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Stress response and expression of fluconazole resistance associated genes in the pathogenic yeast *Candida glabrata* deleted in the *CgPDR16* gene

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ABSTRACT

In yeasts, the *PDR16* gene encodes a phosphatidylinositol transfer protein which belongs to the Sec14 homologue (SFH) family and localizes to lipid droplets, microsomes and at the cell periphery. The loss of its function alters the lipid droplet metabolism and plasma membrane properties, and renders yeast cells more sensitive to azole antimycotics. In this study, the entire chromosomal *CgPDR16* ORF was replaced by the *ScURA3* gene both in azole sensitive and azole resistant strains of *Candida glabrata* bearing a gain-of-function mutation in the *CgPDR1* gene, and their responses to different stresses were assessed. The *CgPDR16* deletion was found to sensitize the mutant strains to azole antifungals without changes in their osmo- and halotolerance. Fluconazole treated *pdr16Δ* mutant strains displayed a reduced expression of several genes involved in azole tolerance. The gain-of-function *CgPDR1* allele as well as the cycloheximide and hydrogen peroxide treatments of cells enhanced the expression of the *CgPDR16* gene. The results indicate that *CgPDR16* belongs to genes whose expression is induced by chemical and oxidative stresses. The loss of its function can attenuate the expression of drug efflux pump encoding genes that might also contribute to the decreased azole tolerance in *pdr16Δ* mutant cells.

1. Introduction

Candida glabrata is an important opportunistic human pathogen which follows *C. albicans* as the second or third most prevalent cause of candidemia worldwide (Pfaller 2012; Silva et al. 2012). Its clinical isolates are often resistant to azole antimycotics due to overexpression of the drug resistance transporter genes (*CgCDR1*, *CgPDH1*, *CgSNQ2*) activated by the *Cg*Pdr1p transcriptional activator (Sanglard et al. 1999; Tsai et al. 2006; Berila et al. 2009; Ferrari et al. 2009). Various approaches have been proposed to increase the susceptibility of yeast cells to fluconazole and other antifungals. Among them can be found combinations of fluconazole with different classes of agents (Liu et al. 2014) or mutations eliminating the function of specific non-essential genes (Kaur et al. 2004; Parsons et al. 2006; Thevissen et al. 2007).

The increased azole susceptibility, apparently due to an enhanced drug uptake by mutant cells, has also been observed after the loss of the *PDR16* (synonym *SFH3*) gene function in different yeast species such as *Saccharomyces cerevisiae* (van den Hazel et al. 1999; Šimová et al. 2013), *Kluyveromyces lactis* (Goffa et al. 2014), *C. albicans* (Saidane et al. 2006) and *C. glabrata* (Kaur et al. 2004; Culakova et al. 2013). In *S. cerevisiae*, *PDR16* is a member of the *PDR1* regulon (DeRisi et al. 2000) and encodes a phosphatidylinositol transfer protein (PITP). This protein belongs to the Sec14 homologue (SFH) family (Li et al. 2000) and localizes to lipid particles, microsomes and at the cell periphery (Schnabl et al. 2003). The recombinant *Sc*Pdr16p purifies as a dimer (Ren et al. 2011) and its structure differs from other monomeric Sec14 homologues (Yuan et al. 2013). Unlike other Sec14 homologues, the overexpressed *PDR16* gene does not complement the *sec14* mutation (Schnabl et al. 2003). Although most PITPs transport both phosphatidylinositol and phosphatidylcholine, *Sc*Pdr16p functions as a non-classical PITP which can bind and transfer only phosphatidylinositol (Ghosh and Bankaitis 2011). The binding of phosphatidylinositol to *Sc*Pdr16p represents an essential feature of this protein for providing yeast protection against azole antifungals

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(Holič et al. 2014). *Sc*Pdr16p can also bind sterol molecules (Holič et al. 2014). Mutants lacking *Sc*Pdr16p exhibit profound sterol handling defects (van den Hazel et al. 1999; Šimová et al. 2013) and the reduced capacity of a yeast population to evolve resistance to fluconazole (Anderson et al. 2009). Recent reports indicate that *Sc*Pdr16p is involved in a developmentally regulated program of meiotic membrane biogenesis and seems to be an inhibitor of lipid droplet utilization based on its ability to bind phosphatidylinositol and to stimulate phosphatidylinositol-4-P production (Ren et al. 2014). The *K. lactis pdr16Δ* deletion mutant displays a reduced ergosterol and phosphatidylserine content, altered plasma membrane properties, increased lithium and fluconazole uptake, and hypersensitivity to alkali metal cations (Li^+, Na^+, K^+) indicating that *Kl*Pdr16p may also be involved in the maintenance of ionic homeostasis (Goffa et al. 2014; Toth Hervay et al. 2015).

The aim of this study was to assess the role of the *CgPDR16* gene in the osmotolerance and halotolerance of *C. glabrata* as well as in the control of expression of the *CgERG11* and major multidrug resistance genes in both the laboratory strain and azole-resistant *C. glabrata* clinical isolates. The response of the *CgPDR16* expression to the absence of transcription factors involved in regulation of multidrug resistance and oxidative stress response in *C. glabrata* was also assessed.

2. Materials and methods

2.1. Strains, culture conditions, plasmids and primers

The laboratory wild type strain *C. glabrata* ΔHTU (*his3Δ trp1Δ ura3Δ*) and its isogenic *Cgpdr1Δ* and *Cgyap1Δ* mutant strains (Roetzer et al. 2011) were provided by Ch. Schüller (Vienna University, Austria). *C. glabrata* clinical isolates DSY562, DSY565 originated from D. Sanglard (Lausanne University, Switzerland) and their properties together with those of the isolate JS1 (Berila et al. 2009; Kołaczkowska et al. 2013) were described previously (Sanglard et al. 1999; Berila et al. 2009; Ferrari et al. 2009). Cells were grown on glucose rich (YPD) medium (2% glucose, 1% yeast extract, 2% bactopeptone), glycerol rich (YPG) medium (as YPD but 2% glycerol was used instead of 2% glucose) or on minimal medium (YNB) containing 0.67% yeast nitrogen base without amino acids, 2% glucose (YNB-D) or 2% glycerol plus 2% ethanol (YNB-GE) and appropriate nutritional requirements. The media were solidified with 2% bactoagar and for evaluation of halotolerance were buffered to pH 5.5 with 20 mM MES and to pH 7.0 with 20 mM MOPS (Krauke and Sychrova 2010). The *Escherichia coli* XL1-Blue strain was used as host for transformation, plasmid amplification and preparation. The bacterial strains were grown at 37 °C in Luria-Bertani medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract, pH 7.5) supplemented with 100 μg/ml ampicillin for selection of transformants. The $pCgPDR1_{4672}$ plasmid containing the gain-of-function mutation in *CgPDR1* has been described previously (Tsai et al. 2006; Berila et al. 2009). The pYES2 plasmid served as the source of the *ScURA3* gene. Sequences of primer pairs are described in Table 1.

2.2. Construction of the Cgpdr16::ScURA3 disruption cassette

The promoter-dependent disruption gene (PRODIGE) method (Edlind et al. 2005) was used to knock-out the chromosomal *CgPDR16* gene in the *C. glabrata* ΔHTU strain and clinical isolates. The *ScURA3* coding sequences were amplified with designed PRODIGE primers using pYES2 plasmid DNA as a template. Reactions included 0.2 mM dNTPs, 1 μM CgPDR16-ScURA3for primer, 1 μM CgPDR16-ScURA3rev primer, 0.2 mM $MgSO_4$, 50 ng plasmid DNA, 1.5 units KOD Hot Start DNA polymerase (Novagen, Germany) and 1 x buffer for KOD polymerase in a total 50 μl volume.

Table 1
Primers used in this study. For PRODIGE primers, *CgPDR16* homology regions are italicized, start and stop codons of the *ScPDR3* marker gene are underlined.

Primer name	Sequence (5′–3′)
CgPDR16-ScURA3for	*GCTAGACTATACAGTTAGCCAACCCAGAAGATTTAATTCTA AAAGAGGTAATCGAACG*ATG TCGAAAGCTACATATAAGG
CgPDR16-ScURA3rev	*TAATTGAATGTTTATTAGTAATCATCCAAAAAAATACAAATAA ATGGGATGATATAATCA*TTA GTTTTGCTGGCCGCATC
CgPDR16uF	GGTGCGATAGGTTGTTAATGC
CgPDR16iR	CTGGAAGAGACTTGATTGGCT
ScURA3iR	CAGCAACAGGACTAGGATGAG
CgACT1-For	GTACCACCATGTTCCCAGGT
CgACT1-Rev	ACCACCGATCCAGACAGAGT
CgPDR1-For	TCAATGCCTTCCTGTACCAA
CgPDR1-Rev	CCGATAAGGGAGATGCAGTT
CgCDR1-For	TGGACCCTACTTCCGATGAG
CgCDR1-Rev	GCGACCAAATCCTTCCAGTA
CgPDH1-For	AATGCTGGGATAACGCTACG
CgPDH1-Rev	CGTCTTGCGAACACTGGTAG
CgSNQ2-For	GCACAGCGACAACAGGAGTA
CgSNQ2-Rev	TCAATGTTACCACCGCTACG
CgERG11-For	ATGCTGCTTCTTCCAGTGCT
CgERG11-Rev	GCGAACAATTCACCGATACAT
CgPDR16-For	CTGCTGAACAACAAGCGAAG
CgPDR16-Rev	TCGATTAAACCCGAGGATTC
CgRDN5.8-For	CTTGGTTCTCGCATCGATGA
CgRDN5.8-Rev	GGCGCAATGTGCGTTCA
CgTRR2-For	ATCACCGAGACCATTGCCAAGG
CgTRR2-Rev	AAGTTTCCTCACCTGGGATG
CgCTA1-For	ACGAAAACATCGCCAAGGG
CgCTA1-Rev	ATCTTACCGACTCTACGCAATG

Amplification involved initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1.25 min; the final extension at 72 °C was for 10 min. The reactions from four separate amplifications were pooled and the PCR product was checked by agarose gel electrophoresis and DNA restriction analysis using *Eco*RV and *Stu*I endonucleases to confirm only one cutting of the *ScURA3* gene.

2.3. Disruption of the CgPDR16 gene

C. glabrata strains auxotrophic for uracil were isolated using 5-fluoroorotic acid (1.4 mg/ml) as described previously (Castano et al. 2003; Culakova et al. 2013). The strains were transformed with the 922 bp Cgpdr16::ScURA3 disruption cassette (Supplementary Fig. S1) using the lithium acetate procedure (Sanglard et al. 1999) and the disruptants were selected for growth on uracil-free minimal medium. Disruption of *CgPDR16* in transformants was monitored by yeast colony PCR analysis using zymolyase, Dream Taq DNA polymerase (Thermo Scientific, UK) and two pairs of primers: CgPDR16uF plus CgPDR16iR and CgPDR16uF plus ScURA3iR, respectively, detecting the intact and disrupted *CgPDR16* gene. Four Ura^+ transformants giving the PCR product of expected size (536 bp) in reaction with the second pair of primers were selected and used for further analyses.

2.4. Quantitative real-time PCR

The levels of gene transcripts were assessed by quantitative real-time PCR. Yeast cells were grown in minimal medium containing glucose to mid-logarithmic phase. Total RNA was isolated by the hot acidic phenol extraction method (Ausubel et al. 1989). One microgram of RNA was used as a template in each sample with a reaction mix (MBI Fermentas, Vilnius, Lithuania) containing 200 U of Revert AidTM H Minus MMuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) in a total volume of 20 μl. The reaction mixture was incubated for 5 min at 37 °C, 60 min at 42 °C and 10 min at 70 °C. For the quantitative PCR, primers were designed using the Primer3 v. 0.4.0 (Rozen and Skaletsky 2000) and PerlPrimer v. 1.1.4a