The GPI-modified proteins Pga59 and Pga62 of *Candida albicans* are required for cell wall integrity

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The fungal cell wall is essential in maintaining cellular integrity and plays key roles in the interplay between fungal pathogens and their hosts. The *PGA59* and *PGA62* genes encode two short and related glycosylphosphatidylinositol-anchored cell wall proteins and their expression has been previously shown to be strongly upregulated when the human pathogen *Candida albicans* grows as biofilms. Using GFP fusion proteins, we have shown that Pga59 and Pga62 are cell-wall-located, *N*- and *O*-glycosylated proteins. The characterization of *C. albicans* *pga59Δ/pga59Δ*, *pga62Δ/pga62Δ* and *pga59Δ/pga59Δ pga62Δ/pga62Δ* mutants suggested a minor role of these two proteins in hyphal morphogenesis and that they are not critical to biofilm formation. Importantly, the sensitivity to different cell-wall-perturbing agents was altered in these mutants. In particular, simultaneous inactivation of *PGA59* and *PGA62* resulted in high sensitivity to Calcofluor white, Congo red and nikkomycin Z and in resistance to caspofungin. Furthermore, cell wall composition and observation by transmission electron microscopy indicated an altered cell wall structure in the mutant strains. Collectively, these data suggest that the cell wall proteins Pga59 and Pga62 contribute to cell wall stability and structure.

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INTRODUCTION

The cell wall is an essential component of fungal cells, preserving cellular integrity and playing a central role in the interaction of fungi with their environment. This is particularly the case for pathogenic fungi such as the opportunistic yeast pathogen *Candida albicans*, where the cell wall has been shown to play central roles in adhesion, virulence, biofilm formation, infection and immunomodulation (Albrecht *et al.*, 2006; Douglas, 2003; Netea *et al.*, 2006; Richard *et al.*, 2002b; Sundstrom, 2002). Because of the essential role of the cell wall in cellular integrity and fungal specificity of some enzymes involved in its biogenesis, it is a recognized target for the development

of novel antifungals (e.g. echinocandins that target the β -1,3-glucan synthase) (Latge, 2007).

The organization of the fungal cell wall has been mainly characterized in the yeasts *Saccharomyces cerevisiae* and *C. albicans* and in the filamentous fungus *Aspergillus fumigatus* (Klis *et al.*, 2006; Latge, 2007; Lesage & Bussey, 2006; Ruiz-Herrera *et al.*, 2006). The yeast cell wall has a bilayered structure. The inner part is composed of a network of β -1,3-glucan molecules linked by hydrogen bonds. These chains can be bound covalently to β -1,6-glucan molecules and to chitin chains. The outer part of the cell wall is composed mainly of mannoproteins (Klis *et al.*, 2006, 2001; Lesage & Bussey, 2006). Most proteins in the cell wall of ascomycetous yeasts are glycosylphosphatidylinositol-anchored proteins (GPI-modified proteins) that become covalently linked to β -1,6-glucan through a remnant of their GPI anchor. As the β -1,6-glucan moiety can be linked to β -1,3-glucan or chitin, the cell wall GPI-modified proteins are strongly linked to the cell wall (De Groot *et al.*, 2005; Klis *et al.*, 2001; Richard & Plaine, 2007).

GPI-modified proteins share a similar structure: an N-terminal sequence for translocation into the endoplasmic

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Abbreviations: CFW, Calcofluor white; CR, Congo red; GPI, glycosylphosphatidylinositol; MM, molecular mass.

reticulum, a mature domain, and a C-terminal hydrophobic region for anchorage to the endoplasmic reticulum membrane. Nine or ten amino acids before this C-terminal sequence the so-called ω site is localized, where the protein is cleaved prior to its ligation to a pre-assembled GPI anchor (Thomas *et al.*, 1990). The core GPI anchor is formed of a lipid group (the membrane anchor), a myoinositol residue, a glucosamine residue, three mannose residues and a phosphoethanolamine group that connects the GPI anchor to the protein via an amide linkage (Tiede *et al.*, 1999). Cell wall GPI-modified proteins are transiently localized in the plasma membrane, the mature domain facing the external milieu. Subsequently, they are removed from the membrane by a cleavage step, which takes place between the glucosamine residue and the first mannose residue of the GPI anchor, and covalently bound to β -1,6-glucan (Kapteyn *et al.*, 1996; Kollar *et al.*, 1997; van der Vaart *et al.*, 1996). Plasma membrane or cell wall localization of GPI-modified proteins can be predicted in *S. cerevisiae*, where dibasic residues (arginine and lysine for instance) upstream of the ω site determine plasma membrane localization of the GPI-modified protein (Caro *et al.*, 1997; Frieman & Cormack, 2003). Cell wall localization of GPI-modified proteins is determined by the absence of this dibasic motif and by the presence of hydrophobic residues in positions ω -2, ω -4 or ω -5 (Caro *et al.*, 1997; Frieman & Cormack, 2003; Hamada *et al.*, 1999). Furthermore, it seems that the sequence of the protein attached to the GPI anchor might be involved in defining the GPI-modified protein localization. In particular, long regions rich in serine and threonine residues in the C-terminal part of mature GPI-modified proteins seem to promote cell wall localization (Frieman & Cormack, 2004). In the case of *C. albicans*, localization of GPI-modified proteins appears to be dependent on similar as well as additional determinants (Richard & Plaine, 2007). For instance, Mao *et al.* (2008) have shown that the occurrence of an aromatic residue and an acidic residue in positions ω -2 and ω -1, respectively, promotes cell wall localization of *C. albicans* GPI-modified proteins. There are 115 putative GPI-modified proteins in *C. albicans* and most of them have unknown functions. However, several have been implicated in processes such as cell wall biogenesis and remodelling, oxidant adaptation and adherence, or have specific enzymic functions (reviewed by Richard & Plaine, 2007).

In a previous study we have shown that the *C. albicans* *PGA59* and *PGA62* genes, encoding two related cell wall GPI-modified proteins, are expressed to much higher levels in biofilms than in planktonic cultures (Garcia-Sanchez *et al.*, 2004). The ability of *C. albicans* to form biofilms – three-dimensional microbial communities attached to a surface and embedded in an extracellular matrix – plays important roles in the virulence of this fungus and in the management of *C. albicans* infections, since biofilm cells show decreased susceptibility to most antifungals. *C. albicans* biofilms are composed of yeast and hyphal cells,

and the ability to make the transition from the yeast form to the hyphal form is crucial for efficient biofilm formation (Ramage *et al.*, 2002; Richard *et al.*, 2005) in addition to being a critical determinant of *C. albicans* virulence (Saville *et al.*, 2003). Here, we have used *C. albicans* deletion mutants in order to evaluate the role of *PGA59* and *PGA62* in biofilm formation. Our results show that Pga59 and Pga62 are cell wall GPI-modified proteins involved in the maintenance of cell wall integrity and to some extent in hyphal morphogenesis but that these proteins are dispensable for biofilm formation.

METHODS

Strains, media, and growth conditions. All *C. albicans* strains used in this study are listed in Table 1. Strains were routinely grown at 30 °C on YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose), or SD minimal medium (0.67 % yeast nitrogen base without amino acids, 0.4 % or 2 % glucose) supplemented with uridine (40 μ g ml⁻¹), arginine (20 μ g ml⁻¹) and histidine (20 μ g ml⁻¹). Lee's (Lee *et al.*, 1975) or RPMI 1640 (Gibco) medium was used to induce hyphal formation in liquid culture. RPMI 1640 medium was buffered with 50 mM HEPES/NaOH, pH 7.3. A 40 h saturated culture in YPD or a 48 h saturated culture in SD 2 % glucose was diluted to an OD₆₀₀ of 0.2–0.5 in the corresponding medium and incubated at 37 °C for 4–5 h with shaking. When hyphal formation in embedded conditions was studied, 50–100 c.f.u. were mixed with 10 ml YPS medium (1 % yeast extract, 2 % peptone, 2 % sucrose, 1 % agar) at 55 °C, plated on a layer of YPS and subsequently overlaid with a second layer of YPS. Plates were incubated at 25 °C for 7–10 days. *C. albicans* was routinely transformed by the lithium acetate-PEG protocol (Walther & Wendland, 2003). When nourseothricine was used as resistance marker, after overnight incubation at 30 °C in PEG, transformants were plated in YPD and replica plated in YPD + 50/100/150 μ g nourseothricine μ l⁻¹ (Werner Bioagent) after 24 h at 30 °C. Transformants were obtained after 48 h incubation at 30 °C.

Growth assays. Growth curves were performed in 96-well plates in a Tecan Infinite M200 microplate reader. Relative fitness (*W*) of a strain was calculated as the ratio of the doubling time of the wild-type strain (*D*_{wt}) to the doubling time of the strain of interest (*D*_x) (*W* = *D*_{wt}/*D*_x), which is equivalent to the ratio of the relative growth rate of the strain of interest and of the wild-type strain (St Onge *et al.*, 2007). Means and standard deviations are presented for fitness values determined in at least two biological replicates, each including at least four technical replicates, with standard deviations not exceeding 5 % of the doubling times obtained for a given strain.

Biofilm formation assays. Biofilms were produced in microfermenters and quantified as described previously (Garcia-Sanchez *et al.*, 2004; Goyard *et al.*, 2008). Alternatively, biofilms were produced in microtitre plates as follows. Saturated cultures in SD 2 % glucose were diluted to OD₆₀₀ 0.05 in 100 μ l RPMI 1640 medium in 96-well microtitre plates and incubated at 37 °C for 24 h. The microtitre plates were then washed with PBS using a HydroFlex platform (Tecan) and 100 μ l of a 1 $\times$ FDA solution (50 $\times$ stock: fluorescein diacetate, 2 g l⁻¹ in acetone; diluted to 1 $\times$ in PBS) was added per well (Honraet *et al.*, 2005). Plates were wrapped in aluminium foil and incubated for 1 h at 37 °C before measuring fluorescence in a Tecan Infinite M200 microplate reader using an excitation filter of 486 $\pm$ 9 nm and an emission filter of 535 $\pm$ 20 nm.

Plasmid and strain constructions. All oligonucleotides used in this study are listed in Table 2.

Table 1. Strains used in this study

Strain	Parent	Genotype	Reference
SC5314		Wild-type strain	Gillum <i>et al.</i> (1984)
CAF2		<i>URA3/ura3::λimm434</i>	Fonzi & Irwin (1993)
HLC54		<i>ura3::λimm434/ura3::λimm434 cph1Δ::hisG/cph1Δ::hisG efg1Δ::hisG/efg1Δ::hisG</i>	Lo <i>et al.</i> (1997)
BWP17		<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	Wilson <i>et al.</i> (1999)
BWP17U	BWP17	<i>ura3::λimm434/URA3 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	Goyard
BWP17AHU	BWP17U	<i>ura3::λimm434/URA3 arg4::hisG/ARG4 his1::hisG/HIS1</i>	Firon <i>et al.</i> (2007)
CEC56	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG PGA59/(PGA59-GFP)-ARG4-PGA59</i>	This work
CEC65	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG PGA62/(PGA62-GFP)-ARG4-PGA62</i>	This work
CEC373	BWP17U	<i>ura3::λimm434/URA3 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga59Δ::HIS1/pga59Δ::ARG4</i>	This work
CEC374	BWP17U	<i>ura3::λimm434/URA3 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga62Δ::HIS1/pga62Δ::ARG4</i>	This work
CEC377	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga59-pga62Δ::HIS1/pga59-pga62Δ::ARG4 RPS1/RPS1::Clp10</i>	This work
CEC832	CEC373	<i>ura3::λimm434/URA3 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga59Δ::HIS1/pga59Δ::ARG4, RPS1/RPS1::ClpSAT::PGA59</i>	This work
CEC837	CEC374	<i>ura3::λimm434/URA3 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga62Δ::HIS1/pga62Δ::ARG4, RPS1/RPS1::ClpSAT::PGA62</i>	This work
CEC922	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/HIS1 PGA59/pga59Δ::ARG4, RPS1/RPS1::Clp10</i>	This work
CEC992	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga59-pga62Δ::HIS1/pga59-pga62Δ::ARG4 RPS1/RPS1::Clp10-PGA59</i>	This work
CEC995	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga59-pga62Δ::HIS1/pga59-pga62Δ::ARG4 RPS1/RPS1::Clp10-PGA62</i>	This work
CEC997	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pga59-pga62Δ::HIS1/pga59-pga62Δ::ARG4 RPS1/RPS1::Clp10-orf19.2766</i>	This work

To study the localization of Pga59 and Pga62, plasmids carrying *PGA59-GFP* and *PGA62-GFP* fusions were constructed. Regions including the *PGA59* or *PGA62* ORFs flanked by 1 kb of the corresponding upstream genomic region and 500 bp or 700 bp of the corresponding downstream genomic region were amplified using oligonucleotides *PGA59.01* and *PGA59.02* or *PGA62.05* and *PGA62.06*, respectively. Amplification products were cloned in pGEM-T, yielding pGEM-T::*PGA59* and pGEM-T::*PGA62*, respectively. The green fluorescent protein (GFP) coding sequence (without stop codon) was amplified from plasmid pMG1602 (Gerami-Nejad *et al.*, 2001) using primers YGFP.01 and YGFP.02. The resulting PCR product was subcloned at *Bam*HI and *Bsr*GI sites into pGEM-T::*PGA59* or pGEM-T::*PGA62* that had been amplified using primers *PGA59Y.01* and *PGA59Y.02* or *PGA62Y.01* and *PGA62Y.02*, respectively. This resulted in plasmids pGEM-T::(*PGA59-GFP*) and pGEM-T::(*PGA62-GFP*), where the GFP coding sequence is inserted in-frame between the codons for the second and third amino acids of the mature Pga59 or Pga62 proteins, as predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The *C. albicans* *ARG4* gene, amplified from plasmid pDDB166 (Enloe *et al.*, 2000) using oligonucleotides *ARG4.01* and *ARG4.02*, was cloned between *Not*I and *Sac*I sites in these plasmids, yielding pGEM-T::(*PGA59-GFP, ARG4*) and pGEM-T::(*PGA62-GFP, ARG4*), respectively. All plasmids were sequenced to verify appropriate amplification of the ORFs. Strains CEC56 and CEC65 were obtained via transformation of strain BWP17 (Table 1) with *Sna*BI- or *Bst*BI-linearized pGEM-T::(*PGA59-GFP, ARG4*) or pGEM-T::(*PGA62-GFP, ARG4*), respectively. Integration of the *PGA59-GFP* or *PGA62-GFP* fusion at the *PGA59* or *PGA62* loci, respectively, was confirmed by PCR

analysis and Southern hybridization. Cells were observed with an ApoTome microscope (Carl Zeiss) or an Eclipse E600 microscope equipped with a Nikon DXM1200F digital camera.

Knockout mutants were constructed in BWP17 or BWP17U by sequential replacement of the complete ORF of both alleles of each gene by PCR-generated *HIS1* or *ARG4* disruption cassettes flanked by 100 bp of target homology region through sequential transformation, as described elsewhere (Gola *et al.*, 2003). Oligonucleotides 5-*PGA59* and 3-*PGA59* were used for the construction of strain CEC373 (*pga59Δ/pga59Δ*) in BWP17U (Table 1). Oligonucleotides 5-*PGA62* and 3-*PGA62* were used for the construction of strain CEC374 (*pga62Δ/pga62Δ*) in BWP17U. Oligonucleotides 3-*PGA5962* and 3-*PGA59* were used for the construction of strain CEC377 (*pga59Δ-pga62Δ/pga59Δ-pga62Δ*) in BWP17. Gene replacements were verified by PCR. Southern analyses were also performed using either a *PGA59* or *PGA62* fragment or an *ARG4* or *HIS1* fragment as probes on *Eco*RI-digested genomic DNA. Prior to phenotypic analysis, strain CEC377 was transformed to uracil prototrophy using the *Stu*I-linearized *Clp10* plasmid, which carries the *URA3* gene and integrates at the *RPS1* locus (Murad *et al.*, 2000).

For reintroduction of the wild-type *PGA59* or *PGA62* alleles in *C. albicans* *pga59Δ* or *pga62Δ* mutants, integrative plasmids that carry the corresponding genomic regions were constructed. Regions including the *PGA59* or *PGA62* ORFs flanked by 1 kb of the corresponding upstream and downstream genomic region were amplified using oligonucleotides *RegPga59-start* and *RegPga59-end* or *RegPga62-start* and *RegPga62-end*, respectively. Amplification products were then cloned in the *Kpn*I and *Sal*I sites of plasmid

100 µl 4% (w/v) acetylacetone in 0.75 M Na₂CO₃, and the mixture was incubated at 100 °C for 20 min. Subsequently, 0.7 ml 96% ethanol and 100 µl 2.7% (w/v) *p*-dimethylaminobenzaldehyde in 50% (v/v) HCl/ethanol were added, and after 1 h incubation at room temperature, glucosamine content was measured as absorbance at 520 nm. Different concentrations of glucosamine treated in the same way were used as standards (Kapteyn *et al.*, 2001).

Plasma membrane isolation. Plasma membranes were isolated as described elsewhere (Niimi *et al.*, 2004). Briefly, overnight cultures in YPD at 30 °C were harvested and pellets were concentrated 20 times and washed with cold H₂O. Cells were then resuspended on ice in 50 mM Tris (pH 7.5), 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and disrupted with 0.25–0.50 mm glass beads (Emergo) in the presence of a protease inhibitor cocktail (Sigma) using a Bio-Savant Fast Prep 120 (Qbiogene). Centrifugation for 10 min at 2000 g removed cell debris and unbroken cells. Centrifugation for 45 min at 30 000 g yielded a crude membrane fraction from the cell-free supernatant. The plasma membrane fraction was enriched by selective precipitation of mitochondria at pH 5.1 as described previously (Goffeau & Dufour, 1988), pelleted by centrifugation for 45 min at 30 000 g, resuspended in 10 mM Tris (pH 7.0)/0.5 mM EDTA/20% (v/v) glycerol, and stored at –80 °C.

Immunodetection of Pga59-GFP and Pga62-GFP. Immunoblots were performed with equivalent amounts from the different cell fractions, referring all to 4 mg dry weight of cell walls. Cell wall fractions were digested with endo-β-1,6-glucanase (ProZyme) (Kapteyn *et al.*, 2001) prior to loading. Proteins were separated using 3–15% gradient SDS-PAGE, and transferred to a nitrocellulose membrane. Anti-GFP antiserum (Molecular Probes) was used at 1:2000 dilution to detect Pga59-GFP and Pga62-GFP.

MS identification of Pga59-GFP and Pga62-GFP and other cell wall proteins. Cell walls were treated with DTT, S-alkylated with iodoacetamide and digested overnight with sequencing-grade trypsin (Roche Molecular Biochemicals) as described by Yin *et al.* (2005). Solubilized peptides were desalted and collected on OMIX µC₁₈ pipette tips (Varian), washed with 0.1% formic acid, and eluted with 60% acetonitrile/0.1% formic acid. For MALDI analysis, the eluates were mixed 1:1 (v/v) with a solution containing 10 mg α-cyano-4-hydroxycinnamic acid ml^{–1} (Sigma-Aldrich) in 50% ethanol/50% acetonitrile/0.3% trifluoroacetic acid. The mixture was spotted on a target plate and allowed to dry at room temperature. Reflectron MALDI-TOF spectra were acquired on a ToFSpec 2E (Waters) and analysed using Masslynx software (Waters) for the presence of GFP-derived peptides.

For MS/MS information, proteolytic digests derived from 30 µg freeze-dried cell walls were fractionated on a 150 mm × 75 µm (length × inner diameter) reversed-phase capillary column (PepMap C18, Dionex) using an Ultimate nano-LC system (Dionex). The peptides were separated over 30 min with a two-step linear gradient of 0–50% acetonitrile + 0.1% formic acid and the flow of 300 nl min^{–1} 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 1500 with a charge state of at least 2+ above a BPI ion count threshold of 40 for collision-induced fragmentation using argon as collision gas. The resulting MS/MS spectra were processed with the Biolynx tool of the Masslynx software, which generated a peak list (.pkl file) with all precursor and product ions calculated to the corresponding MH⁺ charge state. The peak lists (.pkl file) generated with the Masslynx software were submitted to MASCOT (version 2.05, Matrix Science) using the MSDB protein database and an in-house-prepared *C. albicans* database and search parameters as described with *P* < 0.05 for random occurrence of each peptide match (Sosinska *et al.*, 2008).

Freeze-substitution transmission electron microscopy.

Overnight cultures at 30 °C in SD+Arg+His+Uri were concentrated by centrifugation and the pellets were transferred to planchettes of 100 µm depth. The samples were immediately cryoimmobilized with an HPM 010 high-pressure freezer (Bal-tec) and stored in liquid nitrogen. Freeze-substitution of the samples was carried out in an AFS (Leica) in acetone containing 1% osmium tetroxide (Merck). Substitution was carried out for 24 h at –90 °C; then the samples were warmed (5 °C h^{–1}) to –30 °C (3 h) and finally to 0 °C before removal of the substitution medium and embedding in EPON. Contrasted ultrathin sections (60 nm) were observed in a JEM 1010 TEM (JEOL).

RESULTS

PGA59 and PGA62 encode related putative GPI-modified proteins

Genomic transcript profiling has shown that *PGA59* and *PGA62* are overexpressed by 3.7- and 2.4-fold in *C. albicans* biofilm cells relative to planktonic cells (Garcia-Sanchez *et al.*, 2004). As shown in Fig. 1(A), *PGA59* (*orf19.2767*) and *PGA62* (*orf19.2765*) are located in the same region of *C. albicans* strain SC5314 chromosome 4 and transcribed divergently. The 9170 bp region that separates the start codons of *PGA59* and *PGA62* contains a single 309 bp putative ORF, *orf19.2766*, that shows poor coding probability according to a GenMark analysis (Braun *et al.*, 2005; d'Enfert *et al.*, 2005), and is absent from the genome of *C. albicans* strain WO-1 as well as other *Candida* species such as *C. lusitanae*, *C. tropicalis* and *C. guilliermondii* (http://www.broad.mit.edu/annotation/genome/candida_albicans/MultiHome.html; Rossignol *et al.*, 2008). Consistently, no expression of *orf19.2766* was detected by quantitative RT-PCR analysis in YPD or Lee's medium, while *PGA59* and *PGA62* were expressed in these conditions (data not shown).

Comparison of the deduced amino acid sequences of *PGA59* and *PGA62* showed that the 113 aa Pga59 and 213 aa Pga62 proteins share three highly similar domains: a signal peptide (89% identity, 100% similarity), a 42 aa amino-terminal domain (93% identity, 95% similarity) and a carboxy-terminal domain typical of GPI-modified proteins (77% identity and similarity; Fig. 1B, C). The amino acids upstream of the ω sites were also conserved, and the absence of a dibasic motif and occurrence of an acidic residue suggested a cell wall location for both Pga59 and Pga62 (Caro *et al.*, 1997; Mao *et al.*, 2008). Pga62 differed from Pga59 by the occurrence of an additional 42 aa repeat (Fig. 1B, C) and of four 10 aa repeats (Fig. 1B, D). The 42 aa repeat was shared by other *C. albicans* cell wall GPI-modified proteins, including the Cht2 chitinase (Dunkler *et al.*, 2005; McCreath *et al.*, 1995), Hwp1 (Staab & Sundstrom, 1998; Sundstrom, 2002), Hwp2 (Sohn *et al.*, 2003), Rbt1 (Braun *et al.*, 2000), Eap1 (Li & Palecek, 2003, 2008), Ywp1 (Granger *et al.*, 2005; Sohn *et al.*, 2003), and three putative GPI-modified proteins, Pga6, Pga18 and Pga38. In these proteins the amino acid repeat was 42–46 aa

Fig. 1. The *C. albicans* *PGA59* and *PGA62* genes encode putative GPI-modified proteins with conserved motifs. (A) Schematic representation of the region of *C. albicans* chromosome 4 harbouring the *PGA59* (*orf19.2767*) and *PGA62* (*orf19.2765*) genes. Both genes are shown as black arrows while the putative ORF lying in the *PGA59*–*PGA62* intergenic region is shown as a grey arrow. (B) Schematic representation of the Pga59 and Pga62 proteins. The N-terminal signal peptide (SP) and the GPI addition signal (GPI) downstream of the predicted ω sites (Gly92 in Pga59 and Gly194 in Pga62) for GPI anchoring are shown as white boxes. The 42 aa repeats shared by Pga59 and Pga62 are shown as black boxes. The 10 aa repeats specific to Pga62 are shown as grey boxes. Amino acid positions are shown. (C) Multiple alignment of the 42–46 aa repeats identified in Pga59, Pga62 and other *C. albicans* proteins. The location of the repeats in the different proteins is shown. Identical amino acids shared by >50% of the repeats have a black background while similar amino acids shared by >50% of the repeats have a grey background. (D) Multiple alignment of the 10 aa repeats identified in Pga62. Amino acids identified in >75% of the repeats have a black background. (E) Northern blot analysis of the *PGA59* and *PGA62* mRNAs in *C. albicans* strains growing planktonically or as biofilms. Samples (10 µg) of total RNA were blotted onto nylon membranes and hybridized to 32 P-labelled probes for the *PGA59* and *PGA62* genes. A control row showing the methylene-blue staining of the 28S rRNA is included. CAF2, *URA3/ura3*; HLC54, *ura3::λimm434/ura3::λimm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG*; P, Planktonic culture; B, 41 h biofilm obtained in microfermenters.

long (Fig. 1C). The 42 aa repeat was also found in other *C. albicans* proteins, such as Iff11 (Bates *et al.*, 2007) and orf19.3621, that are not GPI-modified proteins. Orthologues of *PGA62* could be identified in other species of the CTG clade, including *C. tropicalis* (CTRG_00154), *C. guilliermondii* (CGUG_04576), *Debaryomyces hansenii* (DEHA0G18986 g, Dujon *et al.*, 2004), *Loderomyces elongisporus* (LELG_04464) and *Pichia stipitis* (PICST_66502, Jeffries *et al.*, 2007). In contrast, no orthologue of *PGA59* could be identified in these and other species. Proteins orthologous to *Pga62* all shared two amino-terminal 42 aa repeats followed by smaller repeats that were not conserved between species.

Because *PGA59* and *PGA62* are highly related, transcript profiling data indicating the overexpression of both genes in biofilm cells might have resulted from cross-hybridization of the corresponding probes on macro- or micro-arrays (Garcia-Sanchez *et al.*, 2004). Thus, expression of *PGA59* and *PGA62* was tested by Northern hybridization of RNA samples prepared from planktonic and biofilm cells. As shown in Fig. 1(E), both *PGA59* and *PGA62* were highly expressed in biofilm cells relative to planktonic cells and this was independent of the presence of functional *EFG1* and *CPH1* genes encoding two transcription factors controlling the yeast-to-hypha switch (Liu *et al.*, 1994; Stoldt *et al.*, 1997), consistent with the transcript profiling data of Garcia-Sanchez *et al.* (2004). Although data in Fig. 1(E) suggested that *PGA59* and *PGA62* are expressed below detectable levels in planktonic cells grown in minimal medium, results published by others and qRT-PCR analysis of RNAs obtained from *C. albicans* cells grown in YPD or Lee's medium indicated that these genes are expressed under different conditions of planktonic growth (Cantero *et al.*, 2007; Castillo *et al.*, 2006; Copping *et al.*, 2005; Garcia-Sanchez *et al.*, 2005; Marcus *et al.*, 2004; Lan *et al.*, 2004; Sohn *et al.*, 2003; and data not shown).

Pga59 and Pga62 are cell wall proteins

As in *C. albicans* predictions about the localization of GPI-modified proteins based on the amino acid environment upstream of the ω site do not always match the experimental data (Richard & Plaine, 2007), we decided to experimentally localize *Pga59* and *Pga62*. For this purpose, plasmids carrying *PGA59-GFP* and *PGA62-GFP* gene fusions were constructed by cloning the GFP coding region immediately downstream of the codons corresponding to the second amino acid in the mature *Pga59* or *Pga62* protein, respectively (Fig. 2A). Because the cloned GFP region lacked a stop codon, the resulting *Pga59-GFP* and *Pga62-GFP* fusion proteins included the signal peptide, GFP, mature domain and GPI-anchor signal peptide of *Pga59* or *Pga62*, respectively (Fig. 2A). Targeting of the *PGA59-GFP* and *PGA62-GFP* fusions at the *PGA59* and *PGA62* loci, respectively, resulted in *C. albicans* strains CEC56 and CEC65. In these two strains, GFP showed cell surface localization as shown in Fig. 2(B). Cell surface localization was independent of whether cells grew as yeasts

or hyphae or in planktonic cultures or biofilms (Fig. 2C and data not shown). However, upon hyphal growth, the *Pga59-GFP* protein appeared evenly distributed in the cell wall while the *Pga62-GFP* protein appeared concentrated in the basal cells of hyphae (Fig. 2C).

Confocal microscopy of *C. albicans* strains CEC56 and CEC65 could not distinguish between localization at the cytoplasmic membrane or association with the cell wall of the *Pga59-GFP* or *Pga62-GFP* proteins. Thus, subcellular fractionation of strains CEC56 and CEC65 was achieved and the presence of the fusion proteins in the cell wall, plasma membrane and intracellular fractions was investigated by immunodetection with anti-GFP antibodies. In both cases, two immunoreactive proteins were detected in the protein fraction released by β -1,6-glucanase from isolated cell walls, one with a molecular mass (MM) above 200 kDa and diffuse migration and one with the MM of GFP and discrete migration (Fig. 3A). The latter protein was likely to represent a degradation product of the *Pga59-GFP* or *Pga62-GFP* fusion proteins. High MM and diffuse migration are landmarks of highly glycosylated proteins. *Pga59* has two putative *N*-glycosylation sites while *Pga62* has only one. GFP does not have any *N*-glycosylation site. Moreover, the mature *Pga59* and *Pga62* are Ser/Thr-rich proteins (*Pga59*, 39.2%; *Pga62*, 37.5%) likely to be modified by *O*-glycosylation. As shown in Fig. 3(B), treatment of the cell-wall-released protein fractions of strains CEC56 and CEC65 with endoglycosidase H (EndoH) converted the GFP-immunoreactive proteins of highest MM to discrete species with lower MM (*Pga59-GFP*, 68 kDa; *Pga62-GFP*, 125 kDa), indicating that both proteins were *N*-glycosylated and subject to outer chain addition. Nevertheless, the MM of the EndoH-treated *Pga59-GFP* and *Pga62-GFP* proteins remained higher than predicted from their amino acid sequence (*Pga59-GFP*, 34.4 kDa; *Pga62-GFP*, 44.6 kDa), suggesting that both were also heavily *O*-glycosylated. Cell wall fractions of strains CEC56 and CEC65 were trypsinized and analysed by mass spectrometry. Abundant peptides corresponding to GFP tryptic products were detected by MALDI-TOF analysis (data not shown). LC/MS/MS analysis of cell wall fractions confirmed the identification of GFP peptides among peptides of common cell wall proteins such as Crh11, Ecm33, Ssr1, Rbt5, Ywp1, Pir1 and Rhd3/*Pga29*, confirming the association of *Pga59-GFP* and *Pga62-GFP* with *C. albicans* cell walls. Taken together, these data indicated that the *Pga59-GFP* and *Pga62-GFP* fusion proteins were *N*- and *O*-glycosylated cell wall proteins, suggesting that the same was true for *Pga59* and *Pga62*. The absence of tryptic peptides for *Pga59* and *Pga62* from trypsinized cell wall extracts of CEC56 and CEC65 might have resulted from their hyperglycosylation. Indeed, deglycosylation of cell wall extracts has enabled the identification of *Pga62* by mass spectrometry (Castillo *et al.*, 2008).

GFP-immunoreactive proteins were also found in the plasma membrane and intracellular fractions of strains

Fig. 2. The Pga59-GFP and Pga62-GFP fusion proteins are located at the cell surface of biofilm and hyphal cells. (A) Schematic representation of the Pga59-GFP and Pga62-GFP fusion proteins. The signal peptide (SP) and GPI-addition signal (GPI) downstream of the ω sites for GPI anchoring are shown as white boxes. GFP was inserted between amino acids 2 and 3 of the mature Pga59 and Pga62 proteins and is shown as a green box. The 42 aa repeats shared by Pga59 and Pga62 are shown as black boxes. The 10 aa repeats specific to Pga62 are shown as grey boxes. (B) Confocal microscopy of *C. albicans* strains CEC56 and CEC65 expressing the Pga59-GFP and Pga62-GFP fusion proteins, respectively. Cells were cultivated under hyphal inducing conditions (Lee's medium) and visualized using an ApoTome microscope. Fluorescence is restricted to the surface of the cells and to the septa of filamentous cells, indicative of an association of the fusion proteins with the plasma membrane or cell wall. (C) Cells of *C. albicans* strains BWP17 (control), CEC56 (Pga59-GFP) and CEC65 (Pga62-GFP) were grown for 24 h in minimal medium at 30 °C (P, planktonic growth), for 24 h on Thermanox under a continuous flow of minimal medium at 37 °C (B, biofilm) or for 2 h in YPD + 10 % bovine serum at 37 °C (F, filamentous growth). Phase-contrast and fluorescence images of the same fields are shown.

CEC56 and CEC65, although in lesser amounts than in the cell wall fraction (Fig. 3). The MMs of these GFP-immunoreactive proteins were consistently lower than those found in the cell wall but higher than that predicted

from the amino acid sequence, suggesting that they were post-translationally modified and might correspond to Pga59-GFP and Pga62-GFP proteins en route to the cell wall through the secretory pathway.

Fig. 3. The Pga59-GFP and Pga62-GFP fusion proteins are *N*- and *O*-glycosylated proteins associated with the cell wall. (A) Detection of the Pga59-GFP and Pga62-GFP proteins in cell-wall-released (CW), plasma-membrane-enriched (PM), and intracellular (M+Cyt) fractions of YPD-grown *C. albicans* strains BWP17AHU, CEC56 and CEC65. The different fractions were separated by SDS-PAGE, transferred to nitrocellulose and GFP-immunoreactive proteins were detected using anti-GFP antibodies. (B) Detection of the Pga59-GFP (lanes 1 and 3) and Pga62-GFP (lanes 2 and 4) proteins in EndoH-untreated (lanes 1 and 2) and -treated (lanes 3 and 4) cell-wall-enriched fractions of YPD-grown *C. albicans* strains CEC56 and CEC65. Treated and untreated samples were fractionated by SDS-PAGE, transferred to nitrocellulose and GFP-immunoreactive proteins were detected using anti-GFP antibodies. In both panels, molecular mass markers are shown in kDa.

Roles of the *PGA59* and *PGA62* genes in growth, filamentation and biofilm formation

In order to investigate the role of *PGA59* and *PGA62*, knockout mutants were constructed in the BWP17U (Table 1) background through replacement of the two alleles of *PGA59* or *PGA62* by the *HIS1* and *ARG4* genes (Gola *et al.*, 2003), yielding strains CEC373 (*pga59Δ/pga59Δ*) and CEC374 (*pga62Δ/pga62Δ*). A double mutant was also constructed, where the whole genomic region harbouring *PGA59* and *PGA62* was deleted. *C. albicans* strain CEC377 (*pga59Δ/pga59Δ pga62Δ/pga62Δ*) was obtained through sequential replacement of the 9040 bp region extending from the stop codon of *PGA59* to the stop codon of *PGA62* by the *HIS1* and *ARG4* genes in BWP17 (Table 1), and then by integration at the *RPS10* locus of the *URA3* gene carried by the CIP10 plasmid (Murad *et al.*, 2000).

Data in Table 3 show relative fitness values (St Onge *et al.*, 2007) for the different strains when grown in rich (YPD), minimal (SD) medium, and minimal medium supplemented with Arg, His and Uri (SD+AHU). Most mutant strains showed a modest reduction in fitness in comparison to the BWP17AHU wild-type strain. Noticeably, the *pga59Δ/pga59Δ* mutant showed a significant decrease in fitness upon growth in SD. This growth defect was alleviated by supplementation of the SD medium with Arg, His and Uri, suggesting a poor expression of the auxotrophic markers in this mutant strain. The observation that a heterozygous *PGA59/pga59Δ* strain also showed a significant fitness reduction in SD medium (Table 3) and that reintroduction of the *PGA59* gene in a *pga59Δ/pga59Δ* strain did not restore wild-type growth in SD medium (data not shown) was consistent with this hypothesis. The

Table 3. Relative fitness of *pga59* and *pga62* deletion strains

Strain	Genotype	Parent	Relative fitness (W)*		
			YPD	SD	SD+AHU
SC5314	Wild-type	–	1.14 ± 0.02	1.07 ± 0.13	1.04 ± 0.15
BWP17AHU	Wild-type	BWP17U	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
CEC373	<i>pga59Δ/pga59Δ</i>	BWP17U	0.89 ± 0.06	0.45 ± 0.00	0.68 ± 0.00
CEC374	<i>pga62Δ/pga62Δ</i>	BWP17U	0.91 ± 0.06	0.89 ± 0.07	0.92 ± 0.03
CEC922	<i>PGA59/pga59Δ</i>	BWP17	0.79 ± 0.07	0.57 ± 0.13	0.78 ± 0.07
CEC377	<i>pga59Δ/pga59Δ pga62Δ/pga62Δ</i>	BWP17	0.81 ± 0.01	0.94 ± 0.06	0.79 ± 0.05

*The relative fitness of a strain was defined by its specific growth rate relative to that of the wild-type BWP17AHU (St Onge *et al.*, 2007). Fitness values are means of those obtained for at least two biological replicates, each including at least four technical replicates.

Fig. 4. Inactivation of *C. albicans* *PGA59* and/or *PGA62* results in a filamentation defect and in flocculation. Cells from 40 h cultures in YPD medium at 30 °C of strains CEC369, CEC373, CEC832, CEC374, CEC377, CEC992, CEC995 and CEC997 were induced to undergo the yeast-to-hypha transition in Lee's medium. Microscopic images (upper panels) and images of cell solutions in reagent tubes (lower panel) are shown. The status of the *PGA59*, *PGA62* and *orf19.2766* genes in each strain is indicated (+/+, homozygous wild-type; -/-, homozygous null; -/-/+, reconstituted).

observation that the *pga59Δ/pga59Δ pga62Δ/pga62Δ* strain did not show such a growth defect might be due to a different configuration of the auxotrophic markers in this strain, where the whole 9040 bp *PGA59*–*PGA62* has been deleted.

The ability of the *C. albicans pga59Δ/pga59Δ* and *pga62Δ/pga62Δ* mutant strains to undergo the yeast-to-hypha transition and to form biofilms was tested. Data shown in Fig. 4 indicated that inactivation of *PGA59* was associated with a severe impairment of filamentation in liquid Lee's medium. A similar, although less pronounced, filamentation defect was observed when *pga59Δ/pga59Δ* mutant cells were exposed to liquid RPMI medium (data not shown). Filamentation was restored upon reintroduction of the *PGA59* gene, indicating that the filamentation defect in Lee's medium of the *C. albicans pga59Δ/pga59Δ* strain was a direct consequence of *PGA59* inactivation (Fig. 4, upper panels). Inactivation of *PGA62* slightly delayed hyphal emergence and elongation in Lee's medium (Fig. 4, upper panels). However, reintroduction of *PGA62* did not restore wild-type filamentation to the *pga62Δ/pga62Δ* mutant (data not shown). No significant impairment of filamentation was observed when the *pga59Δ/pga59Δ* and *pga62Δ/pga62Δ* mutant strains were grown on solid inducing media or under embedded conditions (data not shown). Moreover, we did not observe any reproducible difference when the mutant strains were tested for their ability to form biofilms in two models of biofilm formation. These data suggested that *PGA59* is

necessary for morphogenesis in some conditions and that *PGA59* and *PGA62* are not necessary for biofilm formation despite their elevated expression upon biofilm growth.

Strikingly, when the *C. albicans pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant was incubated in Lee's medium at 37 °C, we observed intense flocculation of germinating cells (Fig. 4, lower panels). Flocculation was reduced upon reintroduction of *PGA59* or *PGA62* in the double mutant while filamentation was still observed. In contrast, reintroduction of *orf19.2766* did not reduce flocculation, suggesting that the flocculation phenotype was a consequence of the simultaneous inactivation of *PGA59* and *PGA62* but not of *orf19.2766*. The observation that the *PGA62*-transformed *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant was able to undergo hyphal morphogenesis in Lee's medium, in contrast to the *pga59Δ/pga59Δ* single mutant, could reflect a difference in fitness in a poor medium such as Lee's medium of the *pga59Δ/pga59Δ* and *pga59Δ/pga59Δ pga62Δ/pga62Δ* strains (Table 3) or a bypass of the *PGA59* requirement for filamentation due to cell-to-cell contact resulting from the simultaneous inactivation of *PGA59* and *PGA62* (see Discussion).

The *PGA59* and *PGA62* genes are necessary for cell wall integrity

Because Pga59 and Pga62 were shown to be constituents of the cell wall, they could play a role in the architecture and

Fig. 5. Inactivation of *C. albicans* *PGA59* and/or *PGA62* results in altered sensitivity to cell-wall-perturbing agents. Overnight cultures in SD medium at 30 °C of strains SC5314, BWP17AHU, *pga59Δ/pga59Δ*, *pga62Δ/pga62Δ* and *pga59Δ/pga59Δ pga62Δ/pga62Δ* were adjusted to OD₆₀₀ 1 (~10⁷ cells ml⁻¹) and fivefold serial dilutions were spotted on (A) YPD, YPD + 300–400 µg ml⁻¹ Congo red and (b) YPD and YPD + drugs (NikkoZ, nikkomycin Z; Caspo, caspofungin; CFW, Calcofluor white; CR, Congo red) at the indicated concentrations. Photographs were taken after 3 days at 30 °C.

integrity of the *C. albicans* cell wall. Thus, sensitivity of the *C. albicans* *pga62Δ/pga62Δ* and *pga59Δ/pga59Δ* mutant strains to various cell-wall-perturbing agents was tested in rich medium. Congo red (CR), Calcofluor white (CFW), nikkomycin Z (a chitin synthase inhibitor), and caspofungin (a β-1,3 glucan synthase inhibitor) sensitivity tests were performed on solid YPD, while sensitivity to Zymolyase (β-1,3 glucanase and β-1,3-glucan laminaripentaohydrolase) and Quantazyme (β-1,3-glucanase) was tested on cells grown in liquid YPD. As shown in Fig. 5(A), both the *pga59Δ/pga59Δ* and *pga62Δ/pga62Δ* strains showed increased sensitivity to CR at concentrations above 300 µg ml⁻¹. No

difference in sensitivity to CFW, nikkomycin Z, caspofungin, Zymolyase or Quantazyme was observed for the *pga62Δ/pga62Δ* and *pga59Δ/pga59Δ* mutant strains (data not shown).

The most striking effect was observed upon deletion of both *PGA59* and *PGA62*. As shown in Fig. 5(B), *C. albicans* strain *pga59Δ/pga59Δ pga62Δ/pga62Δ* showed a dramatically increased sensitivity to CR (25 µg ml⁻¹), nikkomycin Z (20 µg ml⁻¹) and CFW (30 µg ml⁻¹) and resistance to caspofungin (200 ng ml⁻¹).

Reintroduction of the *PGA59* or *PGA62* genes at the cognate loci restored the original phenotype (Fig. 5A). In the case of

Table 4. Cell wall composition of SD-grown cells of the *pga59* and *pga62* deletion strains

Strain	Genotype	Chitin content (% cell wall)	Protein content (% cell wall)	Sugar content (% cell wall)		Ratio glucose : mannose
				Glucose	Mannose	
BWP17AHU	Wild-type	3.8 ± 0.7	3.0 ± 0.1	69.7 ± 3.5	23.1 ± 1.3	3.0 ± 0.0
CEC373	<i>pga59Δ/pga59Δ</i>	5.6 ± 0.61	3.4 ± 0.0	64.3 ± 4.3	20.8 ± 2.0	3.1 ± 0.3
CEC374	<i>pga62Δ/pga62Δ</i>	5.4 ± 0.7	3.3 ± 0.1	68.6 ± 6.4	23.6 ± 2.5	2.9 ± 0.1
CEC377	<i>pga59Δ/pga59Δ pga62Δ/pga62Δ</i>	5.0 ± 0.5	4.0 ± 0.3	77.8 ± 7.7	12.0 ± 1.8	6.5 ± 0.4

the *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant, reintroduction of either *PGA59* or *PGA62* gene at the *RPS1* locus partly alleviated the sensitivity to CR (data not shown).

Altered sensitivity to cell-wall-perturbing agents suggested that inactivation of *PGA59* and/or *PGA62* might result in alterations of the cell wall composition, especially in the case of the *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant. Cell walls were isolated from SD-grown cells of strains BWP17AHU, CEC373, CEC374 and CEC377, and protein, chitin, mannan (measured as mannose) and glucan (measured as glucose) contents were measured as detailed in Methods. Results are presented in Table 4. No differences in protein content were observed. Inactivation of *PGA59* and/or *PGA62* resulted in a 1.3- to 1.5-fold increase in chitin content. No significant variation in glucan and mannan levels nor in glucan:mannan ratio were observed for the *pga59Δ/pga59Δ* and *pga62Δ/pga62Δ* strains. In contrast, the *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant showed a twofold reduction in the relative level of mannose and a concomitant slightly increased level of glucose (1.11). These changes were reflected in the glucose:mannose ratio, which was twice that of the control strain (Table 4). The cell wall of the different mutants was analysed by LC/MS/MS and showed a very similar cell wall proteome to that of the wild-type, with no obvious complementary changes such as the incorporation of uncommon cell wall proteins (data not shown).

In order to get further insights into potential cell wall defects of the *pga59Δ/pga59Δ*, *pga62Δ/pga62Δ* and *pga59Δ/pga59Δ pga62Δ/pga62Δ* strains, they were grown in supplemented minimal medium and observed by electron microscopy following cryofixation. Results presented in Fig. 6 indicated significant differences between the cell wall of the mutant and wild-type strains: the inner layer of the cell wall, composed of chitin and β -1,3-glucan, was less electron-dense while the outer layer, enriched with mannoproteins, appeared disorganized in the three mutants.

Taken together, these data indicated that the cell wall Pga59 and Pga62 proteins are necessary for cell wall integrity in *C. albicans*.

DISCUSSION

In this study, we have investigated the contribution of the *C. albicans* Pga59 and Pga62 GPI-modified cell wall

Fig. 6. Inactivation of *C. albicans* *PGA59* and/or *PGA62* results in altered cell wall morphology. Transmission electron micrographs of *C. albicans* cells grown in SD medium supplemented with arginine, histidine and uridine, showing the glucan/chitin-rich inner layer and mannose-rich fibrillar outer layer of the cell wall. (A, E) Wild-type BWP17AHU. (B, F) CEC373 (*pga59Δ/pga59Δ*). (C, G) CEC374 (*pga62Δ/pga62Δ*). (D, H) CEC377 (*pga59Δ/pga59Δ pga62Δ/pga62Δ*). Scale bars: A–D, 500 nm; E–H, 100 nm.

proteins to morphogenesis, biofilm formation and cell wall biogenesis. Our results show that Pga59 and, to a minor extent, Pga62, are necessary for efficient filamentation in liquid Lee's medium and liquid RPMI medium. Despite this contribution of Pga59 (and Pga62) to morphogenesis, the known link between morphogenesis and biofilm formation (Ramage *et al.*, 2002; Richard *et al.*, 2005) and the elevated expression of the *PGA59* and *PGA62* genes in *C. albicans* biofilms (Garcia-Sanchez *et al.*, 2004), neither of these two genes appears to contribute to biofilm formation. Taken together, these observations suggest a minor requirement of Pga59 and Pga62 for morphogenesis, such that it does not influence biofilm formation under the conditions we have tested.

Strikingly, we observed that simultaneous inactivation of *PGA59* and *PGA62* results in flocculation upon hypha-inductive conditions (Fig. 4), and to some extent in the stationary phase of growth (data not shown), and overrides the filamentation defect associated with the inactivation of *PGA59* alone. The observation that the *pga59Δ/pga59Δ* mutant was defective for filamentation in Lee's medium while the *pga59Δ/pga59Δ pga62Δ/pga62Δ* mutant showed relatively unaltered filamentation might reflect differences in fitness between these two strains when grown in media that are not supplemented with uracil, arginine and histidine such as Lee's medium (Table 3). Thus, the decreased fitness of the *pga59Δ/pga59Δ* mutant could on its own explain the filamentation defect in Lee's medium. However, we favour an alternative hypothesis whereby flocculation resulting from the simultaneous inactivation of *PGA59* and *PGA62* could bypass the requirement for *PGA59* for morphogenesis in Lee's medium. This hypothesis is supported by the observation that the *pga59Δ/pga59Δ* mutant is not defective for contact-induced morphogenesis upon embedded growth or on surfaces. Consistent with this hypothesis, several *C. albicans* mutants (e.g. those in the *SFL1*, *CHK1* and *CRK1* genes) show flocculation and reduced thresholds for hyphal induction (Bauer & Wendland, 2007; Calera & Calderone, 1999; Li *et al.*, 2007; Wang *et al.*, 1999). However, it has not been precisely evaluated whether in these mutants the increased ability to form hyphae triggers flocculation or vice versa.

C. albicans GPI-modified cell wall proteins have been recently classified into the following groups, depending on their functions: cell wall biogenesis and remodelling, oxidant adaptation, adherence, or specific enzymic functions (Richard & Plaine, 2007). Transcript profiling data suggested that Pga59 and Pga62 might play a role in cell wall stability, as both *PGA59* and *PGA62* are upregulated during protoplast regeneration (Castillo *et al.*, 2006). Characterization of a *pga62Δ/pga62Δ* mutant in the framework of a global survey of the function of GPI-modified proteins in *C. albicans* has suggested that Pga62 indeed plays a role in cell wall stability, as inactivation of *PGA62* was associated with Calcofluor white sensitivity, resistance to caspofungin, and an increase in cell wall chitin content and thickness (Plaine *et al.*, 2008). Data presented

in this study confirm the involvement of Pga62 in *C. albicans* cell wall stability and extend this involvement to Pga59. In particular, we have shown that inactivation of *PGA59* and/or *PGA62* is associated with (i) increased levels of chitin in the cell wall, (ii) a cell wall inner layer with decreased electron density and (iii) disorganization of the cell wall outer layer. An increase in chitin levels is known in yeasts to compensate for alterations in the cell wall (Kapteyn *et al.*, 2000; Popolo *et al.*, 2001; Richard *et al.*, 2002a; Walker *et al.*, 2008) and to correlate with increased sensitivity to Congo red, a cell wall binding dye known to interfere with the proper assembly of cell wall components (Herth, 1980; Imai *et al.*, 2005). Consistently, the *pga59Δ/pga59Δ* and *pga62Δ/pga62Δ* mutant strains have increased sensitivity to Congo red. This effect is exacerbated in the double *pga59Δ/pga59Δ pga62Δ/pga62Δ* mutant, which becomes highly sensitive to Congo red, Calcofluor white, a fluorescent stain that binds to nascent chitin molecules and, conceivably, also interferes with the attachment of chitin to β -1,3-glucan (Herth, 1980), and nikkomycinZ, a competitive inhibitor of chitin synthases (Cabib, 1991). These results suggest that chitin biosynthesis is essential to circumvent deleterious effects on the cell wall of mutations in the *PGA59* and *PGA62* genes. Consistent with increased chitin levels and changes in the cell wall is the resistance of the *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant to caspofungin. The echinocandin caspofungin is an inhibitor of the Fks1 glucan synthase, thus interfering with the polymerization of UDP-glucose into the cell wall polymer β -1,3-glucan and in the late stages of β -1,6-glucan synthesis (Kurtz & Douglas, 1997; Stevens *et al.*, 2006). Compensatory upregulation of chitin synthesis is known to protect *C. albicans* cells against caspofungin in a process that involves the protein kinase C-dependent cell wall integrity pathway, the HOG MAP kinase pathway and the Ca^{2+} -calcineurin signalling pathway (Stevens *et al.*, 2004, 2006; Walker *et al.*, 2008; Wiederhold *et al.*, 2005). However, the exact nature of the cell wall modification that triggers increased chitin synthesis in the single and double *pga59Δ/pga59Δ* and *pga62Δ/pga62Δ* mutants remains unclear. Analysis of the cell wall did not indicate any deficiency in glucan synthesis, as only the *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant showed a modest increase in cell wall glucans that may contribute to further compensation of cell wall defects due to the simultaneous inactivation of *PGA59* and *PGA62*. The *pga59Δ/pga59Δ pga62Δ/pga62Δ* double mutant also showed a reduction in mannose while the protein levels and protein composition of the wall remained unaltered. This may reflect an alteration in the level of mannosylation of the manno-proteins found in the outer layer of the cell wall, consistent with the disorganization of this cell wall layer observed upon electron microscopy. It may also reflect the absence of the abundant, highly mannosylated Pga59 and Pga62 proteins from the cell wall. That no obvious difference was seen between the single and double mutants might be due to the inherent limitations of our ultrastructural analysis.

What then could be the function of Pga59 and Pga62 in the cell wall? The cell-wall-bound mature Pga59 and Pga62 proteins are essentially constituted of one or two repeats of a 42 aa domain that is shared by several other *C. albicans* cell wall proteins as well as cell wall proteins in other yeast species (e.g. the *S. cerevisiae* Ccw12 protein, also a short and presumably abundant GPI-cell wall protein as indicated by a high codon adaptation index; Christie *et al.*, 2004; Ragni *et al.*, 2007). Heterologous expression of derivatives of the *C. albicans* Eap1 protein in *S. cerevisiae* suggests that a larger repeated domain encompassing the 42 aa domain is involved in cellular aggregation (Li & Palecek, 2008). Interestingly, the 42 aa domain harbours three conserved cysteine residues along with aromatic residues, serine and threonine residues and hydrophobic residues. It is therefore reminiscent of domains that have been shown to bind cell wall polymers (Boraston *et al.*, 2004) such as those found in the Gas1 family of transglucosidases (Popolo *et al.*, 2008) or Eng1 family of endoglucanases (Martín-Cuadrado *et al.*, 2008). Therefore, the repeated domains in Pga59 and Pga62 might be involved in interacting with cell wall polysaccharides and consequently reinforce the integrity of the cell wall. Alternatively, conserved cysteine residues in the 42-residue repeats might be involved in intermolecular disulfide bridges that could also reinforce the integrity of the cell wall. It has been shown that treatment of yeasts with reducing agents increases the porosity of the cell wall, suggesting that disulfide bridges might contribute to cell wall organization (De Nobel *et al.*, 1990a, b). The 42-residue-repeat-containing cell wall-bound proteins might be responsible for cell wall stability dependent on disulfide bridges, some proteins such as Pga59 and Pga62 performing only this function while proteins such as the Cht2 chitinase and the Hwp1 and Eap1 adhesins perform additional functions. In this regard, a structural role of Pga59 and Pga62 in the cell wall is supported by their probable abundance, as suggested by a high codon adaptation index (0.75; Sharp & Li, 1987; Wu *et al.*, 2005), and an even distribution in the cell wall of yeast, pseudohyphal and hyphal cells. Detailed studies on the role of the cysteine residues in Pga59 or Pga62 function should help test this hypothesis.

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