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Research Article

RTA2 is involved in calcineurin-mediated azole resistance and sphingoid long-chain base release in *Candida albicans*

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Abstract. The calcineurin pathway has been reported to be essential for the development of azole resistance in *Candida albicans*. The depletion or ectopic over-expression of *RTA2* increased or decreased susceptibility of *C. albicans* to azoles, respectively. $CaCl_2$-induced activation of the calcineurin pathway in wild-type *C. albicans* promoted resistance to azoles, while the Ca^{2+} chelator (EGTA), calcineurin inhibitors (FK506 and cyclosporin A) and the deletion of *RTA2* blocked the resistance-promoting effects of $CaCl_2$. Furthermore, we found that *RTA2* was up-regulated in a calcineurin-dependent manner. The depletion of *RTA2* also made the cell membrane of *C. albicans* liable to be destroyed by azoles and *RTA2* over-expression attenuated the destroying effects. Finally, the disruption of *RTA2* caused an increased accumulation of dihydrosphingosine (DHS), one of the two sphingolipid long-chain bases, by decreasing release of DHS. In conclusion, our findings suggest that *RTA2* is involved in calcineurin-mediated azole resistance and sphingoid long-chain base release in *C. albicans*.

Keywords. *Candida albicans*, *RTA2*, azole resistance, calcineurin pathway, sphingoid.

Introduction

The rapid development and spread of multi-drug resistance (MDR) has become an increasingly major threat to public health in recent years [1–3]. Azoles, particularly fluconazole (FLC), have become routine antifungal agents for *Candida albicans* in clinical practice due to their high *in vivo* absorption and fewer adverse effects; unfortunately, resistance of *C. albicans* to azoles, including stable resistance, phenotypic resistance, and tolerance, is commonly seen in clinical isolates [4]. Up to now, only a few MDR efflux genes (*CDR1*, *CDR2* and *CaMDR1*) were confirmed to participate in the development of azole resistance [5–9]. Moreover, overexpression or mutations of *ERG11*, encoding gene of lanosterol 14-α demethylase targeted by azoles, were found capable of causing azole resistance [4, 10, 11].

The calcineurin pathway has also been held responsible for the development of azole resistance in *C. albicans* [12–17]. Calcineurin is a Ca^{2+}-calmodulin-dependent serine/threonine phosphatase consisting of a catalytic subunit A (encoded by *CNA1*) and a regulatory subunit B (encoded by *CNB1*) [18]. The phosphatase activity of calcineurin is activated when calcineurin binds calmodulin in the presence of calcium ions; activated calcineurin then regulates

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Table 1. *C. albicans* strains used in this study.

Strain	Parental strain	Genotype	Reference
RM1000	RM100	*ura3Δ::imm^{434}/ura3Δ::imm^{434}, his1Δ::HisG/his1Δ::HisG, iro1Δ::imm^{434}/iro1Δ::imm^{434}*	[46]
JXM10	RM1000	*RM1000*rta2Δ::hisG-URA3-hisG/RTA2*	This study
JXM11	JXM10	*RM1000*rta2Δ::hisG/RTA2*	This study
JXM100	JXM11	*RM1000*rta2Δ::hisG/rta2Δ::hisG-URA3-hisG*	This study
JXM101	JXM100	*RM1000*rta2Δ::hisG/rta2Δ::hisG*	This study
ER-J9	JXM101	*RM1000*rta2Δ::hisG/rta2Δ::hisG RP10/rp10Δ::pExpRTA2-URA3*	This study
ER-J10	JXM101	*RM1000*rta2Δ::hisG/rta2Δ::hisG RP10/rp10Δ::pExpRTA2-URA3*	This study
Exp-JXM	JXM101	*RM1000*rta2Δ::hisG/rta2Δ::hisG RP10/rp10Δ::pCaExp-URA3*	This study
Exp-RM	RM1000	*RM1000§RP10/rp10Δ::pCaExp-URA3*	This study
CAF2–1	SC5314	*ura3Δ::imm434/URA3*	[23]
DSY2091	CAF4–2	*cnaΔ::hisG/cnaΔ::hisG-URA3-hisG*	[13]
DSY2115	DSY2101	*cnaΔ::hisG/cnaΔ::hisG; LEU2::CNA/URA3*	[15]
DSY2195	DSY2188	*crz1Δ::hisG/crz1Δ::hisG-URA3-hisG*	[13]
MKY268	MKY59	*crz1Δ::hisG/crz1Δ::hisG; LEU2::CRZ1/URA3*	[13]

* RM1000 background.

the downstream gene expression *via* transcriptional factor such as Crz1p [13, 19]. Heat shock protein 90 (Hsp90), a molecular chaperone, can accelerate the evolution of rapid resistance to azoles in *C. albicans*. Furthermore, calcineurin, a client protein of Hsp90, is a key effector of Hsp90-mediated azole resistance [20, 21]. However, the downstream target genes involved in calcineurin-mediated azole-resistance of *C. albicans* are still not characterized.

RTA2 (also known as orf19.24), a potential stress-related gene probably encoding a phospholipid translocase, was investigated in this work. First, we successfully constructed the *rta2* null mutant in *C. albicans* and demonstrated that the depletion of *RTA2* increased susceptibility of *C. albicans* to azoles, while ectopic overexpression of *RTA2* conferred resistance of *C. albicans* to azoles. Finally, we concluded that *RTA2* is responsible for the emergence of calcineurin-mediated azole resistance and sphingoid long-chain base release in *C. albicans*.

Materials and methods

Drugs. FLC was from Pfizer Inc (New York, NY). Fluphenazine, ketoconazole, econazole, cycloheximide, nystatin, EGTA, FK506, cyclosporin A (CsA), fumonisin B1 and myriocin were purchased from Sigma (St. Louis, MO). Brefeldin A and cerulenin were obtained from Serva Electrophoresis (Heidelberg, Germany). Itraconazole and terbinafine were provided as gifts by Professor Liu Chao-mei and voriconazole was a gift from Professor Zhang Wannian of Second Military Medical University (Shanghai, China).

***C. albicans* strains and culture media.** *C. albicans* strains used in this study are listed in Table 1 and cultured in YPD medium or SC medium.

Construction of mutant strains. All the primer sequences are listed in Supplementary Table 1. The construction of plasmid pUC-RTA2-URA3 was done as shown in Figure 1A. Briefly, the fragment containing 5' and 3' ends of *RTA2* gene for homologous recombination was obtained according to the Fusion PCR method [22]. The 4-kb *hisG-URA3-hisG* fragment was from the plasmid p5921[23]. The two fragments were subsequently cloned into plasmid pUCm-t (Sangon, Shanghai, China). The *Xho*I-digested fragment of pUC-RTA2-URA3 was transformed into the *ura3Δ/Δ* mutant (RM1000) by standard methods[24].

Overexpression of *RTA2* in *rta2* mutants. The *RTA2* ORF was amplified by PCR with Pyrobest polymerase (TaKaRa). The *Bam*HI–*Pst*I digested PCR fragment was ligated into pCaExp [25] to obtain recombinant plasmid pEXP-RTA2. DNA sequencing confirmed that the sequence of the insert was identical to *RTA2* sequence reported in the *Candida* Genome Database (http://www.candidagenome.org/). The *rta2Δ/Δ* mutant (JXM101) was transformed with the linearized pEXP-RTA2 by *Stu*I and selected on SC medium lacking uridine, methionine and cysteine.

Susceptibility testing. Drug sensitivities were assayed by microbroth dilution in 96-well plates as described previously [26]. Briefly, cells were inoculated into successive wells of a 96-well microtiter plate containing serial two-fold dilutions of antifungal drugs in RPMI 1640 medium buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid). After incubation at 30°C for 24 h, absorbance at 630 nm was determined in a microplate reader. Drug concentrations inhibiting growth to 80 % of control levels (MIC_{80}) were estimated by interpolation.

Drug sensitivities were also assayed on YPD agar plates containing drugs at the indicated concentrations. DMSO, the drug vehicle, was ≤ 0.5 % in all cases. Five microliters of tenfold serial dilutions of each yeast culture (OD_{600} =1.0) was spotted on the appropriate medium plates and then incubated at 30°C for indicated time.

Relative quantification of differentially expressed genes by quantitative RT-PCR. All the primer sequences are listed in Supplementary Table 1. RNA isolation, cDNA synthesis, and PCR amplification were done as described previously [27]. Triplicate independent quantitative RT-PCRs were performed using the LightCycler System (Roche diagnostics). The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$[27].

Transmission electron microscopy. In the absence or presence of FLC, $CaCl_2$ or FLC plus $CaCl_2$, *C. albicans* cells (RM1000 and JXM101) were collected after 16 h of growth in liquid RPMI 1640 medium supplemented with 0.0025 % uridine, washed twice with PBS solution, fixed at 4°C for 24 h in 500 µl fixative solution (sodium cacodylate buffer, pH 7.2, containing 4 % polyoxymethylene). The samples were then washed with saline and postfixed for 90 min with 1 % phosphotungstic acid. The fixed cells were dehydrated through a graded series of ethanol and embedded with EPON-812. Ultrathin sections were prepared and observed after double staining with uranium and lead under a transmission electron microscope (HITACHI H-800, Japan) with 8×10^4 magnification.

Sterol analysis. Samples for gas chromatography-mass spectrometry were prepared from 100-ml cultures obtained by incubating cells in the presence or absence of drug at the indicated concentrations for 16 h in RPMI 1640 medium supplemented with 0.0025 % uridine. The cell pellet was saponified in 15 % (wt/vol) NaOH in 90 % (vol/vol) ethanol at 80°C for 1 h. Nonsaponifiable lipids (sterols and sterol precursors) were extracted three times with 5 ml hexane and dried under nitrogen. Following silylation for 1 h at 60°C with TMSI (30 µl) in 30 µl pyridine, sterols were analyzed by gas chromatography-mass spectrometry (Finnigan Voyager, USA) by using split injections with a split ratio of 15:1. Sterol identification was by reference to relative retention times and mass spectra, as reported previously [28, 29].

Rhodamine 6G efflux. Efflux of rhodamine 6G was determined according to our previously described protocol [30].

Assaying NBD-DHS uptake and release. *Candida* cells grown at 30°C to 1 OD_{600} unit/ml were treated with 5 µM C_6 NBD labeled D-erythro-dihydrosphingosine (NBD-DHS) (Larodan Fine Chemical AB, Malmö, Sweden), which had been complexed with 1 mg/ml bovine serum albumin (BSA; A-6003; Sigma-Aldrich, St. Louis, MO), and incubated for various time periods. For uptake analysis, cells equivalent to 0.45 OD_{600} were chilled on ice, washed twice, and suspended in 500 µl PBS at 0, 5, 20 and 60 min after addition of NBD-DHS. Living cells were immobilized on 0.1 % poly-L-lysine-coated slides. Specimens were viewed using Leica TCS SP2 confocal microscope (Leica, Germany) with 40×PlanApochromat oil-immersion objectives. A confocal picture was generated with the 488 nm line of the argon laser. The emission light was recorded by a photo multiplier after passing a 520–565-nm band-pass filter under manual gain and black level control.

In the DHS release assay, cells preloaded with 5 µM NBD-DHS for 1 h were washed twice, resuspended in YPD medium supplemented with 1 mg/ml BSA and incubated for the indicated periods (0, 10 min, and 30 min) at 30°C. Cells were pelleted by centrifugation and the concentrations of NBD-DHS in the supernatant were quantified fluorimetrically using a standard curve for NBD-DHS in PBS buffer (excitation wavelength, 475 nm; emission wavelength, 525 nm; PolarStar, BMG LabTechnologies).

Results

Characterization of *RTA2* gene. A BLAST search of the *C. albicans* genome (available at http://www.genolist.pasteur.fr/CandidaDB/) revealed that the *RTA2* gene (also known as orf19.24) encodes a putative polypeptide of 453 amino acids, with a deduced molecular mass of 50.9 kDa. The SOSUI [31] programs predicted Rta2p to be an integral membrane protein with seven membrane-spanning segments. There are five Rta2p homologs (Rsb1p, Rtm1p, Yer185w, Rta1p, and Ylr046c) in the *Saccharomyces cerevisiae* genome database (available at

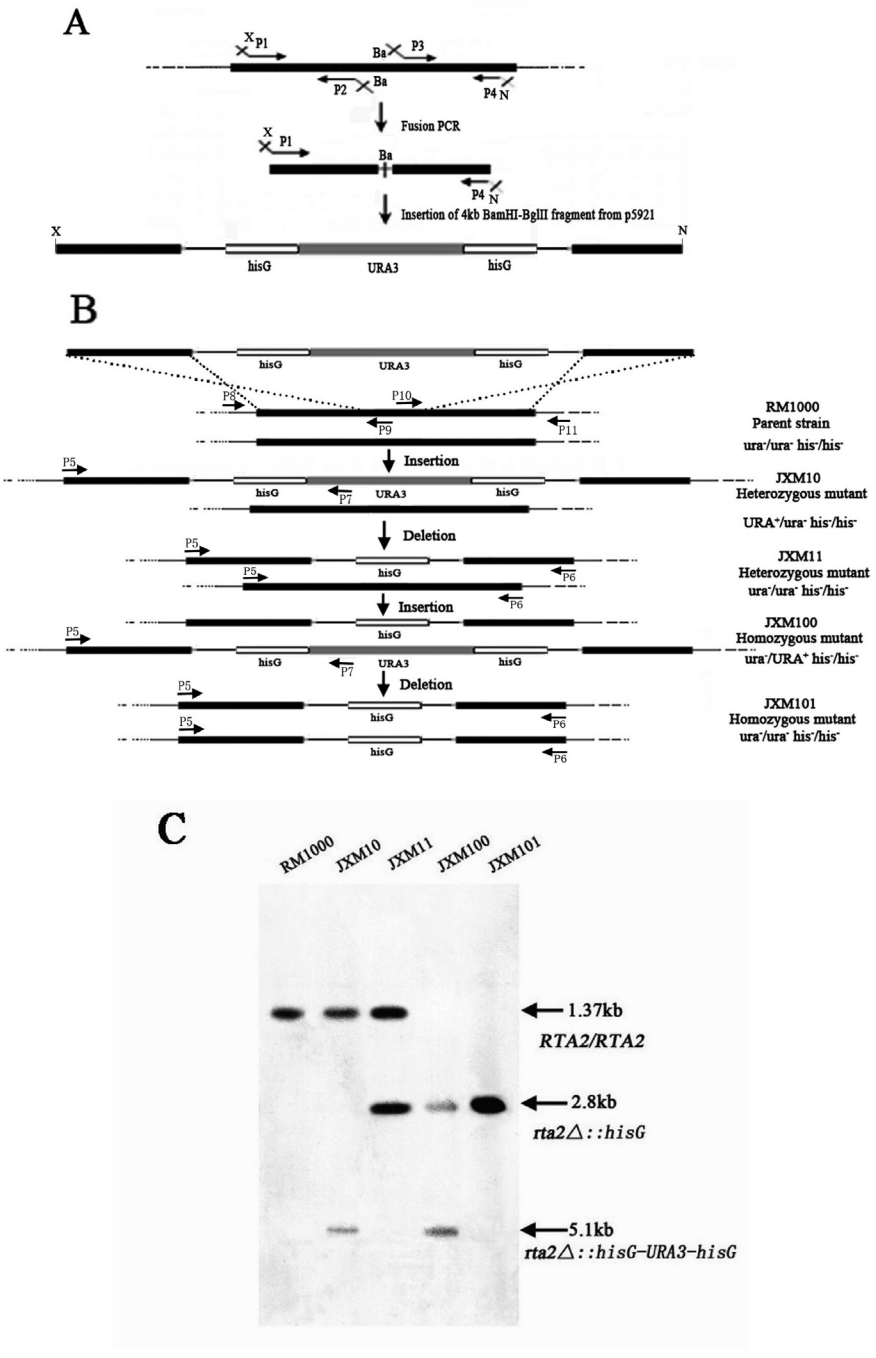

Figure 1. Schematic representation of the plasmid construction and disruption of *RTA2* using the *URA3* selection marker. (*A*) Diagrammatic representation of the plasmid construction of pUC-RTA2-URA3 containing *hisG-URA3-hisG* with flanking portions on either side for homologous recombination. Ba: *Bam*HI, X: *Xho*I, N: *Nde*I. (*B*) Sequential targeted disruption of the two *RTA2* alleles in *Candida albicans* with the disruption cassette. Strain designation is shown on the right. (*C*) Southern analysis of the genomic DNA digested with *Bgl*II and *Sal*I. The exact size and genotype of the expected hybridizing DNA fragment are indicated on the right. The hybridization probe was the 0.686-kb PCR fragment (from –455 to 231 of *RTA2* gene) amplified by Pyrobest polymerase (TaKaRa) from the genomic DNA of RM1000.

http://www.yeastgenome.org/) that have a 25–38% identity and a 44–61% similarity. More importantly, like its *S. cerevisiae* homolog Rsb1p, Rta2p also has a long C-terminal tail and contains an extended loop between the predicted transmembrane domains 5 and 6. Rsb1p is a translocase (floppase) that translocates sphingolipid long chain bases (LCBs) from inside to the outside of the membrane in *S. cerevisiae* [32]. Bioinformatics analysis also revealed that the promoter of *RTA2* gene contained calcineurin-dependent responsive element (CDRE) sequence, which is controlled by either calcineurin and/or Crz1p [13]. However, to now the functions of Rta2p remained unknown.

Disruption of *RTA2* gene in *C. albicans*. To investigate the functions of *RTA2*, we constructed *C. albicans rta2* null mutant using wild-type strain (RM1000). Both *RTA2* alleles were successfully disrupted sequentially using the disruption cassette (Fig. 1A, B). Replacements of the *RTA2* alleles with linear fragments were monitored by PCR with the primers (as

shown in Fig. 1B) for the flanking region of homology to *RTA2* and for *URA3* (Supplementary Fig. 1A–C). The absence of a product using primers (as shown in Fig. 1B) inside and outside the disruption fragment confirmed that *RTA2* sequence had not relocated to another position in the genome (Supplementary Fig. 1D). Further verification of the mutants including JXM10 (*RTA2/ rta2*Δ::*hisG-URA3-hisG*), JXM11 (*RTA2/ rta2*Δ::*hisG*), JXM100 (*rta2*Δ::*hisG-URA3-hisG*/*rta2*Δ::*hisG*) and JXM101 (*rta2*Δ::*hisG*/*rta2*Δ::*hisG*) were undertaken by Southern blot analysis with a probe to the *RTA2* promoter (Fig. 1C).

***C. albicans rta2* mutant displayed an increased sensitivity specifically to azoles *in vitro*.** Drug susceptibility assay demonstrated that the *rta2Δ/Δ* mutant (JXM101) were more susceptible to azoles, including FLC, ketoconazole, econazole, itraconazole, and voriconazole (Fig. 2A, B). Among other medically relevant antifungal agents, terbinafine (an allylamine-inhibiting squalene epoxidase) and nystatin (a polyene antifungal that binds to ergosterol in membranes) did not influence the susceptibility of JXM101 (Fig. 2B). Other metabolic inhibitors such as cycloheximide (a protein biosynthesis inhibitor), brefeldin A (an inhibitor of organelle assembly), fluphenazine (a calmodulin antagonist) and cerulenin (an inhibitor of fatty acid biosynthesis) had a similar effect on the growth of the JXM101 and RM1000 (Fig. 2B).

Ectopic overexpression of *RTA2* conferred resistance to azoles. The *RTA2* ORF was placed under the control of *MET3* promoter in the vector pCaEXP and was successfully integrated into the *rta2Δ/Δ* mutant (JXM101) at the *RP10* locus as determined by PCR (data not shown). Ectopic overexpression of *RTA2* in JXM101 transformed with overexpressing vector pCaEXP-RTA2 was confirmed by quantitative RT-PCR, with its expression in its parental stain (RM1000) harboring empty vector pCaEXP serving as a control (Fig. 3A). The growth of JXM101 transformed with empty vector pCaEXP was severely impaired on SC plates in the presence of azoles, just like those on YPD plates (Fig. 2C). Drug susceptibility assay also showed that the *rta2Δ/Δ* mutant (JXM101) transformed with pCaEXP-RTA2 rendered more resistance to azoles than did its parental strain (RM1000) harboring pCaEXP (Fig. 2C). Together, these results demonstrate a critical role of *RTA2* in the development of azole resistance.

***RTA2* was responsible for the development of calcineurin-mediated azole resistance.** Calcium signaling, which activates the calcineurin pathway, can modulate azole activity in *C. albicans* [16, 17]. The influence of Ca^{2+} on the sensitivities of the wild-type strain (RM1000) and the *rta2Δ/Δ* mutant (JXM101) to FLC was examined. The addition of 1 mmol/l $CaCl_2$ promoted dramatic resistance to FLC in RM1000, with MIC_{80} increased from 0.5 to 128 μg/ml (Table 2). Deletion of *RTA2* completely abolished the promoting effect of $CaCl_2$ on resistance to FLC, with MIC_{80} only increased from 0.125 to 0.25 μg/ml in JXM101 (Table 2). The addition of the Ca^{2+} chelator EGTA (1 mmol/l) removed the resistance-promoting effect of $CaCl_2$ in RM1000. By themselves, neither $CaCl_2$ nor EGTA at the concentrations tested had effects on *C. albicans* growth. Similar effects of $CaCl_2$ and EGTA on the sensitivity to ketoconazole were also observed in RM1000 and JXM101 (Table 2). In contrast to the results presented above, the addition of $CaCl_2$ (1 mmol/l) had no effect on the nystatin (a polyene antifungal antibiotic) activity in the RM1000 and JXM101 strains (Table 3).

A well-characterized target for Ca^{2+}-calmodulin activation is calcineurin and activated calcineurin then regulates the downstream gene expression *via* transcriptional factor Crz1p [13, 16]. The potential role of this phosphatase in mediating the promoting effects of Ca^{2+} on azoles resistance was examined using the calcineurin inhibitors FK506 and CsA in the wild-type strain (RM1000) and the *rta2Δ/Δ* mutant (JXM101). Both FK506 (3 μg/ml) and CsA (3 μg/ml) blocked the resistance-promoting effects of $CaCl_2$ to FLC/ketoconazole in RM1000. However, adding $CaCl_2$ alone or $CaCl_2$ plus FK506 or CsA in JXM101 had little influence on azole resistance (Table 2).

We also examined the influence of Ca^{2+} on the sensitivities of the wild-type strain (CAF2–1), the *cna*Δ/Δ mutant (DSY2091), the *crz1*Δ/Δ mutant (DSY2195), the *CNA* revertant strain (DSY2115) and the *CRZ1* revertant strain (MKY268) (Table 1) to FLC. The addition of 1 mmol/l $CaCl_2$ also promoted dramatic resistance to FLC in CAF2–1, with MIC_{80} increased from 0.5 to 128 μg/ml (Table 2). Similar to the deletion of *RTA2*, the depletion of *CNA* or *CRZ1* also completely abolished the promoting effect of $CaCl_2$ on resistance to FLC, with MIC_{80}s only increased from 0.06 to 0.125 μg/ml in the *cna*Δ/Δ mutant (DSY2091) and 0.125 to 0.25 μg/ml in the *crz1*Δ/Δ mutant (DSY2195) (Table 2). The addition of 1 mmol/l $CaCl_2$ also promoted resistance to FLC in the *CNA* revertant strain (DSY2115) and the *CRZ1* revertant strain (MKY268), with MIC_{80}s increased from 0.5 to 16 μg/ml (Table 2). The addition of the Ca^{2+} chelator EGTA (1 mmol/l) removed the resistance-promoting effect of $CaCl_2$ in the wild-type strain (CAF2–1), the *CNA* revertant strain (DSY2115) and the *CRZ1* revertant strain (MKY268) (Table 2). By themselves, neither $CaCl_2$ nor EGTA at the concen-

A

	MIC_{80}[a] (μg/ml) of				
	fluconazole	econazole	itraconazole	ketoconazole	voriconazole
RM1000	0.5	0.0165	0.125	0.0625	0.001
JXM11	0.25	0.008	0.125	0.0156	0.001
JXM101	0.125	0.002	0.016	<0.002	0.0002

[a]MICs were determined by the method described in Materials and Methods. The results were the means of three times.

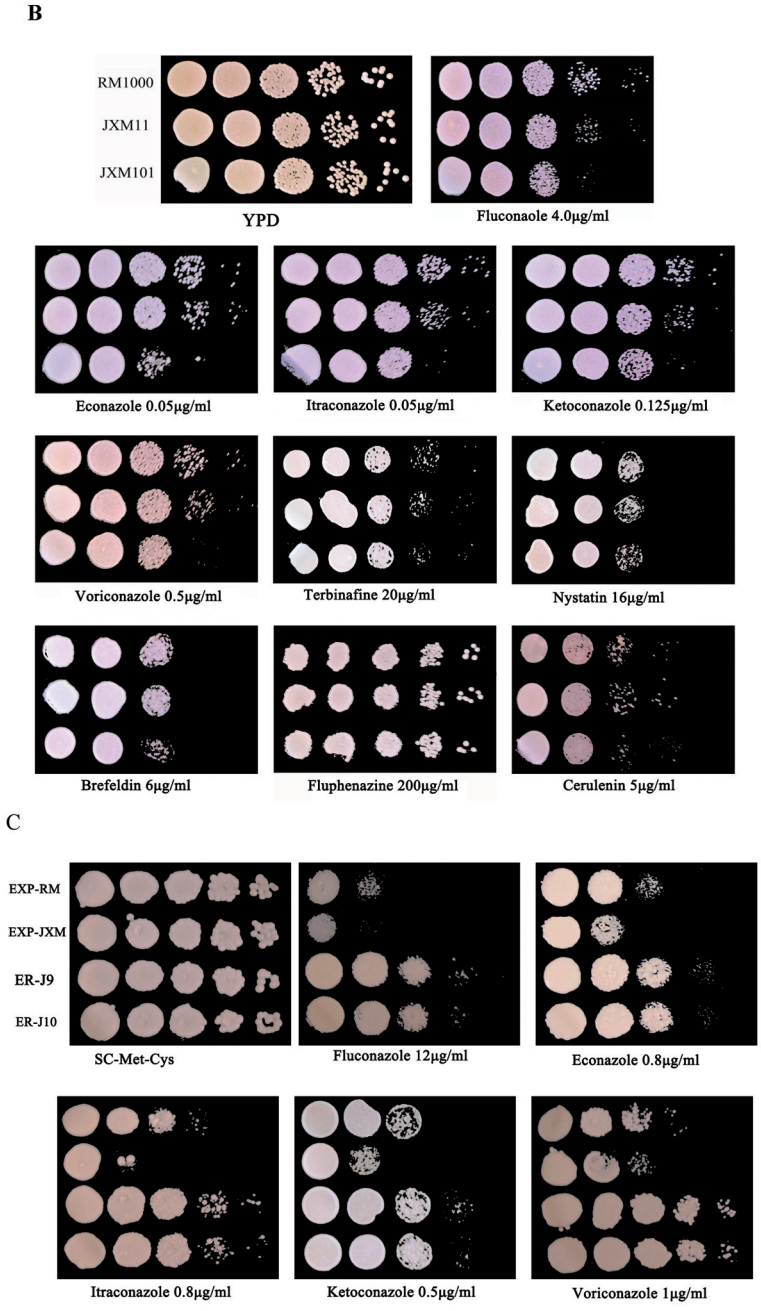

Figure 2. Drug susceptibility profiles of *C. albicans* strains (Table 1). (*A*) Drug susceptibilities profiles of the *C. albicans* wild type (RM1000) and *rta2* mutant (JXM101) determined by broth microdilution assay. (*B*) The *rta2* heterozygous and homozygous mutant strains from the wild-type strain (RM1000) were spotted on YPD agar plates supplemented with 0.0025% uridine with or without different antifungal agents at indicated concentrations. Plates were incubated for 48 h at 30°C. (*C*) The strains indicated on the left were spotted on SC medium plates (lacking methionine, cysteine, and uridine) with or without azoles at indicated concentrations. The strain names were designated as ER-J9 and ER-J10 (JXM101 harboring pCaEXP-RTA2), EXP-JXM (JXM101 harboring pCaEXP) and EXP-RM (RM1000 harboring pCaEXP). Plates were incubated for 72 h at 30°C. The names of strains are indicated on the left.

trations tested had effects on *C. albicans* growth. Similar effects of $CaCl_2$ and EGTA on the sensitivity to ketoconazole were also observed in CAF2–1, DSY2091, DSY2195, DSY2115 and MKY268 (Table 2). In contrast to the results presented above, the addition of $CaCl_2$ (1 mmol/l) had no effect on the nystatin (a polyene antifungal antibiotic) activity in any of the strains mentioned above (Table 2). In

Table 2. Effects of $CaCl_2$, EGTA and calcineurin inhibitors on antifungal activity *versus C. albicans* strains.

Strains	Anifungal MIC_{80} (μg/ml)[a]											
	Fluconazole					Ketoconazole					Nystatin	
	Control	+ $CaCl_2$ (1 mM)	+ $CaCl_2$ (1 mM) + EGTA (1 mM)	+ $CaCl_2$ (1 mM) +FK506 (3 μg/ml)	+ $CaCl_2$ (1 mM) + CsA (3 μg/ml)	Control	+ $CaCl_2$ (1 mM)	+ $CaCl_2$ (1 mM) + EGTA (1 mM)	+ $CaCl_2$ (1 mM) +FK506 (3 μg/ml)	+ $CaCl_2$ (1 mM) + CsA (3 μg/ml)	Control	+ $CaCl_2$ (1 mM)
RM1000	0.5	>128	0.25	0.25	0.25	0.0625	4	< 0.002	< 0.002	< 0.002	3.25	3.25
JXM101	0.125	0.25	0.125	0.25	0.125	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	3.25	3.25
CAF2–1	0.5	>128	0.125	0.125	0.125	0.0625	4	< 0.002	< 0.002	< 0.002	3.25	3.25
DSY2091	0.06	0.125	0.125	0.06	0.06	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	3.25	3.25
DSY2115	0.5	16	0.25	0.5	0.25	0.0625	1	< 0.002	< 0.002	< 0.002	3.25	3.25
DSY2195	0.125	0.25	0.125	0.125	0.125	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	3.25	3.25
MKY268	0.5	16	0.25	0.5	0.25	0.0625	1	< 0.002	< 0.002	< 0.002	3.25	3.25

[a] Minimum inhibitory concentrations inhibiting growth to 80 % of control levels (MIC_{80}s) were determined after 48-h incubation.

conclusion, it is possible that *RTA2* coordinates with calcineurin in response to azoles.

***RTA2* was up-regulated by Ca^{2+} in a calcineurin-dependent manner.** Bioinformatics analysis revealed that the promoter of *RTA2* contained a CDRE sequence and the expression of *RTA2* was found to be up-regulated by 200 mmol/l $CaCl_2$ in a calcineurin-dependent manner [13]. However, the mammalian serum concentration of ionized calcium is about 1–2 mmol/l. In our study, the expression levels of *RTA2* were examined in the wild-type strains (RM1000 and CAF2–1), the *cna*Δ/Δ mutant (DSY2091), the *crz1*Δ/Δ mutant (DSY2195), the *CNA* revertant strain (DSY2115) and the *CRZ1* revertant strain (MKY268) after exposure to 1 mmol/l $CaCl_2$ for 16 h using quantitative RT-PCR (Fig. 3B, C). *RTA2* was up-regulated to 2.35-fold in RM1000 (Fig. 3B) and to 3.82-fold in CAF2–1 (Fig. 3C). The calcineurin inhibitor FK506 at 3 μg/ml removed the effects of $CaCl_2$ on *RTA2* up-regulation in RM1000 (Fig. 3B). The depletion of *CNA* or *CRZ1* also removed the effects of $CaCl_2$ on *RTA2* up-regulation with 1.37-fold change in the *cna*Δ/Δ mutant (DSY2091) and 0.68-fold change in the *crz1*Δ/Δ mutant (DSY2195) (Fig. 3C). However, the re-introduction of *CNA* or *CRZ1* in their null mutant strains restored the effects of 1 mmol/l $CaCl_2$ on *RTA2* up-regulation with 2.85-fold change in the *CNA* revertant strain (DSY2115) and 2.45-fold change in the *CRZ1* revertant strain (MKY268) (Fig. 3C). Taken together, these data support the model that *RTA2* plays an intimate role in calcineurin-mediated resistance to azoles.

Cell membranes of *C. albicans* were destroyed by azoles in a *RTA2*-dependent manner. To determine whether the cell membranes of *C. albicans* were damaged by azoles in a *RTA2*-dependent manner, transmission electron microscopy was performed to observe the ultrastructural changes of the *rta2Δ/Δ* mutant (JXM101) and the wild-type strain (RM1000) in the absence or presence of FLC. High magnification of untreated JXM101 and RM1000 showed intact cell membranes with a continuous triple-layered profile (Fig. 4A, C). No visible changes were seen in the shape or size of the cell membranes of RM1000 and JXM101 after treatment with 1 mmol/l $CaCl_2$ alone (Fig. 4B, D). After being treated with 2 μg/ml FLC, JXM101 showed severely damaged cell membranes with extensive solubilization (Fig. 4G). Ultrastructural images of plasma membrane splitting were seen in JXM101 when treated by 8 μg/ml FLC (Fig. 4K). The addition of 1 mmol/l $CaCl_2$ did not attenuate the destroying effects of FLC (Fig. 4H, L). In contrast, the cell membranes of RM1000 remained intact after treatment with 2 μg/ml FLC and were modestly damaged with partial solubilization when treated by 8 μg/ml FLC (Fig. 4E, I). But the addition of 1 mmol/l $CaCl_2$ completely attenuated the destroying effects of 8 μg/ml FLC (Fig. 4J). Taken together, the depletion of *RTA2* makes the plasma membrane of *C. albicans* liable to destruction by FLC and Ca^{2+}-induced up-regulation of *RTA2* attenuated the destroying effects.

***RTA2* gene did not affected the ergosterol biosynthesis of *C. albicans*.** Since the cell membranes of *C. albicans* were damaged by azoles in a *RTA2*-dependent manner, sterol composition of both wild-type strain (RM1000) and the *rta2Δ/Δ* mutant (JXM101) was analyzed and compared with each

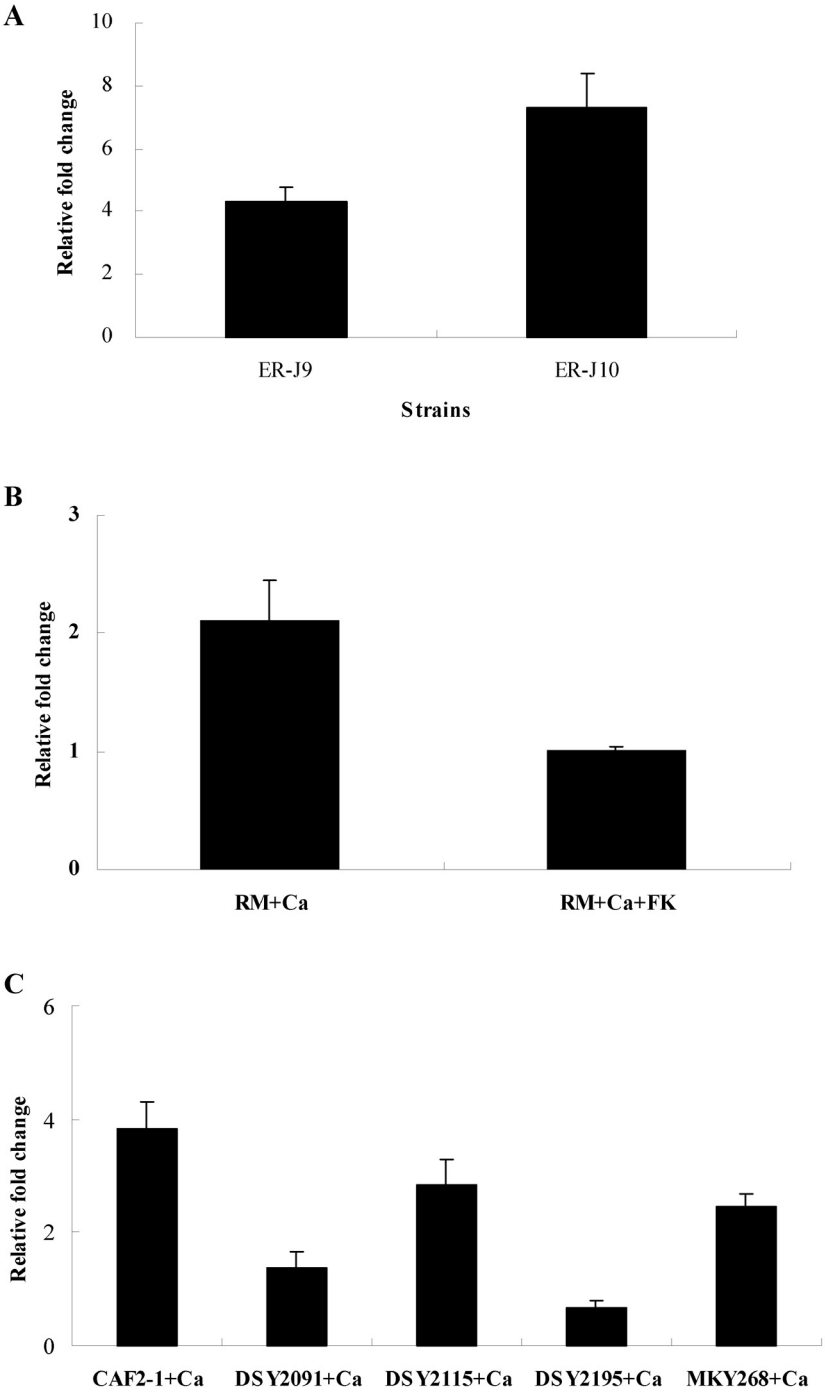

Figure 3. (*A*) Expression levels of *RTA2* in ER-J9 and ER-J10 (JXM101 harboring pCaEXP-RTA2) were compared with those of EXP-RM (RM1000 harboring pCaEXP), respectively. Strains were cultured in SC medium lacking methionine, cysteine, and uridine for 16 h and harvested for quantitative RT-PCR analysis. (*B*) Expression levels of *RTA2* were examined by quantitative RT-PCR in the wild-type strain RM1000 after exposure to 1 mmol/l $CaCl_2$ (RM+Ca, left column) or a combination of 1 mmol/l $CaCl_2$ and 3 μg/ml FK506 (RM+Ca+FK, right column), with drug-free RM1000 serving as a control. (*C*) Expression levels of *RTA2* were examined by quantitative RT-PCR in the wild-type strain (CAF2–1), the *cna*Δ/Δ mutant (DSY2091), the *crz1*Δ/Δ mutant (DSY2195), the *CNA* revertant strain (DSY2115) and the *CRZ1* revertant strain (MKY268) in the presence of 1 mmol/l $CaCl_2$, with their corresponding strains in the absence of $CaCl_2$ as controls. Strains were cultured in RPMI 1640 medium supplemented with 0.0025% uridine for 16 h and harvested for quantitative RT-PCR analysis. Data are represented as means ± SD.

other in the presence or absence of FLC, $CaCl_2$ or FLC plus $CaCl_2$. As shown in Table 3, ergosterol, the major sterol in JXM101, showed no statistically significant change compared with that of RM1000 in the presence or absence of 1 mM $CaCl_2$. After being treated by 2 or 8 μg/ml FLC, there was no significant change in 24-methylene lanosterol (eburicol), the main sterol intermediate, between RM1000 and JXM101(Table 3). The addition of 1 mmol/l $CaCl_2$ did not alter the inhibition of ergosterol biosynthesis by FLC in RM1000 (Table 3). Therefore, we excluded the possibility that the depletion of *RTA2* made the plasma membrane of *C. albicans* liable to destruction by affecting either ergosterol biosynthesis or the inhibitory effects of azoles on ergosterol biosynthesis.

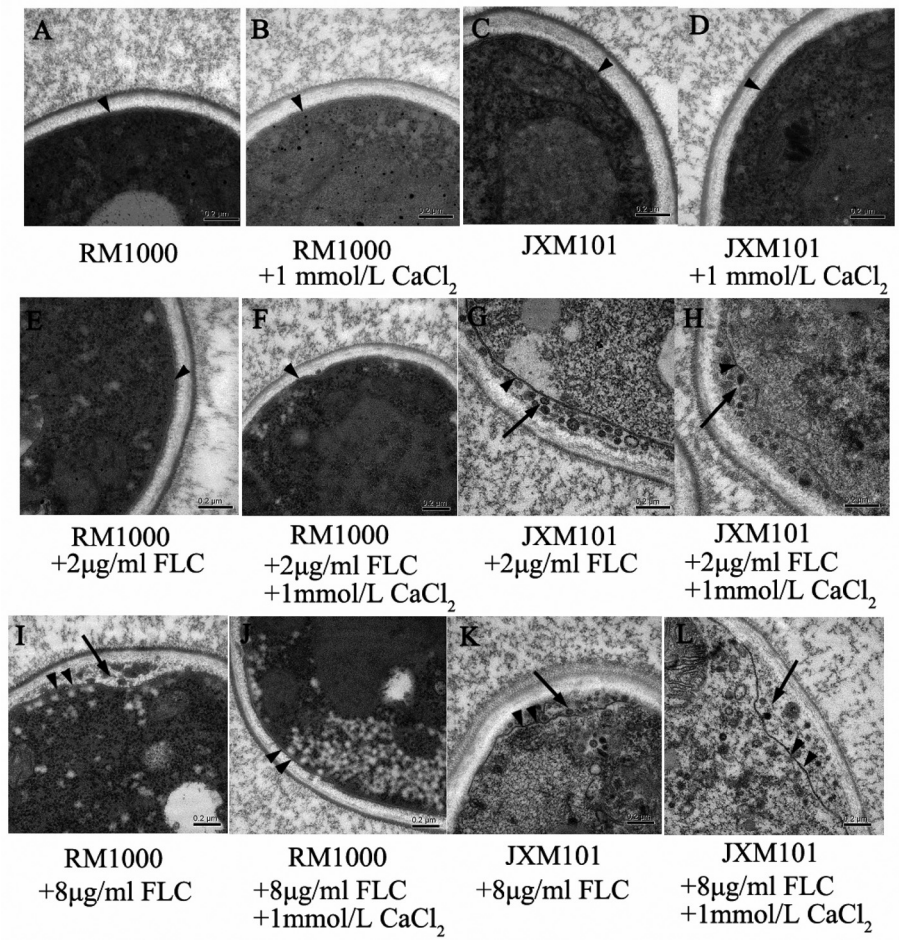

Figure 4. Ultrastructure of *C. albicans* cells. Untreated and treated cells were cultured in RPMI 1640 medium supplemented with 0.0025 % uridine for 16 h and observed by transmission electron microscopy. Strains and agents at indicated concentrations are shown below their corresponding ultrastructural images. Arrowheads indicate triple-layered cytoplasmic membrane and arrows indicate the extensive solubilization of the cytoplasmic membrane. The bar represents 0.2 μm.

Table 3. Relative sterol compositions of *C. albicans* RM1000 and JXM101 after 16 h of incubation with or without indicated drugs.

Sterol	Percentage content[a]											
	RM1000						JXM101					
	Control[b]	Ca[c]	F2[d]	F2Ca[e]	F8[f]	F8Ca[g]	control	Ca	F2	F2Ca	F8	F8Ca
Zymosterol	2.55	2.59	0.0	0.0	0.0	0.0	2.09	2.15	0.0	0.0	0.0	0.0
Ergosterol	90.17	89.87	1.52	1.87	0.0	0.0	83.87	85.68	1.39	1.61	0.0	0.0
obtusifoliol	0.0	0.0	2.84	2.70	3.02	3.48	0.0	0.0	3.54	3.67	3.15	2.68
Fecosterol	3.86	3.95	0.0	0.0	0.0	0.0	5.25	4.63	0.0	0.0	0.0	0.0
Lanosterol	1.74	1.98	16.38	17.52	8.44	14.60	4.38	3.98	13.15	15.36	6.47	6.78
eburicol	0.0	0.0	79.26	77.91	88.54	81.92	0.0	0.0	81.92	79.35	90.39	90.54
Stigmasterol	1.67	1.61	0.0	0.0	0.0	0.0	4.41	3.56	0.0	0.0	0.0	0.0

[a] Sterol proportions varied by less than 10 % in three experiments.
[b] Control (no drug).
[c] Ca, $CaCl_2$ at 1 mM.
[d] F2, fluconazole at 2 μg/ml.
[e] F2Ca, fluconazole at 2 μg/ml combined with $CaCl_2$ at 1 mM.
[f] F8, fluconazole at 8 μg/ml.
[g] F8Ca, fluconazole at 8 μg/ml combined with $CaCl_2$ at 1 mM.

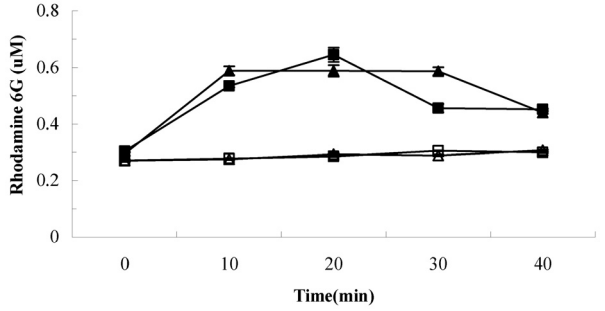

Figure 5. Rhodamine 6G efflux by *C. albicans* wild-type (RM1000, squares) and the *rta2* mutant (JXM101, triangles). Cells were preloaded with 10 μM Rhodamine 6G for 2 h at 30°C, and then treated with (closed symbols) or without (open symbols) 2 mM glucose for 0, 10, 20, 30, 40 min. The cells were then centrifuged and supernatants were quantitated fluorimetrically. The results shown are the means ± SDs of triplicate determinations from one experiment from three independent experiments.

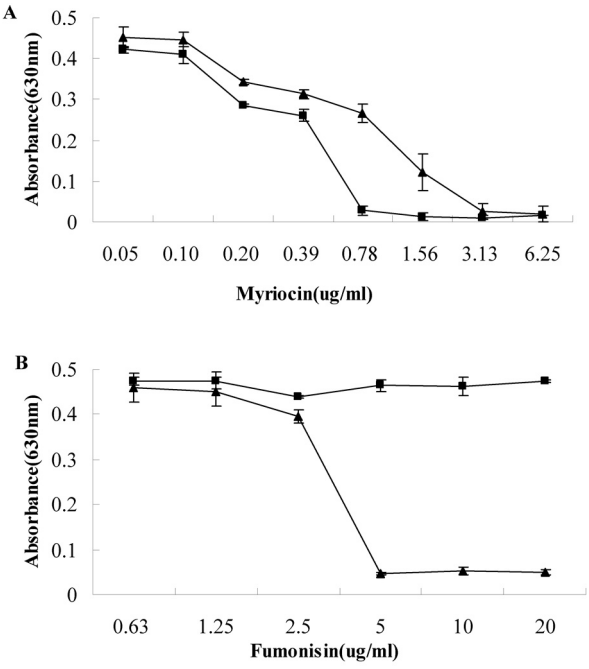

Figure 6. The sensitivities of myriocin (*A*) or fumonisin (*B*) to *C. albicans* wild-type (RM1000, squares) and the *rta2* (JXM101, triangles) cells by microbroth dilution assays. Absorbance was recorded after 24-h incubation. Data are shown as means ± SDs.

Disruption of *RTA2* did not affect efflux of Rhodamine 6G. Since the depletion of *RTA2* made the cell membrane of *C. albicans* vulnerable and increased the susceptibility of *C. albicans* to azoles (the substrates of Cdr1p), Rhodamine 6G efflux analysis was used to investigate the function of Cdr1p. After addition of glucose, no obvious change was observed in the extracellular concentration of Rhodamine 6G between the *rta2Δ/Δ* mutant (JXM101) and wild-type strain (RM1000) (Fig. 5, closed symbols). In addition, de-energized JXM101 had the same passive diffusion of Rhodamine 6G as the de-energized RM1000 (Fig. 5, open symbols). Consequently, we excluded the possibility that disruption of *RTA2* increased sensitivity of *C. albicans* to azoles through impairing the function of Cdr1p.

Disruption of *RTA2* increased accumulation of DHS by reducing its export. Since over-accumulation of LCBs is toxic to yeast cells [33], we tested the susceptibility of the wild-type strain (RM1000) and the ***rta2Δ/Δ*** mutant (JXM101) to myriocin and fumonisin B1, inhibitors of specific steps in sphingolipid biosynthesis. Myriocin decreased, and fumonisin B1 increased, the accumulation of LCBs in *C. albicans*. JXM101 demonstrated more resistance to myriocin than RM1000 (MIC_{80}, 3.13 *versus* 0.78 μg/ml) (Fig. 6A). On the other hand, JXM101 became hypersensitive to fumonisin B1, while RM1000 was hyperresistant to it (MIC_{80}, 5 *versus* >20 μg/ml) (Fig. 6B). Like Rsb1p in *S. cerevisiae*, Rta2p might be a putative LCB-floppase that translocates LCBs from the inner to the outer leaflet of the plasma membrane. To study LCB transport, BSA, which has a high affinity for lipids, was included in the medium to facilitate the release of LCB from the cell surface to the supernatant. We examined the effect of Rta2p on the intracellular accumulation of fluorescence-labeled DHS. When cultured with fluorescence-labeled DHS, JXM101 efficiently accumulated DHS in the cells. The intracellular fluorescence density was increased dramatically at 20–60 min; RM1000, however, showed a much lower accumulation of DHS (Fig. 7A).

To determine whether the increased accumulation of intracellular DHS was caused by its decrease export, we performed an *in vivo* LCB export assay. The JXM101 exported only 0.2 μmol/l DHS into the supernatant by 10 min, which slightly decreased at 30 min, probably because of the homeostasis between import and export reaction (Fig. 7B, triangles). In contrast, RM1000 exported 0.75 μmol/l DHS into the supernatant at 10 min, which was approximately fourfold that in strain JXM101 (Fig. 7B, squares); the export of DHS was slightly increased to 0.86 μmol/l at 30 min (Fig. 7B, squares). Taken together, the disruption of *RTA2* caused an apparent increase in the net accumulation of DHS by decreasing exports of DHS out of the cells.

Discussion

In the present study, we demonstrated that the depletion of *RTA2* increased the susceptibility of *C. albicans* to azoles, whereas ectopic overexpression

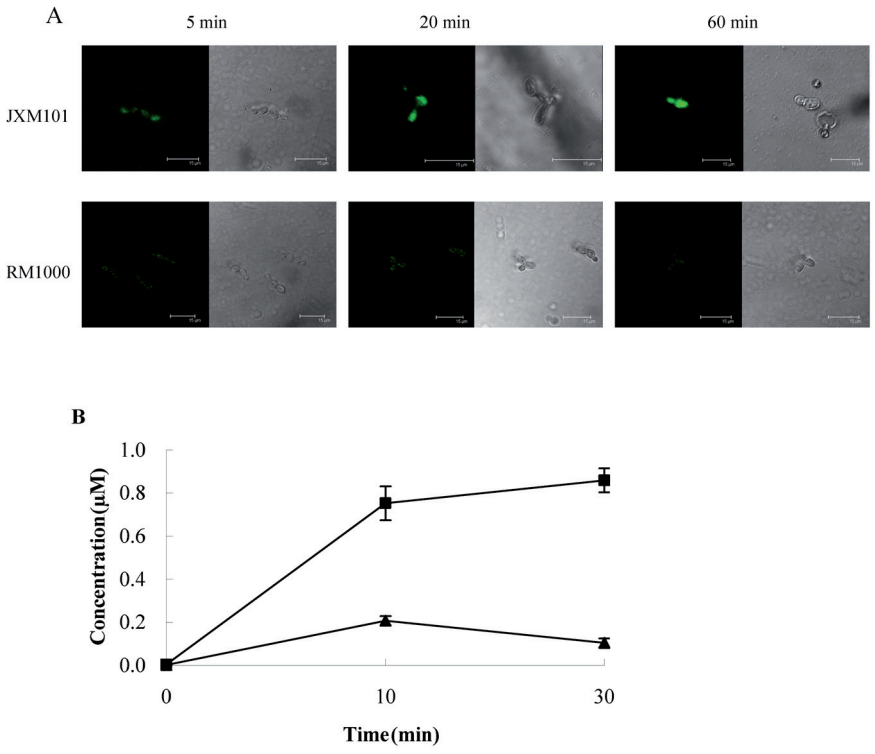

Figure 7. Accumulation and release of dihydrosphingosine (DHS) by Rta2p. The *C. albicans* wild type (RM1000, squares) and the *rta2* mutant (JXM101, triangles) were grown in YPD medium at 30°C. Cells were treated with 5 μM NBD-DHS and 1 mg/ml BSA. (*A*) For uptake experiments, cells were incubated for 5, 20, 60 min. At each time point, living cells were washed twice, resuspended and immobilized on 0.1 % poly-L-lysine-coated slides; confocal pictures were acquired with Leica TCS SP2 confocal microscope (Leica, Germany). (*B*) For release experiments, cells were preloaded with NBD-DHS for 1 h, washed twice, resuspended in YPD medium containing 1 mg/ml BSA, and then incubated for 10 and 30 min. At each time point, cells were pelleted by centrifugation and quantitated fluorimetrically. Data are shown as means ± SDs.

of *RTA2* increased the resistance of *C. albicans* to azoles (Fig. 2). These findings support the hypothesis that *RTA2* is a novel gene responsible for the emergence of azole resistance.

Calcineurin, a Ca^{2+}/calmodulin-activated protein phosphatase, is the target of the immunosuppressive drugs CsA and FK506. These structurally distinct compounds bind to cyclophilin A and FKBP12 (FK506-binding protein 12), respectively. These immunosuppressant drug–receptor complexes subsequently bind to calcineurin and block its enzymatic activity [16]. It has been well documented that the calcineurin pathway could mediate azole resistance [12–16] and *RTA2* was up-regulated by $CaCl_2$ at high concentration in a calcineurin-dependent manner [13]. Our study demonstrated that azole resistance mediated by the calcineurin pathway is completely dependent on the function of *RTA2* through adding $CaCl_2$ alone, $CaCl_2$ plus Ca^{2+} chelator (EGTA), or $CaCl_2$ plus calcineurin inhibitors (FK506 and CsA) in *C. albicans* wild-type, *rta2Δ/Δ* mutant, the *cnaΔ/Δ* mutant, the *crz1Δ/Δ* mutant, the *CNA* revertant and the *CRZ1* revertant strains. In the absence or presence of FK506, we found that *RTA2* was up-regulated by 1 mmol/l $CaCl_2$ in a calcineurin-dependent manner. The depletion of *CNA* or *CRZ1* also removed the effects of $CaCl_2$ on *RTA2* up-regulation, while the reintroduction of *CNA* or *CRZ1* in their null mutants restored the effects. We have proven at the mRNA level that the expression of RTA2 was induced by $CaCl_2$ in a calcineurin-dependent manner, but this result has not been further confirmed at protein level due to lack of an Rta2p antibody. Using transmission electronic microscopy, we found that the cell membrane of the *rta2Δ/Δ* mutant was more liable to being destroyed by FLC than that of wild-type strain. Up-regulation of *RTA2* by 1 mmol/l $CaCl_2$ attenuated the destructive role of FLC in the wild-type strain. The concentration of $CaCl_2$ (1 mmol/l) used in this study was almost equal to that of ionized calcium in mammalian serum (1–2 mmol/l). The results indicate that the effects of *RTA2* on calcineurin-mediated azole resistance have potential clinical significance.

A great concern with antifungal therapy is the development of drug resistance. Inhibition of calcineurin-mediated azole resistance has been proposed as a novel therapeutic approach [17]. FK506 and CsA can chemosensitize *C. albicans* cells to azoles, but they are also immunosuppressive, which can be problematic for candidosis patients because many of them have already been immunocompromised. BLAST search revealed that there are no mammalian or bacterial homologs of Rta2p beyond an E value of $1\times e^{-20}$. It is likely that Rta2p, as the effector of calcineurin-mediated azole resistance in *C. albicans*, would be an ideal new antifungal drug target.

Sphingolipids, along with phospholipids and sterols, are the primary lipids of plasma membrane in

eukaryotic cells. The plasma membrane of *C. albicans* is asymmetric with respect to sphingolipids and phospholipids. Sphingolipids and phospholipids can move within the lipid bilayer, a process termed flip-flop. Because this process is generally slow, it may be driven by proteins termed flippases/floppases in cells. Cdr1p and Cdr2p are involved in the translocation of phospholipids to the outside of the plasma membrane [34, 35]. In yeast such as *Saccharomyces cerevisiae*, ceramide, the backbone of sphingolipids, is composed of an LCB, a fatty acid and a polar head group. The two types of LCBs in yeast are DHS and its 4-hydroxy derivative, phytosphingosine [36, 37]. Sphingolipids are known to be localized in the outer leaflet of the plasma membrane, whereas the distribution of LCBs between the two leaflets has not been determined in *C. albicans*. In *S. cerevisiae,* Rsb1p has been identified as an LCB transporter/ flippase that moves LCBs from the inner to the outer leaflet of the plasma membrane [32]. Our experiments demonstrated that the disruption of *RTA2* caused an apparent increase of intercellular DHS (Fig. 7A); furthermore, we verified that this increase was due to the decrease of DHS release (Fig. 7B). It is suggested that Rta2p acts like a transporter that moves LCBs from the inner to the outer leaflet of the plasma membrane.

In recent years, the idea that cellular membranes contain distinct microdomains has gained considerable momentum. In particular, a class of such domains that is rich in cholesterol or ergosterol as well as sphingolipids, termed lipid rafts, has been the subject of much attention. Lipid rafts are thought to compartmentalize the plasma membrane and to have important roles in cell signaling, polarity and sorting [38–41]. The acquisition of the MDR phenotype in *C. albicans* is not only due to overexpression of the drug efflux pumps but is also accompanied by an up-regulation of genes required for normal lipid metabolism that constitute membrane rafts [42–44]. The depletion of genes involved in the biosynthetic pathways of either ergosterol or sphingolipids could prevent raft formation and increase the drug susceptibilities of *C. albicans* [44, 45]. Therefore, we suggested that the disruption of *RTA2*, altering the distribution of LCBs between the two leaflets of the plasma membrane, rendered *C. albicans* more susceptible to azoles by affecting the raft formation. Further study is needed to determine the precise roles of Rta2p on the formation of lipid rafts.

We found that (1) the disruption and overexpression of *RTA2* in *C. albicans* strains resulted in increased and decreased susceptibility to azoles, respectively; (2) the calcineurin pathway mediated azole resistance in *C. albicans* in a *RTA2*-dependent manner and the up-regulation of *RTA2* expression was calcineurin dependent; (3) the depletion of *RTA2* make the cell membrane of *C. albicans* vulnerable to FLC, and Ca^{2+}-induced up-regulation of *RTA2* attenuated the destructive effects; (4) *RTA2* disruption increased accumulation of DHS due to a decrease in export of DHS. In conclusion, our findings suggest that *RTA2* is involved in calcineurin-mediated azole resistance and sphingoid LCB release in *Candida albicans*.

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