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Differential expression of the *Candida glabrata CgRTA1* and Cg*RSB1* genes in response to various stress conditions

Anna Kołaczkowska [a,*], Mariusz Dyląg [b], Marcin Kołaczkowski [c]

[a] Department of Biochemistry, Pharmacology and Toxicology, University of Environmental and Life Sciences, Wroclaw, Poland
[b] Institute of Genetics and Microbiology, University of Wroclaw, Wroclaw, Poland
[c] Department of Biophysics, Wroclaw Medical University, Wroclaw, Poland

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ABSTRACT

Candida glabrata, a human opportunistic pathogen is characterized by intrinsic, low susceptibility to fluconazole and a high capacity for acquiring high-level azole resistance. This is related to the elevated expression of genes belonging to the CgPdr1-governed regulon, comprising numerous genes, of which the multidrug ABC transporter-encoding *CgCDR1*, *CgCDR2*, *CgSNQ2* are the best characterized. The function of certain PDR loci, such as Cg*RTA1* and Cg*RSB1* is poorly understood. These are homologs of *ScRTA1* and *ScRSB1* from *Saccharomyces cerevisiae*, members of the LTE family of plasma membrane proteins characteristic of fungi. While overproduced, they are involved in tolerance to 7-aminocholesterol or phytosphingosine, respectively. In this report we shed light on the differential regulation of *CgRTA1* and *CgRSB1* in *C. glabrata*. *CgRTA1* expression positively correlated with intrinsic azole tolerance in clinical isolates. In contrast to *CgRSB1*, a high induction of *CgRTA1* was observed upon fluconazole exposure, which was accompanied by a parallel up-regulation of its transcriptional activator *CgPDR1*. Hypoxia or presence of ketoconazole, both leading to ergosterol depletion, resulted in increased level of *CgRTA1* transcript, whereas *CgRSB1* was highly responsive to mitochondrial dysfunction. On the other hand, the expression of *CgRTA1* was suppressed during growth in pseudohyphae formation promoting media. Our results are the first report linking the divergent regulation of LTE family members and azole sensitivity in *C. glabrata*.

1. Introduction

Fungal infections caused by *Candida glabrata* are on the rise [1]. *C. glabrata* colonizes as a commensal in more than 80% of healthy individuals. Weakening of the immune system may turn this species into opportunistic pathogen. Progressive emergence of candidemia caused by *C. glabrata* correlates with increasing population of immunocompromised individuals (surgical patients, chemotherapy receiving patients, elderly). *C. glabrata* exhibits enormous flexibility and easily adapts to occupied niches. In contrast to *C. albicans*, it is recognized as an intrinsically azole-tolerant species and is about eight times more resistant to the widely used antifungal drug fluconazole [2,3]. It can also easily acquire azole resistance during drug exposure [4–6].

The mechanisms of intrinsic as well as acquired azole resistance in *C. glabrata* rely mainly on the overproduction of the ABC type transporters CgCdr1p, CgCdr2p, CgSnq2p extruding drugs out of the cell [4–7]. Expression of the ABC pumps coding genes is regulated by CgPdr1p, comprising the function of both orthologous ScPdr1p and ScPdr3p transcription factors found in *S. cerevisiae*. Wide genome analyses of matched pairs of azole-resistant and azole-sensitive *C. glabrata* clinical isolates deciphered a global picture of the network of genes involved in drug resistance development and highlighted several weakly characterized loci, including *CgRSB1* and *CgRTA1* [8,9]. *CgRTA1* exhibits homology to *ScRTA1*, known as the sole plasma membrane located multicopy suppressor of 7-aminocholesterol toxicity in *S. cerevisiae* [10,11]. *CgRTA1* is one of the members of the *PDR1* regulon in *C. glabrata* [8,9].

To shed more light on the regulation of *CgRTA1* expression and its role in drug resistance, its expression pattern was analyzed along with that of *CgRSB1* in several clinical isolates characterized by various levels of azole susceptibility. The effect of various stress conditions and influence of mitochondrial status on the transcript level of both genes was also investigated.

Abbreviations: ABC, ATP-binding cassette; EtBr, ethidium bromide; PDRE, pleiotropic drug-response element; 7-ACH, 7-aminocholesterol; UTR, untranslated; FCZ, fluconazole; CTZ, clotrimazole; KTZ, ketoconzole.

* Corresponding author. Address: Department of Biochemistry, Pharmacology and Toxicology, University of Environmental and Life Sciences, CK. Norwida 31, 50-375 Wroclaw, Poland. Fax: +48 71 32 05 209.
E-mail address: kolannska@gmail.com (A. Kołaczkowska).

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2. Materials and methods

2.1. Strains and culture conditions

C. glabrata clinical isolates used in this study (Table 1) were recovered from patients in the Laboratory of Molecular Diagnostics "Bio-Genetik" (Wroclaw, Poland) or generously obtained from from J. Subik and D. Sanglard [12–14]. Strains were cultivated in complete YPD media (2% peptone, 1% yeast extract and 2% glucose). For the analysis of various stress conditions, strains were grown in YPD containing appropriate azole drug or 10% FCS, or in VS medium [15]. For testing hypoxic conditions, cells were grown in preconditioned VS medium in the hypoxic chamber (BBL™ GasPack™ Anaerobic System). Petite mutants were generated by incubation of the cells in liquid YPD media containing 25 μg/ml of ethidium bromide for 4 h. The petite phenotype was verified on YPGE solid medium (2% ethanol, 2% glycerol, 1% yeast extract and 2% agar).

2.2. Growth inhibition assays

Resistance to azoles (fluconazole – FCZ, voriconazole – VOR, ketoconazole – KCZ and clotrimazole – CTZ) was verified by the use of previously described microdilution tests [16] according to M27-A2 standard guidelines [17]. Cells were grown in RPMI 1640 MOPS buffered medium. Growth was monitored after 48 h by OD_{600} measurements in the Asys HiTech microplate reader and visual inspection. The lowest drug concentration inhibiting growth in 80% of that of drug-free control was defined as minimal inhibitory concentration (MIC_{80}). Susceptibility to 7-aminocholesterol was determined as described previously [11].

2.3. Quantitative real-time PCR

Overnight cultures were diluted in appropriate medium and incubated for 2 h at 30 °C. For the analysis of drug challenge, exponentially growing cells were split and the same amount of cells were re-grown for 2 h in YPD without drug or YPD containing FCZ or KTZ. Total RNA was isolated by the hot-phenol method [18]. Prior to RT-PCR experiments, total RNA was treated with DNase I (Sigma) and purified on silica containing resin (Qiagen). First strand cDNAs were synthesized from 1.25 μg total RNA using iScript Synthesis kit (Bio-Rad). Independent amplifications were performed on the same cDNA for the gene of interest and the *CgTUB1* reference gene using SYBR Green PCR mix (Bio-Rad) on the iQ5 apparatus (Bio-Rad). Fold change of the analyzed transcript was calculated by the Pfaffl method [19]. The following primers were used in qPCR: CgRTA1f 5′-GCATAGACAGTTCCTGACGCAAT-3′,
CgRTA1r 5′-TTAACGGTATATGGGCAGCAAAT-3′,
CgPDR1f 5′- GGAATGGTGACTCGGAAGAA-3′,
CgPDR1r 5′-ATGGCGTCAATGGATGATTT-3′,
CgRSB1f 5′-CATTGTTCGTTGTTGCTACCGTC-3′,
CgRSB1r 5′-CCAGTGTCACCATCAGGGAATCG-3′,
CgTUB1f 5′-AGAGAAGAATTTCCTGATAGAATGATGG-3′,
CgTUB1r 5′-ACAGACAGAGTGGCGTTATATGG-3′.

3. Results and discussion

3.1. Azole susceptibility of clinical isolates

Recent reports indicate that spontaneous gain-of-function mutations in transcriptional regulator CgPdr1p contribute to azole resistance in clinical isolates [8,9,20]. In addition, several CgPdr1p-governed target genes were shown to be connected with enhanced virulence in animal models [21]. Thus, the PDR network is very likely to facilitate adaptation to the host and to sustain chemical stress. CgPdr1p targets comprise among other two weakly characterized loci, classified by homology as *CgRTA1* and *CgRSB1* in *Candida* Genome Database [8,9]. To shed some light on the regulation of *CgRTA1* expression and its contribution to the development of drug resistance, its expression pattern was analyzed along with that of *CgRSB1* in several clinical isolates characterized by various levels of azole susceptibility. Twenty clinical isolates were recovered from patients (source: Laboratory of Molecular Diagnostics "Bio-Genetik", Wroclaw, isolates MD1 to MD8) or generously obtained from J. Subik and D. Sanglard (strains JS1 to JS30, DSY 92 to DSY 565). Screening for azole resistance of all isolates revealed nine azole-resistant strains with MIC_{80} for fluconazole (FCZ) equal to 128 μg/

Table 1
Drug susceptibility of *C. glabrata* clinical isolates used in this study.

Isolate	MIC_{80} (μg/ml)				Site of isolation or reference
	FCZ	CTZ	KTZ	VOR	
MD 1	16	>2.7	1	0.05	Stool
MD 2	32	0.67	1	0.05	Blood
MD 3	16	0.58	1	0.05	Vagina
MD 4	32	>2.7	1	0.05	Vagina
MD 5	32	2.7	0.5	0.05	Vagina
MD 6	128	5.4	4	>14	Oral cavity
MD 7	32	>2.7	>4	0.05	Oral cavity
MD 8	8	2.7	1	0.05	Oral cavity
JS1	128[a]	>8	8	32[a]	[12]
JS3	128[a]	>4	4	>32[a]	[12]
JS7	128[a]	8	8	32[a]	[12]
JS20	128[a]	>8	8	0.75[a]	[12]
JS21	128[a]	>8	4	>32[a]	[12]
JS22	128[a]	>8	8	>32[a]	[12]
JS27	128[a]	8	8	>32[a]	[12]
JS28	8[a]	2	1	0.38[a]	[12]
JS29	8[a]	4	1	0.38[a]	[12]
JS30	8[a]	1	1	1[a]	[12]
DSY92	1[a]	0.125	0.125	0.05	[13]
DSY94	1[a]	0.125	0.125	0.05	[13]
DSY562	8[a]	4	1	0.2	[13]
DSY565	128[a]	8	4	1.6	[13]
ATCC 90030	64	4	8	0.05	[14]

MIC_{80}, minimal inhibitory concentration was defined as the lowest azole concentration that reduced growth to 80% of that of drug free control.
[a] MIC for FCZ and VOR were in agreement with previously published values [12,13].

ml, six susceptible strains dose dependent with MIC equal to 16–32 μg/ml and five susceptible isolates with MIC equal to 8 μg/ml (Table 1). *C. glabrata* resistant strain ATCC 90030 [14] and hypersusceptible variants DSY 92 and DSY 94 [13] were used as references. Fluconazole resistance profile for isolates JS1, JS3, JS7, JS20, JS21, JS22, JS27, JS28, JS29, JS30 and DSY 92, DSY 94, DSY 562 and DSY 565 was in agreement with the previously published values [12,13]. Isolates MD6, JS1 to JS27, DSY 565 exhibited cross-resistance to clotrimazole (CTZ), ketoconazole (KTZ), and voriconazole (VOR, Table 1). Strains JS28, JS29, JS30, DSY 92, DSY 94, DSY 562 and all MD isolates (apart MD6) were susceptible to all azole drugs tested (Table 1).

3.2. CgRTA1 transcript level correlates with the level of drug resistance

Along with susceptibility tests, relative level of *CgRTA1* transcript was determined as described in Section 2. As shown in Fig. 1A, the level of azole resistance correlated with the level of *CgRTA1* transcript. The most azole-resistant isolates MD6, DSY 565, JS1, JS7, JS27 along with ATCC 90030 exhibited highest *CgRTA1* transcript level as compared to the DSY 562 susceptible strain. MD4 and MD5 showed moderate *CgRTA1* expression that goes in pair with lower azole tolerance. Azole susceptible isolates (e.g. JS28, JS30, MD1 and MD8) were characterized by low *CgRTA1* expression level similar to that of DSY 562. MD6-resistant cells, in addition to elevated *CgRTA1* expression, exhibited also increased *CgCDR1* and *CgPDR1* transcript levels (data not shown). These data indicate positive correlation between azole resistance and the expression of *CgRTA1* in clinical isolates of *C. glabrata* (Fig. 1B) with Pearson correlation coefficient (r) equals to 0.8445. In the evolutionarily related *Saccharomyces cerevisiae*, a higher transcript level of *ScRSB1*, a homolog of *ScRTA1* was observed in drug-resistant strains [22]. Therefore, the expression profile of the *CgRSB1* gene was included for comparison. In contrast to *CgRTA1*, *CgRSB1* expressed at a higher basal level did not correlate with azole susceptibility and no increase was observed even in the most resistant strains (Fig. 1).

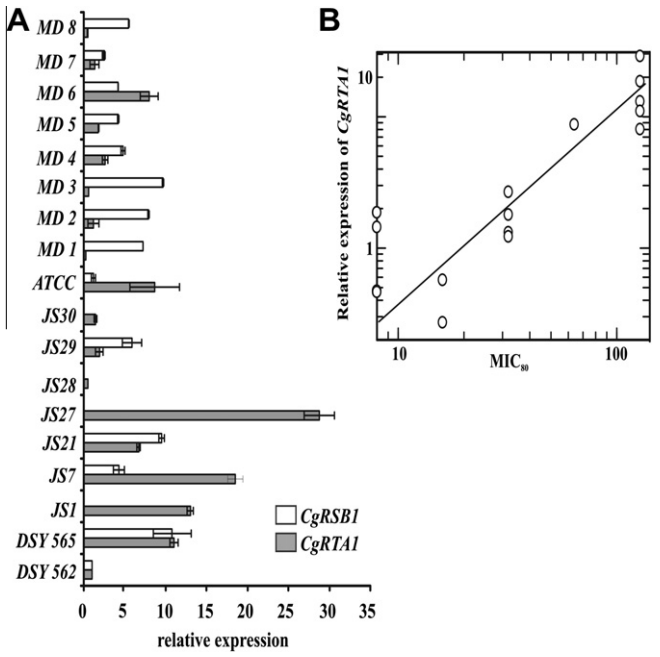

Fig. 1. Expression pattern of *CgRTA1* and *CgRSB1* in clinical isolates. (A) Relative expression of the *CgRTA1* and *CgRSB1* genes. *CgTUB1* was used as reference, the relative *CgRTA1* and *CgRSB1* transcript levels were calculated by the Pfaffl method [19]. *CgRTA1* transcript level in DSY 562 was set as 1. (B) Correlation between *CgRTA1* expression level and the level of drug resistance in *C. glabrata* clinical isolates. Correlation coefficient was calculated with the GraFit program.

Next, we verified whether the expression of *CgRTA1* depends on fluconazole exposure. The indicated cultures of exponentially growing cells were split and the subinhibitory concentration of FCZ was added to one half of the culture (Fig. 2A). Both, drug-treated and drug-free cultures were re-grown for 2 h followed by the isolation of total RNA as described by Smith et al. [18]. Fig. 2A indicates that in the early phase of response to FCZ, the expression of *CgRTA1* increased up to 6-fold, depending on the strain tested and its original level of FCZ resistance. The susceptible isolates were most responsive to FCZ challenge. A nearly 6-fold induction was observed in the susceptible MD5, JS29 and DSY 562 isolates treated with FCZ (Fig. 2A). In contrast, only a weak or no response was observed with resistant strains (Fig. 2A), such as DSY 565 and ATCC 90030. As shown in Fig.2B, expression of *CgRSB1* did not change upon FCZ treatment under conditions tested. Studies by Soustre et al. [11] identified *ScRTA1* as a sole determinant for 7-aminocholesterol (7-ACH) toxicity when overexpressed. In order to differentiate *CgRTA1* and *CgRSB1*, we overexpressed both loci in *S. cerevisiae* and verified their phenotype to 7-ACH. Only *CgRTA1* could suppress 7-ACH toxicity when overproduced (data not shown) suggesting different roles of both loci in response to drug challenge. The observed effect of specific *CgRTA1* induction by FCZ is most probably mediated via PDREs, which are found in the 5′ UTR region of *CgRTA1*. *In silico* analysis revealed the presence of two potential sites recognized by CgPdr1p of type A PDRE (TCCGCGGA) and type B PDRE (TCCGTGGA) located respectively 382 and 303 nucleotides upstream of the initiator ATG codon (Table 2). In contrast, promoter region of a *CgRSB1* (−800 to +1) contains only one degenerated PDRE (−632).

3.3. CgRTA1 and CgRSB1 expression in response to various stress conditions

C.glabrata is recognized as an opportunistic pathogen able to occupy various host's niches characterized by different nutrient and oxygen availability. Its enormous flexibility and successful adaptation to the harsh environment relays on genome plasticity and quick rewiring of the expression of numerous genes. As shown here, *CgRTA1* was responsive to FCZ, the mainstay of antifungal therapy which poses chemical stress. In order to verify whether *CgRTA1* plays a role in adaptation of the cell to other stress conditions, we examined its expression in the presence of serum, in media promoting pseudohyphal growth, and in VS medium (vagina simulating medium) containing lactic acid or under reduced oxygen concentration (hypoxia). The presence of 10% fetal calf serum (FCS) resulted in a marked decrease in *CgRTA1* transcript level in all isolates tested (Fig. 2C). In cells grown in VS medium under reduced oxygen concentration, a slight increase in the *CgRTA1* transcript level was observed (data not shown). The conditions reminiscent of hypoxia may also be induced chemically by KTZ challenge [23]. The presence of KTZ causes ergosterol depletion otherwise occurring under low oxygen concentration. Therefore, we verified *CgRTA1* transcript level in cells treated with sterol lowering drug KTZ. Fig. 2D shows that in the presence of subinhibitory concentration of this drug, a parallel 5-fold increase in *CgRTA1* and its transcriptional activator *CgPDR1* was observed as compared to nontreated cells. Noteworthy, *CgRSB1* transcript level remained unchanged in all isolates tested under these conditions (Fig. 2D). As shown by Thakur et al. [24], the response of yeast cells to various drugs including KTZ was mediated by ScPdr1p and ScPdr3p regulators acting also as xenobiotics receptors. Similarly, CgPdr1p was shown to bind radiolabeled KTZ [24]. Lack of response of *CgRSB1* to drugs (FCZ and KTZ) suggest that the degenerated PDRE site located far upstream of the initiator ATG codon was not functional.

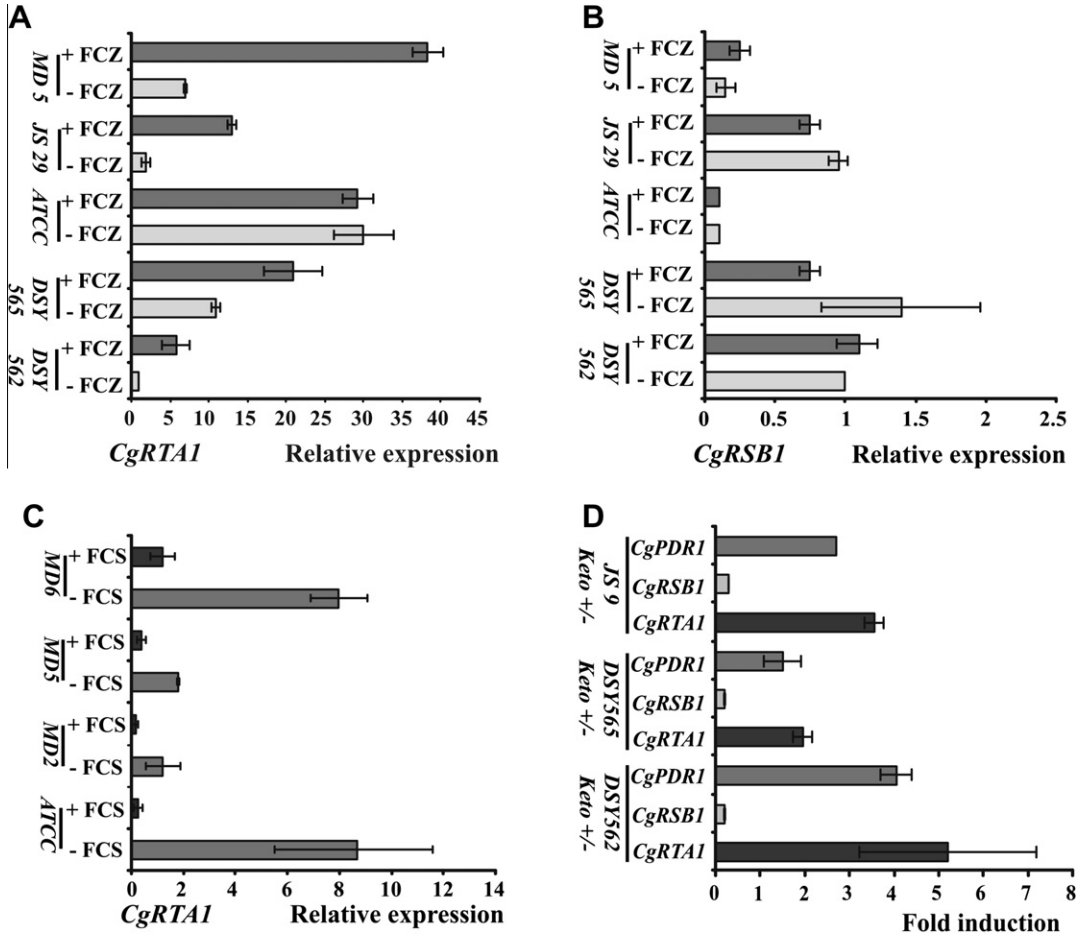

Fig. 2. Expression of *CgRTA1*, *CgRSB1* and *CgPDR1* under various stress conditions. (A) Relative transcript level of *CgRTA1* and (B) *CgRSB1* upon exposure to fluconazole (FCZ), (C) effect of serum (FCS) on *CgRTA1* expression. *CgRTA1* transcript level in DSY 562 was set as 1. (D) Fold change of the *CgRTA1*, *CgRSB1* and *CgPDR1* transcripts in cells treated with sterol lowering ketoconazole versus nontreated cells.

Table 2
Putative transcription factors binding sites within promoters of *Candida glabrata RTA1* and *RSB1* genes.

Regulator	CgPdr1p	CgMot3p	CgCrz1p	CgAce2p	CgCbf1p	CgRtg1/3p	CgMsn2/4p	CgRim101p
CgRTA1	+	+	+	–	–	–	+	–
CgRSB1	+[a]	+	–	+	+	+	+	+

Search for candidate transcription factors was performed on the 5′ UTR region encompassing 800 nucleotides upstream of the initiator ATG codon using the YEASTRACT platform (YEAst Search for Transcriptional Regulators And Consensus Tracking) [29].

[a] Degenerated PDRE.

C. glabrata can produce petite mutants *in vitro* with high frequency [25]. Although rarely, such azole-resistant petites were also isolated from clinical specimen [26]. Petite phenotype can result from mitochondrial dysfunction due to partial or complete loss of mitochondrial DNA. This phenotype can also originate from nuclear mutations. *C. glabrata* petite mutants exhibit decreased azole susceptibility due to overexpression of CgCDR1 and CgCDR2 [5,26]. However, an unusual phenotypic connection between petite mutation and increased susceptibility to azole was also reported [27]. The observed positive correlation between azole resistance and *CgRTA1* transcript level prompted us to investigate the possible influence of the mitochondrial status on *CgRTA1* and *CgRSB1* expression. Petite derivatives of clinical isolates were generated by growth in the presence of ethidium bromide as described (Section 2) and then checked for the inability to grow on glycerol containing media. Cells with compromised mitochondria exhibited petite phenotype due to the inability to respire on nonfermentable carbon sources. Their drug susceptibility was verified along with the analysis of the expression level of *CgRTA1*, homologous *CgRSB1* and *CgPDR1* loci. As shown in Table 3, cells unable to respire exhibited increased drug tolerance to FCZ. In parallel, we tested the susceptibility of the strains to 7-ACH. All mitochondria-deficient variants were able to tolerate highest 7-ACH concentration (1572 ng/ml) as compared to the original isolates (Table 3). MD3 petite derivative was 32-fold more resistant than its wild type counterpart. 7-ACH resistance positively correlated with an increased *CgRTA1* transcript level as revealed by real time PCR analysis (Fig. 3). As shown in Fig. 3, all petite mutants generated in this study overexpressed *CgPDR1* regulator to a different extent, which is in good agreement with studies by Tsai and coworkers [9]. The elevated expression of *CgPDR1* correlated with 5–10-fold upregulation of *CgRTA1*. Noticeably, a higher 10–20-fold increase in *CgRSB1* transcript level was observed in all petites azole-resistant strains. Lack of response of *CgRSB1* to FCZ induction and concomitant in-

Table 3
Drug susceptibilities of *C. glabrata* clinical isolates and their derivatives with petite phenotype.

Isolate	MIC (μg/ml)	
	FCZ	7-ACH
MD2	32	196
MD2 petite	64	1572
MD3	16	49
MD3 petite	32	1572
MD5	32	196
MD5 petite	128	1572
MD6	128	ND[a]
MD6 petite	>128	ND
ATCC 90030	64	393

Minimal inhibitory concentration (MIC) for FCZ was determined in RPMI1640 MOPS buffered medium, MIC for 7-ACH was assessed in SB medium. Growth was monitored after 3 days.
[a] Not determined.

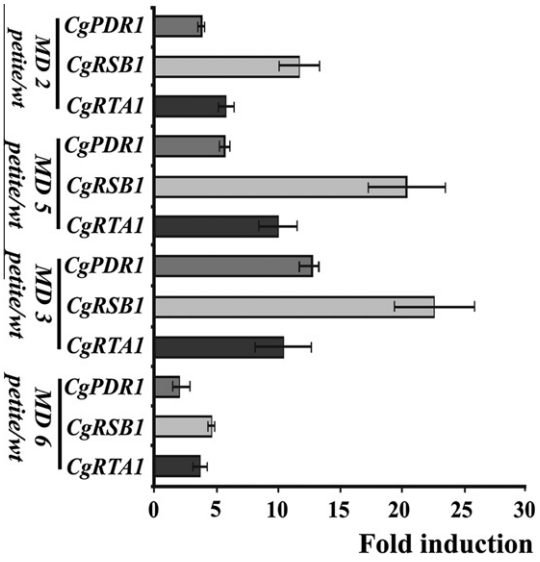

Fig. 3. Expression of *CgRTA1* and *CgRSB1* increases in cells with mitochondrial deficiency. Fold change of the *CgRTA1*, *CgRSB1* and *CgPDR1* genes corresponds to the indicated transcript level in petite mutants versus original isolates.

creased expression of *CgRSB1* in petite mutants argues for other than CgPdr1p-PDRE mediating control. *CgRSB1* might be involved in retrograde signaling pathway from dysfunctional mitochondria regulated by CgRtg1/3 helix–loop–helix/leucin zipper proteins [28]. The putative binding sites of these transcription factors were found in the *CgRSB1* promoter region (Table 2). However, participation of the degenerated PDRE in response to signal from dysfunctional mitochondria cannot be excluded.

In summary, we showed that *C. glabrata* homologous genes *CgRTA1* and *CgRSB1* differentially responded to drug challenge and mitochondrial dysfunction. Transcript level of *CgRTA1* positively correlated with intrinsic azole resistance in *C. glabrata* clinical isolates. *CgRTA1*, but not *CgRSB1*, along with transcriptional regulator *CgPDR1* were responsive to FCZ induction. Reduced oxygen level or presence of KTZ, both leading to ergosterol depletion, resulted in increased transcript level of *CgRTA1*. Growth of cells in media promoting the formation of pseudohyphae inhibited the expression of *CgRTA1*. On the other hand, *CgRSB1* was highly responsive to mitochondrial dysfunction similar to its counterpart from *Saccharomyces cerevisiae ScRSB1*.

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