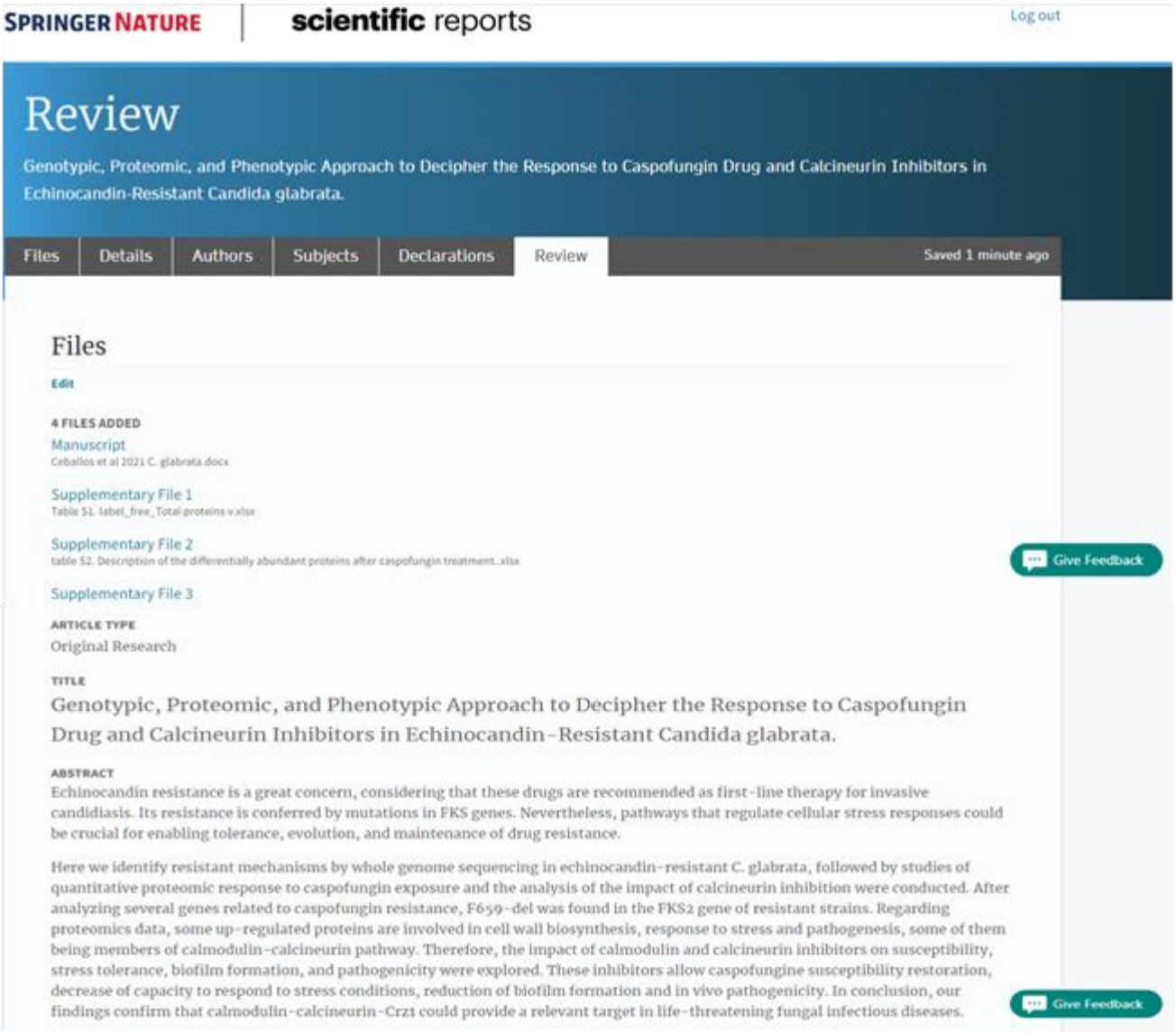

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Genotypic, Proteomic, and Phenotypic Approach to Decipher the Response to Caspofungin Drug and Calcineurin Inhibitors in Echinocandin-Resistant *Candida glabrata*.

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Abstract

Echinocandin resistance is a great concern, considering that these drugs are recommended as first-line therapy for invasive candidiasis. Its resistance is conferred by mutations in *FKS* genes. Nevertheless, pathways that regulate cellular stress responses could be crucial for enabling tolerance, evolution, and maintenance of drug resistance.

Here we identify resistant mechanisms by whole genome sequencing in echinocandin-resistant *C. glabrata*, followed by studies of quantitative proteomic response to caspofungin exposure and the analysis of the impact of calcineurin inhibition were conducted. After analyzing several genes related to caspofungin resistance, F659-del was found in the *FKS2* gene of resistant strains. Regarding proteomics data, some up-regulated proteins are involved in cell wall biosynthesis, response to stress and pathogenesis, some of them being members of calmodulin-calcineurin pathway. Therefore, the impact of calmodulin and calcineurin inhibitors on susceptibility, stress tolerance, biofilm formation, and pathogenicity were explored. These inhibitors allow caspofungine susceptibility restoration, decrease of capacity to respond to stress conditions, reduction of biofilm formation and *in vivo* pathogenicity. In conclusion, our findings confirm that calmodulin-calcineurin-Crz1 could provide a relevant target in life-threatening fungal infectious diseases.

Keywords: *Candida glabrata*, caspofungin resistance, calcineurin inhibitors, proteomic.

Introduction

The echinocandins drugs are recommended as first-line therapy for invasive candidiasis because of their low toxicity and high efficacy, especially against azole-resistant *Candida* isolates [1,2]. Echinocandin resistance in *Candida spp*, being potentially associated with treatment failures, is conferred by mutations in "hot spot" regions of the target *FKS* genes that lead to amino acid substitutions in the 1,3-β-D-glucan enzyme [3,4]. In *C. albicans,* substitutions in Ser641 and Ser645 are the most frequent *FKS* mutations and cause the more pronounced resistance phenotypes [5]. In *C. glabrata,* the principal reported substitutions were Ser629 in Fks1 Ser663 and Phe659 in Fks2 [3,6,7], while natural polymorphisms have been described in *C. parapsilosis* and *C. guilliermondii FKS1* [8]. The ultimate consequence of these mutations is a significant decrease in the echinocandin affinity for the enzyme target and high minimum inhibitory concentrations (MICs) values. It is of importance to highlight that in some resistant isolates, no *FKS* mutations were identified and isolates with the same *FKS* mutations exhibit different resistance profiles indicating that other resistance mechanisms and putative target genes could be implicated [9].

In addition to these described resistance mechanisms, there is also a new hypothesis in which regulators of cellular stress responses could be crucial for enabling the evolution and maintenance of drug resistance [4,10,11]. Indeed, some cellular stress responses are governed by signaling pathways, the most studied being the cAMP, the calmodulin-calcineurin (CaM/CaL), the TOR (target of rapamycin), and the mitogen-activated protein kinase (MAPK) signaling pathways [11–14]. The CaM/Cal pathway formed by a complex of the proteins Cnb1, Cna1, Hsp90 and the transcription factor Crz1 in yeast is involved in calcium homeostasis, sphingolipid and cell wall biosynthesis, protein trafficking, ubiquitin

signaling, autophagy, adaptation to environmental changes and even more importantly, in the response to antifungal drug pressure (11,14-19). Crz1 is found downstream in the CaM/Cal pathway and is one of the main antifungal targets since Crz1 is not present in human cells. Once the transcription factor is activated by dephosphorylation mediated by the CaM/Cal-Hsp90 complex, it moves to the cell nucleus. Crz1 contains a C2H2 zinc finger motif that binds to a specific calcineurin dependent response element (CDRE) in the gene promoters, thus initiating the activation of ~87 genes, among which is the *FKS2* gene, involved in the caspofungin resistance [9,17].

The spread of antifungal resistance and the limited number of available antifungal drugs amplifies the need to identify new fungal targets for development of novel therapeutic alternatives. In this context, bearing in mind the biological importance and the multiple processes in which calcineurin is implicated, this protein has been proposed as a potential target for the design of new antifungals [18].

Calcineurin inhibitors such as Tacrolimus (Fk506) and cyclosporine A (CsA), which bind to the immunophilins FKBP12 and cyclophilin A, respectively, are well recognized as immunosuppressive drugs with potential antifungal properties[19,20].

In attempt to identify protein targets, proteomic approaches should be employed. This approach has been previously applied in *C. albicans* to study many aspects, including adaptive responses to osmotic stress, macrophage interaction, and antifungal exposure. These studies evidenced that pathways such as the MAPK signaling pathway played a significant role in all biological responses [21–26]. More recently, global proteomic analyses defined Hsp90 as a central hub of protein interaction networks, which include nuclear transport, metabolic enzyme and signaling. Furthermore, these studies revealed that a large

group of co-chaperones work together for stress regulation and contribution to drug tolerance [27].

To our knowledge, few proteomic studies have been done in *C. glabrata*. Among them, studies explored the implication of hyperadhesive proteins in host-pathogen interaction and biofilm formation, mechanisms of drug resistance mainly in biofilms and response to antifungal drugs such as clotrimazole and 5-flucytosine [28–31]. However, similar approaches to study antifungal response to caspofungin have not been previously described.

Based on this, the first objective of the present study was to identify resistant mechanisms by whole genome sequencing in clinical echinocandin-resistant *C. glabrata* isolates. We then describe the proteomic response to caspofungin exposure and determine the impact of calcineurin inhibition by FK506 and CsA inhibitors on susceptibility, stress tolerance, biofilm formation, and assess pathogenicity in the *Galleria mellonella* model.

Results

Whole genome sequencing reveals *FKS2* nucleotide deletion

Twenty-five genes associated with antifungal drug resistance were screened for mutations (see Materials and Methods – Molecular Evaluation for the complete gene list) (**Table 1)**. Although some mutations were identified in our resistant isolates (CAGL1256 and CAGL1875), only the *FKS2* gene exhibited a 3-nucleotide deletion (1974-CTT-1976), which has been previously reported to be associated with echinocandin-resistant phenotypes. All other observed mutations were found in both resistant and susceptible isolates. The 3-nucleotide deletion detected (**Figure 1A**), which preserves the same open reading frame, explained the single amino acid deletion at Fks2 (F659-del; see **Figure 1B**)

observed in the two resistant isolates. This amino acid deletion which confers resistance to the echinocandins, resides within the Fks2 hot spot 1 (**Figure 1C**).

Proteomic analysis of resistant *C. glabrata* cells treated with caspofungin reveals an increase of proteins related to stress adaptation and wall organization

A total of 1796 distinct proteins were identified (**Table S1)**. While 1509 of them were encoded by uncharacterized ORFs, 287 were encoded by characterized genes. Among these proteins, 53 were identified as less abundant (i.e., downregulated) and 220 proteins were identified as more abundant (i.e., upregulated) after caspofungine exposure (change in abundance ratio: caspofungin/control).

However, only 21 proteins, including 5 upregulated and 16 downregulated, were significantly different if significance (>1.5-fold change and p-value <0.05). Using gene ontology enrichment tools, several GO terms were found to be enriched from these 21 proteins. In the molecular functions, 14 terms were enriched. Among them, MAP kinase activity, drug binding, calcium-serine/threonine, ATPase activity, and ATP-binding were over-represented. Regarding biological process categories, 18 terms were enriched, including signal transduction, pathogenesis, response to stress/drug and wall biogenesis were over-represented (**Figure 2A**). Downregulated proteins, following caspofungin exposure, were mainly of enzymatic group: CAGL0I03300g (homologue to *Candida albicans* Bud16, here named as (Ca.Bud16), CAGL0K07744g (Ca.Ysa1), CAGL0K05813g (Ca.Ttr1), CAGL0J06952g (Ca.Idi1), CAGL0H09218g (Cg.Sdt1). The protein with the most negative differential ratio was CAGL0M08514g (Cg.Pir5), a protein associated with β-1,3-glucan strengthening. The more abundant proteins after caspofungin exposure were involved in DNA binding i.e., CAGL0M06831g (Cg.Crz1) CAGL0J11440g (Ca.Srp1), CAGL0L10021g (Ca.Dbp5), CAGL0C01683g (Ca.Isw1). Of interest are the proteins

involved in antifungal response CAGL0M06831g (Cg.Crz1) (CaM/Cal pathway), CAGL0J00539g (Cg.Slt2) (PKC pathway), CAGL0J11440g (Ca.Srp1). The corresponding protein abundance is presented in (**Figure 2B)**, and description of these proteins is provided in (**Table S2)**. Additionally, the genes from the 5 most abundant proteins following caspofungin exposure were evaluated for mutations, however, none were observed.

Concerning the 220 more abundant proteins after caspofungin treatment (only change in abundance ratio) (**Table S3**), gene ontology enrichment tools, revealed several enriched GO terms. Eleven molecular function terms were enriched, among them, ATP-binding, drug binding, kinase activator activity, and calmodulin binding. Regarding the biological process categories, 11 terms were enriched, among them, response to stress, growth, and cell wall organization/biogenesis. By using word enrichment, words related to "resistance", "integrity", "calcineurin", "stress" and "kinase" have emerged. Using the STRING tool, the diagram based on the *C. glabrata* proteomic findings after exposure to caspofungin identified relevant members of CaM/Cal (Cna1, Cnb1, Crz1) and PKC (Rho1, Pkc1, Slt2) pathways (**Figure 3**).

Considering the important role of the CaM/Cal pathway in antifungal response, and the significant change of Crz1 (to date there are no Crz1 inhibitors) after caspofungin exposure, we focused this study targeting up-stream CaM/Cal proteins using commercial inhibitors.

Calcineurin inhibition restores susceptibility of caspofungin-resistant *C. glabrata*

Pharmacological inhibition of calcineurin by tacrolimus (Fk506) and cyclosporine (CsA) (15 μg/mL) did not show any statistically significant decrease of susceptible (0916 and ATCC2001) or resistant (CAGL1256 and CAGL1875) *C. glabrata* growth. By contrast, in

presence of caspofungin, the inhibitors Fk506 and CsA allowed susceptibility restoration of resistant clinical isolates with a significance reduction of the >16 µg/mL MIC values to 0.25 and 0.5 µg/mL respectively (**Figure 4**).

Response to oxidative stress is independent of caspofungin-resistance phenotype and not correlated with calcineurin signaling

In order to understand the oxidative stress response in resistant *C. glabrata*, growth was assessed in presence of menadione, a cytotoxic quinone that generates superoxide. The four isolates grew in up to 0.2 mM menadione and addition of Fk506 and CsA did not show any significant modification. The significant growth reduction of the resistant isolate CAGL1875 suggested higher susceptibility to caspofungin in presence of 0.2 mM menadione. Since combinations of caspofungin and calcineurin inhibitors lead to complete growth inhibition out of any stress, the impact of 0.2 mM menadione addition could not be interpreted. In presence of 0.4 mM menadione, PUJ/HUSI 0916 and CAGL1256 strains maintained a similar growth rate, independent of the caspofungin resistance phenotype (**Figure 5A**).

Calcineurin inhibitors compromise growth of caspofungin-resistant *C. glabrata* in heat-shock conditions

The heat stress spot test at 37°C confirmed previous results concerning the effect of calcineurin inhibitors on caspofungin-resistant isolates (**Figure 4**). Heat shock at 40 °C did not have impact on isolate growth for all but ATCC 2021. Interestingly, at this temperature, the growth of caspofungin-resistant isolates was noticeably compromised by calcineurin inhibitors (**Figure 5B**).

Calcineurin inhibitors significantly reduces biofilm-forming capacity

The four isolates had the capacity to form biofilm in polystyrene microplate wells, as on polyurethane catheter pieces, but biofilm formation was lower in the more clinically relevant catheter model. Caspofungin treatment reduced biofilm formation in susceptible isolates especially in catheter piece model whereas no significant activity was detected for resistant isolates (**Figure 6 A-B).** By contrast, addition of calcineurin inhibitors to caspofungin significantly reduced the capacity of biofilm formation of resistant isolates, regardless of the model used ($p < 0.05$).

Calcineurin inhibition reduces *C. glabrata* pathogenicity in the invertebrate *Galleria mellonella.*

C. glabrata isolates typically lead to complete mortality of *G. mellonella*, 4-6 days post-infection. Treatment with caspofungin (1 µg/Larvae) increased the larva survival when infected with susceptible isolates but did not exhibit, as expected, any statistical modification for caspofungin-resistant isolates. However, addition of calcineurin inhibitors to caspofungin proved to be effective in prolonging survival ($P<0.05$). No larval mortality was observed in control larvae injected with an equivalent volume of PBS (**Figure 7**).

Discussion

C. glabrata is one of the most prominent *Candida* species detected in bloodstream isolates worldwide, typically exhibiting intrinsic resistance to azoles [32–35]. Moreover, echinocandin resistance in *C. glabrata* has increased, causing a serious clinical challenge [36]. Different mechanisms of resistance to echinocandins have been described, mainly associated with *FKS* genes alterations [6,9]. In this work, we employ whole genome sequencing to provide a

218 view of mutations involved in clinical caspofungin-resistant isolates targeting genes
219 previously associated with echinocandin resistance [9,37,38]. To date, only a single *FKS2* gene
220 deletion associated with caspofungin resistance was found; however, a larger
221 comprehensive comparative analysis is ongoing.

222 Herein, we describe the first proteome description of resistant *C. glabrata* after caspofungin
223 exposure, allowing us to identify 21 upregulated proteins. Taking into account that
224 inhibition of β-1,3-glucan biosynthesis is the principal mode of action of caspofungin,
225 which results in osmotic disruption of the fungal cell [39], enrichment of molecular function
226 and biological processes pathways were associated, as expected, with antifungal response,
227 cell wall biogenesis, PKC and CaM/Cal pathways modulation. These results, similar to
228 those previously reported in *C. albicans* [13,40], confirm the association of these processes in
229 the tolerance of *C. glabrata* to echinocandin exposure. Furthermore, we observed that
230 caspofungin exposure resulted in increased GO annotations related to stress adaptation such
231 as chemical and cation homeostasis, wall organization or biogenesis and response to heat.
232 This feature has been described by Hoehamer et al.,[21] as the important changes in the *C.*
233 *albicans* proteome in response to ketoconazole, amphotericin B, and caspofungin
234 treatments although in that study only antifungal-susceptible strains were included.

235 *C. glabrata* exposure to caspofungin resulted in an increased abundance of MAP kinase
236 Slt2 and Crz1 proteins which, being part of PKC and CaM/Cal pathways, respectively,
237 have been implicated in cell wall biogenesis and integrity. This compensation phenomenon,
238 also observed in *Saccharomyces cerevisiae* and *C. albicans* (Mkc1), constitutes a
239 mechanism of tolerance to caspofungin [13,14,41,42]. Mutants lacking *SLT2/MKC1* and *CRZ1*
240 are both sensitive to echinocandins in *in vitro* assays [14,43]. Nevertheless, Slt2 mutants have

been described as hypervirulent [44]. Other 3 proteins (CAGL0C01683g, CAGL0L10021g, CAGL0J11440g) were found with higher abundance after exposure to caspofungin, however, these proteins have not been characterized in *C. glabrata* to date. The protein CAGL0C01683g (Isw1) in *C. albicans* and *S. cerevisiae* has been described as a chromatin remodeling factor involved in the repression of the initiation of transcription. Isw1 also works in parallel with the NuA4 and Swr1 complexes in the repression of stress-induced genes [45]. The protein CAGL0L10021g (C.a, S.c Dpb5) is an ATP-dependent cytoplasmic RNA helicase involved in translation termination along with Sup45p (eRF1); it also has a role in the cellular response to heat stress [46]. Finally, the protein CAGL0J11440g (C.a, Sc Srp1) (importin-α) has nuclear import signal receptor activity and is involved in the degradation of proteins. Loss of Srp1 is lethal, although several temperature-sensitive mutants have been described [47,48]. Conversely, Pir5 protein was decreased in abundance in response to caspofungin exposure. Pir proteins are a structural constituent of cell wall and are associated with cell wall organization due to linkage to multiple β-1,3-glucan chains. The changes in cell wall composition of the Pir proteins are a consequence of the cell wall integrity pathway activation [49–51].This decrease of Pir5 abundance appears to be part of a general compensatory mechanism in response to cell wall weakening caused by caspofungin; consequently, the cell increases chitin and/or mannan production, a phenomenon reported in *S. cerevisiae* and *Candida spp* [49,52]. In a recently published study, all *Candida* species tested, except *C. glabrata* and *C. parapsilosis*, demonstrated a compensatory increase in cell wall chitin content in response to caspofungin treatment, therefore in *C. glabrata* the increase in the mannan content appears to be a compensatory mechanism [53].

Given the importance of CaM/Cal-Crz1 pathway in several biological processes, the impact of Fk506 and CsA calcineurin inhibitors was studied in temperature and oxidative stress conditions. Similar to previous studies, we confirm that CaM/Cal pathway is involved in thermotolerance, mainly at higher temperatures [54]. By contrast, according to our results, the inhibition of calcineurin does not appear to affect the growth of *C. glabrata* in oxidative stress. It is important to emphasize that the antioxidant capacity of *C. glabrata*, mainly associated with the catalase Cta1, is higher than that of *S. cerevisiae* and *C. albicans*. This catalase Cta1 is controlled by the transcription factors Yap1, Skn7, Msn2, and Msn4 and modulated by pathways other than the CaM /Cal pathway[55].

Biofilm formation is another important factor in the understanding of cellular disruption. Biofilms are thought to provide ecologic advantages such as protection from the environment, nutrient availability, metabolic cooperation, and acquisition of new traits. In general, *C. glabrata* biofilms possessed a higher density of cells comparatively to *C. tropicalis* and *C. parapsilosis* (hyphal and blastoconidia mix) biofilms. This may be implicated in the typical high degree of resistance of *C. glabrata* biofilms to azole antifungals and amphotericin B [56]. Biofilm eradication as a therapeutic approach is generally effective using echinocandins, as long as the isolate is drug susceptible[57]. In our study, planktonic caspofungin-resistant isolates maintain this characteristic in biofilm community state, even in the presence of high doses of caspofungin. Nevertheless, this situation can be reversed by addition of CaM/Cal inhibitors, as we demonstrated in the clinical-relevant model using polyurethane catheter pieces.

In a repurposing strategy as alternative to conventional antifungal therapy against caspofungin-resistant *C. glabrata*, our results establish an important role for CaM/Cal

inhibitors. These highly encouraging results could be supported by the well-recognized regulation of multiple biological processes governed by the CaM/Cal-Crz1, including over-expression of the *FKS2* gene which is involved in the resistance to the echinocandins. Effect of Fk506 and CsA on heat-shock tolerance or susceptibility restoration of biofilm to caspofungin could contribute to their *in vivo* activity. Indeed, in an invertebrate *Galleria* model of disseminated caspofungin-resistant *C. glabrata* infection, addition of CaM/Cal inhibitors to caspofungine enhances its efficacy, allowing a significant increase in larval survival. This is in concordance with previous data showing the role of CaM/Cal pathway in virulence of fungal species [54,58]. Despite these promising findings, non-immunosuppressive analogs of both FK506 and CsA with no cross-activity with calcineurin in human cells must be developed [19,59,60]. With regards to the CaM/Cal pathway, the challenge also will lie in focusing on the transcription factor Crz1, as recently explored for *Rhizoctonia solani* [61]. Transcription factors are now attractive as antifungal drug targets since they are evolutionarily divergent between fungi and humans and therefore can be exploited as selective targets [62].

In conclusion, this is the first work on caspofungin-resistant *C. glabrata* clinical isolates that includes phenotypic, proteomic, and genomic analysis of impact of CaM/Cal pathway inhibition. Our study provides proteomic evidence that select proteins in this pathway, such as Crz1, are more abundant after caspofungin exposure. In addition, inhibition of this pathway in the clinical isolates with an *FKS2* gene mutation changed their planktonic and biofilm susceptibility, thermotolerance, and finally pathogenicity. Furthermore, addition of CaM/Cal inhibitors to caspofungin restored the susceptibility of resistant isolates in an *in vivo Galleria* model. Synthesis of more specific antifungal compounds targeting this stress

response could be a successful therapeutic strategy for fighting life-threatening fungal diseases and increase of echinocandin resistance.

Materials and Methods

Microorganisms

One susceptible and two caspofungin-resistant *C. glabrata* isolates were studied. The first one, *C. glabrata* PUJ/HUSI 0916 was recovered from a blood culture of a hematopoietic stem cell transplant recipient admitted to the Hospital Universitario San Ignacio Bogota, Colombia. The caspofungin-resistant isolates CACG1875 and CACG1256 were obtained from blood and urine cultures of hospitalized patients in intensive care unit of Centre Hospitalier Universitaire de Nantes, France and identified by ITS sequencing in a previous study by our research team [11]. Isolates were categorized as susceptible or resistant to caspofungin according to the interpretative breakpoints of CLSI M60 2017 criteria (> 0.5 μg/mL resistant strain). In addition, the reference *C. glabrata* ATCC2001, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used in specific experiments, as described in results. In this investigation, no ethical approval was required. All patients are anonymized and only the code of strains was transferred, therefore, no informed consent was required. Our study confirmed that all methods were performed in accordance with the relevant guidelines and regulations.

Whole genome sequencing and identification of molecular resistance mechanism

The whole genome of the susceptible *(*PUJ/HUSI 0916) and resistant *(*CACG1875 and CACG1256) *C. glabrata* isolates were sequenced using Illumina paired-end sequencing platform with a read length of 300bp and an average read depth coverage of 300X. The

obtained raw read sequences were cleaned using *fastp* [63] and assembled with SPAdes v3.12.0 [64]. Gene prediction was carried out with Prodigal V2.6.3 [65] and the coding sequences (CDS) obtained were annotated using *blastn* [66], against the genome of *C. glabrata* ATCC2001 reference strain.

In this study, we decided to focus on the following twenty-five genes associated with antifungal drug resistance, including twenty previously described and five we identified as upregulated proteins following caspofungin exposure (Table 1): *i*) *CEK1* (CAGL0K04169g), *ii*) *CDC55* (CAGL0L06182g), *iii*) *CDC6* (CAGL0K00605g), *iv*) *DOT6* (CAGL0I05060g), *v*) *FKS1* (CAGL0G01034g), *vi*) *FKS2* (CAGL0K04037g), *vii*) *FKS3* (CAGL0M13827g), *viii*) *MKT1* (CAGL0J05566g), *ix*) *MOH1* (CAGL0F04631g), *x*) *MPH1* (CAGL0F04895g), *xi*) *MRPL11* (CAGL0J09724g), *xii*) *MSH2* (CAGL0I07733g), *xiii*) *PDR1* (CAGL0A00451g), *xiv) PHO4* (CAGL0D05170g), *xv*) *SNQ2* (CAGL0I04862g), *xvi*) *SUI2* CAGL0B03795g, *xvii*) *TCB1* (CAGL0J08591g), *xviii*) *TCB3* (CAGL0L11440g), *xix*) *TOD6* (CAGL0A04257g), *xx*) *TPK2* (*CAGL0M08404g*), and *xxi*) *CRZ1* (CAGL0L00605g), *xxii*) *SLT2* (CAGL0J00539g), *xiii*) *SRP1* (CAGL0J11440g), *xiv*) *DBP5* (CAGL0L110021g), *xv*) *SWI1* (CAGL0C01683g). For each of these genes, multiple sequence alignment (nucleotide and amino acid) and mutation identification was performed using T-Coffee [67] and JalView [65] for visualization.

Computational transmembrane regions predictions were carried out using RaptorX [68,69], Sable [70–73], TMHMM v. 2.0 [74], TOPCONS [75], TMpred [76], CCTOP [77,78], HMMTOP [79], and Phobius [80]. InterProScan 5 [81] was used for primary protein structure analysis.

Proteomic analysis

Resistant CAGL1875 isolate was grown for 7h at 30°C with shaking in YPD and YPD with caspofungin (0,5 µg/mL), four biological replicates were performed to each condition. For viability assay, propidium iodide was made. Cell extracts were obtained suspending cells in lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.5 mM PMSF, and 10 % of a mix of protease inhibitors (Pierce TM)) and disrupted by centrifugation adding glass beads (0.4-0.6 mm diameter) in a Fast-Prep system (Bio101, Savant) applying five 20s rounds at 5.5 speed with intermediate ice cooling. Cell extracts were separated from glass beads by centrifugation and the supernatant was collected and cleared by centrifugation. Protein concentration was measured by Bradford protein assay. Digestion and desalting of peptides were carried out in gel with trypsin, according to Sechi and Chait [82].

The desalted protein digest was analyzed by RP-LC-ESI-MS/MS in an EASY nLC 1000 System coupled to the Q-Exactive HF mass spectrometer through the Nano-Easy spray source (all from Thermo Scientific, Mississauga, ON, Canada). Peptide identification from raw data were carried out using Mascot v. 2.6.1 search engine through the Protein Discoverer 2.2 Software (Thermo Scientific). Database search was performed against SwissProt. Mascot Scores were adjusted by a percolator algorithm. The acceptance criteria for protein identification were an FDR < 1% and at least one peptide identified with high confidence (CI>95%). To determine the abundances of the identified peptides and proteins, a processing free label workflow was initiated in the first step. The recalibration of the masses was performed through a rapid search in Sequest HT against the database. Based on the positive identifications, an alignment of the chromatograms of all the samples with a tolerance of up to 10 min were established[83,84].

Subsequently, alignment of the retention times between the different analyzed samples for the quantification of the precursor ions was performed, taking into account the unique peptides and the razor peptides (i.e., peptides that can be assigned to more than one protein). Finally, the results were normalized to the total amount of the peptides, equaling the total abundance among the different samples. After the analyses were finalized, a final report presented the list of peptide groups and proteins with scaled abundances and selected ratios. The Proteome Discoverer application includes a feature for assessing the significance of differential expression by providing p-values for those ratios (p-value <0.05). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [85,86] partner repository with the dataset identifier PXD021578 and 10.6019/PXD021578.

Antifungal susceptibility testing

Antifungal susceptibility testing was carried out using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI-BMD), following the M27-A3 guidelines with slight modifications for the combination of caspofungin with the calcineurin inhibitors [87]. Briefly, isolates were subcultured on yeast extract peptone dextrose agar (YPD) and grown for 24 h at 35 °C. The yeast suspensions were prepared in liquid RPMI 1640 medium (Sigma-Aldrich) to a final concentration of $0.5x10^3$ -2.5 x 10^3 cells/mL. Yeast inoculum (100-µl) was added to a 96-well plate containing serial two-fold dilutions of caspofungin with or without inhibitors tacrolimus (Fk506) or cyclosporin A (CsA) (15 µg/mL). MICs were visualized, and densitometry was determined as the lowest concentration of drug that caused a significant decrease (MIC-2 or ≥50%) compared with that of the drug-free growth control after 48 h of incubation. Quality control was ensured by

testing the CLSI-recommended strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 [88].

Stress-related phenotypic assays

To examine the potential role of calcineurin in cellular protection of *C. glabrata* from heat and oxidative stresses, we assessed the impact of Fk506 and CsA, with or without caspofungin. For heat-shock stress, drop tests were performed by spotting serial dilutions of *C. glabrata* cells (10^6 to 10^3 cells/mL) onto YPD agar plates with Fk506 or CsA (15 µg/mL), caspofungin (1 µg/mL) or both compounds. The plates were incubated at 37°C or 40°C for 24 h. For oxidative stress, YPD plates were prepared as previously except that the medium was supplemented with the naphtoquinone menadione (0.2 and 0.4 mM). The plates were incubated at 37°C for 24 h[11,26].

Biofilm formation

The *C. glabrata* isolates were grown on Sabouraud medium (Biomerieux, France) and incubated at 30 °C for 24 h. Two hundred µL of *Candida* cell suspensions (10^6 cells/mL) in RPMI-1640 with MOPS adjusted to pH 7 were seeded in 96-well microdilution wells with or without GDHK-1325 250mm Gam polyurethane catheter pieces (Hechingen, Germany) and allowed to adhere for 24 h at 37°C. The non-adherent cells were then removed by gently washing twice with 300 µL PBS or by redisposing catheter pieces in new microplates wells. Caspofungin was added at 1 µg/mL with and without 15 µg/mL of the calcineurin inhibitors for 24 h incubation at 37 °C for biofilm adhesion phase. Then wells or catheter pieces were washed twice with PBS and finally 100µL of RPMI-1640 plus 10µL of 700µM resazurin (Sigma-Aldrich) was added to each well and incubated at 37°C for 4 h.

[89]. Fluorescence was measured at 560nm with an emission at 590nm. The results are expressed in arbitrary fluorescence unit (AU).

Invertebrate *Galleria mellonella* model

Killing assays were performed in *G. mellonella* as described by Fallon, 2012 [90]. Briefly, the larvae were obtained from a Scientia breeding facility (Cali-Colombia); larvae of late stages (fifth and sixth) between 250 to 330 mg and a length of approximately 2 cm were selected. A group of 10 larvae was used for each of the controls: absolute control, disinfection, and inoculation. To compare mortality, three biological replicates were performed with 10 larvae for each isolate evaluated. The isolates were grown in Sabouraud dextrose agar and incubated for 48 hours at 35 °C. Suspensions adjusted to $1x10^9$ UFC/mL using Neubauer chamber were used to inoculate 10 larvae per *Candida* isolate. Larvae receive 10µL of inoculum and caspofungin (1 µg/Larvae-100mg/L), Fk506 and CsA (15µg/mL), or their combination by injection into the last left and right proto-leg using a 0.5mL gauge insulin syringe. After inoculation, larvae were placed in Petri dishes and incubated in darkness at 37°C, the number of dead larvae was recorded daily.

Statistical Analysis

All experiments were performed on three independent biological replicates; survival curves were constructed using the method of Kaplan and Meier, then the curves were compared using the Log-Rank (Mantel-Cox) test. Statistical models were constructed and analyzed using PRISM software version 7.

Data availability

All experimental data are provided in the manuscript and in supplemental files, or available via ProteomeXchange with identifier PXD021578 (10.6019/PXD021578) and in the NCBI BioProject database with the accession number PRJNA648794.

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Authors' contributions

AC-G performed experiments and wrote the main manuscript, AC-G, LM, CG designed proteomic experiments and analyzed proteomic data, CA-M analyzed clinical implications, NEV-V conducted bioinformatics analysis of genomic data, and prepared figure 1, DME, JB isolates sequencing, AC, PLP, CP-G designed the experiments and wrote the final manuscript, and PLP, CP-G conceived the experiments and managed the resources. All authors have read and agreed to the published version of the manuscript.

Competing interest

P.L.P received grants from Astellas, Basilea, MSD and Pfizer and speaker’s fees from Gilead, Basilea, Pfizer and MSD. The other authors declare no conflict of interest.

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Tables and figures

Table 1. Genes associated with caspofungin resistance.

Figure 1. Multiple alignment of Fks2 (Beta-1,3-glucan synthase catalytic subunit 2) nucleotide and amino acid sequences among *C. glabrata* resistant and susceptible strains. Multiple nucleotide (A) and amino acid sequence alignments (B) that show a 3-nucleotide deletion (1974-CTT-1976) and a single amino acid deletion (F659-Del), respectively, occurring only in *C. glabrata* resistant strains. C.) A consensus *C. glabrata* Fks2 membrane protein structure and topology model predicted by seven different membrane protein secondary structure prediction servers and visualized with Protter [91]; hot spots 1 and 2 are showed in red, as well the position F659 in which occurs the single amino acid deletion associated with echinocandins resistance.

Figure 2.Label-free quantitative proteomics results. **(A)** Gene Ontology (GO) analysis of the proteins considered differentially abundant after caspofungine treatment. (M) molecular function and (P) biological process . The X-axis indicates the number of associated proteins **(B)** Protein abundance profile. Inside the brackets *Candida albicans* Orthologues names. Down-regulated proteins are marked green and up-regulated are marked red.

Figure 3. Analysis of proteins identified in caspofungin condition **(A).** Gene Ontology (GO) analysis of all the proteins whose abundance was modified after caspofungin treatment., MF (molecular function) and BP (biological process) (-) **(B)** Word enrichment that was created using the P-values and the full terms from the enrichment analysis via a program called GO summaries available at the FungiDB website: https://fungidb.org/fungidb/app/ **(C)** STRING protein-protein interaction diagram constructed with proteins of interest found more abundant after caspofungin exposure (confidence score 0.400, STRING V.11) * indicate the proteins predicted by STRING software. Filled protein nodes that signify the availability of protein 3D structural information is known or predicted

Figure 4. Caspofungin minimal inhibitory concentrations under basal and calcineurin inhibition. Resistant strains > 0.5 µg/mL (1875 and 1256).

Figure 5. Stress responses under CaM/Cal inhibition. (A) Strains were grown in the presence of 0.2 and 0.4 mM menadione (Med) (B) Heat shocked at 37°C and 40°C, with or without 1 µg/mL caspofungin (Cas) with or without 15 µg/mL Fk506, CsA. YPD* at 37°C, free of the drug was considered as growth control

Figure 6. Biofilm formation of *C. glabrata* isolates in microplate wells (A) and on catheter pieces (B), exposed to CsA, FK506, caspofungin and their combinations. The data are expressed as arbitrary fluorescence unit.

Figure 7. *Galleria* time-kill curves of caspofungin-susceptible (A, B) and resistant (C, D) *C. glabrata* isolates exposed to, caspofungin, calcineurin inhibitors and their combinations. The data are expressed as the percentages of survival. Log-rank (Mantel–Cox) test with p-values of < 0.05 was used to indicate statistical significance. As follows, $p < 0.05$* $p < 0.02$** and $p < 0.001$***.

Supplementary

Table S1. Total identified proteins.

Table S2. Description of the differentially abundant proteins after caspofungin treatment.

Table S3. Description and analysis of the proteins identified in YPD or CAS conditions.

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776 **Table 1.** Genes associated with caspofungin resistance.

Gene Symbol	Systematic name	Mutations	Mutation in resistant strains (1875-1256)	Resistance-associated mutation (+/-)
CEK1	CAGL0K04169g	-	-	-
CDC55	CAGL0L06182g	-	-	-
CDC6	CAGL0K00605g	R117K, V163A, K268R, R80K	-	-
DOT6	CAGL0I05060g	P104S	-	-
FKS1	CAGL0G01034g	G14S	-	-
FKS2	CAGL0K04037g	**F659-Del**, T926P	**F659-Del**	+
FKS3	CAGL0M13827g	A42V, T1676S	-	-
MKT1	CAGL0J05566g	N512K, A643T	-	-
MOH1	CAGL0F04631g	S15N	-	-
MPH1	CAGL0F04895g	-	-	-
MRPL11	CAGL0J09724g	-	-	-
MSH2	CAGL0I07733g	-	-	-
PDR1	CAGL0A00451g	V91I, L98S, D243N	-	-
PHO4	CAGL0D05170g	S327N	-	-
SNQ2	CAGL0I04862g	P1104H	-	-
SUI2	CAGL0B03795g	-	-	-
TCB1	CAGL0J08591g	Q437E, K585R, N622K	-	-
TCB3	CAGL0L11440g	-	-	-
TOD6	CAGL0A04257g	P64S, D81N, N85D	-	-
TPK2	CAGL0M08404g	T132A, T158A	-	-
*CRZ1**	CAGL0M06831g	-	-	-
*SLT2**	CAGL0J00539g	-	-	-
*SRP1**	CAGL0J11440g	-	-	-
*DBP5**	CAGL0L110021g	-	-	-
*SWI1**	CAGL0C01683g	-	-	-

* Genes of proteins found in this study

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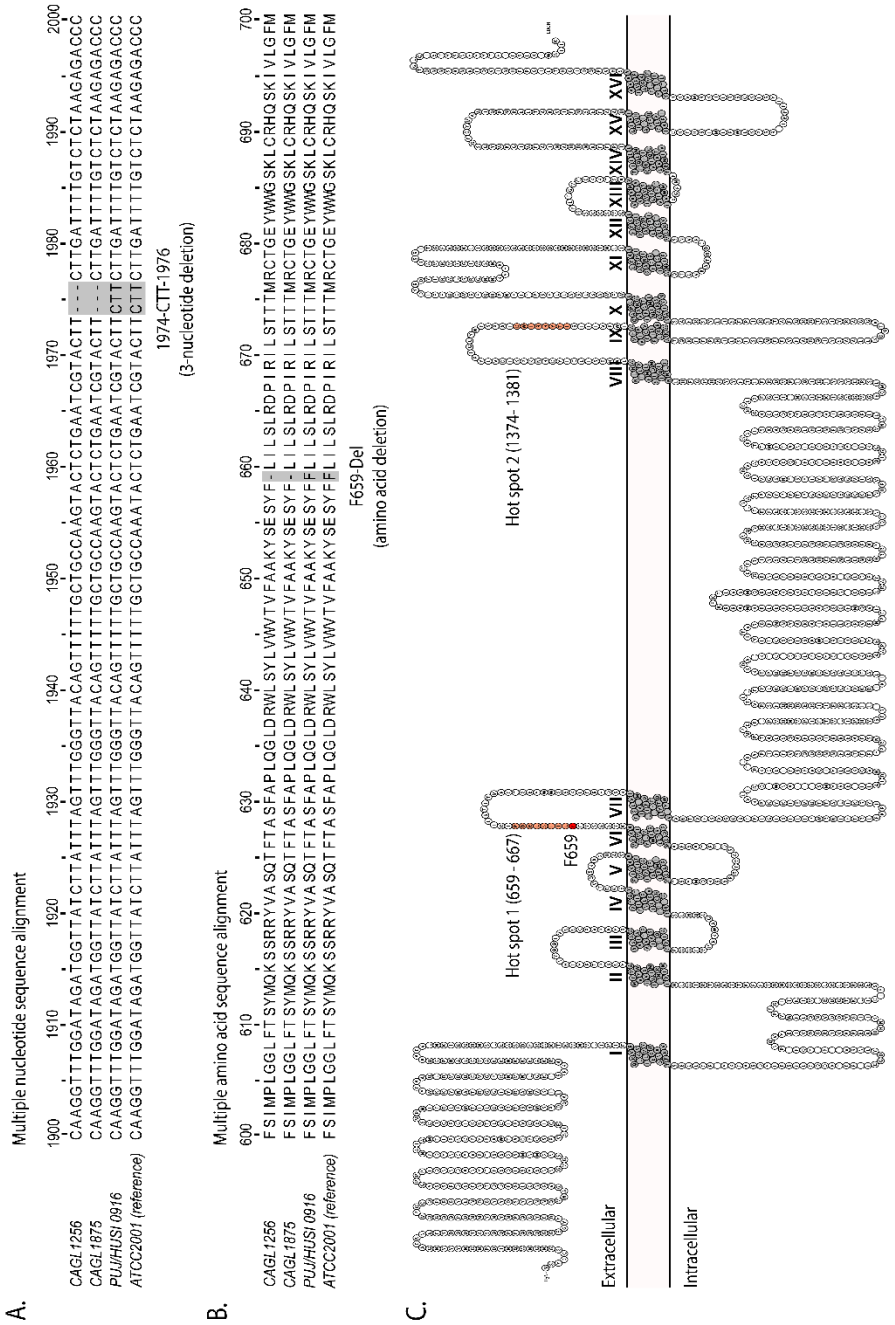

779 **Figure 1**. Multiple alignments of Fks2 (Beta-1,3-glucan synthase catalytic subunit 2) nucleotide and amino
780 acid sequences from *C. glabrata* resistant and susceptible strains. Multiple nucleotide (A) and amino acid
781 sequence alignments (B) that show a 3-nucleotide deletion (1974-CTT-1976) and a single amino acid deletion
782 (F659-Del), respectively, occurring only in *C. glabrata* resistant strains. (C) A consensus *C. glabrata* Fks2
783 membrane protein structure and topology model predicted by seven different membrane protein secondary
784 structure prediction servers and visualized with Protter [91]. Hot spots 1 and 2 are showed in red, as well the
785 position F659 in which occurs the single amino acid deletion associated with echinocandins resistance.

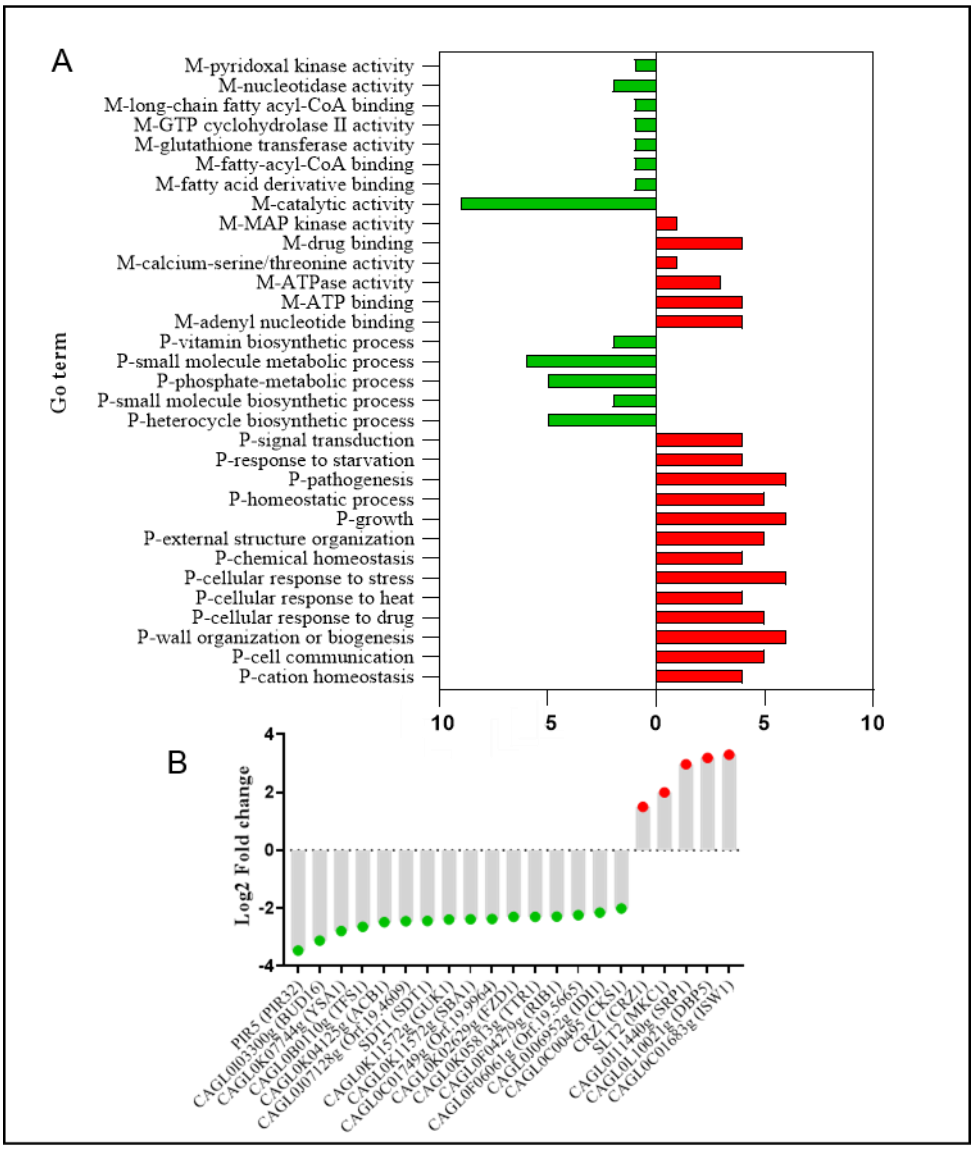

Figure 2. Label-free quantitative proteomics results. **(A)** Gene Ontology (GO) analysis of the proteins considered differentially abundant after caspofungine treatment. M = molecular function; P = biological process; X-axis indicates the number of associated proteins. **(B)** Protein abundance profile. Down-regulated proteins are marked green and up-regulated are marked red (*Candida albicans* orthologue names are inside the parantheses).

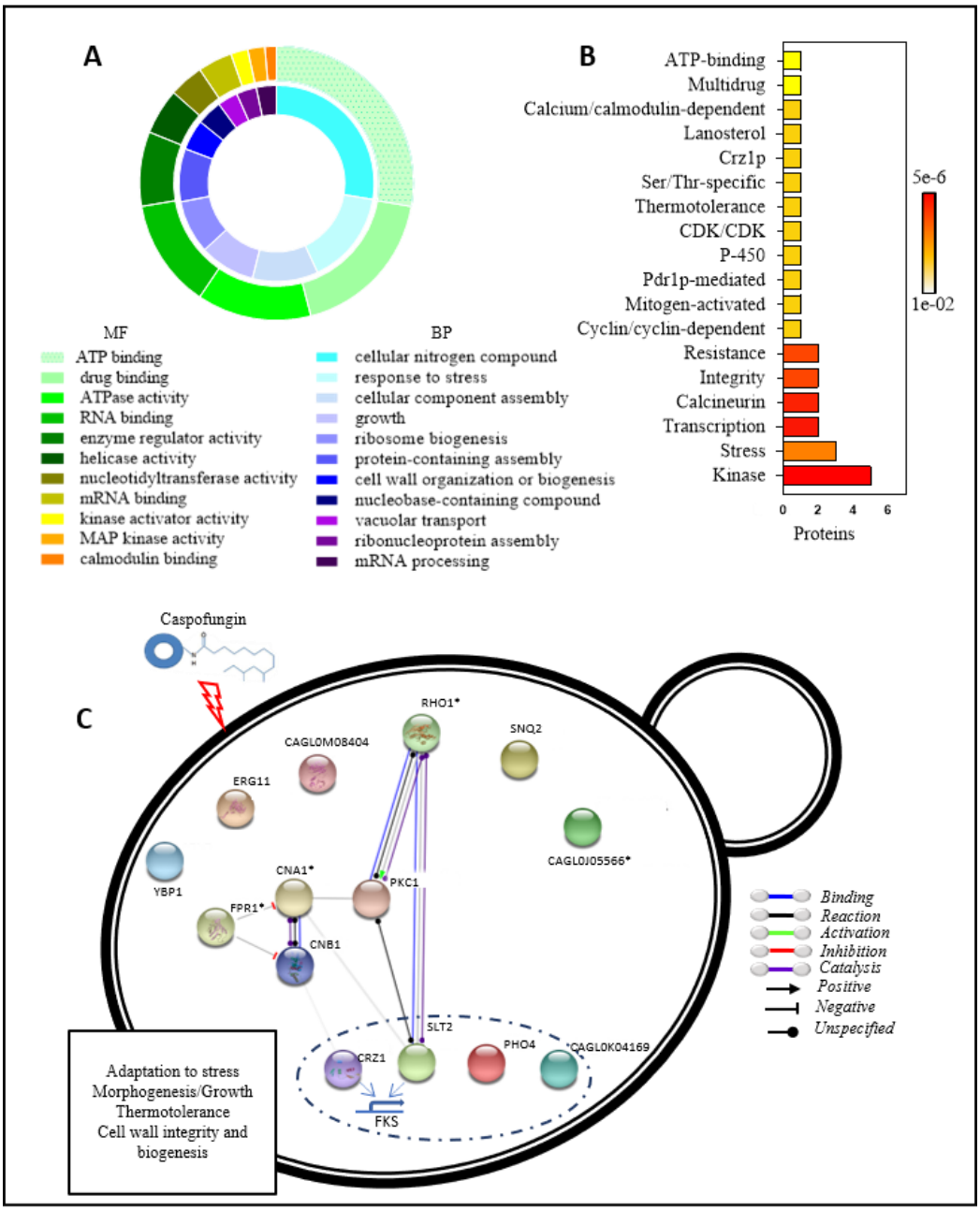

Figure 3. Analysis of proteins identified in caspofungin condition **(A).** Gene Ontology (GO) analysis of all the proteins whose abundance was modified after caspofungin treatment. MF = molecular function and BP = biological process. **(B)** GO Summary word enrichment created using the P-values and the full terms from the enrichment analysis. **(C)** STRING protein-protein interaction diagram constructed with upregulated proteins following caspofungin exposure (confidence score 0.400, STRING V.11). * indicate the proteins predicted by STRING software. Filled protein nodes that signify the availability of known or predicted protein 3D structural information.

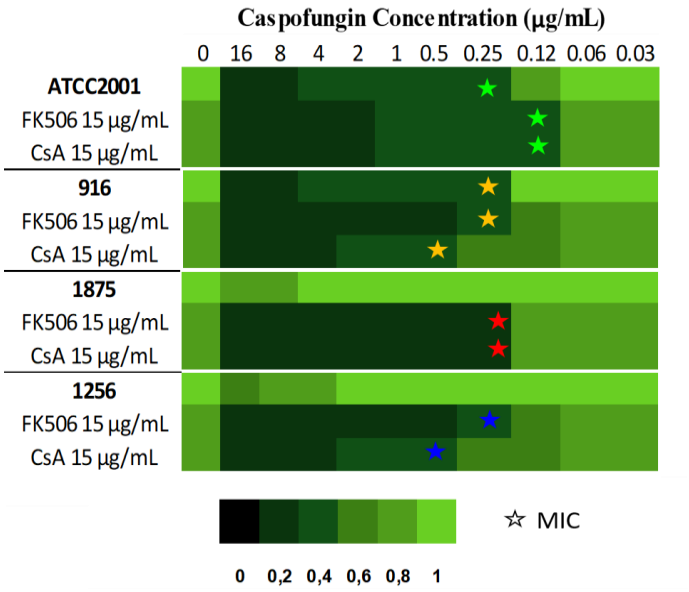

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Figure 4. Caspofungin minimal inhibitory concentrations under basal and calcineurin inhibition, resistant strains > 0.5 µg/mL (1875 and 1256).

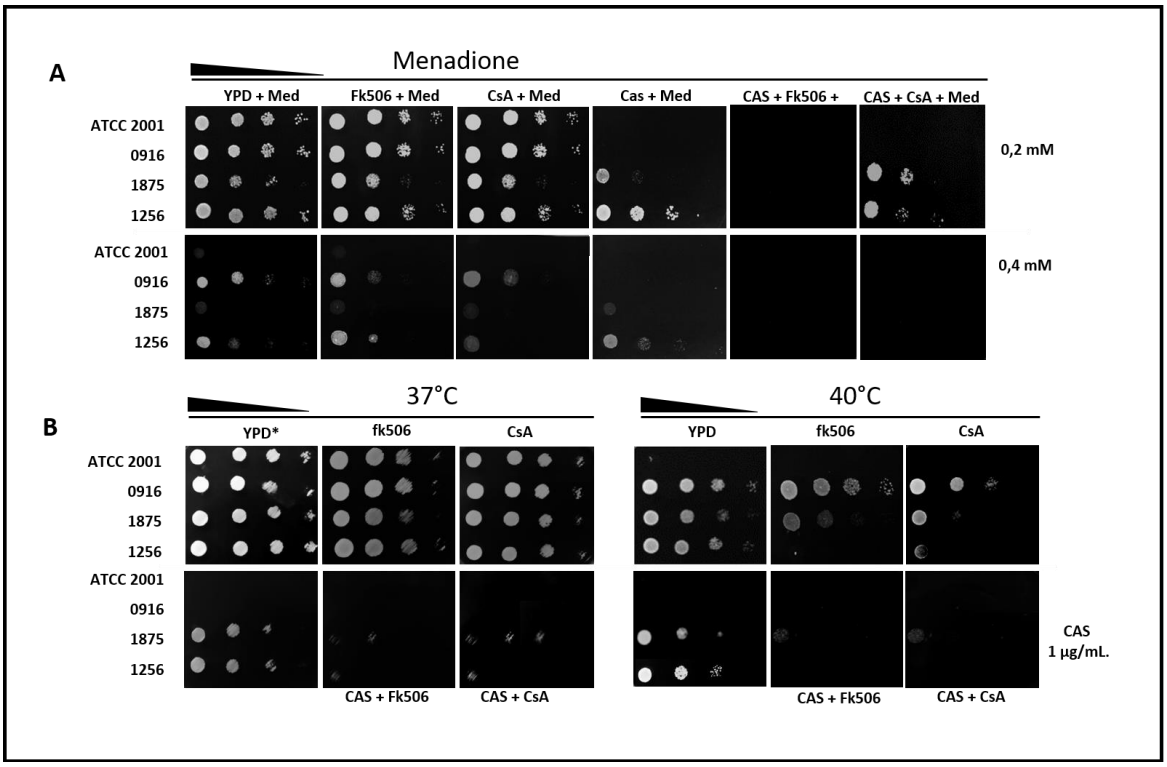

Figure 5. Stress responses under CaM/Cal inhibition. (A) Strains grown in the presence of 0.2 and 0.4 mM menadione (Med). (B) Strains heat shocked at 37°C and 40°C, with or without 1 µg/mL caspofungin (CAS), with or without 15 µg/mL Fk506, CsA. *YPD at 37°C was used as no drug growth control.

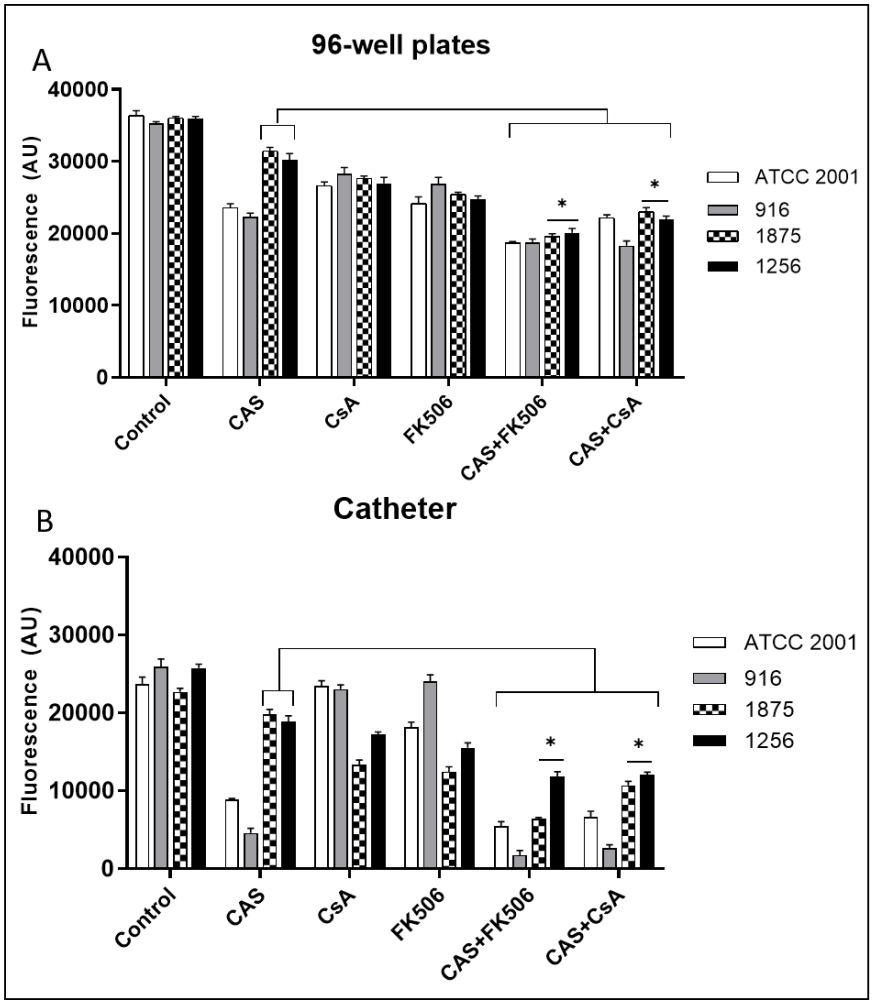

Figure 6. Biofilm formation of *C. glabrata* isolates grown (A) in microplate wells and (B) on catheter pieces; exposed to CsA, FK506, caspofungin and their combinations. Data are expressed in arbitrary fluorescence unit (AU).

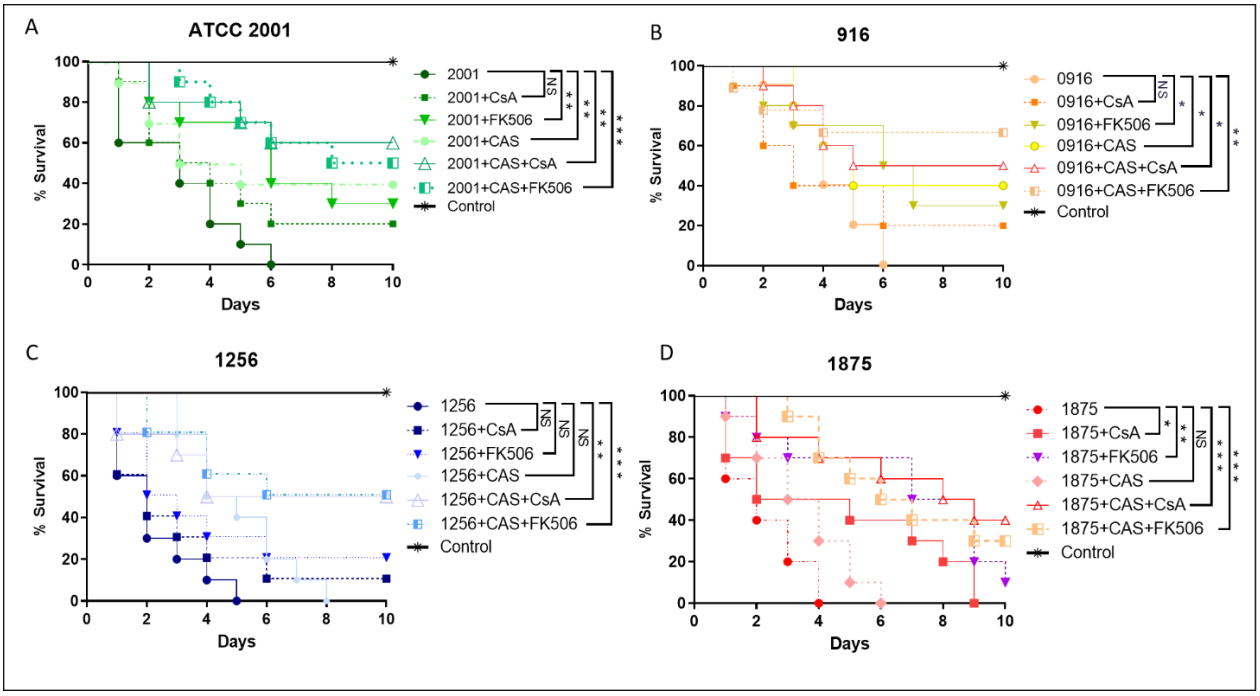

Figure 7. *Galleria* time-kill curves of caspofungin-susceptible (A, B) and resistant (C, D) *C. glabrata* isolates exposed to, caspofungin, calcineurin inhibitors and their combinations. The data are expressed as the percentages of survival. Log-rank (Mantel–Cox) test with p-values of < 0.05 was used to indicate statistical significance. As follows, *p < 0.05, **p < 0.02 and ***p < 0.001.