Membrane proteome-wide response to the antifungal drug clotrimazole in *Candida glabrata*: role of the transcription factor CgPdr1 and the Drug:H^+ Antiporters CgTpo1_1 and CgTpo1_2

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Abstract

Azoles are widely used antifungal drugs, which include triazoles, mostly used in the treatment of systemic infections, and imidazoles, such as clotrimazole, often used in the case of superficial infections. *Candida glabrata*, which is the second most common cause of candidemia worldwide, presents higher levels of intrinsic azole resistance when compared to *Candida albicans*. Since resistance often relies on the action of membrane transporters, including drug efflux pumps from the ATP-Binding Cassette (ABC) family or from the Drug:H^+ Antiporter (DHA) family, an iTRAQ-based membrane proteomics analysis was performed to identify all the proteins whose abundance changes in *C. glabrata* cells exposed to the azole drug clotrimazole. 53 proteins were identified, among these, the DHA transporter CgTpo1_2 was identified as overexpressed in the *C. glabrata* membrane in response to clotrimazole. Functional characterization of this putative drug:H^+ antiporter, and of its homolog CgTpo1_1, allowed the identification of these proteins as localized to the plasma membrane. They were found to be overexpressed under clotrimazole stress, therefore conferring azole drug resistance in this fungal pathogen by actively extruding clotrimazole to the external medium. The role of a cell wall remodeling protein, CgGas1, in azole drug resistance was also assessed. This membrane proteomics approach was also used to determine the overall role of the transcription factor CgPdr1 in the observed expression patterns, highlighting the existence of additional unforeseen targets of this transcription factor, recognized as a major regulator of azole drug resistance in clinical isolates.

Keywords: *Candida glabrata*, membrane proteomics, antifungal drug resistance, clotrimazole, CgPdr1, CgTpo1_1 and CgTpo1_2

Introduction

Candidemia represents the 4th most common nosocomial infection in humans [1]. The most common pathogenic yeast species is *Candida albicans*, with prevalence up to 55% in all *Candida* infections. Although the use of antifungal drugs has been relatively effective in fighting fungal infections by *C. albicans*, the continuous and extensive use of fungicides certainly contributed to the appearance of other relevant species, such as *Candida glabrata* [2]. Over the past 2 decades there has been an increase in the number of infections concerning non-

albicans species, with *C. glabrata* arising as the second most frequent pathogenic yeast in mucosal and invasive fungal infections in humans, representing 15-20% of all infections caused by *Candida* species, depending on the geographical region [3-6]. Together, *C. albicans* and *C. glabrata* represent 65%-75% of all systemic candidiasis, followed by *C. parapsilosis* and *C. tropicalis* [5].

C. glabrata is an haploid ascomycete yeast, closely related to *Saccharomyces cerevisiae*, both originating from a common ancestor who has undergone whole genome duplication [3, 5]. It is phylogenetically, genetically and phenotypically very different from *C. albicans*, and usually is not found in the environment but rather adapted to survival in mammals [7]. It is a ubiquitous commensal yeast, asymptomatically colonizing the gastrointestinal tract and genital tract of healthy hosts [5, 8], but it also acts as an opportunistic pathogen in immunocompromised patients [9], being known to cause mucosal and blood stream infections [10] in patients suffering from advanced HIV or cancer [8] in which their immune system is impaired locally or systematically.

Azoles are the main family of drugs presently used to treat *Candida* infections, including vaginal or oral candidemia and skin infections [11]. They inhibit fungal growth by interfering with the biosynthesis of ergosterol in the cell membrane [12, 13], more specifically, azole drugs bind the cytochrome P450 dependent enzyme lanosterol 14-α-sterol-demethylase encoded by *ERG11* in yeasts, which is involved in the conversion of lanosterol into ergosterol; the main sterol in fungal cell membranes. As a result, fungal cell membranes will be defective in ergosterol, resulting in defective structural properties, loss of fluidity and altered functions such as signaling, transport, exocytosis, endocytosis, and inhibition of cell growth due to accumulation of a toxic sterol produced by Erg3 [3, 12, 14].

C. glabrata presents higher levels of intrinsic resistance to azoles in comparison with *C. albicans* and develops further resistance during prolonged azole therapy. It is becoming clear that *C. glabrata* is highly prone to multidrug resistance development; therefore the dissemination and resistance mechanisms of this increasingly relevant pathogen should be carefully monitored in an effort to develop new solutions to treat fungal infections by *Candida* species.

One of the mechanisms postulated to be responsible for yeast cells becoming resistant to drugs is the expression of membrane proteins called multidrug resistance (MDR) transporters. An MDR transporter is an efflux pump that catalyses the extrusion of a variety of dissimilar toxic compounds from the cell, rendering cells resistant to multiple drugs. Multidrug resistance is a ubiquitous phenomenon causing several difficulties in the treatment of fungal infections. The most prominent MDR mechanism observed is the overexpression of membrane-associated transporters, responsible for the efflux of structurally and functionally unrelated compounds out of the cell. The most commonly found MDR transporters belong to the ATP-binding cassette (ABC) superfamily, such as the human P-glycoprotein (P-gp) [15]; and the Major Facilitator Superfamily (MFS). As major effectors of pleiotropic drug resistance in *C. glabrata*, expression of several of these transporters was shown to be dependent on the transcription factor CgPdr1 [16-18].

Antifungal drug resistance is an alarming clinical problem in the treatment of infections by fungal pathogens. Current treatments and antifungal agents presently used are becoming inefficient, making it imperative to study and understand the molecular mechanisms underlying multidrug resistance phenotypes in order to develop new antifungal therapeutics.

Materials and Methods

Strains, plasmids and growth media

Saccharomyces cerevisiae parental strain BY4741 (*MAT*a, ura3Δ0, leu2 Δ 0, his3 Δ 1, met15 Δ 0) was obtained from Euroscarf (http://web.uni-rankfurt.de/fb15/mikro/euroscarf/). Cells were batch-cultured at 30ºC, with orbital agitation (250 rpm) in MM4 medium, which consists in basal medium (BM) with the following composition (per liter): 1.7 g yeast nitrogen base without amino acids or $NH4^+$ (Difco), 20 g glucose (Merck) and 2.65 g $(NH4)_2SO_4$ (Merck), supplemented with 20 mg/L methionine, 20 mg/L histidine, 60 mg/L leucine, and 20 mg/L uracil (all from Sigma). The parental strain BY4741 was also batch-cultured at 30ºC, with orbital agitation (250 rpm), in rich Yeast Extract-Peptone-Dextrose (YPD) medium for transformation purposes, containing (per liter): 20 g glucose (Merck), 20 g peptone (HIMEDIA) and 10 g yeast extract (HIMEDIA).

Candida glabrata parental strain KUE100 [19] and derived single deletion mutants KUE100_*Δcggas1,* KUE100_*Δcgtpo1_1* and KUE100_*Δcgtpo1_2*, kindly provided by Hiroji Chibana, Chiba University, Japan; as well as the *C. glabrata* strains 66032u and 66032u_*Δcgpdr1* [20], kindly provided by Thomas Edlind, from the

Department of Microbiology and Immunology, Drexel University, College of Medicine, Philadelphia, PA, were batch-cultured at 30ºC, with orbital agitation (250 rpm) in BM medium, with the following composition (per liter): 1.7 g yeast nitrogen base without amino acids or $NH4^+$ (Difco), 20 g glucose (Merck) and 2.65 g $(NH4)_2SO_4$ (Merck). *C. glabrata* strain L5U1 (cgura3 Δ 0, cgleu2 Δ 0) [21], kindly provided by John Bennett, from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA, was grown in BM medium supplemented with 20 mg/L uracil and 60mg/L leucine. To maintain selective pressure over the recombinant strains, the addition of uracil to this medium was only carried out to grow the host yeast cells. For transformation purposes, L5U1 strain was batch-cultured at 30ºC, with orbital shaking (250 rpm) in rich YPD medium, with the following composition (per liter): 20 g glucose (Merck), 20 g peptone (HIMEDIA) and 10 g yeast extract (HIMEDIA). Solid media contained, besides the above-indicated ingredients, 20 g/L agar (Iberagar).

The plasmid pGREG576, used for gene and promoter cloning was obtained from the Drag&Drop collection [22].

CgTpo1_1 and CgTpo1_2 subcellular assessment

The subcellular localization of the CgTpo1_1 and CgTpo1_2 proteins was determined based on the observation of BY4741 *S. cerevisiae* or L5U1 *C. glabrata* cells transformed with the pGREG576_*CgTPO1_1* and pGREG576_*CgTPO1_2* or pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2* plasmids, respectively. These cells express the CgTpo1_1_GFP and CgTpo1_2_GFP fusion proteins, whose localization may be determined using fluorescence microscopy. *S. cerevisiae* cell suspensions were prepared in MM4-U medium containing 0.5% glucose and 0.1% galactose, at 30ºC, with orbital shaking (250 rpm), until a standard culture OD_{600nm} = 0.4 ± 0.04 was reached. At this point, cells were transferred to the same medium containing 0.1% glucose and 1% galactose, to induce protein expression. *C. glabrata* cell suspensions were prepared in BM medium, supplemented with 60 mg/L leucine, until a standard culture OD_{600nm} = 0.4 ± 0.04 was reached, and transferred to the same medium supplemented with 50 µM $CuSO_4$ (Sigma), to induce protein overexpression. After 5h of incubation, 2 mL of cell suspension were centrifuged and resuspended in distilled water. The distribution of CgTpo1_1_GFP or CgTpo1_2_GFP fusion proteins in *S. cerevisiae* or *C. glabrata* living cells was detected by fluorescence microscopy in a Zeiss Axioplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelength of 395 and 509 nm, respectively.

Antifungal susceptibility assays in C. glabrata

Susceptibility assays were performed based on spot assays in solid media. KUE100 and L5U1 *C. glabrata* cell suspensions used to inoculate the agar plates were mid-exponential cells grown in BM medium (KUE100) or BM supplemented with leucine and 50 µM $CuSO_4$ (Sigma), to induce protein overexpression (L5U1 harboring the recombinant plasmids) until culture OD_{600nm} = 0.4 ± 0.02 was reached and then diluted in sterile water to obtain suspensions with OD_{600nm} = 0.05 ± 0.005. These cell suspensions and subsequent dilutions (1:5; 1:25) were applied as 4 µL spots onto the surface of solid BM medium supplemented with adequate chemical stress concentrations. The tested drugs included the following compounds, used in the specified concentration ranges: the azole antifungal drugs ketoconazole (10 to 60 mg/L), fluconazole (20 to 80 mg/L), miconazole (0.08 to 0.14 mg/L), tioconazole (0.2 to 0.9 mg/L), itraconazole (5 to 40 mg/L), and clotrimazole (2.5 to 10 mg/L), the polyene antifungal drug amphotericin B (0.12 to 0.19 mg/L), the fluoropyrimidine 5-flucytosine (0.010 to 0.017 mg/L), the pesticide mancozeb (0.5 to 2.5 mg/L), and the polyamines spermine (2 to 4.5 mM) and spermidine (3 mM to 5 mM) (all from Sigma).

CgTPO1_1 and CgTPO1_2 expression measurements

The levels of *CgTPO1_1* and *CgTPO1_2* transcripts in *C. glabrata* 66032 and 66032_*Δcgpdr1* cells were assessed by quantitative real-time PCR. Total-RNA samples were obtained from cell suspensions harvested under control conditions (mid-exponential phase cells in the absence of drugs) or upon 1h of exposure to 60 mg/L clotrimazole.

cDNA for real-time reverse transcription-PCR (RT-PCR) experiments was synthesized from total RNA samples by using the MultiScribe Reverse Transcriptase kit (Applied Biosystems) and the 7500 RT-PCR thermal cycler block (Applied Biosystems). In the synthesis of cDNA, a reaction mixture of 10 µL was prepared using 1.0 µL TaqMan RT Buffer (10x), 2.2 µL $MgCl_2$ (25 mM), 2.0 µL dNTPs (2.5 mM), 0.5 µL Random hexamers (50 µM), 0.2 µL RNase inhibitor (20 $U.L^{-1}$), 0.25 µL MultiScribe reverse transcriptase (50 U. μL^{-1}), 2 µL RNA samples (500 ng. μL^{-1}) and 1.85 µL ddH_2O DEPC treated; while the program used for the cDNA synthesis consisted in an incubation (10 min, 25ºC), reverse transcription (30 min, 48ºC) and reverse

transcription inactivation (5 min, 95ºC) steps. The quantity of cDNA for subsequent reactions was diluted and kept at 10 ng. Before preparation of the second step reaction mixture, each cDNA sample was diluted 1:4 for a final volume of 40 µL. The subsequent real-time PCR step was carried out using SYBR Green® reagents (Applied Biosystems) in a mixture with the following composition: 12.5 µL SYBR® PCR Master Mix (2x), 2.5 µL forward primer (4 pmol. μL^{-1}), 2.5 µL reverse primer (4 pmol. μL^{-1}), 2.5 µL cDNA sample and 5.0 µL ddH_2O. The real-time PCR was carried out by 40 cycles of PCR amplification using the following setup: 10 min at 95ºC, 15 sec at 95ºC, and 1 min at 60 º C. Primers were designed using Primer Express® Software (Applied Biosystems).

[^{3}H]-clotrimazole accumulation assays

^{3}H-clotrimazole accumulation assays were carried out has described previously [23]. To estimate the accumulation of clotrimazole (Intracellular/Extracellular) from yeast cells, the parental strain KUE100 and the mutant strains KUE100_*Δgas1*, KUE100_*Δcgtpo1_1* and KUE100_*Δcgtpo1_2* were grown in BM to mid-exponential phase and were harvested by filtration. Cells were washed and were resuspended in BM to obtain cell suspensions with OD_{600nm} = 0.5 ± 0.05, equivalent to approximately 0.22 mg [(dry weight) mL^{-1})]. Readily, 0.1 µM ^{3}H-clotrimazole (1mCi/mL; American Radiolabelled Chemicals) and 30 mg/L of unlabeled clotrimazole were added to the cell suspensions. Incubation proceeded for an additional period of 30 min. The intracellular accumulation of ^{3}H-clotrimazole was monitored by filtering 200 µL of cell suspension, at adequate time intervals (1, 3, 6, 10, 20 and 30 minutes), through pre-wetted glass microfiber filters (Whatman GF/C). The filters were washed with ice-cold TM buffer (0.1 M MES hydrate, 41 mM Tris base, pH 4.5). To calculate the intracellular concentration of labeled clotrimazole, the internal cell volume (v_i) of the exponential-phase cells, grown in the absence of drug and used for accumulation assays, was considered constant and equal to 2.5 µL/mg dry weight, while the amount of cells was considered to be 0.45 mg/mL dried biomass [24]. Extracellular ^{3}H-clotrimazole was estimated by radioactivity assessment of 50 µL of the supernatant recovered by centrifugation.

B-1,3-glucanase susceptibility assay

To monitor structural changes at the cell wall level, a lyticase (β-1,3-glucanase, Sigma) susceptibility assay was conducted as described before [25]. The susceptibility of the parental strain KUE100 to an inhibitory concentration of the azole antifungal drug clotrimazole was compared to that of the deletion mutant KUE100_*Δcggas1*. Cells from both strains were grown in BM medium without aminoacid complementation, in the presence of 60 mg/L clotrimazole, and harvested following 0h or 30 min of cell incubation, during the period of early adaptation to stress, and at the exponential growth phase, until a culture OD_{600nm} of 1 ± 0.1 was reached. The harvested cells were washed with distilled water and resuspended in 0.1 mM sodium phosphate buffer (pH 7). After the addition of 10 µg/mL lyticase from *Arthrobacter luteus* (Sigma), cell lysis was monitored by measuring the percent decrease of the initial OD_{600nm} of the cell suspensions every 30 min for a total period of 3 hours.

Results

Membrane proteome-wide changes occurring in response to clotrimazole in C. glabrata

As expected, all 4 proteins present in the multidrug resistance transporters cluster are up-regulated upon exposure to the drug (Table 1). This observation is consistent with the previous characterization of these proteins as multidrug transporters, although several molecules have been found to be extruded by the proteins present in this cluster; the role in specific clotrimazole exposure had only been previously described for CgQdr2 [11]. CgCdr1 appears as the most up-regulated protein, which is consistent with the function of its *S. cerevisiae* homolog, Pdr5, as a determinant of clotrimazole extrusion [26] and with its characterization as an azole resistance determinant [27]. CgSnq2 has been characterized as a multidrug transporter [28], as well as its *S. cerevisiae* homolog [29, 30]. The up-regulation of CgSnq2 observed in this study predicts a possible role for this transporter in clotrimazole extrusion. *ORF CAGL0E03674g* (CgTpo1_2) was further found to be up-regulated in this functional group. Its *S. cerevisiae* homolog is known to confer resistance to spermine, putrescine and spermidine; catalyzing the extrusion of polyamines in *S. cerevisiae* [31]. The up-regulation of this transporter in clotrimazole exposed *C. glabrata* cells raises the possibility that this predicted MDR transporter plays a role in imidazole transport in *C. glabrata*. CgTpo1_2 is the less characterized protein present in this group, a fact that allied with the suggested role in clotrimazole response, makes this transporter an interesting object of further study, to deepen current knowledge on azole drug resistance mechanisms in *C. glabrata*. Additionally, a cell wall

remodeling protein, CgGas1, was also found to be overexpressed upon clotrimazole stress.

Table 1 - Set of multidrug resistance transporters found to have significant expression changes in *C. glabrata* in the presence of clotrimazole. Protein clustering was performed based on the role of their predicted *S. cerevisiae* homolog.

Multidrug resistance transporters			
C. glabrata ORF	***S. cerevisiae homolog***	**Description**	**Fold change**
CAGL0E03674g	*TPO1*	Uncharacterized. *S. cerevisiae* homolog encodes a polyamine transporter of drug:H(+) antiporter DHA1 family	1.44
CgQDR2 (CAGL0G08624g)	*QDR2*	Drug:H+ antiporter of the Major Facilitator Superfamily, confers imidazole drug resistance; activated by Pdr1p and in azole-resistant strain	4.29
CgSNQ2 (CAGL0I04862g)	*SNQ2*	Plasma membrane ATP-binding cassette (ABC) transporter; involved in Pdr1p-mediated azole resistance	2.78
CgCDR1 (CAGL0M01760g)	*PDR5*	Multidrug transporter of ATP-binding cassette (ABC) superfamily; involved in Pdr1p-mediated azole resistance; increased abundance in azole resistant strains	6.40

Effect of CgPdr1 deletion in the membrane proteome-wide changes occurring in response to clotrimazole in C. glabrata

Particularly interesting in this context are the 4 proteins which were found to be induced by clotrimazole in the dependence of CgPdr1: the multidrug transporters CgQdr1, CgSnq2 and CgCdr1; and the hexadecenal dehydrogenase Hfd1 (Table 2). These results are consistent with the characterization of several of these proteins as efflux pumps and their expression to be dependent on CgPdr1 in response to other chemical stress inducers [11, 27, 28]. The expression changes here obtained reinforce CgPdr1 as a major pleiotropic drug resistance mediator, and its role in mediating the expression of multidrug transporters in response to clotrimazole. The observation regarding the lipid metabolism related protein CgHfd1 is consistent with previous microarray studies, reporting the activation of CgHfd1 upon exposure to fluconazole induced stress in the dependence of CgPdr1 [16]. It would be interesting to assess whether this result may relate to the imidazole mode of action on lipid raft binding, with Hfd1 possibly intervening in plasma membrane lipid destabilization as a resistance mechanism dependent on CgPdr1. Interestingly, at least one CgPdr1-binding site is found in the promoter regions of these 4 genes, suggesting that the action of CgPdr1 in their expression may be direct.

Table 2 - Assessment of the fold change of multidrug resistance transporters in *C. glabrata* cells exposed to clotrimazole, in the dependence of the transcription factor CgPdr1. Protein clustering was performed based on the role of their predicted *S. cerevisiae* homolog.

Multidrug resistance transporters			
C. glabrata ORF	***S. cerevisiae homolog***	**Description**	**Fold change**
CAGL0E03674g	*TPO1*	Uncharacterized. *S. cerevisiae* homolog encodes a polyamine transporter of drug:H(+) antiporter DHA1 family	1.09
CgQDR2 (CAGL0G08624g)	*QDR2*	Drug:H+ antiporter of the Major Facilitator Superfamily, confers imidazole drug resistance; activated by Pdr1p and in azole-resistant strain	0.73
CgSNQ2 (CAGL0I04862g)	*SNQ2*	Plasma membrane ATP-binding cassette (ABC) transporter; involved in Pdr1p-mediated azole resistance	0.26
CgCDR1 (CAGL0M01760g)	*PDR5*	Multidrug transporter of ATP-binding cassette (ABC) superfamily; involved in Pdr1p-mediated azole resistance; increased abundance in azole resistant strains	0.03

CgTpo1_1 and CgTpo1_2 subcellular localization in C. glabrata and S. cerevisiae

Candida glabrata cells harboring the pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2* plasmids were grown to mid-exponential phase in minimal medium, and then transferred to the same medium containing 50 µM $CuSO_4$, to promote expression in moderate controlled levels through the *MTI* promoter. At a standard OD_{600nm} of 0.5 ± 0.05, cells were analyzed by fluorescence microscopy. The incubation time was defined as 5 hours to allow detectable protein expression levels, but not a high degree of overexpression which could lead to mis-localization. In *C. glabrata* cells, the CgTpo1_1_GFP and CgTpo1_2_GFP fusion proteins were found to be localized to the cell periphery (Figure 1).

These findings represent a first step into the functional characterization of the putative transporters CgTpo1_1 and CgTpo1_2, as their localization confirms their predicted role as plasma membrane proteins, while

showing some similarity to the better-known *S. cerevisiae* Tpo1.

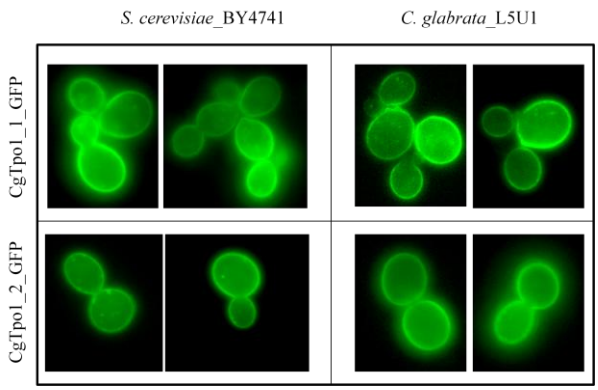

Figure 1 - Fluorescence of exponential phase *S. cerevisiae* and L5U1 *C. glabrata* cells, harboring the cloning vectors pGREG576_*CgTPO1_1* and pGREG576_*CgTPO1_2*; and pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2*, after galactose or copper-induced recombinant protein production, respectively.

CgTpo1_1 and CgTpo1_2 confer resistance to azole drugs

In order to determine possible chemicals to which CgTpo1_1 and CgTpo1_2 confer resistance to, the effect of their expression in *C. glabrata* susceptibility to several compounds was assessed by spot assays. The deletion of *CgTPO1_1* and *CgTPO1_2* in *C. glabrata* was found to increase the susceptibility of this pathogen to several families of antifungal drugs, including the azole antifungal drugs clotrimazole, miconazole, ketoconazole, tioconazole (imidazoles); and fluconazole and itraconazole (triazoles) (Figure 2). On the other hand, the overexpression of these transporters increases fitness under azole drug stress in *C. glabrata* (data not shown).

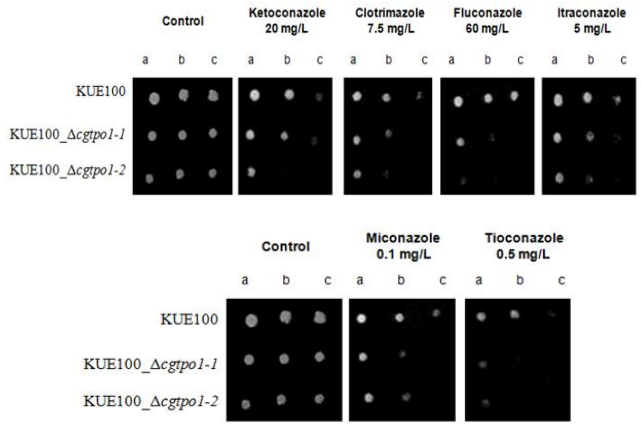

Figure 2 - Comparison of the susceptibility to inhibitory concentrations of several chemical stress inducers, at the indicated concentrations, of the *C. glabrata* KUE100, KUE100_*Δcgtpo1_1* and KUE100_*Δcgtpo1_2* strains, in BM plates by spot assays. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The displayed images are representative of at least three independent experiments.

CgTpo1_1 and CgTpo1_2 play a role in reducing the intracellular accumulation of ^{3}H-clotrimazole in C. glabrata

The accumulation of radiolabelled clotrimazole in non-adapted *C. glabrata* cells suddenly exposed to the presence of 30 mg/L clotrimazole during 30 minutes was seen to be two times higher in cells devoid of CgTpo1_1 (Figure 3.A) and two and a half times higher in cells devoid CgTpo1_2 (Figure 3.B) when compared to the KUE100 wild-type cells, correlating with the previously observed effect of *CgTPO1_1* and *CgTPO1_2* deletion, which appears to be consistent with the seeming relatively higher susceptibility of the *Δcgtpo1_2* cells in comparison to the *Δcgtpo1_1* cells.

These findings strongly suggest that CgTpo1_1 and CgTpo1_2 increase *C. glabrata* resistance towards clotrimazole by effectively reducing its accumulation within yeast cells, possibly by catalyzing the extrusion of this antifungal drug, corroborating their action as efflux pumps.

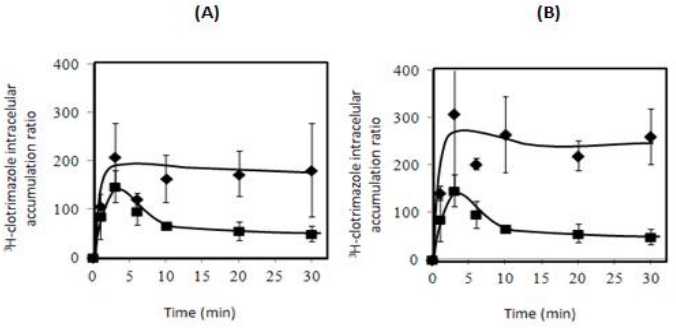

Figure 3 - Time-course accumulation of clotrimazole in strains KUE100 (▪) wild-type and KUE100_*Δcgtpo1_1* (♦) (A) and KUE100 (▪) and KUE100_*Δcgtpo1_2* (♦) (B), in the presence of radiolabelled ^{3}H-clotrimazole. Accumulation values are the average of at least three independent experiments. Error bars represent the corresponding standard deviation.

CgTPO1_1 and CgTPO1_2 transcript levels are up-regulated during clotrimazole stress

The effect of *C. glabrata* cell exposure to inhibitory concentrations of clotrimazole, to which CgTpo1_1 and CgTpo1_2 were found to confer resistance to, in *CgTPO1_1* and *CgTPO1_2* transcription, was evaluated. The transcript levels of *CgTPO1_1* were seen to have a 1.5-fold up-regulation upon 1h of exposure of an un-adapted *C. glabrata* population to inhibitory concentrations of clotrimazole; whereas transcript levels of *CgTPO1_2* were found to have a more significant change (5-fold) upon clotrimazole exposure (Figure 4.A and B).

Given the attained results, *CgTPO1_1*, but especially *CgTPO1_2* were seen to be responsive to clotrimazole

exposure. The higher transcript levels associated with *CgTPO1_2* appear to be consistent with the higher susceptibility of the correspondent mutant observed in the spot assays, and also with the higher accumulation ratio of intracellular clotrimazole in cells devoid of the same gene.

Additionally, *CgTPO1_1* appears to be independent on the transcription factor *CgPDR1* (Figure 4.A). *CgTPO1_2* levels do not appear to be dependent on this transcription factor, given that transcript levels of such gene remain significantly high in mutant cells devoid *CgPDR1* (Figure 4.B).

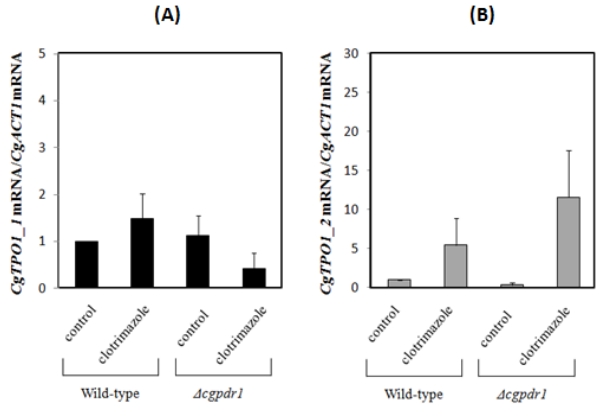

Figure 4 - Comparison of the variation of the *CgTPO1_1* and *CgTPO1_2* transcript levels before (control) and after 1h of incubation with clotrimazole induced stress in *C. glabrata* wild-type cells and cells devoid the transcription factor CgPdr1. The presented transcript levels were obtained by quantitative RT-PCR and the relative *CgTPO1_1/CgACT1* and *CgTPO1_2/CgACT1* mRNA values, considering the value registered in control conditions, equal to 1. The obtained values are the average of at least two independent experiments. Error bars represent the corresponding standard deviations.

CgGas1 contributes to azole drug resistance

In an effort to determine if CgGas1 confers azole drug resistance in *C. glabrata* cells, spot assays were done to evaluate the susceptibility of *Δcggas1* cells in comparison to the corresponding parental strain.

In fact, the deletion of *CgGAS1* in *C. glabrata* was found to increase the susceptibility of this pathogen to several azole antifungal drugs, such as clotrimazole, miconazole, ketoconazole, tioconazole (imidazoles); and fluconazole and itraconazole (triazoles) (Figure 5). As it is clear, the wild-type strain (KUE100) is capable of growing in the tested concentrations, while the *Δcggas1* mutant displays slower growth when compared to the wild-type, therefore showing a higher degree of susceptibility.

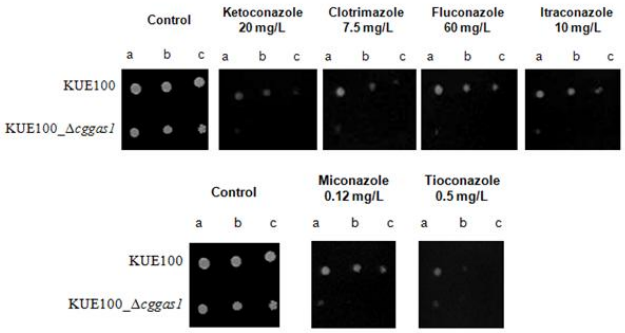

Figure 5 - Comparison of the susceptibility to azole antifungal drugs, at the indicated concentrations, of the *C. glabrata* KUE100 and KUE100_*Δcggas1* strains, in BM agar plates by spot assays. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The displayed images are representative of at least three independent experiments.

CgGas1 is required for cell wall stability

The susceptibility to lyticase of exponential wild-type (KUE100) cells was seen to be lower in comparison with the *Δcggas1* deletion mutant cells grown in the absence of clotrimazole (Figure 6.A and B). This indicates that even without any stress, the cell wall of *Δcggas1* cells is more fragile than that of wild-type cells, apparently resulting in a more susceptible wall structure.

In wild-type or *Δcggas1* cells sudden exposure to clotrimazole during 30 minutes leads to similarly increased susceptibility to lyticase, showing that clotrimazole appears to have a drastic effect at the level of the cell wall structure. Interestingly, once adapted to exponential growth in the presence of clotrimazole, either wild-type or *Δcggas1* cells exhibited levels of lyticase resistance which are even higher than those exhibited by non-stressed cells. This result suggests that adaptation to clotrimazole includes cell wall remodeling. Altogether, the lack of the CgGas1 putative cell wall assembly protein increases lyticase susceptibility at the level of cell wall in control conditions, eventually helping the *C. glabrata* cells to cope with sudden stress exposure. However, this protein appears not to be crucial for the cell wall remodeling taking place during adaptation to clotrimazole (Figure 6.A and B).

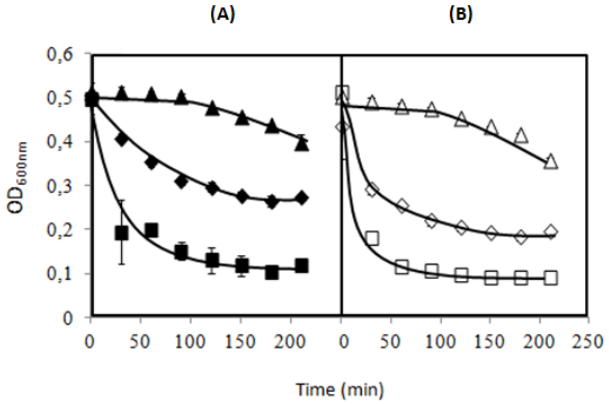

Figure 6 - Lyticase sensitivities from cells of KUE100 wild-type (A) and KUE100_*Δcggas1* (B) strains grown in the absence of clotrimazole (♦), after 30 minutes of clotrimazole exposure (▪) or after exponential growth resumption in the presence of clotrimazole (▲). The different cell populations were washed and resuspended in 0.1 M sodium phosphate buffer, pH 7. After addition of 10 µg of lyticase (Sigma) per mL, the decrease in the OD_{600} of the cell suspensions was measured periodically. The obtained values are the average of at least two independent experiments. Error bars represent the corresponding standard deviations.

CgGas1 plays a role in reducing the intracellular accumulation of ^{3}H-clotrimazole in C. glabrata

In the search for a plausible mode of action that allows CgGas1 to act as clotrimazole resistance determinant, its possible involvement in reducing the intracellular accumulation of this azole drug was examined. The accumulation of radiolabelled clotrimazole in non-adapted *C. glabrata* cells suddenly exposed to 30 mg/L clotrimazole was seen to be approximately 2.5 times higher in cells devoid CgGas1 when compared to the KUE100 wild-type cells (Figure 7).

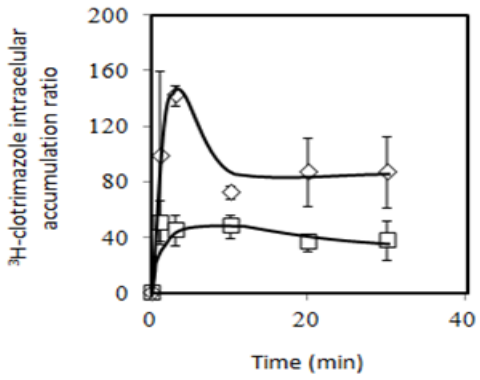

Figure 7 - Time-course accumulation of clotrimazole in strains KUE100 (▪) wild-type and *Δcggas1* (♦), in the presence of radiolabelled ^{3}H-clotrimazole. Accumulation values are the average of at least three independent experiments. Error bars represent the corresponding standard deviation.

Discussion

In this study, the first membrane proteomics study focused on the fungal pathogen *Candida glabrata* was undertaken, leading to functional characterization of the *C. glabrata* CgTpo1_1 and CgTpo1_2 drug:H^{+} antiporters, as well as the cell wall assembly protein CgGas1, in the context of clotrimazole drug resistance.

Using a membrane proteomics analysis, several proteins from distinct functional groups were found to be differentially expressed in *C. glabrata* clotrimazole response. Ribosomal proteins were among the down-regulated ones, in accordance with the environmental stress response described by Gasch *et al.* [32, 33], in which ribosomal proteins have a stress-dependent repression as a mechanism to conserve mass and energy while redirecting transcription to genes whose expression is induced by stress. Following this assumption, proteins involved in stress (clotrimazole) response should be up-regulated. The up-regulated proteins encompass glucose metabolism, also in accordance with the predicted environmental stress response [33] and therefore were considered to be part of a general response. On the other hand, the multidrug transporter CgTpo1_2 and CgGas1 up-regulation appear to implicate these proteins in a specific response to clotrimazole. The participation of these proteins in clotrimazole resistance was not previously described. Based on the knowledge that CgPdr1 is a major regulator of MDR in *C. glabrata*, a possible dependence of the quantified proteins on CgPdr1 was assessed. In fact, the verified down-regulation of the multidrug transporters functional group, upon clotrimazole stress, in the absence of CgPdr1 is consistent with the described role of this transcription factor, suggesting the action of these transporters may also have a relevant role in clinical context. Concerning *CgTPO1_1* and *CgTPO1_2*, mRNA expression results confirm the expression of these transporters in presence of clotrimazole stress, and appear to imply that none of them is regulated by CgPdr1.

The MFS transporters CgTpo1_1 and CgTpo1_2 were found to confer azole drug resistance in *C. glabrata.* So far, CgTpo3 was the only transporter from the Tpo1-4 group to be associated with azole drug resistance in *C. glabrata* [34]. CgTpo1_1 and CgTpo1_2 (ORFs *CAGL0G03927g* and *CAGL0E03674g*, respectively) are described herein as the fourth and fifth in a family of 10 members from the DHA1 family to be associated with azole drug resistance; after CgQdr2, CgAqr1 and CgTpo3 [11, 23, 34]. Similarly to what happens with their *S. cerevisiae* counterpart, these

predicted transporters were found to be localized to the plasma membrane, confirming them to be integral plasma membrane proteins, also accordingly to what was observed in the case of their *S. cerevisiae* ortholog [35] and of *C. glabrata* CgTpo3 [34].

In *C. glabrata*, resistance to fluconazole among clinical isolates has been shown to often depend on the action of ABC transporters, especially *CgCDR1* and *CgCDR2* [36, 37]. Furthermore, the ABC drug efflux pump CgSnq2 was also reported as an important fluconazole resistance determinant in *C. glabrata* clinical isolates [28, 37]. However, the participation of DHA family members in imidazole drug resistance in *C. glabrata* still needs further study. Although many of the characterized drug efflux pumps are known to confer resistance to a wide variety of compounds [31, 38, 39], the molecular mechanisms behind their substrate diversity are very much unknown [31, 38, 40]. Given the high degree of homology with *S. cerevisiae TPO1* gene, a possible role in *C. glabrata* survival to inhibitory concentrations of spermine and spermidine [41] was assessed. Indeed, *CgTPO1_1* and *CgTPO1_2* deletion increased susceptibility to spermine, whereas overexpression increased tolerance to that compound, demonstrating a role of the *C. glabrata* transporters in polyamine resistance as well.

Given the referred promiscuity of drug efflux pumps, the ability of CgTpo1_1 and CgTpo1_2 to confer resistance to additional drugs was investigated. Azole antifungal drugs to which CgTpo1_1 and CgTpo1_2 were found to confer resistance to include the imidazoles clotrimazole, miconazole, ketoconazole and tioconazole, used in the treatment of localized skin infections such as vaginal or oral candidiasis, and also triazoles, including itraconazole and fluconazole, applied in the treatment of systemic fungal infections; joining the previously characterized transporter from this family, CgTpo3, in imidazole drug resistance [34]. CgTpo1_1 and CgTpo1_2 have close homologs in other pathogenic *Candida* species, which may also play a role in azole drug resistance in these related pathogenic yeasts, including those encoded by *C7_01520W_A* in *C. albicans*, *CPAR2_300730* in *C. parapsilosis*, *Cd36_71360* in *C. dubliniensis*; and *orf19.6577* in *C. albicans*, *CPAR2_300740* in *C. parapsilosis*, *Cd36_71360* in *C. dubliniensis*, respectively. In this study *Δcgtpo1_1* and *Δcgtpo1_2* deletion mutants display susceptibility phenotypes, especially *Δcgtpo1_2*, while overexpression of such genes in wild-type cells increases fitness upon exposure to inhibitory concentrations of the tested azole drugs. Furthermore, the effect of these transporters in response to the polyene amphotericin B, the fluorinated pyrimidine analog flucytosine and the pesticide mancozeb was also assessed. Interestingly, the related DHA CgTpo3 does not appear to confer resistance to flucytosine or amphotericin B [34], while CgAqr1 is involved in flucytosine resistance [23]. These observations seem to imply that CgTpo1_1 and CgTpo1_2 are gifted with extraordinary substrate variety, even within their own DHA1 family [42, 43]. Consistent with these results, their *S. cerevisiae* homolog was demonstrated to confer resistance to, at least, five different drugs besides polyamines, including the fungicide cycloheximide, the antiarrythmic drug quinidine, the polyene nystatin and the herbicides MCPA and 2,4-D [44-47].

In order to test the role of CgTpo1_1 and CgTpo1_2 as efflux pumps, the accumulation of clotrimazole was assessed. *C. glabrata* cells exposed to inhibitory concentrations of clotrimazole were found to accumulate this metabolite intracellularly. A higher accumulation of clotrimazole was verified in the *Δcgtpo1_1* or *Δcgtpo1_2* cells, when compared to the wild-type strain, consistent with a role for these transporters in imidazole drug extrusion, therefore decreasing drug intracellular concentration. Interestingly, the homolog of these transporters in *C. albicans*, *FLU1*, is extensively studied as a fluconazole resistance determinant, possibly by catalyzing its extrusion out of challenged cells. However, disruption of this gene in *C. albicans* appears to only have a slight effect on fluconazole susceptibility and no relevant drug extrusion effect was detected [48]. In contrast, *C. glabrata* cells displayed 2-2.5 times higher accumulation rates in the absence of the transporters studied herein. These results solidify the position of *C. glabrata* as an increasingly relevant pathogen, displaying higher levels of azole drug resistance than the well known *C. albicans* [49-51].

A protein required for cell wall assembly, CgGas1, was also studied as a possible determinant of clotrimazole resistance. The deletion of *CgGAS1* resulted in higher susceptibility of exposed cells to several azole drugs (miconazole, ketoconazole, clotrimazole, tioconazole, fluconazole and itraconazole), supposing some effect in azole drug resistance. So far, *C. glabrata* Gas1 was found to be constitutively expressed, probably due to an important role in cell wall homeostasis, since its deletion was found to result in the formation of cell aggregates and growth defects, much in tune with the observed *S. cerevisiae* mutant phenotype [52]. The formation of aggregates has been also reported for the *C. albicans* homolog gene deletion mutant [53]. Having this in mind, a

lyticase susceptibility assay was done, revealing higher cell wall susceptibility in *Δcggas1* cells than wild-type cells in control conditions. The observed profile of cell wall resistance to lyticase suggests that clotrimazole induces cell wall damage and that adaptation to this azole drug includes cell wall remodeling. Altogether, the obtained results suggest that CgGas1 may have a protective effect in sudden exposure to clotrimazole, but it appears to have no significant role in the observed clotrimazole-induced cell wall remodeling. Interestingly, *Δcggas1* mutant cells were found to accumulate more intracellular clotrimazole in comparison to wild-type cells, possibly suggesting that the expression of CgGas1 may play a role in clotrimazole resistance by making the cell wall less permeable to this compound.

Altogether, the results described in this study testify the importance of multidrug transporters from the MFS in overall resistance phenotypes. The characterization of *C. glabrata* Tpo1_1 and Tpo1_2 multidrug transporters involved in azole drug resistance reinforce the need for study remaining members of this family in this increasingly relevant pathogenic yeast, given these transporters are likely to have clinical impact, thus impairing treatment of azole-resistant fungal infections. This work also highlights the importance of genome/proteome-wide approaches in the study of possible resistance determinants, such as CgGas1, and regulators like CgPdr1; as global approaches are very useful in identifying previously unforeseen or uncharacterized genes relevant for drug resistance phenotypes.

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