Genomic evolution towards azole resistance in *Candida glabrata* clinical isolates unveils the importance of CgHxt4/6/7 in azole accumulation

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The increasing prevalence of candidosis caused by *Candida glabrata* is related to its ability to acquire azole resistance. Although azole resistance mechanisms are well known, the mechanisms for azole import into fungal cells have remained obscure. In this work, we have characterized two hexose transporters in *C. glabrata* and further investigate their role as potential azole importers. Three azole susceptible *C. glabrata* clinical isolates were evolved towards azole resistance and the acquired resistance phenotype was found to be independent of *CgPDR1* or *CgERG11* mutations. Through whole-genome sequencing, CgHXT4/6/7 was found to be mutated in the three evolved strains, when compared to their susceptible parents. CgHxt4/6/7 and the 96% identical CgHxt6/7 were found to confer azole susceptibility and increase azole accumulation in *C. glabrata* cells, strikingly rescuing the susceptibility phenotype imposed by *CgPDR1* deletion, while the identified loss-of-function mutation in CgHXT4/6/7, leads to increased azole resistance. In silico docking analysis shows that azoles display a strong predicted affinity for the glucose binding site of CgHxt4/6/7. Altogether, we hypothesize that hexose transporters, such as CgHxt4/6/7 and CgHxt6/7, may constitute a family of azole importers, involved in clinical drug resistance in fungal pathogens, and constituting promising targets for improved antifungal therapy.
Candida species are one of the most common causes of fungemia in humans, responsible for >400,000 life-threatening infections yearly worldwide with an associated 46–75% mortality rate. Clinical isolates resistant to all three classes of antifungals available have been found in hospitalized patients. C. glabrata, particularly, is a prominent cause of invasive candidosis, best known for its ability to overcome azole antifungal therapy.

Azoles act by inhibiting the 14α-demethylase Erg11 in the ergosterol biosynthesis pathway, causing the accumulation of a toxic sterol that permeabilizes the plasma membrane. These drugs have been used for decades as the standard treatment against candidosis and are still the only oral treatment option. Instead of developing new drugs to overcome antifungal resistance, a process which is both very costly and time-consuming, therapeutic strategies can be improved by enhancing the efficacy of existing drugs, such as azoles. Therefore, it is worth identifying the genes and cellular pathways involved in the development of drug resistance.

The main mechanism of acquired azole resistance is the up-regulation of ABC (ATP-binding Cassette) drug transporters, mainly CgCdr1 and, and, to a lower extent, of the major facilitator superfamily (MFS) drug:H + antiporters, which catalyse the efflux of azoles preventing their intracellular accumulation. Generally, the constitutive up-regulation of the ABC efflux pumps is due to the emergence of gain-of-function (GOF) mutations in the CgPDR1 gene, encoding the key regulator of multidrug resistance in C. glabrata. In contrast to what is observed in C. albicans, several studies suggest that mutations in CgERG11 is not a relevant mechanism of acquired azole resistance in C. glabrata clinical isolates. Although CgPDR1 GOF mutations are considered the major mechanism of clinical acquisition of azole resistance, several resistant clinical isolates do not display any of the known mechanisms of resistance, suggesting the presence of yet unknown alternative paths to acquired azole resistance in C. glabrata.

In this study, prolonged exposure to posaconazole was used to induce in vitro C. glabrata azole susceptible clinical isolates to acquire azole resistance. The underlying molecular mechanisms were evaluated through whole-genome sequencing. Genes found to exhibit non-synonymous single-nucleotide polymorphisms (nsSNPs) in the resistant strains, when compared to their susceptible counterparts, were identified and evaluated for a possible role in azole resistance. The putative hexose transporter CgHxt4/6/7 (ORF CAGL0A02233g) stood out in the analysis, being hypothesized to work as an azole importer. Although little is known about how these drugs enter pathogenic fungal cells to exert their antifungal action, it was demonstrated that azoles are imported by facilitated diffusion rather than passive diffusion or ATP-dependent import. Moreover, fluconazole import differs among C. albicans resistant clinical isolates, which suggests that altered facilitated diffusion is possibly a previously uncharacterized mechanism of resistance to azole drugs.

In this context, the role of CgHxt4/6/7 and of the 96% identical CgHxt6/7 (ORF CAGL0A02211g) as hexose transporters was confirmed, while their role as potential azole importers was further explored leading to the characterization of an alternative mechanism of acquired azole drug resistance in C. glabrata.

Results

In vitro evolution towards posaconazole resistance in C. glabrata clinical isolates. Four isolates, previously found to display azole susceptibility, the CBS138 reference strain and three clinical isolates (040, 044 and OL152), were subjected to an in vitro directed evolution approach, to generate azole-resistant populations. After prolonged exposure to a therapeutic plasma concentration of the triazole posaconazole (1 mg L⁻¹), the CBS138, 040 and OL152 isolates evolved towards posaconazole, fluconazole and clotrimazole resistance following 10 days of incubation, while the 044 isolate acquired resistance to clotrimazole, fluconazole, voriconazole and posaconazole after 25 days of incubation (Supplementary Table 1). Interestingly, MIC₅₀ levels for voriconazole never reached resistance levels for CBS138, 040 and OL152 isolates (Supplementary Table 1). The evolved multiazole-resistant strains were denominated CBS138_Psc, 040_Psc, 044_Psc and OL152_Psc. The resistance pattern remained stable in all the evolved resistant isolates following sub-culture in drug-free medium for up to 30 days.

Acquisition of azole resistance can be independent of mutations in CgPDR1 or CgERG11. To understand whether the evolved resistance phenotype relied on the major mechanism of acquired azole resistance, the acquisition of Pdr1 GOF mutations, the transcription factor encoding gene CgPDR1 was sequenced in the four evolved resistant strains (CBS138_Psc, 040_Psc, 044_Psc and OL152_Psc). Remarkably, no sequence changes were detected in 040_Psc, 044_Psc or OL152_Psc and so the molecular basis underlying the development of azole resistance on these three evolved strains remained to be clarified. In the azole-resistant strain derived from the CBS138 strain, CBS138_Psc, a mutation in the CgPDR1 gene sequence was found. In this single case, a non-synonymous mutation leading to a Trp297Leu substitution was observed, which corresponds to a previously characterized GOF mutation in CgPDR1. This mutation is likely underlying the observed resistance phenotype in the evolved strain, which was therefore excluded from further analysis. Furthermore, no changes in the sequence or expression (Supplementary Fig. 1) of the drug target, CgERG11, were found in the four evolved strains, reinforcing the assumption that alteration of the drug target is not a usual mechanism of acquired azole resistance in C. glabrata clinical isolates. Potential resistance-related mutations in the promoter regions of both CgPDR1 and CgERG11 were also not found (Supplementary Data 1).

Identification of nsSNPs acquired during posaconazole exposure. In the search for insights into the mechanism(s) underlying the development of azole resistance in the evolved strains 040_Psc, 044_Psc and OL152_Psc, their genomes were sequenced and compared to that of the corresponding susceptible parents 040, 044 and OL152, respectively (Supplementary Data 2; Supplementary Data 3). Of 40, 70 and 103 nsSNPs were registered for the 040_Psc, 044_Psc and OL152_Psc strains, respectively. Most of the mutated genes were found to encode adhesins or adhesin-like proteins, specifically 70%, 92% and 70% of the mutated genes in the 040_Psc, 044_Psc and OL152_Psc strains, respectively. This is likely a direct consequence of adhesin encoding genes being mostly located in subtelomeric regions and including tandem-repeat regions, which favours increased random mutation rates. Nonetheless, it is interesting to point out that at least the CgEPA3 adhesin has been implicated in azole resistance.

Despite no common nsSNPs selected during prolonged posaconazole exposure among the 040_Psc, 044_Psc and OL152_Psc strains (Supplementary Fig. 2a) were registered, 5 genes were found to display non-synonymous mutations in the three resistant strains, when compared to the susceptible
counterparts. These genes included 4 adhesin-like proteins, including CgEPA3, plus the putative hexose transporter CgHXT4/6/7 (Supplementary Fig. 2b), which was selected for further analysis.

CgHxt4/6/7 and CgHxt6/7 are plasma membrane hexose transporters. The subcellular localization of CgHxt4/6/7 and CgHxt6/7 to import the hexoses glucose, mannose, fructose and galactose into yeast cells was assessed using the hexose transporter-null S. cerevisiae strain (Schxt0), which grows normally on maltose as carbon source29. The Schxt0 strain is unable to grow on glucose, mannose or fructose as sole carbon sources and grows very poorly on galactose29. Results demonstrate that Schxt0 cells expressing either CgHxt4/6/7 or CgHxt6/7 were able to grow on glucose and mannose and slightly on fructose as carbon sources, while cells harbouring the cloning vector were not (Fig. 1b). Together, these results demonstrate that CgHxt4/6/7 and CgHxt6/7 are plasma membrane hexose transporters, which was expected considering the role of their S. cerevisiae orthologues ScHxt6 and ScHxt7, respectively30.

CgHXT4/6/7 and CgHXT6/7 promote azole drug susceptibility in C. glabrata, probably by facilitating azole entrance into fungal cells. Since the so far uncharacterized hexose transporter encoding gene CgHXT4/6/7 stood out in the genome-wide analysis of the evolved resistant strains 040_Psc, 044_Psc and OL152_Psc, the potential impact of this transporter in C. glabrata tolerance to azoles was further evaluated, along with its close homologue CgHXT6/7. By comparing the susceptibility of wild-type, single deletion strains (Δcghxt4/6/7 and Δcghxt6/7) and double deletion strain (Δcghxt4/6/7Δcghxt6/7), the absence of either HXT gene was found to increase C. glabrata resistance towards fluconazole and posaconazole (Fig. 2a). Accordingly, MIC50 values for fluconazole and posaconazole were found to be lower for the wild-type strain (16 and 0.5 mg L\(^{-1}\), respectively) when compared to the single deletion strains Δcghxt4/6/7 (32 and 1 mg L\(^{-1}\), respectively) or Δcghxt6/7 (32 and 1 mg L\(^{-1}\), respectively), and to the double deletion strain Δcghxt4/6/7Δcghxt6/7 (64 and 2 mg L\(^{-1}\), respectively).

The results obtained for either CgHxt4/6/7 or CgHxt6/7 are consistent, but moderate, likely because 10 other hexose transporters. The subcellular localization of CgHxt4/6/7 and CgHxt6/7 was inspected by fluorescence microscopy in both C. glabrata and S. cerevisiae cells. As expected, the CgHxt4/6/7_GFP and the CgHxt6/7_GFP fusion proteins were found to be predominantly localized to the cell periphery, which is consistent with its predicted plasma membrane localization and transporter function, in both C. glabrata and S. cerevisiae cells (Fig. 1a).

The ability of the putative hexose transporters CgHxt4/6/7 and CgHxt6/7 to import the hexoses glucose, mannose, fructose and galactose into yeast cells was assessed using the hexose transporter-null S. cerevisiae strain (Schxt0), which grows normally on maltose as carbon source29. The Schxt0 strain is unable to grow on glucose, mannose or fructose as sole carbon sources and grows very poorly on galactose29. Results demonstrate that Schxt0 cells expressing either CgHxt4/6/7 or CgHxt6/7 were able to grow on glucose and mannose and slightly on fructose as carbon sources, while cells harbouring the cloning vector were not (Fig. 1b). Together, these results demonstrate that CgHxt4/6/7 and CgHxt6/7 are plasma membrane hexose transporters, which was expected considering the role of their S. cerevisiae orthologues ScHxt6 and ScHxt7, respectively30.
transporters are predicted to be encoded by the *C. glabrata* genome, potentially displaying functional overlap. In fact, the double deletion strain \( \Delta cghxt4/6/7 \\Delta cghxt6/7 \) was found to display a fourfold increase in the MIC 50 for both fluconazole and posaconazole, comparing to the wild-type. To assess the concept of functional overlap, the hexose transporter-null \( Schxt0 \) mutant cells are indeed less susceptible to the azoles tested (Fig. 3). Although this finding needs to be further assessed in *C. glabrata*, this result strongly supports the hypothesis that several of the *HXT* transporters might play a key role as azole importers, therefore contributing to azole susceptibility in fungal cells. Hexose transporter-null \( Schxt0 \) cells are indeed less susceptible to the azoles tested (Fig. 3). Although this finding needs to be further assessed in *C. glabrata*, this result strongly supports the hypothesis that several of the *HXT* transporters might play a key role as azole importers, therefore contributing to azole susceptibility in fungal cells. Accordingly, the accumulation of \(^{3}\text{H}\)-labelled fluconazole was evaluated in the single deletion strain \( \Delta cghxt4/6/7 \) comparing to the wild-type. Under these conditions, cells lacking \( CgHXT4/6/7 \) accumulate significantly less fluconazole than the parental wild-type cells (Fig. 2b). Moreover, the same effect was observed when measuring the accumulation of the \(^{3}\text{H}\)-labelled imidazole clotrimazole in cells lacking \( CgHXT4/6/7 \), comparing to wild-type cells (Supplementary Fig. 3), further supporting the hypothesis that \( CgHxt4/6/7 \) affects azole uptake in *C. glabrata*.

Deletion of *CgHXT4/6/7* in the 040 susceptible clinical isolate promotes azole resistance. The relevance of *CgHXT4/6/7* in the azole resistance phenotype developed by the 040 clinical isolate when exposed to posaconazole was further evaluated. Susceptibility testing demonstrates that the deletion of *CgHXT4/6/7* in the 040 isolate decreases its susceptibility towards posaconazole and fluconazole (Fig. 4a). MIC \(_{50}\) values of fluconazole and posaconazole were found to be lower for the azole susceptible

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**Fig. 2** *CgHxt4/6/7 and CgHxt6/7 promote azole susceptibility in *C. glabrata*. a) Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the *C. glabrata* KEU100 wild-type strain or derived KUE100_\(\Delta cghxt4/6/7\), KUE100_\(\Delta cghxt6/7\) or KUE100_\(\Delta cghxt4/6/7\_\Delta cghxt6/7\) mutant cells on YPD agar plates by spot assays. The inoculum was prepared as described in the Methods section. Cell suspensions used to prepare the spots correspond to 1:5 (b) and 1:25 (c) dilutions of the cell suspensions used in (a). The displayed images are representative of at least three independent experiments. b) Time-course accumulation ratio of \(^{3}\text{H}\)-fluconazole in non-adapted KUE100 wild-type cells (black circles) or derived KUE100_\(\Delta cghxt4/6/7\) mutant cells (grey squares) during cultivation in liquid YPD medium in the presence of unlabelled fluconazole. The accumulation ratio values are averages of, at least, \( n = 3 \) independent experiments. Error bars represent the corresponding standard deviations. Significance levels are attributed as follows: \(* p\) value < 0.05; \(** p\) value < 0.01; \(*** p\) value < 0.001.
isolate 040 (16 and 0.125 mg L\(^{-1}\), respectively) when compared to the derived mutant strain \(040_{\text{Δcghxt4/6/7}}\) (32 and 2 mg L\(^{-1}\), respectively), while for the evolved \(040_{\text{Psc}}\) resistant strain the values were even higher (\(≥\)64 and \(≥\)8 mg L\(^{-1}\), respectively). Accordingly, the accumulation of \(^{3}H\)-labelled fluconazole was shown to be significantly lower in the \(040_{\text{Δcghxt4/6/7}}\) strain when comparing to the susceptible parent isolate 040 (Fig. 4b). Although it is noteworthy that the overall accumulation of \(^{3}H\)-labelled fluconazole appears to be higher in the 040 clinical isolate, when compared to the KUE100 lab strain, likely due to extensive genomic background differences, the impact of \(CgHXT4/6/7\) expression in azole accumulation is clearly strain-independent. Additionally, the G361A missense mutation found in the \(CgHXT4/6/7\) gene from the evolved 040\(_{\text{Psc}}\) strain, e.g. displaying lower MIC\(_{50}\) values for fluconazole (MIC\(_{50}=32\) mg L\(^{-1}\)) and posaconazole (MIC\(_{50}=1\) mg L\(^{-1}\)), while its complementation with the pGREG576_PDC1\(_{\text{mut}}\) plasmid in the single deletion mutant strain \(\Delta cghxt4/6/7\) did not affect the MIC\(_{50}\) values for fluconazole (MIC\(_{50}=32\) mg L\(^{-1}\)) and posaconazole (MIC\(_{50}=1\) mg L\(^{-1}\)), its complementation with the pGREG576_PDC1\(_{\text{mut}}\) plasmid, expressing the wild-type \(CgHXT4/6/7\) transporter, resulted in promoted susceptibility of the \(\Delta cghxt4/6/7\) strain, e.g. displaying lower MIC\(_{50}\) values for fluconazole (MIC\(_{50}=16\) mg L\(^{-1}\)) and posaconazole (MIC\(_{50}=0.5\) mg L\(^{-1}\)) (Fig. 4c).

Altogether, these results strongly suggest that \(CgHXT4/6/7\) plays a role in \(C. glabrata\) azole drug susceptibility and that the nsSNP selected in \(CgHXT4/6/7\) of the 040 isolate during the in vitro evolution experiment impaired of the transporter function, contributing to the final resistant phenotype observed in the evolved strain \(040_{\text{Psc}}\).

Deletion of either \(CgHXT4/6/7\) or \(CgHXT6/7\) rescues the susceptibility phenotype imposed by the absence of the drug resistance regulator \(CgPDR1\). \(CgPdr1\) is the major regulator of azole resistance in \(C. glabrata\), controlling the overexpression of azole drug efflux pumps. Consequently, cells lacking the \(CgPDR1\) gene are highly susceptible to azoles. Surprisingly, the deletion of either \(CgHXT4/6/7\) or \(CgHXT6/7\) was found to rescue the azole susceptibility phenotype of \(CgPDR1\) disrupted cells (Fig. 5a).

Strikingly, MIC\(_{50}\) values for fluconazole and posaconazole were found to be similar for the wild-type strain (16 and 0.5 mg L\(^{-1}\), respectively) and for the double deletion strains \(\Delta cgpdr1\Delta cghxt4/6/7\) or \(\Delta cgpdr1\Delta cghxt6/7\) (16 and 0.5 mg L\(^{-1}\), respectively), while for the \(\Delta cgpdr1\) strain the MIC\(_{50}\) values were found to be 4-fold lower for fluconazole (4 mg L\(^{-1}\)) and 32-fold lower for posaconazole (0.015625 mg L\(^{-1}\)). Accordingly, growth curves obtained in the presence of inhibitory concentrations of posaconazole (4 mg L\(^{-1}\)) demonstrate that the deletion of either \(CgHXT4/6/7\) or \(CgHXT6/7\) in the \(\Delta cgpdr1\) strain fully rescues its azole susceptibility phenotype (Fig. 5b).

These results further support the hypothesis that these putative hexose transporters play a crucial role in \(C. glabrata\) azole drug susceptibility, probably by facilitating azole entrance into fungal cells. More importantly, this points out the critical relevance of these transporters in azole drug mode of action and in the consequent mechanism of drug resistance.

\(CgHxt4/6/7\) is predicted to display high affinity for fluconazole binding. To strengthen our current hypothesis that \(CgHxt4/6/7\) may act as an azole importer, the affinity of this hexose transporter to azoles was evaluated in silico. Since there is no available structure for \(CgHxt4/6/7\), the \(CgHxt4/6/7\) structure was modelled based on the 3D structure of the homologous \(E. coli\) transporter XylE (PDB ID 4GBZ) (Supplementary Fig. 4a). This was possible due to the sequence identity between both proteins, which is of 30% for all residues and 50% for the residues exposed to the transporting channel (Supplementary Fig. 4c). The crystal structure of \(E. coli\) XylE was solved with a resolution of 2.89 Å and features a D-glucose bound to its internal cavity (Supplementary Fig. 4b), providing information about the positioning of the ligands to be transported. Most of the structural differences between our model and the \(E. coli\) XylE crystal structure are found in the loops that connect the transmembrane helices (Fig. 6a). These loops are unstructured and exposed to the solvent regions that are far from the substrate binding regions, therefore not accountable for the quality of the model at the transmembrane region (Fig. 6a). As observed for the \(E. coli\) XylE (PDB ID 4GBZ) protein, our model is in an outward-facing, partially occluded conformation with a D-glucose molecule captured in the transmembrane channel. The ligand is trapped within the centre of the transmembrane domain, completely occluded from the intracellular side, yet solvent-accessible from the extracellular side through a channel that is too narrow (5.2 Å diameter) to allow the escape of the ligand[3].

Using the modelled structure of \(CgHxt4/6/7\), its affinity to fluconazole was predicted through in silico docking, using AutoDock Vina, and compared to that of glucose, the natural substrate of this transporter. This study revealed that \(CgHxt4/6/7\) is predicted to have strong affinity to glucose (−5.5 kcal/mol), as expected, and an even stronger predicted affinity to fluconazole (−7.3 kcal/mol) (Fig. 6b, c). Similar binding affinities to glucose and fluconazole were observed for the \(E. coli\) XylE transporter (Supplementary Fig. 5a,b). Although occupying the same region in the internal cavity, fluconazole and D-glucose revealed distinct patterns of interactions with the protein in our docking studies. D-glucose participates in more hydrogen bonds than fluconazole, whereas desolvation effects seem to play a major role in the binding affinity of the latter. Indeed, fluconazole shows a higher shape complementarity to the binding pocket compared to D-glucose (Fig. 6b, c). Taken together, our results suggest that fluconazole is predicted to be a binder of \(CgHxt4/6/7\). Despite these results, posaconazole docking failed to bind to the transporter, what would be expected given the size difference between the fluconazole and posaconazole. However, it is important to consider that this modelling was made from a partially occluded conformation, and, as such we can hypothesize that posaconazole may be accommodated and transported in an open conformation. In order to test this possibility, we decided to make a second modelling from the structure of \(Homo sapiens\) XylE transporter.
glucose transporter (PDB ID 4ZWC)\textsuperscript{32} that was obtained in an outward-open conformation (Supplementary Fig. 6). Indeed, docking studies showed that the open configuration allows binding of both posaconazole and fluconazole. Interestingly, both drugs bind in the same region in the outward-open structure (Supplementary Fig. 7). It is important to mention that posaconazole is a large molecule with a higher number of possible rotations, which makes it difficult to predict the precise mode of binding, beyond the base structure common to fluconazole, through molecular docking.

### Discussion

In this study, two hexose transporters in \textit{C. glabrata} were identified and characterized. More importantly, a possible role for hexose transporters as drug carriers is proposed in \textit{C. glabrata}

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**Fig. 4 CgHxt4/6/7 promotes azole susceptibility in \textit{C. glabrata} 040 clinical isolate by mediating azoles uptake.**

**a** Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the \textit{C. glabrata} 040 clinical isolate, the derived mutant strain 040\_Δcghxt4/6/7 and the evolved resistant strain 040\_Psc on YPD agar plates by spot assays. The inoculum was prepared as described in the Methods section. Cell suspensions used to prepare the spots correspond to 1:5 (b) and 1:25 (c) dilutions of the cell suspensions used in (a). The displayed images are representative of at least three independent experiments.

**b** Time-course accumulation ratio of \[^{3}H\]-fluconazole in non-adapted (black circles) 040 or derived (grey squares) 040\_Δcghxt4/6/7 mutant cells during cultivation in liquid YPD medium in the presence of unlabelled fluconazole. The accumulation ratio values are averages of, at least, \(n = 3\) independent experiments. Error bars represent the corresponding standard deviations. Significance levels are attributed as follows: *\(p\) value < 0.05; **\(p\) value < 0.01.

**c** Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the \textit{C. glabrata} KUE100::URA\_Δcghxt4/6/7 mutant strain harbouring the pGREG576_PDC1\_CgHXT4/6/7 or pGREG576_PDC1\_mut\_CgHXT6/7 on BM agar plates (without uracil, for plasmid maintenance) by spot assays. The inocula were prepared as described in the Methods section. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspensions used in (a). The displayed images are representative of at least three independent experiments.
shedding light into an important feature ofazole drug mode of action: its uptake into fungal cells, possibly through hexose transporters. An in vitro directed evolution experiment was used to drive susceptible clinical isolates towards multi azole resistance, leading to the functional characterization of the CgHxt4/6/7 hexose transporter as a determinant of azole susceptibility in C. glabrata. The in vitro evolved resistant isolates 040_Psc, 044_Psc and OL152_Psc acquired multi azole resistance through previously unknown mechanisms. No CgPDR1 GOF mutations were found in these evolved strains, the main mechanism of acquired azole resistance in C. glabrata clinical isolates4, 20. Moreover, according to the general notion that alterations on the drug target are not a frequent mechanism of acquiredazole resistance in C. glabrata clinical isolates5, 9, 21, we confirmed that no mutations arose in the CgERG11 gene during posaconazole exposure. Although there were no common nsSNPs among the three evolved resistant strains, CgHXT4/6/7 gene was found to be mutated in all three evolved resistant strains (Supplementary Fig. 2b). Our results show that, although CgPDR1 GOF mutations are a frequent event leading to acquisition of azole resistance, they are not the only mechanism. Whether the posaconazole-driven evolution, when compared to evolution driven by otherazole drugs, or the genetic background of the used strains may underlie the results obtained in this study remains unclear and is certainly worthy of further scrutiny.

CgHxt4/6/7 was, so far, an uncharacterized putative hexose transporter in C. glabrata. Based on knowledge gathered for S. cerevisiae homologous transporters, CgHxt4/6/7 belongs to the big family of HXT genes, specifically to the high-affinity glucose transporter cluster33. With 96% amino acid sequence identity to CgHxt4/6/7, CgHxt6/7 was also characterized. According to their predicted function, CgHxt4/6/7 and CgHxt6/7 were found to be localized to the cell plasma membrane (Fig. 1a), and to be able to import hexoses (Fig. 1b).

Several studies have covered a panoply of drug resistance mechanisms that depend on drug efflux pumps belonging to the ABC and MFS. Nonetheless, despite some efforts25, 34, the study of drug uptake mechanisms has been, to some extent, overlooked in pathogenic fungi24. Besides being an important mechanism of drug effectiveness acknowledged in human parasites24, decreased accumulation of drugs has been associated with azole resistance in Candida clinical isolates25. Nonetheless, the players underlying drug uptake have remained elusive for at least 20 years. HXT genes belong to the MFS class of membrane proteins and were associated with the multidrug resistance phenomenon in S. cerevisiae in 1997, being implicated in cycloheximide, sulfomethuron methyl and 4-NQO (4-nitroquinoline-N-oxide) susceptibility35. In fact, CgHxt4/6/7 and CgHxt6/7 S. cerevisiae orthologues (ScHx6 and ScHx7, respectively) were previously shown to be major transporters of arsenic import in yeast35. Our study shows that at least some of S. cerevisiae HXT transporters also play a relevant role in yeast azole susceptibility, probably by being hijacked by these antifungals serving as channels for drug entry into fungal cells (Fig. 3). Consistently, CgHxt4/6/7 or CgHxt6/7 expression was found to confer azole susceptibility to C. glabrata cells (Fig. 2a).

The accumulation of radiolabelled fluconazole was shown to be higher in wild-type C. glabrata cells when compared to cells devoid of CgHxt4/6/7 (Fig. 2b), suggesting that fluconazole can enter C. glabrata cells through facilitated diffusion, mediated by this transporter. Furthermore, in silico molecular docking results

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Fig. 5 Deletion of either CgHXT4/6/7 or CgHXT6/7 in a Δcgpdr1 background rescues the azole susceptible phenotype imposed by the absence of CgPdr1. a Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the KUE100 C. glabrata wild-type strain and the derived mutant strains KUE100_Δcgpdr1, KUE100_Δcgpdr1Δcghxt4/6/7, and KUE100_Δcgpdr1Δcghxt6/7 on YPD agar plates by spot assays. The inoculum was prepared as described in the “Methods” section. Cell suspensions used to prepare the spots correspond to 1:5 (b) and 1:25 (c) dilutions of the cell suspensions used in (a). The displayed images are representative of, at least, n = 3 independent experiments. b Comparison of the growth curves of the C. glabrata wild-type strain KUE100 and the derived mutant strains KUE100_Δcgpdr1, KUE100_Δcgpdr1Δcghxt4/6/7, and KUE100_Δcgpdr1Δcghxt6/7, in liquid YPD, in the absence or presence of 4 mg/L posaconazole, measured in terms of variation in OD600. The displayed growth curves are representative, at least, n = 3 independent experiments. Error bars represent the corresponding standard deviation. Significance levels are attributed as follows: ****p value <0.0001.
Drug resistance involves CgPdr13. Once this transcription factor but also in the 040 clinical isolate (Fig. 4a, b), suggesting that the similar point mutations may be used as clinically relevant transporter inactive in terms of azole susceptibility, suggesting that the Val121Ile mutation that arose in the 040 clinical isolate upon role of this transporter is not strain dependent. Additionally, the phenotypes were observed in the leading to the disappearance of other subpopulations and to the corrosion this hypothesis, as they predict that CgHxt4/6/7 has affinity to its natural substrate, glucose, but also affinity to fluconazole and posaconazole. Interestingly, the CgHxt4/6/7 related phenotypes were observed in the C. glabrata lab strain KUE100, but also in the 040 clinical isolate (Fig. 4a, b), suggesting that the role of this transporter is not strain dependent. Additionally, the Val121Ile mutation that arose in the 040 clinical isolate upon prolonged exposure to posaconazole was found to render this transporter inactive in terms of azole susceptibility, suggesting that similar point mutations may be used as clinically relevant mechanisms of evolution towardsazole resistance (Fig. 4c).

In wild-type C. glabrata cells, the prime mechanism of azole drug resistance involves CgPdr13. Once this transcription factor acquires GOF mutations, induced by drug exposure, natural selection favours cells harbouring these mutations, ultimately leading to the disappearance of other subpopulations and to the development of fully azole-resistant cells20, 28. Therefore, CgPDR1 disruption in C. glabrata cells restores azole susceptibility8. Since there were no GOF mutations in CgPDR1 in the evoluted resistant strains 040_Psc, 044_Psc and OL152_Psc, we assessed the impact of the proposed resistance mechanism, involving the reduced uptake of fluconazole, in a strain devoid of CgPDR1. While in wild-type cells the single deletion of CgHxt4/6/7 or CgHxt6/7 results only in a moderate increase in azole resistance, in cells devoid of CgPdr1 the single deletion of either HXT gene fully reverses the strong azole susceptibility phenotype imposed by the absence of CgPdr1 (Fig. 5a, b). This further supports the concept that azoles may enter C. glabrata cells through Hxt proteins and that the impairment of this pathway constitutes an important mechanism of resistance, that, when triggered, renders alternative azole resistance pathways, based on drug extrusion, almost irrelevant.

Overall, we show that hexose transporters affect azole accumulation in C. glabrata and play a role in the acquisition of resistance by clinical isolates as an alternative to the main mechanism of acquired azole resistance involving CgPDR1. Despite the extensive knowledge on the mechanisms of action/ resistance to azoles, little was known about how these drugs enter fungal cells to exert their antifungal action. Although indirect effects of HXT transporters in azole susceptibility, eventually affecting energy availability or the expression of drug efflux pumps, cannot be fully discarded, our results show that CgHxt4/6/7 and CgHxt6/7 transporters not only play a role in the uptake ofglucose, but also appear to mediate azole uptake into C. glabrata cells. Being two in a family of 11 HXT transporters in C. glabrata, it is likely that their role inazole uptake is just the tip of the iceberg. It will be important to assess how far other homologous hexose transporters may contribute to azole uptake and whether mutations acquired in additional hexose transporter encoding genes may contribute to azole resistance in fungal pathogens, in general.

Methods

Strains and growth media. The reference strain CBS138 and three Candida glabrata clinical isolates (named OL152, 040 and 044) obtained from patients admitted to Centro Hospitalar de S. Joao (CHSJ) in Porto, Portugal were used in this study27. Isolates 040 and 044 were recovered from blood cultures, while isolate OL152 was recovered from urine. These isolates were characterized by Vitek YBC identification cards (BioMérieux, Paris, France) and mass-spectrometry (Maldi TOF). Until testing, the strains were stored in Brain–Heart broth (Difco) with 5% glycerol at −70 °C. For each experiment, the strains were subcultured twice on Sabouraud agar (Difco) at 35 °C for 48 h. C. glabrata parental strain KUE10027 and derived KUE100_Dcgdr138 were kindly provided by Hiroji Chibana, Medical Mycology Research Center at Chiba University, Japan. KUE100_Dcgdr1Δcghxt4/6/7, KUE100_Dcgdr1Δcghxt6/7, KUE100_Dcgdr1Δcghxt4/6/7Δcghxt6/7, KUE100_Dcgdr1Δcghxt4/6/7Δcghxt6/7Δcghxt4/6/7 and KUE100 ΔURA-, were all constructed in this study. Cells were batch-cultured at 30 °C, with orbital agitation (250 rpm) in Basal Medium (BM) or Yeast Extract-Peptone-Dextrose (YPD) medium. BM has the following composition (per litre): 1.7 g yeast nitrogen base without amino acids or NH4+ (Difco), 20 g glucose (Merck) and 2.65 g (NH4)2SO4 (Merck). YPD has the following composition (per liter): 20 g glucose, 20 g Peptone (Merck) and 10 g Dextrose (YPD) medium. BM has the following composition (per litre): 1.7 g yeast nitrogen base without amino acids or NH4+ (Difco), 20 g glucose (Merck) and 2.65 g (NH4)2SO4 (Merck). YPD has the following composition (per litre): 20 g glucose (Merck), 20 g Peptone (Merck) and 10 g Yeast extract (Merck).

S. cerevisiae parental strain BY4741 (MATa, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0) was obtained from the Euroscarf collection. Wild-type yeast cells harbouring pGREG576 derived plasmids were batch-cultured at 30 °C, with orbital agitation (250 rpm) in BM supplemented with 20 mg L−1 histidine, 20 mg L−1 methionine, 20 mg L−1 tryptophan and 60 mg L−1 leucine (all from Sigma). 

S. cerevisiae parental strain CEN.PK2-1C39 and derived hexose transporter-null (Schizosaccharomyces pombe) strain EBY.VW400029 were kindly provided by Eckhard Boles, Institute of Molecular Biological Sciences, Goethe University, Frankfurt, Germany. Cells were batch-cultured at 30 °C, with orbital agitation (250 rpm) in Yeast Extract-Peptone-Maltose (YPM) medium containing (per liter): 20 g maltose (Merck), 20 g Peptone (Merck) and 10 g Yeast extract (Merck). CEN.PK2-1C and EBY.VW4000 cells harbouring pGREG576 derived plasmids were grown overnight in BM-Maltose medium containing (per liter): 1.7 g yeast nitrogen base without amino acids or NH4+(Difco), 20 g maltose (Merck) and 2.65 g (NH4)2SO4 (Merck), supplemented

Fig. 6 Fluconazole displays a strong predicted affinity for the glucose binding site of CgHxt4/6/7. a Overlap of the best DOPE score model and the template E. coli XylE (PDB ID 4GBZ) (pink). Loop regions that differ from the template are represented in green. b Docking of fluconazole on the modelled CgHxt4/6/7 structure, best pose. c Docking of D-glucose on the modelled CgHxt4/6/7 structure, best pose. Figures were prepared using PyMOL2.5.
with 20 mg L\(^{-1}\) tryptophan, 20 mg L\(^{-1}\) histidine and 60 mg L\(^{-1}\) leucine (all from Sigma).

Solid media contained, besides the above indicated ingredients, 20 g L\(^{-1}\) agar (Iberagel).

**Gene expression analysis.** The transcript level of the CgERG11 gene in the initial azole susceptible clinical isolates was compared to the evolved resistant strains using quantitative real-time PCR (qRT-PCR). Total-RNA samples were obtained from cell suspensions harvested upon reaching an OD\(_{600nm}\) = 0.8 ± 0.08 in rich YPD medium. cDNA for real-time reverse transcription-PCR was synthesized from total-RNA samples by using the MultiScribe\textsuperscript{TM} reverse transcriptase kit (Applied Biosystems) and the 7500 RT-PCR thermal cycle block (Applied Biosystems) according to the manufacturer's instructions. The quantity of cDNA for subsequent reactions was kept at ca. 10 ng. The subsequent RT-PCR step was carried out using SYBR green reagents. Primers for the amplification of the CgERG11 and CgACT1 genes were designed using Primer Express software (Applied Biosystems) and are summarized in Supplementary Table 3. The RT-PCR was carried out using a thermal cycle block (7500 real-time PCR system, Applied Biosystems). Default parameters established by the manufacturer were used, and fluorescence was detected by the machine and recorded in an amplification plot (7700 SDS software, Applied Biosystems). The CgACT1 mRNA level was used as an internal control. The relative values obtained for the initial isolates expressing the lower gene expression level were set as 1 and the remaining values are presented relative to that control. To avoid false-positive signals, the absence of nonspecific amplification by the chosen primers was confirmed by the generation of a dissociation curve for each pair of primers.

**Plasmids.** For CgHXT4/6/7 or CgHXT6/7 gene cloning, the plasmid pREG576 was obtained from the Drag\&Drop collection\(^40\). For CgHXT4/6/7 or CgHXT6/7 gene disruption, the plasmid pYCA4 (Addgene plasmid \#63903) was used; for expression of the flipase enzyme, the plasmid pLS10 was used. For CgURA3 gene disruption, the plasmid pV1382\(^2\) was obtained from Addgene (Addgene plasmid \#111436).

**Disruption of the CgHXT4/6/7 (ORF CAGLA0A2223g) or CgHXT6/7 (ORF CAGLA0A2221g) genes.** The wild-type strain KUE100, derived KUE100_Accgd1 mu1, and 040 clinical isolate were transformed by electroporation with the deletion cassette (a nourseothricin cassette flanked by FRT sites and a -500 bp region flanking the targeted gene). The deletion cassette was constructed by Gibson Assembly using the pYCA4 plasmid, previously cut with Xhol and NotI, -300 bp of the CgHXT6/7 promoter region and -500 bp of the CgHXT6/7 terminator region, or -500 bp of the CgHXT6/7 promoter region and -500 bp of the CgHXT6/7 terminator region. Cells were plated on YPD agar medium supplemented with 200 mg L\(^{-1}\) nourseothricin. Transformants were checked for insertion of the deletion cassette by PCR using the control primers. Correct strains were subsequently transformed with plasmid pLS10 to induce expression of the flipase enzyme (300 mg L\(^{-1}\) hygromycin selection). Removal of the nourseothricin cassettes of the transformants was checked by PCR. The plLS10 plasmid was lost by growth on non-selective YPD medium and checked by replicating on YPD supplemented with 300 mg L\(^{-1}\) hygromycin. All the primers used for the construction of the mutant strains are depicted in Supplementary Table 3.

**Cloning of the CgHXT4/6/7 (ORF CAGLA0A2223g) or CgHXT6/7 (ORF CAGLA0A2221g) genes.** The pREG576 plasmid from the Drag\&Drop collection was used to clone and express CgHXT4/6/7 or CgHXT6/7 genes, as described before\(^14,17,19\). pREG576 contains a galactose inducible promoter (GAL1), the yeast selectable marker URA3 and the GFP gene, encoding a Green Fluorescent Protein (GFP\(_{65S6T}\)), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. CgHXT4/6/7 and CgHXT6/7 DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 C. glabrata strain. The designed primers contain, besides a region with homology to the 5' and 3' UTRs of the probe, a 5'-GGATCC-3' and 5'−GGATCC−3' nucleotide sequences of the CgERG11 gene from C. glabrata strain. The designed primers are depicted in Supplementary Table 3.

**Site-directed mutagenesis.** The CgHXT4/6/7 gene sequence in the recombinant pREG576_PDC1_CgHXT4/6/7 plasmid was mutated by site-directed mutagenesis. The designed primers contain the mutation occurred in the 040_Psc resistant isolate during posaconazole exposure, resulting in the production of the mutated sequence by PCR amplification to obtain the pREG576_PDC1_Psc_CgHXT4/6/7 plasmid. The original template was then degraded by DpnI digestion. All primers used are present in Supplementary Table 3.

**Disruption of the CgURA3 (ORF CAGL0I03080g) gene.** The disruption of the C. glabrata URA3 gene, encoded by ORF CAGL0I03080g, was carried out in the KUE100 parental strain as described previously\(^41\), using the CIRSPR-Cas9 system from Vyas et al.\(^41\). Briefly, a CgURA3 gRNA sequence selected from the resources made available by Vyas et al.\(^41\) was cloned in the pV1382 plasmid, previously linearized with the restriction enzyme BamBI. The CgURA3 gRNA was obtained by oligonucleotide annealing and the product ligated into the previously linearized pV1382 plasmid to obtain the pV1382_CgURA3 vector. The construct was verified by DNA sequencing. The plasmid was transformed into C. glabrata cells which were then directly plated on 5-Fluoroorotic acid (5-FOA) to select URA- cells. Sequential passages in nonselective medium (YPD) were performed to avoid detrimental effects of further Cas9 expression and CgURA3 loss of function was further confirmed by the inability to grow in medium without uracil. The introduction of pREG576 derived plasmids in the edited strains was able to rescue the growth impairment in the absence of uracil. Sequencing of the CgURA3 gene from the selected candidates revealed the existence of frameshifts within the ORF, thus resulting in premature stop codons as it is expected from Non-Homologous End Joining (NHEJ) correction of double-strand breaks. All primers used are present in Supplementary Table 3.

**In vitro induction of antifungal resistance in C. glabrata clinical isolates and CBS138 strain and assessment of its stability.** Standard powders of fluconazole (Pfizer, Groton, CT), voriconazole (Pfizer, New York, NY), posaconazole (Scher−Plough, Kenilworth, NJ) and clotrimazole (Sigma, St. Louis, MO) were obtained from the respective manufacturers. A stock solution of fluconazole was prepared in distilled water, while voriconazole, posaconazole and clotrimazole were prepared with dimethyl sulfoxide (DMSO) and stored at -20 °C. L-789,150 (Merck, Germany). Antifungal treatment samples were diluted afterwards with Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholine propenalsulfonic acid buffer (MOPS; Sigma) and stored at ~70 °C until further use. The culture medium used for resistance induction assays was RPMI 1640 with 0.165 M MOPS, pH 7.0. A single, randomly selected, colony from each C. glabrata strain (040, 044, OL152 and CBS138) was incubated in 10 mL of RPMI 1640 overnight in a rotating drum at 150 rpm and 35 °C. An aliquot of this culture, containing 10\(^{6}\) yeast cells, was transferred to different vials, each containing 10 mL of culture medium with or without posaconazole and incubated overnight as described above. The following day, aliquots from each culture containing 10\(^{8}\) yeast cells were again transferred into fresh medium containing the same antifungal and re-incubated as described. Each day, for the 30 days of the assay, a 1 mL aliquot from each sub-culture was mixed with 0.5 mL of 50% glycerol and frozen at ~70 °C for later testing. Induction took place for 30 days with constant concentrations of posaconazole (1 mg L\(^{-1}\)). The draft genome sequences of the three C. glabrata clinical isolates used in this study, 040, 044 and OL152, had been characterized previously, as described in Pais et al.\(^46\). The genome sequences of the selected in vitro evolved resistant strains 040_Psc, 044_Psc and OL152_Psc were obtained from a CgHXT4/6/7 deletion clinical isolates, respectively, were obtained and characterized in this study. Raw sequencing data and genome assemblies can be found accessing Bioproject No. PRJNA325402 (isolates) and BioProject no. PRJNA694431 (evolved isolates).

**Genome sequencing and data analysis.** The draft genome sequences of the three azole susceptible C. glabrata clinical isolates used in this study, 040, 044 and OL152, had been characterized previously, as described in Pais et al.\(^46\). The genome sequences of the selected in vitro evolved resistant strains 040_Psc, 044_Psc and OL152_Psc were obtained from a CgHXT4/6/7 deletion clinical isolates, respectively, were obtained and characterized in this study. Raw sequencing data and genome assemblies can be found accessing Bioproject No. PRJNA325402 (isolates) and BioProject no. PRJNA694431 (evolved isolates).
reads. Reads were mapped against the C. glabrata reference genome yielding >97% of aligned reads for each isolate. Genomes were assembled using SPAdes20 and scaffolds <500 bp discarded to attain final draft genomes. Each assembly was aligned against the reference genome and found to align >97.6% in each case. The high sequencing depth was leveraged to perform Single Nucleotide Polymorphism (SNP) identification with increased sensitivity and sensibility across the genome. Briefly, reads were aligned against the reference genome and variant identification was performed using GATK46. Low-quality variants were subsequently filtered out with BCTools43 as described previously. For subsequent analysis, only variants occurring within gene coding sequences and resulting in missense or non-sense mutations in the encoded protein were considered.

Antifungal susceptibility assays

**Clinical isolates.** The Minimal Inhibitory Concentration (MIC$_\text{C. glabrata}$ of voriconazole, fluconazole, clotrimazole and posaconazole antifungal drugs was determined according to the M27-A3 protocol of the Clinical Laboratory Standards Institute44, for all the C. glabrata isolates under study. The MICs were determined after 24 h. Interpretative criteria for fluconazole were those of the CLSI document M62-1: susceptible-dose dependent (S-DD) MIC$_\text{C. glabrata}$≤52 mg L$^{-1}$ and resistance (R) MIC$_\text{C. glabrata}$>64 mg L$^{-1}$. Although susceptibility breakpoints have not yet been established for voriconazole, posaconazole or clotrimazole for C. glabrata, strains inhibited by ≤2 mg L$^{-1}$, ≤1 mg L$^{-1}$ and ≤2 mg L$^{-1}$, respectively, were considered to be susceptible. Every 5 days of incubation, with or without antifungal, MIC$_\text{C. glabrata}$ values were redetermined for the four antifungals tested. The antifungal concentrations ranged from 0.03125 to 16 mg L$^{-1}$ for posaconazole and voriconazole, and 0.125-64 mg L$^{-1}$ for fluconazole and clotrimazole. C. glabrata reference strain CBS138 was used in each testing assay, as recommended.

**Lab strains.** C. glabrata cells susceptibility to toxic concentrations of the selected azoles was evaluated by spot assays. Cell suspensions used to inoculate agar plates were prepared with mid-exponential cells grown in YPD, YPM or BM until an azoles was evaluated by spot assays. Cell suspensions used to inoculate agar plates supplemented or not with 4 mg L$^{-1}$ of 4% glucose medium at 30 °C, 250 rpm. New cultures were made in fresh YPD (all from Sigma).

The subcellular localization of the CgHxt4/6/7 or CgHxt6/7 protein was determined based on the observation of KUE100::URA- or pGREG576_TEF_ and pGREG576_PDC1_ plasmids, respectively. These cells express either the CgHxt4/6/7-GFP or the CgHxt6/7-GFP fusion protein, whose localization may be determined using fluorescence microscopy. C. glabrata or S. cerevisiae cells grown in BM without uracil, for plasmid maintenance, until mid-exponential phase, OD$_{600nm}$ = 0 ± 0.05. The distribution of fusion protein was assessed by fluorescence microscopy in a Zeiss Axiosplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelength of 395 and 509 nm, respectively. Fluorescence images were captured using a cooled Zeiss AxioCam 503 colour (Carl Zeiss Microscopy).

Hexose utilization assays

The ability of CgHxt4/6/7 and CgHxt6/7 to import glucose, mannose, fructose or galactose into fungal cells was assessed in S. cerevisiae by using the hexose transporter-null EBY.VW4000 (Scekpt) strain, which grows normally on maltose, and barely on galactose as carbon sources. EBY.VW4000 cells harboring pGREG576 Empty vector or the pGREG576_TEF_CgHxt4/6/7 or pGREG576_TEF_CgHxt6/7 plasmids were grown overnight at 30 °C, with orbital agitation (250 rpm) in BM-Maltose, without uracil. Cell suspensions used to inoculate agar plates were mid-exponential cells grown in OD$_{600nm}$ = 0.5 ± 0.05, and then diluted in sterile water to an OD$_{600nm}$ = 0.05 ± 0.005. These cell suspensions and subsequent 1:5 and 1:25 dilutions were applied as 4 µL spots onto the surface of solid BM media plates containing the indicated proportion of carbon sources, supplemented with 20 mg L$^{-1}$ tryptophan, 20 mg L$^{-1}$ histidine and 60 mg L$^{-1}$ leucine.

(3H)-fluconazole and (3H)-clotrimazole accumulation assays.

The intracellular accumulation of fluconazole or clotrimazole in the different C. glabrata strains was compared resorting to radiolabelled (3H)-fluconazole and (3H)-clotrimazole, respectively. The internal accumulation was determined by calculating the ratio between the value measured within yeast cells and in the external medium ([Intracellular]/[Extracellular]). The parental strains and the derived mutant strains were grown in YPD medium until mid-exponential phase and harvested by filtration. Cells were washed and resuspended in YPD medium, to obtain dense cell suspensions (OD$_{600nm}$ = 0.5 ± 0.1, equivalent to approximately 1.57 mg (dry weight) ml$^{-1}$)

The external accumulation of (3H)-fluconazole or (3H)-clotrimazole was estimated by radiotracer assay of 0.5 ml of the supernatant. To calculate the intracellular concentration of radio-labelled fluconazole, the internal cell volume (V) of the exponential cells, grown in the absence of drug and used for accumulation assays, was considered constant and equal to 2.5 μL mg$^{-1}$ (dry weight)$^{-1}$.

Protein modelling and molecular docking

The homology modelling of the CgHxt4/6/7 membrane protein was performed using MODELLER version 9.234. The crystal structure of E. coli XylE (PDB ID 4GBZ)31 was used as a template for the outward-facing, partially occluded conformation and the crystal structure of the H. sapiens glucos transporter (PDB ID 4ZWC)32 was used for the outward-open conformation. For each modulation, twenty independent models were generated. Subsequent analysis were carried on the model with the lowest DOPE score32.

The docking calculations were performed using AutoDock Vina32, with an exhaustiveness value of 50 and in a search box centralized at the ligand of the PDB ID 4GBZ2 and with sizes of 16, 14 and 14 Å. For each docking calculation, the docking pose with lowest energy was used in subsequent analysis. Figures of docking results were prepared using PyMOL2.57.

Statistics and reproducibility.

All experiments represent the average of three or more independent experiments. Error bars represent the standard deviation. Statistical analysis was performed using one-way analysis of variance with Tukey’s correction. Significance levels are attributed as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw sequencing data and genome assemblies can be found accessing NCBI BioProjects PRJNA525402 (initial isolates) and PRJNA694431 (evolved isolates). All other data are available from the corresponding author on reasonable request. Raw data underlying all graphs have been provided as Supplementary Data 4.

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Author contributions
M.G. conducted most of the experiments. R.V., C.S.S. and C.M.S. conducted the protein modelling and molecular docking experiments. P.P. contributed to the genome sequencing data generation and analysis. A.S.-D., I.M.M., M.C., C.C. and J.B. contributed to in vitro evolution experiments. R.V., M.C., P.P. and M.V.E. contributed to genetic manipulation and strain construction. M.C.T., A.G.R. and P.V.D. designed the experiments and M.C.T. and A.G.R. coordinated the underlying project. The manuscript was written by M.G., A.G.R. and M.C.T. with contributions from all authors. All authors have given approval to the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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