Clp10 integration. The arrows indicate the primer binding positions. *EcoRI* cuts in the *URA3* ORF at position 133 and in the *RP10* untranslated region (3 prime) at position +2122. Sizes of the knock-out cassette and untranslated regions are indicated in base pairs (bp).

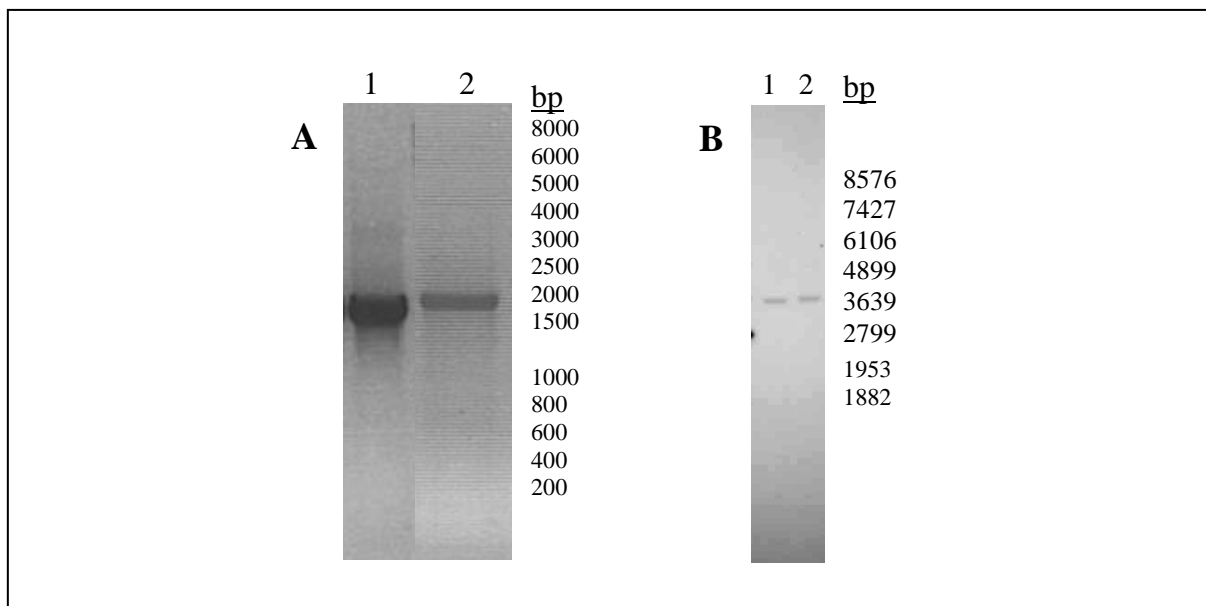


Figure 15A and B. Verification of the integration of Clp10-*PGA29* with PCR and Southern blot analysis. (A) Two different PCRs were performed which resulted in products that included the genome and Clp10-*PGA29* sequences. The PCR products were visualised by agarose gel electrophoresis. Lane 1) PCR of 5 prime region. Lane 2) PCR of 3 prime region. (B) Detection of the 3422 bp *EcoRI* fragment with Southern blot analysis with *URA3* probe. Lane 1 and 2) *EcoRI* digestion of genomic DNA of two transformants. The numbers at the right indicate the size of standard markers in base pairs.

In order to confirm that the deletion mutants were no longer able to produce a *PGA29* gene product, we performed Northern blot (not shown) and immunoblot analysis. These analyses showed that the $\Delta pga29/\Delta pga29$ mutants lacked *PGA29* expression and the reconstituted strains properly expressed *PGA29* (Figure 16).

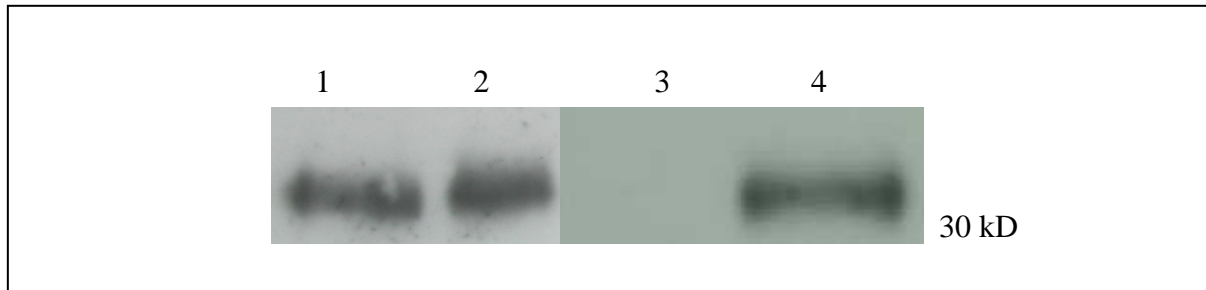


Figure 16. Generation of *PGA29* deletion mutants.

Cell walls were isolated from mid-log phase cells (OD_{600} of 2.0) and were treated with β -1,6-glucanase. Proteins were separated by SDS-PAGE (11%) and analysed by immuno-blotting using anti-Pga29p polyclonal antibodies. Lane 1) Wild type SC5314. Lane 2) $\Delta pga29/PGA29$. Lane 3) $\Delta pga29/\Delta pga29$. Lane 4) $\Delta pga29/\Delta pga29/PGA29$.

3.6 Cell wall composition of the $\Delta pga29/\Delta pga29$ mutants

Pga29p is abundantly present in yeast cell walls, therefore its absence in the deletion mutants might effect the cell wall composition. In order to unravel morphological differences in the structure of the cell wall between the $\Delta pga29/\Delta pga29$ and control strains, mid-log phase yeast cells were high-pressure frozen (cryofixation), and analysed with transmission electron microscopy. Structural differences were quantified by measuring the thickness of the fibrillar and glucan layer (Figure 17). No differences were observed between the cell walls of the mutant and revertant strain, which implies the cell wall structure is not affected upon *PGA29* deletion (Table 12). Additionally, differences were found in the thickness of both cell wall layers between the wild type SC5314 and $\Delta pga29/\Delta pga29/PGA29$ strain (Table 12), which is probably due to the background strain used and could also be related to the ectopic expression of the auxothropic markers *ARG4*, *HIS1* and *URA3* (see **4. Discussion**).

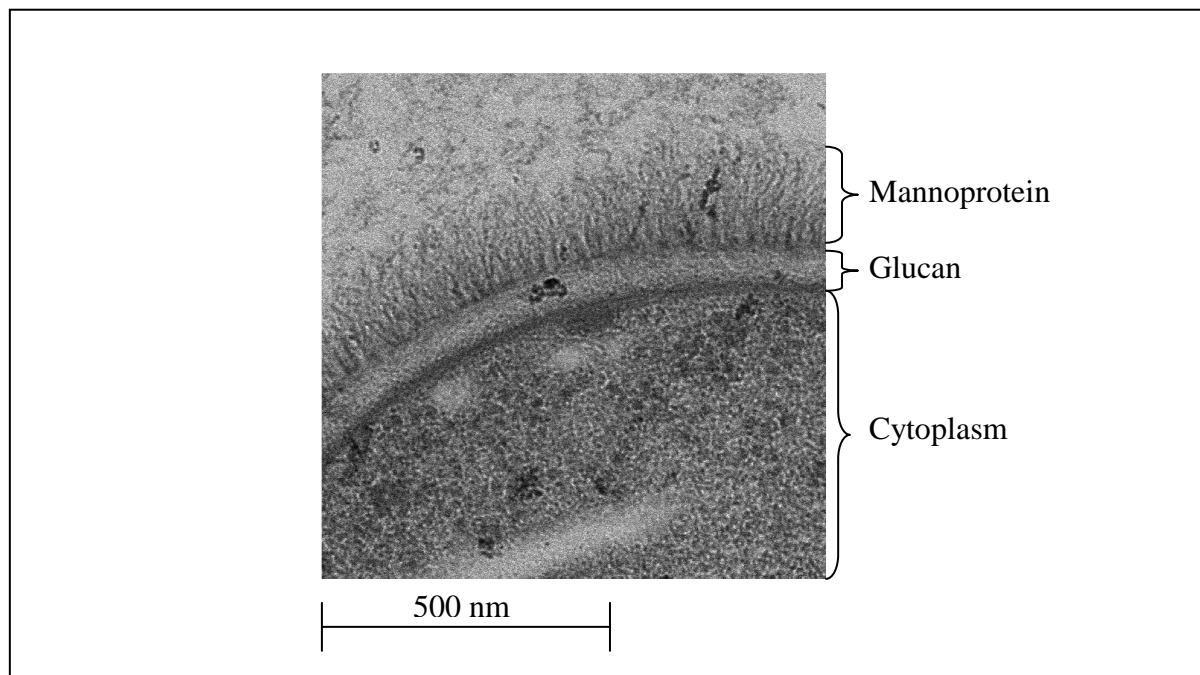


Figure 17. EM photograph of *C. albicans* cell wall.

The upper fibrillar layer consists of mannoproteins, whereas the inner layer consists mainly of glucan. The thickness of the two layers was measured in a total of 100 individual cells of the mutant, revertant and wild type strain, respectively

Table 12. Thickness of the glucan and mannoprotein layer

Of each strain 100 cells were selected to measure the thickness of the glucan and mannoprotein layer. The numbers indicate the mean values in nm.

<u>Strain</u>	<u>Glucan</u>	<u>Mannoprotein</u>
<i>Δpga29/Δpga29</i>	114.31	51.71
<i>Δpga29/Δpga29/PGA29</i>	115.01	50.47
SC5314	98.19	76.85

Next, we determined the composition and possible changes in the concentration of chitin, protein, and the mannose/glucose ratio in the cell wall of the *Δpga29/Δpga29* strain in comparison to the control strains. To quantify the chitin concentration, cell walls were treated with HCl which resulted in the release of N-acetylglucosamine residues that were colorimetrically measured. A difference in chitin concentration was not observed for our strains (Table 13; Figure 18A).

To determine the protein concentration, cell walls were treated with a boiling NaOH solution. This treatment hydrolyses CWPs and releases single amino acids, that can be quantified using a colorimetric assay. We did not observe any significant differences in the CWP concentration between the strains (Table 13; Figure 18A).

Next, we measured the mannose/glucose ratio, by treating cell walls with H₂SO₄ which hydrolyses polysaccharides. The samples were analysed with HPLC on an organic acid analysis column and the ratios between the different sugars were determined. The *Δpga29/Δpga29* mutants showed a significant reduction in the mannose/glucose ratio compared with the control strains (Table 13; Figure 18B), which indicates a reduction of mannan in the mutants cell wall.

Table 13. Cell wall composition.

Cell walls of each strain were isolated from two different cultures. The samples were analysed in triplo from which the mean values were calculated. The chitin and protein concentration was determined in percentage. Mannose and glucose values were divided in order to obtain the mannose/glucose ratio (M/G). SD indicates the sample standard deviation.

	<i>Δpga29/Δpga29</i>		<i>Δpga29/Δpga29/PGA29</i>		SC5314	
	mean	SD	mean	SD	mean	SD
Chitin (%)	5.15	0.34	5.37	0.65	4.66	1.02
Protein (%)	10.60	0.69	9.53	1.12	9.14	0.73
M/G	0.309	0.0025	0.513	0.0033	0.470	0.0105

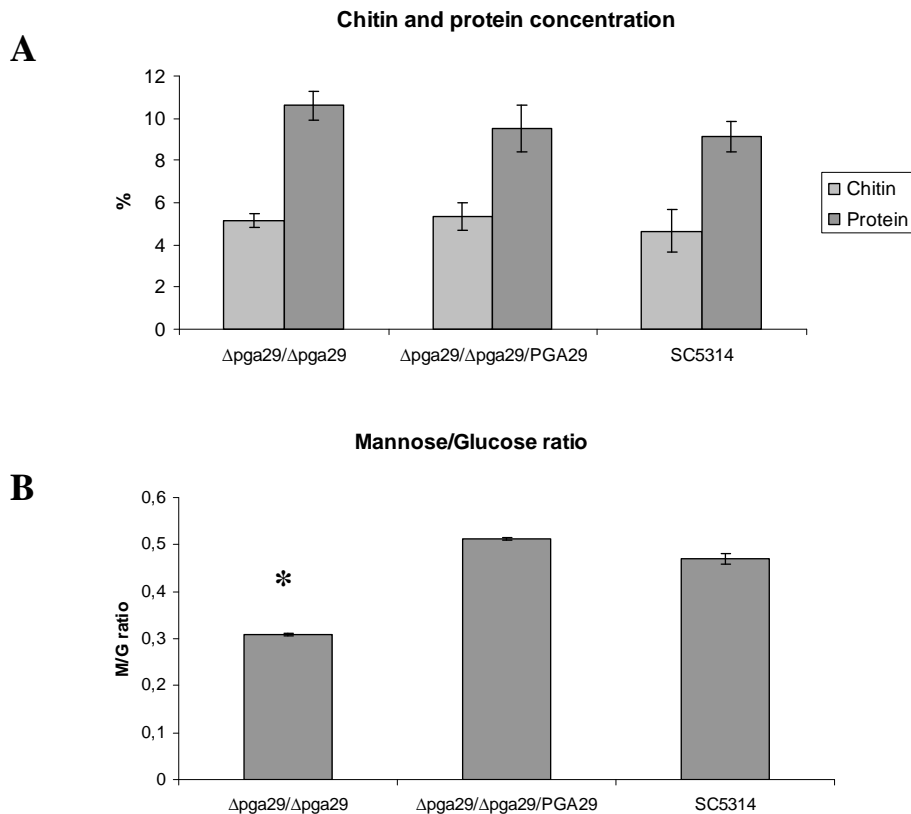


Figure 18. Cell wall composition analysis of the $\Delta pga29/\Delta pga29$ strain.

The *C. albicans* strains were cultured under conditions in which *PGA29* is abundantly expressed (YPD medium at 30°C) and harvested at an OD₆₀₀ of 2.0. The isolated cell walls were treated with NaOH, to release protein, with HCl (Chitin) and with H₂SO₄ (mannan, glucan) to release monosaccharides. Protein and chitin were quantified using a colorimetric assay. Mannose and glucose were determined with HPLC.

(A) Chitin and protein concentration (indicated in %) and (B) mannose/glucose ratio in cell walls of $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and wild type SC5314. The asterisk indicates a significant lower mannose/glucose ratio compared with the control strains (*T*-test, *P*<0.05)

3.7 Phenotypic analysis of *PGA29* deletion mutants

3.7.1 Growth rate of $\Delta pga29/\Delta pga29$ mutants

We determined whether deletion of *PGA29* affected the growth rate in the $\Delta pga29/\Delta pga29$ strain. Because *C. albicans* is capable to grow in a wide pH range, we tested our strains for growth in a pH range from pH 4.0 to pH 9.0. These assays showed that *PGA29* deletion did not alter the growth rate at the different pH values, as indicated by a comparison of the $\Delta pga29/\Delta pga29$ deletion mutant and the revertant (Figure 19).

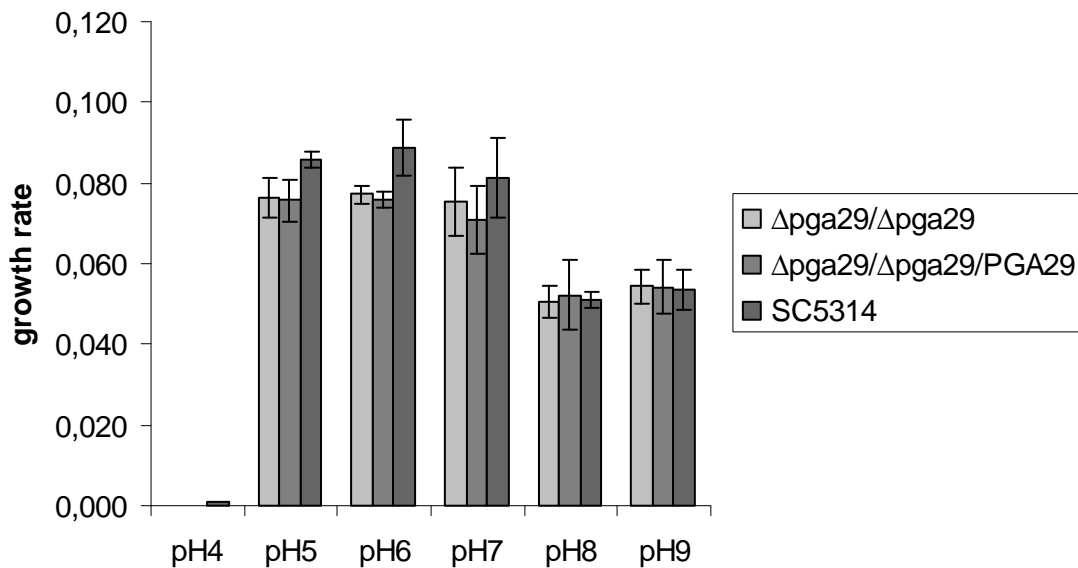


Figure 19. Growth rate determination of the $\Delta pga29$ mutants.

Growth rate of mutant, revertant and wild type was determined at different pH values. From overnight cultures, cells were inoculated in 96-well plates and incubated at 30°C. Two independent $\Delta pga29/\Delta pga29$ strains and two $\Delta pga29/\Delta pga29/PGA29$ strains were used. From each strain 6 cultures were grown. The OD₆₀₀ was measured at 15 minutes time intervals from which the average slope was calculated.

3.7.2 CFW and other cell wall stress

The observed upregulation of Pga29p under CFW stress (Figure 6) could imply a compensatory effect induced by *C. albicans* in order to fortify its cell wall. To analyse this possibility, $\Delta pga29/\Delta pga29$ mutants were cultured under different CFW concentrations on YPD or YNB agar (Figure 20) in order to monitor the impact on the growth. Interestingly, no increased CFW-sensitivity was observed between the mutants and the revertant strains. Applying additional stresses, for example an increase (40°C) or decrease (25°C) in temperature and an increase in osmolarity, did not significantly alter the sensitivity of the mutant for CFW. Subsequently, the $\Delta pga29/\Delta pga29$ strain was tested for SDS-sensitivity as well, which did not reveal an altered phenotype of the $\Delta pga29/\Delta pga29$ strain. These data suggest that Pga29p is not involved in the fundamental maintenance of the cell wall integrity in *C. albicans*. In all microdilution spot assays the wild type SC5314 appeared more resistant than the mutant and revertant strains. As mentioned above in the cell wall composition analysis of $\Delta pga29/\Delta pga29$ mutants (see 3.6), we speculate that this could be explained by the ectopic expression of the auxotrophic markers in the mutant and revertant.

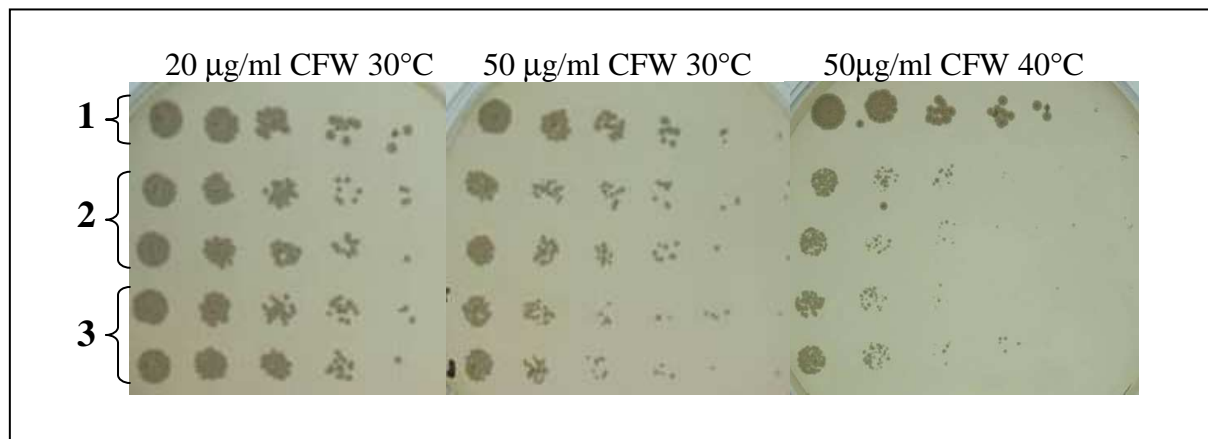


Figure 20. Microdilution spot assay screening for CFW sensitivity in Δ pga29/ Δ pga29 mutants. For each strain six different concentrations of stationary phase or mid-log phase cells were spotted on YPD or YNB agar supplemented with concentrations of CFW that were ranging between 0-75 μ g/ml. Cells were incubated at different temperatures (25°C -not shown-, 30°C, 37°C -not shown- and 40°C). Additional stress was induced by adding high concentrations (0.7-1.0 M) of NaCl or by incubating at low (25°C) or high (40°C) temperatures. Two independent Δ pga29/ Δ pga29 strains and two independent Δ pga29/ Δ pga29/PGA29 strains were used in each experiment. (1) wild type SC5314. (2) Δ pga29/ Δ pga29/PGA29 strain. (3) Δ pga29/ Δ pga29 strain.

3.7.3 β -1,3-glucanase sensitivity assay

The mannoprotein layer can serve as a protective entity against foreign macromolecules and degrading enzymes. As Pga29p is abundantly present in yeast cells, we investigated the possibility whether this protein might serve as a protective shield for the inner glucan layer. Our strains were grown to the mid log-phase and were incubated in 50 mM Tris-HCl with β -1,3-glucanase (Quantazyme), a recombinant enzyme that degrades the polysaccharide backbone of the cell wall. Over time in a hypo-osmolaric solution this leads to cell lysis that can be monitored photospectrometrically. Lysis was determined and compared by measuring the OD₆₀₀ over a time period of 60 minutes (Figure 21). The Δ pga29/ Δ pga29 mutants did not show increased sensitivity to β -1,3-glucanase, suggesting that Pga29p does not play a role as a protective entity in the mannoprotein layer in this respect.

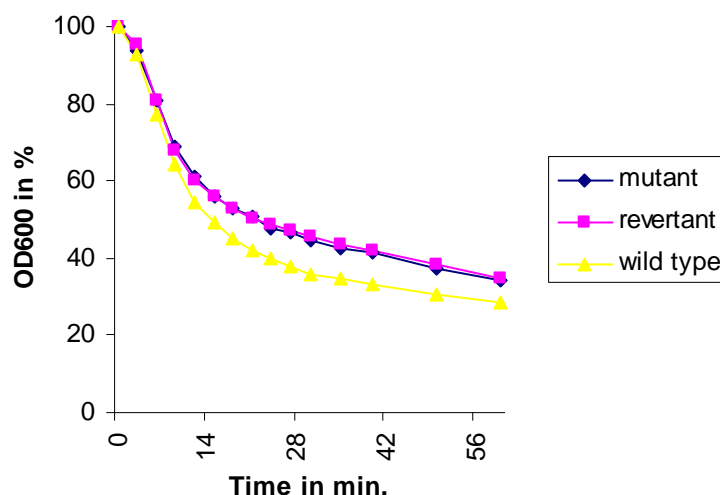


Figure 21. β -1,3-glucanase sensitivity assay

Strains were grown to the mid-log phase till an OD_{600} of 2.0 was reached. For the assay cells were adjusted to an OD_{600} of 0.7, which is indicated as 100% in the graph. After adding Quantazym, the decrease in the OD_{600} was measured over a time period of 60 min. The experiment was repeated two times and the mean values were used to generate the graph.

3.7.4 Sensitivity against antimycotics

We tested whether deletion of Pga29p affected the sensitivity against selected clinically relevant antimycotics using the E-test format: Fluconazole, Intraconazole, Voriconazole, 5-Fluocytosin and Amphotericin B and the cell wall active substance Caspofungin. The mutant strain showed similar sensitivity compared with the revertant strain (Table 17, Appendix).

3.8 Is Pga29p redundant?

3.8.1 *PGA30* and *PGA31* expression in Δ *pga29*/ Δ *pga29* mutants

In order to investigate whether the lack of obvious cell wall phenotypes in the Δ *pga29*/ Δ *pga29* mutants is based on the fact that *PGA29* is a redundant gene, we examined the expression of *PGA30* and *PGA31* (two identified homologs of *PGA29*) in the Δ *pga29*/ Δ *pga29* strain by using Northern analysis. *PGA31* was shown to be highly upregulated in protoplasts that regenerate their cell wall and is therefore a potential candidate for taking over the function of *PGA29*. Increased expression of *PGA30* and *PGA31* was not observed in mutant cells grown in YPD medium at 30°C (Figure 22, 1a) as well as in cells grown under CFW stress (Figure 22, 1b). In case of CFW stress, *PGA31* transcription was upregulated in both, the mutant and in the wild type strain, whereas *PGA30* transcripts could not be detected in both conditions.

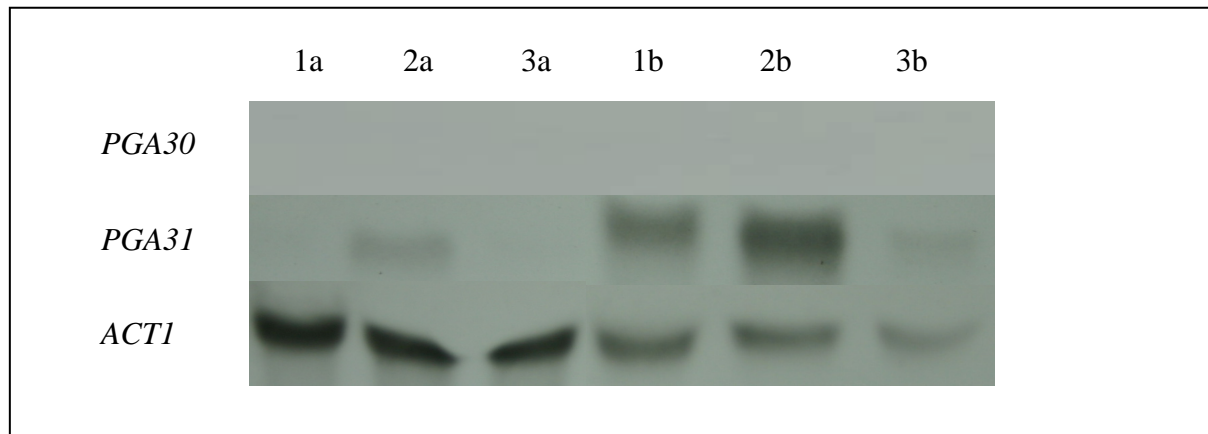


Figure 22. Comparison of *PGA30* and *PGA31* expression between *Δpga29/Δpga29* mutants and in wild type strains

C. albicans cells were grown to the mid-log phase (OD₆₀₀ of 2.0) and harvested or incubated with CFW for 15 min. Northern blot analysis was performed with *PGA30*, *PGA31* and *ACT1* digoxigenin probes. *ACT1* was used as a loading control.

1a) *Δpga29/Δpga29*. 2a) *Δpga29/Δpga29/PGA29*. 3a) Wild type SC5314. 1b) *Δpga29/Δpga29* grown in 25 μg/ml CFW. 2b) *Δpga29/Δpga29/PGA29* grown in 25 μg/ml CFW. 3b) Wild type SC5314 grown in 25 μg/ml CFW

3.8.2 Cell wall proteome of the *Δpga29/Δpga29* mutants

To determine whether *C. albicans* attempts to compensate for *PGA29* deletion by upregulating other CWPs or by incorporating a different set of CWPs into the cell wall, we analysed the full cell wall proteome of the *Δpga29/Δpga29* mutant with the aid of liquid chromatography-tandem mass spectrometry (LC/MS/MS) (Table 14). We extracted cell walls from stationary phase-yeast cells that were cultured in YPD medium at 25°C or 37°C. CWPs were released from the cell wall by using the ‘cell wall shaving method’, in which extracted cell walls are directly digested by trypsin (Yin et al., 2008).

In total 24 CWPs were identified, which included GPI-adhesins (Als1p, Als2p, Als3p and Als4p), proteins that are involved in cell wall integrity or assembly (Cht2p, Crh11p, Ecm33p, Pga4p, Phr1p, Phr2p, Pir1p, Sap9p, Scw1p, Ssr1p and Utr2p), proteins that are involved in iron acquisition (Rbt51p/Pga10p and Rbt5p), superoxide dismutases (Sod4p/Pga2p and Sod5p/Pga3p) and proteins with unknown functions (Csa1p, Hyr1p, Pga29p and Pga36p). The yeast form wall protein 1 (Ywp1p) seems to play a role in dispersal of *C. albicans* in the host (Granger et al., 2005). It should be noted that Csa1p was identified with only one peptide sequence, ‘TYDQLPECAK’, which occurs seven times in this protein, but is also present one time in Rbt5p and Rbt51/Pga10p. In Csa1p, these peptide sequences are followed by a proline

residue, which can prevent cleavage of trypsin, suggesting the identification of Csa1p has not been fully proven.

Interestingly, several CWPs were shown to be specifically upregulated at 25°C or 37°C, as indicated by the number of identified peptides. Als2p peptides were exclusively identified in cell walls from cells that were cultured at 25°C, whereas the proteins Als3p and Hyr1p were only found at 37°C.

Twenty tryptic peptides from Pga29p were identified, confirming that it is abundantly present in the cell wall of *C. albicans*. As expected, the MS results confirmed that the cell walls of the deletion mutant $\Delta pga29/\Delta pga29$ did not contain Pga29p, whereas the control strains did. The family members Pga30p and Pga31p were not identified in any strain, which coincides with our data deduced from Northern blot analysis.

Although the performed LC/MS/MS analysis is a qualitative rather than a quantitative method, the data for the cell walls of the $\Delta pga29/\Delta pga29$ strain that was grown at 25°C indicate that the mutant contains less Als4p and Pga4p. In $\Delta pga29/\Delta pga29$ cell walls, only three tryptic peptides from Als4p were identified, whereas nine and ten peptides from this protein were identified in the revertant and wild type strains, respectively. In addition, only one peptide from Pga4p was found in $\Delta pga29/\Delta pga29$ cell walls, compared to four peptides in the revertant and wild type strains. These data might indicate that Als4p and Pga4p are downregulated upon *PGA29* deletion. However, this has to be confirmed with a quantitative approach. Upregulation of other CWPs in the $\Delta pga29/\Delta pga29$ strain was not observed, suggesting *C. albicans* does not compensate for *PGA29* deletion.

Table 14. LC/MS/MS analysis of the cell wall proteome from $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and SC5314 cell walls. Cell walls were extracted from yeast cells that were grown for 24 hours at 25°C or 37°C. Cells wall samples were treated with trypsin and the supernatants containing the solubilised peptides were analysed with LC/MS/MS. Identified peptides are indicated a '+’.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Als1	68-76	TLNMPCVFK	+					
	77-92	YTTSQTSVDLTADGVK	+		+	+		+
	176-188	STVDPSGYLYASR	+	+	+	+	+	+
	237-254	GLNDWNYPVSSSEFSYTK	+	+		+	+	+
	265-279	YQNVPAgyRPFIDAY			+		+	+
	303-311	SQSKPFTLR		+		+		+
Als2	18-34	KVITGVFNSFDSLWTR	+		+		+	
	35-41	AGNYAYK			+		+	
	42-76	GPNRPTWNAVLGWSLDGTSANPGDTFTLNMPCVFK			+			
	45-76	RPTWNAVLGWSLDGTSANPGDTFTLNMPCVFK			+			
	77-92	FITDQTSVDLTAEGVK	+		+		+	
	93-111	YATCQFYSGEEFTTFSSLK			+			
	112-123	CTVSNTLTSSIK	+					
	153-166	AGTNTVTFNDGDKK	+		+		+	
	166-175	KISIDVDFEK	+		+		+	
	291-305	YTNDYACVGSSLQSK	+		+		+	
	291-310	YTNDYACVGSSLQSKPFNLR	+		+		+	
Als3	50-76	AVLGWSLDGTSASPGDTFTLNMPCVFK						+
	77-92	FTTSQTSVDLTAHGVK		+		+		+
	108-123	STLTCTVSNTLTPSIK				+		
	124-149	ALGTVTLPLAFNVGGTGSSVDLEDSK		+		+		+
	133-149	AFNVGGTGSSVDLEDSK		+				+
	135-149	NVGGTGSSVDLEDSK		+				+
	150-166	CFTAGTNTVTFNDGGKK		+				+
	166-175	KISINVDFER		+		+		+
	167-175	ISINVDFER						

Table 14. Continued.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Als3	182-188	GYLTDSR				+		+
	196-236	VSTLFVAPQCANGYTSGMTMGFANTYGDVQIDCSNIHVGITK						+
	208-236	GYTSGMTMGFANTYGDVQIDCSNIHVGITK		+		+		+
	217-236	ANTYGDVQIDCSNIHVGITK		+				+
	237-254	GLNDWNYPVSSESFSYTK		+		+		+
	255-266	TCSSNGIFITYK		+		+		+
	289-305	TLSYANEYTCAGGYWQR		+		+		+
	291-305	SYANEYTCAGGYWQR						+
	295-305	EYTCAGGYWQR						+
	307-312	APFTLR		+		+		+
	1344-1350	VIPSLNK		+		+		+
Als4	18-38	KVITGIFDSFNSLTWTNAASY				+		
	55-76	SLDGATASAGDTFTLDMPCVFK				+		+
	77-91	FITDQTSIDL VADGR	+	+		+		+
	92-107	TYATCNLNSAEFFTF				+		
	113-123	TVTTTMTADTK						+
	157-175	TVTFNDGDTSISTTVDFEK				+		
	217-254	STAGTGATIDCSTVHVGISNGLNDWNYPISSESFSYTK						+
	237-254	GLNDWNYPISSESFSYTK						+
	255-273	TCTSTSVLVTYQNPAGYR						+
	255-275	TCTSTSVLVTYQNPAGYRPF				+		
	255-279	TCTSTSVLVTYQNPAGYRPFVDAY						+
	255-284	TCTSTSVLVTYQNPAGYRPFVDAYVSATR	+					
	260-284	SVLVTYQNPAGYRPFVDAYVSATR				+		
	266-284	QNPAGYRPFVDAYVSATR				+		+
	268-284	VPAGYRPFVDAYVSATR						+
	274-284	PFVDAYVSATR				+		
	285-291	VSSYAMR	+			+		+

Table 14. Continued.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Cht2	20-39	SASNQVALYWGQNGAGGQER			+			
	22-39	SNQVALYWGQNGAGGQER	+	+	+		+	+
	75-91	TFESGLLHCSQIGADIK	+		+		+	
	99-121	TVLLSLGGGVGDYGFSDVASATK	+				+	
	122-129	FADTLWNK	+					
	185-200	PQCPYPDASLGDLLSK						+
	229-236	FADSAPNK	+					
	240-258	LFVGV PATSNIAGYVDTSK	+	+		+		
	259-267	LSSAIEEIK	+		+		+	+
	268-290	CDSHFAGVSLWDASGAWLNTDEK					+	
	268-299	CDSHFAGVSLWDASGAWLNTDEKGENFVVQVK			+			
	278-299	WDASGAWLNTDEKGENFVVQVK			+			
Crh11	29-45	SSDCSPVPALGSSFLEK	+	+	+	+		+
	45-58	KFDNGLGPHFESLK				+		
	46-58	FDNGLGPHFESLK		+				+
	46-59	FDNGLGPHFESLKK		+				+
	60-75	QGTIDSGSNGLSLTMK		+				
	77-84	RFDNPSFK	+	+	+	+	+	+
	78-84	FDNPSFK			+		+	
	148-158	GGYHDIANPLK	+	+	+	+	+	+
	159-169	DYHTYVIDWTK	+	+		+		
	170-182	DAVTWSVDG SVIR	+	+	+		+	
	236-246	SVLVADYSSGK	+	+				+
	247-261	QYSYSDQSGSWESIK	+	+	+	+		
	271-279	YDQAQDDIK					+	+

Table 14. Continued.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
(Csa1)	53-62 (244-253) (576-585)	IYDQLPECAK	+	+	+			
	55-62 (246-253) (416-423) (578-585)	DQLPECAK			+			+
Ecm33	139-168	TGLTAGITSAESVVISDTGLSSLTGINVFK			+			
	246-262	VELAELTSIGNSLTINK			+			
	246-273	VELAELTSIGNSLTINKNDDLTELDFPK			+		+	
	276-290	TIGGALQISDNSELR	+	+	+	+	+	
	291-297	SFSGFPK	+	+	+		+	+
	321-329	RVSGGFILK		+		+		+
	334-341	LSCSAFNK	+	+	+	+	+	+
Hyr1	31-41	GGIQGFHGDVK		+		+		+
	135-142	STAYLYAR		+				
	143-157	EWTNNGLIVAYQNQK		+				+
	181-189	HQDFVPATK		+		+		+
	192-205	GTGCVTADEDTWIK						+
	206-222	LGNTILSVEPTHNFYLK		+		+		
	278-286	AAALPQYFK		+		+		+
Pga4	24-37	VEGNAFWDESESGDR						+
	42-65	GVDYQPGGSSELEDPLADTNVCER						+
	69-79	YFQELGINTIR		+	+	+	+	+
	99-116	AGIYVILDVNTPHSSITR		+		+		+
	199-214	LPSGLYFNCGDDDMAR	+	+			+	+
	215-241	IDMYGINDYSWCGDASMTTSQYSQQMK						+
	302-315	GDSVTTNDDFDNLK			+		+	+
	321-332	TKNPSGDGGYLK		+	+	+	+	

Table 14. Continued.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Pga4	343-357 365-385	SNIWNVTEEIPDTPK GLAEPTGHGFDAYVQGNCNAK			+			+
Pga10/Rbt51	47-56 50-56 61-92	IYDQLPECAK QLPECAK QSTSNTPCPYWDTGCLCVMPQFGGAIGDCVAK	+	+		+		
					+	+	+	+
Pga24/Ywp1	331-339 329-339	TVVACDEHK VITVACDEHK	+		+			
			+		+	+	+	+
Pga29	16-25 26-39 27-39 29-39 31-39 40-58 64-71 72-78 65-71 72-78 79-103 82-103 104-114 104-119 106-119 120-126 127-137 127-138 138-150 144-150	TISSIQLFAK SDDSKVDGLGLYSK DDSKVDGLGLYSK SKVDGLGLYSK VDGLGLYSK HEGAAIDYLFLGKNGADLK KQIFQELK TSSITVR QIFQELK TSSITVR QSFTLGGDVYELGATDNFIPVTINK TLGGDVYELGATDNFIPVTINK DGTLSTGDDK DGTLSTGDDKVYASK TSLSTGDDKVYASK NVNDPYR YSESEYAVSNK YSESEYAVSNKK KTDDSAPIVAK PITIVAK			+	+		+
					+		+	
					+	+		
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					+		+	

Table 14. Continued.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Pga36	20-34	ADQICNDHCSNAYQK						+
	35-52	QQSCGGTDDVSSQSATLK		+				+
Phr1	21-35	KFESSTPPVEVVGNK		+		+		+
	49-70	GIA YQQDAAGSVSSGYDADPNR		+		+		+
	99-113	VYAIDPKDHEECMK						+
	138-147	PEWNL DLYKR						+
	175-184	SNTDASAFVK		+	+	+		+
	201-215	QIPVGYSSNDDEEIR		+				+
	280-291	LFQEIGTLYSDK	+	+	+	+		
	387-423	SLECVVADDVVKEDYGD LFGQVCGYIDCSAISADGSK						+
	424-437	GEYGVASFCSDKDR		+		+		+
	438-452	LSYVLNQYYLDQDKK		+		+		+
439-452	SYVLNQYYLDQDKK						+	
Phr2	23-34	EDLPAIEIVGNK	+	+		+		
	164-174	KSNTDASAFVK		+	+			+
	165-174	SNTDASAFVK	+		+		+	
	191-205	SIPVGY SANDDSAIR	+	+	+	+	+	+
	193-205	PVGYSANDDSAIR						+
	228-238	MYEWCGDSSYK			+			
	239-250	ASGYESATNDYK		+		+		+
	270-292	KFTEVATLFGDQMPVWSSGIVY					+	
	316-325	DYSYYSSEIK		+		+		+
Pir1	249-266	ACSSANNLEMTLHDSVLK	+	+	+	+	+	+
	251-266	SSANNLEMTLHDSVLK	+	+	+	+		
	253-266	ANNLEMTLHDSVLK			+			
	255-266	NLEMTLHDSVLK	+	+	+			

Table 14. Continued.

Protein Name	Residues	Peptide sequence	$\Delta pga29/\Delta pga29$		$\Delta pga29/\Delta pga29/PGA29$		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Pir1	272-281	WGAIIVANHQF					+	
	272-284	WGAIIVANHQFQFD					+	
	272-294	WGAIIVANHQFQFDGPIQAGTIY					+	
	272-301	WGAIIVANHQFQFDGPIQAGTIYSAGWSIK	+					+
	295-301	SAGWSIK	+		+		+	
	316-331	CLSGDFYNLYDENVAK	+		+		+	
	317-331	LSGDFYNLYDENVAK	+					+
Rbt5	47-56	IYDQLPECAK	+	+		+		
	49-56	DQLPECAK				+		+
	61-92	QSTSSTPCPYWDTGCLCVMPQFAGAVGNCVAK		+	+	+		+
	71-92	WDTGCLCVMPQFAGAVGNCVAK		+	+	+		+
	78-92	VMPQFAGAVGNCVAK		+		+		+
	82-92	FAGAVGNCVAK		+		+	+	+
Sap9	307-319	AGSILFGAIDHAK					+	
Scw1/MP65	128-141	GITYSPYSDNGGCK		+	+	+		+
	142-160	SESQIASEIAQLSGFDVIR						+
	311-322	NVLITETGWPSR						+
	334-344	SNQQAASSIK	+		+			
Sod4/Pga2	105-117	TPAALELGDLSGR					+	+
Sod5/Pga3	118-127	HGNIMGESYK		+		+		+
	118-140	HGNIMGESYKTEYDDSYISLNEK						+
Srr1	23-31	APPACLLAC		+	+	+	+	+
	24-34	PPACLLACVAK	+	+				
	25-34	PACLLACVAK					+	
	41-51	CSGLNDLSCICTTK	+	+	+	+	+	+

Table 14. Continued.

Protein Name	Residues	Peptide sequence	<i>Δpga29/Δpga29</i>		<i>Δpga29/Δpga29/PGA29</i>		SC5314	
			25°C	37°C	25°C	37°C	25°C	37°C
Srr1	64-79	EICPNGDADTAISAFK	+	+	+	+		+
Utr2	34-61	HCPEDKPCCSQFGICGTGAYCLGGCDIR						+
	75-85	MSTFQESFDSK					+	
	245-253	YDYPQTPSR		+	+	+	+	+
	254-268	IQFSLWPGGDSSNAK			+			
	269-286	GTIEWAGGLINWDSEDIK						
	269-287	GTIEWAGGLINWDSEDIKK			+			+
	273-287	WAGGLINWDSEDIKK						+
288-296	YGYYYAHIK	+	+	+	+	+		

3.9 Pga29p is involved in systemic candidosis

Our phenotypic screens do not support the hypothesis that Pga29p plays a major role in maintaining the cell wall integrity of *C. albicans*. However, we experimentally confirmed that Pga29p is one of the most abundant GPI-dependent CWP in the yeast form of *C. albicans* and, as shown above, we found homologs of Pga29p in other pathogenic *Candida spp.* To investigate whether Pga29p might be required for establishing *C. albicans* infections, we examined the $\Delta pga29/\Delta pga29$ strain in a mouse model of systemic candidosis. As systemic candidosis starts with *Candida* entering into the bloodstream, BALB/c mice were intravenously infected with the $\Delta pga29/\Delta pga29$ strain and control strains. Standard death curves were obtained after the infection (Figure 23), which showed that the virulence of the $\Delta pga29/\Delta pga29$ strain is slightly but significantly reduced compared with the control strains, indicating that Pga29p does play a role in the establishment of *C. albicans* infections.

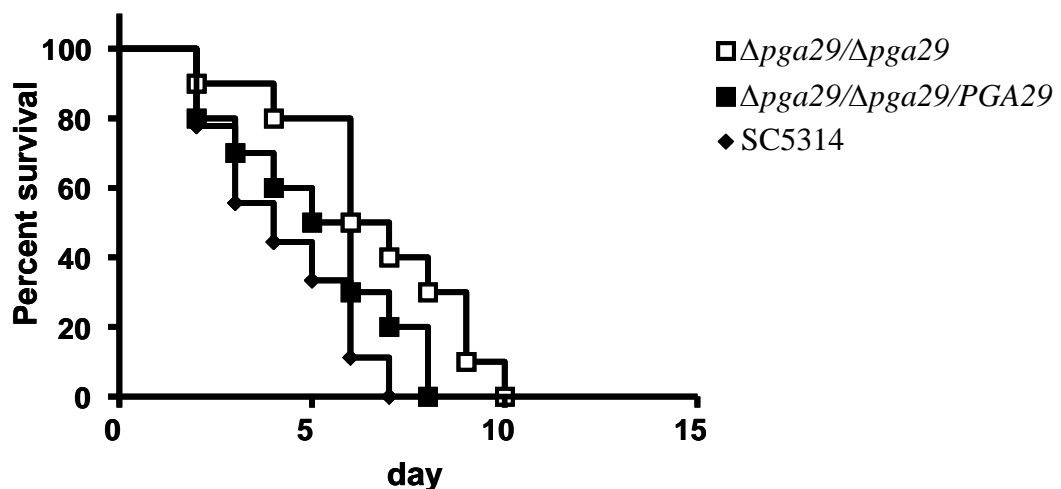


Figure 23. Systemic infection model.

Ten BALB/c mice were intravenously infected via the lateral tale vein with 1.0×10^6 *C. albicans* cells. The median survival times were 6.5, 5.5 and 4.5 days for the $\Delta pga29/\Delta pga29$ strain, $\Delta pga29/\Delta pga29/PGA29$ and SC5314 strains, respectively.

3.10 PGA29 deletion does not affect morphology or adhesion of *C. albicans*

Our next objective was to better elucidate the role of Pga29p in the infection process. The ability of *C. albicans* to transit between different morphological forms and to adhere to host cells is essential for establishing a systemic infection. We investigated whether the reduced virulence of the $\Delta pga29/\Delta pga29$ strain was caused by a deficiency in morphological transition or by a reduced ability to adhere to different plastic surfaces and host cells.

C. albicans is a polymorphic fungus that is able to grow as yeast, pseudohyphae, true hyphae or chlamydo spores. This ability for morphological transition has been related to the virulence of *C. albicans*. To study a possible role of Pga29p in the morphological transition and morphological plasticity of *C. albicans*, we tested the ability of the $\Delta pga29/\Delta pga29$ strain to grow in the above mentioned growth forms in various liquid and agar media. Different inducers of hyphal growth were used to test the yeast-to-hyphae transition: serum (YPD medium with 10% serum), neutral pH (Lee medium), N-acetylglucosamine (SB medium), nutrient deprivation (Spider medium) and low concentrations of farnesol, which is a quorum sensing molecule that is excreted by *C. albicans* and prevents mycelium development (Hornby et al., 2001.). The formation of yeast cells from hyphae was tested by incubating serum-induced hyphae in YPD medium at 30°C for several hours. The ability to form chlamydo spores was tested on rice-Tween agar medium. The $\Delta pga29/\Delta pga29$ strain showed normal development for all growth forms in these assays. An example is shown in figure 24, which depicts our *C. albicans* strains that were grown on Spider agar.

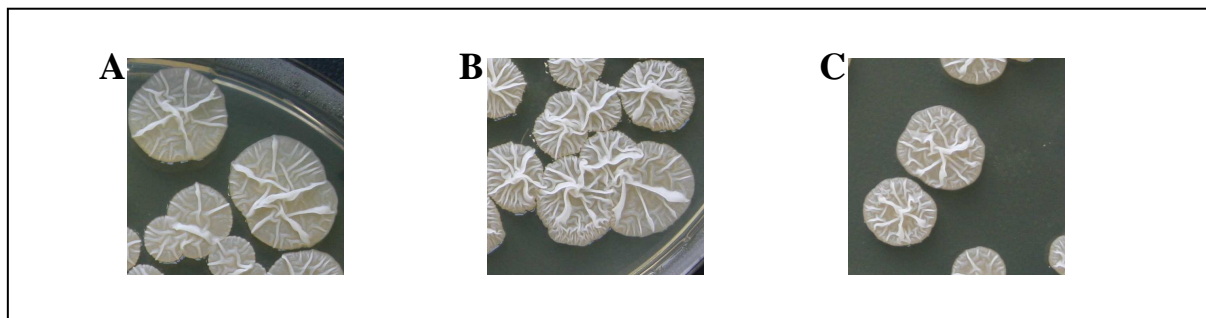


Figure 24. Morphology assay to screen for the ability of the $\Delta pga29/\Delta pga29$ mutants to develop hyphae.

From an overnight culture *C. albicans* cells were plated out on Spider agar, in order to induce nutrient-deprived hyphal development. The cells were incubated at 30°C or 37°C for 5-7 days. (A) $\Delta pga29/\Delta pga29$ strain. (B) $\Delta pga29/\Delta pga29/PGA29$ strain. (C) wild type SC5314.

The ability of the mutants to adhere to several surfaces, which included plastic and epithelial cells, was tested in three different assays. In the first assay, we incubated *C. albicans* yeast cells with Caco-2 cells and quantified number of non-adherent and adherent cells (Dieterich et al, 2002). After 15, 30, 45, 60 and 120 minutes, non-adherent cells were aspirated and adherent cells were scraped off the bottom of the ELISA wells. To determine the adherence ability of the strains, non-adherent and adherent cells were plated out on YPD agar and after 24 hours the colony forming units were counted. No significant differences were observed between the strains (not shown).

C. albicans can adhere to a variety of plastic materials, including polystyrene, which is a model system to study the adhesion of this fungal pathogen to medical devices. It has been shown that the adherence ability of *C. albicans* germ tubes to polystyrene is correlated with the presence of a fibrillar layer which contained adhesins (Tronchin et al., 1988). The adherence ability of $\Delta pga29/\Delta pga29$ stationary-phase yeast cells to plastic was tested with tissue culture-treated and non-treated polystyrene Petri dishes (Granger et al., 2005). The cells were incubated at 30°C or 37°C for 3, 4, 6 and 24 hours and were stained with the dye crystal violet (CV) (Figure 25). In the assays no significant differences were observed between the $\Delta pga29/\Delta pga29$ and control strains.

To study the adhesion of hyphae to polystyrene, stationary phase yeast cells were diluted in modified RPMI medium and were distributed in tissue culture treated 24-well plates. During an incubation period of 3 days, the adhesion of hyphal colonies was checked by gently shaking the plates, thereby distinguishing the number of non-adherent colonies from the adherent ones. No significant differences in this assay were observed (data not shown). Taken together, the data obtained by the adhesion assays suggest that the adherence is not altered by the deletion of *PGA29* in *C. albicans*.

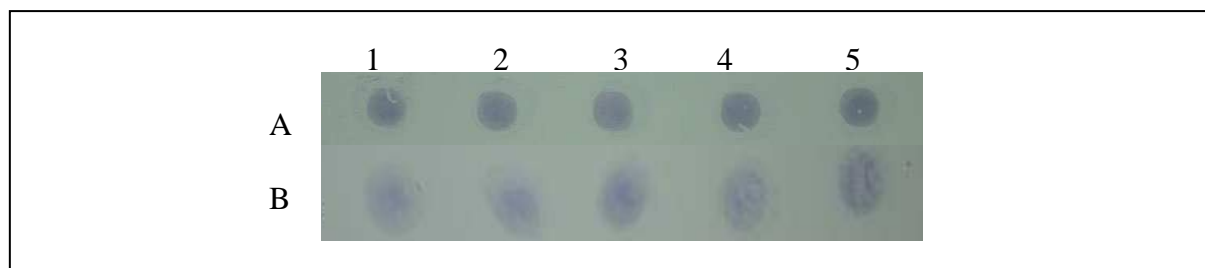


Figure 25. Adhesion assay, CV staining of *C. albicans* on polystyrene. Stationary phase cells were spotted on non-treated (A) or tissue culture treated (B) polystyrene Petri dishes and incubated for different time periods at 30°C or 37°C. Cells were stained with 0.1% CV. 1+2.) $\Delta pga29/\Delta pga29$ 3+4.) $\Delta pga29/\Delta pga29/PGA29$. 5) wild type SC5314

3.11 Reconstituted Human Epithelial as model for oral candidosis.

We used the Reconstituted Human Epithelium (RHE) as an infection model, to study more in detail the interaction of the $\Delta pga29/\Delta pga29$ strain with host cells. The model is developed by culturing transformed human keratinocytes of the cell line TR146 derived from a carcinoma of the oral epithelium (Rupniak et al., 1985). This three-dimensional epithelial tissue simulates very well the oral mucosa *in vivo* and can be supplemented with polymorphonuclear leukocytes (PMNs), which enhance the upregulation of TH1-cytokines and protect the

epithelium by inhibiting *C. albicans* growth (Schaller et al., 2004). After 12 hours of infection, light microscopic pictures showed that the $\Delta pga29/\Delta pga29$ strain caused a reduced tissue damage to the epithelium (Figure 26B) compared with the $\Delta pga29/\Delta pga29/PGA29$ strain (Figure 26C) and the wild type strain SC5314 (Figure 26D). To more accurately quantify and compare the degree of epithelial damage, an assay was performed in which the release of lactate dehydrogenase (LDH) from damaged epithelial cells measured. These analyses revealed a statistical discrepancy in the LDH release between $\Delta pga29/\Delta pga29$ strain and the control strains (Table 15 and Figure 27). The quantitative difference between $\Delta pga29/\Delta pga29$ strain and the control strains, although statistically significant, is moderate, which is in accordance with the data obtained from the mouse model. The LDH activity from RHE supplemented with PMNs was overall lower than RHE without PMNs, which confirms the protective effect of these immune cells against *C. albicans* (Table 15 and Figure 27). Taken together, these data from the RHE-model confirm that Pga29p plays a role in the establishment of *C. albicans* infection.

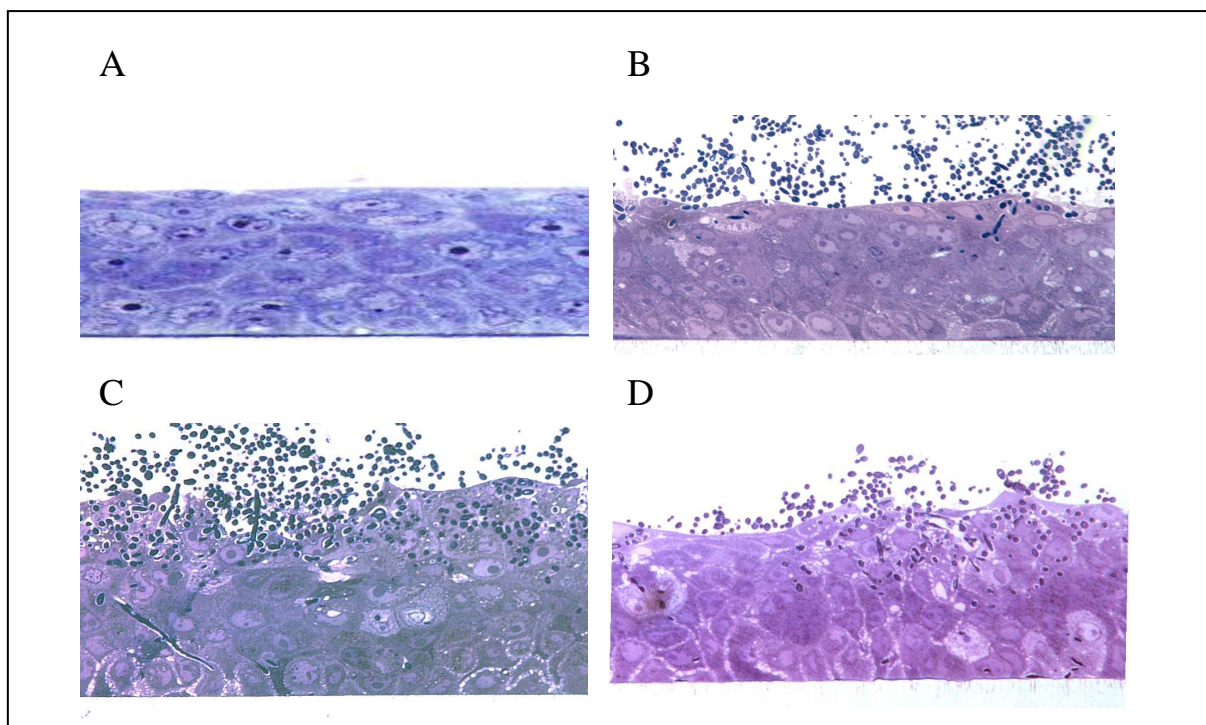


Figure 26. $\Delta pga29/\Delta pga29$ mutants show an attenuated virulence in a RHE model of oral candidosis.

RHE was infected with 2.0×10^6 *C. albicans* cells and was examined by light microscopy after 12 hours. (A) Non-infected (B) $\Delta pga29/\Delta pga29$ strain. (C) $\Delta pga29/\Delta pga29/PGA29$ strain. (D) SC5314 wild type. The experiment was three times performed with two independent $\Delta pga29/\Delta pga29$ strains and two independent $\Delta pga29/\Delta pga29/PGA29$ strains. RHE infected with the mutant showed a reduced damage. In case of the revertant and wild type strain tissue damage was severe, showing clear oedema and vacuolisation of the epithelium.

Table 15. Determination of LDH activity in the RHE model.

The activity of LDH that is liberated from the damaged epithelial cells was measured for RHE infected 24 hours with $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and wild type SC5314. Experiments were three times performed, from which the mean values and sample standard deviations (SD) were determined

	LDH (U/l) without PMNs		LDH (U/l) with PMNs	
	mean	SD	mean	SD
Control (PBS)	18	2.65	19	4.24
$\Delta pga29/\Delta pga29$	134	8.14	97	8.89
$\Delta pga29/\Delta pga29/PGA29$	190	7.02	142	8.96
SC5314	197	9.45	152	4.95

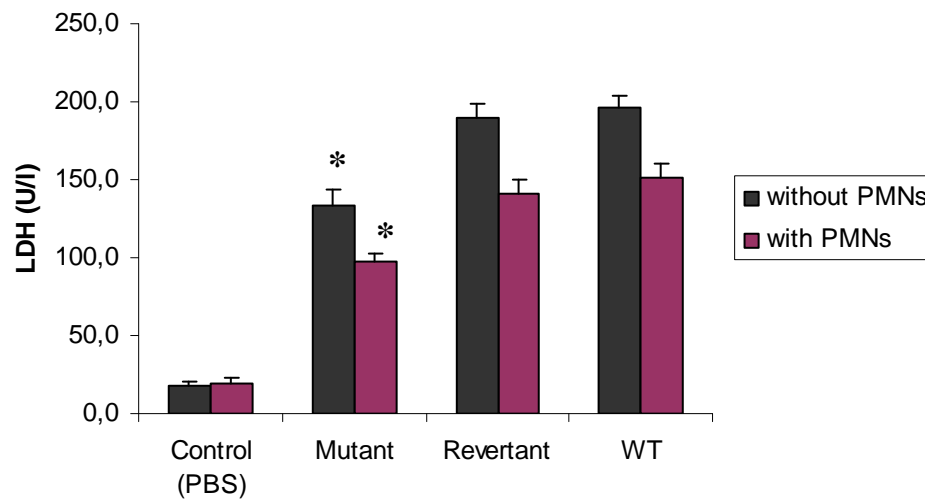


Figure 27. Determination of LDH activity in the RHE model.

The activity of LDH that is liberated from the damaged epithelial cells was measured for RHE infected with $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and wild type SC5314. RHE that was supplemented with PMNs after 12 hours of infection, showed an overall lower LDH activity. The asterisks indicate significant lower LDH activity compared with the control strains (T -test, $P < 0.05$)

3.11.1 *PGA29* expression in *C. albicans* infected RHE

The observed reduced virulence of the *Δpga29/Δpga29* strain is clearly caused by the deletion of the *PGA29* gene. In order to obtain insight whether *PGA29* is directly or indirectly involved in virulence, the expression of *PGA29* in the RHE model was examined by isolating mRNA from 24 hours infected tissue and subsequently generating cDNA with reverse transcriptase PCR. Using this cDNA as a template, a PCR with specific primers for the open reading frame of *PGA29* was performed. Analysis of the PCR by agarose gel electrophoresis did not show any signal (not shown), corroborating the data of Zakikhany et al. (2007) who performed genome wide transcript profiling of *C. albicans* RHE infection, which showed the lack of *PGA29* gene expression. As *C. albicans* yeast cells rapidly develop germ tubes during the incubation with RHE, it is not surprising that expression of the yeast-specific *PGA29* was decreased in this model. This result indicates that Pga29p might be important during a short period of time in the initial stage of infection, prior to the yeast-to-hyphae transition.

3.12 Pga29p is a potential PAMP

Important structural features by which GPI-proteins can be identified are serine and threonine rich domains that are heavily O-glycosylated with mannan (Richard and Plaine, 2007). O-linked mannan is an important PAMP that is recognised by the host via the TLR4 receptor, which in turn induces the release of TH1-cytokines (Netea et al., 2008). The Pga29p amino acid sequence consists of 18% serine and threonine residues, which could serve as O-glycosylation sites. Pga29p does not contain the sequence N-X-S (X is any amino acid except proline), excluding that it is N-glycosylated. The fact that Pga29p (expected size of the mature protein is 18 kDa) migrates with SDS-PAGE at approximately 30 kDa and that cell walls of *Δpga29/Δpga29* mutants show a reduced mannose/glucose ratio (Figure 14), suggests that several serine/threonine residues are indeed mannosylated. In order to investigate whether Pga29p is O-mannosylated and therefore could be a potential PAMP, we analysed β -1,6-glucanase CWP extracts of defined mutants that are deficient in O-glycosylation (Figure 28). The selected mutants have deletions in *PMT1*, *PMT2*, *PMT4*, *PMT5* or *PMT6*, which encode protein mannosyltransferases that are responsible for adding the first mannose to a serine or threonine residue. Immunoblot analysis of the β -1,6-glucanase-released CWPs showed a downward shift of the Pga29p band (lane 2), which confirms O-mannosylation by Pmt1p. No downward shift was observed in case of the other Pmt mutants (lane 3-6), which indicates that Pmt2p, Pmt4p, Pmt5p and Pmt6p do not target Pga29p.

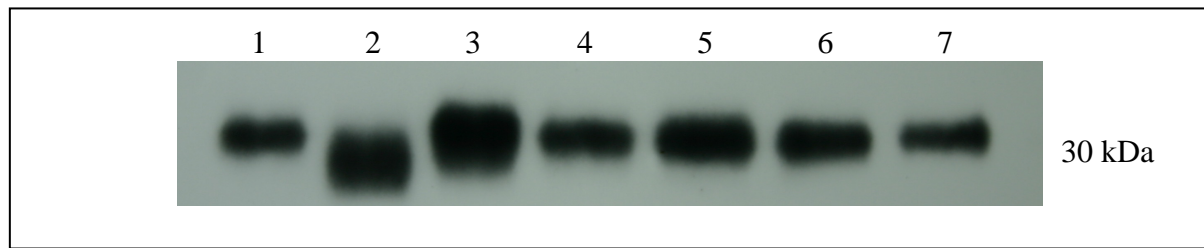


Figure 28. O-glycosylation of Pga29p in *C. albicans* Pmt deletion mutants.

β -1,6-glucanase cell wall protein extracts were separated by SDS-PAGE (11%) and analysed by immuno-blotting using anti-Pga29p polyclonal antibodies. Lane 1 and 7) wild type strain SC5314. Lane 2) $\Delta pmt1/\Delta pmt1$. Lane 3) $\Delta pmt2/PMT2$. Lane 4) $\Delta pmt4/\Delta pmt4$. Lane 5) $\Delta pmt5/\Delta pmt5$. Lane 6) $\Delta pmt6/\Delta pmt6$

3.13 Immune response against the $\Delta pga29/\Delta pga29$ strain

We showed that Pga29p is glycosylated with O-mannan (Figure 29), a sugar that was shown to be recognised by the host immune system with the PRR TLR4. Deletion of *PGA29* probably results in a reduction of O-linked mannan (Figure 18B), that could cause an altered host-pathogen cross-talk, which may result in a diminished immune response. In addition to that, it seems also possible that *PGA29* deletion and consequent lack of the abundant Pga29p in the cells wall leads to the exposure of other PAMPs to the outside environment and therefore could be readily recognised by the immune system. This in turn could lead to an increased immune response. In order to study the role of Pga29p during host cell interactions in more detail, we examined the immune response of the RHE upon infection with the *PGA29*-deletion and the control strains. In addition, the $\Delta pga29/\Delta pga29$ strain was tested in interaction assays with different immune cells.

3.13.1 Immune response of *C. albicans* infected RHE

The immune response of human oral epithelia has been well studied in the past. Using the RHE as a model, it has been shown that *C. albicans* stimulates a strong expression of TH1-cytokines at the mRNA and protein level. The expression of these cytokines is correlated with the degree of virulence (Schaller et al., 2002). Supplementing this model with polymorphonuclear leukocytes (PMNs) revealed that this immune response is associated with the chemoattraction of the PMNs, which protect the epithelial cells and enhance the TH1-type immune response (Schaller et al., 2004).

To more closely examine the interaction of the $\Delta pga29/\Delta pga29$ mutant with the RHE, we studied the immune response of the epithelial cells against our strains by quantifying mRNA

levels for the TH1 cytokines: granulocyte-monocyte colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF α), interleukin-1 alpha (IL-1 α), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6) and interleukin-8 (IL-8). As expected, epithelial cells that were infected with the reconstituted and wild type strains showed high expression levels of *GM-CSF*, *TNF- α* , *IL-8* and moderate levels of *IL-1 α* , *IL-1 β* and *IL-6* (Figure 29A). Challenging the RHE with the Δ *pga29*/ Δ *pga29* mutant strains resulted in a significant reduced expression for these cytokines (Figure 29A). In order to investigate whether the cytokine mRNA levels coincide with their translational level, we determined the levels of GM-CSF, IL-6, IL-8 (Figure 29B) and TNF α (Figure 29C) with ELISA. RHE infected with the mutant strain released significant reduced levels for these cytokines, which coincides with the mRNA levels. In addition, we studied the expression and cytokine levels of infected RHE supplemented with PMNs. In both experiments only minor discrepancies were observed between the Δ *pga29*/ Δ *pga29* and control strains, which suggests a protective effect of the PMNs (Figure 30).

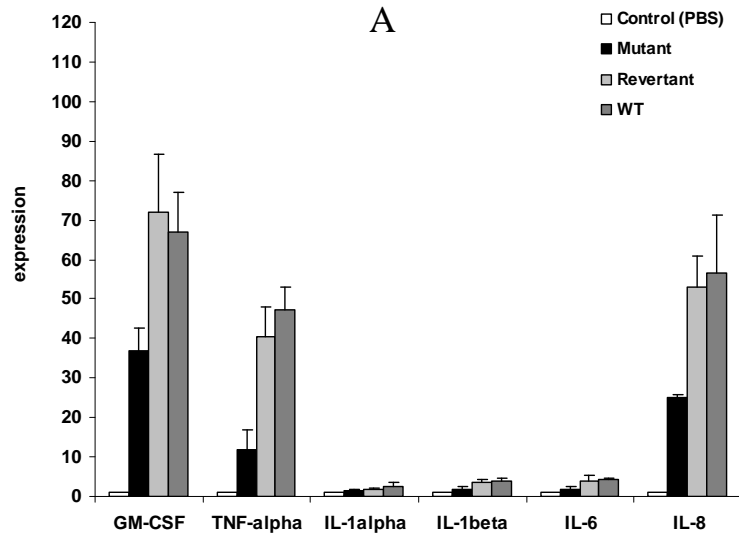
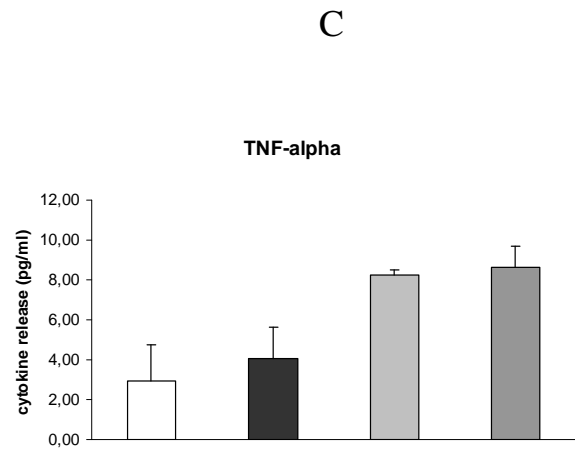
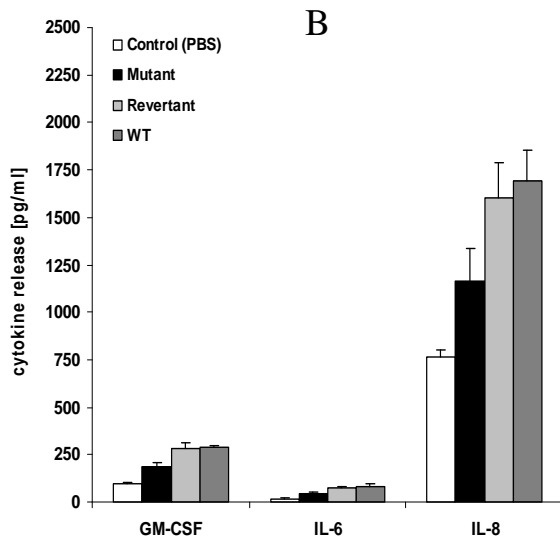


Figure 29. Cytokine expression in RHE. (A) RNA was isolated from RHE that was infected with *C. albicans* for 24 hours. Gene expression was examined for several cytokines that are known to be expressed in keratinocytes upon *Candida* infection. (B) GM-CSF, IL-6, IL-8 and (C) TNF α protein release was quantified from 24 hours infected RHE.



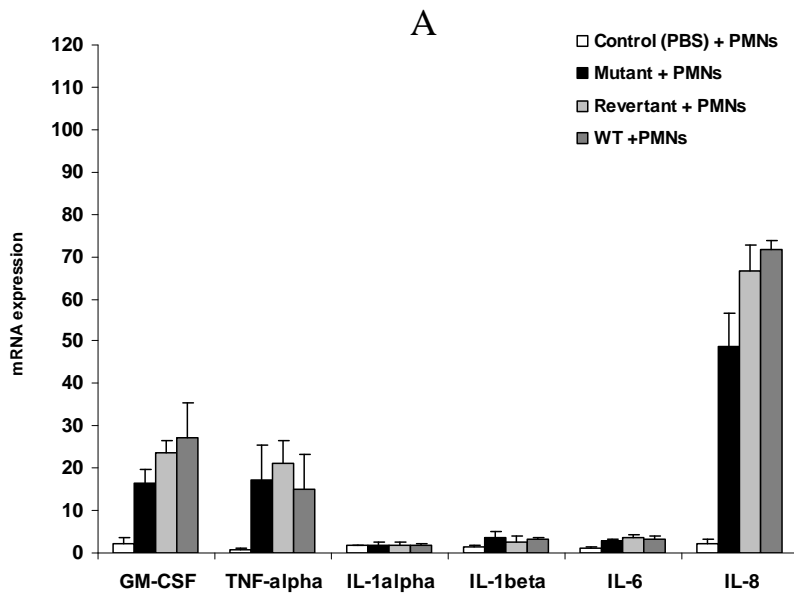
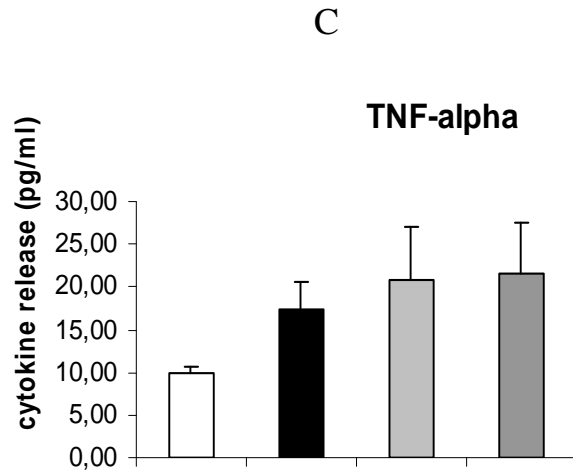
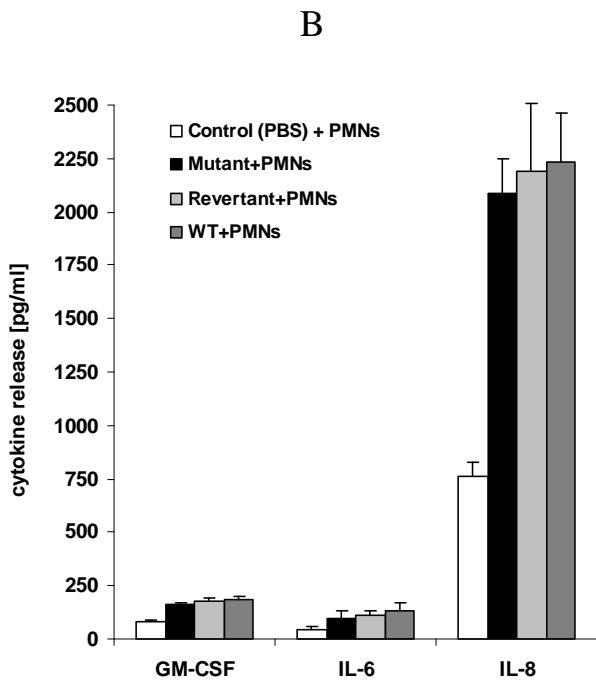


Figure 30. Cytokine expression in RHE supplemented with PMNs.

A) After 24 hours of infection, RNA was isolated from RHE that was supplemented with PMNs. Gene expression was examined for several cytokines that are known to be expressed in keratinocytes upon *Candida* infection. (B) GM-CSF, IL-6, IL-8 and (C) TNF α protein release was quantified from 24 hours infected RHE that was supplemented with PMNs.



3.13.2 Interaction of *C. albicans* with PMNs and primary dendritic cells

We further investigated whether the $\Delta pga29/\Delta pga29$ strain induced a different immune response in interaction assays with two different types of immune cells: murine primary dendritic cells and human PMNs. Dendritic cells (DCs) are antigen presenting cells that are able to promote the initiation of T-cell-dependent immunity and can stimulate the immune response by the secretion of cytokines (Netea et al., 2008) Due to their location at sites where *C. albicans* invasion frequently occurs, such as mucosal surfaces and in the skin, it is believed that dendritic cells play an important role in immunity against *C. albicans*.

Primary DCs were obtained by isolating DC precursors from mice bone marrow that were cultured for 7 days in mDC medium. We determined cytokine expression of murine primary dendritic cells stimulated with $\Delta pga29/\Delta pga29$ mutants. The cytokine expression was examined by quantifying mRNA levels of TH1 cytokines IFN- β , TNF- α , IL-12, IFN- γ and the TH2 cytokine IL-10 of primary dendritic cells that were incubated with our strains for 20 or 110 minutes. No significant differences were observed between the strains (example given for IFN- β , Figure 31).

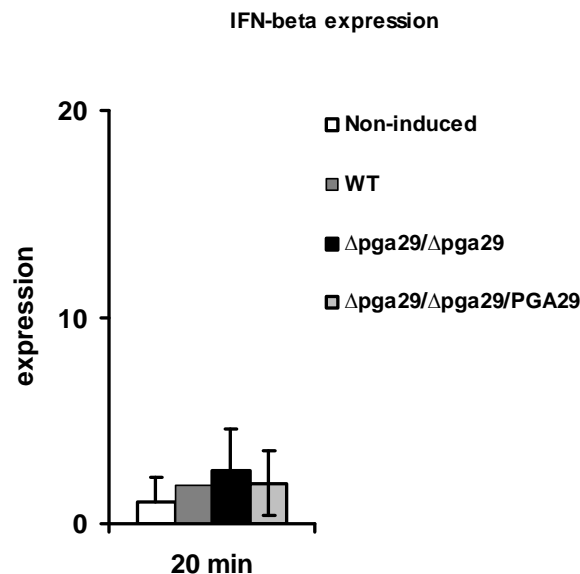


Figure 31. Cytokine expression of primary DCs upon interaction with the $\Delta pga29/\Delta pga29$ strain.

Primary DCs were stimulated for 20 minutes with the $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and wild type strain. The *Candida*- DCs interaction was performed at a 2:1 MOI in a 2 ml volume in 6 cm dishes. RNA was isolated and cytokine expression was examined by RT-PCR.

The oxidative burst is an important mechanism of host immune cells to kill *C. albicans* (Netea et al., 2008). Consequently, we measured the oxidative burst of human PMNs upon interaction with the $\Delta pga29/\Delta pga29$ and $\Delta pga29/\Delta pga29/PGA29$ strains (Figure 32), that were grown at 28°C or 37°C. *C. albicans* grown at 37°C were stimulating the PMNs more than cells grown at 28°C, however no significant difference was observed between the $\Delta pga29/\Delta pga29$ and $\Delta pga29/\Delta pga29/PGA29$ strains. Our data indicate that the reduced virulence may not be related to an altered pathogen host cross-talk via the mannans.

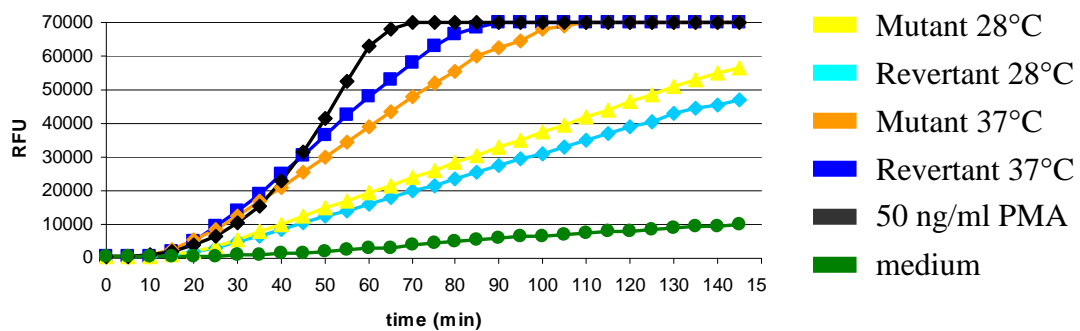


Figure 32. Determination of oxidative burst in interaction assay between PMNs and the $\Delta pga29/\Delta pga29$ mutant strain.

C. albicans strains that were grown at 28°C or 37°C were incubated with human PMNs and the oxidative burst was measured at 37°C over a time period of 145 minutes. As a positive control 50 ng/ml phorbol 12-myristate 13-acetate (PMA) was used. Relative fluorescence units (RFU) were measured every 5 minutes.

4. Discussion

C. albicans is a human pathogenic fungus that normally exists in the host as a commensal organism, but can cause severe infections when the immune system is weakened.

Infections can occur superficially on the skin or mucosa, but can emerge systemically as well, causing damage to organs, which can lead to the decease of the patient.

Since the completion of the *C. albicans* Sequencing Project at the Stanford Genome Technology Center in 1998, molecular analyses of this pathogen have been considerably facilitated. As a consequence of this, the generation of gene deletion mutants and development of DNA-microarray and proteome projects rapidly increased, which resulted in a ampleness of data that already have started to give answers to several questions regarding the function of particular genes.

C. albicans research has been mainly focussing on the identification of virulence factors and their role in host-pathogen interactions. Cell wall proteins (CWPs) are known to be involved in virulence and several of them are necessary for establishing an infection. In this study, we characterised the GPI-CWP Pga29p, an abundant protein that is covalently linked in the cell wall of the yeast form of *C. albicans*, with the aid of different molecular techniques.

4.1 Pga29p incorporation into the cell wall

There are two types of CWPs that are covalently linked to the cell wall of *C. albicans*: ASL-CWPs and GPI-CWPs. ASL-CWPs are directly attached to the β -1,3-glucan framework, presumably via an alkali sensitive ester-linkage (Ecker et al., 2006). GPI-proteins contain at their C-terminal part a hydrophobic domain, which is cleaved off and replaced by a glycolipid, the GPI-anchor. After being processed through the secretory pathway, GPI-proteins finally arrive in the extracellular space while they remain attached to the plasma membrane via their GPI-anchor. In *C. albicans*, GPI-proteins can be further processed to be linked to the cell wall via a β -1,6-glucan- β -1,3-glucan heteropolymer (Kapteyn et al., 1995, 2000).

De Groot et al. (2004) could identify Pga29p in extracts from cell walls treated with HF-pyridine and β -1,3-glucanase, thereby proving that this protein is incorporated into the cell wall. In this study, we attempted to reveal to which polysaccharides in the cell wall Pga29p is covalently linked. First, a recombinant form of Pga29p was produced and isolated from *E. coli*, which was used to generate an anti-Pga29p polyclonal antibody that specifically recognised *C. albicans* Pga29p. To release CWP fractions from SDS-extracted cell walls of

the wild type SC5314, the enzymes β -1,3-glucanase, β -1,6-glucanase and a 30 mM solution of NaOH were used. The supernatants were analysed by immuno-blotting and showed that cell walls that were treated with β -1,6-glucanase released Pga29p, resulting in a single band on the membrane at 30 kDa. In contrast, supernatant from cell wall samples that were treated with β -1,3-glucanase resulted in a high molecular smear. This β -1,3-glucanase extract was additionally treated with β -1,6-glucanase, which resulted in a migration of the signal to a size of 30 kDa. This proved that Pga29p is linked to the β -1,3-glucan layer via β -1,6-glucan. Treatment of cell walls with NaOH did not release Pga29p, which showed that the protein is not linked via a mild alkali sensitive linkage. This result is in agreement with the lack of Pir-like sequences (QIGDGQVQ) in the amino acid sequence of Pga29p, through which presumably the GPI-CWP Cwp1p of *S. cerevisiae* and *C. glabrata* is linked to the β -1,3-glucan (Kapteyn et al., 2001; Weig et al., unpublished data).

4.2 Pga29p is a yeast-specific protein

4.21 *PGA29* expression during the yeast-to-hyphal transition

Under certain conditions, the yeast form of *C. albicans* is able to develop hyphae, a process that is regulated via several pathways and transcription factors (reviewed by Liu, 2001). The morphological transition from the yeast to the hyphal form strongly affects the composition of the cell wall proteome. For example, Sohn et al. (2003) showed with microarrays that the cell wall genes *YWP1* and *HWP2* are specifically expressed in the yeast and hyphal form, respectively.

In this study, we were able to show that Pga29p is a yeast-specific protein in *C. albicans*, by monitoring the downregulation of the gene products in time during yeast-to-hyphal transition. Northern analysis showed that the *PGA29* transcript rapidly decreased over a time period of 45 minutes, indicating that the expression of the gene is particularly related to the yeast form of *C. albicans*. In addition, we confirmed with immuno-blot analysis that this decrease occurs also on translational level, though over a much longer time period. After 2 hours of hyphal induction, Pga29p levels in the cell wall start to significantly decrease and could even be detected after 4 hours, which coincides with study of Ruiz-Herrera et al. (2002) who observed in a pulse-chase experiment that cell wall protein recycling did not occur within a time period of 4.5 hours. However, it should be noted that the Pga29p signal that is detected after 4 hours of hyphal growth might originate from yeast cells that are still in a small number present in the

culture. From these data, we conclude that Pga29p is a yeast-specific CWP and that its turnover is probably very slow upon hyphal induction.

4.2.2 Expression in the *Δefg1/Δefg1* and *Δefg1/Δefg1 Δcph1/Δcph1* mutants

The transcription factors *EFG1* and *CPH1* encode transcription factors that play a major role in hyphal development and virulence in *C. albicans* (Lo et al., 1997). Transcription analysis revealed that *PGA29* expression is increased in *Δefg1/Δefg1* and *Δefg1/Δefg1 Δcph1/Δcph1* mutants (Nantel et al., 2002). We proved that higher amounts of Pga29p are incorporated in the cell wall of these mutants in comparison with the wild type strain SC5314. This suggests that both transcription factors act as repressors of *PGA29* transcription in yeast cells of *C. albicans*. Transcription profiling of putative cell wall genes in a *Δefg1/Δefg1* mutant revealed that Efg1 controls the expression of yeast as well as hyphae specific genes and acts as a strong repressor of cell wall genes, which particularly was shown for *RBE1* (Sohn et al., 2003). It has been reported that Efg1 specifically binds to an E-box *in vitro*, which has the consensus sequence CANNTG (Leng et al., 2001). We determined the number of E-box sequences by searching in the regulatory region for the consensus sequence CANNTG 2 kb upstream after the start codon of *PGA29*. In our search we found 8 E-boxes, which suggests that Efg1 directly regulates *PGA29* via these sites. However, it should be noted that the E-box consensus sequence occurs frequently by chance (Argimon et al., 2007) and the *in vivo* transcriptional regulation of Efg1 via E-boxes has never been proven, which suggest that *PGA29* may be indirectly repressed by Efg1.

Sohn et al. (2003) showed in the *Δefg1/Δefg1 Δcph1/Δcph1* mutant that cell wall gene expression depends more on Efg1 and Cph1 plays only a minor role. However, in our study we show that Pga29p incorporation is two times more elevated in the *Δefg1/Δefg1 Δcph1/Δcph1* than in the *Δefg1/Δefg1* strain, indicating Cph1 strongly influences the expression of this gene. Cph1 is the homologue of Ste12 from *S. cerevisiae*, which binds to the specific sequence TGAAACA (Dolan et al., 1989). These motifs could be putative binding sites for Cph1 in *C. albicans*. In order to determine the presence of potential binding sites for Cph1 in the promoter region *PGA29*, we searched for Ste12 binding motifs. Although we did not find any Ste12 motifs, we cannot exclude that Cph1 is able to directly affect *PGA29* expression by binding to other motifs. A recent study showed that overexpression of *CPH1* under the control of the *ADH1* promoter results in a increased expression of *PGA29* under hyphal growth conditions (YPD with 10% serum at 37°C), which refutes the idea that

Cph1 solely acts as a repressor for *PGA29* expression (Huang et al., 2008). Moreover this result shows that the transcriptional regulation of *PGA29* by Cph1 is rather complex.

4.4 Expression under different conditions

Transcription analysis of cell wall genes may give a strong indication about their function. For example when the cell wall is experimentally weakened in *S. cerevisiae* by incubating the cells with the enzyme β -1,3-glucanase or is genetically altered in deletion mutants, several cell wall genes that have a role in cell wall integrity or modelling were found to be upregulated (Jung and Levin, 1999; De Nobel et al., 2000; Lagorce et al., 2003; Boorsma et al., 2004; García et al., 2004). Another example is that under iron starvation *C. albicans* upregulates a number of genes that encode GPI-proteins, suggesting they may be involved in the uptake of iron (Lan et al., 2004). Cell wall gene expression can also be investigated on the translational level for example by studying protein incorporation using Western analysis. For example, Weissman and Kornitzer (2004) generated antibodies against Rbt5p, a GPI-protein that facilitates the uptake of haemin and haemoglobin, and subsequently showed with Western analysis that this protein is strongly induced by iron starvation.

4.4.1 Expression under cell wall stress

Pga29p is a small CWP and it does not consist of conserved domains that propose enzymatic or adhesive properties. Therefore, it is possible that this protein has a structural purpose by maintaining the cell wall integrity. Consequently, we investigated Pga29p incorporation in the cell wall of cells that underwent cell wall stress. Pga29p incorporation was increased in cells that were grown in YPD medium which was supplemented with CFW, a well-known cell wall perturbant that binds to chitin. Culturing *C. albicans* cells in YPD with the membrane perturbant SDS did not result in elevated levels of the protein in the cell wall. It has been shown for *C. albicans* and other fungi that a reduced cell wall porosity leads to a decrease in SDS sensitivity (Turchini et al., 2000; Coca et al., 2000; Rowbottom et al., 2004). Reducing the cell wall porosity can be established by increasing the concentration of cell wall components, such as mannoproteins (De Nobel et al., 1990). These data suggest that Pga29p upregulation is more related to cell wall stress that directly affects the cell wall organisation, as shown for CFW stress. It is likely that *C. albicans* attempts to compensate the disorganisation of the cell wall by increasing the Pga29p incorporation, which might indicate that this CWP plays a role in maintaining the stability of the cell wall. This suggestion is

supported by the study of Castillo et al. (2006) who showed with micorarrays that *PGA29* is among the seven highest expressed genes in protoplasts that regenerate their cell wall.

4.5 Generation of *PGA29* deletion mutants

In order to generate a *PGA29* deletion mutant and a reconstituted strain, we used PCR-based transformation, a technique that is based on the amplification of the knockout cassette by PCR. Gene deletion was performed in strain BWP17 (Wilson et al., 1999), a widely used derivative of the *C. albicans* wild type isolate SC5314 which has a triple auxotrophy due to the deletion of *ARG4*, *HIS1* and *URA3*. We used the marker genes *ARG4* and *HIS1* for deleting both alleles of *PGA29* and the subsequent deletion mutants were complemented with *URA3* in order to restore the uracil auxotrophy. The revertant strain was generated by directing one copy of *PGA29* together with *URA3* in the *RPS10* locus of $\Delta pga29/\Delta pga29$ mutants. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *C. albicans* deletion mutants (Staab and Sundstrom, 2003). This problem can be solved by targeting *URA3* at the *RPS10* locus, from which it is well expressed (Brand et al., 2004). Phenotype related problems have not been reported for *ARG4* and *HIS1* marker genes.

4.6 Cell wall composition of the $\Delta pga29/\Delta pga29$ mutants

As described above, Pga29p is abundantly present in the yeast cell wall where it can play a role maintaining the cell wall stability. Its absence in $\Delta pga29/\Delta pga29$ mutants could induce compensatory mechanisms that result in the increase or decrease of cell wall components. In order to unravel any possible differences in the cell wall structure, we analysed the $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and wild type SC5314 strains with transmission electron microscopy. Prior to electron microscopic analysis, the yeast cells were fixed with the high pressure freezing technique (cryofixation), which is an essential fixation method to maintain the upper mannoprotein layer of the cell wall intact (Tokunaga et al., 1986). By measuring the thickness of the glucan and mannoprotein layer we observed no significant difference between the $\Delta pga29/\Delta pga29$ and $\Delta pga29/\Delta pga29/PGA29$ strain, suggesting *PGA29* deletion does not lead to gross changes in the cell wall structure in *C. albicans*. A more detailed analysis of the cell wall composition was carried out by measuring the concentrations of cell wall components. It is known that deletion of cell wall genes that are involved in cell wall integrity or assembly can result in altered levels of chitin, glucan and cell wall proteins. For example, deletion of the glycosidases *PHR1* and *PHR2*, which are part of

the cell wall assembly in *C. albicans*, result in a 5-fold increase of chitin (Fonzi, 1999). In contrast, deletion of a family member of *PGA29*, *PGA31*, leads to a 2-fold decrease of the chitin concentration in the in the cell wall (Plaine et al., 2008). An increase in the amount of β -1,3-glucan was detected in deletion mutants lacking *SSR1*, which encodes a GPI-protein that has a structural purpose in the cell wall (Garcerá et al., 2003, 2005).

To determine the levels of chitin, glucan, mannan and protein in the Δ *pga29*/ Δ *pga29* strain, cell walls were treated with the chemicals HCl or H₂SO₄ and NaOH in order to hydrolyse glycosidic and amide bonds, respectively. The concentrations of monosaccharides and amino acids were measured in a colorimetric assay or with HPLC. No changes in the concentration of chitin or protein were detected in the cell wall of Δ *pga29*/ Δ *pga29* mutants, however, the mannose/glucose ratio was significantly reduced in Δ *pga29*/ Δ *pga29* strain, compared with the revertant and wild type strain. This suggests that either the mannan amount is decreased or the glucan amount is increased in the cell wall. It is more plausible that the mannan amount is decreased since Pga29p is O-mannosylated (see **4.9 Pga29p is a potential PAMP**) and Δ *pga29*/ Δ *pga29* mutants did not show an increased sensitivity for the enzyme β -1,3-glucanase. In case of a decrease in mannan, one could assume that the structure of the fibrillar mannoprotein layer should be strongly affected by the reduced mannan level. Indeed, the N-glycosylation mutants Δ *och1*/ Δ *och1* and Δ *pmr1*/ Δ *pmr1* are almost lacking their fibrillar layer, however, a Δ *mnt1*/ Δ *mnt1* Δ *mnt2*/ Δ *mnt2* mutant that lacks the four terminal O-linked mannosyl residues, shows a normal fibrillar layer compared to the wild type strain (Netea et al., 2006). These data indicate that O-mannosylation is not responsible for the appearance of the fibrillar layer.

It should be noted that in our electron microscopic analysis of the cell wall structure, a significant difference was observed in the thickness of the cell wall layers between the wild type SC5314 and the generated mutant strains Δ *pga29*/ Δ *pga29* and Δ *pga29*/ Δ *pga29*/*PGA29*. This discrepancy might be explained by the ectopic expression of the auxotrophic markers which might change the cell wall composition in the mutant and revertant. Although this phenomenon has never been directly shown, several indications are described in previous studies for the *URA3* marker. For example, adherence to host cells, a factor that is dependent on the CWP composition, is affected by changing the location or by the absence of *URA3* (Cheng et al., 2003; Bain et al., 2001). The increased awareness among scientists about the problem of misleading phenotypes (Lay et al, 1998; Bain et al.,2001; Sundstrom et al, 2002; Cheng et al., 2003; reviewed by Staab and Sundstrom, 2003), led to the development of a new

gene knockout methodology (Brand et al., 2004). In this method the *URA3* gene is solely used to select revertant strains, by directing the CIp10, that contains *URA3* and the gene of interest, to the high-expression locus *RP10*. Although BWP17 has genetic differences (such as aneuploidy) compared to its parental strain SC5314 (Forche et al. 2004; Selmecki et al., 2005; Ahmad et al., 2008), this strain together with the CIp10 system is still considered as a valuable model system to knockout genes and study the respective mutants.

4.7 Phenotypic analysis of *PGA29* deletion mutants

Deleting cell wall genes that are implicated in cell wall stability or organisation may lead to changes in cell wall composition (described above), cell morphology and increased sensitivity to cell wall stress. For instance, deletion of one single allele of *PIRI* results in elongated and abnormal formed cells that have a tendency to aggregate. This mutant was highly sensitive to CFW and transformants that lack both alleles could not be obtained, indicating that this cell wall protein is necessary for the viability of *C. albicans* (Martinez et al., 2004). Examples for GPI-proteins are the *CRH* family (*CRH11*, *CRH12* and *UTR2*) (Pardini et al., 2006), *CHT2* (Dünkler et al., 2005) *ECM33* (Martinez-Lopez et al., 2004) and *SRR1* (Garcerá et al., 2003) of which the respective deletion mutants showed changes in cell morphology or increased hypersensitivity to agents that effect cell wall assembly.

The upregulation of Pga29p during growth in YPD supplemented with the cell wall perturbant CFW indicated that the protein might stabilise the cell wall upon cell wall stress. However, the Δ *pga29*/ Δ *pga29* strain did not induce compensatory mechanisms that lead to alterations of chitin or glucan levels in the cell wall, nor did it exhibit any cell wall defective phenotypes or changes in morphology . Furthermore the mutant did not show increased sensitivity to β -1,3-glucanase, which excludes the possibility that Pga29p is needed to limit cell wall porosity in order to protect the β -1,3-glucan layer (Zlotnik et al., 1984; De Nobel et al., 1990). To determine whether Pga29p is redundant, the expression of two family members, *PGA30* and *PGA31*, was analysed under normal growth conditions and CFW stress. *PGA31* seemed to be a potential candidate for taking over the function of *PGA29*, since this homolog is the second highest upregulated gene (18.2 fold increase) in regenerating protoplasts (Castillo et al., 2006) and its deletion results in hypersensitivity to CFW and caspofungin, an antifungal drug that is inhibiting the synthesis of beta-1,3-glucan (Plaine et al., 2008). Northern analysis showed that *PGA31* was expressed under CFW stress and *PGA30* expression was undetectable under both conditions. However, we did not observe an increased *PGA30* or *PGA31* expression in our

mutant strains, indicating these genes do not compensate for the *PGA29* deletion. Moreover, Pga30p and Pga31p were not identified in our LC/MS/MS analysis of the *Δpga29/Δpga29* cell wall proteome (see below), neither upregulation of other CWPs was detected. Conclusively, our data show that Pga29p is not involved in maintaining the cell wall integrity in *C. albicans*.

4.8 Pga29p is involved in the virulence of *C. albicans*

C. albicans is a polymorphic fungus that is able to grow in a yeast as well as in a hyphal form. The formation of hyphae is strongly related to the virulence of the fungus, which has been shown by the reduction of virulence of mutants that are defective in hypha formation (reviewed by Kumamoto and Vines, 2005). Co-regulation of numerous virulence factors during hyphal morphogenesis plays an important role in the establishment of a *C. albicans* infection. For example, Hwp1p, a GPI-adhesin that has a major role in *C. albicans* infection (Tsuchimori et al., 2000), is expressed in germ tubes and hyphae, but not in yeast cells and pseudohyphae (Staab et al., 2003b). Although hyphal development has been shown to be crucial to cause tissue damage that leads to subsequent death (Bendel et al., 2003; Saville et al., 2003), little is known about the role of the yeast form of *C. albicans* in virulence. Indications about a significant contribution of the yeast form have been deduced experimentally by mutants that were unable to develop hyphae, that still could enter tissues via blood vessels (Bendel et al., 2003; Saville et al., 2003) and could cause death in severely immunocompromised mice (Saville et al., 2008). It has been proposed that yeast cells, because of their compact size and shape, may be adapted for free dissemination within the circulation (Gow et al., 2002).

PGA29 deletion mutants exhibited a moderate, but significant reduction in virulence in a mouse model for systemic candidosis, which suggests that Pga29p is involved in the virulence of *C. albicans*. To study more in detail the role of Pga29p in virulence, we selected the RHE model. This model is widely used (Schaller et al., 1998, 1999) to test *C. albicans* deletion mutants for their ability to establish oral candidosis. In this model, the virulence of *C. albicans* strain can be monitored microscopically and quantified accurately by measuring levels of LDH activity from damaged epithelial cells. Furthermore, RNA can be readily isolated from the RHE, thereby enabling to investigate gene transcription from the host or pathogen during infection (Schaller et al., 1998; Green et al., 2004; Schaller et al., 2002; Zhao et al., 2005; Zakikhany et al., 2007). Another advantage of using this model is that defective

growth, such as diminished hyphae formation, can be studied with microscopy during infection.

Light microscopic pictures showed that after 12 hours of infection the *Δpga29/Δpga29* mutants induced significantly less tissue damage than the revertant and wild type SC5314 strains. This reduction of tissue damage was accompanied by a moderate, but significant reduction of LDH activity, confirming that the *Δpga29/Δpga29* strain is reduced in virulence. We propose that Pga29p is the first yeast-specific GPI-CWP that is directly involved in the virulence of *C. albicans*.

PGA29 transcript levels could not be detected in 24 hours infected epithelium, which is in agreement with the data of Zakikhany et al. (2007) who did not observe *PGA29* expression in a genome wide transcript profiling of *C. albicans* RHE infection at different time points. During RHE infection most of the *C. albicans* cells germinate and produce hyphae, which coincides with a rapid downregulation of *PGA29* transcript levels as we showed with Northern analysis. These data suggest that Pga29p is involved in the initial stage of infection, prior to germ tube development. In the literature mutants have been described that are not able to form hyphae while infecting RHE, for example the *EEDI* deletion mutant showed a superficial invasion of epithelial cells, but remained trapped intracellularly due to its inability to germinate (Zakikhany et al., 2007). We exclude such scenario for the *PGA29* deletion; after several hours of infection the majority of the *Δpga29/Δpga29* cells was able to develop normal hyphae, which is in accordance with the results from the yeast-to-hyphae transition assays. These results provide support to the hypothesis that the yeast form plays a significant role in the pathogenesis of *C. albicans*.

Another possible cause for the reduced virulence could be a change in the cell wall protein pool, which for example could alter the ability of the mutant to adhere to the epithelium. With the aid of LC/MS/MS analysis, we identified less peptides of the GPI-CWPs Als4p and Pga4p in *Δpga29/Δpga29* cell walls that were extracted from stationary phase cells cultured at 25°C, which might indicate *PGA29* deletion results in a downregulation of these proteins. Although *ALS4* and *PGA4* are transcribed in *C. albicans* during the RHE infection (Green et al., 2004; Eckert et al., 2007), deletion of these genes does not result in a reduced virulence in this oral model (Zhao et al., 2005; Eckert et al., 2007), which excludes a role for Als4p and Pga4p in the observed reduction in virulence. Moreover, at 37°C, the temperature used during the RHE experiment, no significant differences were found in the CWP composition of our strains, which shows the diminished virulence of the *Δpga29/Δpga29* strain is the result of the lack of

Pga29p in the cell wall. We cannot exclude the possibility that the cell wall proteome of hyphal cell walls, however, since Pga29p is a yeast-specific protein this seems rather unlikely. In order to pinpoint the mechanism that is responsible for the reduced virulence, the ability of $\Delta pga29/\Delta pga29$ strain to adhere to Caco-2 epithelial cells and plastic was determined. In case of the adherence assay with Caco-2 cells, short incubation periods (15, 30 and 45 minutes) were selected as well, in order to study the adhesion of yeast cells prior to their germ tube development. To study the adherence ability of yeast cells and hyphae to plastic, untreated and cell culture-treated polystyrene was used as a surface. These assays showed no differences in adherence between the $\Delta pga29/\Delta pga29$ strain and control strains, indicating that not altered adherence, but another factor is responsible for the reduced virulence.

4.9 Pga29p is a potential PAMP

The Pga29p amino acid sequence consists of 18% serine and threonine residues and migrates with SDS-PAGE at a higher molecular weight than its expected size. Furthermore, deletion of *PGA29* resulted in a reduced mannose/glucose ratio in the cell wall, which gave us a hint that Pga29p is mannosylated.

We investigated further whether the mannosylation of Pga29p could be the essential factor that determines the interaction with host cells and the virulence phenotype. In *C. albicans*, Pmt proteins are responsible for O-glycosylation by adding mannose to serine or threonine residues. The O-mannosylation of 7 *C. albicans* proteins have been studied previously by using *PMT* deletion mutants. These analyses showed that the cell surface proteins Als1, Pir2p, Kre9p and Hwp1p are mannosylated by the enzyme Pmt1 (Timpel et al., 1998; Prill et al., 2005; Staab et al., 2004), the ER membrane protein Sec20p by Pmt1 and Pmt4 (Weber et al., 2004) and the membrane protein Axl2p by Pmt4 (Prill et al., 2005). In *S. cerevisiae*, the yeast that originally was used as model for *C. albicans*, 14 proteins have been shown to be O-mannosylated by different Pmt proteins. The majority of these proteins are targeted by Pmt1p and/or Pmt2p (reviewed by Lengler et al., 2008).

We studied the O-mannosylation of Pga29p by analysing CWP extracts of *PMT1*, *PMT2*, *PMT4*, *PMT5* and *PMT6* deletion mutants using immuno-blot analysis. These studies revealed that Pga29p is indeed O-mannosylated and targeted by Pmt1p. Previous studies reported that *pmt1* and *pmt4* double mutants show a cell separation defect, an aggregation phenotype, a decreased hydrophobicity and an altered cell wall composition (Timpel et al., 1998; Prill et al., 2005; Kapteyn et al., 2000). Moreover, these mutants as well as the $\Delta pmt2/PMT2$ strain

show an increased sensitivity against several antifungal drugs and cell wall-destabilising agents (Prill et al., 2005). These data suggest that Pmt1p, Pmt2p and Pmt4p are responsible for most of the O-glycosylation activity in *C. albicans*, while Pmt5p and Pmt6p play a less prominent role and may specifically target a small group of proteins (Lengler et al., 2008). Pmt1p accounts probably for most O-mannosylation of proteins that reside in the cell wall, which is supported by the fact that most cell wall proteins studied so far in *C. albicans* are mannosylated by Pmt1p (Timpel et al., 1998; Prill et al., 2005; Staab et al., 2004 and this study).

4.10 Immune response against the *Δpga29/Δpga29* strain

O-mannan is a major PAMP of *C. albicans*, which can induce cytokine production in immune cells upon TLR4 recognition (Tada et al., 2002; Netea et al., 2006). TLR4 activation results in the induction of two pathways that initiate transcription via the transcription factors NF-κB or IRF3, which leads to a strong stimulation of pro-inflammatory cytokines (Netea et al., 2008). TLR4 recognition is lost during the yeast to hyphae transition, which results in a TLR2 dependent anti-inflammatory response (Van der Graaf et al., 2005). This implies that TLR4 is involved in the recognition of yeast cells, the growth form in which Pga29p is abundantly present. As shown above, Pga29p is O-mannosylated by Pmt1, which designates this GPI-protein as a potential PAMP.

4.10.1 Immune response of the RHE

In order to protect itself against *Candida* infection, the RHE induces a TH1 type immune response by expressing proinflammatory cytokines such as GM-CSF and IL-8 (Schaller et al., 2002), which play a role in the recruitment of neutrophils *in vivo*. To examine the immune cross-talk between the epithelial cells and the *Δpga29/Δpga29* strain, we determined the transcript and protein levels of TH1 cytokines that are usually expressed in RHE upon *C. albicans* infection (Schaller et al., 2002). Compared with the control strains, we observed a decrease of all studied cytokines in RHE that was infected with the *PGA29* deletion mutant. There are two possible explanations for this reduction: (1) As a consequence of the reduced mannose level in the cell wall, the TLR4 receptors on the epithelia cells are less activated, which in turn leads to a decrease in pro-inflammatory cytokine production. (2) Another possibility is that the degree of virulence is related to the degree of cytokine expression; the *Δpga29/Δpga29* strain causes a diminished epithelial damage, therefore a lower cytokine level

is needed to protect the tissue. In view of the data of Schaller et al. (2002), who showed that a reduced cytokine expression in the RHE seem to correlate with the degree of virulence of different *Candida* species, the last explanation is very plausible. Moreover, it could be that cell wall components are not involved in inducing cytokine expression in epithelial cells, since heat-killed *C. albicans* failed to induce an immune response in RHE (Schaller et al., 2002). However, these *C. albicans* cells were heated at 90°C for 30 minutes, which could cause denaturation of CWPs and lead to an altered cell wall structure that fails to initiate an immune response in the epithelial cells.

The addition of PMNs to *Candida*-infected RHE results in an enhanced TH1 type immune response and a downregulation of the TH2 immune response. This is associated with a protective effect against *Candida*-induced tissue damage (Schaller et al., 2004). The addition of PMNs to RHE that was infected for 12 hours with the $\Delta pga29/\Delta pga29$ strain and control strains resulted in high cytokine levels of IL-8, which is known a chemoattractant and promotes degranulation and other antifungal activities of PMNs (Djeu et al., 1990; Kullberg et al., 1999). However, in this experiment the $\Delta pga29/\Delta pga29$ strain did not show great discrepancies between the cytokine expression or protein levels compared with the controls. This is might be the effect of the direct immunological crosstalk between the epithelium and PMNs (Schaller et al., 2004, Weindl et al., 2007).

4.10.2 Immune response of immune cells

The decreased cytokine expression in the RHE that was infected with the $\Delta pga29/\Delta pga29$ strain, urged us to investigate further the possibility of a link between *PGA29* deletion and a reduction of TLR4 mediated cytokine expression. Moreover, the lack of Pga29p in the cell wall could lead to the exposure of other PAMPs, that are hidden in wild type strains and thereby stimulating the immune response. To investigate more in detail the immune response of the innate immune system against the $\Delta pga29/\Delta pga29$ strain, interaction assays were performed with two types of immune cells, DCs and PMNs. The expression of the cytokines IFN- β , TNF- α , IL-12, IFN- and IL-10 were examined in DCs that were stimulated for 20 or 110 minutes with the $\Delta pga29/\Delta pga29$, $\Delta pga29/\Delta pga29/PGA29$ and SC5314 strain. No differences were observed in expression between the mutant and control strains, indicating Pga29p does not have influence on the immune response of DCs. At the time of writing, it has been reported that human DCs recognise N-linked mannan with the lectin receptors DC-SIGN and MR, which results in binding and internalisation of *C. albicans* (Cambi et al., 2008). In

addition, interaction assays with *C. albicans* glycosylation mutants showed that only N-linked mannan stimulates the production of the pro-inflammatory IL-6 in human DCs (Cambi et al., 2008). Hence, it is likely that the lack of O-linked mannan in our mutant strain does not change the immune response of the DCs.

Upon β -glucan recognition, the PRR Dectin-1 induces the oxidative burst against fungi (Kennedy et al., 2007). To determine whether the glucan layer in cell walls of the $\Delta pga29/\Delta pga29$ strain are more exposed to the outside environment, we incubated $\Delta pga29/\Delta pga29$ mutants with human PMNs and measured the oxidative burst. No differences in this assay were observed, indicating Pga29p does not shield the β -1,3-glucan layer against Dectin-1 recognition. These data are in line with the β -1,3-glucanase sensitivity assay, in which was shown that the $\Delta pga29/\Delta pga29$ strain was equally sensitive to β -1,3-glucanase compared to the control strains. Our interaction assays suggest that the reduced virulence may not be related to an altered pathogen host cross-talk via the mannans. However we cannot fully exclude that Pga29p is recognised by other types of immune cells.

4.11 Conclusion

In this study the abundant GPI-CWP Pga29p of the human fungal pathogen *C. albicans* was characterised. Homologs of this protein are widespread among well-known virulent *Candida* species, but so far their functional characteristics have not been investigated. Pga29p was shown to be covalently linked in the cell wall to the β -1,3-glucan framework via β -1,6-glucan. During the yeast-to-hyphae transition this GPI-CWP is strongly downregulated, which proves that Pga29p is a yeast-specific protein. Although Pga29p was upregulated in cells that were grown in the presence of the cell wall perturbant CFW, it is unlikely that this protein has a major role in maintaining the cell wall integrity of *C. albicans*. This was shown by the unchanged sensitivity of a $\Delta pga29/\Delta pga29$ deletion strain against agents that induce cell wall stress, such as CFW, SDS and β -1,3-glucanase. In addition, the $\Delta pga29/\Delta pga29$ strain did not exhibit an altered thickness of the inner cell wall layer or a changed chitin concentrations, a phenomenon that is frequently observed after deletion of cell wall genes that play a role in cell wall integrity or assembly. Furthermore, Northern and LC/MS/MS analysis of the $\Delta pga29/\Delta pga29$ cell wall proteome showed that *C. albicans* did not compensate for *PGA29* deletion by upregulating the homologs, *PGA30* and *PGA31*, or other CWPs. By using two virulence models (systemic infection model and the RHE oral model), we could clearly show that the $\Delta pga29/\Delta pga29$ strain exhibited a significant diminished virulence. The

environmental conditions in these two virulence models stimulate filamentation of *C. albicans*, a growth form in which Pga29p is downregulated. We have therefore reason to believe that Pga29p is involved in the initial stage of infection, prior to germ tube development from the yeast cells. To our knowledge Pga29p is the first yeast-specific CWP in *C. albicans* that directly plays a role in virulence. Additionally, this study supports the theory that the yeast cell of *C. albicans* is not merely a commensal growth form, but is also involved in pathogenesis. However, the role of Pga29p in virulence is not related to an altered adherence ability or a changed morphological plasticity, indicating another mechanism is responsible. For instance, Pga29p might facilitate the endocytosis of *C. albicans* yeast cells by endothelial or epithelial cells via host cell receptors that recognise particular amino acid sequences of Pga29p.

More research is needed in order to extend our insights into the exact function of Pga29p and to understand the role of the yeast form in *C. albicans* pathogenesis. In view of this, further characterisation of Pga29p homologs that are present in cell walls of other *Candida* species, might be helpful to answer the remaining questions about Pga29p and its role in virulence.

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Appendix

Table 16. Homologs of Pga29p

<u>Species/proteins</u>	<u>Description</u>	<u>Identity in %</u>	<u>E-value</u>
<u>C. dubliniensis</u>			
Cd36_43810	uncharacterized cell wall protein, putative	69	2e-71
Cd36_43780	uncharacterized cell wall protein, putative	52	2e-33
Cd36_43790	uncharacterized cell wall protein, putative	46	2e-30
Cd36_24390	putative protein	36	7e-22
Cd36_72060	<i>Candida</i> conserved, putative	39	2e-21
Cd36_46120	conserved putative protein	40	3e-19
Cd36_72070	<i>Candida</i> conserved, putative	34	4e-19
Cd36_46110	conserved hypothetical protein	38	1e-16
Cd36_62260	conserved hypothetical protein	31	4e-13
Cd36_05950	putative	28	3e-05
<u>C. guilliermondii</u>			
PGUG_01942	predicted protein	33	6e-09
<u>C. tropicalis</u>			
CTRG_00299.3	conserved hypothetical protein	46	1e-31
CTRG_00352.3	conserved hypothetical protein	44	8e-27
CTRG_00350.3	conserved hypothetical protein	43	1e-21
CTRG_00348.3	conserved hypothetical protein	35	1e-14
CTRG_03792.3	conserved hypothetical protein	36	1e-13
CTRG_00349.3	conserved hypothetical protein	33	3e-13
CTRG_02903.3	conserved hypothetical protein	34	8e-09
CTRG_03785.3	conserved hypothetical protein	33	1e-13
CTRG_03793.3	conserved hypothetical protein	33	4e-08
CTRG_04251.3	conserved hypothetical protein	31	2e-06
CTRG_02904.3	conserved hypothetical protein	25	1e-06
CTRG_04264.3	conserved hypothetical protein	30	2e-05
CTRG_04263.3	conserved hypothetical protein	24	2e-05
CTRG_04252.3	conserved hypothetical protein	26	7e-05
<u>C. lusitaniae</u>			
CLUG_02844.1	conserved hypothetical protein	34	1e-09
<u>C. parapsilosis</u>			
CPAG_04045.0	conserved hypothetical protein	45	4e-27
CPAG_04046.0	conserved hypothetical protein	44	3e-28
CPAG_00197.0	conserved hypothetical protein	34	2e-16
CPAG_00195.0	conserved hypothetical protein	34	3e-15
CPAG_00196.0	conserved hypothetical protein	30	7e-13
CPAG_00194.0	conserved hypothetical protein	30	5e-09
<u>D. hansenii</u>			
DEHA0D08261g	hypothetical protein	35	9e-15
<u>L. elongisporus</u>			
LELG_04425	conserved hypothetical protein	43	2e-30
LELG_04426	conserved hypothetical protein	42	4e-28
LELG_02548	conserved hypothetical protein	36	9e-23
LELG_05729	conserved hypothetical protein	37	6e-20
LELG_04428	predicted protein	33	2e-07
LELG_04429	predicted protein	33	4e-06

Table 16. Continued.

Species/proteins	Description	Identity in %	E-value
<i>P. stipitis</i>			
PICST_43383	hypothetical protein	43	5e-20
PICST_57358	hypothetical protein	37	4e-15

Table 17. Sensitivity against antimycotics.

Sensitivity against antimycotics was tested by using the E-test format. The *Δpga29/Δpga29* *Δpga29/Δpga29/PGA29* cells were diluted to 0.5 McFarland units and were streaked out on RPMI agar medium supplemented with 0.05% methylene blue. E-test strips were laid on top of the agar medium and the plates were incubated for 24 hrs at 35°C. The minimal inhibitory concentrations were determined visually (μg/ml).

	<i>Δpga29/Δpga29</i>	<i>Δpga29/Δpga29/PGA29</i>
Fluconazole	0.5	0.5
Intraconazole	0.0275	0.023
Voriconazole	0.012	0.008
5-Fluocytosin	0.0275	0.032
Amphotericin B	0.047	0.0395
Caspofungin	0.125	0.125

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