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Overcoming the heterologous bias: An *in vivo* functional analysis of multidrug efflux transporter, CgCdr1p in matched pair clinical isolates of *Candida glabrata*

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ABSTRACT

We have taken advantage of the natural milieu of matched pair of azole sensitive (AS) and azole resistant (AR) clinical isolates of *Candida glabrata* for expressing its major ABC multidrug transporter, CgCdr1p for structure and functional analysis. This was accomplished by tagging a green fluorescent protein (GFP) downstream of ORF of *CgCDR1* and integrating the resultant fusion protein at its native chromosomal locus in AS and AR backgrounds. The characterization confirmed that in comparison to AS isolate, CgCdr1p-GFP was over-expressed in AR isolates due to its hyperactive native promoter and the GFP tag did not affect its functionality in either construct. We observed that in addition to Rhodamine 6 G (R6G) and Fluconazole (FLC), a recently identified fluorescent substrate of multidrug transporters Nile Red (NR) could also be expelled by CgCdr1p. Competition assays with these substrates revealed the presence of overlapping multiple drug binding sites in CgCdr1p. Point mutations employing site directed mutagenesis confirmed that the role played by unique amino acid residues critical to ATP catalysis and localization of ABC drug transporter proteins are well conserved in *C. glabrata* as in other yeasts. This study demonstrates a first *in vivo* novel system where over-expression of GFP tagged MDR transporter protein can be driven by its own hyperactive promoter of AR isolates. Taken together, this *in vivo* system can be exploited for the structure and functional analysis of CgCdr1p and similar proteins wherein the arte-factual concerns encountered in using heterologous systems are totally excluded.

1. Introduction

Haploid *Candida glabrata* is a common cause of *Candida* infections and exhibits intrinsically low susceptibility to azoles [1,2]. Similar to *C. albicans*, one of the predominant mechanisms of azole resistance in *C. glabrata* is the increased expression of multidrug transporter genes *CgCDR1* and *CgPDH1* under the transcriptional regulation of *CgPDR1*, a single Zn(2)-Cys(6) transcription factor (TF), a homologue of *Saccharomyces cerevisiae PDR1/PDR3* [1,3]. Gain of function (GOF) mutations (W297S, F575L, P927L) in *CgPDR1* conferring hyperactivity to CgPdr1p are responsible for its constitutive high expression as well as its auto-regulation thereby resulting in increased drug resistance [3]. It is well documented that CgCdr1p and CgPdh1p of PDR family in *C. glabrata* efflux azoles and other structurally unrelated compounds, however, in comparison to other yeasts, very limited information is available about the structure and function of these efflux proteins [1,4,5].

So far, the yeast ABC transporters including that of *Candida* have been expressed in heterologous systems such as in *S. cerevisiae* wherein strong promoters of the host have been exploited to over-express the protein under consideration [6–8]. While these studies have proved to be extremely insightful yet, the arte-factual concerns pertaining to heterologous background could never be excluded from such studies. *C. glabrata* being a haploid organism provides an advantage over the diploid *C. albicans* as it can easily be used to develop a vector model system for *in vivo* studies.

In this study, we have exploited the natural milieu of matched pair azole sensitive (AS) and azole resistant (AR) isolates of *C. glabrata*. A GOF mutation (L280F) in *CgPDR1* regulator is responsible for relatively higher expression of *CgCDR1* and drug resistance in AR isolate [3]. CgCdr1p was tagged with a fluorescent protein GFP (CgCdr1p-GFP) and integrated at its native chromosomal locus in AR isolate as well as its matched pair AS isolate. Thus, expression of CgCdr1p-GFP in the AS isolates of *C. glabrata* was driven by its native promoter while in AR background over-expression was regulated by its native hyperactive promoter [3]. The results of this study demonstrate an *in vivo* novel system where structure and functional analysis of the multidrug transporter CgCdr1p can be performed in its native environment.

Abbreviations: ABC, ATP binding cassette; MF, major facilitator; AR, azole resistant; AS, azole sensitive; NR, Nile Red; GOF, gain of function.

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2. Methods

2.1. Bacterial and yeast strains

Plasmids and *C. glabrata* strains used in this study are listed in Supplementary Tables 1 and 2, respectively. Bacterial and yeast cultures were grown and maintained as described previously in Puri et al., [9].

2.2. Plasmid construction and yeast transformation

Plasmid pCPG7 containing the *GFP* reporter gene fused in frame with the last codon of *CgCDR1* open reading frame (ORF) *viz.* P_{CgCDR1}-*CgCDR1-GFP* was constructed as follows. A *CgCDR1* downstream fragment was amplified from genomic DNA of DSY565 with primers *CgCDR1*-3′UTR-F and *CgCDR1*-3′UTR-R (Supplementary Table 1), subcloned in pGEM®T-Easy vector generating p*CgCDR1*-3′UTR, digested at the introduced *PstI* and *SacII* sites, ligated with the *PstI*/*SacII*-digested plasmid vector pCPG2 to generate pCPG6. The *CgCDR1 ORF* was amplified from DSY565 with the primers *CgCDR1*-F and *CgCDR1*-R (Supplementary Table 1), digested at the introduced *KpnI* and *BamHI* sites, ligated together with *KpnI* and *BamHI*-digested plasmid vector pCPG6, resulting in pCPG7 containing *CgCDR1*-ORF in translation fusion with *GFP*, followed by *ACT1* transcription termination sequence, and the *SAT1* marker. For, in-frame translational fusion of the *CgCDR1* ORF with *GFP*, pCPG7 was generated in such a way that the *CgCDR1* stop-codon was substituted by *BamHI* site, GGA TCC, encoding Pro-Trp. All constructs were confirmed by appropriate restriction digestion analysis. The flanking *CgCDR1* sequences in the plasmid pCPG7 served for genomic integration of the P_{CgCDR1}-*CgCDR1-GFP* translational reporter fusion cassettes at the native *CgCDR1* locus and the dominant *SAT1* marker Reuss et al., [10] was used to select nourseothricin-resistant (Nou^R) transformants. *KpnI*/*SacII* digested, gel-purified, linearised DNA fragment was used for transformation by electroporation protocol described in [10,11].

2.3. Site specific mutagenesis of CgCdr1p

Site specific mutagenesis was performed by using the QuickChange Mutagenesis kit from Stratagene (La Jolla, CA) as described previously [9]. The mutations were introduced into the plasmid NR-pCPG7 according to the manufacturer's instructions and the desired nucleotide sequence alterations were confirmed by DNA sequencing of the ORF. The primers used for the purpose are listed in Supplementary Table 1. The mutated plasmid, after digesting with *Kpn1* and *SacII*, was used to transform NRY565 cells using electroporation protocol as described in [10,11]. All constructs were confirmed by appropriate restriction analysis and by sequencing.

2.4. Drug susceptibility assay

The susceptibilities of *C. glabrata* cells to different drugs were determined using filter disk assay, microtiter plate and spot assays essentially as previously described [8,9,12].

2.5. Efflux assays

Efflux of R6G and [^{3}H] FLC was determined essentially using a previously described protocol [8,9]. Accumulation of NR by *C. glabrata* cells was measured by flow cytometry with a FACsort flow cytometer (Becton–Dickson immunocytometry systems, SanJose, CA) as described by [13].

3. Results and discussion

3.1. CgCdr1p-GFP is over-expressed in AR isolates

The gene encoding fluorescent GFP was tagged in frame at the C-terminal end of *CgCDR1* and the resultant recombinant *CgCDR1-GFP* was reintroduced at its native genomic locus by homologous recombination in both AS and AR clinical isolates using *SAT1* as the selection marker as shown in Fig. 1A [10]. The strains DSY562 (AS) and DSY565 (AR) expressing GFP tagged CgCdr1p were designated as NRY562 (AS) and NRY565 (AR), respectively (Fig. 1).

Confocal images revealed that CgCdr1p-GFP was expressed and properly targeted to the plasma membrane (PM) in both AS and AR isolates (Fig. 1B). However, intensity of rim-staining due to CgCdr1p-GFP fluorescence was higher in AR isolates as compared to its AS counter-part and the fluorescence intensity was highest at 4 h of growth of AR isolates which subsequently declined with time (Fig. 1B). Northern and Western analyses also confirmed that CgCdr1p-GFP was transcribed and expressed optimally at 4 h in AR isolates (Fig. 1C). The insets in Fig. 1C reflected similar growth rates for CgCdr1p-GFP in NRY562 and NRY565 backgrounds. There was also no difference in growth rate and expression of CgCdr1p between untagged DSY562 and DSY565 and GFP tagged strains NRY562 and NRY565 (data not shown). All the subsequent functional characterisation assays were performed at 4 h of growth. It should be pointed out that the cells harvested at 4 h time point corresponded to 10^6 ml^{-1}. For functional assays, larger volumes of culture were used to obtain the desired number of cells (10^7 ml^{-1}).

3.2. CgCdr1p-GFP is fully functional like native protein

We assessed the functionality of CgCdr1p by examining drug susceptibilities, ATPase activity and energy dependent efflux of R6G and [^{3}H] FLC of cells harbouring GFP-tagged version of CgCdr1p (CgCdr1p-GFP) in AS and AR isolates. Expectedly, in comparison to AS isolates, AR cells expressing CgCdr1p-GFP showed decreased susceptibility to FLC which was comparable to native protein (Fig. 2A). Likewise, the ATPase activities and drug efflux abilities of R6G and FLC of tagged and untagged proteins were comparable (Fig. 2B–D). It was thus established that cells over-expressing CgCdr1p and its GFP tagged versions are functionally equivalent.

3.3. Nile Red (NR) is a new substrate of CgCdr1p

While FLC and R6G are established substrates of CgCdr1p, during the course of this study, we observed that lipid soluble, fluorescent dye NR which is widely used as a probe of intracellular lipids and hydrophobic domains of proteins is also a good substrate of CgCdr1p [14,15]. Recently, NR has been identified as substrate of drug efflux pumps Cdr1p, Cdr2p and Mdr1p of *C. albicans* [13]. The ability of CgCdr1p to efflux NR was tested by measuring the fluorescence of AR (NRY565) cells, relative to AS (NRY562) cells, when incubated under energized conditions [13]. As compared to AS (NRY562) cells, significantly lower levels of NR fluorescence were observed in AR cells (NRY565) (Fig. 2E and F). Our results showed that, in addition to R6G and FLC, NR is also a substrate of CgCdr1p [1].

3.4. CgCdr1p could confer resistance to variety of structurally unrelated compounds

We exploited CgCdr1p-GFP to assess its substrate promiscuity which is a predominant property of yeast MDR transporters [12]. For this, we evaluated the drug sensitivities of AR strain NRY565

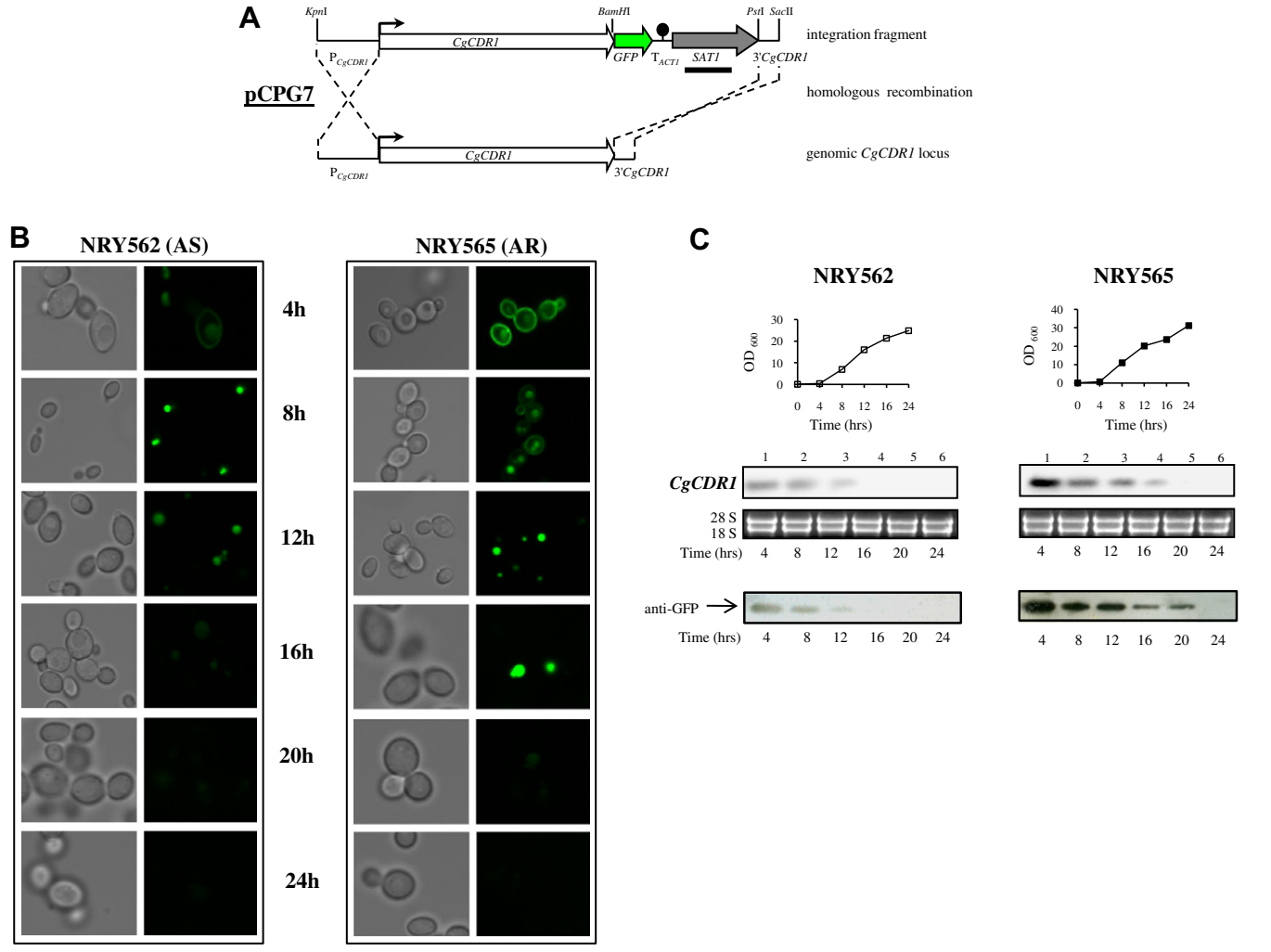

Fig. 1. Schematic depiction of *CgCDR1-GFP* reporter fusion integrants and its expression in AS and AR isolates. (A) Structure of the DNA cassette that was used to integrate the P_{CgCDR1}-*CgCDR1-GFP* translational reporter fusions at the *CgCDR1* locus of the clinical *C. glabrata* isolates. The *CgCDR1* and *GFP* coding regions are represented by white and green arrows respectively, the *SAT1* marker by grey arrow and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle. *CgCDR1* upstream and downstream regions are represented by solid lines, the *CgCDR1* promoter (P_{CgCDR1}) is symbolized by the bent arrow. The straight arrow indicates the direction of transcription. The probe used to verify the correct integration is indicated by a thick line. Only relevant restriction sites are shown. (B) Nomarski (left) and corresponding Confocal pictures (right) of the chromosomally integrated transformants harbouring the P_{CgCDR1}-*CgCDR1-GFP* reporter construct (translational fusion) depicting plasma membrane localization of chimeric CgCdr1p in clinical AS and AR isolates at indicated time point of growth. (C) Time kinetics of *CgCDR1* expression in clinical AS and AR isolates by Northern and Western blot analysis. Chromosomally integrated transformants harbouring the P_{CgCDR1}-*CgCDR1-GFP* reporter construct (translational fusion) AS and AR isolates were subjected to Northern blotting up to 24 h as shown. Equal RNA loading and transfer were monitored by staining the gel with ethidium bromide prior to blotting. The blot was hybridized with [α-^{32}P] dATP labelled *CgCDR1* specific probe. Time points in hours are indicated below the equally loaded RNA gels. The inset depicts the corresponding growth curves of clinical AS and AR isolates. Lower panel shows Western analysis depicting proper membrane localisation of CgCdr1p in the clinical isolates.PM proteins (25 μg) were used for equal loading in Western analysis [8] (For interpretation of references to color in this fifure legend, the reader is referred to web version of this article.)

against AS strain NRY562 by employing agar disc diffusion assay for 65 structurally unrelated compounds and difference in zone of inhibition between NRY562 and NRY565 was measured (Table 1 and Supplementary Table 3). To avoid any ambiguity, the compounds resulting in a difference in zone-of-inhibition of less than 0.2 cm were categorized as non-substrates while compounds having difference in zone-of-inhibition greater than 0.2 cm were grouped as substrates (Table 1 and Supplementary Table 3). With this criteria, out of 65 compounds tested, 38 compounds appeared to be as potential substrates of CgCdr1p (Table 1A), while the rest were categorised as non-substrates (Table 1B). Similar results were obtained for the corresponding untagged strains DSY562 and DSY565 (data not shown).

3.5. CgCdr1p has multiple drug binding sites

We used substrates such as R6G, NR and [^{3}H] FLC to further get an insight into the drug binding pocket of CgCdr1p. For this, we used 5× concentrations of competing compounds and tested their ability to inhibit the efflux of known substrates. Due to limited availability of compounds, we could only test 31 out of 38 possible substrates. Two non-substrate compounds of CgCdr1p (4-NQO and disulfiram) were also tested during competition assays as negative controls. Results from competition assays suggest that 10 compounds namely, chlorbromuron, itraconazole (ITR), yohimbine, ketoconazole (KTC), Miconazole (MIC), clotrimazole (CLO), DE-11, tamoxifen (TAM), quinidine, verapamil could compete for R6G's binding site(s) while compounds such as propanil, chloramphenicol, benomyl, voriconazole (VRC), tritylimidazole, KTC, MIC, TAM, gefitinib shared binding site(s) with FLC. In comparison, NR mediated efflux appeared to be relatively more specific since only five compounds such as ZW3-12, rhodamine 123, MIC, CLO, and ITR could inhibit its accumulation as shown in Fig. 3A–C. Notably, similar to Cdr1p of *C. albicans*, CgCdr1p also shares binding site of KTC, MIC and ITR with R6G [9] and also exhibits similarity in its substrate profile with CaCdr1p (Supplementary Table 4).

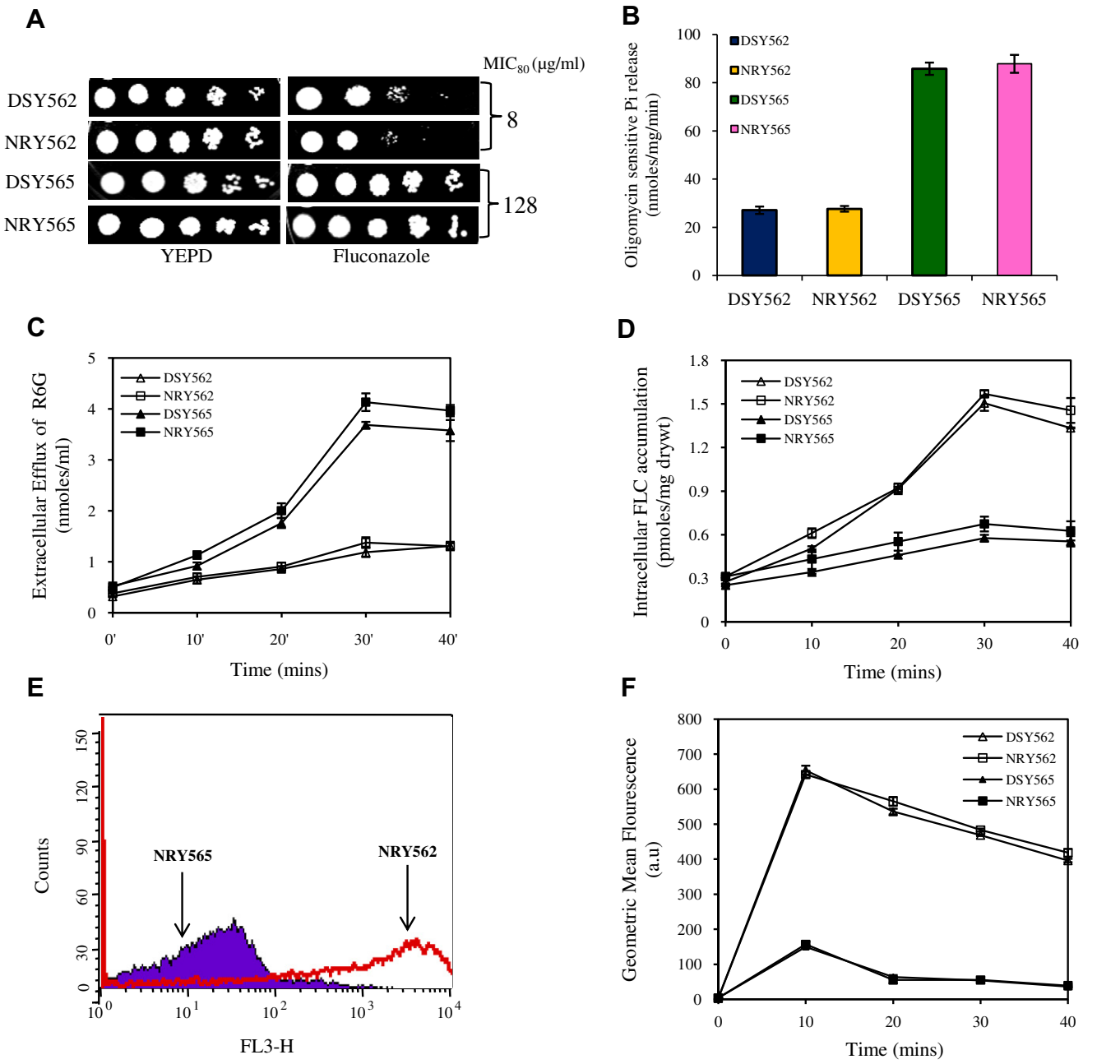

Fig. 2. Characterisation of CgCdr1p in clinical isolates of *C. glabrata*. (A) Spot test showing that *CgCDR1* (untagged, without GFP) and *CgCDR1-GFP* mediated resistance for FLC. Cells were spotted on YEPD plates in the absence of drug (control) and in presence of FLC (20 μg/ml). (B) Oligomycin-sensitive ATPase activity in the PM proteins of cells expressing CgCdr1p and CgCdr1p-GFP. The ATPase activity of CgCdr1p and CgCdr1p-GFP was determined in the PM fraction as described [8]. (C) R6G efflux in *C. glabrata* clinical isolates DSY562, DSY565, NRY562 and NRY565. (D) FLC accumulation in *C. glabrata* clinical isolates DSY562, DSY565, NRY562 and NRY565. (E) NR content of *C. glabrata* cells expressing CgCdr1p in NRY562 and NRY565 strains. (F) NR accumulation in *C. glabrata* clinical isolates DSY562, DSY565, NRY562 and NRY565. All results are means ± standard deviations for three independent experiments.

Notwithstanding the domain-based differences and low level of sequence identity outside conserved stretches, the presence of multiple drug binding sites appears to be a characteristic shared by CgCdr1p with other ABC transporters including *CaCDR1*, *ScPDR5* and human *MDR1*. The multidrug transporter *Sc*Pdr5p from *S. cerevisiae* is proposed to have at least three different substrate binding sites, each of which appears to use different chemical properties to transport compounds [16]. In the absence of availability of photoaffinity radio-labelled compounds, competition assays present an indirect method for evaluating the nature and number of drug binding sites on a protein. Our assays using NR, [^{3}H] FLC and R6G revealed presence of at least three substrate recognition sites on CgCdr1p that interact with structurally unrelated drugs (Fig. 3D). One of the site(s) is probably responsible for the translocation of R6G and the azoles like KTC, MIC and ITR while another site(s) can only interact and transport substrate such as FLC and yet another site extrudes compounds similar to NR in structure and other physicochemical parameters. Fig. 3D also suggests that compounds such as ITR, CLO, KTC, MIC and TAM appear to interact at more than one substrate binding site (s). Structure activity relationships of these substrates along with their competing compounds need to be explored with respect to this promiscuous, multidrug transporter, CgCdr1p.

Table 1
Summary of 65 compounds that have been classified as either substrates or non-substrates of CgCdr1p using filter disk assay.

Compounds	Compounds	Compounds	Compounds
(A) Compounds that are substrates of CgCdr1p			
Daunorubicin	AT-12	Cycloheximide	Gefitinib
Anisomycin	Tricyclohexyltin chloride	Crystal violet	Dexamethazone
Benomyl	Chloramphenicol	Niguldipine	Yohimbine
Clotrimazole	DE-11	Tamoxifen	Topotecan
Chlorbromuron	Diuron	Verapamil	Quinidine
Fluconazole	DM-11	ZW3	Rhodamine 6G
Itraconazole	Doxorubicin	Tritylimidazole	Rhodamine123
Ketoconazole	Terbinafine	Linuron	Propanil
Miconazole	Etoposide	Progesterone	
Voriconazole	Fluphenazine		
(B) Compounds that are non-substrates of CgCdr1p			
5-Fluorocytosine	Mycophenolic Acid	DM-13	Quercitin
8-hydroquinoline	Nefidipine	Estradiol	Rifampicin
Actinomycin D	Nimodipine	FK520	Lincomycin
4-Nitro-quinoline-oxide	Ouabain	Fumetrimorgin C	Novobiocin
Disulfiram	Paclitaxel	Hygromycin B	Mitoxantrone
CCCP	0-Phenanthroline	Cyclophophamide	Indomethacin
Colchicine	Thiolutin	Imidazole	

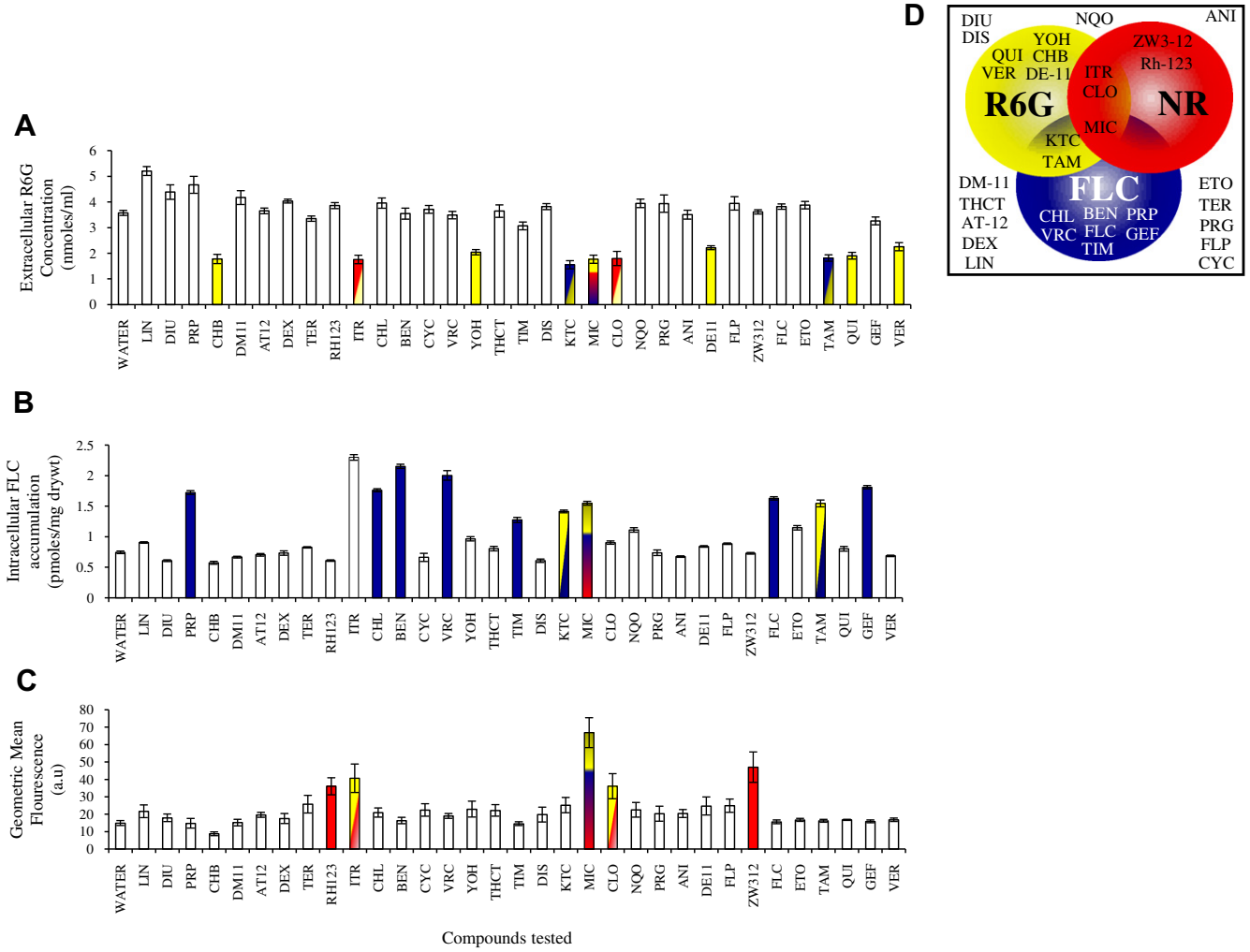

Fig. 3. Competition of various compounds with three substrates R6G, [^{3}H] FLC and NR tested in cells expressing CgCdr1p-GFP in NRY565 background. (A) For competition between R6G and various drugs, R6G was used at a final concentration of 10 μM and a fivefold concentration of each competing drug was added. (B) For competition between FLC and various drugs, FLC was used at a final concentration of 10 μM and a fivefold concentration of each competing drug was added. (C) For competition between NR and various drugs, NR was used at a final concentration of 3.5 μM and a fivefold concentration of each competing drug was added. The results are means ± standard deviations for three independent experiments. (D) Venn diagram depicting compounds competing for FLC, R6G and NR binding sites on CgCdr1p. Bars in Yellow represent compounds that are efficient competitors for R6G, bars in Red compete for NR and the bars in blue represent competitors for FLC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. The expression of mutant variants of CgCdr1p-GFP in AS and AR isolates

Taking advantage of the *in vivo* nature of our expression system, we explored if it can also be exploited to carry out structure and functional studies. We subjected CgCdr1p-GFP to site directed mutational analysis by replacing predicted critical residues with alanines. Sequence alignment of CgCdr1p with its close homologue CaCdr1p of *C. albicans* helped in identification of equivalent critical amino acid residues of *C. glabrata* protein. For example, the N-terminal NBD of CaCdr1p contains the conserved Walker A (GRPGAGCST) and B (IQCWD) motifs, and an ABC signature sequence (VSGGERKRVSIA) [17], the commonly conserved lysine residue within the Walker A motif is replaced by a cysteine [18]. This replacement appears to be a unique feature of the N-terminal NBD of all the known fungal ABC transporters. We found that CgCdr1p also has an equivalent cysteine at amino acid position 188 in Walker A of N-terminal NBD. Considering the importance of the role played by specific amino acid residues, we subjected some of the equivalent and equi-positional residues to mutational analysis which are shown to be critical for CaCdr1p function [8,9,18]. The three equi-positional selected residues were C188 (C193 in CaCdr1p), F773 (F774 in CaCdr1p) and S660 (T661 in CaCdr1p) [8,9,18]. These were either replaced with alanines (C188 and S660) or deleted (F773) for assessing the feasibility of our expression system for structure and functional analysis (Fig. 4A).

Notably, confocal analysis and immunoblot analysis showed that the expression of CgCdr1p-GFP in all the mutant variants was comparable to wild type NRY565 except in NRY565-ΔF773 which was mislocalized and thus was not detected in the PM (Fig. 4B and C). Spot assays in Fig. 4D showed significant loss of resistance to the tested drugs in cells over-expressing mutant variants of CgCdr1p-GFP (NRY565) with three point mutations namely; C188A, ΔF773 and S660A corresponding to results observed for CaCdr1p-GFP [8,9,18].

To test the possibility that the introduced mutations in CgCdr1p-GFP have, in any way, altered the catalytic cycle of CgCdr1p, which might have resulted in impaired efflux of substrates, we checked the basal ATPase activity in the cells expressing the mutant variants. ATPase activities of the mutant variants

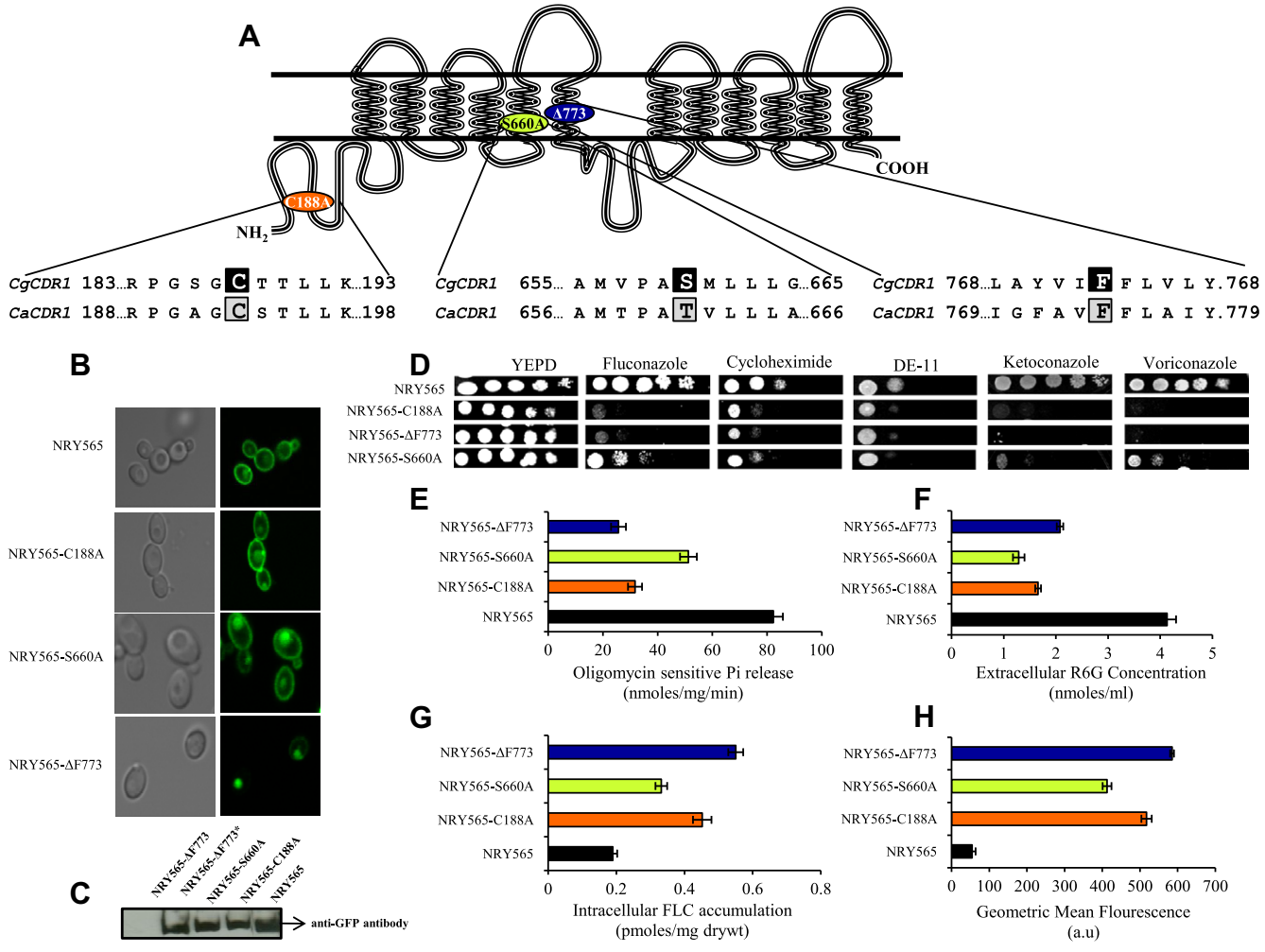

Fig. 4. Predicted topology of CgCdr1p and functional characterization of mutant variants of NRY565 (A) The highlighted amino acid residues have been mutated to alanine or have been deleted. The sequence alignment of protein Cdr1p from *C. glabrata* has been done with Cdr1p from *C. albicans*. (B) Confocal analyses of NRY565 (AR) and mutant CgCdr1p-GFP strains. (C) Western Analysis showing proper membrane localization of NRY565 and mutant CgCdr1p-GFP strains. (D) Drug resistance profiles of NRY562, NRY565 and mutant CgCdr1p-GFP strains determined by Spot assay. For spot assay following drugs were used: FLC (10 μg/ml), CYC (1 μg/ml), DE-11 (50 μg/ml), KTC (0.1 μg/ml) and VRC (4 μg/ml). Growth was not affected by the presence of the solvents used for the drugs. (E) Oligomycin-sensitive ATPase activity in the PM proteins of cells expressing CgCdr1p-GFP and mutant CgCdr1p-GFPs. The ATPase activity of CgCdr1p-GFP and mutant variants of CgCdr1p-GFP was determined in the PM fraction as described previously [8]. (F) R6G efflux in CgCdr1p-GFP and mutant CgCdr1p-GFP variant expressing cells. The assay was performed as described in [9]. (G) NR accumulation in CgCdr1p-GFP and mutant CgCdr1p-GFP variant expressing cells. The assay was performed as described in [13]. (H) FLC accumulation in CgCdr1p-GFP and mutant CgCdr1p-GFP variant expressing cells. The assay was performed as described in [9]. The results for all efflux assays are means ± standard deviations for three independent experiments.

NRY565-C188A and NRY565-ΔF773 were drastically reduced in comparison to the parent strain NRY565 while the loss in ATPase activity for the mutant variant NRY565-S660A was not drastic, yet noteworthy (Fig. 4E). Whether the enhanced drug sensitivity of the mutants was associated with reduced efflux of substrates was also assessed by measuring efflux of R6G. Notably, a 70% decrease in the extra-cellular concentration (due to low efflux) of R6G was observed in the mutant variants NRY565-C188A and NRY565-ΔF773 while a 50% reduction was seen in the mutant variant S660A-NRY565 in as compared to that in the native CgCdr1p-GFP cells, which clearly indicated impaired efflux activity by the mutant cells (Fig. 4F). Accumulation due to passive diffusion of R6G in the de-energized cells in the mutant variants was comparable to that in the native CgCdr1p-GFP (data not shown). A similar observation was made for other substrates tested namely [^{3}H] FLC and NR (Fig. 4G and H). Results of this study corroborated the previously observed role for equivalent residues of CaCdr1p-GFP, thus, validating that these conserved residues are critical for the structure and function of both ABC transporters belonging to two different yeasts.

Taken together, this study provides an *in vivo* hyper-expression system where structure and functional analysis of the multidrug transporter CgCdr1p can be performed in its native environment. We demonstrate that this system can also be used for high throughput screen for compounds that overcome fungal resistance. Considering the clinical importance of MDR in cancer patients and fungal infections, structure and function study of CgCdr1p in native background paves ways to develop similar hyper-expression system in other pathogenic and diploid organism like *C. albicans*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.123.

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