RESEARCH ARTICLE

The putative ABC transporter encoded by the orf19.4531 plays a role in the sensitivity of Candida albicans cells to azole antifungal drugs

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One sentence summary: ROA1 encodes a putative ABC transporter and is involved in the sensitivity of Candida albicans cells to azole antifungal drugs.

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ABSTRACT

ATP-binding cassette (ABC) transporters constitute a large superfamily of integral membrane proteins in prokaryotic and eukaryotic cells. In the human fungal pathogen Candida albicans, there are 28 genes encoding ABC transporters and many of them have not been characterized so far. The orf19.4531 (also known as IPF7530) encodes a putative ABC transporter. In this study, we have demonstrated that disruption of orf19.4531 causes C. albicans cells to become tolerant to azoles, but not to polyene antifungals and terbinafine. Therefore, the protein encoded by orf19.4531 is involved in azole sensitivity and we name it as ROA1, the regulator of azole sensitivity 1 gene. Consistently, we show that the expression of ROA1 is responsive to treatment of either fluconazole or ketoconazole in C. albicans. In addition, through a GFP tagging approach, Roa1 is localized in a small punctuate compartment adjacent to the vacuolar membrane. However, ROA1 is not essential for the in vitro filamentation of C. albicans cells.

Keywords: Candida albicans; ABC transporter; antifungals;azole; ROA1

INTRODUCTION

ATP-binding cassette (ABC) transporters, functioning as both importers and exporters, constitute a large superfamily of integral membrane proteins with more than 3000 members in prokaryotic and eukaryotic cells (Jungwirth and Kuchler 2006; Golin and Ambudkar 2015). ABC proteins are ubiquitous in prokaryotes and eukaryotes. ABC proteins are energy-dependent transporters of a variety of substrates, which include nutrients, herbicides, anticancer agents and cytotoxic drugs (Kartner and Ling 1989; Dassa et al. 1999). Candida albicans is the predominant human fungal pathogen of invasive candidiasis, a leading cause of mycosis-associated mortality (Chauhan, Latge and Calderone 2006; Pfaller and Diekema 2007; Mayer, Wilson and Hube 2013). Development of drug resistance in C. albicans is a well-documented phenomenon for immunocompromised patients with recurrent oropharyngeal candidiasis. ABC proteins are involved in the secretion of mating factors and quorum-sensing molecules, which affect biofilm structure and behavior that can result in increased fungal drug resistance (Cannon and Holmes 2015). Overexpression of genes encoding ABC
transports represents one of the predominant drug-resistant mechanisms in clinical strains of C. albicans, which is a major threat to antifungal therapy (Morschhäuser et al. 2002; Sanglard and Odds 2002; Anderson 2005; Higgins 2007; Goffeau 2008; Monk and Goffeau 2008; Prasad et al. 2015).

There are 28 ABC transporter proteins in the genome of C. albicans, with the largest group being encoded by the CDR gene family (Prasad et al. 2015). Cdr1 and Cdr2 are involved in multidrug resistance of clinical isolates, while Cdr3 and Cdr4 function as phospholipid flippases (Smriti et al. 2002; Holmes et al. 2008). Most of the ABC transporter genes in C. albicans have been previously given names through their closest homologs in Saccharomyces cerevisiae (Braun et al. 2005). However, functions of many ABC transporter genes in C. albicans remain to be elucidated.

ABC transporter proteins share highly conserved one or two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP to provide energy for their functions (Schneider and Hunke 1998). A NBD domain consists of approximately 200 amino acids and displays two major conserved motifs: the ‘Walker A’ ([AG]-x(4)-G-K-[ST]) and the ‘Walker B’ (D-E-x(5)-D), separated by about 120 amino acid residues and an additional ‘AT motif’ (LSGGQ) situated between the two motifs (Gaur, Choudhury and Prasad 2005). Recent studies show that the purified N-terminal NBD of Cdr1p exhibits similar biochemical features of ATP hydrolysis to those of the full transporter and the Cys-193 in Walker A is crucial for its catalytic activity (Jha et al. 2003a,b).

The orf19.4531 (also known as IPF7530) encodes a DNA fragment, containing the GFP ORF, with primers GFP-F (5′-ACGCGTCGAC AAACCATTTA ACTCTGACGT AAATTG-3′) and GFP-R (5′-ATGAGTAAAG GAGAAGAACT TTTC-3′), yielding pCR4-GFP. The GFP insert sequence in pCR4-GFP was confirmed by DNA sequencing. Next, this DNA insert fragment was amplified by PCR from the genomic DNA of RM1000 with primers IPF7530GFP-F (5′-CGCCAGCTCTTGGTACCGAG ACCAA-3′, BamHI added) and CaABC-IR (5′-AACCAGCTGC AACCATGTC TACACC-3′, Pst I added) into the BamHI and PstI sites of pRC2312 (Liu et al. 2007; Wang et al. 2007), which yields pRC2312-ABC1. A 3095-bp fragment, containing the second part of ROA1, was amplified by PCR from the genomic DNA of RM1000 with primers CaABC-IF (5′-CAGTCCAGCG AACCACAGG 3′) and CaABC-IR (5′-GAAGATCTGC ATGGCAGCTG ATAACCTG-3′, BglII added), cut with BglII and cloned into the BglII site in pCR2312-ABC1, which yielded pRC2312-ROA1 containing the 783-base promoter region, the 3825-base ORF and the 250-base 3′ untranslated region of ROA1. This insert was confirmed by DNA sequencing.

To visualize the subcellular localization of Roa1, we constructed pCR4-ROA1-GFP, expressing the ROA1-GFP fusion protein under the control of its own promoter. First, we PCR amplified a DNA fragment, containing the GFP ORF, with primers GFP-F (ACCGGTTCGAC ATGAGTAAAG GAGAAGAACT TTTC) and GFP-R (CCCCCCAGGT TATTTGTGATAAATCCAT GC, HindIII site underlined) using pRC2312-CaPTC7-GFP (Wang et al. 2007) as template. This fragment was then digested with SalI and HindIII enzymes, and cloned into the C. albicans expression vector pCR4 (Rocha et al. 2001; Li et al. 2010; Jiang et al. 2012), yielding pCR4-GFP. The GFP insert sequence in pCR4-GFP was confirmed by DNA sequencing. We then PCR amplified the 3′ end 1212-bp DNA fragment of the ROA1 ORF without its stop codon using primers IF7530GFP-F (5′-CGGGATCCG ATGAGTAAAG GAGAAGAACT TTTC, BamHI site underlined) and IF7530GFP-R (5′-ACCGGTTCGAC AAACCAGATTA CACTCTGAGC AAATTTG 3′, SalI site underlined). This fragment was cloned into the BamHI and SalI sites in pRS316, yielding pRS316-IFPC, and its sequence was confirmed by DNA sequencing. Next, this DNA insert fragment was excised with SalI and SalI and subcloned into the SacI and SalI sites in pCR4-GFP, yielding pCR4-IPFC-GFP. Finally, the 3394-bp fragment in pRC-ABC containing the promoter and the 5′ end of pUC19 to obtain pUCIPF. The 4.0-kb fragment containing the hisG-URA3-hisG cassette in p5921 was excised with BamHI and BglII and cloned into the BglII site in pUCIPF (this BglII site is present within the 899-bp DNA fragment in pUCIPF), which yielded pABCD containing the disruption cassette. The pABCD DNA was cut with SacI and KpnI and used for transformation of RM1000 cells for subsequently disrupting the two alleles of the orf19.4531 (Fig. S1A, Supporting Information). Genotypes of the heterozygous mutant CNS2 and the homozygous mutant CNS4 were confirmed with PCR (Fig. S1B, Supporting Information).

**Materials and Methods**

**Strains and media**

C. albicans strains were maintained at 30°C on YPD plates. The synthetic drop-out medium was supplemented with appropriate nutrients for plasmid selection and maintenance (Jiang et al. 2002). Rapamycin (A. G. Scientific, USA) stock solution (1 mg/ml) was prepared in ethanol containing 10% Tween-20. Candida albicans cell transformation was described previously (Lee et al. 2004; Wang et al. 2007). Genomic DNA was extracted from the C. albicans strain RM1000 as described (Jiang, Whiteway and Shen 2002; Anderson 2005; Higgins 2007; Goffeau 2008; Monk and Goffeau 2008; Prasad et al. 2015).

**Disruption of the C. albicans gene orf19.4531**

To generate a disruption cassette for the orf19.4531 (also known as IPF7530, ROA1), we amplified the 899-bp DNA region within this open reading frame (ORF) from the genomic DNA of C. albicans strain RM1000 with primers of IF7530-IF (5′-CGGGATCCG TTCATTGGCAGG AGGAAG, Sall site underlined) and IF7530-IR (5′-CGGGATCCG ATGGTGGCAG TGGAC 3′, KpnI site underlined). This fragment was digested with SacI and KpnI, and cloned into pUC19 to obtain pUCIPF. The 4.0-kb fragment containing the hisG-URA3-hisG cassette in p5921 was excised with BamHI and BglII and cloned into the BglII site in pUCIPF (this BglII site is present within the 899-bp DNA fragment in pUCIPF), which yielded pABCD containing the disruption cassette. The pABCD DNA was cut with SacI and KpnI and used for transformation of RM1000 cells for subsequently disrupting the two alleles of the orf19.4531 (Fig. S1A, Supporting Information). Genotypes of the heterozygous mutant CNS2 and the homozygous mutant CNS4 were confirmed with PCR (Fig. S1B, Supporting Information).

**Plate assay and filamentation test**

Plate assay and filamentation test were carried out as described previously (Liu et al. 2010; Zhao et al. 2012; Feng et al. 2013; Xu et al. 2015). Phenotypes were recorded after plates were incubated for 2–3 days at 30°C. Filamentation was assessed by growing cells into liquid YPD medium containing 10% fetal bovine serum, SLAD medium or Lee’s medium, which were incubated with shaking at 37°C for 6 h. Colony morphology was examined on agar plates of these media. Twenty cells of each strain were spread onto one plate, and plates were incubated for 5–7 days at 37°C before colonies were photographed.

**Gene cloning**

We first cloned the 1984 bp fragment, containing the first part of ROA1, from the genomic DNA of C. albicans strain RM1000 with primers CaABC-IF (CGGGATCCG GTTATCATCG CATCCAC 3′, BamHI added) and CaABC-IR (5′-AACCAGCTGC AACCATGTC TACACC 3′, PstI added) into the BamHI and PstI sites of pRC2312 (Liu et al. 2007; Wang et al. 2007), which yields pRC2312-ABC1. A 3095-bp fragment, containing the second part of ROA1, was amplified by PCR from the genomic DNA of RM1000 with primers CaABC-IF (5′-CAGTCCAGCG AACCACAGG 3′) and CaABC-IR (5′-GAAGATCTGC ATGGCAGCTG ATAACCTG 3′, BglII added), cut with BglII and cloned into the BglII site in pRC2312-ABC1, which yielded pRC2312-ROA1 containing the 783-base promoter region, the 3825-base ORF and the 250-base 3′ untranslated region of ROA1. This insert was confirmed by DNA sequencing.
the ROA1 ORF was excised with BamHI and PstI and subcloned into pCR4-IPFC-GFP, which yielded pCR4-ROA1-GFP.

RNA isolation

For drug treatment experiments, C. albicans SC5314 strain was grown to log phase and challenged with 2 μg/ml fluconazole or 2 μg/ml ketoconazole, and cells were collected in 20, 40 and 60 min, respectively. For RNA extraction, collected cells were washed once with ice-cold PBS. Further RNA isolation was carried out with the Fungal RNAout kit (TIANDZ, Mianyang, China) according to the manufacturer’s instructions. Isolated total RNA was digested with RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) to remove contaminated genomic DNA and further purified with the Column puriﬁed RNAclean kit (TIANDZ) to remove proteins before being used for RT-PCR.

Quantification by real time RT-PCR

First-strand cDNAs were synthesized from 1 μg of total RNA in a 20-μl reaction volume using the cDNA synthesis kit for RT-PCR (TaKaRa) following the manufacturer’s instructions. Quantitative real time PCRs were performed in triplicates using the Light Cycler System (Roche diagnostics, GmbH Mannheim, Germany). SYBR Green I (TaKaRa) was used to visualize and monitor the ampliﬁed product in real time. The ROA1 and 18S rRNA gene primers were IPF7530-F (5’ TGAATATTG GACCCTCAAG G3’) and IPF7530-R (5’ TGATCCAAAG GCAAACGAAG 3’) as well as 18S-F (5’ TCTTTTTGTA TTGGGTTG GGT 3’) and 18S-R (5’ TCATACTC TTCAGAAAG TG 3’).

The PCR protocol consisted of denaturation program (95°C for 5 min), 40 cycles of amplification and quantiﬁcation program (95°C for 10 s, 60°C for 20 s, 72°C for 15 s with a single ﬂuorescence measurement), melting curve program (60°C – 95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement) and ﬁnally a cooling step to 40°C. The change in ﬂuorescence of SYBR Green I dye in every cycle was monitored by the Light Cycler system software (Roche Diagnostics), and the threshold cycle (CT) above background for each reaction was calculated. The CT value of 18S rRNA was subtracted from that of the ROA1 gene to obtain a ΔCT value. The ΔCT value of an arbitrary calibrator (e.g. untreated sample) was subtracted from the ΔCT value of each sample to obtain a ΔΔCT value. The gene expression level relative to the calibrator was expressed as 2−ΔΔCT.

Viability assay

C. albicans cells pretreated with ﬂuconazole or ketoconazole for 3 h at 30°C were stained with the morbidity dye bis-[1,3-dibutylbarbituric acid] trimethine oxonol (DiBAC) (DOJINDO Laboratories, Japan) as described (Shirazi and Kontoyiannis 2015).

Statistical analysis

Signiﬁcant differences were analyzed by GraphPad Prism version 4.00. P values of < 0.05 were considered to be signiﬁcant.

RESULTS

Sequence analysis

We obtained the nucleotide sequence of the C. albicans orf19.4531 (ROA1) from the C. albicans genomic database (www.candidagenome.org). ROA1 encodes a protein of 1274 amino acids that contains two ‘AAA’ domains, two ‘ABC2_membrane’ domains and two independent transmembrane domains predicted by SMART analysis (http://smart.embl-heidelberg.de) (Fig. S1C, Supporting Information). These domains are highly conserved in the PDR subfamily members of ABC transporter proteins (Gaur, Choudhury and Prasad 2005). Roa1 has 94.7%, 61.4%, 33.0% and 31.4% sequence identities with its homologs in the human fungal pathogen C. dubliniensis (GenBank accession No. CAX44448), the lignocellulose-bioconverting and xylose-fermenting yeast Pichia stipitis (Jeffries et al. 2007), the yeast Debaryomyces hansenii (Dujon et al. 2004), Penicillium marneffei (Accession No. XP.002143557) causing a high incidence of penicilliosis in patients with AIDS in Southeast Asia (Sirisanthana and Supparatpinyo 1998) and the budding yeast S. cerevisiae, respectively (data not shown). These yeast species all belong to the CTG subclade of ascomycetous fungi, suggesting that Roa1 is conserved in this group of fungi.

Disruption of ROA1 causes Candida cells to be tolerant to azole drugs

To examine the functions of ROA1 in C. albicans cells, we generated its heterozygous mutants CNS1 (IPF7530/ipf7530::hisG-URA3-hisG) and CNS2 (IPF7530/ ipf7530::hisG) as well as its homozygous mutants CNS3 (ipf7530::hisG/ipf7530::hisG-URA3-hisG) and CNS4 (ipf7530::hisG / ipf7530::hisG) (Fig. S1, Supporting Information). As compared to the wild-type and the heterozygous mutant CNS2, the homozygous mutant CNS4 was tolerant to three types of imidazoles, 4 μg/ml ketoconazole (KCZ), 1 μg/ml econazole and 0.4 μg/ml clotrimazole, as well as two kinds of triazoles, 16 μg/ml fluconazole (FLU) and 2 μg/ml voriconazole (Fig. 1). Introduction of the orf19.4531 gene back to the homozygous mutant cells reversed theseazole-tolerant phenotypes (Fig. 1). However, no phenotype was observed for the homozygous mutant to 5 μg/ml tunicamycin and 300 μg/ml paromomycin sulfate, 10 μg/ml amphotericin B, 10 μg/ml nystatin and 5 μg/ml terbinafine as well as H2O2 and CdCl2 (Fig. 1; data not shown). Taken together, these results suggest that Roa1 is involved in azole sensitivity in C. albicans.

Roa1 localizes to a small compartment associated with the vacuolar membrane

To visualize the cellular localization of Roa1, we generated the pCR4-ROA1-GFP plasmid, expressing the ROA1-GFP fusion protein, and introduced it into the homozygous mutant cells. The ROA1-GFP fusion protein reverted the tolerant phenotype of the homozygous mutant to azole drugs (Fig. 2A), indicating its functionality. In log-phase growing cells, the ROA1-GFP protein localized to an unknown compartment represented by one intensely fluorescent small punctate structure, which immediately abuts the vacuolar membrane stained with the lipophylic dye FM4-64 (Fig. 2B). Taken together, these results suggest that Roa1 is present in a small compartment adjacent to the vacuolar membrane.

Expression of ROA1 is responsive to drug treatment

By comparing the cDNA microarray profiles of gene expression between a matched pair of fluconazole-susceptible and -tolerant bloodstream C. albicans isolates from bone marrow transplanted patients TL1/TL3, we previously found that the expression of the gene orf19.4531 (ROA1) was upregulated by 4.5 times in the
Figure 1. Disruption of IPF7530 (ROA1) causes C. albicans cells to be tolerant to azole antifungal drugs. RM1000 cells containing the vector, the heterozygous mutant CNS2 containing the vector and the homozygous mutant CNS4 cells containing the vector or the vector + IPF7530 gene were grown overnight in SD-URA medium, serially diluted by 10 times and spotted onto YPD plate and YPD plate containing various drugs including ketoconazole (KCZ), fluconazole (Flu), voriconazole (Vori), econazole (Econ), clotrimazole (Clo), tunicamycin (Tuni) and paromomycin sulfate (Paro), at indicated concentrations. Cells were incubated at 30 °C for 3–5 days before photos were taken.

Fluconazole-tolerant isolate (Marr et al. 2001; Xu et al. 2006). Here, we examined if expression of ROA1 could be induced by fluconazole azole treatment in the clinical strain SC5314 cells. In response to both 2 μg/ml fluconazole and 2 μg/ml ketoconazole, mRNA expression of ROA1 in log-phase growing SC5314 cells was significantly increased after 40 min treatment and then followed a decline trend in 60 min (Fig. 3). Expression of ROA1 reached the maximal level 20 min post ketoconazole treatment but 40 min post fluconazole treatment (Fig. 3), suggesting the transcriptional expression of ROA1 has an earlier response to ketoconazole than fluconazole.

Deletion of ROA1 increases the plasma membrane potential

To explore the mechanism by which the deletion of ROA1 increases the tolerance of C. albicans cells to theazole antifungals, we stained C. albicans cells with DiBAC, an anionic lipophilic dye sensitive to the plasma membrane potential (Liao, Rennie and Talbot 1999). In normal YPD medium, the heterozygous mutant and the homozygous mutant showed similar percentages of fluorescent cells, which were dramatically lower than those in the wild type and the complemented strain (CNS4+pRC2312-ROA1) (Fig. 4). Similarly, in the presence of azoles, both the heterozygous mutant and the homozygous mutant showed lower percentages of DiBAC-fluorescent cells than the wild type and the complemented strains (Fig. 4). These data indicate that deletion of ROA1 increases the membrane potential of C. albicans cells independent of theazole antifungals. Consistent with a previous observation thatazole treatment did not increase the intensity of fluorescence in C. albicans cells after DiBAC staining (Teixeira-Santos et al. 2012), we found that neither ketoconazole nor fluconazole increased percentages of fluorescent cells for both the wild type and the complemented strains after DiBAC staining.

Figure 1. Disruption of IPF7530 (ROA1) causes C. albicans cells to be tolerant to azole antifungal drugs. RM1000 cells containing the vector, the heterozygous mutant CNS2 containing the vector and the homozygous mutant CNS4 cells containing the vector or the vector + IPF7530 gene were grown overnight in SD-URA medium, serially diluted by 10 times and spotted onto YPD plate and YPD plate containing various drugs including ketoconazole (KCZ), fluconazole (Flu), voriconazole (Vori), econazole (Econ), clotrimazole (Clo), tunicamycin (Tuni) and paromomycin sulfate (Paro), at indicated concentrations. Cells were incubated at 30 °C for 3–5 days before photos were taken.

Figure 2. Subcellular localization of ROA1-GFP. (A) Functional complementation of the ROA1-GFP fusion protein. RM1000 cells containing the pCR4 vector and the homozygous mutant cells containing pCR4 or pCR4-ROA-GFP were grown overnight and serially diluted before being spotted onto YPD plate and YPD plate containing 4 μg/ml ketoconazole or 16 μg/ml fluconazole. Plates were incubated at 30 °C for 2–3 days before photos were taken. (B) Subcellular localization of ROA1-GFP. The homozygous mutant cells containing the pCR4-ROA1-GFP, expressing a ROA1-GFP fusion protein, under the control of its own promoter were grown to log-phase at 30 °C in SD-URA medium. FM4-64 dye was added to stain the vacuolar membrane 30 min before cells were taken for examination under fluorescent microscope using appropriate filters with a Nikon microscope at a 1000 × magnification. Arrows indicate the intensely fluorescent small punctate structure, which immediately abuts the vacuolar membrane.
mutation on the membrane potential of ROA1 (orf19.4513) is coordinately unregulated with ergosterol and ERG1 in C. albicans. The pathogenesis of the human pathogen C. albicans is linked to its morphological switch from budding yeast form to hyphal form under certain conditions (Chauhan, Latge and Calderone 2006). We have not observed any defect for C. albicans cells lacking ROA1 in vitro filamentation in solid and liquid hyphal induction media. Therefore, Roa1 is not essential for the in vitro

[Image 54x498 to 288x724]
Figure 4. Visualization and quantification of DiBAC-fluorescent cells. (A) Overnight cultures of the wild type (WT) RM1000, the heterozygous mutant CNS2 and the homozygous mutant CNS4, containing the vector, as well as the complemented strain CN4 containing pRC2312-ROA1 in SD-URA medium were grown to log phase at 30°C in YPD medium, and treated with either 4 μg/ml ketoconazole or 16 μg/ml fluconazole for 3 h before they were stained with the dye DaBAC. Stained cells were visualized under a Nikon ECLIPSE 80i fluorescent microscope equipped with Plan flour 100/1.3 oil objective and cube filter FITC (EX 465-495, EM515-555). Representative images of each strain were presented. (B) Percentages of DiBAC-fluorescent cells from a total of approximately 500 cells in a bright field were calculated from each strain. Values were means of three independent experiments. The asterisk (*) shows statistically significant differences (P < 0.05) between the wild type and each of the two mutants and the complemented strain in the absence or presence of the azoles. The number sign (#) indicates statistically significant differences (P < 0.05) between the heterozygous mutant and the homozygous mutant.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSyr online.

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Conflict of interest. None declared.

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