# 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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Thesis to obtain the Master of Science Degree in

**Biotechnology**

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# Abstract

Azoles are widely used antifungal drugs. This family of compounds includes 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*, thus being an interesting subject for the study of azole resistance mechanisms in fungal pathogens. Since multidrug 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. Proteins found to have significant expression changes in this context were clustered into functional groups, namely: glucose metabolism, oxidative phosphorylation, mitochondrial import, ribosome components and translation machinery, lipid metabolism, multidrug resistance transporters, cell wall assembly and stress response, comprising a total of 53 proteins. 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 also found to be overexpressed under clotrimazole stress, 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

# Resumo

Azóis são drogas antifúngicas largamente usadas atualmente. Esta família de compostos inclui os triazóis, usados no tratamento de infeções sistémicas, e os imidazóis, tal como o clotrimazol, frequentemente usado no tratamento de infeções superficiais. A levedura *Candida glabrata* representa a segunda causa mais comum de candidémia no mundo e apresenta níveis de resistência intrínseca a azóis mais elevados aos de *Candida albicans*, sendo por isso um bom modelo para o estudo de mecanismos de resistência a azóis em fungos patogénicos. Uma vez que a resistência depende frequentemente da ação de transportadores de múltiplas drogas na membrana plasmática, incluindo bombas de efluxo da família ATP ou da família dos transportadores antiporte droga:$H^+$, uma análise do proteoma de membrana, determinado por iTRAQ, foi feita de forma a identificar todas as proteínas diferencialmente expressas em células de *C. glabrata* quando expostas ao azol clotrimazol. Proteínas que apresentam diferenças de expressão significativas foram agrupadas em grupos funcionais, nomeadamente: metabolismo de glucose, fosforilação oxidativa, importe mitocondrial, componentes ribossomais e maquinaria de tradução, metabolismo de lípidos, transportadores de resistência de múltiplas drogas, formação da parede celular e resposta ao stresse, num total de 53 proteínas. Entre estas, o transportador CgTpo1_2 foi identificado como tendo expressão elevada em *C. glabrata* como resposta ao clotrimazol. A caracterização funcional deste transportador droga:$H^+$ putativo, e do seu homólogo CgTpo1_1, permitiu localizar estas proteínas na membrana plasmática e determinar que são sobre-expressas em exposição ao clotrimazol, conferindo resistência a azóis neste patogénico por expulsão da droga para o exterior das células. Esta avaliação ao nível do proteoma de membrana permitiu igualmente avaliar o papel do fator de transcrição CgPdr1, destacando a existência de alvos adicionais sobre o seu controlo que não estavam previstos até ao momento, confirmando assim a sua posição como um regulador principal de resistência a azóis em isolados clínicos.



**Palavras-chave:** *Candida glabrata*, proteómica de membrana, resistência a drogas antifúngicas, clotrimazol, CgPdr1, CgTpo1_1 e CgTpo1_2

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# Acronyms

5-FC – 5-fluorocytosine

5-FU – 5-fluorouracil

2,4-D – 2,4-dichlorophenoxyacetic acid

4-NQO – 4-nitroquinoline-1-oxide

ABC – ATP-Binding Cassette

BM – Basal medium

*C. glabrata* – *Candida glabrata*

*S. cerevisiae* – *Saccharomyces cerevisiae*

*C. albicans* – *Candida albicans*

*C. parapsilosis* – *Candida parapsilosis*

*C. tropicalis* – *Candida tropicalis*

*C. krusei* – *Candida krusei*

*C. dubliniensis* – *Candida dubliniensis*

cDNA – Complementary Desoxirribonucleic Acid

CDR – Candida Drug Resistance

CiAP – Calf Intestinal Alkaline Phosphatase

DHA – Drug:$H^+$ Antiporter

DRE – Drug Response Elements

DNA – Desoxirribonucleic Acid

DEPC – Diethyl Dicarbonate

dNTP – Deoxynucleotide Triphosphates

DMSO – Dimethyl Sulfoxide

EDTA – Ethylene Diamine Tetraacetic Acid

ER – Endoplasmic Reticulum

GOF – Gain-of-function

GPI – Glycosylphosphatidylinositol

GFP – Green Fluorescent Protein

HIV – Human Immunodeficiency Virus

iTRAQ – Isobaric Tag for Relative and Absolute Quantification

ICAT – Isotope-Coded Affinity Tag

MDR – Multidrug Resistance

MFS – Major Facilitator Superfamily

MCPA – 2-methyl-4-chlorophenoxyacetic acid

MAP – Mitogen-Activated Protein

MS – Mass Spectrometry

MMTS – Methyl Methane Thiosulfonate

mRNA – Messenger Ribonucleic Acid

NBD – Nucleotide Binding Domain

ORF – Open Reading Frame

$OD_{600nm}$ – Optical Densitometry at 600 nm

PDR – Pleiotropic Drug Resistance

PDRE – Pdr Response Elements

PG – Phosphatydil Glycerol

PMSF – PhenylMethylSulfonyl Fluoride

P-gp – Mammalian Glicoprotein

PCR – Polymerase Chain Reaction

RT-PCR – Reverse Transcription Polymerase Chain Reaction

Rpm – Revolution Per Minute

RNase – Ribonuclease

rRNA – Ribosomal Ribonucleic Acid

SDS – Sodium Dodecyl Sulfate

SPM – Spermine

SPMD – Spermidine

TEAB – Tetraethylammonium Bromide

TCEP – Tris(2-carboxyethyl)phosphine

YPD – Yeast Extract-Peptone-Dextrose

# 1. Introduction

## 1.1. Thesis outline

This dissertation is organized in five chapters. The first chapter offers an overview on the increasing relevance of the opportunistic pathogenic yeast *Candida glabrata*, followed by some insight into the currently used families of antifungal drugs in clinical practice, together with their modes of action. Special attention is given to azoles, one of the main families of drugs presently used, with information concerning modes of action and resistance mechanisms for imidazoles and triazoles. Since Pdr1 is a major regulator of pleiotropic drug resistance, including response to azoles, an overview concerning the participation of this transcription factor in drug response is described in the yeast model *Saccharomyces cerevisiae*, in the pathogenic yeast *Candida glabrata* and its counterpart in the primary pathogen *Candida albicans*. Given that resistance phenotypes are largely dependent on multidrug resistance (MDR) transporters, the role of ATP-Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) transporters in this context is reviewed.

The second chapter contains all the materials and methods used during the fulfillment of this work.

The third chapter describes the results attained with this study, comprising the analysis of the membrane proteome-wide changes occurring in *C. glabrata* cells upon exposure to clotrimazole. Based on these results, the functional characterization of the MFS drug:$H^+$ antiporters (DHA) CgTpo1_1 and CgTpo1_2 and their role in resistance to several chemical stress inducers, including azoles, is described. The results achieved include study the effect of the deletion of these transporters, as well as their overexpression in *C. glabrata* drug susceptibility, their subcellular localization, the determination of their expression levels in the presence of clotrimazole, and their role in the extrusion of that specific azole. This chapter also includes characterization of the participation of CgGas1 in clotrimazole resistance, comprising the effect of its deletion in azole drug susceptibility and weakening of the cell wall, as well as its role in cell wall remodeling, thus affecting the accumulation of clotrimazole inside the cells.

In the fourth chapter, the results obtained with this project are discussed and compared with the current knowledge about azole resistance mechanisms.

In the fifth and last chapter, final remarks considering the work developed and future perspectives are made, together with references to what contributions this work offered in the comprehension of acquired resistance mechanisms in *C. glabrata*.

## 1.2. Emergence of *Candida glabrata*

From a clinical perspective, antifungal drug resistance refers to the lack of susceptibility of a fungus or yeast to an antifungal agent, in which the minimum inhibitory concentration (MIC) value of the drug exceeds the susceptibility breakpoint of that organism [1]. A concerning number of patients with invasive mycoses experience treatment failure because of clinical resistance, which is critical to the outcome of a fungal infection [1]. Table 1.1 summarizes the main factors determining antifungal clinical resistance. In immunocompromised patients therapy is even more difficult, since the net state of immunosuppression can be so negative that antifungals simply cannot overcome the deficiency [11].

**Table 1.1 - Possible factors underlying antifungal clinical resistance [1].**

| Factor | Implication |
|---|---|
| Wrong diagnosis | Weak diagnostics and/or IRIS |
| Net state of immunosuppression | Improvement in immunity of host is essential |
| High burden of fungus at initiation of treatment | Earlier treatment intervention improves outcome |
| Strain acquisition of increased virulence | Probably less of a problem than host factors but can be measured |
| Pharmacokinetics and/or pharmacodynamics | Drug toxicity, drug–drug interaction, drug levels |
| Site of infection | Drug penetration, tissue necrosis, foreign body |
| Length of treatment and/or compliance | Precision is not certain; patient and clinician may lose focus on long-term drug administration |
| Underlying disease | Final arbitrator in most invasive mycoses |

Mechanisms of antifungal resistance can be primary (intrinsic) or secondary (acquired). Primary or intrinsic resistance is characteristic of yeasts that naturally have resistance to a certain compound without previously being exposed to it, e.g. resistance of *Candida krusei* to fluconazole [1]. On the other hand, secondary or acquired resistance develops in previously susceptible strains after exposure to the antifungal agent and is usually dependent on altered gene expression, e.g. development of fluconazole resistance by *Candida albicans* [1, 3].

Candidemia represents the 4$^{th}$ most common nosocomial infection in humans [12]. 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 extensive use of antifungal drugs both as treatment and prophylaxis contributed to the appearance of other relevant species, such as *Candida glabrata* [13]. 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 [2, 14-16]. Together, *C. albicans* and *C. glabrata* represent 65%-75% of all systemic candidiasis, followed by *C. parapsilosis* and *C. tropicalis* [15].

*C. glabrata* is an haploid ascomycete yeast, closely related to *Saccharomyces cerevisiae*, both originating from a common ancestor who has undergone whole genome duplication [2, 15] (Figure 1.1). 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 [17]. It is a ubiquitous commensal yeast, asymptomatically colonizing the gastrointestinal tract and genital tract of healthy hosts [15, 18], but it also acts as an opportunistic pathogen in immunocompromised patients [19], being known to cause mucosal and blood stream infections [20] in patients suffering from advanced HIV or cancer [18], in which their immune system is impaired locally or systematically. Other risk factors for disseminated infections are neutrophil dysfunction, disruption of mucosal barriers, intravascular catheters, diabetes, extreme age and long-term hospitalization [15, 18].

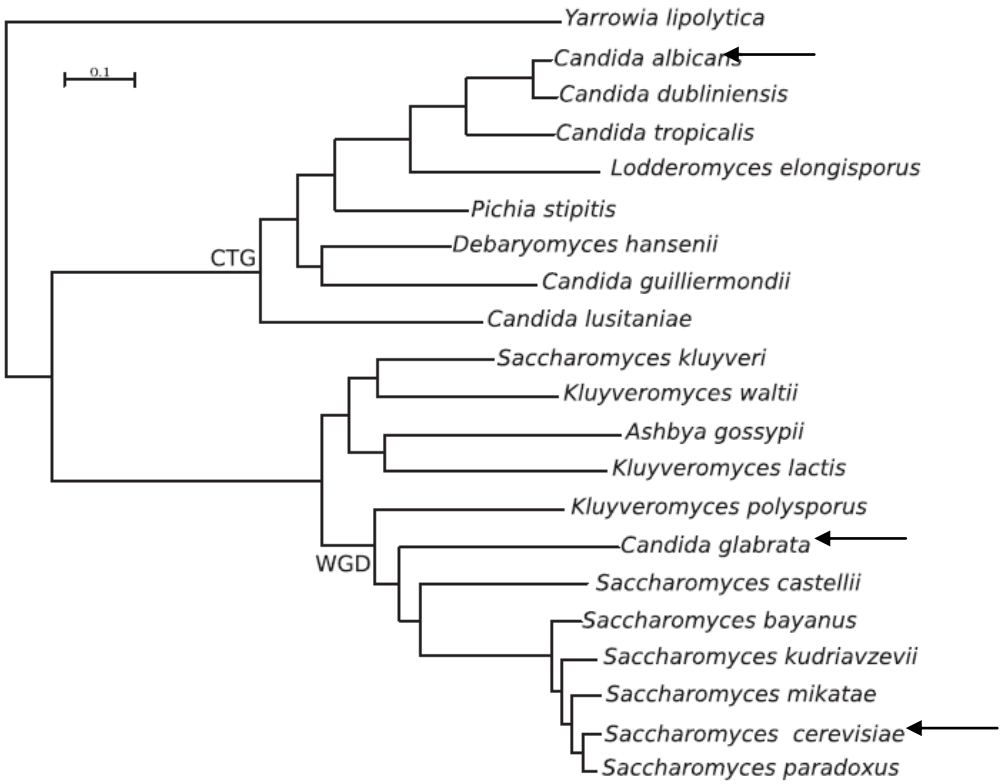


**Figure 1.1 - Phylogenetic tree representing the evolutionary relationships between sequenced *Candida* and *Saccharomyces* species. Adapted from Jandric *et al.*, [2].**

*C. glabrata* infections are usually treated with several antifungal drugs, mainly polyenes, echinocandins and azoles [21-23]. However, it presents higher levels of intrinsic resistance to azoles than *C. albicans* and develops further resistance during prolonged azole therapy. Two studies, from Borst *et al.* [24] and Sanglard *et al.* [25], determined that the average fluconazole MIC for *C. glabrata* was 32-fold higher than *C. albicans*. Additionally to intrinsic resistance, *C. glabrata* can also develop acquired antifungal resistance, especially to azole drugs.

Although not being as much disseminated as *C. albicans*, 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. Despite still being disregarded as relevant human pathogens, fungi infect billions of people every year, and some fungal diseases are as relevant as tuberculosis or malaria [26].

One of the mechanisms postulated to be responsible for yeast cells becoming resistant to drugs is the expression of membrane proteins called multidrug resistance transporters. A 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.

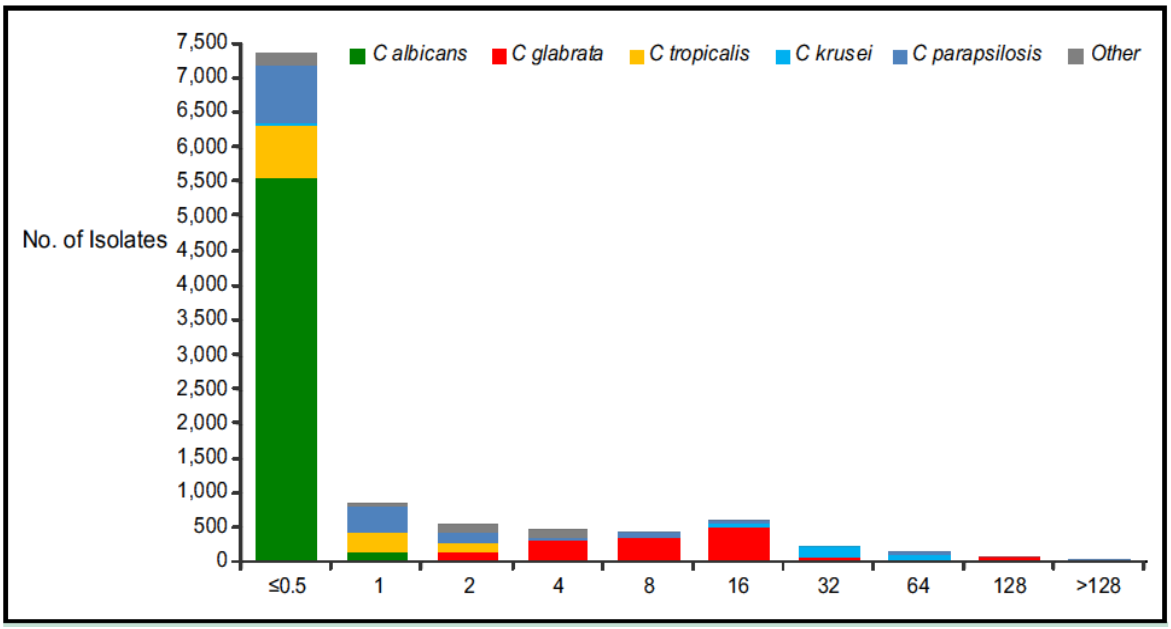


**Figure 1.2 - Fluconazole $MIC_{50}$ distribution of 10,803 isolates from invasive *Candida* species [3].**

## 1.3. Antifungal drugs

Antifungals are drugs used to treat infections by fungi, also known as mycosis, which can be divided in two groups according to their effects: fungicides or fungistatic agents. Fungicides are substances that are able to kill or inhibit fungi or fungal spores, whereas fungistatic agents are capable of inhibiting the growth and reproduction of fungi without destroying them [27].

There are 5 major classes of antifungal agents developed until today, they include: echinocandins, polyenes, allylamines, fluorinated pyrimidines and azoles [7]. These compounds have different mechanisms of action, targeting different molecules and disrupting different processes inside the cells.

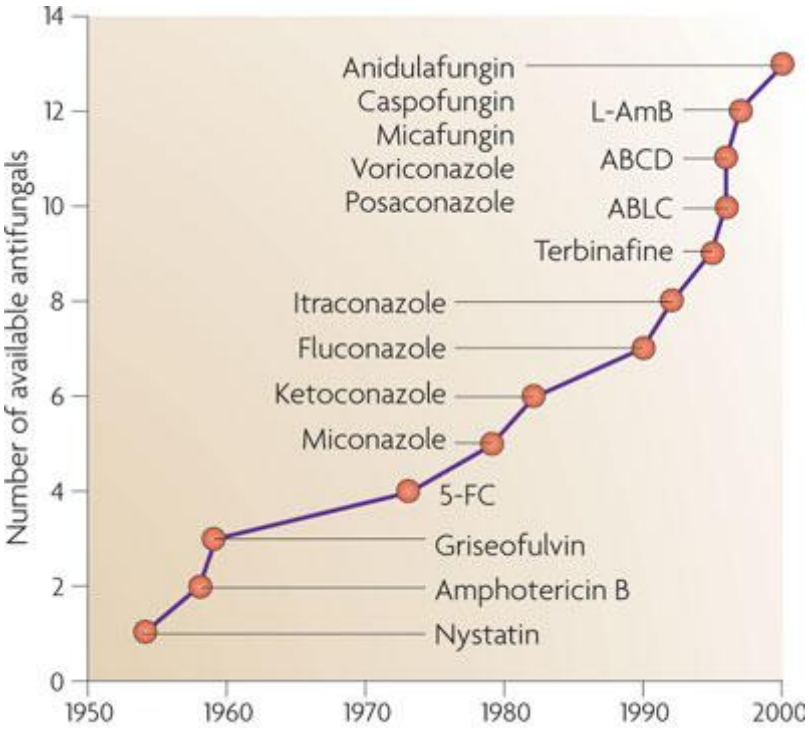


**Figure 1.3 - Name, number and rate of development of available systemic antifungal agents from the 1950s to 2000s [4].**

Echinocandins inhibit β-1,3-glucan synthase, the enzyme which catalyzes the production of glucan, the major component in *Candida* cell walls [28]. It is responsible for transferring sugar moieties from activated donor molecules to specific acceptor molecules forming glycosidic bonds [29]. The inhibition of this enzyme by echinocandin drugs disrupts the structure of the cell wall, leading to osmotic instability and consequent death of yeast cells [30]. As a result of its unique mechanism of action, cross-resistance between echinocandins and other classes of drugs is rare [31].

Echinocandin resistance was associated with mutations in *FKS1*, one of three genes encoding one of the two subunits of the β-1,3-glucan synthase in *C. albicans*, *C. tropicalis* and *C. krusei* [32]. Accordingly, mutations in *FKS1* and *FKS2* were also found to be responsible for echinocandin resistance in *C. glabrata*. Moreover, echinocandins are a poor substrate for multidrug transporters [31], which undoubtedly contributes to rare cases of resistance reported.

Polyenes are amphipathic drugs which bind to ergosterol, creating drug-lipid complexes intercalated into the fungal membrane to form a membrane-spanning channel. Cellular ions can leak out of the cell destroying the proton gradient, rendering the cells unviable [4, 33]. The most widely used polyene is amphotericin B and it is very effective against systemic infections, however, it possesses high host toxicity, probably due to similarities between ergosterol and the cholesterol in mammalian cells [34].

Allylamines are a class with activity against a wide range of pathogenic fungi. The mechanism of action of these drugs is the specific inhibition of fungal squalene epoxidase. Fungi treated with these

drugs accumulate squalene and become deficient in ergosterol in the cell membrane. Allylamine exposure results in high intracellular squalene concentrations, interfering with membrane function and cell wall synthesis. An advantageous feature is that squalene epoxidase is not an enzyme of the cytochrome P-450; therefore allylamines have no risk of intrinsic inhibition of this class of enzymes [35].

Fluorinated pyrimidines are chemicals which interfere with the use of a metabolite. 5-fluorocytosine (5-FC) is a synthetic antimycotic that is converted into 5-fluorouracil (5-FU) inside fungal cells. Then it is further converted to metabolites that inhibit fungal RNA and DNA synthesis [36]. Flucytosine started by being applied in combination with amphotericin B to treat severe systemic mycoses and surpass acquired resistance. Later on it started being applied in combination with azoles. Unfortunately, flucytosine displays high hepatotoxicity, limiting its use [36].

**Table 1.2 - Mechanisms of action and resistance to major antifungal agents [27].**

| Antifungal agent | Mechanism(s) of action | Mechanism(s) of resistance |
|---|---|---|
| *Antimetabolites* | | |
| Flucytosine | Cell death: inhibition of DNA and RNA synthesis | Enzymatic modifications: cytosine permease (*FCy2* gene); cytosine deaminase (*FCy1* gene); and phosphoribosyl transferase (*FUR1* gene) |
| *Azoles* | | |
| Fluconazole, itraconazole, posaconazole and voriconazole | Perturbation of fungal membrane: inhibition of ergosterol synthesis by blocking P-450 14-α demethylase leads to accumulation of lanosterol | Decreased drug concentration by activation of efflux pumps (*CDR, MDR* and *atrF* genes); decreased affinity to the binding site (*ERG11* and *Cyp51A* genes); upregulation of target enzyme (*ERG11* and *Cyp51A* genes)<br>Bypass pathways (*ERG3* gene) |
| *Echinocandins* | | |
| Anidulafungin, caspofungin and micafungin | Formation of a defective cell wall leads to cell rupture (yeasts) or aberrant hyphal growth (molds): inhibition of β-1,3-D-glucan synthesis | Point mutations (*FKS1* and *FKS2* genes) |
| *Polyenes* | | |
| Amphotericin B | Cell death: intercalation of pores across the membrane formed by eight polyene molecules linked to the ergosterol, through which the fungal cell leaks cytoplasmic components (protons and monovalent cations); oxidative damage of fungal membrane | Decreased access to the drug target by altered membrane ergosterol content, accumulation of other sterols and reduced intercalation (*ERG3* gene); decreased oxidative damage by increased catalase activity |

### 1.3.1. Azoles

Azole drugs are a group of fungistatic agents with broad-spectrum activity, including both imidazoles and triazoles. An azole is constituted of a nitrogen heterocyclic ring with at least one other non-carbon atom of nitrogen, sulfur or oxygen. These drugs have been the most widely used group of antifungals for approximately 2 decades [5].

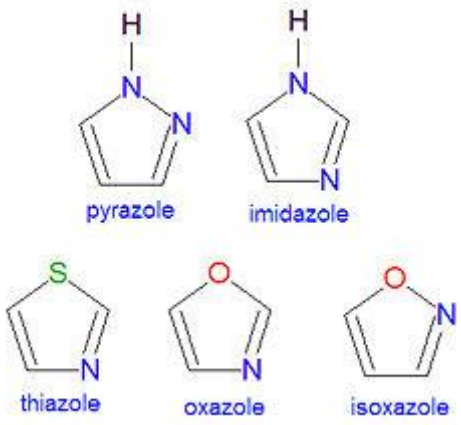


**Figure 1.4 - Basic azole compounds structure (en.citizendium.org).**

Azoles are the main family of drugs presently used to treat *Candida* infections, including vaginal or oral candidemia and skin infections [37]. They inhibit fungal growth by interfering with biosynthesis of ergosterol present in the cell membrane [1, 38], 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 [1, 2, 5]. More recently, it was also shown that azoles impair the function of vacuolar membrane $H^+$ ATPases, therefore disrupting cation homeostasis within the cell [39].

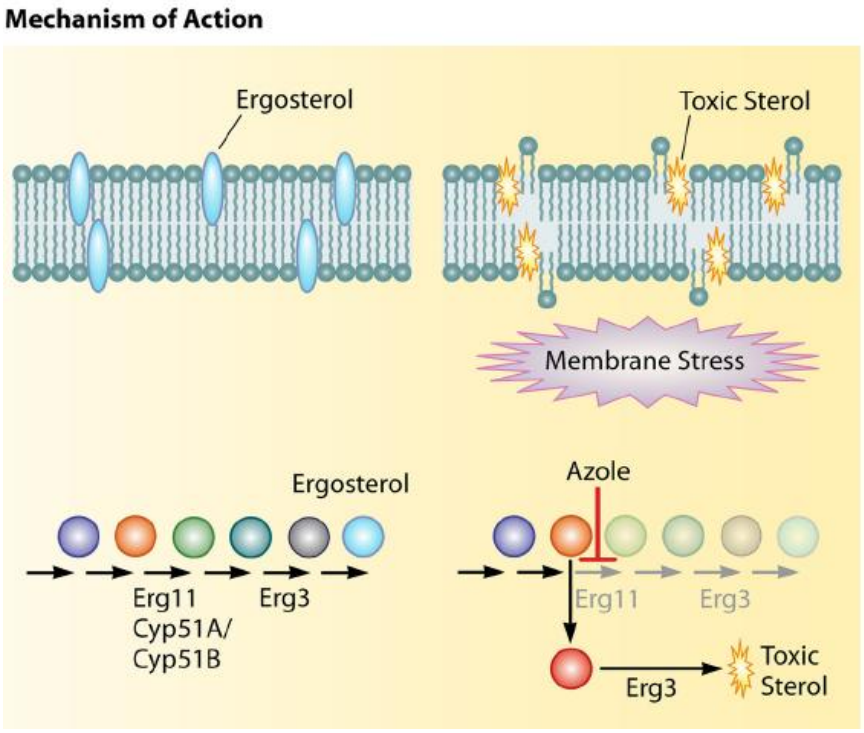


**Figure 1.5 – Mode of action of azole drugs in ergosterol biosynthesis inhibition, adapted from Shapiro *et al.* [5].**

## 1.3.2. Azole resistance mechanisms in *Candida* species

Azoles differ in their affinities to their target, which may account for differences in their spectrum of activity. Using three-dimensional models, Xiao *et al.* [40] determined that the long side chain of posaconazole and itraconazole would result in tighter affinities to their target-protein by making extensive hydrophobic contacts along their entire lengths, arguing for a more significant role of the side chains in determining drug activity. On the contrary, compact azoles without extended side chains, such as fluconazole or voriconazole, display less affinity to their target, and consequently have inherently higher propensity to be less effective and more easily affected by acquired resistance strategies.

Furthermore, variations in their structure are thought to be responsible for cross-resistance patterns among *Candida* species [41, 42], for example, while cross-resistance among triazoles has been observed in *C. glabrata*, no such pattern seems to exist in *C. krusei* [1, 43].

There are four major mechanisms responsible for resistance to azoles in *Candida* species. The first mechanism is the expression of efflux pumps, resulting in a decrease of drug susceptibility by decreasing the concentration of drug inside the cell. Efflux pumps are encoded by *Candida* drug resistance (CDR) genes of the ATP-binding Cassette (ABC) family and the MDR genes of the Major Facilitator Superfamily (MFS) [1, 6]. While *CDR* genes up-regulation confers resistance to all azoles, MFS-encoded transporters were described to have a narrower spectrum [1, 31] of activity. Increased

expression of *CDR1* and *CDR2* genes has been proposed as one cause of azole resistance in *C. albicans* and *C. glabrata*, together with overexpression of *MDR1* in *C. albicans* [31, 44, 45]. Additionally, other genes such as *CdCDR1* and *CdMDR1* in *Candida dubliniensis* and *CgCDR1*, *PDH1* (*CgCDR2*) and *CgQDR2* in *C. glabrata* have also been reported as important transporter genes in azole-resistance strains [37, 46].

The core mechanisms behind overexpression of MDR transporters are mutations in the transcription factor *CgPDR1*, which regulates multidrug response; and defects in the mitochondrial function. Under stress, *CgPDR1* can undergo gain-of-function (GOF) mutations resulting in increased transcription of efflux pumps. Different single point mutations cause activation of different regulons, and several dozens of mutations in *CgPDR1*have been identified in fluconazole-resistance isolates. The mutations can take place in several domains of *CgPDR1*, including the putative activation domain, the xenobiotic binding domain and the putative inhibition domain [2]. Moreover, cells displaying mitochondrial DNA deficiency designated petite mutants, present increased azole resistance. Petite mutants have shown to have enhanced virulence and better fitness, but mitochondrion-associated acquired fluconazole resistance in *C. glabrata* is not necessarily associated with the loss of mitochondrial genome, showing a dedicated retrograde signaling pathway [47]. Mutants defective in biosynthesis of anionic phospholipids that are necessary for mitochondrial function have also enhanced levels of ABC transporters and decreased susceptibility to azoles in *C. glabrata* [48].

Another strategy is target site alteration in the *ERG11* gene. It was demonstrated that mutations in this gene prevent binding of azole drugs to the enzymatic site [49]. Also, different mutations can coexist with additive effects; in fact, more than 80 amino acid substitutions in Erg11 have been detected in *C. albicans* [50]. Low affinity of azoles to Erg11 is considered to be one of the primary reasons for intrinsic resistance to fluconazole, as it was shown by Orozco *et al.* [50] in *C. krusei* isolates.

A complementary approach is the up-regulation of the target enzyme. Some *Candida* isolates show higher intracellular concentrations of Erg11, resulting in reduced susceptibility to azoles [51]. Gene up-regulation can be achieved through gene amplification, increased transcription rate or decreased degradation of the product [1]. As a result of the increased number of targets, the antifungal agent can no longer be effective in routine therapeutic concentrations in inhibiting ergosterol synthesis. However, this effect is thought to have a somewhat secondary effect, since only modest increases in enzyme levels have been described [1].

The last resistance mechanism is the development of bypass pathways. Exposure to azole drugs leads to depletion of ergosterol and accumulation of the toxic product 14α-methyl-3,6-diol produced by Erg3, resulting in growth inhibition [52]. Mutations in the *ERG3* gene prevent formation of the toxic product [52], leading to functional membranes and negating the action of azoles on the ergosterol biosynthesis pathway; plus conferring resistance to polyenes in some *Candida* strains as well [1].

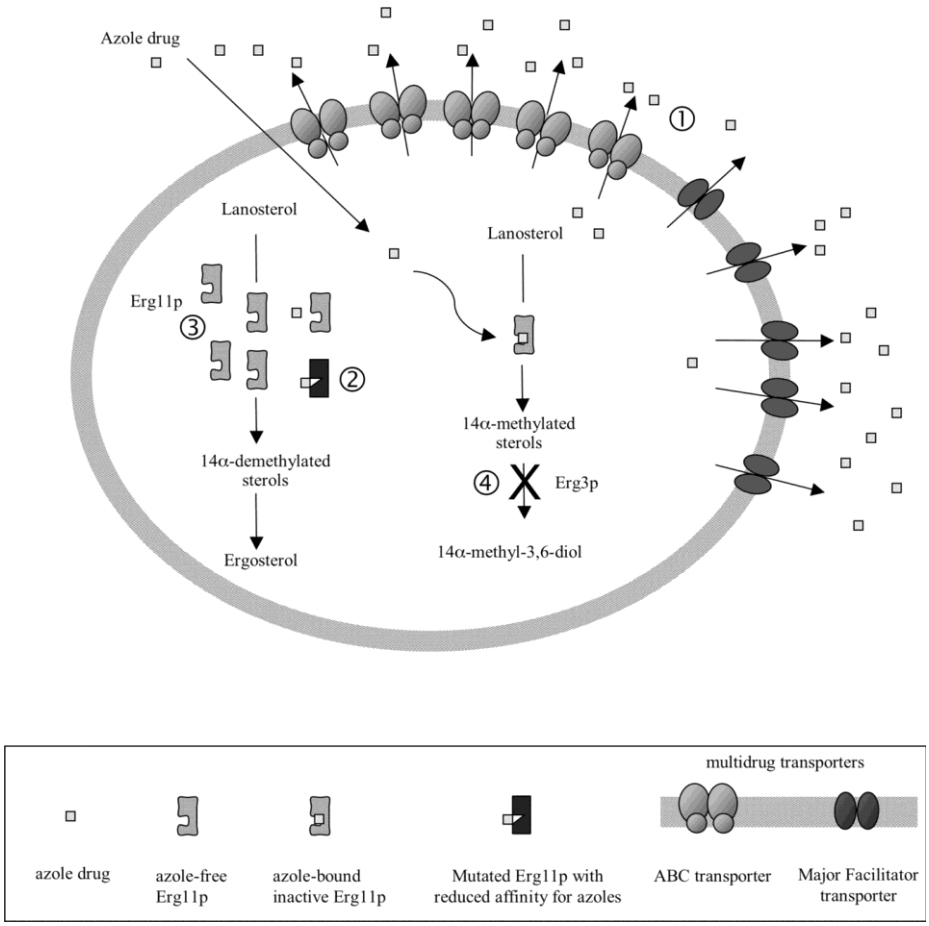


**Figure 1.6 – Schematic representation of resistance mechanisms to azole antifungal agents in *Candida* spp. (1) Decreased accumulation of drug due to up-regulation of ABC and MFS transporters, (2) decreased affinity of azoles to the target enzyme Erg11, (3) increased cellular content of Erg11, (4) alteration of the ergosterol biosynthetic pathway by inactivation of Erg3 (from www.biochemsoctrans.org).**

## 1.3.3. Imidazoles modes of action and resistance mechanisms

Despite the fact that azole resistance mechanisms are well established, studies addressing this question focus greatly in the use of fluconazole as "model drug". Since fluconazole is a triazole, much information can be obtained concerning resistance mechanisms for this class of drugs. Logically, much less studies are currently contemplating possible resistance mechanisms specific for other classes of azoles, namely imidazoles, which are widely used in topical applications. From these drugs, ketoconazole and miconazole are the most studied ones. For clotrimazole, very little information is available about its action and resistance mechanisms in *C. glabrata*.

In a study by Calahorra *et al.* [53] the mode of action and resistance mechanisms of ketoconazole and miconazole (both imidazoles) were studied in the model *S. cerevisiae*. Although the primary mode of action involves the inhibition of sterol synthesis common to all azoles, these two drugs seem to also have an effect on cation transport systems. Ketoconazole and miconazole produced an efflux of $K^+$ at low concentrations (~200 μM) leading to an almost depletion of that ion from the cells. The drugs appear to bind to the surface of the cell due to their amphipathic and cationic nature, decreasing the

surface charge of the membrane. As a consequence, both antifungals can stimulate efflux of $K^+$ at low concentrations, once at higher concentrations the uptake of $H^+$ can be stimulated. The change of the surface charge was yet hypothesized to lead to a disruption of the membrane structure due to the interaction of the antifungals with lipid rafts. These results were not found with two triazolic antifungals, fluconazole and itraconazole, indicating that the active group of the molecules is the imidazole moiety of the molecule, thus showing a clear difference in imidazoles and triazoles mode of action. Ketoconazole and miconazole were found yet to affect respiration, probably related to the cationic nature of the imidazolic portion of the molecule. More studies highlighted the importance of mitochondrial function for tolerance to antifungal drugs and virulence, with functions in lipid homeostasis likely to be the center of mitochondrial action in tolerance to antifungal drugs, with some crosstalk between mitochondria and cell wall integrity; but the exact molecular mechanisms are not fully understood [54, 55].

François *et al.* [56] also reported an interaction between miconazole and lipid rafts. In this study, mutants affected in sphingolipid biosynthesis were found to be miconazole-resistant, suggesting a possible role for membrane rafts in miconazole antifungal action. Disruption of these rafts interfered with miconazole antifungal action with reduced intracellular accumulation of miconazole in yeast cells. However, whether the reduced intracellular accumulation of miconazole is caused by a reduced uptake in yeast cells or by increased efflux was not determined.

Miconazole tolerance genes were also reported to be implicated in tryptophan biosynthesis, membrane trafficking including endocytosis and regulation of actin cytoskeleton expression, in a study by Thevissen *et al.* [57]. Apparently, miconazole is capable of inducing changes in actin cytoskeleton for increased filament stability in yeast, resulting in actin clumping prior to induction of endogenous ROS and cell death by the drug. This is supported by the prior knowledge that miconazole induces ROS generation in susceptible fungi, and actin cytoskeleton has been implicated in mitochondrial activity and regulation of endogenous ROS levels and apoptosis in yeast [58].

Most of what is known about clotrimazole has been focused in terms of research into fermentative process. Clotrimazole resistant *S. cerevisiae* strains show improved fermentative activity in the brewing process for Japanese sake [59], and also exhibit PDR phenotypes [60]. ScPdr5 is known to be responsible for clotrimazole transport outside the cell and clotrimazole resistant strains have *ScPDR1* and *ScPDR3* mutant alleles which enhance expression of *ScPDR5*. Tsujimoto *et al.* [61] described the functional roles of *ScYPT31* and *ScYPT32* in drug resistance, as *ScYPT31*, which encodes a Rab family small GTPase, was identified as a clotrimazole resistance gene (among other azole drugs). ScYpt31 and ScYpt32 are involved in the trafficking of proteins to the plasma membrane, suggesting that they are involved in drug influx through the regulation of cell membrane integrity (*Δypt31* and *Δypt32* results in higher influx), and that they regulate trafficking of one or more transporters to the cell membrane. Although ScPdr5 is known to excrete clotrimazole, the action of ScYpt31 and ScYpt32 appears to mediate sorting of ABC transporters other than ScPdr5 and/or are involved in clotrimazole resistance through mechanisms that do not involve transporters. These hypotheses were confirmed by the resistant phenotype seen in cells lacking seven major ABC

transporters besides ScPdr5, by demonstrating that vacuoles were involved in resistance to clotrimazole mediated by *ScYPT7*, a gene encoding a key protein involved in vacuole inheritance and by observing abnormal vacuoles in *Δypt31* cells [61].

Similarly to what was reported for ketoconazole and miconazole, clotrimazole also shows an effect in ion flux. Bartolommei *et al.* [62] show that the Na,K-ATPase activity can be progressively inhibited by the addition of increasing concentrations of clotrimazole, demonstrating that clotrimazole interacts some way with the sodium pump. Interference with the H,K-ATPase was reported as well [63]. Clotrimazole likely interferes with ion pumps near the interface of the lipid phase and membrane domain. As a high hydrophobic molecule, clotrimazole accumulates significantly in the membrane, inhibiting enzyme activity and modifying the ion binding kinetics of ion pumps [62].

## 1.4. The Pleiotropic Drug Resistance network

Communication and sensing of environmental parameters is a fundamental process for cells to adjust and redirect their resources to purposes of protection or proliferation, in order to adapt to changing conditions outside the cell, either in the environment or inside a host. Gene transcription regulation in response to external conditions is one of the major strategies used by fungal cells, including in drug response [2].

Drug resistance is an alarming clinical problem in the treatment of infections not only by bacteria, but also 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.

Pdr1 is known to be one of the major pleiotropic drug resistance regulators in yeast, either in *S. cerevisiae*, but also in *Candida* species, by controlling the expression of genes responsible for multidrug resistance phenotypes [64-66]. The next sections will present an overview in the PDR networks of the yeast model *S. cerevisiae* and the pathogenic species *C. glabrata* and *C. albicans*, with special emphasis on the role of the transcription factor Pdr1.

### 1.4.1. PDR network: *Saccharomyces cerevisiae*

In *S. cerevisiae*, two zinc-cluster transcription factors, ScPdr1 and ScPdr3, are associated with regulation of multidrug resistance [39]. It involves two classes of transporter proteins consisting on members of the ABC and MFS families. The multidrug resistance phenotype is also known as pleiotropic drug resistance and is mainly attributed to the overexpression of ABC transporters like ScPdr5, ScYor1 and ScSnq2 [39]; or DHA transporters such as ScTpo1 and ScFlr1 [67] from the MFS family. The genes encoding these important transporters are regulated by transcription factors such as ScPdr1, ScPdr3 and ScYrr1; which regulate the expression of their target genes by binding to

consensus sequences in their promoter region known as Pdr1/Pdr3 response elements (PDRE) [68]. ScPdr3 has PDREs in its own promoter region; therefore being under positive autoregulation [69]. Figure 1.7 represents the pathways regulating the expression of these multidrug resistance genes.

Besides ScPdr1 and ScPdr3 regulating similar target genes, it was also found that they can form both homo and heterodimers [70], and that single point mutations in the N- or C-terminal region of the regulatory domain and in the activation domain of both ScPdr1 and ScPdr3 confers them hyperactivity [71].

ScPdr5 is the most commonly encountered ABC transporter responsible for pleiotropic drug resistance in *S. cerevisiae*, harboring three PDRE sequences (TCCGCGGA) in its promoter region, therefore being highly responsive to ScPdr1/3 levels [72, 73]. It was no surprise when Dexter *et al*. and Meyers *et al*. [74, 75] verified that hyperactive alleles of Sc*PDR1* or Sc*PDR3* led to overexpression of ScPdr5 and a consequent increase in drug resistance.

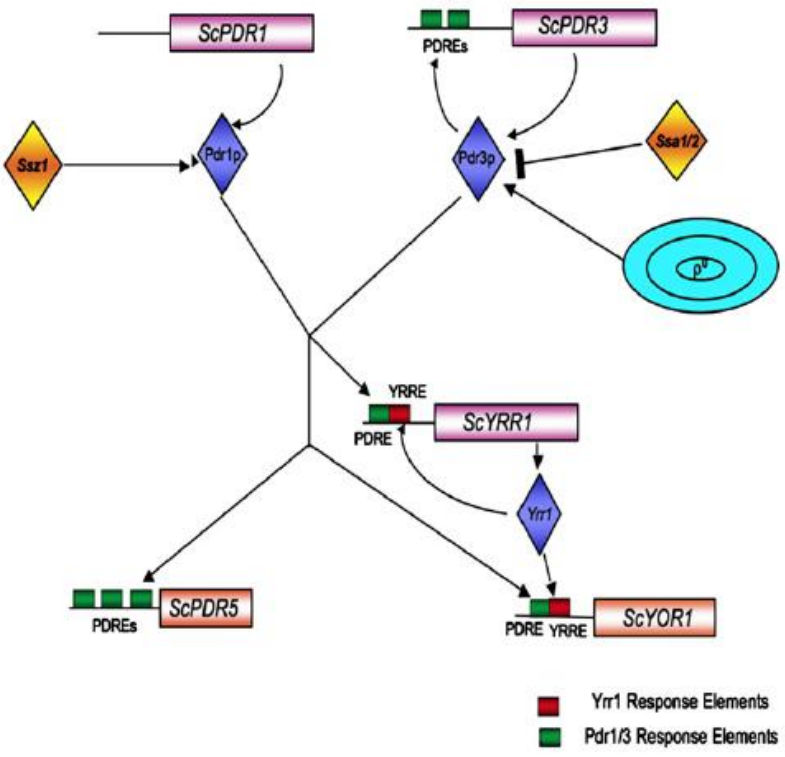


**Figure 1.7 - Control of multidrug resistance in *S. cerevisiae*. Positive interactions are depicted as arrows, while negative effects are represented as a T-bar. Loss of mitochondrial DNA gives rise to ρ0 cells that activate ScPdr3 post-transcriptionally. ScYrr1 binds to its target element which is juxtaposed with the Pdr1/3 response elements (PDRE) in some promoters [6].**

Also, cells that have lost their mitochondrial genome ($\rho^0$ cells) strongly induce levels of *ScPDR5* in a pathway dependent on ScPdr3 (Figure 1.7). It was found that a nuclear protein, ScLge1, is involved in histone ubiquitination and although its function does not appear to be involved in the regulation of

*ScPDR5* directly, signals from dysfunctional mitochondria to the nucleus were blocked by deletion of *ScLGE1* [76]. Additionally, the protein ScPsd1 was also found to be important in mitochondrial to nucleus retrograde regulation of *ScPDR5* in a ScPdr3-dependent manner. This protein is the mitochondrial phosphatidylserine decarboxylase and loss of the *ScPSD1* gene prevents normal retrograde induction of *ScPDR5* in $\rho^0$ cells [6]. It is clear that there is some degree of mitochondrial control over ScPdr3 activity.

Other than upstream regulators of ScPdr1/3 action, some downstream effectors were also described [77]. ScPdr1/3 were identified has xenobiotic receptors, with their central regions directly binding to drugs. This binding is responsible for a remodeling of their structure, allowing them to bind to a key domain in the RNA polymerase II, especially ScPdr1. As a result, RNA polymerase II has enhanced recruitment to target promoters [78]. Furthermore, ScPdr1 is post-translationally regulated by ScPdr13, the unique member of the Hsp70 protein family [78]. Interestingly, ScPdr13 regulates the function of ScPdr1, but not ScPdr3, by a gain-of-function mutation or overproduction of this transcriptional regulator [79].

Not only ScPdr1/3 are responsible for PDR genes regulation. Other $Zn_2Cys_6$ transcription factors, such as *ScYRR1* were demonstrated to bind to PDR genes in *S. cerevisiae* as well [6]. ScYrr1 was first identified as a determinant of resistance against reveromycin A, a cell cycle inhibitor [80]. Later, this transcription factor was also found to induce the expression of the ABC transporter genes *ScYOR1* and *ScSNQ2*, as well as induce its own transcription [6]. ScYrr1 has some similarities with ScPdr3, namely its autoregulation characteristic and mutations causing hyperactivity [6]. A second transcription factor, ScYrm1, presents also high sequence similarity and binding specificity when compared to ScYrr1. ScYrm1 seems to recognize the same DNA element in promoter regions as ScYrr1, and it appears to become more active when ScYrr1 is removed from the cell, showing some complex regulatory relationship [81]. Like ScPdr1 and ScPdr3 show some common features and a close relationship, the same goes for ScYrr1 and ScYrm1.

In *S. cerevisiae*, ScPdr1 has been shown to form an heterodimer with another transcription factor, ScStb5 [82]. ScStb5 is a $Zn_2Cys_6$ transcription factor as well, which regulates genes involved in the oxidative stress response by increasing the supply of NADPH through the pentose phosphate pathway [83]. Furthermore, *STB5* seems to have a role in azole resistance, once ScPdr1 and ScStb5 dimerize and directly bind the promoter of *ScPDR5* [82].

### 1.4.2. PDR network: *Candida albicans*

Despite the emergence of *C. glabrata* in the clinical setting, *C. albicans* still represents the major species associated with candidiasis, which makes this pathogen one of the yeasts with better understanding in resistance mechanisms.

The first drug resistance gene isolated from *C. albicans*, on the contrary from *S. cerevisiae* and *C. glabrata*, was a MFS protein-encoding gene, *CaMDR1* [84]. Only after that, the first ABC-encoding

gene, *CaCDR1* was found and showed high similarity degree with *ScPDR5*. Interestingly, searching for a *C. albicans* homolog of ScPdr1/3 was more difficult than expected, with several transcription factors being able to complement *Δpdr1* or *Δpdr3* in *S. cerevisiae* mutant strains, but having no role in *C. albicans* drug resistance phenotype [85]. Unlike an homolog *PDR1* gene as it happens in *S. cerevisiae* and *C. glabrata*, the main transcription factor responsible for multidrug resistance in *C. albicans* is *CaTAC1*. CaTac1 protein was shown to be of unparallel importance for azole drug resistance and expression of the *CaCDR2* gene. Likewise *ScPDR3* and *CgPDR1*, *CaTAC1* is autoregulated, with drug response elements (DREs) in its own promoter region (Figure 1.8) [86].

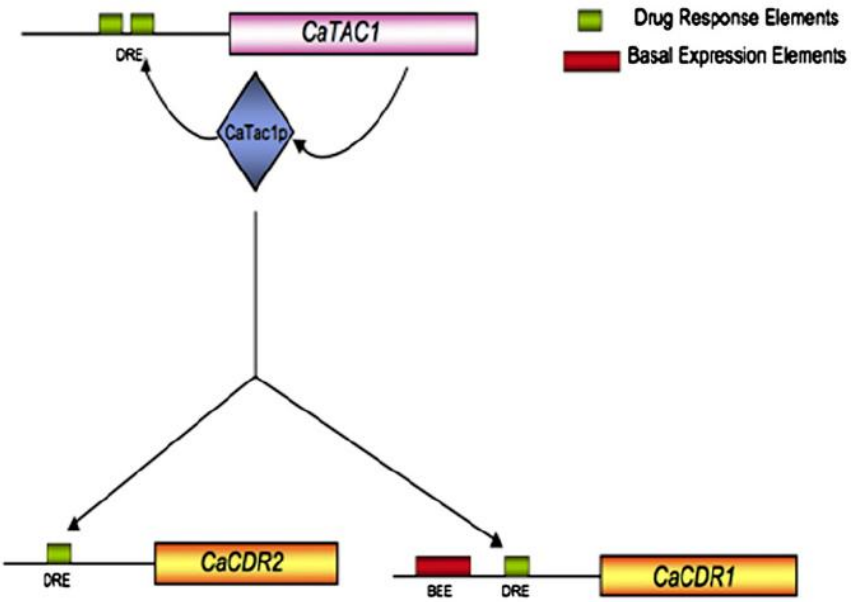


**Figure 1.8 – Regulation of multidrug resistance in *C. albicans*, based on the transcription factor CaTac1 [6].**

Besides CaTac1, two other transcription factors have proven to be relevant in expression of multidrug resistance genes. Associated with *CaMDR1* overexpression in resistant isolates, the CaMrr1 transcription factor is necessary in azole resistant strains [87]. However, the regulatory network involving CaMrr1 seems to be distinct from that of CaTac1. The second transcription factor also showing some importance is the regulator of sterol biosynthetic gene expression called CaUpc2 [88]. It is concordant to what was expected, given that azole drugs target a sterol biosynthetic pathway. What is surprisingly though, is the fact that CaUpc2 also binds to a variety of multidrug resistance gene promoters, such as *CaCDR1*/*CDR2* and *MDR1*, unveiling an unexpected link between sterol biosynthesis and multidrug resistance cross-regulation [6].

### 1.4.3. PDR network: *Candida glabrata*

Ever since *C. glabrata* arose as an important pathogenic yeast in the clinical picture, several studies have addressed its intrinsic and acquired multidrug resistance. A link between high azole resistance and the overexpression of ABC transporters was quickly discovered, in a way very closely related to the resistance pathways described in Section 1.4.1 for *S. cerevisiae*.

The first ABC transporter-encoding gene identified in *C. glabrata* was *PDH1*, also known as *CgCDR2* and related to the transporter ScPdr15 in *S. cerevisiae* [89]. Later on, a second ABC transporter, CgCdr1, was found and demonstrated to be more related to ScPdr5 [6]. CgSnq2 closes the main ABC transporters responsible for high azole resistance in clinical isolates of *C. glabrata* [6].

In the search for important transcription regulators in *C. glabrata*, an homolog of ScPdr1 was found. This key regulator of multidrug resistance gene expression, showed striking sequence similarity with ScPdr1, being denominated *CgPDR1*. It was found to be a major regulator element in multidrug resistance in *C. glabrata*, confirmed by loss of the high level drug resistance in clinical isolates that suffered loss of *CgPDR1* and by having a PDRE sequence (TTCCGTGGAA) at the 5' upstream region (-558 to -549) [66]. Similarly to *ScPDR1* and *ScPDR3*, *CgPDR1* is prone to mutations in its DNA sequence, which are likely to behave as hyperactive transcriptional regulators. Gain-of-function mutations in *CgPDR1* are responsible for intrinsic high expression of ABC transporters and therefore constitute the molecular basis of azole resistance [20]. More than 67 mutations conferring azole drug resistance are described so far [12], resulting in increased transcription of *CgCDR1*, *CgPDH1* and *CgSNQ2*. It is noteworthy to point out that this high variability in CgPdr1 mutations results in the overexpression of different targets. For instance, Torelli *et al.* [90] found that one given mutation led to the overexpression of *CgSNQ2* but not *CgCDR1* or *CgPDH1*. Ferrari *et al.* [91] also demonstrated high variability in these mutations and showed that they have not only a role in azole resistance but also virulence, by infecting mice with *C. glabrata* strains less susceptible to azoles than wild-type isolates. The azole-resistant strains, harboring *CgPDR1* GOF mutations, effectively took over the azole-susceptible isolates in the absence of drug selection, suggesting a gain in fitness translated into higher virulence. On the opposite to known *C. glabrata* mutants (*Δace2*) for which hypervirulence was associated with formation of pseudo-hyphae [92], the morphology of azole-resistant isolates tested was not altered and therefore GOF mutations in *CgPDR1* are likely to account for the observed phenotype. Therefore, CgPdr1 is uniquely important to the ability of *C. glabrata* to not only resist antifungal therapy, but also cause disease [93]. Similar to what happens in *S. cerevisiae* with PDREs, *C. glabrata* possesses similar regions also called drug response elements that are required for multidrug resistance gene expression in a drug-induced manner (Figure 1.9).

A study in which the analysis of the transcriptome under CgPdr1 control was determined by DNA microarray, enlightened about many targets up-regulated by this transcription factor in response to fluconazole. Interestingly, significant overlap was seen with genes regulated by ScPdr1/3 in *S. cerevisiae* [94]. Among the up-regulated targets in an azole-resistant clinical isolate, 9 genes were homologs of *S. cerevisiae* ScPdr1/3 targets. Five of these nine genes encode putative membrane

proteins with functions related to small molecule transport or lipid metabolism, including *CgYOR1* involved in oligomycin efflux, *CgRSB1* involved in sphingolipid base-resistance, *CgRTA1* involved in 7-aminocholesterol resistance and of course *CgCDR1* and *CgPDH1*. On the other hand, the remaining 4 genes include *CgPDR1* itself, *CgRPN4* involved in proteasome gene transcription, and two open reading frames (*ORF*s) involved in signaling proteins such as MAP kinase Slt2, which is mitochondria-localized, suggesting a possible role in mitochondria to nucleus retrograde signaling [94].

In the same study, additional 69 other genes were specifically up-regulated and did not appear to be similarly regulated in *S. cerevisiae*. From these genes, more small molecule transport genes involved in multidrug and bile acid efflux were detected, the DHA1 CgQdr2 and CgYbt1 encoding genes. Also, lipid metabolism and steroid detoxification genes including *CgARE1* and *CgATF2* were found to be up-regulated in response to azoles. Among other notable up-regulated genes were also *CgSUT1* encoding a transcription factor involved in sterol uptake, *CgYIM1* and *CgMEC3* involved in DNA damage response, *CgHSP12* and *CgCTA1* involved in cell stress response, *CgMKC7* and *CgMNT3* involved in cell wall function, *CgOCH1* and *CgGSF2* involved in Golgi-ER functions, *CgUFD1* and *CgRPN8* in proteasome-related proteins and *CgFMP48* involved in a quinine reductase enzyme [94]. Despite several studies have been performed using a genome-wide approach for CgPdr1 targets, there is no membrane proteome studies to see how that transcriptional response translates into membrane proteins.

Once CgPdr1 is prone to substitution mutations which convert this transcription factor to a hyperactive state, it is probably under negative control. Furthermore, it seems to be autoregulated, such as ScPdr3, for which there is no clear homolog in *C. glabrata*. Also, and accordingly to what was seen *S. cerevisiae*, $\rho^0$ *C. glabrata* cells display high drug resistance, inducing CgCdr1 overexpression and being accompanied and dependent on CgPdr1 expression [66]. Also, CgPdr1 seems to bind to RNA polymerase II, like it was described above in *S. cerevisiae* response. This shows that although its sequence is more similar to that of ScPdr1, it actually may be regulated more like ScPdr3. The hypothesis that CgPdr1 appears to be a combination of characteristics from ScPdr1 and ScPdr3 is taking root.

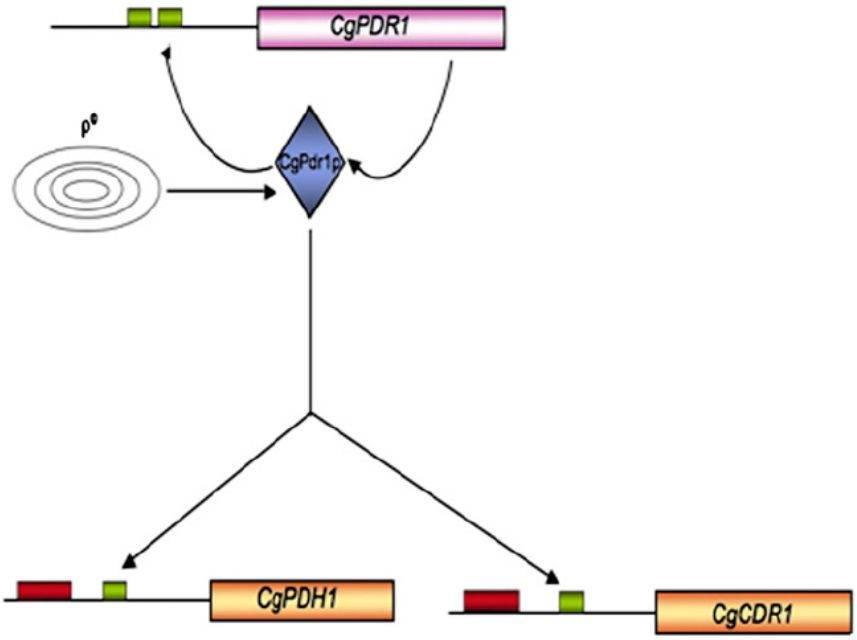


**Figure 1.9 - Control of multidrug resistance in *C. glabrata*. Green marks represent Drug Response Elements (DREs), red ones represent basal expression elements [6].**

As it was stated for the regulation of the PDR network in *S. cerevisiae*, also in *C. glabrata* there is a *CgSTB5* gene, encoding a $Zn_2Cys_6$ transcription factor homolog to that of *S. cerevisiae*, sharing 50 amino acid similarity and 38.8% identity, and being able to complement the *Δstb5 S. cerevisiae* strain [23]. *CgSTB5* was found to be a negative regulator of drug resistance; decreasing resistance to fluconazole, fluphenazine, cycloheximide and brefeldin A [85]. Also, *CgSTB5* is a negative regulator of the major azole transporter-encoding gene *CgCDR1*. The function of this gene is analogous to that of the negative regulator of drug resistance in *S. cerevisiae*, *RDR1* [95]. Noble *et al.* [23] found that *CgPDR1* and *CgSTB5* have a shared regulon, hence genes up-regulated by *CgPDR1* overexpression were up-regulated by deletion of *CgSTB5*, specially the major ABC transporter targets of *CgPDR1*: *CgCDR1*, *CgPDH1* and *CgYOR1*.

## 1.5. The Multidrug Resistance phenomenon

Multidrug resistance is an 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 ABC superfamily, such as the human P-glycoprotein (P-gp) [96]; and the MFS. Both families are found across prokaryotes, eukaryotes and plants, involved in transport of solutes across the plasma membrane, representing a physiological function beyond drug transport. Nevertheless, as major effectors of pleiotropic drug

resistance, expression of several of these transporters was shown to be dependent on CgPdr1 [66, 93, 94].

ABC transporters have been extensively characterized, while MFS transporters have been studied to less extent. In the following sections these two main transporter families will be described, with special emphasis in the Drug:$H^+$ Antiporters (DHA) of the MFS superfamily particularly focusing on the *TPO1-4* genes.

### 1.5.1. Multidrug resistance: ABC transporters

ATP-binding cassette transporters require ATP hydrolysis in order to have energy available to transport a wide variety of molecules across the cell membrane. ATP molecules bind to a Nucleotide Binding Domain (NBD) and are hydrolyzed, providing energy for a conformational change that allows the substrate to be translocated to the other side of the lipid membrane [7].

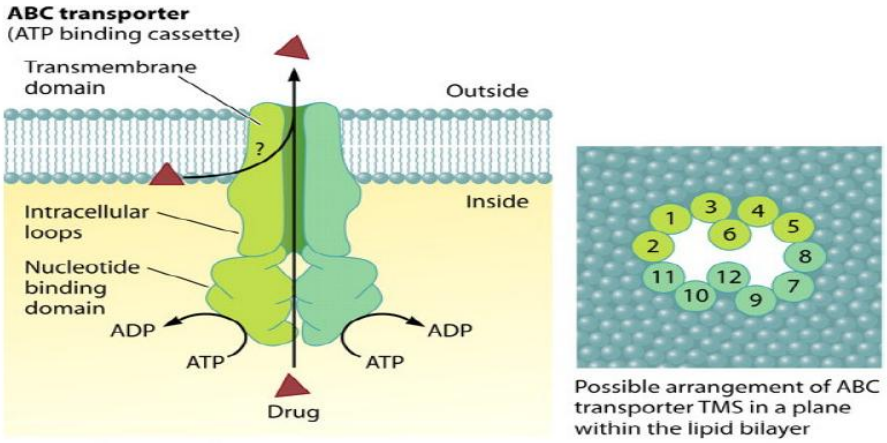


**Figure 1.10 - Schematic representation of an ABC transporter structure [7].**

This family of transporters harbors ATP-dependent drug efflux pumps with a broad specificity of substrates. In *S. cerevisiae*, 22 genes are classified as transporters of the ABC family [8]. Several studies have shown a wide range of drug sensitivity phenotypes upon loss of ABC transporters. Furthermore, these transporters were implicated in the control of phospholipid asymmetry across the plasma membrane [6]. Experiments performed with ScPdr5 shown that the protein can act to control the flop of phospholipids from the inner leaflet of the plasma membrane to the outer leaflet. Studies with the mouse transporter Mdr2 in mammalian cells also exhibited an influence on phospholipid transport [97]. Accordingly, Cabrito *et al.* [98] proposed that the ABC transporter ScPdr18 plays a direct role in the incorporation of ergosterol in the plasma membrane, since its deletion in *S. cerevisiae* cells was found to lead to an accumulation of the precursors of the ergosterol biosynthetic pathway,

influencing sterol homeostasis; thus confirming that this function is likely a conserved role of ABC transporters.

ABC transporters were the first class of membrane proteins found to be involved in fungal multidrug resistance, starting with *S. cerevisiae PDR5*. In turn, *C. glabrata* expresses its homolog, designated *CDR1*. *CgCDR1* is capable of complementing the hypersensitivity phenotype of *Δpdr5* strains in *S. cerevisiae*, proving its high similarity with *ScPDR5*. Furthermore, considering that all ABC transporter sequences share high sequence similarity, it is legit to assume that these proteins carry out similar actions in their native environments. *CgCDR1* expression seems to be dramatically induced in the presence of cycloheximide and oligomycin, beyond fluconazole response [66]. Another important ABC transporter is *CgPDH1*, also denominated *CgCDR2*. Just as *CgCDR1*, *CgCDR2* harbors a PDRE (TTCCGTGGAA) in its upstream region (-560 to -511). *CgCDR1* and *CgCDR2* complement each other in the resistant phenotype. In the event of loss of one of these genes, the efflux capacity can be somewhat maintained by the other transporter [66].

Another well-known ABC transporter is ScSnq2, with an ortholog in *C. glabrata*. A consensus PDRE sequence 5'-TCCACGGA-3' was found in *CgSNQ2* promoter region, suggesting that it may be regulated by *CgPDR1* [90]. This transporter was characterized as conferring azole resistance among *C. glabrata* clinical isolates, as the deletion mutant *Δsnq2* is more susceptible to azole antifungals, while reintroduction of *CgSNQ2* restores azole resistance. Furthermore, it seems to be able to compensate low levels of *CgCDR1/2* expression [90]. It was also found that *CgSNQ2* is able to complement the susceptibility phenotype of *Δpdr5 S. cerevisiae* to 4-NQO, confirming the homology with *ScSNQ2* and its role in resistance to this quinoline derivative [90].

### 1.5.2. Multidrug resistance: MFS transporters

The Major Facilitator Superfamily harbors more than a dozen families of transporters, each one of them with its own substrate pool and transport mechanism. It is present ubiquitously in bacteria, fungi and more complex eukaryotes [99] and includes members that can function by solute uniport, solute/cation symport, solute/cation antiport and solute/solute antiport [7]. The MFS transporters found to confer resistance in fungi are multidrug resistance pumps which belong to the DHA1 and DHA2 families, comprising a total of 22 transporters identified in *S. cerevisiae* [100]. These transporters have the ability to extrude drugs, but also physiological substrates including amino acids, polyamines and $K^+$, conferring resistance to a large number of unrelated chemicals [7].

DHA families differ in the number of transmembrane domains their transporters have. As depicted in Figure 1.11, DHA1 family transporters have 12 transmembrane spanners, while DHA2 family has 14 transmembrane spanners. Interestingly, the range of organisms in which DHA1 family is encountered is larger than that of the DHA2 family. Thus, DHA1 MDR pumps are found in animals as well as in yeasts and bacteria [7]. The DHA2 family was found to be more variable between genomes than the DHA1 family, while both are more variable than the ABC transporter family [8].

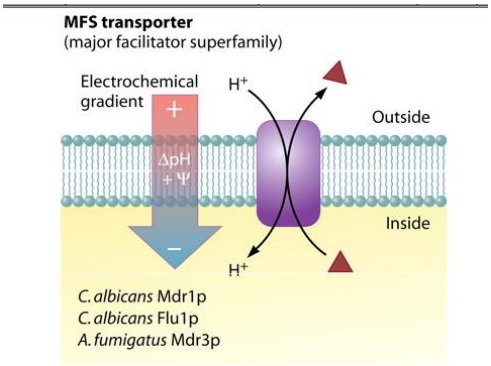


**Figure 1.11 – Mode of action of DHA1 and DHA2 families from the MFS [7].**

The major energy source used by MFS transporters is the proton motive force. Therefore, most MFS transporters, including DHA1 and DHA2 families, function as drug:$H^+$ antiporters [99] (Figure 1.11). MFS transporters couple the free energy released from the flux of protons into the cell to the extrusion of the drugs against their concentration gradient [7].

While ABC transporters have been extensively studied in conferring multidrug resistance phenotypes, the role of transporters from the MFS family has received much less attention. The first MFS transporter identified was the *C. albicans* drug:$H^+$ antiporter Mdr1, an homolog of *S. cerevisiae FLR1* gene, responsible for azole drug resistance [101]. CgFlr1 from *C. glabrata* also have been found to confer benomyl resistance [102]. In *C. glabrata* there are 15 predicted DHA transporters, 10 from the DHA1 family and 5 belonging to the DHA2 family [100].

Besides these transporters, also a recently characterized one, under transcription control of CgPdr1, was characterized in *C. glabrata*, CgQdr2. This is an homolog of the *S. cerevisiae QDR1* gene, which is known to confer resistance to the antimalarial/antiarrhythmic drug quinidine and ketoconazole. It was also implicated in resistance of yeast cells to the anticancer drugs cisplatin and bleomycine [103], and toxic concentrations of spermine, spermidine and putrescine [104]. Additionally, it was found to maintain intracellular $K^+$ levels in stress conditions such as limiting $K^+$ concentrations and presence of quinidine [105]. Surprisingly, in *S. cerevisiae*, it appears that *ScQDR1* is not regulated by the PDR network, once its expression is not induced upon exposure to drugs to which it confers resistance to. However, in *C. glabrata*, it was found to be under CgPdr1 control and its expression up-regulated upon clotrimazole and quinidine exposure, additionally conferring resistance to tioconazole, miconazole and ketoconazole [37]. This fact is confirmed by the two predicted CgPdr1 binding sites found in the *CgQDR2* promoter region (GCCATCATT and GCCGATAGA), suggesting that *CgQDR2* is a direct target of *CgPDR1*. *CgQDR2* was the first transporter of the DHA1 family to be associated with antifungal drug resistance in *C. glabrata*, specifically imidazole drug resistance [37].

Another newly relevant transporter characterized was CgAqr1. In *S. cerevisiae*, ScAqr1 was shown to confer resistance to short chain mono carboxylic acids such as acetic and propionic acids, but also to

chemical stress inducers such as quinidine and ketoconazole [106]. CgAqr1 in *C. glabrata* shows a dual role in acetic acid and antifungal drug resistance, conferring resistance in this species to the antifungal drug flucytosine, and to a lower level, resistance to azole drugs, including the imidazoles miconazole, tioconazole and clotrimazole. Also, a possible physiological role linked to the presence of inhibitory concentrations of acetic acid was described, although the exact role in acetic acid resistance remains to be established, it seems to be indirectly due to the transport of a still unidentified physiological substrate, possibly amino acids, as it is observed for ScAqr1 [106].

The most recently characterized MFS transporter in *C. glabrata* is CgTpo3. This transporter was found to play a direct role in extrusion of several azole antifungals including clotrimazole, as well as conferring resistance to the polyamine spermidine, similarly to what is observed in its *S. cerevisiae* homolog [107]. It was also found to have a role in acetic acid resistance [107], which is an interesting feature since *C. glabrata* cells often have to surpass acidic stress in the vaginal mucosa environment. The expression of *CgTPO3* was found to be dependent on the transcription factor CgPdr1 [107], corroborating this transcription factor as a major regulator of multidrug resistance response in *C. glabrata*, including several transporters from different families.

#### 1.5.2.1 The MFS *TPO1-4* genes

The TPO transporters have been studied in the yeast model *S. cerevisiae*, while little is known about these transporters in the pathogenic *C. glabrata*. In *S. cerevisiae*, this family comprises genes *TPO1* to *TPO4*, which are characterized as a group of proton-motive-force-dependent multidrug transporters from the drug:$H^+$ antiporter DHA1 family, harboring 12 predicted transmembrane-spanning domains [108, 109].

These transporters are characterized as determinants of polyamine resistance [110], detoxifying excess of spermidine and putrescine [111]. Initially, these transporters were purposed to be localized in the yeast vacuolar membrane [112], but were ultimately found to be located in the plasma membrane [111].

Tpo1 is the best characterized member of this family, displaying typical multidrug resistance transporter behavior, being able to extrude at least eight different substrates, among them polyamines (physiological substrate), quinidine, cycloheximide and nystatin [109]. Moreover, the *Δtpo1* mutant exhibits increased sensibility to nystatin and higher ergosterol content in the plasma membrane [113]. After that, ScTpo1 was also found to confer resistance to the herbicides 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) [114]. The *ScTPO1* gene was found to be regulated by the transcription factors ScPdr1 and ScPdr3 upon exposure to the previous herbicides [114], proving the role of this transporter as an important multidrug resistance determinant, once it is regulated by a main drug response regulator. Interestingly, there are two *ScTPO1* orthologs in *C. glabrata* (*CAGL0E03674g* and *CAGL0G03927g*) [8] (Figure 1.12).

The only transporter from the Tpo1-4 group to be characterized in *C. glabrata* is encoded by *CgTPO3*, the single ortholog of the *S. cerevisiae TPO2* and *TPO3* gene pair [107].

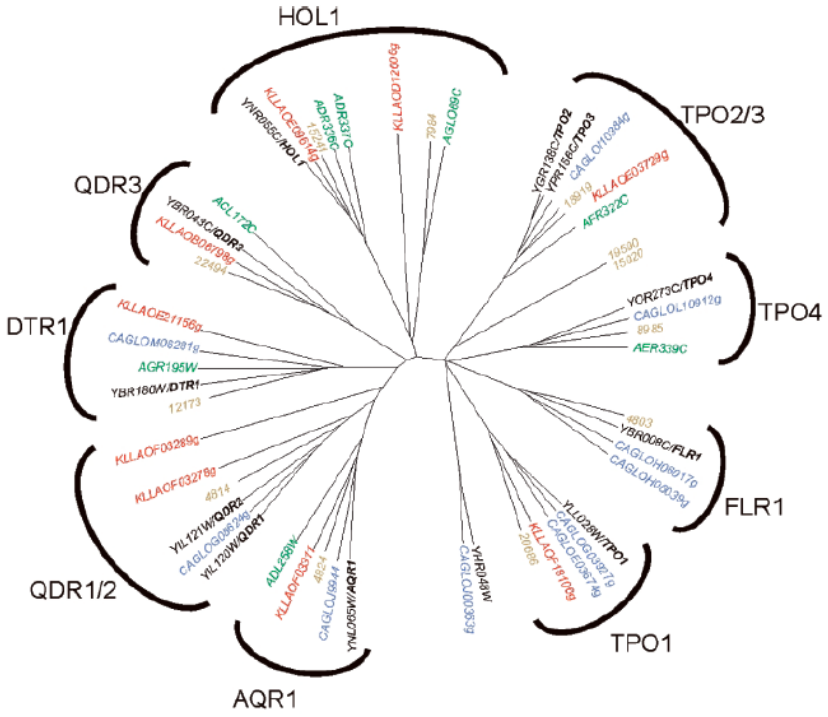


**Figure 1.12 - Phylogenetic relationship of the DHA1 family of the drug:H$^+$ antiporters (Major Facilitator Superfamily) [8].**

## 1.6. *CgGAS1* and its role in cell wall biogenesis

The cell wall is a supramolecular structure with primary functions in determining cell shape and mechanical strength [115]. Yeast cell walls are comprised of four main elements, including chitin, β-1,3-glucan, β-1,6-glucan and mannoproteins [116]. One of the most relevant components is the glucose polymer β-1,3-glucan, the main component in yeast cell walls and the one responsible for the mechanical strength properties [117].

β-1,3-glucan chains belong to the hollow helix family and can exist in various states of extension [117], conferring elasticity to the cell wall. These molecules are constituted by several hundred glucose monomers [117] and differential degrees of polymerization can be found depending on environmental conditions, since yeasts produce β-1,3-glucan to different extents depending on the growth phase and carbon source [118].

Given its elastic properties, the cell wall is not a tight and sealant structure, but a porous one, explaining why the permeability of the inner wall is only limiting for very large molecules [117]. Despite this fact, β-1,3-glucan chains are synthesized as linear chains, but mature molecules possess

branches, implying some level of post-production processing activity. ScGas1, a β-1,3-glucanosyltransferase was identified to be involved in the formation and maintenance of β-1,3-glucan, resulting in its chain elongation [119].

Upon protein translation, the Gas1 precursor is modified by removal of the C- terminal domain and attached to the glycosylphosphatidylinositol (GPI) moiety, allowing the transport of the protein from the endoplasmic reticulum (ER) and Golgi to the cell surface. This way, Gas1 is attached to the phospholipid bilayer via GPI anchor [120].

In *S. cerevisiae*, the GAS family harbors 5 distinct genes (*GAS1*, *GAS2*, *GAS3*, *GAS4*, *GAS5*), sharing significant similarity with *Aspergillus fumigates GEL1/2* and with *Candida albicans PHR1/2*. In *C. glabrata*, there is also a family of genes, *CgGAS1-3*, with deduced amino acid sequences and predicted structural homology with the Gas1 protein from *S. cerevisiae* and the Pdhr1-2 proteins of *C. albicans*, suggesting similar functions of the Gas proteins in *C. glabrata* [121].

Confirming its role in cell wall biogenesis, the study by Weig *et al.* [121] have shown that deletion of CgGas1 in *C. glabrata* leads to reduced growth and morphological aberrations. The affected cells formed aggregates and their doubling time was increased, in comparison to *S. cerevisiae Δgas1* cells, definitely showing the importance of CgGas1 in cell wall integrity. Despite no sensitivity assays have been done to attest CgGas1 role in resistance to chemical stress inducers, it was already shown that lack of Gas1 in *S. cerevisiae* increases sensitivity to cell wall affecting drugs and elevated temperatures, together with abnormally round cells with reduced viability, possibly underlying a complementary defensive effect of cell wall against antifungal drugs.

In *C. albicans*, a possible link involving cell wall integrity in azole resistance was reported [55]. Mitochondria have been identified as an important contributor for drug tolerance in fungal pathogens [122], as mitochondrial mutants display higher drug susceptibility, especially to azoles and polyenes [123]. Apparently, mitochondrial function in membrane lipid homeostasis is a common denominator underlying the observed effects of mitochondrial in drug tolerance and cell wall integrity [55]. In the study by Singh *et al.* [55], isolates with lower levels of mitochondrial phosphatydil glycerol (PG) also possessed damaged cell wall integrity. Interestingly, isolates with lower PG levels were susceptible to cell wall disrupting agents, while isolates with normal PG levels were resistant to azoles, unveiling a link between mitochondrial defect and cell wall integrity. Additional reports concluded that phospholipid imbalance is causative in cell wall defects, despite the way phospholipids affect cell wall is unknown. Dagley *et al.* [123] proposed that phospholipid imbalance affects the activity of β-glucan synthase, which is an integral membrane protein apparently dependent on the phospholipid environment in ensuring β-glucan activity.

## 1.7. Identification of azole drug resistance determinants using membrane proteomics approaches

Genome or proteome-wide studies are extremely useful to identify which genes or proteins are being expressed in a given condition, such as in drug response. They are particularly helpful in identifying unforeseen resistance determinants which could not be associated with a certain function if not for this kind of wide survey. Specifically, in the case of proteome-wide studies, mass spectrometry techniques are imperative in order to identify and quantify the proteins with altered expressed levels in a certain condition, allowing a better understanding of cell responses to each specific stress.

Mass spectrometry (MS) is a technique in which proteins are enzymatically digested until a peptide mixture is obtained. The mixture is then applied to the spectrometer and the mass of each peptide is determined. A spectrum of masses of the molecules (peptides) comprising the sample is produced, and it is used to determine the mass of the peptides. MS works by ionizing the compounds to generate charged molecules and the peptides (now ionized) are separated and identified according to their mass-to-charge ratios. Ions are detected by a mechanism capable of detecting charged particles. Signal processing results are displayed as spectra of the relative abundance of ions as a function of the mass-to-charge ratio.

There are several ways of making the separation and identification of peptides by MS, one of them is by peptide tagging. The first method using this approach was ICAT (Isotope-Coded Affinity Tag), which relies on tagging cysteine residues and isolating peptides containing these tagged residues by affinity chromatography [124]. On the other hand, in iTRAQ (isobaric tags for relative and absolute quantification), which is a variation of ICAT, tagging is on primary amines. The iTRAQ method is based on tagging each peptide covalently to the N terminus or to the lateral amine groups of lysines (global tagging), eliminating the dependence on relatively non-abundant cysteine containing peptides intrinsic to ICAT, thus potentially allowing the tagging of most tryptic peptides [124].

Other noteworthy features of the iTRAQ technology are that relative quantification is performed using MS/MS and that it utilizes several different tags, which allow multiplexing of different samples at the same time in a single experiment. Initially it started by using with four tags [9]: the isobaric tags used are formed by a reporter group (114-117 Da) based on *N*-methylpiperazine, a mass balance group (28-31 Da) and a peptide-reactive group (NHS ester), in a way that all of the four tags have a combined mass equal to 145 Da (Figure 1.13). These tags have identical mass as a result of differences in other parts of the tag structure, meaning that an identical peptide in the four samples will have an identical mass after tagging [9].

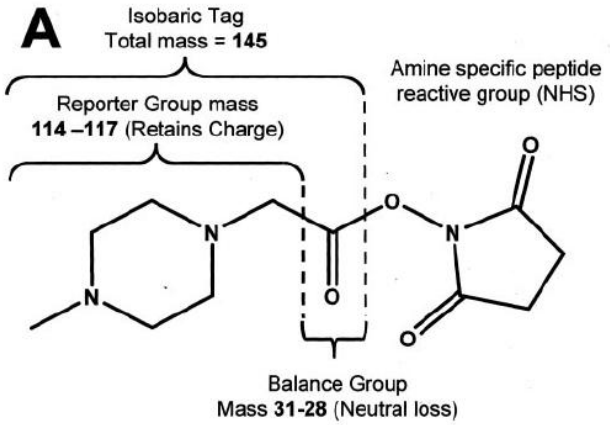


**Figure 1.13 - Structure of the isobaric tags used on iTRAQ. The overall mass of reporter and balance components of the molecule are kept constant using differential isotopic enrichment with 13C, 15N and 18O atoms [9].**

The samples are mixed with the isobaric tags. A first MS step serves the purpose of attesting a good labeling reaction, yielding indistinguishable peaks with the same mass-to-charge ratio. Subsequently, the amide linkages between reporter and balance groups are fragmented. Following fragmentation of the tag amide bond, the balance (carbonyl) moiety is lost (neutral charge loss), while charge is retained by the reporter group fragment. In a second MS step, quantification can then be performed via the differences in abundances of four product ions (114, 115, 116, and 117 Da) that are each cleaved from one of the four possible tags [9] (Figure 1.14). The multiplex isobaric tags produce abundant MS/MS signature ions, and the relative areas of the peaks correspond with the proportions of the labeled peptides [9].

The multi-sample capability of iTRAQ technology is ideal for proteomics studies in which several experimental conditions are tested [124]. Presently, more than four tags are used, allowing the study of more samples simultaneously. However, MS/MS spectra must be acquired, which requires more analysis time than performing result-dependent analysis only on differentially expressed peptide pairs in MS (e.g. with ICAT). Nevertheless it simplifies analysis and increases analytical accuracy and precision, having the ability to identify more proteins with increased confidence and greater peptide coverage [9].

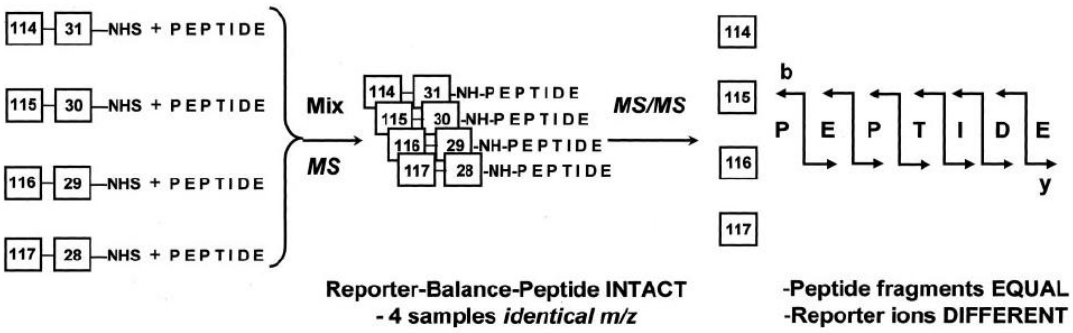


**Figure 1.14 - Illustration of the isotopic tagging used to arrive at four isobaric combinations with four different reporter group masses [9].**

# 2. Materials and Methods

## 2.1. 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-frankfurt.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 $NH_4^+$ (Difco), 20 g glucose (Merck) and 2.65 g $(NH_4)_2SO_4$ (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 [125] and derived single deletion mutants KUE100_*Δcggas1,* KUE100_*Δcgtpo1_1* or KUE100_*Δcgtpo1_2*, kindly provided by Hiroji Chibana, Chiba University, Japan; as well as the *C. glabrata* strains 66032u and 66032u_*Δcgpdr1* [126], 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, 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 $(NH_4)_2SO_4$ (Merck). *C. glabrata* strain L5U1 (cgura3 Δ 0, cgleu2 Δ 0) [102], kindly provided by John Bennett, from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA, was grown in BM 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 (described below) was obtained from the Drag&Drop collection [10].

## 2.2. Membrane proteome-wide analysis of *C. glabrata* response to clotrimazole

Wild-type 66032 *C. glabrata* strain and the derived 66032_*Δcgpdr1* deletion mutant were cultivated in liquid BM at 30ºC with orbital agitation (250 rpm) in the absence of stress until the standardized culture $OD_{600nm}$ of 0.8 ± 0.08 was reached. Cells were then transferred to fresh medium in the absence of stress (control conditions) or in the presence of 100 mg/L clotrimazole, with an initial $OD_{600nm}$ of 0.4 ± 0.05. Upon 1h of cultivation, cells were harvested by centrifugation and resuspended in A Buffer (50 mM Tris, pH 7.5, with 0.5 mM EDTA and 20% glycerol), with protease inhibitors (10 mg/L leupeptine, 1 mg/L pepstatine A, 20 mg/L aprotinine, 2 mg/L trypsin/quimotrypsin inhibitor, 1.5 mg/L benzamindine and 1 mM PhenylMethylSulfonyl Fluoride (PMSF) – all obtained from Sigma). Cell lysis was accomplished by consecutive steps of vortexing and cooling in the presence of glass beads. The mixture was centrifuged for clarification (8000 rpm, 5 min, 4ºC) and the top phase collected. A Buffer was added to a final volume of 8 mL, this mix ultra-centrifuged on a Beckman XL-90 ultracentrifuge (24000 rpm, 90 min, 4ºC) and the pellet washed with 8 mL of 0.1 M $Na_2CO_3$ and incubated on ice for 30 min with orbital agitation (60 rpm). The mixture was then ultracentrifuged (26000 rpm, 60 min, 4ºC) and the pellet was washed with 8 mL of 50 mM TEAB (Tetraethylammonium bromide, Sigma) and ultracentrifuged again (26000 rpm, 60 min, 4ºC). This procedure was repeated 2 more times and, finally, the pellet was resuspended in 325 µL of 50 mM TEAB with 8 M urea (Sigma).

Expression proteomics analysis of the obtained membrane-enriched fraction was carried out using and iTRAQ-MS procedure, carried out as a paid service at the Keck Foundation Biotechnology Resource Laboratory, Yale University, USA (http://medicine.yale.edu/keck/proteomics/index.aspx). Briefly, samples were sonicated and proteins reduced by adding 50 mM TCEP (tris(2-carboxyethyl)phosphine), followed by 200 mM MMTS (methyl methane thiosulfonate). Protein digestion was achieved by adding 10 µL of a solution of 1 mg/mL Lys-C, followed by incubation at 37ºC for 3h, and 10 µL of 1 mg/mL trypsin, followed by overnight incubation at 37ºC. Macro-spin desalt of the digests with C18 spin columns for cleanup and quantitation was carried out, followed by dissolution in 65 µL of 500 mM TEAB. iTRAQ labelling was carried out based on the AAA quant protocol. iTRAQ experiments were carried out through the SCX cartridge and experiments run on 5600.

Proteomics data analysis started from 3 iTRAQ sets. The samples present in each set were randomized to prevent bias, and in different sets distinct labels were used to tag the samples, ensuring that protein identification in the MS step is not biased by the tags. For each sample in a given set, protein quantification was only considered for P-value<0.05. Protein expression changes above 1.5-fold or below 0.66-fold were considered relevant. Protein identification was considered reliable for a Protein Score > 2, corresponding to a confidence level of 99%. Protein classification into functional groups was achieved based on their predicted function, according to the Candida Genome Database (www.candidagenome.org), or based on the function of their closest *S. cerevisiae* homolog, according to the *Saccharomyces* Genome Database (www.yeastgenome.org).

## 2.3. *S. cerevisiae* and *C. glabrata* transformation

All transformation reactions were performed using the Alkali-Cation Yeast Transformation Kit (MP Biomedicals), according to the manufacturer's instructions. Mid-exponential cells from *S. cerevisiae* strain BY4741 and *C. glabrata* strains KUE100 and L5U1 were batch-cultured at 30ºC with orbital shaking (250 rpm) in 30 mL YPD liquid medium until a standard $OD_{600nm}$ 0.4 ± 0.05 was reached. The cells were harvested by centrifugation at 7000 rpm for 5 min at 4ºC and the resulting pellets were resuspended in 2.7 mL of TE buffer, pH 7.5. After a second centrifugation step, the cells were harvested and rinsed with 1.5 mL of 0.15 M Lithium Acetate solution and shaken gently (100 rpm) at 30ºC for 25 minutes. Cells were harvested by centrifugation (7000 rpm, 5 min, 4ºC) and resuspended in 300 µL TE buffer, pH 7.5. Cells were then transferred to 1.5 mL tubes, combining: 100 µL yeast cells, 5 µL Carrier DNA, 5 µL Histamine Solution and 100-200 ng plasmid DNA. Cells were gently mixed and incubated at room temperature for 15 min. A mixture of 0.8 mL PEG and 0.2 mL TE/Cation MIXX solution was added to each transformation reaction, followed by 10 min incubation at 30ºC and heat shock at 42ºC for 10 minutes. Cells were then pelleted in a microcentrifuge and resuspended in 100 µL YPD liquid medium before plating in solid YPD agar plates.

## 2.4. Cloning of the *C. glabrata CgTPO1_1* and *CgTPO1_2* genes (*ORFs CAGL0G03927g* and *CAGL0E03674g*, respectively)

The pGREG576 plasmid from the Drag & Drop collection [10] was used to clone and express the *C. glabrata ORFs CAGL0G03927g* and *CAGL0E03674g* in *S. cerevisiae*, as described before for other heterologous genes [127]. pGREG576 was acquired from Euroscarf and contains a galactose inducible promoter (*GAL1*), the yeast selectable marker *URA3* and the *GFP* gene, encoding a Green Fluorescent Protein (GFPS65T), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. The plasmid was restricted with the restriction enzyme SalI (Takara) in the cloning site harboring the *HIS3* gene and treated with CiAP (Invitrogen) during 45 minutes at 37ºC to prevent recircularization (Figure 2.1). The *CgHIS3*, *CAGL0G03927g* or *CAGL0E03674g* DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the specific primers present in Table 2.1.

**Table 2.1 - Primer sequences used to obtain *CAGL0G03927g* or *CAGL0E03674g* DNA for pGREG576 cloning procedure. The sequences present a region with homology to the cloning site flanking regions of the vector (underlined) and homology regions to the genes to be amplified (italic).**

| Gene | Primer | Sequence |
|---|---|---|
| ***CgTPO1_1*** | Forward | 5'-GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGGTGGAAGAGATATCGCC*-3' |
| | Reverse | 5'-GCGTGACATAACTAATTACATGACTCGAGGTCGAC*TTAAGCGTAGTAAGCATCC*-3' |
| ***CgTPO1_2*** | Forward | 5'-GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGTCCTCCACTAGTAGCG*-3' |
| | Reverse | 5'-GCGTGACATAACTAATTACATGACTCGAGGTCGAC*TTATAACGAATATGCGTAC*-3' |

The designed primers contain, besides a region with homology to the first 20 and the last 19 nucleotides of the *CAGL0G03927g*, and with homology to the first and last 19 nucleotides of the *CAGL0E03674g* coding regions (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragments were co-transformed into the parental *S. cerevisiae* strain BY4741 with the pGREG576 vector, previously cut with the restriction enzyme SalI, to obtain the pGREG576_*CgTPO1_1* or pGREG576_*CgTPO1_2* plasmids. Since the *GAL1* promoter only allows a slight expression of downstream genes in *C. glabrata*, to visualize by fluorescence microscopy the subcellular localization of the CgTpo1_1 or CgTpo1_2 proteins in *C. glabrata*, new constructs were obtained. The *GAL1* promoter present in the pGREG576_*CgTPO1_1* and pGREG576_*CgTPO1_2* plasmids was replaced by the copper-induced *MTI C. glabrata* promoter, giving rise to the pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2* plasmids. The *MTI* promoter DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the specific primers present in Table 2.2.

**Table 2.2 - Primer sequences used to obtain *MTI* promoter DNA for pGREG576 cloning procedure. The sequences present a region with homology to the cloning site flanking regions of the vector (underlined) and homology regions to the target (italic).**

| Target | Primer | Sequence |
|---|---|---|
| ***MTI* promoter** | Forward | 5'-<u>TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTC</u>*TGTACGACACGCATCATGTGGCAATC*-3' |
| | Reverse | 5'-<u>GAAAAGTTCTTCTCCTTTACTCATACTAGTGCGGC</u>*TGTGTTTGTTTTTGTATGTGTTTGTTG*-3' |

The designed primers contain, besides a region with homology to the first 26 and the last 27 nucleotides of the first 1000 bp of the *MTI* promoter region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental strain BY4741 with the pGREG576_*CgTPO1_1* or pGREG576_*CgTPO1_2* plasmids, previously cut with SacI and NotI (Takara) restriction enzymes to remove the *GAL1* promoter, generating the pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2* plasmids.

The PCR amplification reactions of *CgTPO1_1*, *CgTPO1_2* and *MTI* promoter were performed using a C1000 Thermal Cycler (Bio-Rad) and the following program present in Table 2.3. The reaction mixture used for genes and promoter DNA attainment was prepared as depicted in Table 2.4.

**Table 2.3 - PCR program used for amplification of *CgTPO1_1*, *CgTPO1_2* and *MTI* promoter for pGREG576 cloning procedure.**

| Step | Time | Temperature (ºC) | Cycles |
|---|---|---|---|
| **Initial denaturation** | 30 sec | 98 | 1 |
| **Denaturation** | 10 sec | 98 | |
| **Annealing** | 20 sec | 56 | 30 |
| **Extension** | 1 min | 72 | |
| **Final extension** | 7 min | 72 | 1 |

**Table 2.4 - Reaction mixture composition for PCR amplification of *CgTPO1_1*, *CgTPO1_2* and *MTI***

| Component | Volume per reaction (µL) |
|---|---|
| **10x HF buffer** | 10 |
| **Primer forward (50 pmol)** | 1 |
| **Primer reverse (50 pmol)** | 1 |
| **dNTPs (10 mM)** | 1 |
| **$MgCl_2$ (50 mM)** | 2 |
| **DMSO** | 1.5 |
| **DNA template** | 2 |
| **$ddH_2O$** | 31 |
| **Taq Phusion (2 $U.\mu L^{-1}$)** | 0.5 |
| **TOTAL** | 50 |

The recombinant plasmids pGREG576_*CgTPO1_1*, pGREG576_*CgTPO1_2*, pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2* were obtained through homologous recombination in *S. cerevisiae* using the Alkali-Cation Yeast Transformation Kit (MP Biomedicals) according to the manufacturer's instructions, and verified by DNA sequencing.

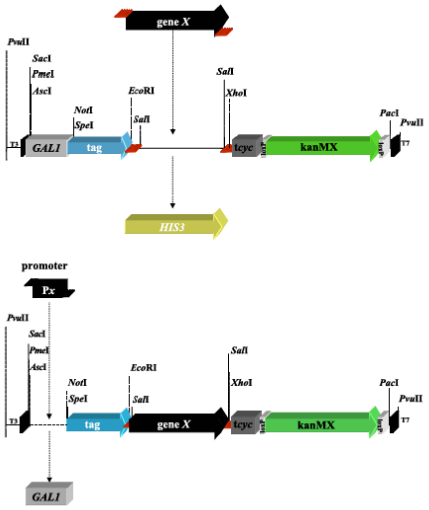


**Figure 2.1 - Schematic representation of the cloning procedure using pGREG576. The gene cloning site harbors a *HIS3* tag flanked by SalI restriction sites. The promoter cloning site harbors the *GAL1* promoter, flanked by SacI and NotI restriction sites [10].**

## 2.5. CgTpo1_1 and CgTpo1_2 subcellular localization 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, 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 over-expression. After 5h of incubation, 2 mL of cell suspension were centrifuged at 13500 rpm for 2 minutes, and the pelleted cells were resuspended in 7 µL 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. Fluorescence images were captured using a cooled CCD camera (Cool SNAPFX, Roper Scientific Photometrics).

## 2.6. Antifungal susceptibility assays in *C. glabrata*

The susceptibility of the parental strain KUE100 towards toxic concentrations of the selected drugs was compared to that of the deletion mutants KUE100_*Δcggas1*, KUE100_*Δcgtpo1_1* and KUE100_*Δcgtpo1_2* by spot assays. The ability of *CgTPO1_1* and *CgTPO1_2* gene expression to increase wild-type resistance to the tested chemical stresses was also examined in the $URA3^-$ strain L5U1 *C. glabrata*, using the pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2* centromeric plasmids.

KUE100 *C. glabrata* cell suspensions used to inoculate the agar plates were mid-exponential cells grown in BM, 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 plates, supplemented with adequate chemical stress concentrations. L5U1 *C. glabrata* cell suspensions used to inoculate the agar plates were mid-exponential cells grown in BM, supplemented with 60 mg/L leucine and 50 µM $CuSO_4$ (Sigma), to induce protein overexpression, and without uracil when using the L5U1 strain harboring the pGREG576 derived 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 plates, without uracil for strains transformed with the pGREG576 derived plasmids, supplemented with 60 mg/L leucine, 50 µM $CuSO_4$ and 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).

## 2.7. *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.

Cells were resuspended in AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.3, 0.1% DEPC) with posterior SDS 10% addition. The samples were treated with Phenol solution for RNA purification (Sigma) and heat shocked at 65ºC for 4 minutes, followed by cooling in dry ice (ice + ethanol + calcium chloride) until the appearance of phenol crystals occurred. The samples were then centrifuged at 15000 rpm during 5 minutes and the resulting upper phase was collected, a double extraction with a phenol/chloroform mixture (chloroform = 24:1, chloroform:isoamilic alcohol) was performed. After one new extraction with chloroform:isoamilic alcohol, the upper phase was collected and a solution of sodium acetate 3 M, pH 5.3 was added, followed by addition of 2.5x the resulting volume in ethanol and the samples were stored at -20ºC during 20 minutes. The samples were then pelleted by centrifugation (15000 rpm, 15 minutes) and the pellets were dried during 15 min at 45ºC in SpeedVaccum. Total RNA was then resuspended in DEPC treated water and diluted until a final concentration of 500 ng/µL was reached.

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 was prepared as present in Table 2.5; while the program used for the cDNA synthesis is shown in Table 2.6. The quantity of cDNA for subsequent reactions was diluted and kept at 10 ng.

**Table 2.5 - First step reaction mixture for Real Time RT-PCR (Applied Biosystems).**

| Component | Volume per reaction (µL) |
|---|---|
| **TaqMan RT Buffer (10x)** | 1.0 |
| **$MgCl_2$ (25 mM)** | 2.2 |
| **dNTP's (2.5 mM)** | 2.0 |
| **Random hexamers (50 µM)** | 0.5 |
| **RNase inhibitor (20 $U.L^{-1}$)** | 0.2 |
| **MultiScribe reverse transcriptase (50 $U.\mu L^{-1}$)** | 0.25 |
| **RNA samples (500 $ng.\mu L^{-1}$)** | 2 |
| **$ddH_2O$ DEPC treated** | 1.85 |
| **TOTAL** | 10.0 |

**Table 2.6 - First step thermal cycling parameters for Real Time RT-PCR (Applied Biosystems).**

| Step | Time (min) | Temperature (ºC) |
|---|---|---|
| **Incubation** | 10 | 25 |
| **Reverse transcription** | 30 | 48 |
| **Reverse transcriptase inactivation** | 5 | 95 |

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) as it is present in Table 2.7.

**Table 2.7 - Second step reaction mixture for Real Time RT-PCR (Applied Biosystems).**

| Component | Volume per reaction (µL) |
|---|---|
| **SYBR Green® PCR Master Mix (2x)** | 12.5 |
| **Forward primer (4 $pmol.\mu L^{-1}$)** | 2.5 |
| **Reverse primer (4 $pmol.\mu L^{-1}$)** | 2.5 |
| **cDNA sample** | 2.5 |
| **$ddH_2O$** | 5.0 |
| **TOTAL** | 25.0 |

Real -time PCR was carried out using a thermal cycler block (7500 real-time PCR system; Applied Biosystems) following the program depicted in Table 2.8. Default parameters established by the manufacturer were used, and fluorescence was detected by the instrument and registered in an amplification plot (7500 System SDS Software; Applied Biosystems). The *CgACT1* mRNA level was used as an internal control. The relative values obtained for the wild-type strain under control conditions were set at 1, and the remaining values are presented relative to that control. To avoid false-positive signals, the absence of non-specific amplification with the chosen primers was confirmed by the generation of a dissociation curve for each pair of primers. The primers used for the amplification of the internal control *CgACT1*, and the target mRNAs from *CgTPO1_1* and *CgTPO1_2* are shown in Table 2.9.

**Table 2.8 - Second step thermal cycling parameters for Real Time RT-PCR (Applied Biosystems).**

| Step | | Time | Temperature (ºC) |
|---|---|---|---|
| **AmpliTaq® gold DNA polymerase activation** | | 10 min | 95 |
| **PCR (40 cycles)** | Reverse Transcription | 15 sec | 95 |
| | Reverse transcriptase inactivation | 1 min | 60 |

**Table 2.9 - Designed primers for Real Time RT-PCR expression measurements of the target genes *CgACT1* (internal control), *CgTPO1_1* and *CgTPO1_2* using Primer Express software (Applied Biosystems).**

| Gene | Primer | Sequence |
|---|---|---|
| ***CgACT1*** | Forward | 5'-AGAGCCGTCTTCCCTTCCAT-3' |
| | Reverse | 5'-TTGACCCATACCGACCATGA-3' |
| ***CgTPO1_1*** | Forward | 5'-CGCTGCTTCCCCAGTTATCT-3' |
| | Reverse | 5'-CTAGCACACCACGTCTACCGT-3' |
| ***CgTPO1_2*** | Forward | 5'-AGGACCCGCTCTATCGAAAAA-3' |
| | Reverse | 5'-GCTGCGACTGCTGACTCAAC-3' |

The quantification is possible by registration of the signal level in an amplification plot. From it, a Ct value is estimated and normalized using an internal control:

$$\Delta C_t = C_t(target) - C_t(control) \qquad (2.1)$$

The normalized value is then compared to each target gene:

$$\Delta\Delta C_t = \Delta C_t(sample) - \Delta C_t(calibrator) \quad (2.2)$$

Gene expression levels can be calculated using the values from the previous expression:

$$2^{-\Delta\Delta C_t} \qquad (2.3)$$

## 2.8. $^{3}$H-clotrimazole accumulation assays

$^{3}$H-clotrimazole accumulation assays were carried out has described previously [106]. 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 the 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

1.57 mg [(dry weight) $mL^{-1}$)]. Readily, 0.1 µM $^{3}$H-clotrimazole (1 mCi/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), and the radioactivity was measured in a Beckman LS 5000TD scintillation counter. To calculate the intracellular concentration of labelled 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 [128]. Extracellular $^{3}$H-clotrimazole was estimated by radioactivity assessment of 50 µL of the supernatant after collecting 70 µL of cell suspension. Non-specific $^{3}$H-clotrimazole adsorption to the filters and to the cells (less than 5% of the total radioactivity) was assessed and taken into consideration.

## 2.9. β-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 [129]. 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 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.

# 3. Results

## 3.1. Proteome-wide identification in *C. glabrata* membrane-enriched fraction

840 proteins were identified in the membrane-enriched fraction in *C. glabrata*, 88 encoded by characterized genes, while 752 are encoded by non-characterized genes. To obtain a global perspective of the functional distribution of the 840 proteins identified in the membrane-enriched fraction of *C. glabrata*, the online Gene Ontology term-based grouping tool GoToolBox (http://genome.crg.es/GOToolBox/) was used, considering their *S. cerevisiae* homologs. Figure 3.1 highlights several enriched GO terms related with transmembrane transport functions and membrane associated metabolic processes, as expected.

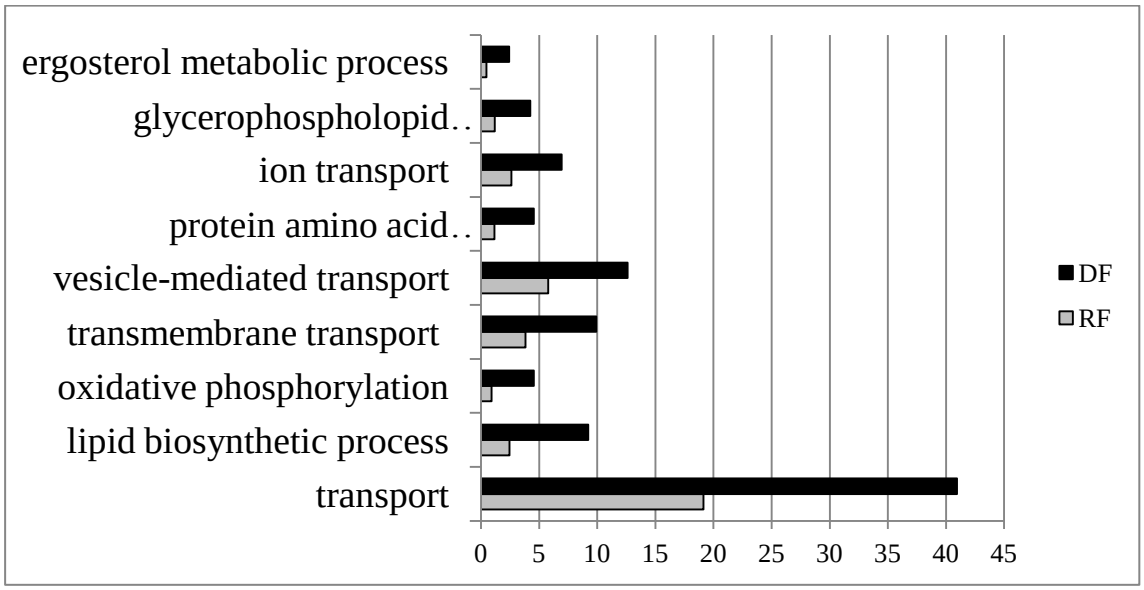


**Figure 3.1 - Main GO terms enriched in the submitted data set. DF - Frequency of genes annotated for each term in the submitted set. RF - Frequency of genes annotated for each term in the reference set.**

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

With the objective of understanding which proteins and functional groups respond to clotrimazole stress, an analysis of the membrane-enriched fraction of the *C. glabrata* proteome obtained from cells exposed to clotrimazole when compared to control conditions was made.

This study allowed the identification of 37 proteins being differentially expressed in the presence of clotrimazole. Among those, 12 proteins have shown an increase in their content, while 25 proteins exhibit decreased expression upon exposure to the drug. To better understand the response profile to clotrimazole, and since only 4 of these 37 proteins had been previously characterized, they were clustered into 6 functional groups based on the role of their predicted *S. cerevisiae* homologs: Glucose

metabolism, Ribosome components and translation machinery, Oxidative phosphorylation, Lipid and cell wall metabolism, Mitochondrial import and Multidrug resistance transporters (Table 3.1 and Figure 3.2).

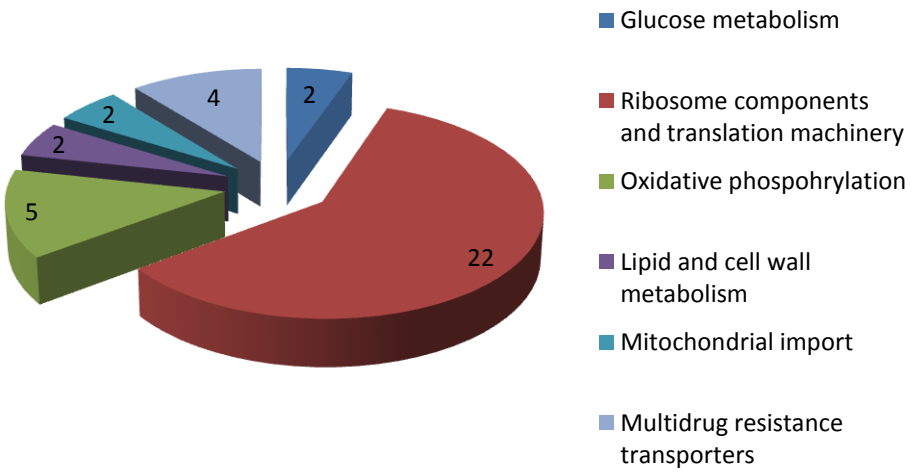


**Figure 3.2 - Major functional groups found to have significant expression changes in the membrane-enriched fraction proteome upon exposure to clotrimazole in *C. glabrata*.**

Among the obtained clusters, the most populated one is Ribosome components and translational machinery, with 2 ribosomal proteins being up-regulated, while the remaining 20 are down-regulated. This observation appears consistent with a decreased translation rate, which has been considered part of the so-called “Environmental Stress Response” [130]. This suggested decrease in translational activity can be related with growth arrest conditions resultant from subjacent stress background [130]. It is also possible to be an attempt to only produce the exclusively necessary proteins to surpass stress and maintain cell viability. The two up-regulated proteins belong to the ribosomal 60S and 40S subunits, respectively. The first, *CAGL0A03388g*, was previously described to respond to DNA replication stress [131]. As stated in Section 1.3.3, imidazoles, in which clotrimazole is included, were described to induce endogenous ROS in exposed cells, and these reactive oxygen species are known to cause oxidative damage to DNA and other cellular constituents [132]. This could explain why an overexpression of these proteins is verified over the rest underexpressed proteins in the same functional group. Many rRNA genes have coordinate regulation [133, 134], possibly the second up-regulated protein from *ORF CAGL0E02013g* has some degree of joint regulation with *ORF CAGL0A03388g*, what would be acceptable giving their similar expression change.

The oxidative phosphorylation cluster is also well represented, with 4 proteins being down-regulated, while only 1 is up-regulated. These results appear to be consistent with previous microarray studies focusing on fluconazole response, in which down-regulation of genes related with oxidative

phosphorylation pathway was reported [94]. Given the described effect of endogenous ROS generation by other imidazoles [58], a possible similar effect could be argued, thus limiting oxidative phosphorylation activity due to excessive oxidative burden.

As expected, all 4 proteins present in the Multidrug resistance transporters cluster are up-regulated upon exposure to the drug. 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 [37]. 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 [135] and with its characterization as an azole resistance determinant [46]. CgSnq2 has been characterized as a multidrug transporter [90], as well as its *S. cerevisiae* homolog [136, 137]. 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* [109]. 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*.

Clusters comprising glucose metabolism, mitochondrial import and lipid and cell wall metabolism are represented by only two proteins each. The proteins present in the glucose metabolism cluster are both up-regulated, which is consistent with the energy requirements necessary for drug extrusion and typical from the ESR [130]. Concordantly, is the observed up-regulation of *ORF CAGL0L01485g*, a putative homolog of *GSF2* in *S. cerevisiae* which is required for the targeting of glucose transporters to the plasma membrane [138], possibly underlying an increase in energy source requirements.

On the other hand, mitochondrial import proteins have a mixed response, with one being up-regulated while the other is down-regulated. The up-regulated one is an homolog of *S. cerevisiae* Phb1, a protein characterized as chaperone required for optimal mitochondrial morphology and function [139, 140], implying a response for mitochondrial preservation and protection, especially in the case of higher oxidative burden due to clotrimazole exposure. The down-regulated protein, on the other hand, is a translocase involved in import steps for mitochondrially directed proteins [141]. Given the attained low-oxidative phosphorylation activity already verified, such an occurrence in decreasing mitochondrial import is plausible.

A protein related with cell wall metabolism, CgGas1, was found to be up-regulated in response to clotrimazole. As stated in Section 1.6, this protein is necessary for cell wall assembly, raising the hypothesis that a response at the level of the cell wall may underlie an important aspect of

clotrimazole response and resistance mechanisms exhibited by *C. glabrata* cells. The up-regulation of Gas1 can represent a way to maintain cell wall integrity under stress.

**Table 3.1 - Set of 37 proteins 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.**

| ***C. glabrata* protein (ORF) name** | ***S. cerevisiae* homolog** | **Description of the function of the *C. glabrata* protein or of its *S. cerevisiae* homolog[a]** | **Wild-type fold change** |
|---|---|---|---|
| ***Glucose Metabolism*** | | | |
| ***CAGL0L01485g*** | *GSF2* | Uncharacterized. *S. cerevisiae* homolog encodes a ER localized integral membrane protein that may promote secretion of certain hexose transporters, including Gal2 | 2.26 |
| ***PGK1 (CAGL0L07722g)*** | *PGK1* | Uncharacterized. *S. cerevisiae* homolog encodes a 3-phosphoglycerate kinase; key enzyme in glycolysis and gluconeogenesis | 6.31 |
| ***Oxidative phosphorylation*** | | | |
| ***CAGL0H05489g*** | *ATP4* | Uncharacterized. *S. cerevisiae* homolog encodes a subunit b of the stator stalk of mitochondrial F1F0 ATP synthase | 1.68 |
| ***CAGL0F04565g*** | *COR1* | Uncharacterized. *S. cerevisiae* homolog encodes a core subunit of the ubiquinol-cytochrome c reductase complex, a component of the mitochondrial inner membrane electron transport chain | 0.32 |
| ***RIP1 (CAGL0I03190g)*** | *RIP1* | Uncharacterized. *S. cerevisiae* homolog encodes a ubiquinol-cytochrome-c reductase; a Rieske iron-sulfur protein of the mitochondrial cytochrome bc1 complex | 0.10 |
| ***CAGL0G10131g*** | *QCR2* | Uncharacterized. *S. cerevisiae* homolog encodes a subunit 2 of the ubiquinol cytochrome-c reductase complex, a component of the mitochondrial inner membrane electron transport chain | 0.40 |
| ***CAGL0G10153g*** | *QCR7* | Uncharacterized. *S. cerevisiae* homolog encodes a subunit 7 of the ubiquinol cytochrome-c reductase complex, a component of the mitochondrial inner membrane electron transport chain | 0.21 |
| ***Mitochondrial import*** | | | |
| ***CAGL0I10472g*** | *PHB1* | Uncharacterized. *S. cerevisiae* homolog encodes a subunit of the prohibitin complex (Phb1p-Phb2p), a 1,2 MDa ring-shaped inner mitochondrial membrane chaperone that stabilizes newly synthesized proteins | 3.09 |
| ***CAGL0L12936g*** | *TOM70* | Uncharacterized. *S. cerevisiae* homolog encodes a component of the TOM (translocase of outer membrane) complex; involved in the recognition and initial import steps for all mitochondrially directed proteins | 0.57 |
| ***Ribosome components and translation machinery*** | | | |
| ***CAGL0A03388g*** | *RPL13B* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L13B; not essential for viability | 2.22 |

| | | | |
|---|---|---|---|
| ***CAGL0E02013g*** | *RPL28A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 2.21 |
| ***CAGL0E03938g*** | *RPL8B* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L4B | 0.16 |
| ***CAGL0F07073g*** | *RPS2* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) subunit | 0.45 |
| ***CAGL0F09031g*** | *RPS4A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.47 |
| ***CAGL0G00990g*** | *RPP0* | Uncharacterized. *S. cerevisiae* homolog encodes a conserved ribosomal protein P0 of the ribosomal stalk | 0.59 |
| ***CAGL0G01078g*** | *RPL26A* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L33B | 0.22 |
| ***CAGL0G06490g*** | *RPS7A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.22 |
| ***CAGL0H00462g*** | *RPS5* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.55 |
| ***CAGL0J03234g*** | *RPS24B* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.33 |
| ***CAGL0K06567g*** | *RPL27A* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L27A | 0.13 |
| ***CAGL0K07414g*** | *RPL20B* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L20A | 0.36 |
| ***CAGL0K11748g*** | *RPS11A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.47 |
| ***CAGL0L08114g*** | *RPS22A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.52 |
| ***CAGL0L12870g*** | *TMA19* | Uncharacterized. *S. cerevisiae* homolog encodes a protein that associates with ribosomes | 0.26 |
| ***CAGL0M02695g*** | *RPL5* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L5 | 0.45 |
| ***EFT2 (CAGL0A03234g)*** | *EFT1* | Uncharacterized. *S. cerevisiae* homolog encodes an elongation factor 2 | 0.43 |
| ***CAGL0H08976g*** | *RPL1A* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L15A | 0.41 |
| ***CAGL0H03773g*** | *RPL7* | Uncharacterized. *S. cerevisiae* homolog encodes a nucleolar protein with similarity to large ribosomal subunit L7 proteins; constituent of 66S pre-ribosomal particles | 0.36 |
| ***CAGL0I00792g*** | *RPS16A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 0.20 |

| | | | |
|---|---|---|---|
| ***SSB1 (CAGL0C05379g)*** | *SSB2* | Uncharacterized. *S. cerevisiae* homolog encodes a cytoplasmic ATPase that is a ribosome-associated molecular chaperone | 0.58 |
| ***TEF3 (CAGL0B03487g)*** | *TEF3* | Uncharacterized. *S. cerevisiae* homolog encodes a gamma subunit of translational elongation factor eEF1B | 0.58 |
| ***Lipid and cell wall metabolism*** | | | |
| ***HFD1 (CAGL0K03509g)*** | *HFD1* | Uncharacterized. Gene is upregulated in azole-resistant strain. *S. cerevisiae* homolog encodes a hexadecenal dehydrogenase | 6.74 |
| ***GAS1 (CAGL0G00286g)*** | *GAS1* | Putative glycoside hydrolase of the Gas/Phr family; predicted GPI-anchor; *S. cerevisiae* homolog is a beta-1,3-glucanosyltransferase, required for cell wall assembly | 3.92 |
| ***Multidrug resistance transporters*** | | | |
| ***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 |

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

The membrane-enriched fraction of the *C. glabrata* proteome obtained from cells devoid of CgPdr1 upon exposure to clotrimazole was also analyzed. The obtained results show that, among the clotrimazole-affected proteins in the wild-type strain, 11 are differentially affected by clotrimazole in *Δcgpdr1 C. glabrata* cells. CgPdr1 appears to mediate the repression of the expression of 6 proteins, possibly in an indirect way, and the activation of 5 proteins, upon clotrimazole challenge.

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 CgQdr2, CgSnq2 and CgCdr1; and the hexadecenal dehydrogenase Hfd1. 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 [37, 46, 90]. The expression changes obtained in this set 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 [66].

It would be interesting to assess whether this result may relate to the referred 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 (Figure 3.3), suggesting that the action of CgPdr1 in their expression may be direct.

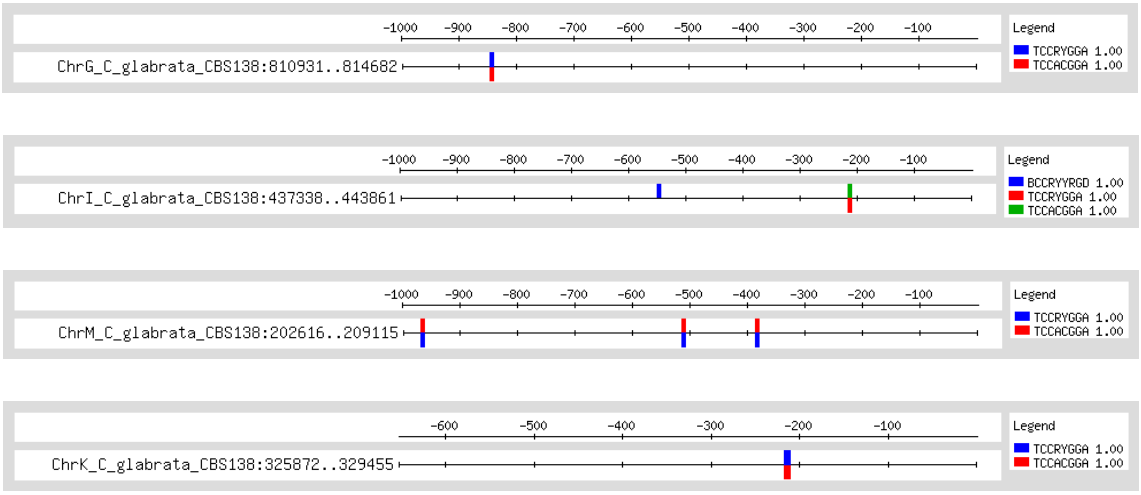


**Figure 3.3 – Presence and position of PDRE consensus sequences recognized by CgPdr1. From top to bottom, *CgQDR2* harbors one CgPdr1-binding site (pos -848 to -841), *CgSNQ2* harbors two CgPdr1-binding sites (pos -552 to -544 and -218 to -211), *CgCDR1* harbors three CgPdr1-binding sites (pos -969 to -962, -515 to -508 and -387 to -380), and *CgHFD1* harbors one CgPdr1-binding site (pos -218 to -211) (http://rsat.ulb.ac.be/).**

Surprisingly, 3 ribosome associated proteins and 3 oxidative phosphorylation components which are repressed by clotrimazole in wild-type cells, were found to be activated upon clotrimazole exposure in *Δcgpdr1 C. glabrata* cells. The CgPdr1-dependent repression of these proteins is probably indirect. However, it suggests that this transcription factor may affect besides multidrug resistance proteins, also proteasomal and mitochondrial activity. Interestingly, in *S. cerevisiae*, the transcription factors ScPdr1/3 participate together with the major regulator of ribosome biosynthesis – Rpn4 – in the regulation of ribosomal proteins [142] and ScPdr3 is known to be responsive to mitochondrial dysfunction [143].

**Table 3.2 - Set of 12 proteins found to have significant expression changes in *C. glabrata* 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.**

| ***C. glabrata* protein (ORF) name** | ***S. cerevisiae* homolog** | **Description of the function of the *C. glabrata* protein or of its *S. cerevisiae* homolog[a]** | ***Clotrimazole induced changes in Δcgpdr1 vs clotrimazole induced changes in wt (fold-change)*** |
|---|---|---|---|
| ***Oxidative phosphorylation*** | | | |
| ***CAGL0F04565g*** | *COR1* | Uncharacterized. *S. cerevisiae* homolog encodes a core subunit of the ubiquinol-cytochrome c reductase complex, a | 14.75 |

| | | | |
|---|---|---|---|
| | | component of the mitochondrial inner membrane electron transport chain | |
| ***CAGL0G10131g*** | *QCR2* | Uncharacterized. *S. cerevisiae* homolog encodes a subunit 2 of the ubiquinol cytochrome-c reductase complex, a component of the mitochondrial inner membrane electron transport chain | 1.68 |
| ***CAGL0G10153g*** | *QCR7* | Uncharacterized. *S. cerevisiae* homolog encodes a subunit 7 of the ubiquinol cytochrome-c reductase complex, a component of the mitochondrial inner membrane electron transport chain | 5.91 |
| ***Ribosome components and translation machinery*** | | | |
| ***CAGL0G01078g*** | *RPL26A* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein L33B | 0.51 |
| ***CAGL0G06490g*** | *RPS7A* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 1.68 |
| ***CAGL0H00462g*** | *RPS5* | Uncharacterized. *S. cerevisiae* homolog encodes a protein component of the small (40S) ribosomal subunit | 1.54 |
| ***CAGL0K06567g*** | *RPL27A* | Uncharacterized. *S. cerevisiae* homolog encodes a ribosomal 60S subunit protein | 1.89 |
| ***Lipid metabolism*** | | | |
| ***HFD1 (CAGL0K03509g)*** | *HFD1* | Uncharacterized. Gene is upregulated in azole-resistant strain. *S. cerevisiae* homolog encodes a hexadecenal dehydrogenase | 0.18 |
| ***Multidrug resistance transporters*** | | | |
| ***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 |

## 3.2. Functional characterization of the CgTpo1_1 and CgTpo1_2 multidrug resistance transporters

Given the attained membrane proteomics results, two multidrug resistance transporters from the MFS were selected for further studies. CgTpo1_2 is the only MDR transporter present in the previous proteomics analysis lacking characterization in *C. glabrata*. Unlike its *S. cerevisiae* counterpart, there are two orthologs in *C. glabrata*: CgTpo1_1 and CgTpo1_2 (consult Section 1.5.2.1). Therefore, the functional characterization was undertaken for the two *C. glabrata* homologs: CgTpo1_1 (*ORF CAGL0G03927g*) and CgTpo1_2 (*ORF CAGL0E03674g*).

### 3.2.1. CgTpo1_1 and CgTpo1_2 expression and subcellular localization in *C. glabrata* and *S. cerevisiae*

Since the *S. cerevisiae* homolog Tpo1 is a plasma membrane multidrug resistance transporter, the subcellular localization of CgTpo1_1 and CgTpo1_2 was assessed in *C. glabrata* cells, to determine if these proteins also act as integral membrane efflux pumps. The location of the *C. glabrata* transporters was also assessed in *S. cerevisiae* cells, in an effort to study their functional similarity with the *S. cerevisiae* homolog.

To observe the subcellular localization of these proteins, *S. cerevisiae* cells harboring the pGREG576_*CgTPO1_1* and pGREG576_*CgTPO1_2* plasmids were grown to mid-exponential phase in minimal medium containing 0.5% glucose and 0.1% galactose, and then transferred to the same medium containing 0.1% glucose and 1% galactose, to promote protein overexpression. At a standard $OD_{600nm}$ of 0.5 ± 0.05, cells were analyzed by fluorescence microscopy and plasma membrane localization was verified (Figure 3.4 A).

*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 3.4 B).

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.

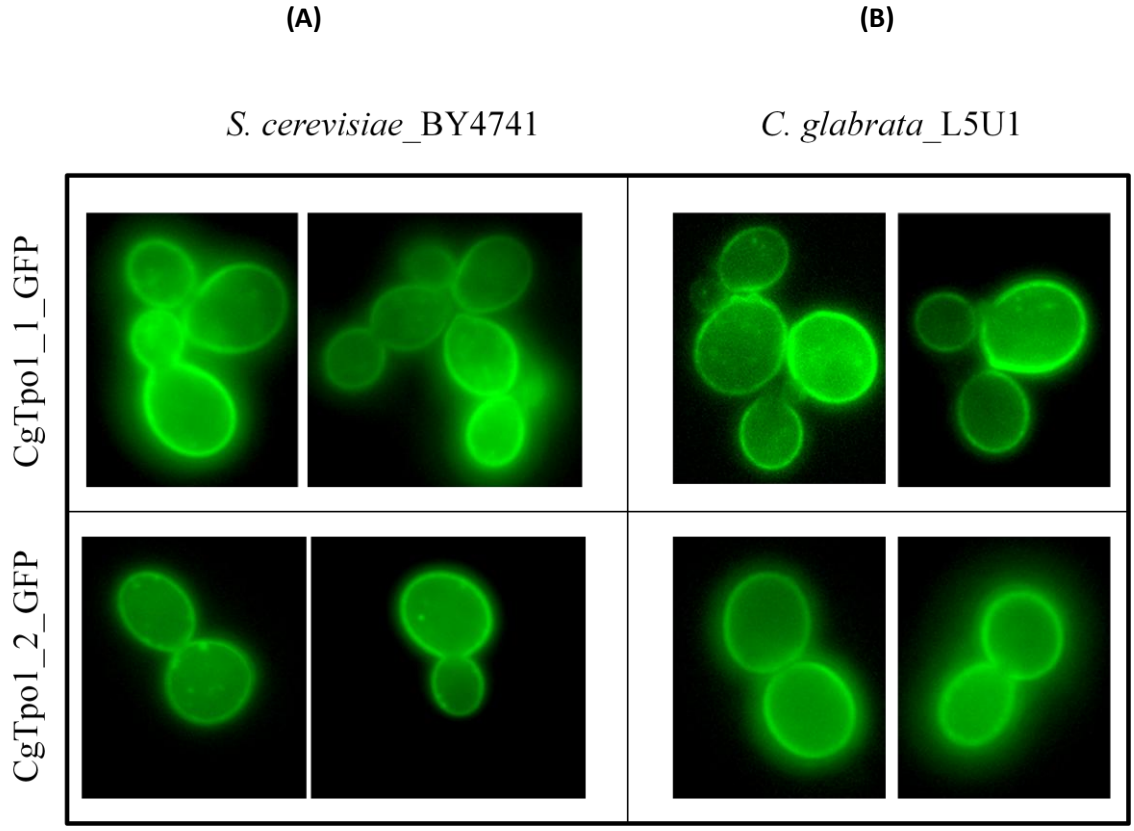


**Figure 3.4 - Fluorescence of exponential phase *S. cerevisiae* (A) and L5U1 *C. glabrata* (B) cells, harboring the cloning vectors pGREG576_*CgTPO1_1* and pGREG576_*CgTPO1_2*; and pGREG576_*MTI_CgTPO1_1* and pGREG576_*MTI_CgTPO1_2*, after galactose or copper-induced recombinant protein production, respectively.**

### 3.2.2. CgTpo1_1 and CgTpo1_2 expression confer resistance to several chemical stress inducers and azole antifungal 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, such as amphotericin B, flucytosine and the azole antifungal drugs clotrimazole, miconazole, ketoconazole, tioconazole (imidazoles); and fluconazole and itraconazole (triazoles), as well as to the fungicide mancozeb (Figure 3.5).

Similar to the effect registered for their homolog in *S. cerevisiae*, *CgTPO1_1* and *CgTPO1_2* deletion increases *C. glabrata* susceptibility to the polyamine spermine.

As it is clear in Figure 3.5, the wild-type strain KUE100 is capable of growing in the tested concentrations, while the *Δcgtpo1_1* and *Δcgtpo1_2* mutants display reduced growth when compared to wild-type, therefore being more susceptible.

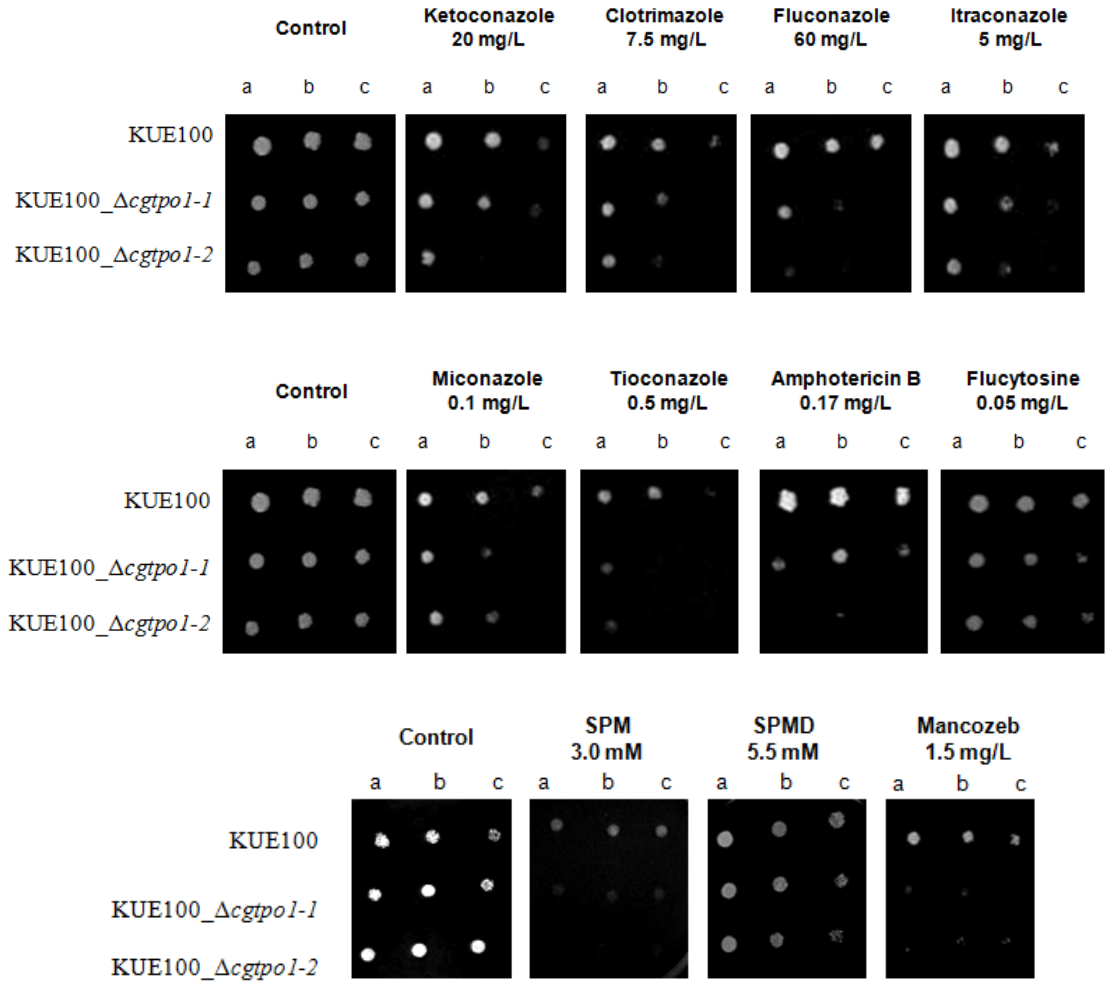


**Figure 3.5 – Comparison of the susceptibility to inhibitory concentrations of several chemical stress inducers, at the indicated concentrations, of the *C. glabrata* wild-type KUE100, KUE100_*Δcgtpo1_1* and KUE100_*Δcgtpo1_2* strains, in YPD agar plates (SPM and SPMD) and BM plates (remaining drugs) by spot assays. The inocula were prepared as described in Section 2.6. 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.**

To complement these findings, the introduction of a recombinant plasmid expressing *CgTPO1_1* or *CgTPO1_2* was found to increase *C. glabrata* natural resistance towards the tested drugs when compared to the same strain harboring the corresponding cloning vector (Figure 3.6).

This way, additional evidence was gathered to support the predicted role of these proteins as multidrug resistance transporters, acting as determinants of polyamines and azole drug resistance in *C. glabrata*, with an apparent role in the extrusion of additional compounds as well, exhibiting the exceptional substrate variety characteristic of multidrug transporters.

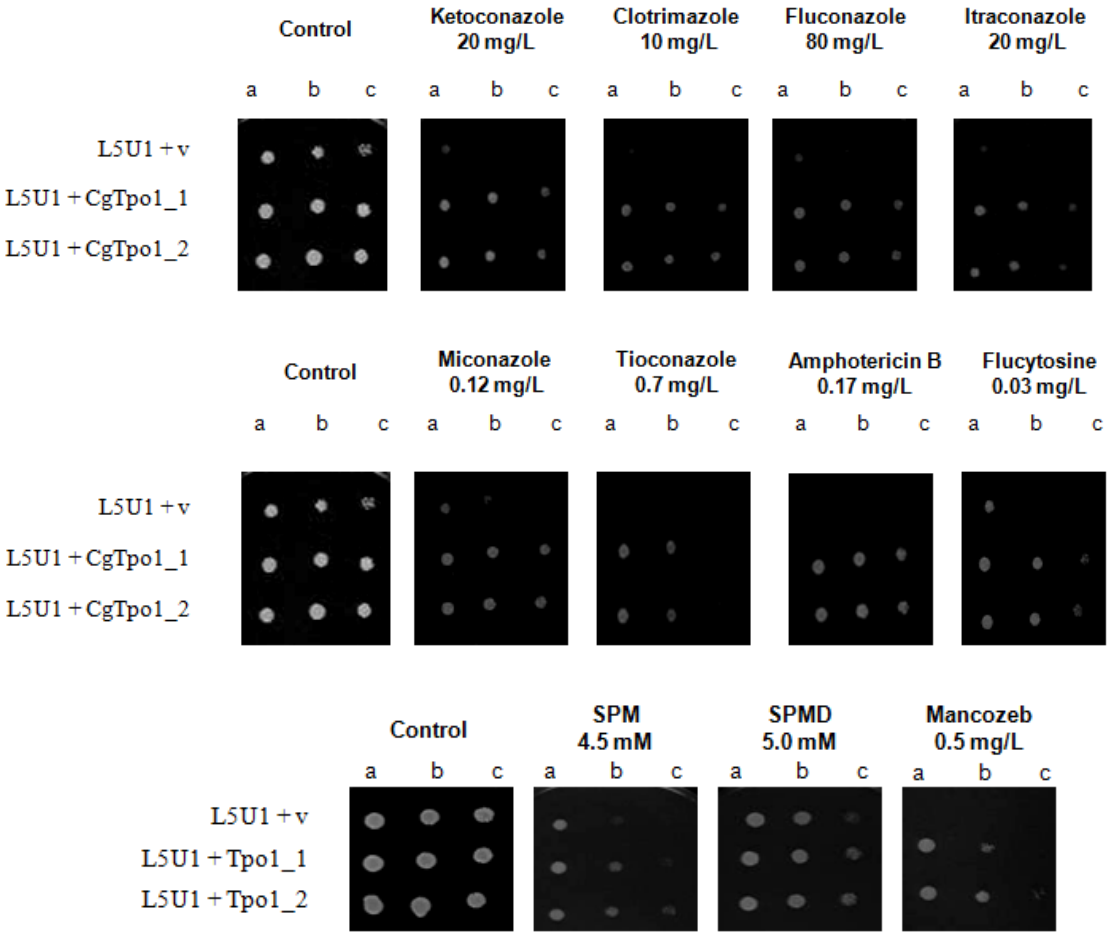


**Figure 3.6 - Comparison of the susceptibility to several drug stress inducers, at the indicated concentrations, of the *C. glabrata* L5U1 strain, harboring the pGREG675 cloning vector (v) or the pGREG576_*MTI_CgTPO1_1* or pGREG576_*MTI_CgTPO1_2* plasmids in YPD agar plates (SPM and SPMD) and BM agar plates (remaining drugs), without uracil, by spot assays. The inocula were prepared as described in Section 2.6. 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.**

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

Since the *C. glabrata* genes *CgTPO1_1* and *CgTPO1_2* were demonstrated to encode drug resistance transporters of the plasma membrane, and to act as determinants of resistance to several azole drugs including clotrimazole, their 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 the presence of 30 mg/L clotrimazole during 30 minutes was seen to be two times higher in cells devoid of CgTpo1_1 (Figure 3.7 A) and two and half times higher in cells devoid CgTpo1_2 (Figure 3.7 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 toward clotrimazole by effectively reducing its accumulation within yeast cells, possibly by catalyzing the extrusion of this antifungal drug, corroborating their action as efflux pumps.

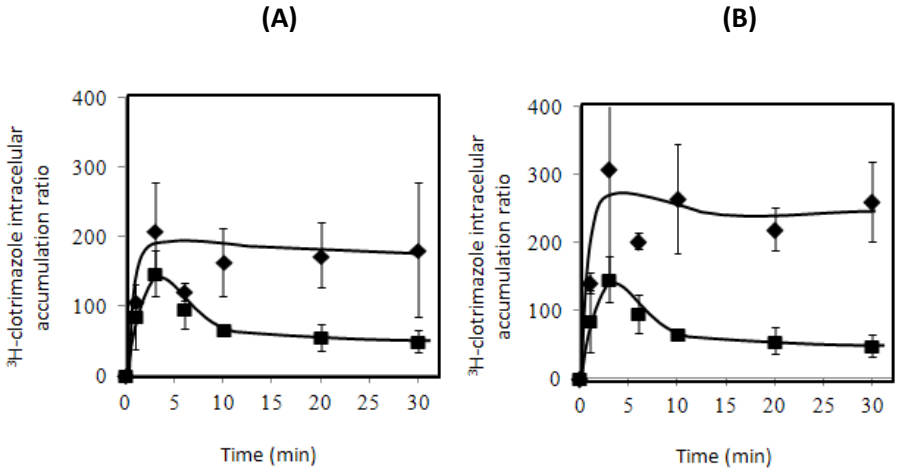


**Figure 3.7 - Time-course accumulation of clotrimazole in strains KUE100 (▪) wild-type and KUE100_*Δcgtpo1_1* (♦) (A) and KUE100 (▪) and KUE100_*Δcgtpo1_2* (♦) (B), in the presence of radiolabelled $^{3}$H-clotrimazole. Accumulation values are the average of at least three independent experiments. Error bars represent the corresponding standard deviation.**

## 3.2.4. *CgTPO1_1* and *CgTPO1_2* transcript levels are up-regulated under 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 to an unadapted *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 3.8).

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 (Section 3.2.2), and also with the higher accumulation ratio of intracellular clotrimazole in cells devoid of the same gene (Section 3.2.3).

Additionally, *CgTPO1_1* appears to be independent on the transcription factor *CgPDR1* (Figure 3.8 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 3.8 B).

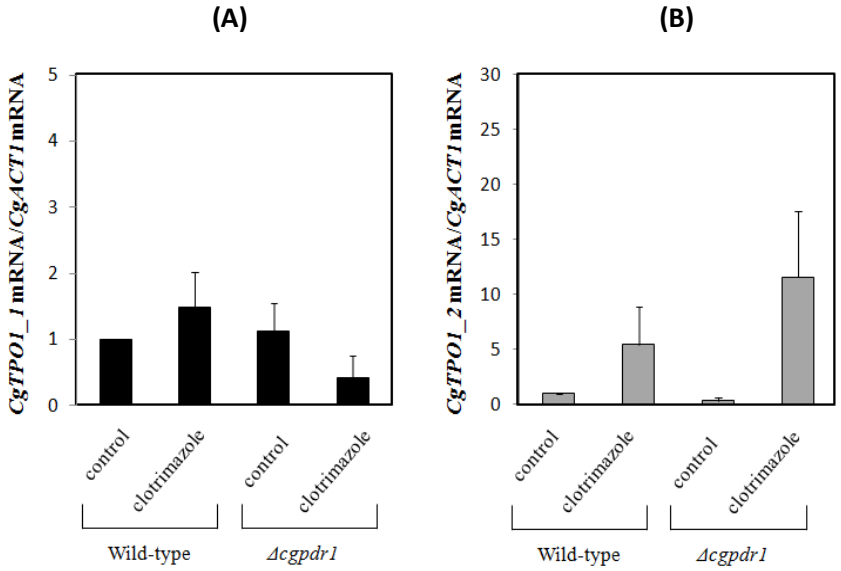


**Figure 3.8 - Comparison of the variation of the *CgTPO1_1* and *CgTPO1_2* transcript levels before (control) and after 1h of incubation with clotrimazole induced stress in *C. glabrata* wild-type cells and cells devoid the transcription factor CgPdr1. The presented transcript levels were obtained by quantitative RT-PCR, as described in Section 2.7, 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.**

## 3.3. The possible role of CgGas1 (*ORF CAGL0G00286g*) in clotrimazole resistance

The cell wall assembly protein CgGas1, up-regulated in response to clotrimazole stress exposure, was also selected for further studies. Its *S. cerevisiae* homolog was found to be required for cell shape, viability and resistance to some cell wall disturbing substances [116, 144]. However, this protein was not yet described as a determinant of antifungal drug resistance, and the characterization of its *C. glabrata* counterpart is summarized to intervene in cell shape and growth rate; not from the pleiotropic drug response optic. Therefore, the observed up-regulation of CgGas1 upon clotrimazole exposure was assessed, in order to understand the participation of this protein in the resistance process.

### 3.3.1. 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 3.9). 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.

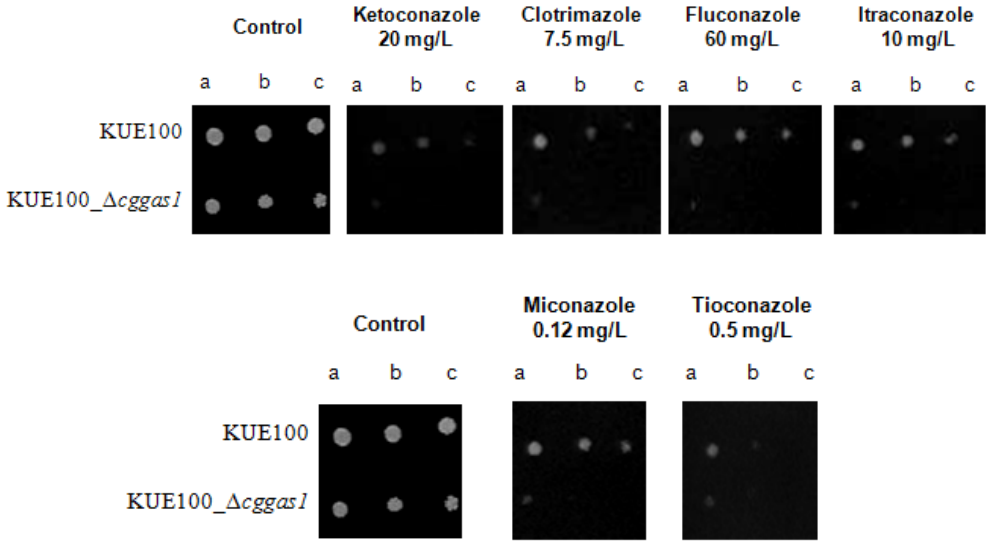


**Figure 3.9 - Comparison of the susceptibility to azole antifungal drugs, at the indicated concentrations, of the *C. glabrata* KUE100 wild-type and KUE100_*Δcggas1* strains, in BM agar plates by spot assays. The inocula were prepared as described in Section 2.6. Cell suspensions used to prepare the 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.**

### 3.3.2. CgGas1 is required for cell wall stability

Despite the fact that CgGas1 only has a predicted role as a relevant protein in cell wall assembly, the comparison of the deduced amino acid sequences showed homologies to Gas1 from *S. cerevisiae*, and the Phr1 or Phr2 proteins from *C. albicans* [121], proteins necessary for β-1,3-glucan extension and cross-linking [119]. Based on those observations, the possible role of CgGas1 in cell wall resistance was assessed through the evaluation of lyticase susceptibility assay in yeast cells before and after adaptation to clotrimazole.

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 3.10 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 3.10 A and B).

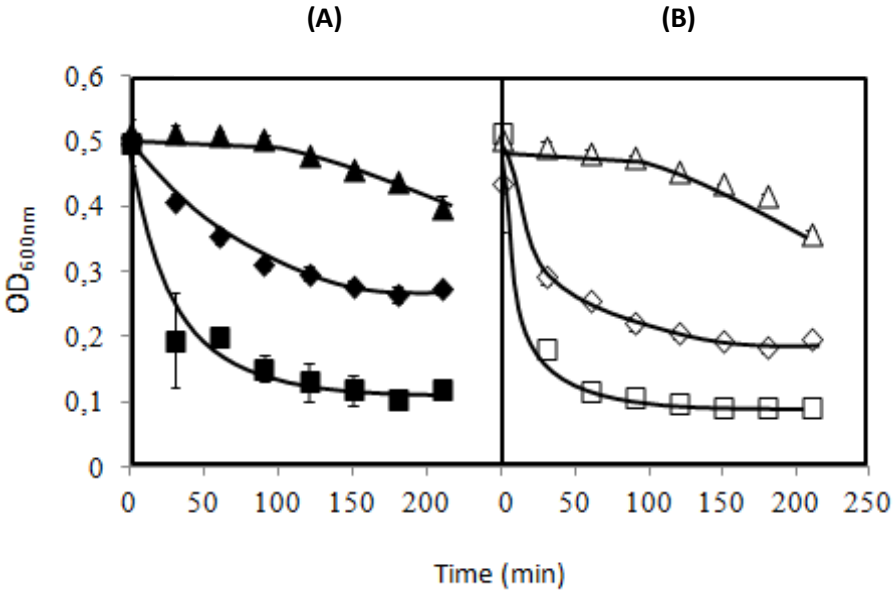


**Figure 3.10 – Lyticase sensitivities of cells of KUE100 wild-type (A) and KUE100_*Δcggas1* (B) strains grown in the absence of clotrimazole (♦), after 30 minutes of clotrimazole exposure (▪) or after exponential growth resumption in the presence of clotrimazole (▲). The different cell populations were 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.**

### 3.3.3. 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 3.11). These results appear to be consistent with the higher susceptibility of the *Δcggas1* cells observed in Sections 3.1.1 and 3.3.2.

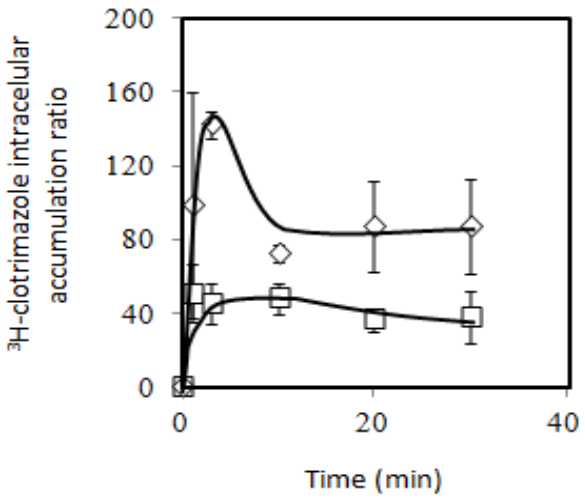


**Figure 3.11 - Time-course accumulation of clotrimazole in strains KUE100 (▪) wild-type and KUE100_*Δcggas1* (♦), in the presence of radiolabelled $^{3}$H-clotrimazole. Accumulation values are the average of at least three independent experiments. Error bars represent the corresponding standard deviation.**

These findings strongly suggest that CgGas1 contributes to *C. glabrata* resistance towards clotrimazole by effectively reducing its accumulation within yeast cells. These results show CgGas1 to be an important factor for cell wall composition, apparently necessary for clotrimazole resistance by catalyzing glucan linkages and chain elongation, thus reducing drug diffusion through cell wall into the citosol. *CgGAS1* is therefore found to be an unforeseen target for clotrimazole resistance.

# 4. 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.* [130, 145], 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 [145] and therefore were considered to be part of a general response. On the other hand, the multidrug transporter CgTpo1_2 and CgGas1 up-regulation appears 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 overexpression 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* [107]. 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 [37, 106, 107]. 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 [111] and of *C. glabrata* CgTpo3 [107].

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* [7, 146]. Furthermore, the ABC drug efflux pump CgSnq2 was also reported as an important fluconazole resistance determinant in *C. glabrata* clinical isolates [90, 146]. 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 [67, 108, 109], the molecular

mechanisms behind their substrate diversity are very much unknown [67, 109, 147]. 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 [112] was assessed. Indeed, *CgTPO1_1* and *CgTPO1_2* deletion increased susceptibility to spermine, whereas overexpression increased resistance 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 [107]. 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 [107], while CgAqr1 is involved in flucytosine resistance [106]. These observations seem to imply that CgTpo1_1 and CgTpo1_2 are gifted with extraordinary substrate variety, even within their own DHA1 family [8, 100]. 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 [113, 114, 148, 149].

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 have only a slight effect on fluconazole susceptibility and no relevant drug extrusion effect was detected [150]. In contrast, *C. glabrata* cells displayed 2-2.5 times higher accumulation rates in the absence of the transporters studied herein. These results may underlie the position of *C. glabrata* as an increasingly relevant pathogen, displaying higher levels of azole drug resistance in comparison to the more studied *C. albicans* [3, 24, 25].

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 [121]. The formation of aggregates has been also reported for the *C. albicans* homolog gene deletion mutant [151]. 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* CgTpo1_1 and CgTpo1_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.

# 5. Concluding remarks

The goal of this study was to analyze the *C. glabrata* membrane enriched proteome in order to identify differentially expressed proteins in clotrimazole exposed cells, in the dependence or not of the transcription factor CgPdr1. This way, a general picture could be created relative to which proteins are required for azole drug response, and which functional groups are represented in that cellular response. Given the increasingly appearance of multidrug resistance phenomenon, and the lack of information concerning resistance mechanisms to azole drugs in *C. glabrata*, it is imperative to understand how is this pathogenic yeast able to cope with azole drug treatment in clinical practice.

With this work, some light was shed into the MDR phenotype in *C. glabrata* resistant cells. The MFS efflux pumps CgTpo1_1 and CgTpo1_2 were characterized as relevant azole drug resistance determinants, shown to be required for clotrimazole drug resistance by effectively extruding the drug to the external medium. This represents one more step in the study of MFS transporters, clearly showing their relevance for multidrug resistance phenotypes, as these transporters are able to extrude a great variety of compounds and chemical stress inducers. These findings are even more relevant in the case of *C. glabrata* infections, which often present higher levels of acquired and intrinsic azole resistance. Not only two more MDR transporters were herein characterized under the scope of multidrug resistance, but also a complementary mechanism based on the reinforcement of the cell wall was unveiled. The relevance of these findings in the clinical acquisition of azole drug resistance needs to be addressed.

In short, this dissertation contributes to elucidate clotrimazole resistance mechanisms in *C. glabrata*. Results provided evidence of two new DHA transporters relevant for clotrimazole drug resistance and a role of cell wall in drug resistance, which is very relevant given that there is very few information about specific imidazole resistance mechanisms. The attained results demonstrate the great variability of strategies involved in clotrimazole drug resistance, highlighting the relevance for further studies focused on directed responses to specific families of antifungal drugs.

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