Functional analysis of *Candida albicans* GPI-anchored proteins: Roles in cell wall integrity and caspofungin sensitivity

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**Abstract**

The outer layer of the *Candida albicans* cell wall is enriched in highly glycosylated proteins. The major class, the Glycosylphosphatidylinositol (GPI)-anchored proteins are tethered to the wall by GPI-anchor remnants and include adhesins, glycosyltransferases, yapsins and superoxide dismutases. In silico analysis suggested that *C. albicans* possesses 115 putative GPI anchored proteins (GpIPs), almost twice the number reported for *Saccharomyces cerevisiae*. A global approach to characterise in silico predicted GpIPs has been initiated by generating a library of 45 mutants. This library was subjected to a screen for cell wall modifications by testing the cell wall integrity (SDS and Calcofluor White sensitivity) and response to caspofungin. We showed that, when caspofungin sensitivity was modified, in more than half of the cases the susceptibility can be correlated to the level of chitin and cell wall thickness: sensitive strains have low level of chitin and a thin cell wall. We also identified, for the first time, genes that when deleted lead to decreased caspofungin sensitivity: *DFG5, PHR1, PGA4* and *PGA62*. The role of two unknown GpIPs, Pga31 and Pga62 in the cell wall structure and composition was clearly demonstrated during this study.

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1. Introduction

*Candida albicans* is the most common fungal pathogen of humans. A crucial feature of this microorganism is its ability to survive in the host and to infect several anatomically distinct sites. This fungus has elaborated numerous mechanisms to adapt to a diversity of niches (mouth, digestive tract, skin, vagina, etc.) and to live in the host and to infect several anatomically distinct sites. As an opportunistic pathogen, *C. albicans* possesses 115 putative GPI anchored proteins (GpIPs), almost twice the number reported for *Saccharomyces cerevisiae*. A global approach to characterise in silico predicted GpIPs has been initiated by generating a library of 45 mutants. This library was subjected to a screen for cell wall modifications by testing the cell wall integrity (SDS and Calcofluor White sensitivity) and response to caspofungin. We showed that, when caspofungin sensitivity was modified, in more than half of the cases the susceptibility can be correlated to the level of chitin and cell wall thickness: sensitive strains have low level of chitin and a thin cell wall. We also identified, for the first time, genes that when deleted lead to decreased caspofungin sensitivity: *DFG5, PHR1, PGA4* and *PGA62*. The role of two unknown GpIPs, Pga31 and Pga62 in the cell wall structure and composition was clearly demonstrated during this study.

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have provided evidence of GPI-anchoring in *C. albicans* (Grimme et al., 2004; Kapteyn et al., 2000; Kapteyn et al., 1994). Kapteyn and collaborators have described the cross-linkage of GPI proteins to cell wall 1-1,6-glucan in *C. albicans* (Kapteyn et al., 1994). Grimme et al. (2004) demonstrated that the *C. albicans* mannosyltransferase Smp3, involved in the synthesis of the GPI-anchor glycan, retained the same function as its *S. cerevisiae* ortholog. In addition, the inability to generate a *CaSMP3* null mutant suggested the synthesis of GPI-anchors is essential in *C. albicans* as it is in *S. cerevisiae* (Grimme et al., 2004).

A previous study in our laboratory established that GpiPs were no longer normally linked to the cell wall in a *C. albicans gpi7–* mutant affected in GPI-anchor side chain addition. This mis-localization of numerous cell wall proteins had dramatic consequences on cell wall composition and the *in vivo* virulence of the mutant (Richard et al., 2002a; Richard et al., 2002b). These data highlight the importance of GpiPs in the interaction with the host, but does not differentiate a direct role from an indirect role. A better understanding of the functions associated with this class of protein might thus lead to the identification of potential targets for therapeutic treatment of *C. albicans*.

To date, several strategies have permitted the identification of GpiPs in *C. albicans*, including proteomics, functional expression in *S. cerevisiae*, cloning of surface antigens, and identification of *C. albicans* sequence homologs of characterized *S. cerevisiae* GpiPs (de Groot et al., 2004; Garcera et al., 2003; Lamarre et al., 2000). Here, we report the first systematic functional analysis of a large number of putative GpiPs in the human pathogen *C. albicans*. A total of 45 GpiP mutants were constructed or gathered from different laboratories and submitted to a series of phenotypic tests in order to characterize their involvement in cell wall structure. Our results extend the functional analyses of putative GpiPs characterized elsewhere and identify several novel putative GpiPs that contribute to cell wall integrity.

### Table 1

| Gene name | Orf19 number | Similarity group | Function | Source |
|-----------|--------------|------------------|----------|--------|
| CHT1      | 7517         | 1                | Chitinase | This work |
| CHT2      | 3895         | 1                | Chitinase | This work |
| PGA7      | 3923         | 2                | Putative GPI-anchored protein of unknown function | This work |
| PGA57     | 4689         | 2                | Putative GPI-anchored protein of unknown function | This work |
| SAP9      | 6928         | 3                | Asparyl proteinase, yapsin | This work |
| SAP10     | 3839         | 3                | Asparyl proteinase, yapsin | Albrecht et al. (2006) |
| PGA2/SOD4 | 2062         | 4                | Copper- and zinc-containing superoxide dismutase | This work |
| PGA5/SOD5 | 2060         | 4                | Copper- and zinc-containing superoxide dismutase | Fadin et al. (2005) |
| PGA4/SOD6 | 2108         | 4                | Copper- and zinc-containing superoxide dismutase | This work |
| HWP1      | 1321         | 5                | Hyphal cell wall protein | Staab et al. (1999) |
| RBT1      | 1327         | 5                | Putative cell wall protein with similarity to Hwp1 | Braun et al. (2000) |
| PGA8      | 3380         | 5                | Putative GPI-anchored protein of unknown function | This work |
| PGA9      | 5303         | 6                | Putative GPI-anchored protein of unknown function | This work |
| PGA10     | 5302         | 6                | Putative GPI-anchored protein of unknown function | This work and Santendreu’s laboratory (unpublished) |
| PGA32     | 6784         | 6                | Putative GPI-anchored protein of unknown function | This work |
| CSA1      | 7114         | 7                | Surface antigen on elongating hyphae and buds | This work and Lamarre et al. (2000) |
| PGA7      | 5635         | 7                | Putative GPI-anchored protein of unknown function | This work |
| PGA10     | 5674         | 7                | Putative GPI-anchored protein of unknown function | This work |
| RB5       | 5636         | 7                | GPI-anchored cell wall protein | This work and Braun et al. (2000) |
| PGA4      | 4035         | 8                | Putative GPI-anchored protein of unknown function | This work |
| PGA5      | 3693         | 8                | Putative GPI-anchored protein of unknown function | This work |
| PRR5      | 3829         | 8                | Glycosidase; role in cell wall structure | Saporito-Irwin et al. (1995) |
| DFG5      | 6081         | 8                | N-linked mannosprotein of cell wall and membrane | Fonzi et al. (1999) |
| ECM331    | 4255         | NS               | Putative GPI-anchored protein of unknown function | This work |
| HYR1      | 4975         | NS               | Nonessential, GPI anchored, predicted cell wall protein | This work and Bailey et al. (1996) |
| MDD1      | 3212         | NS               | Putative component of the high affinity calcium uptake system | This work |
| PGA6      | 4705         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA17     | 893          | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA23     | 3740         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA24     | 3816         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA27     | 2044         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA33     | 876          | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA36     | 3760         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA39     | 6302         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA40     | 1616         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA42     | 2907         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA43     | 2910         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA45     | 2451         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA46     | 3638         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA50     | 1824         | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA56     | 207          | NS               | Putative GPI-anchored protein of unknown function | This work |
| PGA62     | 2705         | NS               | Putative GPI-anchored protein of unknown function | This work |
| SPR1      | 2237         | NS               | Similar to ScSpr1, a sporulation-specific exo-1,3-beta-glucanase | This work |
| SRR1      | 7030         | NS               | Beta-glucan associated cell-wall protein | This work and Garcera et al. (2003) |

**Similarity group:** genes are classified in the table into 8 families according to their amino acid sequence similarity; **NS** means no similarity within the groups of genes.
BWP17 (ura3::imm434/ura3::imm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG) (Wilson et al., 1999). In all experiments, two independent mutant clones were tested twice and compared to DAY286 (ura3::imm434/ura3::imm434, his1::hisG/his1::hisG, pARG4::URA3::hisG/arg4::hisG), a Ura+, Arg+, His− derivative of BWP17 (Davis et al., 2000), unless otherwise indicated. Mutants obtained by the classical URA-blaster method (Fonzi and Irwin, 1993) and lacking either CSA1, HWP1, HYR1, PHR1, PHR2, RBT1, RBT5, SAP10, SOD5 and SSR1 along with the corresponding complemented or reference strains, were obtained from the groups who generated them (see Table 1). For these latter strains, all experiments were performed two to four times and SC5314 or CAI-4 (Fonzi and Irwin, 1993) was used as reference strains according to their genetic background.

2.2. Culture media

DH5α was grown at 37 °C in Luria–Bertani medium supplemented with 100 μg ampicillin mL−1 (Amp) and 20 μg kanamycin mL−1 (Kan) as required. C. albicans strains were grown in YPD plus uridine (2% dextrose, 2% Bacto peptone, 1% yeast extract and 80 μg uridine mL−1) at 30 °C. Following transformation, clones were selected on synthetic medium (SC; 2% glucose, 0.67% yeast nitrogen base without amino acid plus ammonium sulphate, 1.7 g amino acid drop out mix L−1, supplemented for auxotrophic requirements).

2.3. Mutant library construction

All C. albicans sequences were obtained from the Candida Database and the Candida Genome Database web sites (http://genolist.pasteur.fr/CandidaDB/ and http://www.candidagenome.org/). The open reading frame (ORF) of all genes to be disrupted was amplified by PCR and ligated into the vector pGEM-T (Promega, Madison, WI, USA) to generate the target plasmid for subsequent in vitro transposition using the GPS-M mutagenesis kit (New England Biolabs, Beverly, MA, USA, see Fig. 1). The donor plasmid, pAED98 contains Tn7-UAU1 (Davis et al., 2002), a modified Tn7 transposon carrying the UAU1 cassette (Enloe et al., 2000). In vitro transposition was performed using 20 ng of pAED98 and 80 ng of

![Fig. 1. Strategy used for gene interruptions (the ORF to be disrupted is depicted as a black box, the ARG4 marker as a grey box, the URA3 marker as a vertically hatched box and the IR of the Tn7 transposon as horizontally hatched boxes). (a) Construction of the disruption cassette by in vitro transposition. The integration site of the Tn7-UAU1 cassette was estimated by double digestion (PmeI and NotI). The target plasmid which had integrated the Tn7-UAU1 transposon in the middle of the ORF was selected and transformed in the C. albicans strain BWP17. (b) Selection of double disruptions by colony PCR. Colonies isolated on SC-Arg-Ura plates were screened for the absence of the wild-type allele and the presence of the UAU1 insertion allele. Primers 5’ and 3’ detect were the primers used to amplify the ORF in order to create the target plasmid.](image-url)
the target plasmid. Transposon-mutagenized plasmids were transformed into DH5α and transformants were selected on LB + Amp + Kan plates. For each transposition, 24 plasmids were isolated from individual colonies using the Millipore Montage miniprep system (Millipore, Bedford, USA). In order to map the integration site of the Tn7-UAU1 in the target plasmid, we digested the mutated plasmid with NotI and PmeI. One target plasmid, which had integrated the Tn7-UAU1 transposon near the middle of the ORF was selected for each gene (see Fig. 1a).

2.4. Construction of homozygous mutants

 Interruption of both alleles of each C. albicans gene was achieved following the UAU1 method (Enloe et al., 2000). Briefly, the Tn7-UAU1 mutagenized plasmid was digested with NotI to release the UAU1 disruption cassette which was then used to transform C. albicans strain BWPe17 (Wilson et al., 1999). Arg⁷ transformants were selected. Genotypes were analysed by colony PCR to detect the wild-type allele and the Tn7-UAU1 insertion (see Fig. 1b). For each plasmid, 24 heterozygous strains were patched onto YPD plates, grown at 30°C for 2 days. After replica-onto SC-Arg-Ura plates, the colonies were screened by colony PCR for the absence of the wild-type allele and the presence of the UAU1 insertion allele. Genotypes of all transformants showing a phenotype different from the control strain were further confirmed by Southern analysis.

2.5. Phenotypic analysis by drop tests

The phenotypes of the 45 GpiP mutants were monitored using drop tests. All media were supplemented with histidine. Strains were grown overnight in YPD at 30°C and diluted with H₂O to an OD₆₀₀ of 1. Then, 5 µl of serial 1/10 dilutions was spotted onto the following solid media: YPD supplemented with 0.025% SDS or 30 µg Calcofluor White ml⁻¹ (Sigma–Aldrich, Steinheim, Germany). Plates were monitored over 2 days for altered growth.

2.6. Antifungal tests

The in vitro susceptibility of the mutants was tested against caspofungin. The Minimal Inhibitory Concentration (MIC) was determined using the microbroth dilution method M27-A2 (NCCLS, 2002). Each drug was obtained from its respective manufacturer as pure compound. RPMI medium (Sigma–Aldrich, Steinheim, Germany) buffered at pH 7 with morpholinepropanesulfonic acid (MOPS; Sigma–Aldrich, Steinheim, Germany) was used. Additional tests with caspofungin were performed using the drop test method on SC plates buffered at pH 7.0 with HEPES 150 mM and containing sub-inhibitory concentrations of caspofungin. Plates were incubated for two days at 37°C.

2.7. Chitin content quantification

Cell walls were prepared from exponential C. albicans yeast cultures grown in YPD and the chitin content was measured as described previously with modifications (Munro et al., 2007). Briefly, cell walls were extracted by disrupting cells with glass beads (Sigma, G9268) using a Fastprep cell breakage machine (Thermo Savant, Middlesex, UK). Samples were then washed five times with 1 M NaCl and cell walls were extracted using SDS-MerOH buffer (50 mM Tris, 2% sodium dodecyl sulphate (SDS), 0.3 M β-mercaptoethanol, 1 mM EDTA; pH 8.0) at 100°C for 10 min. Cell wall pellets were resuspended in sterile dH₂O, freeze dried, and the dry weight of recovered cell walls was measured. Chitin contents were determined by measuring the glucosamine released by acid hydrolysis of purified cell walls (Kapteyn et al., 2000).

2.8. Monitoring of PKC pathway activation

Western analysis was performed following the method described in Munro et al. with some modifications (Munro et al., 2007). Mid-log phase cultures were harvested by centrifugation (1500g, 5 min, 4°C) and washed in 1 ml cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 2 g ml⁻¹ Leupeptin, 2 µg ml⁻¹ Pepstatin, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF). Cells were collected by centrifugation (800g, 5 min, 4°C) and resuspended in 250 µl cold lysis buffer. Cells were broken using a Fast-Prep machine in the presence of acid-washed glass beads (4 × 15 s bursts at speed 6.5 with 1 min on ice between bursts). The extracts were clarified by centrifugation (16 000g, 5 min, 4°C). Protein concentration in the cleared lysate was estimated using the method described by Bradford with BSA as a standard (Bradford, 1976).

Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using the XCell SureLock™ Mini-Cell system (Invitrogen) with NuPAGE™Novex Bis–Tris 4–12% pre-cast gels (Invitrogen) in NuPAGE™ MOPS-SDS Running Buffer (Invitrogen) containing NuPAGE™ Antioxidant (Invitrogen) as per the manufacturer’s instructions. Approximately 15 µg of protein was loaded in each lane. The proteins were transferred to Invitrolon™ PVDF Membranes (Invitrogen) in NuPAGE™ Transfer Buffer containing methanol using the XCell II™ Blot Module (Invitrogen) following the manufacturer’s instructions.

Following transfer, the membranes were rinsed in PBS and blocked in PBS-T-1% BSA (PBS, 0.1% Tween 20, 10% (w/v) BSA, 50 mM NaF) for 30 min at RT. The membranes were then incubated overnight at 4°C in PBS-T-5% BSA (PBS, 0.1% Tween 20, 5% (w/v) BSA, 50 mM NaF) containing a 1:1000 dilution of Phospho-p44/50 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology). The membranes were washed five times for 5 min in PBS–T (PBS, 0.1% Tween 20) and then incubated for 1 h at RT in PBS–T+5% BSA containing a 1:2000 dilution of Anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology). The membranes were washed three times for 5 min in PBS–T and the signal was detected using LumiGLO™ Reagent and Peroxide (Cell Signaling Technology) as per the manufacturer’s instructions.

2.9. Transmission electron microscopy

Mid-exponential phase yeast cells were grown in YPD harvested by centrifugation and the pellets were fixed in 3% glutaraldehyde (v/v) in 0.1 M sodium phosphate buffer pH 7.4. Secondary fixation was performed in 1% osmium tetroxide in dH₂O and cells were then embedded in TAAB resin before ultrathin silver or gold defracting sections were cut on a Leica UC6 ultramicrotome and stained with uranyl acetate and lead citrate and examined with Philips CM10 transmission microscope (FEI UK Ltd., Cambridge, UK) and the images recorded with a Gatan BioScan 792 (Gatan UK, Abingdon, UK). Average cell wall thickness was measured manually for at least 20 individual yeast cells.

2.10. Analysis of the cell wall β-glucan content

The β-glucan content was determined by hydrolysis of this polymer and quantification of glucose. Yeast cells were grown in YPD medium at 30°C, broken and hydrolyzed as described (Mora-Montes et al., 2007). Hydrolyzed samples were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in a carbohydrate analyzer system from Dionex (Surrey, UK), equipped with a ED50 electrochemical detector with gold electrode, a G5050 pump gradient, and a CarboPac PA200 analytical column (3 × 250 mm) with a CarboPac PA200 guard column (3 × 50 mm). In order to elute the samples, an isocratic gradient of 3.2 mM NaOH with a flux rate of
0.15 ml/min was applied for 20 min. The column was washed with 200 mM NaOH and equilibrated with 3.2 mM NaOH prior to the next analysis.

3. Results

3.1. Creation of the library

Mutants were constructed using an insertional mutagenesis strategy (Davis et al., 2002) based on the UAU1 marker cassette (Enloe et al., 2000). The UAU1 cassette (ura3Δ33·ARG4·ura3Δ55) includes a functional ARG4 gene that can be excised by recombination between flanking repeats of an interrupted URA3 gene to regenerate a functional URA3 gene. This cassette, present in the Tn7-UAU1 transposon (see Materials and methods), was inserted by in vitro transposition into each of the 39 target ORFs encoding putative GpiPs (Fig. 1a). We selected insertions where the UAU1 cassette had inserted near the middle of the ORF. Each of the 39 disruption cassettes was transformed into C. albicans strain BW217 after excision from the vector. Following disruption of the first allele, spontaneous recombination events gave rise to ArgΔ, UraΔ, HisΔ alleles and generate phenotypes not linked to the interruption of the target gene. We believe nevertheless that this bias might be limited since: (i) we tested at least two independent mutants, (ii) in the case of previously studied genes, the phenotypes observed in our strains matched the published ones (DFG5, SSR1); (iii) when strains obtained by the UAU1 method and the classical Ura-Blaster method were compared (e.g. PCA31, SSR1) identical phenotypes were observed.

Our current library, composed of 39 UAU1 insertional mutants plus six mutants provided by different laboratories, represents 39.1% of the 115 putative GpiPs of C. albicans. The genes disrupted were not selected using any particular criteria. This library represents the first part of our study of all predicted GpiPs of C. albicans and will be followed in time by the characterization of the remaining genes. The construction of each of the 39 mutants, even using the UAU1 method, was time consuming thus we decided to screen this library before completion of all the gene disruptions. With a little less than 40% of the genes deleted we unveiled important roles for some of these GpiPs, giving an overview of the biological importance of these proteins. This is of particular interest since these proteins are predicted to be at the cell surface and more than 60% are of unknown function.

3.2. Primary screening on the 45 mutants

This library was initially screened for various biological functions including morphogenesis, ability to respond to diverse stresses, cell wall related phenotypes and susceptibility to antifungal drugs. In this publication, we will mainly focus on the cell wall and how deletion of specific predicted GpiPs affects the structure, composition and resistance to stress of the cell wall. The additional results obtained from the whole screen are supplied in Supplementary data S1.

Thus, in order to evaluate the impact of the disruption of genes encoding putative GpiPs on the structural integrity of the cell wall, mutants were tested for sensitivity to a number of agents that target the cell wall or membrane. These included Calcofluor White (CFW) a fluorescent optical brightener with chitin-binding properties and caspofungin a drug inhibiting the synthesis of β-1,3-glucan, the main component of the cell wall. In addition we measured growth in the presence of SDS a detergent that compromises the integrity of the cell membrane, this tests the accessibility of SDS to the membrane through the cell wall.

Hypersensitivity to CFW was observed with six mutants rbt1Δ/Δ, hwp1Δ/Δ, pga31Δ/Δ, pga62Δ/Δ, phr1Δ/Δ and ssr1Δ/Δ using serial...
dilutions on plates containing different concentrations of CFW (Fig. 2). In the presence of SDS, using the same protocol, hypersensitivity was observed with two mutants \( pga31/C0/C0 \) and \( hwp1/C0/C0 \) (data not shown).

In order to evaluate the \textit{in vitro} susceptibility of the GpiP mutants to caspofungin, MICs of caspofungin were determined using the CLSI (former NCCLS) microbroth dilution method M27-A2 (NCCLS, 2002). Several strains had different susceptibilities to caspofungin compared to the reference strain DAY286. The bank of 45 mutants were then screened for altered growth on SC-plates containing caspofungin. Strains \( mid1/C0/C0 \), \( ssr1/C0/C0 \) and \( pga31/C0/C0 \) were hypersensitive to caspofungin and \( pga62/C0/C0 \), \( dfg5/C0/C0 \), \( phr1/C0/C0 \) and \( pga4/C0/C0 \) showed reduced susceptibility (Fig. 2).

Out of the 45 mutants tested, 9 mutants were affected by the cell wall perturbing agents including 7 that had altered caspofungin sensitivity in comparison to the parental strain (Table 2).

### 3.3. Analysis of the GpiP mutants with caspofungin-susceptibility phenotypes

The seven mutants with altered caspofungin sensitivity were characterized more precisely by monitoring three distinct features: (i) the overall cell wall architecture using electron microscopic imaging; (ii) activation of the PKC signalling pathway by monitoring the phosphorylation of Mkc1 and (iii) the cell wall chemical composition by quantifying the glucan and chitin content.

### 3.4. Cell wall architecture

Transmission electron microscopy (TEM) was used to look for gross changes in the cell wall structure of the mutants with altered caspofungin sensitivities. Although subtle changes in cell wall architecture may be missed by this approach significant changes in the thickness of the cell wall layer were observed. Strains that were less susceptible to caspofungin had thicker walls (Fig. 3) than the reference strain DAY286, and strains with increased caspofungin susceptibility had thinner walls. In the \( phr1/C0/C0 \) mutant, with a significantly thicker cell wall; the TEM images show an increase of

| Category | Description of mutant | Interrupted gene |
|----------|-----------------------|------------------|
| I        | Hypersensitive to CFW  | RBT1, HWP1       |
| II       | Hypersensitive to caspofungin | MID1 |
| III      | Hypersensitive to CFW and caspofungin | PGA31, SSR1 |
| IV       | Hypersensitive to CFW and | PGA62, PHR1 |
| V        | Only resistant to caspofungin | DFG5, PGA4 |

**Fig. 3.** TEM reveals GpiPs mutants have different overall cell wall thicknesses. Mid-exponential phase phase yeast cells grown in YPD were harvested, fixed, sectioned and examined by TEM (wild-type: 0.15 \( \mu m \)). Cell wall thicknesses were measured manually for at least 20 individual yeast cells. The asterisks indicate significantly different thicknesses (T-test, \( p < 0.05 \)). Caspofungin-resistant and sensitive strains are clustered on the chart.
the central low density layer, but not of the peripheral high density layers (mainly composed of mannoproteins) on the outer face of the cell wall. This implies that the chitin/glucan core is affected by the lack of Phr1. This is compatible with the predicted β-1,3-glucanosyl transferase function for Phr1.

3.5. Activation of the PKC pathway and caspofungin susceptibility

It has been suggested that C. albicans and S. cerevisiae respond to caspofungin by activating the protein kinase cell integrity pathway (PKC) (Bruno et al., 2006; Lesage et al., 2004; Liu et al., 2005; Reinoso-Martín et al., 2003; Walker et al., unpublished). In order to investigate this further, we examined by western analysis the phosphorylation status of Mkc1, the MAP kinase of the PKC integrity pathway in the GpiP mutants with altered caspofungin susceptibility (Fig. 4). Mkc1 was phosphorylated in dfg5–/–, phr1–/– and pga31–/– and little or no phosphorylation was observed in pga4–/–, pga62–/–, mid1–/–, ssr1–/– and the reference strain DAY286 (Fig. 4). The anti-phospho p42/44 antibody used to monitor Mkc1 phosphorylation serendipitously cross-reacts with the phosphorylated form of Cek1, the MAP kinase of the SVG pathway (Eisman et al., 2006). Cek1 phosphorylation was observed in phr1–/– and pga62–/– compared to the reference strain. As shown in Fig. 4, phosphorylated Mkc1 was detected in mutants with reduced and elevated susceptibility to caspofungin. This suggests that the differential sensitivities to caspofungin are not due to alterations in the status of the PKC pathway alone.

3.6. Measurement of cell wall chitin and glucan content

Altered sensitivities to cell wall perturbing agents indicate modified cell wall properties. The crucial role of the cell wall in fungal viability implies that the cell has developed ways to cope with most alterations of its envelope. It has been shown that one way cells respond to cell wall defects is to modify their cell wall composition (Popolo et al., 2001). We investigated cell wall remodelling in the GpiP mutants by quantifying their cell wall chitin and glucan content. The phr1–/– and pga62–/– mutants have a significant increase in cell wall chitin content (Fig. 5). In contrast, mid1–/– and pga31–/– have significantly decreased chitin levels. Whereas, pga4–/–, dfg5–/– and ssr1–/– have wild type chitin levels. In general terms such marked differences were not observed in glucan levels however, phr1–/–, pga62–/–, pga4–/–, dfg5–/– and ssr1–/– have significantly increased cell wall glucan content, while the mutants mid1–/– and pga31–/– conserve their wild type glucan levels (Fig. 5).

4. Discussion

The ability to disrupt genes rapidly in C. albicans is a powerful tool to study gene function. The availability of C. albicans genome sequences allows reverse genetics strategies to be employed on a large scale. Equivalent approaches have been carried out to analyse chlamydospore formation, fluconazole resistance and biofilm formation (Bruno and Mitchell, 2005; Nobile et al., 2003; Nobile and Mitchell, 2005; Richard et al., 2005). The approach described here was not focussed on a specific biological process or a definite function, but on a specific cellular component: the cell wall. Using the UAU1 method and the resources of the Candida community, we obtained a library of 45 mutants representing almost 40% of the candidate GpiP genes identified in silico (Richard and Plaine, 2007). This represents the largest library of GpiP mutants and thus is a significant step towards unveiling the roles of all the GpiPs of C. albicans. In this study, we considered all the predicted GpiPs but a complete analysis supported by localization and biochemical data will be necessary to ascertain whether these are true GPI-anchored cell wall proteins.

The mutant library was subjected to a series of phenotypic tests that evaluated their ability to grow in a variety of conditions including altered pH, high temperature and oxidative stresses. In total 13 conditions were tested and 15 mutants were identified as showing a phenotype different to the reference strains (see Supplementary Data S1). We observed distinct phenotypes in mutants of only some members of small gene families such as PAG29/PAG30/PAG31, PAG4/PAG5/PHR1/PHR2 and HW1P1/RBT1/PAG8 suggesting that members of GpiP families have often evolved specific functions. We decided to focus on the mutants affected in cell wall integrity and antifungal sensitivity. Nine mutants tested displayed a phenotype different to the reference strain under at least one condition tested. Moreover, seven of the nine mutant strains exhibited altered caspofungin susceptibility. Further characterization of these mutants revealed significant changes in chitin and glucan levels accompanied by cell wall thickness changes. Activation of the PKC cell integrity signalling pathway was another indication of the important role of GpiPs in maintaining a robust cell wall. Thus, this preliminary survey in C. albicans allowed us to identify some novel GpiPs involved in cell wall structure and resistance to cell wall perturbing agents. These putative GpiPs may have a structural role, or may contribute to the biosynthesis or assembly of the major cell wall components or may function in cell wall remodelling.
4.1. Calcofluor sensitivity and its relation with cell wall composition

In the majority of cases hypersensitivity to CFW can be correlated with an increase in the cell wall chitin content. Chitin synthesis is activated in order to counteract a weakening of the cell wall as demonstrated in gpi7/C0/C0/C0 mutants (Richard et al., 2002b). To examine the relationship between CFW sensitivity and elevated chitin content we quantified the chitin content of the mutant strains that were hypersensitive to CFW and examined the level of fluorescence of CFW-stained cells. The pga62/C0/C0/C0, phr1/C0/C0/C0 (Fig. 5) and rbt1/C0/C0/C0 (L. Walker, N. Gow and C. Munro, unpublished data) mutants had a significant increase in cell wall chitin content. However, ssr1/C0/C0/C0 (Fig. 5) and hwp1/C0/C0/C0 (L. Walker, N. Gow and C. Munro, unpublished data) had wild type chitin levels. Pga62 was also hypersensitive to CFW. PGA62 and its paralog PGA59, both encode small GPI-proteins of unknown function. This result suggests that Pga62 is involved in cell wall integrity and that Pga59 cannot compensate for the absence of Pga62 under the conditions tested. Pga59 and Pga62 may be capable of functional redundancy but they may be expressed under completely different conditions thus disabling any functional compensation as is the case for Phr1 and Phr2. The overexpression of Pga59 in a pga62/C0/C0/C0 mutant background would be an interesting experiment to question the redundancy of function in this small family. The pga62/C0/C0/C0 mutant in our screen had increased chitin levels and decreased glucan levels, probably as a consequence of cell wall remodelling. Therefore, these results suggest that Pga62 is likely to play a role in cell wall biosynthesis but we cannot define a specific function from this analysis.

Fig. 5. Measurement of cell wall chitin and glucan content Cell wall preparations of three replicates of each strain were subjected to different hydrolysis in order to quantify chitin and glucan levels (see Section 2). The asterisks indicate significant results using the T-test with p < 0.05. In addition CFW-stained cells are included as a qualitative measure of cell wall chitin. Caspofungin-resistant and sensitive strains are clustered on the chart.
been suggested that Ssr1 might participate in the assembly of cell wall components to give the final organization of the cell wall (Garcera et al., 2005). The cell wall defects of the ssr1Δ/Δ, reflected in the lower level of glucan, may not be sufficient to trigger the PKC cell wall salvage pathway or activate compensatory chitin synthesis.

Interestingly, in the pga31Δ/Δ mutant the chitin content was significantly lower than the reference strain despite CFW and SDS hypersensitivity and activation of the PKC pathway (see below), which is unusual knowing the normal course of events during cell wall remodelling. This suggests alternative hypotheses, which are not mutually exclusive, that Pga31 is part of the cell wall salvage pathway or that Pga31 is involved in the regulation or assembly of chitin. In the latter case the decrease of chitin would be a direct consequence of the absence of Pga31, and the hypersensitivity to CFW could be explained by a weakened cell wall in which the low residual chitin content is essential for integrity. The pleiotropic phenotypes of pga31Δ/Δ (supplementary data S1) suggest an important role in cell wall biosynthesis and integrity. This is supported by the finding that PGA31 gene expression was induced during protoplast regeneration (Castillo et al., 2006). Pga31 is a member of a three-protein family of unknown function, sharing 59% and 54% amino acid sequence similarity with Pga30 and Pga29, respectively. A mutant lacking Pga29 is not currently available, but the pga30Δ/Δ mutant contained in our library, was tested and did not display any of the pga31Δ/Δ—phenotypes. This implies that Pga31 has a unique function that cannot be compensated by its paralogs in the conditions tested.

The rbt1Δ/Δ (Regulated By Tup1) mutant was hypersensitive to CFW but not to any other cell wall perturbing agent (Fig. 2). Rbt1 has been characterized in silico as being cell wall localized (Alberti-Segui et al., 2004) and prior studies indicated that mutants lacking RBT1 have significantly reduced virulence (Braun et al., 2000). Interestingly, RBT1 and HW1P1 share high identity over the entire sequence except in the extracellular domain, which in the case of Hwp1, is a substrate for transglucosaminases (Staab et al., 1999). Both of these proteins are hypha-induced (Ihmels et al., 2005) and regulated by Tup1 (Braun et al., 2000), and both may have adhesion properties to different substrates. We show here that inactivation of both genes results in CFW hypersensitivity, but rbt1Δ/Δ—was neither hypersensitive to SDS nor affected in hyphal formation contrary to hwp1Δ/Δ—(data not shown). This suggests that the two paralogs perform related, but non-redundant functions. Staab et al. (1999) reported that Hwp1, might mediate the stable attachment of C. albicans hyphae to human buccal epithelium cells by acting as a substrate for mammalian transglucosaminase. The SDS and CFW hypersensitivity of hwp1Δ/Δ— imply a marked defect in cell wall composition and structure, which may also explain the filamentation defect. In conclusion, there are reasons to suggest that the role of Hwp1 is not only to serve as a substrate for transglucosaminase, but also to participate in cell wall architecture and in hyphal differentiation.

### 4.2. Caspofungin susceptibility and its relation to cell wall composition and thickness

Microarray studies reported that caspofungin induces expression of several genes encoding cell wall proteins and cell wall maintenance proteins both in S. cerevisiae and C. albicans (Bruno et al., 2006; Lesage et al., 2004; Liu et al., 2005; Reinoso-Martín et al., 2003).

Echinocandins target cell wall biosynthesis by inhibiting the production of β-1,3-glucan, which in S. cerevisiae and C. albicans represents the main structural skeleton of the cell wall to which all other components are cross-linked (Klis et al., 2002). Consequently echinocandins are fungicidal to C. albicans. We found no absolute correlation between caspofungin and CFW sensitivity or PKC pathway activation; but we speculate that in some cases at least the strains (phr1Δ/Δ and pga62Δ/Δ) that were more resistant to caspofungin may have triggered a cell wall salvage mechanism that resulted in elevated chitin levels. Increased chitin synthesis has already been associated with reducing caspofungin susceptibility (Stevens et al., 2006; Walker et al., 2008). The opposite situation is also observed with strains that have a weaker cell wall because they have low chitin content, and thus are more susceptible to caspofungin treatment like pga31Δ/Δ, and mid1Δ/Δ. However, in other mutants with altered caspofungin sensitivity (dfg5Δ/Δ, pga4Δ/Δ and ssr1Δ/Δ) chitin levels were similar to the parental strain DAY286 (see Fig. 3).

Interestingly, the glucan composition does not give a clear clue to explain the caspofungin susceptibility phenotypes, this may be because the glucan is not the target of caspofungin but the product of the enzyme targeted.

Interestingly, we found the same correlation between caspofungin susceptibility and cell wall thickness and between caspofungin susceptibility and chitin content: pga31Δ/Δ and mid1Δ/Δ— have a thin cell wall and are sensitive whereas phr1Δ/Δ and pga62Δ/Δ— have thick cell wall and are more resistant. Consequently, thickness and chitin content are here tightly linked: low chitin content is associated with thin cell wall and vice versa.

We observed that the disruption of MID1 blocked the growth of the fungus only in presence of caspofungin. Mid1 plays a role in the calcineurin response in S. cerevisiae and C. albicans (Brand et al., 2007). It is required for the normal response to iron stress in both yeasts (supplementary data S1). Moreover, in C. albicans the growth of calcineurin mutants are affected not only by cell wall disturbing agents but also osmotic stress agents and azole treatment (Sanglard et al., 2003), a phenotype not shared by the Camid1Δ/Δ mutant. In addition, the very low amount of chitin in the mutant cells indicates that Camid1Δ/Δ— is unable to produce a mature cell wall, suggesting the function of C. albicans Mid1 might have diverged from that of S. cerevisiae. Our work also suggests an essential and specific role of CaMid1 in caspofungin tolerance.

In S. cerevisiae, the screening of a yeast mutant collection for altered sensitivity to caspofungin identified 39 resistant mutants (Lesage et al., 2004). The genes deleted in these mutants encoded proteins involved in cell wall assembly such as Fks2, Slg1 (a sensor for the cell integrity pathway) and Tus1 (a GDP-TP exchange factor for Rh1); but no GpiPs were included. In our study, two of the mutants conferring reduced sensitivity to caspofungin belong to components affecting glucan assembly and remodelling: phr1Δ/Δ— and pga4Δ/Δ—. As described above, Phr1 is required for the proper cross-linking of β-1,3- and β-1,6-glucans. These results suggest that Phr1 and Pga4 could be additional targets for caspofungin due to their roles in β-1,3-glucan assembly and maturation, which explains the phenotypes observed. The pga62Δ/Δ— and dfg5Δ/Δ— mutants were also less susceptible to caspofungin. Pga62 and Dfg5 participate in the maintenance of cell wall integrity but their precise functions remain unknown. Dfg5 and its paralog Dcw1 have some homology to bacterial mannosidases and may participate in the translocation of GpiPs from the membrane to the wall. One possibility is that Pga62 and Dfg5 mediate effects of the drug or that the effects of cell wall remodelling due to their absence makes the cell more resistant to caspofungin.

### 5. Conclusion

Our results highlight three aspects related to caspofungin sensitivity: (i) chitin levels greatly influence the sensitivity to this drug, high chitin content confers reduced susceptibility while low chitin content confers hypersensitivity; (ii) in the majority of the cases a link can be drawn between thickness of the cell wall, chitin level...
and sensitivity to caspofungin treatment: thick cell wall have a higher concentration of chitin and confers resistance and vice versa; (iii) surprisingly very little correlation can be found between cell wall glucan content or PKC pathway activation and the sensitivity to caspofungin treatment. These observations cannot be generalized for all strains but give new insights in the study of caspofungin resistance.

In terms of gene discovery, this work emphasizes the importance of two new proteins for C. albicans cell wall integrity Pga31 and Pga62. Pga31 mutant strain shows pleiotropic phenotypes with large modifications in cell wall composition and caspofungin sensitivity, suggesting a function in the early steps of cell wall bio-synthesis or in cell wall salvage pathways. The absence of Pga62 strongly alters the cell structure and composition in the opposite way to Pga31 and consequently renders the strain more resistant to caspofungin. The less pronounced phenotypes suggest a function in the late stage of cell wall assembly. This library allowed the identification of three genes that when deleted lead to increased caspofungin sensitivity Ssr1, Mid1 and Pga31. We also identified four genes that when deleted lead to decreased caspofungin sensitivity: Dfg5, Phr1, Pga4 and Pga62. In the literature, no other examples of deletion mutants resistant to caspofungin have been found in C. albicans apart from strains with point mutations in the FKS1 target gene (Park et al., 2005). This work gives new leads to study cell wall assembly mechanisms and caspofungin resistance emergence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.08.003.

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