INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Characterization of ABC-type transporters in *Candida glabrata*.

Tesis que presenta

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Para obtener el grado de

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Director de la Tesis:
Alejandro De Las Peñas, Ph. D.

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Acta de Examen

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Abbreviations

ABC	**A**TP-**B**inding **C**assettes
ATP	**A**denosine **t**ri**p**hosphate
CDR1	***C****andida* **D**rug **R**esistance 1 protein
CDR2	***C****andida* **D**rug **R**esistance 1 protein
PDR1	**P**leiotropic **D**rug **R**esistance 1 protein
SNQ2	**S**ensitivity to 4-**N**itro**q**uinoline-N-oxide
YCF1	**Y**east **C**admium **F**actor
YOR1	Yeast Oligomycin Resistance
PDR12	**P**leiotropic **D**rug **R**esistance 1 protein
Cd	**C**a**d**mium
As	**A**r**s**enic
OSR	**O**xidative **S**tress **R**esponse
GSH	Glutathione
GOF	**G**ain-**O**f-**F**unction
PDRE	**P**leiotropic **D**rug **R**esponse **E**lement
EPIs	**E**fflux **P**ump **I**nhibitor**s**
YPD	**Y**east extract-**P**eptone-**D**extrose
SC	**S**ynthetic **C**omplete
CAA	**C**as**a**mino **a**cids
LB	**L**uria-**B**ertani
WT	**W**ild**t**ype
CFU	**C**olony **F**orming **U**nits
bp	**B**ase **p**airs
Kb	**K**ilo**b**ases
$CdSO_4$	Cadmium sulphate
Na_2HAsO_4	Sodium arseniate
LiOAc	**Li**thium **Ac**etate
SSDNA	**S**almon **s**perm DNA
PEG	**P**oly**e**thylen**g**lycol

ORF	**O**pen **R**eading **F**rame
Hyg^{R}.	**Hyg**romycin **r**esistant
FRT	**F**lp **R**ecombination **T**argets
FLP	**Fl**i**p**pase
OD	**O**ptical **D**ensity
GFP	**G**reen **F**luorescent **P**rotein

Resumen

Caracterización de los transportadores tipo ABC en *Candida glabrata*.

Candida glabrata es un hongo patógeno oportunista causante de aproximadamente 20% de casos de candidiasis invasiva. Su tasa de morbilidad y mortalidad varía del 40% al 60% debido a su resistencia tanto innata como adquirida a azoles, el tipo de antifúngicos más utilizado en hospitales. Esta resistencia está mediada por el factor de transcripción Pdr1, el cual regula positivamente los genes que codifican los transportadores tipo ABC (del inglés, **A**TP-**b**inding **c**assette) como *CDR1*, *CDR2, PDR11*, *PDR12*, *YOR1*, *YCF1,* and *SNQ2*, los cuales funcionan como bombas de eflujo de xenobióticos. Es esencial caracterizar estos transportadores y buscar antifúngicos alternativos para tratar la candidiasis, como son los fitoquímicos, que pueden actuar como inhibidores de estas bombas, y mejorar así el efecto de los azoles. En este trabajo construimos mutantes nulas en los genes que codifican para los transportadores tipo ABC y encontramos que Cdr1 es el principal transportador que media la resistencia a fluconazol (FLC), mientras que Pdr12 y Yor1 juegan un papel menor. Sorprendentemente, descubrimos que la mutante *snq2*Δ es resistente a FLC en medio rico, lo que sugiere que Snq2 puede estar transportando moléculas del exterior de la célula. Llama la atención que este fenotipo se pierde en medio sintético completo. Además, confirmamos que la tolerancia a cadmio (Cd) es conferida específicamente por Ycf1 y encontramos una mejor tolerancia a $CdSO_4$ en medio sintético completo, en comparación a medio rico. Finalmente, demostramos que el diterpeno Amarisolida A, proveniente de *Salvia amarissima* no inhibe la actividad de las bombas de eflujo.

Palabras clave: *Candida glabrata*, *Nakaseomyces glabratus,* transportadores tipo ABC, resistencia a azoles.

Abstract

Characterization of ABC-type transporters in *Candida glabrata*.

Candida glabrata is an opportunistic fungal pathogen that accounts for approximately 25% of cases of invasive candidiasis. Its morbidity and mortality rates are around 40-60% due to its innate and acquired resistance to azoles, the most widely used antifungals in hospitals. This resistance is mediated by the transcription factor Pdr1, which up-regulates the **A**TP-**b**inding **c**assette (ABC) transporter encoding genes (*CDR1*, *CDR2, PDR11*, *PDR12*, *YOR1*, *YCF1,* and *SNQ2*), which are xenobiotic efflux pumps. It is essential to characterize these transporters and search for alternative antifungals to treat candidiasis, such as phytochemicals that could act as efflux pump inhibitors, thus enhancing the effect of azoles. We constructed mutants in all ABC transporter genes and found that Cdr1 is the main ABC transporter that mediates resistance to fluconazole (FLC), while Pdr12 and Yor1 play a minor role. Surprisingly, we discovered that the *snq2*Δ mutant is resistant to FLC in rich media, suggesting that Snq2 could be importing molecules from the exterior. Interestingly, this phenotype is lost in synthetic complete media. Furthermore, we confirm that tolerance to cadmium (Cd) is specifically conferred by Ycf1 and found an increased tolerance to $CdSO_4$ in synthetic complete media compared to rich media. Finally, we showed that Amarisolide A, a diterpene from *Salvia amarissima*, does not inhibit efflux pump activity.

Key words: *Candida glabrata*, *Nakaseomyces glabratus,* ABC-type transporters, azole resistance.

Running title: **Characterization of ABC-type transporters in *Candida glabrata*.**

Key words:

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1. Abstract

Candida glabrata is an opportunistic fungal pathogen that accounts for approximately 25% of cases of invasive candidiasis. Its morbidity and mortality rates are around 40-60% due to its innate and acquired resistance to azoles, the most widely used antifungals in hospitals. This resistance is mediated by the transcription factor Pdr1, which up-regulates the **A**TP-**b**inding **c**assette (ABC) transporter encoding genes (*CDR1*, *CDR2, PDR11*, *PDR12*, *YOR1*, *YCF1,* and *SNQ2*), which are xenobiotic efflux pumps. It is essential to characterize these transporters and search for alternative antifungals to treat candidiasis, such as phytochemicals that could act as efflux pump inhibitors, thus enhancing the effect of azoles. We constructed mutants in all ABC transporter genes and found that Cdr1 is the main ABC transporter that mediates resistance to **fl**u**c**onazole (FLC), while Pdr12 and Yor1 play a minor role. Surprisingly, we discovered that the *snq2*Δ mutant is resistant to FLC in rich media, suggesting that Snq2 could be importing molecules from the exterior. Interestingly, this phenotype is lost in synthetic complete media. Furthermore, we confirm that tolerance to cadmium (Cd) is specifically conferred by Ycf1 and found an increased tolerance to $CdSO_4$ in synthetic complete media compared to rich media. Finally, we showed that Amarisolide A, a diterpene from *Salvia amarissima*, does not inhibit efflux pump activity.

2. Introduction

Fungal pathogens are responsible for causing several human diseases that range from allergic syndromes to life-threatening invasive forms. These fungal infections affect more than 1 billion people worldwide annually, particularly those with a compromised immune system (Rayens and Norris, 2022). One of the most common opportunistic infections is candidiasis, caused by several species of the yeast *Candida*. *Candida* spp. is part of the commensal microbiota present in the human oral cavity, gastrointestinal, and genitourinary tracts, however, when individuals become immunocompromised, the homeostasis in the microbiota is disrupted, leading to their pathogenic state (Lass-Flörl et al., 2024).

Candida glabrata (*Nakaseomyces glabratus*) is an asexual haploid non-hypha forming yeast, phylogenetically related to *Saccharomyces cerevisiae*. It is an opportunistic pathogen that causes superficial and invasive infections. *C. glabrata* accounts for approximately 25% of invasive clinical cases, with a morbidity and mortality of around 40-60%. There has been a clear increase in the incidence of *C. glabrata* infections in recent years, and it is now considered the second most common cause of candidiasis, after *C. albicans* (Fisher et al., 2022).

C. glabrata can adapt to several microenvironments to grow efficiently within the host. It has been isolated from abiotic surfaces in both clinical settings, and non-clinical settings such as coffee beans and bird feces. Virulence factors in *C. glabrata* are essential for its pathogenicity, since they allow colonization, adhesion, invasion, dissemination, and evasion of the host's immune response (Frías-De-León et al., 2021).

Adhesion to epithelial and endothelial cells is one of the main virulence factors of *C. glabrata*. This ability is mediated by the Epa adhesins, a family of cell wall proteins encoded by the *EPA* genes (Hernández-Hernández et al., 2021). Additionally, *C. glabrata* has hydrolytic enzymes that facilitate the invasion of the host cells, such as phospholipases, lipases, and proteases, such as yapsins, aspartyl proteases that belong to the *YPS* family. Yapsins are important for the

yeast's ability to survive within human macrophages, as well as for biofilm formation (López-Fuentes et al., 2018).

C. glabrata has been shown to have a robust **o**xidative **s**tress **r**esponse (OSR) to survive the oxidative stress generated by the host's macrophages during infections. It neutralizes **r**eactive **o**xygen **s**pecies (ROS) by inducing enzymatic (catalase, **s**uper**o**xide **d**ismutases [SODs] and peroxidases) and non-enzymatic (glutathione [GSH]) antioxidant defenses (Gutiérrez-Escobedo et al., 2020). *Candida glabrata* OSR goes beyond neutralizing and surviving to the host's immune response. Even though heavy metals such as copper and zinc are essential micronutrients with physiological purposes, metals such as cadmium (Cd), mercury (Hg), arsenic (As) and lead (Pb) are highly toxic and reactive, hence producing oxidative stress. To reduce the toxic effects of metals such as Cd, *C. glabrata* enzymatically synthesizes cadmium chelates conjugated to cysteine-rich peptides derived from GSH, called phytochelatins. However, the genes encoding for these phytochelatin synthases are still unknown (Briones-Martin-Del-Campo et al., 2014; Mehra et al., 1994)

One of the most concerning virulence factors in *C. glabrata* is its innate and acquired antifungal resistance (Cavalheiro et al., 2021; Hassan et al., 2021). Currently, there are only 4 groups of drugs that can treat fungal infections: polyenes, echinocandins, the pyrimidine analog 5-flucytosine, and azoles, such as **fl**u**c**onazole (FLC). There are multiple mechanisms by which multidrug resistance can be acquired. Resistance to azoles is mediated via **g**ain-**o**f-**f**unction (GOF) mutations in the **p**leiotropic **d**rug **r**esponse transcription factor Pdr1 (Vu et al., 2019). Pdr1 is a positively acting transcription factor that binds to DNA recognition sites, known as PDRE (**P**DR **R**esponse **E**lement) and induces the up-regulation of **A**TP-**b**inding **c**assette (ABC) transporter encoding genes (*CDR1*, *CDR2, PDR11*, *PDR12*, *YOR1*, *YCF1,* and *SNQ2*) (Hassan et al., 2021; Paul et al., 2018).

The ABC-type transporter superfamily function as high affinity nutrient importers in bacteria, as well as exporters in higher eukaryotes. They are described

as ‘promiscuous’ translocators, since they import and export a wide variety of substrates, including peptides, sugars, other metabolites, toxins, and xenobiotics, including drugs and metals across biological membranes, requiring ATP hydrolysis. The architecture of a typical ABC transporter consists of four domains: two **t**rans**m**embrane **d**omains (TMDs), which provide the actual substrate translocation, and two **n**ucleotide **b**inding **d**omains (NBDs) that bind and hydrolyze ATP to provide energy for the active transport (Kumari et al., 2018; Wen and Tajkhorshid, 2011). In. *C. glabrata*, ATP transporters contribute to the development of drug resistance by extruding azoles from the cell.

Azoles are fungistatic drugs that inhibit ergosterol biosynthesis by blocking the enzyme lanosterol 14α-demethylase encoded by *ERG11*. More specifically, the free N atom of the azole ring binds to the iron atom within the heme group of the enzyme, which prevents the demethylation of lanosterol and, therefore, inhibits the synthesis of ergosterol. Although azoles have several disadvantages, such as hazardous drug-drug interactions with the P450 enzyme system, erratic absorption that requires therapeutic drug monitoring, cardiac adverse events, and many levels of organ toxicity, they are safer than other antifungals and are relatively affordable. Hence, their indiscriminate use and prescription has led to increasing levels of resistance worldwide (Pfaller et al., 2015).

The lack of discovery and development of new antifungals, makes it urgent to search for new therapeutic solutions (Vanreppelen et al., 2023). Considering the importance of azoles as the main class of antifungal drugs and ABC transporters that are targets of resistance mechanisms, research is now focused on finding inhibitors or modulators that block the extrusion activity of these transporters, known as **e**fflux **p**ump **i**nhibitor**s** (EPIs).

Medicinal plants are an important source of a vast diversity of bioactive secondary metabolites of therapeutic value, known as phytochemicals. Examples of such molecules are terpenoids, alkaloids, and phenolic compounds, and they have been used empirically since ancient times. In addition, these molecules are of low

cost, easy availability, and accessibility, and can be used in combination with antifungal drugs to improve their effectiveness. Phytochemicals can interact with specific targets within the cell, such as transcription factors, specific enzymes, or transporters (Tegos et al., 2002). Hence, the use of phytochemicals in combination with conventional antifungal therapy might be a potential solution for antifungal resistance while re-using the same therapeutic molecules.

In this study, we characterized ABC-type transporters in *Candida glabrata*. We determined that the efflux pumps Pdr12, Yor1 and Ycf1 have a minor role in azole resistance in comparison to Cdr1. Whereas Snq2 provides fluconazole resistance in the BG14 background. Furthermore, we confirm that tolerance to cadmium (Cd) is specifically conferred by Ycf1 and found an increased tolerance to $CdSO_4$ in synthetic complete media compared to rich media. Finally, we showed that Amarisolide A, a diterpene from *Salvia amarissima*, does not inhibit efflux pump activity.

3. Materials and Methods

3.1 Strains

All strains are described in Table S1.

3.2 Primers

All oligonucleotides are listed in Table S2.

3.3 Media and growth conditions

Yeast cultures were grown at 30ºC in standard yeast medium (**Y**east Extract-**P**eptone-**D**extrose), contains: 10 g/L yeast extract, 10 g/L peptone and supplemented with 2% glucose and 25 mg/L uracil. 2% agar was used for plates (Sherman et al., 1986). **S**ynthetic **c**omplete (SC) medium contains: 1.7 g/L of a mixture of yeast nutrient base (without $(NH_4)_2SO_4$ and amino acids) 5 g/L $(NH_4)_2SO_4$ or 1 g/L glutamic acid, 0.6% **c**as**a**mino **a**cids (CAA) and supplemented with 2% glucose. 25 mg/L uracil was added when needed. YPD plates were supplemented with Hygromycin B (A.G. Scientific) at 460 µg/mL when needed.

Bacteria were grown in **L**uria-**B**ertani (LB) medium as previously described (Ausubel, 2002). LB media was prepared as following: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl. All plasmid constructs were introduced into *Escherichia coli* DH10 by electroporation and selected with 50 µg/mL carbenicillin (Invitrogen™). For plates, 1.5% agar was used.

3.4 Construction of null mutants in *PDR12*, *SNQ2* and *YCF1* and double mutants in *PDR12*, *SNQ2*, *YCF1* and *YOR1* in the *cdr1*Δ background.

To construct the knockout mutations in *PDR12*, *YCF1,* and *SNQ2* that encode ABC transporters, we used fusion PCR (Kuwayama, 2002) (**Fig. S1**). Briefly, from the **w**ild**t**ype strain (WT), we amplified the ORF of each gene plus the 5' and 3' flanking regions of each (~ 1kb upstream and 1 kb downstream. For *PDR12* 5' primers #3272 and #3274; for PDR12 3' primers #3277 and #3278, for *YCF1* 5' primers #3295 and #3294; for *YCF1* 3' primers #3298 and #3299, for *SNQ2* 5' primers #3284 and #3285; for *SNQ2* 3' primers #3289 and #3290, and for *URA3* primers #604 and #605. **Table S1**). All PCR products were purified using the Qiagen PCR Purification Kit. These three fragments were linked by fusion PCR. *C. glabrata* was then transformed with the fusion PCR fragment and transformants were selected on SC (-) Ura plates. PCR analysis was performed to confirm the 5' and 3' ends of each deletion with primers outside to the fusion PCR fragment and internal primers of *URA3* (For *PDR12* primers #3272 and #3279, for *YCF1* primers #3303 and #3438 and for *SNQ2* primers #3282 and #3291, for *URA3* primers #113 and #114 **Table S1**). The absence of each deleted gene was also verified by the inability to amplify an internal fragment of the gene by PCR (For *PDR12* primers #3280 and #3281, for *YCF1* primer #3301 and #3437 and for *SNQ2* primer #3286 and #3292, **Table S1**) (**Fig. S2)**.

For double mutants, we amplified the deleted *cdr1Δ* cassette (*cdr*1Δ::*hph*), primers #745 and #748 (**Table S1**) and transformed it into the knockout *C. glabrata* strains *pdr12Δ* (CGM5024), *ycf1Δ* (CGM5052), *yor1Δ* (CGM1474)

and *snq2Δ* (CGM5026). Transformants were selected on YPD-Hygromycin plates. PCR analysis was performed to confirm the 5' and 3' ends of each deletion with primers outside to the fusion PCR fragment and internal primers of *HPH* (primers #15 and #16 **Table S1**) The absence of the HPH gene was also verified by the inability to amplify an internal fragment of the gene by PCR (primers #750 and #751 **Table S1**) (**Fig. S2**).

All mutants were proven to have normal mitochondrial function (Gly+) by streaking them on YPG media plates.

3.5 Yeast transformation

Yeast transformations with linear/supercoiled plasmid DNA or PCR product was performed as previously described using the LiOAc/ssDNA/PEG method (Castaño et al., 2003) (**Fig. S3**).

3.6 Growth assays in liquid media

Cells were grown to stationary phase for 48 h in YPD, or SC media. Stationary phase cultures were adjusted to an OD600 of 0.01 in the SC media and 300 µL were dispensed in a honeycomb plate. Growth was automatically recorded using Bioscreen C analyser (Oy, Growth Curves) at 30 ºC. with constant shaking and OD measurements taken every 15 minutes during a period of 48 h (Gutiérrez-Escobedo et al., 2013) (**Fig. S4**).

3.7 Growth assays in solid media

For solid media experiments, stationary phase cultures (48h) were adjusted to an OD600 of 0.5 with sterile water, and 10-fold serial dilutions were made in 96-well plates. 5 µL of each dilution were spotted on to YPD plates with and without fluconazole, $CdSO_4$ or Na_2HAsO_4, then incubated at 30ºC and photographed 48 h later.

3.8 Cell viability assay

To determine how many *C. glabrata* cells survive after chronic exposure to cadmium, BG14, CGM976 (*gsh2*Δ) and CGM5052 (*ycf1*Δ) were grown to stationary phase in SC media supplemented with uracil for 48h, then adjusted to an OD600 of 1. In test tubes, we prepared 5mL chronical treatments with cadmium using SC media supplemented with uracil and 0, 0.03, and 1 mM $CdSO_4$ for CGM976 (*gsh2*Δ), 0, 0.05 and 1 mM $CdSO_4$ for CGM5052 (*ycf1*Δ) and 0, 1 and 2.5 mM $CdSO_4$. for BG14. These tubes were then inoculated with the necessary volume of adjusted cells to have a final OD600 of 0.05. Treated cells were incubated at 30ºC in constant shaking for 6 consecutive days. At day 1, 2, 4 and 6, we took a 100µL sample of each treatment and made 10-fold serial dilutions and plated them onto solid YPD media. This experiment was performed three times. The number of **c**olony-**f**orming **u**nits (CFU) was calculated as following:

$$\frac{CFU}{mL} = \frac{CFU}{DF} * \frac{1000\ \mu L}{V\ (\mu L) * 1mL}$$

Where,

CFU/mL: Total number of colony-forming units per mL

CFU: Colony-forming units.

DF: Dilution factor

V (µL): volume of the sample in µL used to make the serial dilution.

The cell viability percentage was calculated as following,

$$Cell\ viability\ (\%) = \frac{\bar{x}_{[x]}}{\bar{x}_{[0]}} * 100$$

Where,

Cell viability (%): Percentage of viable cells at a determined $CdSO_4$ concentration.

$\bar{x}_{[x]}$: Average number of cells at a determined $CdSO_4$ concentration (0.03, 0.05, 1 mM).

$\bar{x}_{[0]}$: Average number of cells without $CdSO_4$ (0 mM).

4. Results

4.1 Pdr12, Yor1 and Ycf1 have a minor role in fluconazole resistance.

Given that ABC transporters are important for antifungal resistance (Whaley et al., 2018), we constructed null mutations in *PDR12*, *YOR1*, *YCF1* and *SNQ2* and double mutants with *cdr1*Δ (*cdr1*Δ *pdr12*Δ, *cdr1*Δ *ycf1*Δ, and *cdr1*Δ *yor1*Δ). We tested the single mutants and double mutants for FLC sensitivity. Consistent with previous results, *cdr1*Δ has an increased susceptibility to FLC (Guerrero-Serrano, 2011) (**Fig.1A,** 2 and 4 µg/mL). The single mutants *pdr12*Δ, *ycf1*Δ, and *yor1*Δ show the same susceptibility to FLC as BG14 (**Fig. 1A**). Under these FLC concentrations *cdr1*Δ *pdr12*Δ and *cdr1*Δ *ycf1*Δ susceptibility to FLC is epistatic to *cdr1*Δ (**Fig. 1A**). However, when we broadened the FLC concentration windows, we found that Pdr12 and Ycf1 play a minor role in FLC resistance (**Fig. 1B**, 2.5 µg/mL). Interestingly, *cdr1*Δ *yor1*Δ is slightly more susceptible to FLC at 2 µg/mL compared to *cdr1*Δ and the other double mutants (**Fig. 1A**, 2 µg/mL). These results confirm that Cdr1 is the main ABC transporter that mediates resistance to FLC and indicate that Pdr12, Ycf1 and Yor1 play a minor role in FLC resistance.

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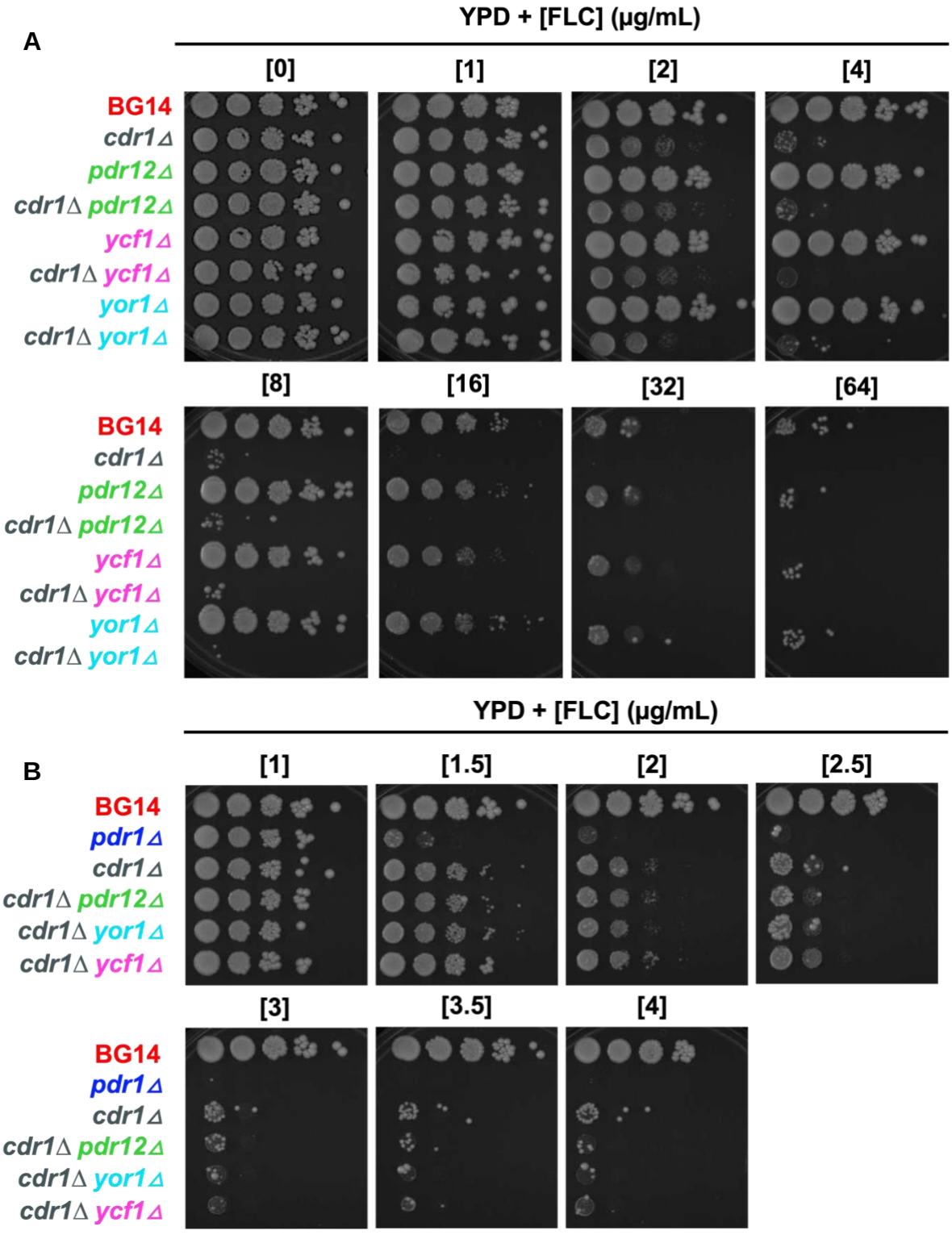

Figure 1. Role of Pdr12, Yor1, and Ycf1 in fluconazole resistance.

Cultures of BG14, *pdr1*, *cdr1*Δ, *pdr12*Δ, *ycf1*Δ, *yor1*Δ, *cdr1*Δ *pdr12*Δ, *cdr1*Δ *ycf1*Δ, and *cdr1*Δ *yor1*Δ were grown for 48h at 30˚C in YPD. 10-fold dilutions were made from each culture and spotted onto YPD plates with increasing fluconazole concentrations. Plates were incubated at 30˚C for 48h. See Materials and Methods.

4.2 *snq2*Δ is resistant to fluconazole.

In addition to the mutants that encode the ABC-type transporters described above, we also constructed a null mutation in *SNQ2*, which encodes a transporter described in resistance to fluconazole (Torelli et al., 2008) and the double mutant *cdr1*Δ *snq2*Δ. We used *pdr1*Δ and *hst1*Δ as sensitivity and resistance controls, respectively. Unexpectedly, *snq2*Δ is resistant to FLC 32µg/mL compared to BG14 (**Fig. 2A**). The double mutant *cdr1*Δ *snq2*Δ is slightly more resistant to FLC 2µg/mL than *cdr1*Δ (**Fig. 2A**). Surprisingly, the resistance phenotype of *snq2*Δ is lost if cells are grown in SC media media (compare **Fig. 2A** FLC 32µM and **Fig. 2B** FLC 32µM) and the double mutant *cdr1*Δ *snq2*Δ is now more susceptible to FLC 2µg/mL (**Fig. 2A** compare *cdr1*Δ with *cdr1*Δ *snq2*Δ **2B**). These results suggest that in the BG14 background and dependent on the media, Snq2 could be importing molecules (like FLC) from the exterior of the cell.

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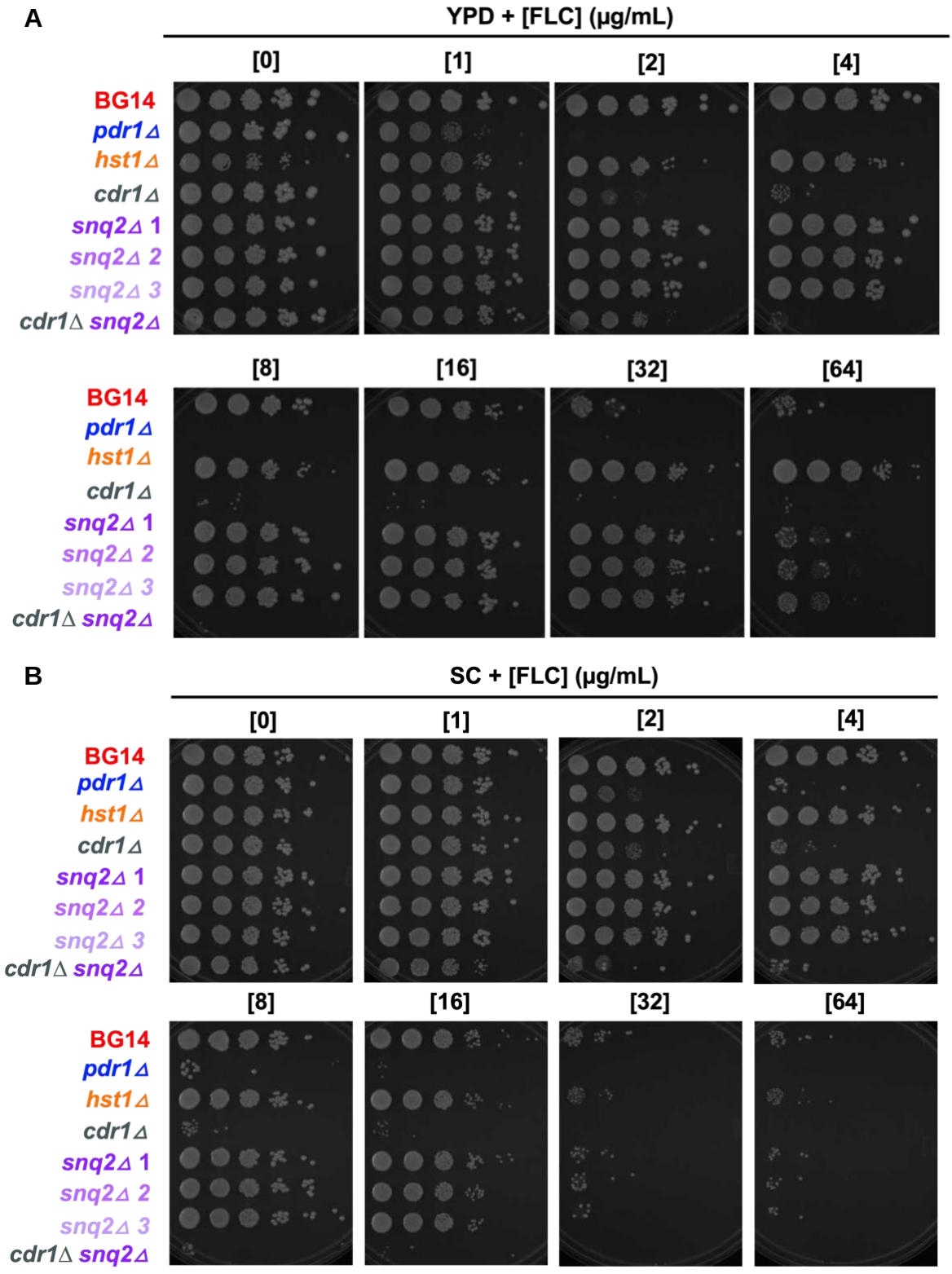

Figure 2. Role of Snq2 in fluconazole resistance.

Cultures of BG14, *pdr1*Δ, *hst1*Δ, *cdr1*Δ, and *snq2*Δ (3 independent mutants) and *cdr1*Δ *snq2*Δ were grown for 48h at 30˚C. 10-fold dilutions were made from each culture and spotted onto (**A**) YPD and (**B**) uracil-supplemented SC media plates with increasing FLC concentrations. See Materials and Methods.

4.3 Ycf1 is required for cadmium resistance.

Heavy metals, such as cadmium (Cd), arsenic (As), and mercury (Hg), are highly cytotoxic to many organisms, including yeast, rodents, and humans. Cd has been shown to induce oxidative stress and misfolding and aggregation of cytosolic proteins in yeast (Jacobson et al., 2017). Here, we evaluated whether the ABC-type transporters play a role in the tolerance to cadmium ($CdSO_4$). We exposed BG14, *pdr1*Δ, *cdr1*Δ, *yor1*Δ, *pdr12*Δ and *snq2*Δ mutants to $CdSO_4$ and used the glutathione synthase 2 gene mutant (*gsh2*Δ) as the sensitivity control. Only *ycf1*Δ is highly sensitive to $CdSO_4$ 0.03 mM (**Fig. 3** $CdSO_4$ 0.03 mM), while there is no difference in $CdSO_4$ tolerance between BG14 and *yor1*Δ, *pdr12*Δ and *snq2*Δ (**Fig. 3**). Interestingly, BG14 and all other mutants except *ycf1*Δ and *gsh2*Δ, increased their resistance to $CdSO_4$ when plated onto SC media, (**Fig. 3A and 3B**). These data indicate that Ycf1 is required to confer resistance to cadmium.

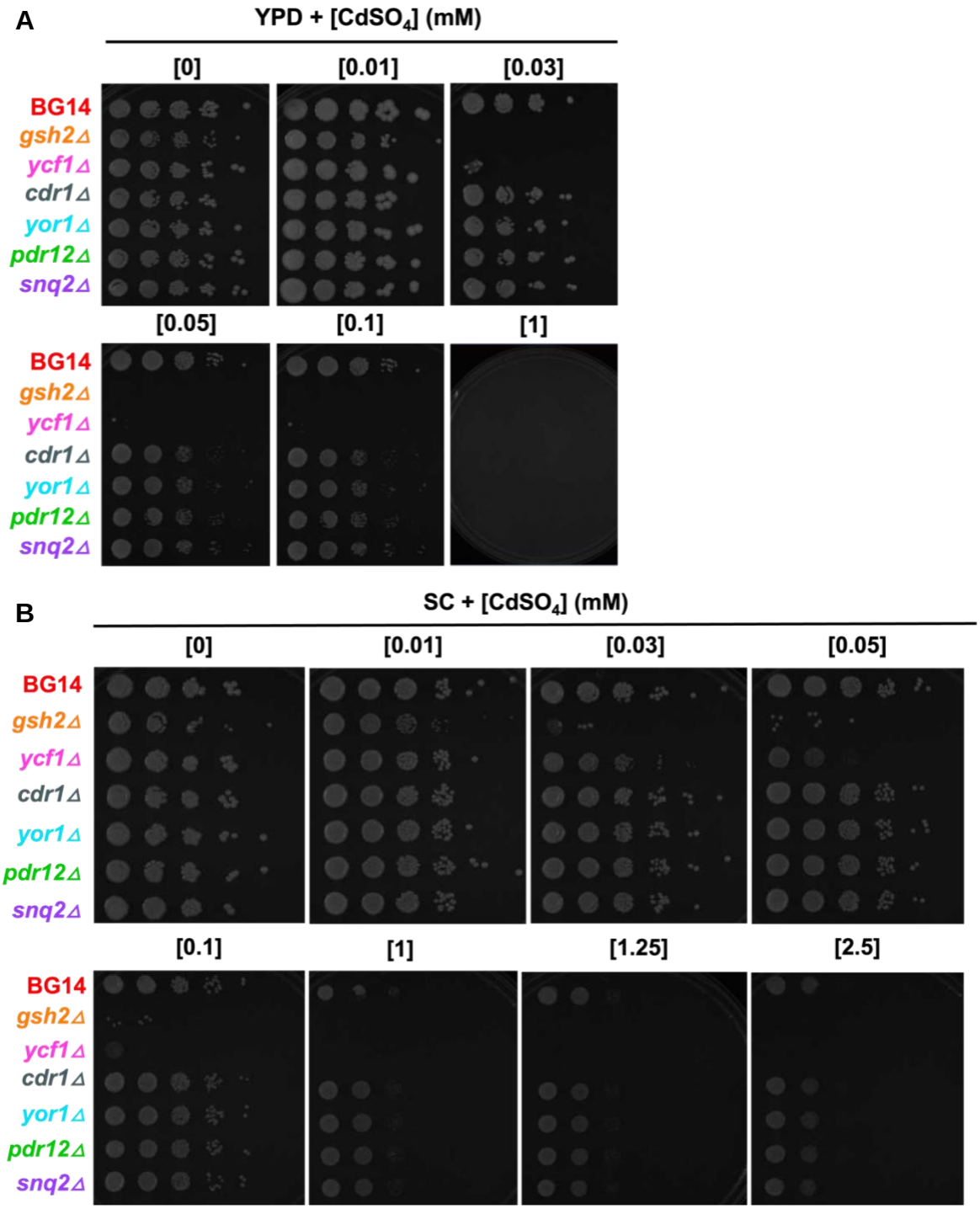

Figure 3. Role of ABC transporters in the resistance to cadmium in *C. glabrata*.

Cultures of BG14, *pdr1*Δ, *gsh2*Δ, *cdr1*Δ, *yor1*Δ, *pdr12*Δ and *snq2*Δ, were grown for 48h at 30˚C in YPD and SC media. 10-fold dilutions were made from each culture and spotted onto YPD (**A**) and uracil-supplemented SC media (**B**) plates with increasing $CdSO_4$ concentrations. See Materials and Methods.

4.4 *C. glabrata* adapts to chronical exposure to cadmium.

GSH has been described to bind Cd and is essential for defense against metal-induced oxidative stress in *C. glabrata* (Brennan and Schiestl, 1996). To determine the effect of chronical exposure to cadmium, we exposed BG14 to 0, 1 and 2.5 mM $CdSO_4$, *gsh2*Δ to 0, 0,03 and 1 mM $CdSO_4$ and *ycf1* to 0, 0.05 and 1mM $CdSO_4$ for 6 days. For 1 mM, only 29% of BG14 survive to the initial cadmium exposure, while *gsh2*Δ and *ycf1*Δ lose almost all viability (**Fig 4A**, day 1). After day 1, surviving cells either adapt or suppressors arise in the cell population. Growth is resumed up to day 4 where BG14 recovers to 80% and *gsh2*Δ and *ycf1*Δ between 35% - 40%. (**Fig. 4A**). Interestingly, after plating for CFU, there are small and regular-size colonies during the 'adaptation period' (day 1 to day 2). A similar tendency is observed for *gsh2*Δ at 0.03 mM and for *ycf1*Δ 0.05 mM (**Fig. 4B** and **4C**). However, since these concentrations are not as damaging as 1 mM, cells survive up to 50% after day 2. At 2.5 mM $CdSO_4$ cell viability drops below 20% since day 1. These results confirm that Ycf1 is required for cadmium tolerance and indicates that *C. glabrata* can adapt to chronical exposure to cadmium.

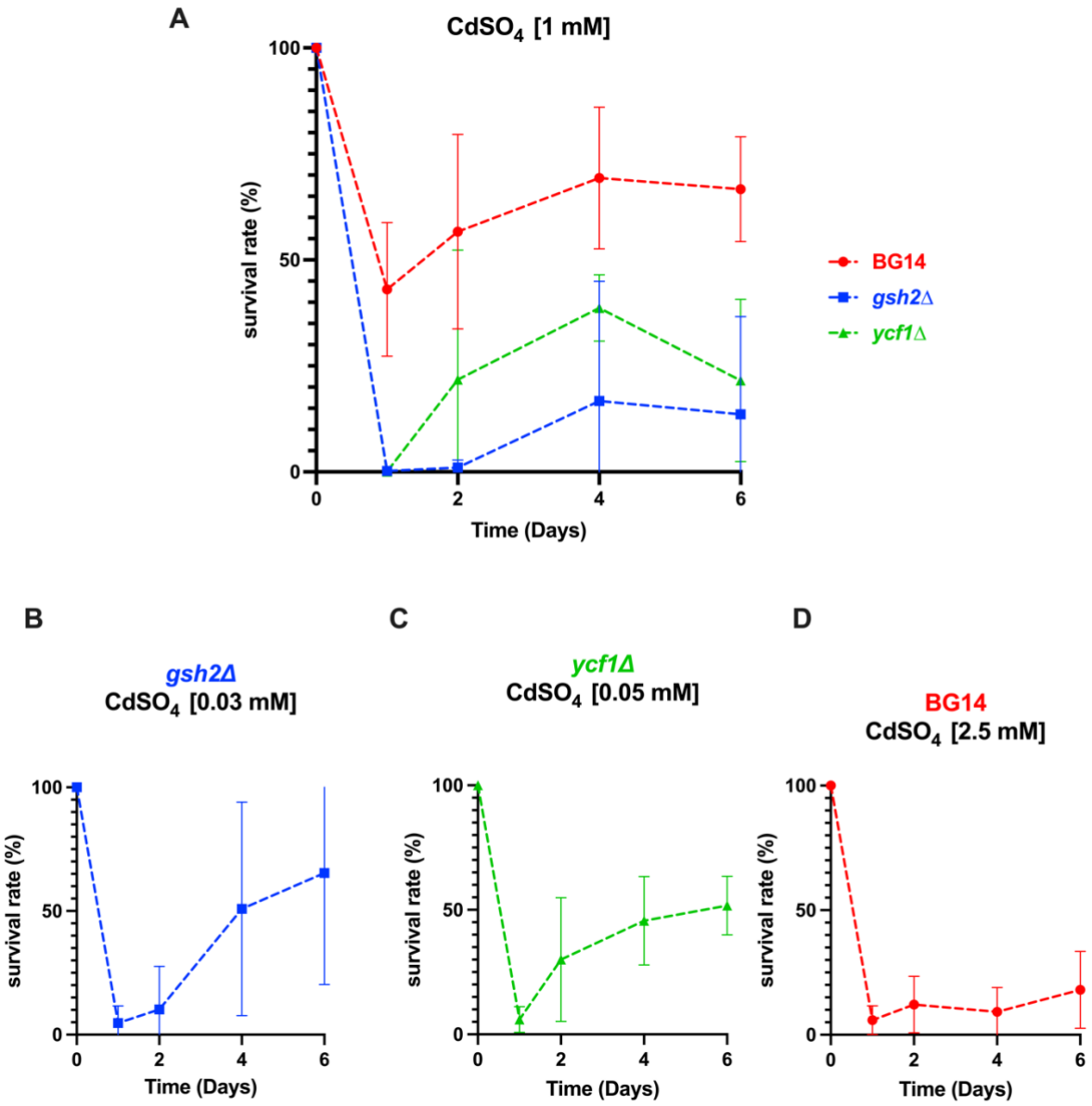

Figure 4. *C. glabrata* response to chronical exposure to cadmium.

Cultures of BG14, *gsh2*Δ and *ycf1*Δ were grown for 6 days at 30˚C in SC media with $CdSO_4$ 0, 0.03, 0.05 and 1 mM. Dilutions from each culture were plated onto YPD plates and CFU were counted after 24h at 30ºC. Survival rates were calculated as the total number of colonies at different concentrations of $CdSO_4$ divided by the total number of colonies without $CdSO_4$. See Materials and Methods.

4.5 The diterpene glycoside Amarisolide A does not have antifungal activity against *C. glabrata*.

Diterpenes, diterpenoids and their derivatives are common secondary metabolites that have been reported to have antimicrobial activity (Ivanov et al., 2021). To evaluate the antifungal activity of the diterpene glycoside Amarisolide A (García-Nava et al., 2023), we determined its minimum inhibitory concentrations. Cultures of BG14, *cdr1*Δ, *pdr1*Δ, *yor1*Δ, *pdr12*Δ, *snq2*Δ, and *ycf1*Δ, were grown and exposed to 0, 50, 100 and 200 µM of the diterpene glycoside Amarisolide A and growth curves were monitored for 48h. Amarisolide A has no antifungal effect. However, *ycf1*Δ at 200 µM has an increase in growth compared to the parental strain BG14 (**Fig. 5**). These results indicate that the diterpene glycoside Amarisolide A has no antifungal activity.

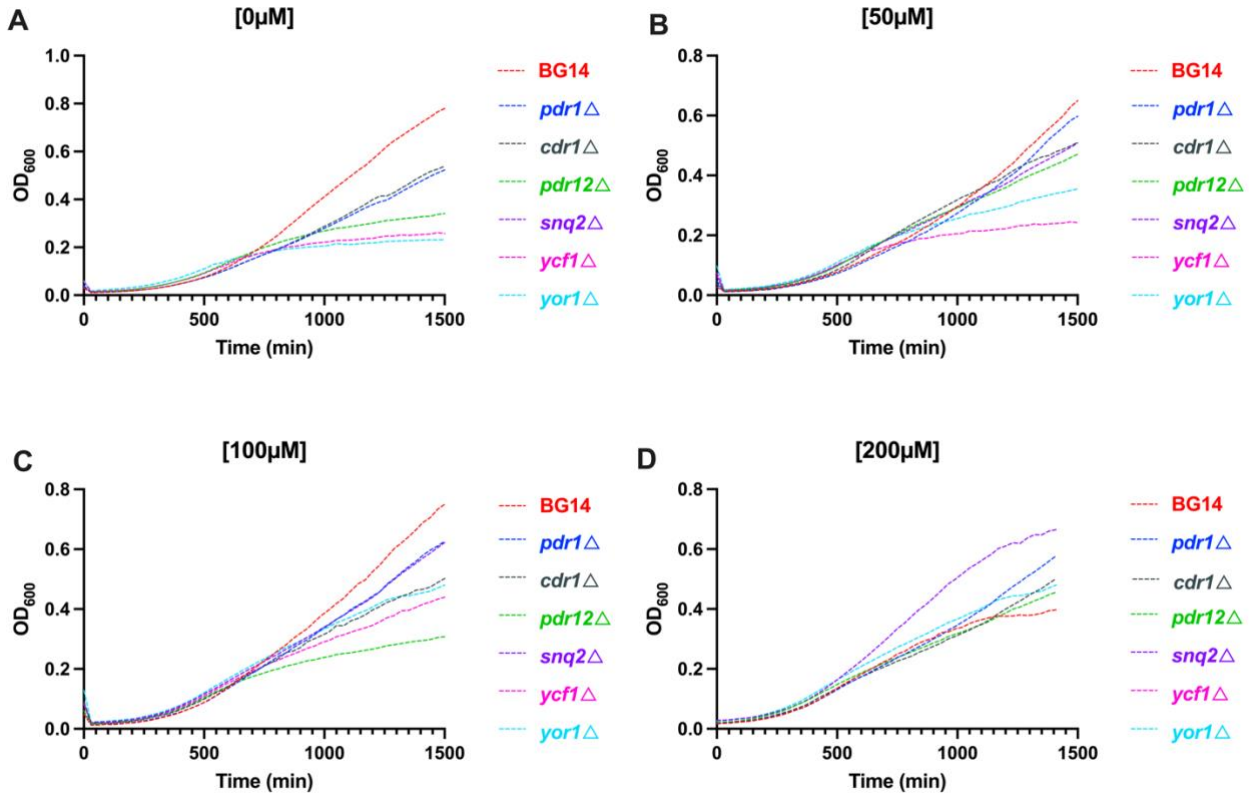

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Figure 5. Antifungal activity of Amarisolide A.

Cultures of BG14, *pdr1*Δ, *cdr1*Δ, *yor1*Δ, *pdr12*Δ, *snq2*Δ, and *ycf1*Δ, were grown in YPD at 30˚C for 48h. Cells were collected and adjusted to an OD_{600} of 0.01 in fresh SC media and Amarisolide A was added to a final concentration of 0, 50, 100 and 200 µM. 300 µL of each cell dilution were added to each well of the Honeycomb plate in triplicates for each strain. Growth was automatically monitored for 48h at 30 ºC with OD measurements every 15 minutes with a Bioscreen C analyzer (Oy, Growth Curves).

5. Discussion

5.1 Cdr1 is the main ABC-type transporter in *Candida glabrata*.

*Cg*Cdr1 was first described in 1999, when Sanglard et al., discovered a similar gene in *C. albicans*. They later established that in *C. glabrata* azole-resistant clinical isolates, *CDR1* was up-regulated by a factor of 5 to 8. To demonstrate this, they deleted *Cg*CDR1 from an isolate, and susceptibility was rendered. Additionally, azole resistance was restored when the mutants was complemented with *CDR1* in a plasmid. Thus, the correlation between the upregulation of this gene and azole-resistance was established (Sanglard et al., 1999). Since then, several ABC transporters that are involved in the efflux of xenobiotics have been identified in *C. glabrata*, including Pdr12, Snq2, Ycf1 and Yor1.

Our results confirm that *CDR1* is the main efflux pump related to azole-resistance. The role of Ycf1, Yor1 and Pdr12 became slightly evident only when *CDR1* was deleted. This data has been described before, since Ycf1 is primarily involved in the detoxification of cadmium and other metal conjugates through the vacuole (Li et al., 1996). Whereas Yor1 is mainly involved in resistance to oligomycin, an antimicrobial agent produces by *Streptomyces* that inhibits the mitochondrial ATP-synthase (Katzmann et al., 1995). However, transcript levels of *YOR1* were found to be upregulated in azole-resistant clinical isolates and lab-generated mutants (Ferrari et al., 2011). It has also been described that under nitrogen depletion conditions, a null mutant in *YOR1* showed an azole-susceptible phenotype that was independent of Cdr1 (Kumari et al., 2018). On the other hand, *PDR12* has also been described to be downregulated in the presence of azoles (Vermitsky et al., 2006). We have yet to test if the deletion of these genes in clinical isolates show any characteristic phenotype that could demonstrate an abnormal function of these transporters, particularly if there is mitochondrial damage.

5.2 Snq2 confers sensitivity to FLC.

Snq2 has been widely described as an ABC transporter that is directly linked to azole resistance in *C. glabrata* both in lab-made mutants and in clinical isolates *(Kumari et al., 2018; Torelli et al., 2008)*. Surprisingly, we discovered that in the BG14 background and in three independent mutants, the deletion of *SNQ2* confers resistance to fluconazole. A similar phenotype has only been reported once before, when Whaley et al., showed that in the SM1 background, *SNQ2* only had a decrease in MIC when both *CDR1* and *CDR2* were deleted. Interestingly, in a growth curve with increasing concentrations of fluconazole their *snq2*Δ mutant grew slightly better than their parent strain (Whaley et al., 2018). We have yet to determine if this resistance phenotype is unique to the BG14 background by making a *snq2*Δ mutant in the CBS138 background. Additionally, to confirm azole-sensitivity, we will overexpress *SNQ2* in BG14. Furthermore, we will heterologously express *SNQ2* in *E. coli* to determine the direction of transport of xenobiotic molecules. Lastly, we will construct a null mutant in the putative hexose transporter gene HXT4/6/7, which has been previously described to import azoles in *C. glabrata* (Galocha et al., 2022). By deleting this gene, we expect to still observe fluconazole sensitivity. Whereas with a double mutant in *hxt4/6/7*Δ ***snq2***Δ*,* we expect to observe an increased resistance to azoles.

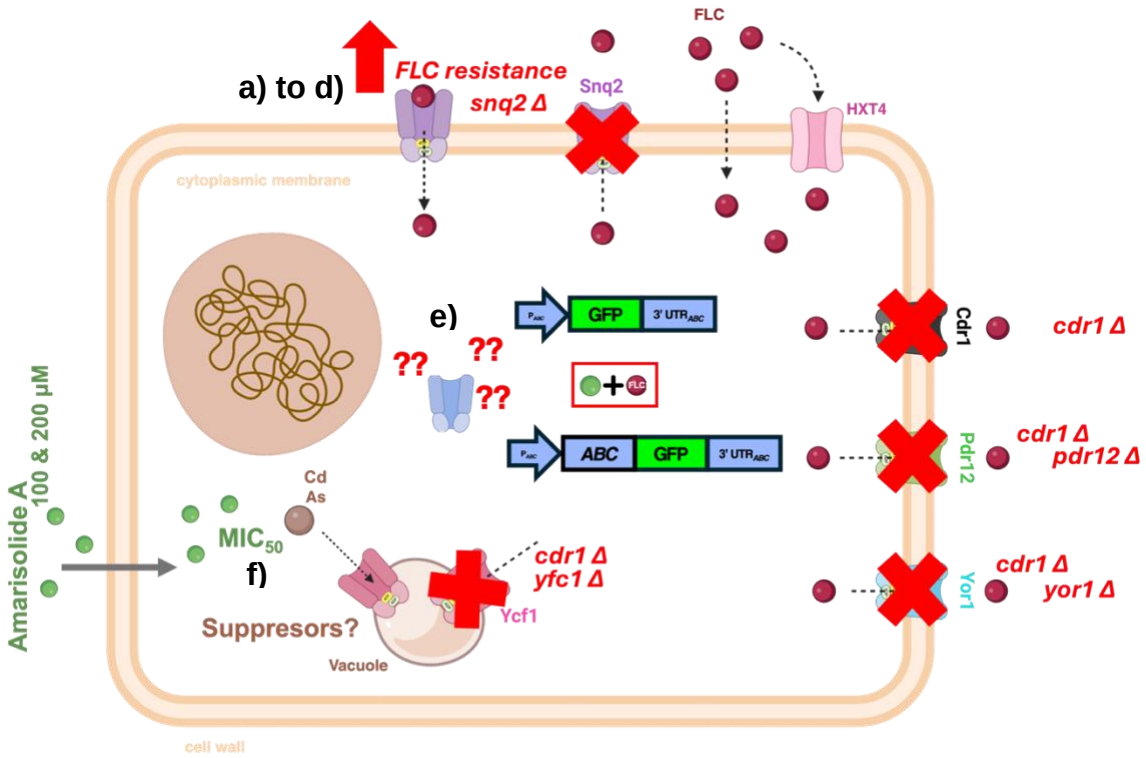
One possible explanation for this phenotype is that perhaps the ATPase subunits in Snq2 are switching their activity from exporting to importing molecules, since ATP transporters have been previously described as ‘promiscuous’. Alternatively, the actual transporter has flipped directions. These phenomena has been previously described in bacteria (Elston et al., 2023). To determine this activity, we are to structurally analyze if the electrostatic gradient is different than other ABC transporters that have been already crystalized, such as Cdr1. One of the main limitations to show this is that Snq2 has not been crystalized yet, therefore, we will be relying on predicted structures. Experimentally, we could use fluorescent probes to quantify the amount that enters or exits the cell. Additionally, we could tag the

carboxyl-terminal end of the protein with GFP, to determine the orientation of the protein and to verify which side anchors to the cytoplasmic membrane.

5.3 *ycf1*Δ grows in the presence of Amarisolide A.

Our preliminary growth curves showed that the exposure of our *C. glabrata* mutant strains to the diterpene glycoside Amarisolide A does not have antifungal activity on its own. Contrary to what we expected, the growth curves showed that for *ycf1*Δ, exposure to 200 µM Amarisolide A enhances its growth, rather than diminish it. However, the experiment could only be performed once with triplicates. We are yet to analyze if FLC along with this and several other compounds have EPI activity. The main limitation for this experiment is the amount of natural compound that could be extracted from the plants. Therefore, we decided to use that limited amount to determine whether these compounds affect either the transcription or translation of the transporter genes by constructing transcriptional and translational fusions with GFP. Although not all phytoactive compounds have EPI activity, many have been described to inhibit growth. Hence, if we find any activity through transcriptional or translational fusions, we could be looking at potential drugs either for humans or even to slow down biofilm formation. Additionally, these compounds could be tested against other drug-resistance targets, such as *FKS* genes, or ergosterol biosynthesis genes.

6. Graphical Abstract



6.1 Perspectives

a) To determine *snq2*Δ azole-resistance in the CBS138 background.

b) To overexpress *SNQ2* in *C. glabrata* and in *E. coli* to determine sensitivity to fluconazole.

c) To construct a single null mutant in *HXT4/6/7* and a double mutant *hxt4/6/7*Δ *snq2*Δ to further confirm the role of snq2 as an azole importer, as well as a

d) To chemically tag fluconazole to quantify the amount that gets into the cell through Sqn2.

e) To tag the C-terminal end of Snq2 to verify the orientation of the transporter.

f) To determine whether Amarisolide A and other phytochemicals act as EPIs through transcriptional and translational fusions.

g) To determine MIC_{50} of the phytochemicals in liquid media.

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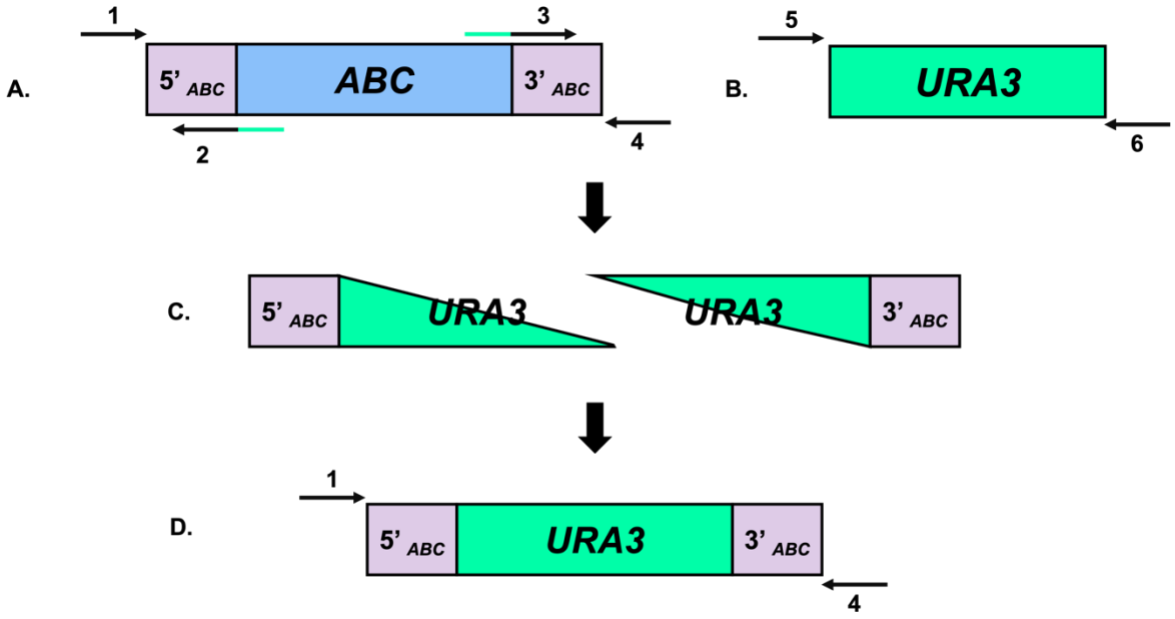
8. Supplementary Data

8.1 *ScARR2* does not confer arsenic resistance to C*. glabrata.*

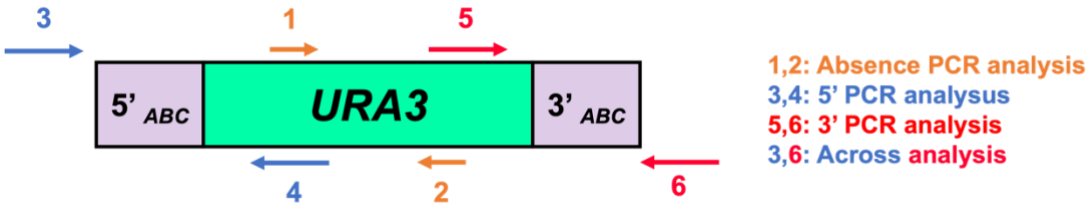
Arsenic is a highly reactive, toxic heavy metal. However, some microorganisms, such as *S. cerevisiae*, have been described to be arsenic resistant. Particularly, *S. cerevisiae* has three genes that confer this resistance: *ARR1*, *ARR2* and *ARR3.* These genes are contiguous gene clusters that encode the transcription factor Arr1 that regulates the transcription of arsenate reductase 2 (Arr2), and the arsenite extrusion transporter (Arr3), respectively (Ghosh et al., 1999). In *C. glabrata* there are no gene clusters that confer arsenic resistance. Nevertheless, it has been demonstrated that extracellular sulfate metabolizes to sulfides, which acts as electron donor for arsenate reduction. Additionally, it has been suggested that arsenite (As(III)) forms a complex with GSH and is transported into the vacuole for detoxification (Tsai et al., 2009)

Therefore, to evaluate if *ScARR2* gene confers arsenic resistance to *C. glabrata*, we used four independent strains containing a plasmid overexpressing *S. cerevisiae ARR2* (pGE481/pGE482). Nevertheless, we observed that *ScARR2* does not confer arsenic resistance, even after 72h of growth (**Fig. S6,** 0.5mM).

9. Supplementary figures

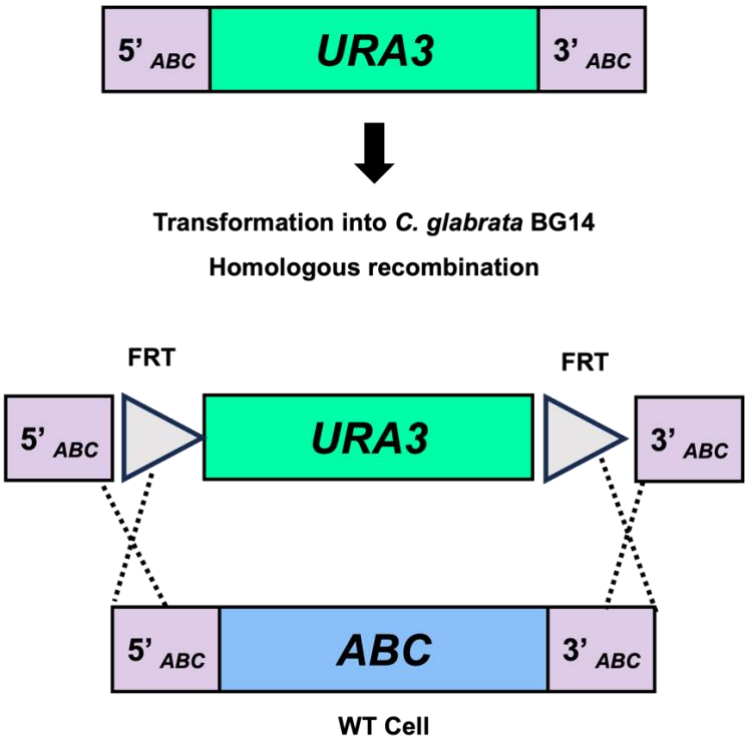


9.1 Supplementary figure 1. Schematic representation of Fusion PRC. Step **A** and **B** consists of 3 individual PCR reactions. The 5'- and 3'- flanking regions are amplified with primers 1/2 and 3/4, respectively. Additionally, primers 5/6 are used to amplify the selectable marker *URA3*. **C.** Primers 2 and 3 contain annealing sequences of the selectable marker *URA3* in addition to the 5'- and 3'- flanking regions, respectively. Therefore, chimeric products of the 5' and 3' and *URA3* annealing sequences are obtained. **D.** For the fusion PCR, both 5'- and 3'- flanking regions are joined to *URA3*, and the final PCR product is amplified with the outermost primers 1 and 4.



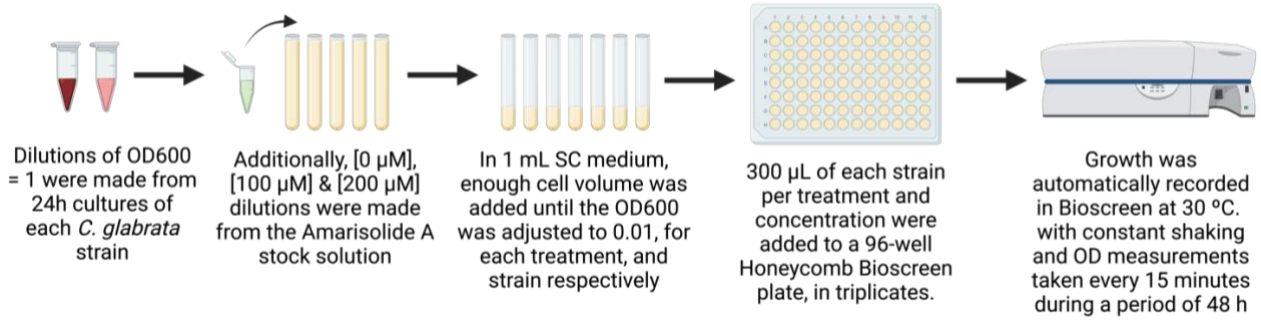
9.2 Supplementary figure 2. Schematic representation of PCR analysis for mutant verification.

All used primers are described in Materials and Methods, and in Table S2. Briefly, primers 1 and 2 are used to verify the absence of the knocked-out gene. Primers 3,4 and 5,6 are used to verify that the 5' and 3' regions are fused to the URA3 cassette. Primers 3 and 6 are then used to amplify the complete knock-out construction.



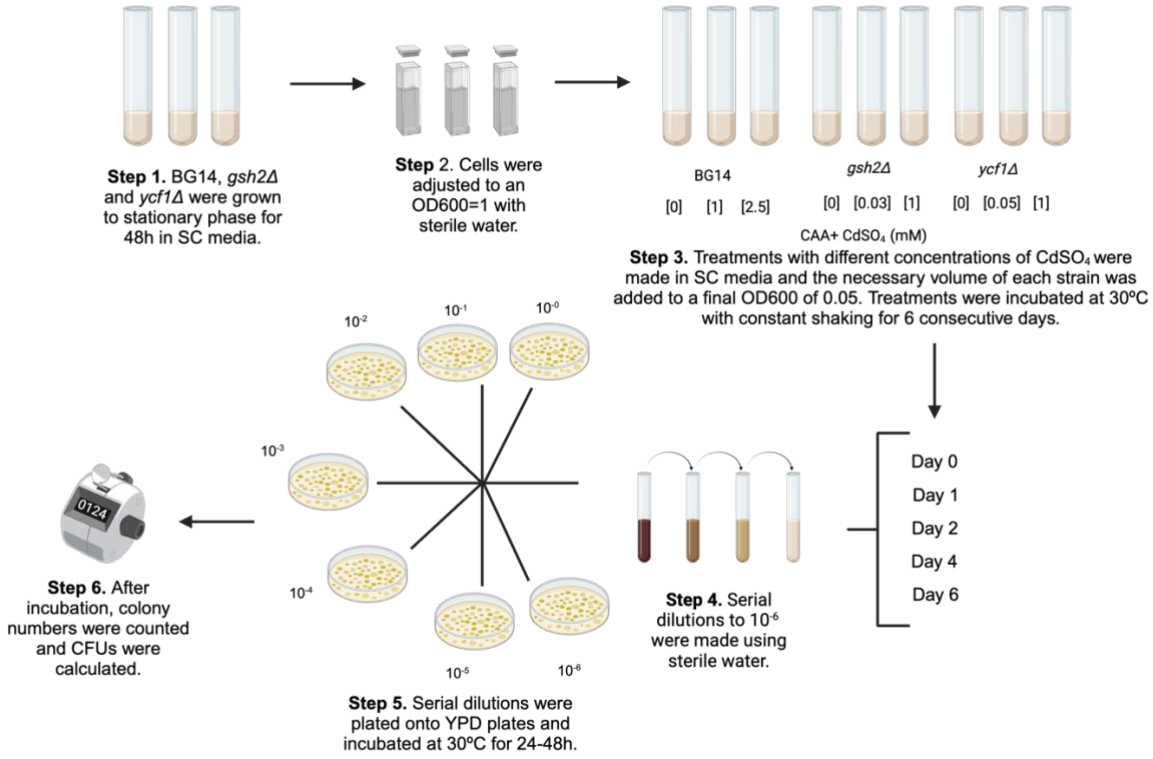
9.3 Supplementary figure 3. Schematic representation of homologous recombination.

The fusion PCR product is transformed into *C. glabrata*. The *URA3* cassette contains *S. cerevisiae* FRT sequences in order to obtain homologous recombination. Briefly, this system involves using the flippase recombinase (FLP), which recognizes the FLP recombinase target sequences, FRT that flank a genomic region of interest.



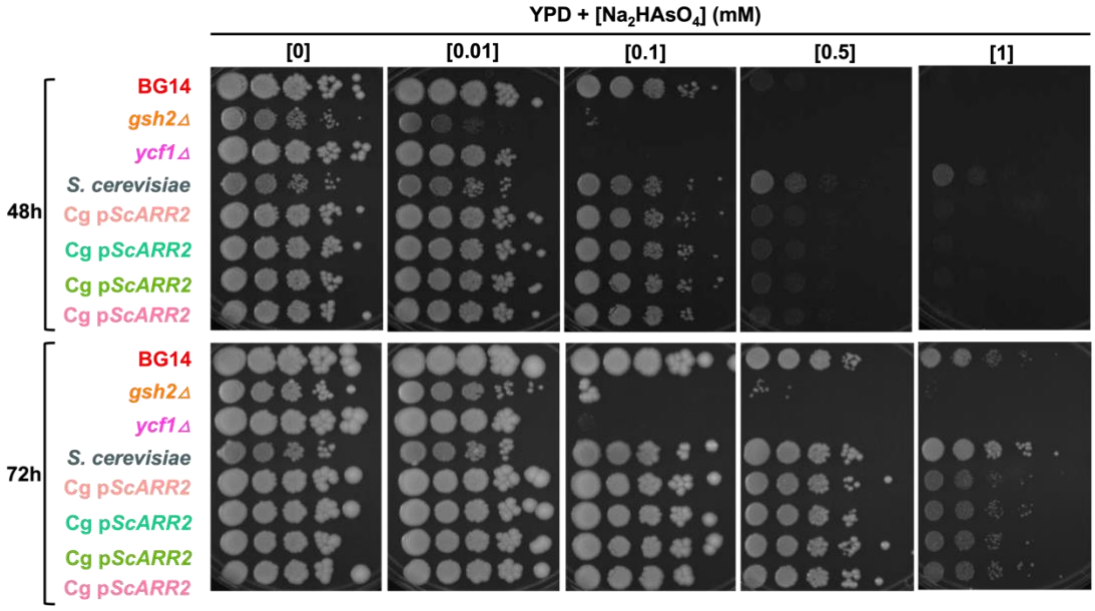
9.4 Supplementary figure 4. Growth assay in liquid media.

Growth curves are explained in detail in section 3.6. Cells were grown to stationary phase for 48 h in YPD, or SC media. Stationary phase cultures were adjusted to an OD600 of 0.01 in the SC media and 300 µL were dispensed in a honeycomb plate. Growth was automatically recorded using Bioscreen C analyser (Oy, Growth Curves) at 30 ºC. with constant shaking and OD measurements taken every 15 minutes during a period of 48 h (Gutiérrez-Escobedo et al., 2013).



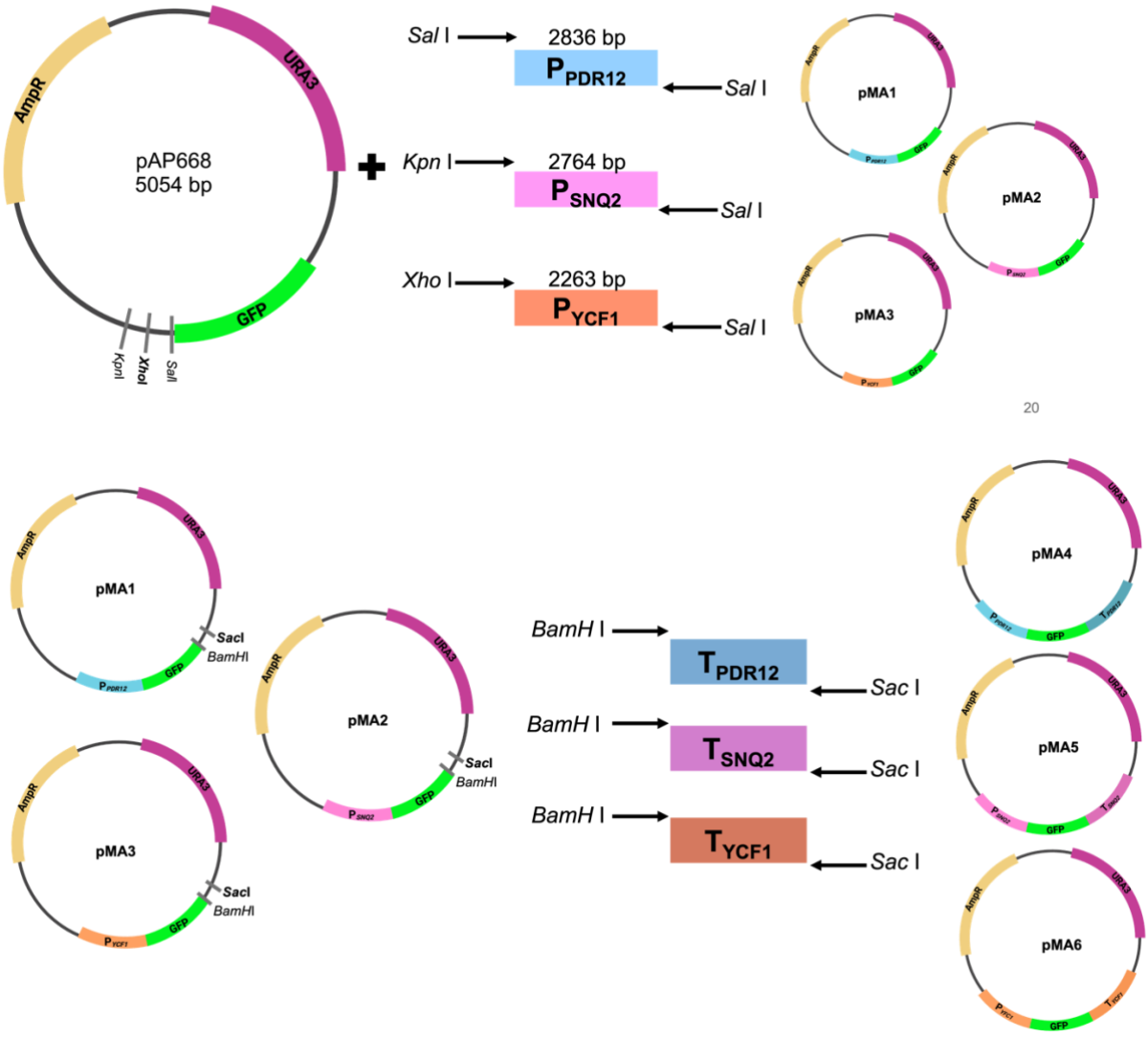
9.5 Supplementary figure 5. Viability assay.

To determine how many *C. glabrata* cells survive after chronic exposure to cadmium, BG14, CGM976 (*gsh2*Δ) and CGM5052 (*ycf1*Δ) were grown to stationary phase in SC media supplemented with uracil for 48h, then adjusted to an OD600 of 1. In test tubes, we prepared 5mL chronical treatments with cadmium using SC media supplemented with uracil and 0, 0.03, and 1 mM $CdSO_4$ for CGM976 (*gsh2*Δ), 0, 0.05 and 1 mM $CdSO_4$ for CGM5052 (*ycf1*Δ) and 0, 1 and 2.5 mM $CdSO_4$. for BG14. These tubes were then inoculated with the necessary volume of adjusted cells to have a final OD600 of 0.05. Treated cells were incubated at 30ºC in constant shaking for 6 consecutive days. At day 1, 2, 4 and 6, we took a 100µL sample of each treatment and made 10-fold serial dilutions and plated them onto solid YPD media. This experiment was performed three times.



9.6 Supplementary figure 6. *ScARR2* does not confer increased arsenic resistance to *C. glabrata*.

Cultures of BG14 (parental strain), *gsh2*Δ, *ycf1*Δ, S. cerevisiae and 4 independent mutants of Cg p*ScARR2* were grown for 48h at 30˚C in YPD. For the arsenic sensitivity assay, 10-fold dilutions were made from each culture and spotted onto YPD plates with increasing Na_2HAsO_4 concentrations. See Materials and Methods.



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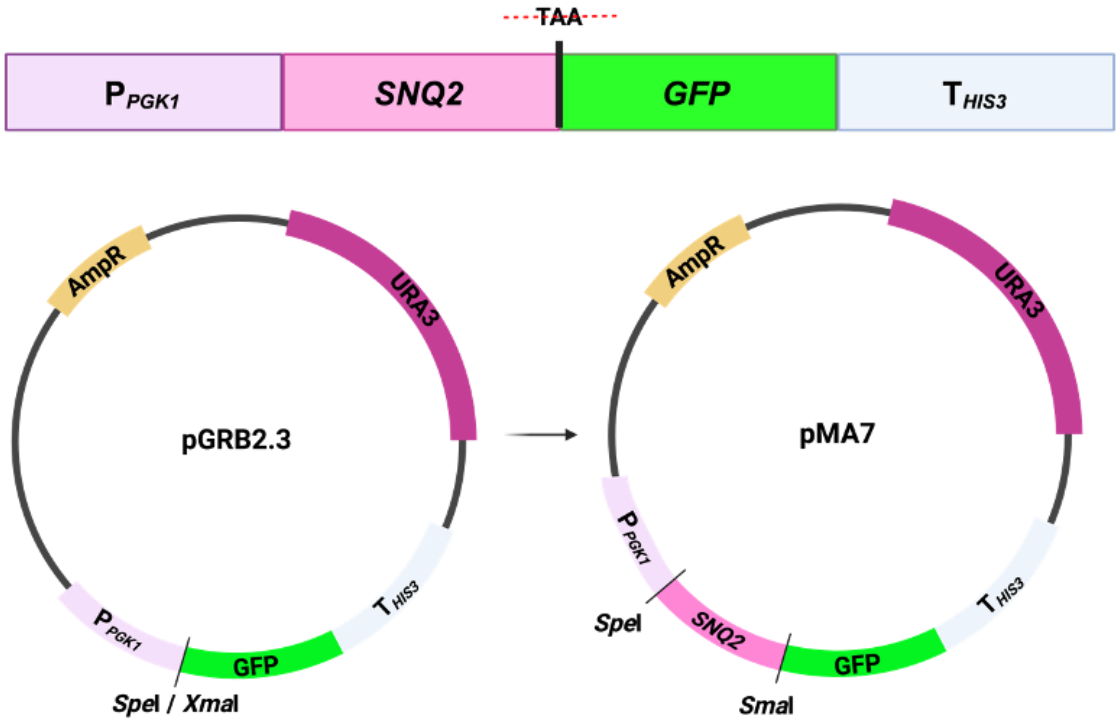
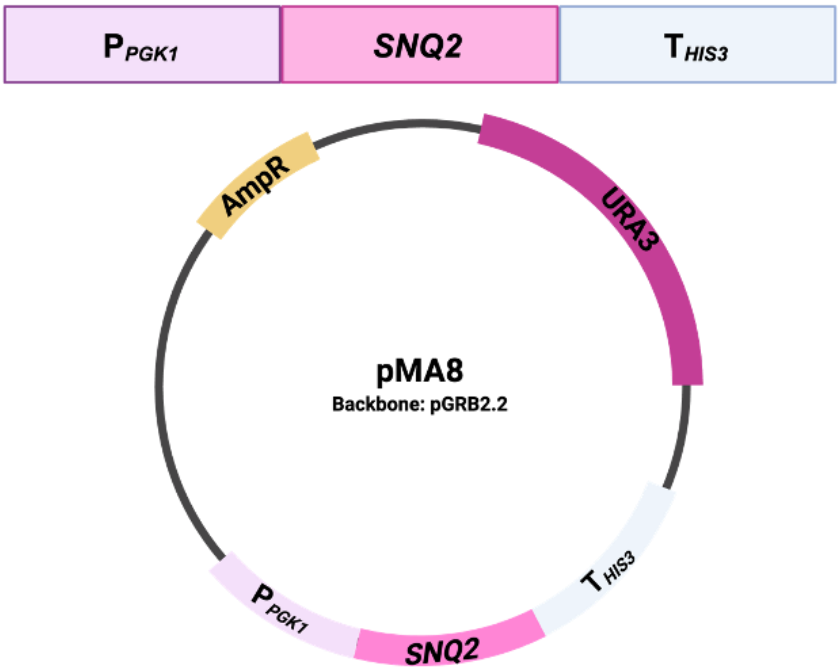
694 **9.7 Supplementary figure 7. Strategy for transcriptional fusions**

695 Briefly, for the 5'-flanking regions of the promoters, *Sal*I, *Kpn*I and *Xho*I will be
696 used to digest P*PDR12*, P*SNQ2*, and P*YCF1*, respectively. For the 3'-flanking regions
697 of the promoters, *Sal*I will be used to digest all promoters. These digested
698 products will be cloned onto pAP668. For the terminators, the previously
699 constructed plasmids (pMA1, pMA2 and pMA3) will be digested with *BamH*I for
700 the 5'-flanking regions and with *Sac*I for the 3'-flanking regions. These plasmids
701 (pMA4, pMA5, and pMA6) will be transformed into *C. glabrata* to analyze the
702 transcriptional response to the combination of FLC and Amarisolide A.



9.8 Supplementary figure 8. Strategy for translational fusions

Briefly, pGRB2.3 will be digested with *Spe*I and *XmaI*, and the *SNQ2* ORF without the termination codon (TAA) will be cloned on the N-terminal region of the *GFP* gene. This plasmid will then be transformed into *C. glabrata* to analyze the localization of Snq2 in response to a combination of FLC and Amarisolide A and other phytochemicals.



9.9 Supplementary figure 9. Overexpression of *SNQ2* in *C. glabrata.*

Briefly, *SNQ2* will be cloned into pGRB2.2 that contains the constitutive promoter *PGK1* and the *HIS3* terminator. This plasmid will be cloned into *C. glabrata* to overexpress Snq2.

10. Supplementary Tables

10.1 Table S1. Strains

Strain	Genotype	Phenotype	References
C. glabrata			
BG14	*ura3Δ::Tn903 Neo^R* Ura$^-$	Control	Cormack & Falkow, 1999
CGM1094 (*pdr1*Δ)	*ura*3Δ::Tn*903* G418^R *pdr*1Δ::*hph* Hyg^R	FLC-sensitive	Guerrero-Serrano, 2011
CGM1096 (*cdr1*Δ)	*ura*3Δ::Tn*903* G418^R *cdr*1Δ::*hph* Hyg^R	FLC-sensitive	Guerrero-Serrano, 2011
CGM84 (*hst1*Δ)	*ura3Δ::Tn903* G418^R *hst1*Δ::*ura3*	FLC-resistant	Guerrero-Serrano, 2011
CGM976 (*gsh2*Δ)	*ura3*Δ*::Tn903 G418^R gsh2*Δ*::hph*	Cd sensitive	Gutiérrez-Escobedo et al.,2013
CGM1074 (*yor1*Δ)	*ura*3Δ::Tn*903* G418^R *yor1*Δ::*ura3* Ura$^+$	FLC-sensitive	Gutiérrez-Escobedo et al., 2012
CGM5024 (*pdr12*Δ)	*ura*3Δ::Tn*903* G418^R *pdr12*Δ::*ura3* Ura$^+$	FLC-sensitive	This work
CGM5026 (*snq2*Δ) 1	*ura*3Δ::Tn*903* G418^R *snq2*Δ::*ura3* Ura$^+$	FLC-resistant	This work
CGM5027 (*snq2*Δ) 2	*ura*3Δ::Tn*903* G418^R *snq2*Δ::*ura3* Ura$^+$	FLC-resistant	This work

CGM5052	(*ycf1*Δ)	*ura*3Δ::Tn*903* G418^R *ycf1*Δ::*ura3* Ura$^+$	FLC-sensitive	This work
CGM5166	(*cdr1*Δ *pdr12*Δ)	*ura*3Δ::Tn*903* G418^R *pdr12*Δ::*ura3* Ura$^+$ *cdr*1Δ::*hph* Hyg^R	FLC-sensitive	This work
CGM5168	(*cdr1*Δ *snq2*Δ)	*ura*3Δ::Tn*903* G418^R *snq2*Δ::*ura3* Ura$^+$ *cdr*1Δ::*hph* Hyg^R	FLC-sensitive	This work
CGM5208	(*cdr1*Δ *yor1*Δ)	*ura*3Δ::Tn*903* G418^R *yor1*Δ::*ura3* Ura$^+$ *cdr*1Δ::*hph* Hyg^R	FLC-sensitive	This work
CGM5209	(*cdr1*Δ *ycf1*Δ)	*ura*3Δ::Tn*903* G418^R *ycf1*Δ::*ura3* Ura$^+$ *cdr*1Δ::*hph* Hyg^R	FLC-sensitive, Cd sensitive	This work
CGM5239 3	(*snq2*Δ)	*ura*3Δ::Tn*903* G418^R *snq2*Δ::*ura3* Ura$^+$	FLC-resistant	Robledo-Márquez et al., 2022
CGM5455	(*Cg pScARR2)*	*Cg pScARR2* (pGE481) Ura$^+$		This work
CGM5456	(*Cg pScARR2)*	*Cg pScARR2* (pGE481) Ura$^+$		This work
CGM5457	(*Cg pScARR2)*	*Cg pScARR2* (pGE482) Ura$^+$		This work

CGM5458 (*Cg pScARR2)*	*Cg pScARR2* (pGE482) Ura$^+$	This work
E. coli		
DH10	F-*mcrA*Δ (*mrr-hsdRMS-mcrBC*) F80 Δ*lacZ* Δ*M*15 Δ*lacX74 deoR recA1 endA1 araD*139 Δ (*ara*,*leu*)*7697 galU galK* l- *rpsL nupG*	Calvin and Hanawalt, 1988
pGE80	PCR product (1.353kb) corresponding to the *URA3* gene from the strain L-105. Cloned into pMB11 and digested with *Stu* I. Cm^R	Gutierrez-Escobedo, 2013
pGE481	PCR product corresponding to *ScARR2* (390bp) cloned into pGRB2.2. Digested with *BamH*I and *Xho*I. Cb$^+$ Ura$^+$	Lab collection
pGE482	PCR product corresponding to *ScARR2* (390bp) cloned into pGRB2.2. Digested with *BamH*I and *Xho*I. Cb$^+$ Ura$^+$	Lab collection

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10.2 Table S. Primers

Number	Name	Sequence	Site
For single null mutants			
PDR12			
3272	*PDR12 @-1337 Fw*	CATGAAAAATTGGCACCTCC	-
3274	*PDR12 @ -1 URA3*	cgaattcaggaacttgatattttttTCTAAGTCAG ATTCTTTACTTATTATT	-
3275	*PDR12 @ -2836 Fw*	CGCgtcgacCCTCCCAATTAACTAGTT TATGC	*Sal*I
3277	*PDR12 @* +1Fw *URA3*	ggctaccacatcgtctttgACTCTTTCTTTCA CCTAACTAATGAC	-
3278	*PDR12 @ +837 Rev*	CCTGGCCCAGCGATAAGG	-
3279	*PDR12 @ +967 Rev*	CAAGgagctcCCTTTTCCGCTAACTGT TTGTTC	*Sac*I
3280	*PDR12 @ 935 Fw*	GCGTGTGTCTTTAGTAGAAGC	-
3281	*PDR12 @1479 Rev*	CCAGTAATTGACAACATAACGGG	-
SNQ2			
3282	*SNQ2 @ -2764 Fw*	CGGggtaccGTTTACCTGTGAGATTCC GCGGG	*Kpn*I
3284	*SNQ2 @ -1 Rev URA3*	cgaattcaggaacttgatattttttTGTTTCACTC GTTATTGCAGTATTTTTACC	-
3285	*SNQ2 @ -996 Fw*	CGCGGAGAGTAGGCGGCGGAG	-
3286	*SNQ2 @ 1156 Fw*	CACTGGTAGACAGATATACTTTGGC C	-
3287	*SNQ2 @ + 940 Rev*	CCTGACATAGTGGAGTGTTACTCG	-
3289	*SNQ2 @+1 Fw URA3*	ggctaccacatcgtctttgATGCACTGTACTG CATTTTTACAACACC	-
3290	*SNQ2 @ +851 Rev*	CTTCACTACTGCTGAGAAAGTTCCC	-
3291	*SNQ2 @ +940 Rev*	CAAGgagctcCCTGACATAGTGGAGT GTTACTCG	*Sac*I

3292	*SNQ2 @ 1675 Rev*	GGACACCACCTCTGGAAAATGCACC	-
YCF1			
3294	*YCF1 @ -1 URA3*	cgaattcaggaacttgatattttTGTTAAAACC CAATAGGAATATATTAAGC	-
3295	*YCF1 @ -1096 Fw*	GGTTAAGGCACCGTGCTAATAAC	-
3296	*YCF1 @ -2263 Fw*	GTTGctcgagCCATTTTGTAAACACTG TTTATTAAACCG	*Xho*I
3298	*YCF1 @ +1 URA3*	ggctaccacatcgtctttgAGAAATGTGTATA ATACCGCAAATGATATATAG	-
3299	*YCF1 @ +1042 Rev*	GTCTCCCACGTATCGACCC	-
3301	*YCF1 @ 650 Fw*	GGAAGCCCTCCCAAGTAAGCC	-
3303	*YCF1 @ -1487 Fw*	GTTGctcgagCTTGGTTGTGTCTTGAC TATCC	*Xho*I
3438	*YCF1 @ +1511 Rev*	CAAGgagctcGGATCTACGCAGGAAG AAATCCC	*Sac*I
3437	*YCF1 @ 1409 Rev*	CCAACCCACATAGAATGACC	-
URA3			
604	*URA3 Annealing Fw*	GGCTACCACATCGTCTTTG	-
605	*URA3 Annealing Rv*	CGAATTCAGGAACTTGATATTTTT	-
114	*URA3 @137 Rv*	TGGGACCTAATGCTTCAACTAAC	-
113	*URA3 @729 Fw*	GCTAAGGTAGAGGGTGAACGTTACA G	-
For double mutants			
745	*PDR5 @-778bp FW*	CGCGTAATAGGACTGCAAAG	-
746	*PDR5 @-821bp FW*	GGAAAGGATGAGTAGAAATCG	-
748	*PDR5 @+753pb RV*	GCTGATTTACCAGATAAC	-
749	*PDR5 @+790bp Rev*	GGACAACTTGTTTGTATTCACAA	-
750	*PDR5 @294pb FW*	GTATTGGTCTGGCCTATGTG	-
751	*PDR5 @565pb RV*	GGTTTATCTCAATCTGAAGCC	-
15	*#2292 PGK-P Rev-out*	CATAAAGCACGTGGCCTCTTATCG	-

16	*#2293 HIS3 FW-out*	AGAAATACGCACGAACACGATATAG AGG	-
For transcriptional fusions			
PDR12			
3273	*PDR12 @ -1 Rv*	CGCgtcgacTCTAAGTCAGATTCTTTA CTTATTATT	*Sal* I
3275	*PDR12 @ -2836 Fw*	CGCgtcgacCCTCCCAATTAACTAGTT TATGC	*Sal* I
3276	*PDR12 @ +1 Fw*	CGCggatccACTCTTTCTTTCACCTAA CTAATGAC	*BamH*I
3279	*PDR12 @ +967 Rev*	CAAGgagctcCCTTTTCCGCTAACTGT TTGTTC	*Sac*I
SNQ2			
3282	*SNQ2 @ -2764 Fw*	CGGggtaccGTTTACCTGTGAGATTCC GCGGG	*Kpn*I
3283	*SNQ2 @ -1 Rev*	CGCgtcgacTGTTTCACTCGTTATTGC AGTATTTTTACC	*Sal*I
3288	*SNQ2 @ +1 Fw*	CGCggatccATGCACTGTACTGCATTT TTACAACACC	*BamH*I
3291	*SNQ2 @ +940 Rev*	CAAGgagctcCCTGACATAGTGGAGT GTTACTCG	*Sac*I
YCF1			
3293	*YCF1 @ -1 Rev*	CGCgtcgacTGTTAAAACCCAATAGGA ATATATTAAGC	*Sal*I
3296	*YCF1 @ -2263 Fw*	GTTGctcgagCCATTTTGTAAACACTG TTTATTAAACCG	*Xho*I
3297	*YCF1 @ +1 Fw*	CGCggatccAGAAATGTGTATAATACC GCAAATGATATATAG	*BamH*I
3438	*YCF1 @ +1511 Rev*	CAAGgagctcGGGATTTCTTCCTGCGT AGATCC	*Sac*I

3303	*YCF1 @ -1487 Fw*	GTTGctcgagCTTGGTTGTGTCTTGAC TATCC	*Xho*I
For translational fusions			
SNQ2			
3675	*SNQ2@ATG SpeI FW*	gtgactagtaaaATGAGTTCTTCTTCAGA GATCTCG	*Spe*I
3676	*SNQ2 @4521 SmaI Rv*	gtgcccgggGTTGGACTTCTTCCCCCTC CT	*Sma*I
3677	*SNQ2 @+1 XhoI Rv_HIS3*	ctcgagTTAGTTGGACTTCTTCCC	*Xho*I
3678	*SNQ2 @TAA SmaI Rv*	gtgcccgggTTAGTTGGACTTCTTCCCC CTCCT	*Sma*I

721