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RTA2, a novel gene involved in azole resistance in *Candida albicans*

Xin-Ming Jia [a], Zhi-Ping Ma [b], Yu Jia [a], Ping-Hui Gao [a], Jun-Dong Zhang [a], Yan Wang [a], Yong-Gang Xu [a], Lin Wang [a], Ying-Ying Cao [a], Yong-Bing Cao [a,*], Li-Xin Zhang [c], Yuan-Ying Jiang [a,*]

[a] *Department of Pharmacology, School of Pharmacy, Second Military Medical University, Shanghai 200433, China*
[b] *The Third People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 201900, China*
[c] *Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China*

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ABSTRACT

Widespread and repeated use of azoles, particularly fluconazole, has led to the rapid development of azole resistance in *Candida albicans*. Overexpression of *CDR1*, *CDR2*, and *CaMDR1* has been reported contributing to azole resistance in *C. albicans*. In this study, hyper-resistant *C. albicans* mutant, with the above three genes deleted, was obtained by exposure to fluconazole and fluphenezine for 28 passages. Thirty-five differentially expressed genes were identified in the hyper-resistant mutant by microarray analysis; among the 13 up-regulated genes, we successfully constructed the *rta2* and *ipf14030* null mutants in *C. albicans* strain with deletions of *CDR1*, *CDR2* and *CaMDR1*. Using spot dilution assay, we demonstrated that the disruption of *RTA2* increased the susceptibility of *C. albicans* to azoles while the disruption of *IPF14030* did not influence the sensitivity of *C. albicans* to azoles. Meanwhile, we found that ectopic overexpression of *RTA2* in *C. albicans* strain with deletions of *CDR1*, *CDR2* and *CaMDR1* conferred resistance to azoles. *RTA2* expression was found elevated in clinical azole-resistant isolates of *C. albicans*. In conclusion, our findings suggest that *RTA2* is involved in the development of azole resistance in *C. albicans*.

The rapid development and spread of antifungal multi-drug resistance (MDR) has become an increasingly serious public health problem in recent years [1–3]. Fungal drug resistance is particularly of great economic and biomedical importance because eukaryotic pathogenic microbes, whose cellular machinery is similar to that of our own cells. Treatment of drug resistant fungal infection is also much more difficult and expensive than that of sensitive fungal pathogens. Understanding of the complex mechanisms of evolutional resistance would enable new drugs, to prevent or delay the emergence of resistant fungal pathogens like the case of Augmentin for anti-bacteria [4–6].

Resistance to drug(s) can be visualized as a gradually evolving process wherein various mechanisms may appear during the course of chemotherapy [7]. The evolution of resistance to azoles, particularly fluconazole (FLC), which are the most broadly used antifungals in the clinic, is all but inevitable in *Candida albicans*. However, only limited MDR genes have been discovered to be responsible for azole resistance, such as *ERG11, CDR1, CDR2,* and *CaMDR1* [8–13]. There are also instances where known MDR genes are not over-expressed in experimentally induced azole-resistant *C. albicans* strains and clinical resistant isolates [14–16], implying the contribution of yet other unknown genes of resistance.

Recently, DNA microarray and proteomic analysis has revealed many up-regulated and down-regulated genes in both laboratory [14,17] and clinical [18–21] *C. albicans* isolates. But none of these new observations had convincing genetic back up because fungi are difficult to study experimentally due to their asexual diploid nature and variant genetic code.

To find novel resistant genes other than previously reported, we employed a mutant strain of *C. albicans* DSY1024 (with deletions of *CDR1*, *CDR2*, and *CaMDR1*) to avoid the influences of known MDR genes. Hyper-resistance to azoles in DSY1024 was obtained by long time exposure to fluconazole-fluphenazine FLP combination. After large-scale gene profile analysis and *RTA2* disruptions or overexpressions in *CDR1*, *CDR2*, and *CaMDR1* deleted *C. albicans* strains, we concluded that *RTA2* is involved in the emergence of azole resistance in *C. albicans*.

Materials and methods

Drugs. FLC was from Pfizer Inc (New York, NY). FLP, ketoconazole, econazole, cycloheximide, and nystatin were purchased from Sigma (St. Louis, MO). Brefeldin A and cerulenin were obtained from Serva Electrophoresis (Heidelberg, Germany). Itraconazole

Abbreviations: Mdr, multi-drug resistance; Cdr, *Candida* drug resistance; Flc, fluconazole; Flp, fluphenazine; Mic, minimum inhibitory concentration.

* Corresponding authors.
E-mail addresses: ybcao@vip.sina.com.cn (Y.-B. Cao), jiangyy@smmu.edu.cn (Y.-Y. Jiang).

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and terbinafine were provided as gifts by Professor Liu Chao-mei and voriconazole was a gift from Professor Zhang Wan-nian of Second Military Medical University, Shanghai, China).

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

Development of azole resistance and its identification. A randomly selected colony of *C. albicans* strain DSY1024 was incubated in YPD medium overnight at 30 °C [22]. An aliquot containing 10^6 cells was then serially transferred to fresh YPD broth containing FLC (12 μg/ml), or FLP (25 μg/ml), or FLC-FLP combinations for 28 passages. At an interval of 7 passages, a 1-ml aliquot of the culture suspension, mixed with 30% glycerol, was frozen at −70 °C for the following experiments. The susceptibility of *C. albicans* was tested in each aliquot by the NCCLs microdilution method [23] . The strains were defined as FLC susceptible if the MIC_{80} was ⩽8 μg/ml and FLC resistant if the MIC_{80} were ⩾64 μg/ml. The treated strains from FLC-FLP combinations were named as DSF (DSF7, DSF14, DSF21, and DSF28 representing the 7th, 14th, 21st, and 28th passage, respectively).

Culture growth for microarrays. A single colony of the azole resistant strain, DSF28, was selected on YPD agar with 10 μg/ml FLC. DSY1024 and DSF28 were cultured overnight in drug-free YPD medium at 30 °C, diluted to an OD_{600} of 0.1 in fresh YPD and were allowed to grow to an OD_{600} of 1.0. Then cells were harvested, flash-frozen and stored in liquid nitrogen. The above procedures were conducted twice with each sample for the coming microarray experiments.

Microarray experiments. Candida albicans 3136 cDNA microarray preparation, RNA isolation, probe labeling, hybridization, and signal analysis were conducted by United Gene Holdings, Ltd. (Shanghai, PR China) as described previously [24]. Transcript profiling of strain DSF28 was compared with its parental hypersusceptible strain DSY1024. Each experiment had a corresponding dye swap to avoid dye bias. Statistical analysis was performed using Student's *t* test for replicate samples. The differentially expressed genes were defined when the expression of a gene had a statistically significant changes (FDR < 5%, $P < 0.05$) and a 2-fold or more change in four data sets. DNA sequences were annotated on the basis of the results of BlastN and BlastX searches using the *Candida*DB database (http://genolist.pasteur.fr/CandidaDB/) and the *Candida* Genome Database (http://www.candidagenome.org/).

Relative quantification of differentially expressed genes by quantitative RT-PCR. All the primer sequences are listed in Supplementary Table 2. RNA isolation, cDNA synthesis, and PCR amplification were done as described previously [24]. Triplicate independent quantitative RT-PCRs were performed using the LightCycler System (Roche diagnostics). The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$ [24].

Construction of mutant strains. All the primer sequences are listed in Supplementary Table 2. The construction of plasmid pUC-RTA2-URA3 was done as follows: Briefly, the fragment containing 5' and 3' ends of *RTA2* gene for homologous recombination was obtained according to the Fusion PCR method [25]. The 4-kb *hisG-URA3-hisG* fragment was from the plasmid p5921 [26]. The two fragments were subsequently cloned into plasmid pUCm-t (Sangon, Shanghai, China). The XhoI digested fragment of pUC-RTA2-URA3 was transformed into the *ura3* mutant strain (DSY9u) by standard methods [27]. Southern blot analysis was used to confirm the absence of *RTA2* gene. The hybridization probe was the 0.686-kb PCR fragment amplified by Pyrobest polymerase (TaKaRa) from the genomic DNA of *C. albicans* strain DSY1024.

Overexpression of RTA2 in rta2 mutants. The *RTA2* ORF was amplified by PCR with Pyrobest polymerase (TaKaRa). The BamHI-PstI digested PCR fragment was ligated into pCaExp [28] 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 *rta2* mutant (DSJ102) were transformed with the linearized pEXP-RTA2 by StuI and selected on SC medium lacking uridine, methionine and cysteine.

Susceptibility testing. The sensitivities of the mutant strains were also tested on agar plates containing different concentrations of azoles (FLC, ketoconazole, econazole, itraconazole, and voriconazole) or other agents (terbinafine, nystatin, brefeldin A, FLP and cerulenin). 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.

Results

In vitro development of resistance to FLC

To begin our systemic investigation of additional factors contributing to azole resistance, we examined *in vitro* development of FLC resistance in *C. albicans* strain DSY1024 (Supplementary Table 1). The stepwise acquisition of azole resistance was examined by exposing the strain to FLC, FLP, or FLC-FLP combination for 28 passages (indicated as DSF 7, 14, 21, and 28, with each number reflecting the number of passages).

In the presence of FLC or FLP alone, DSY1024 retained its FLC susceptible phenotype (Fig. 1A). In contrast, when exposed to FLC-FLP combination, DSY1024 developed FLC resistance (MIC_{80}, 64 μg/ml) at passage 7 and gradually reached its maximum (MIC_{80}, 128 μg/ml) at passage 28 (Fig. 1A). DSF28, the 28th passage of DSY1024 strain after exposure to FLC-FLP combination, regained its susceptible phenotype after twenty-one passages in drugs-free medium (Fig. 1B). We also found that DSF28 had cross-resistance to ketoconazole, itraconazole, econazole and voriconazole (Table 1). These data suggested that DSF28 acquired hyper-resistance to azoles.

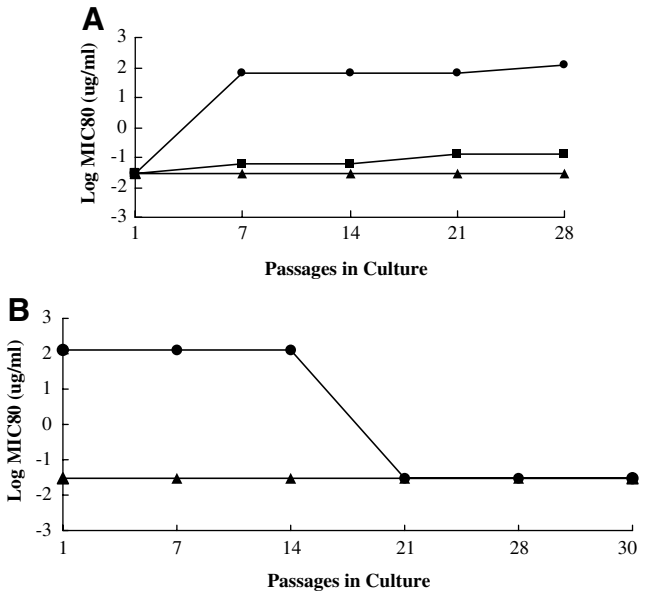

Fig. 1. (A) Variation of fluconazole resistance determined by microdilution methods in *C. albicans* mutant DSY1024 exposed to fluconazole (12 μg/ml, squares) or fluphenazine (25 μg/ml, triangles), or the combination of two (circles). (B) The stability of fluconazole resistance determined by microdilution methods in DSY1024 (triangles) and DSF28 (circles) serially passed for 30 passages in drugs-free YPD medium. Results represent the averages from at least two separate experiments.

Table 1
In vitro activities of azoles against *C. albicans* strains DSY1024 and DSF28

Strain	MIC_{80}[a] (μg/ml)				
	Fluconazole	Itraconazole	Econazole	Ketoconazole	Voriconazole
DSY1024	0.03	0.03	<0.002	0.003	<0.004
DSF28	128	4.48	2	>3.5	>12.5

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

cDNA microarray analysis of experimental strains

To explore those additional genes responsible for azole resistance, we examined the changes in a large-scale gene expression profile of DSY1024 and DSF28. Two biological replicates were conducted. To avoid dye-associated effects on cDNA synthesis, RNA from the hyper-resistant strain DSF28 was labeled with Cy5-dUTP and with Cy3-dUTP separately. In each hybridization experiment, duplicate spots were measured as usual. For further analysis, only the statistically significant (FDR<5%, $P < 0.05$) genes whose expression level changed by at least 2-fold in all data sets were selected. The data generated from these independent microarray experiments reflected a high level of reliability and reproducibility due to the presented ratios obtained from repeated assays of the same samples and of samples from independent experiments.

Compared to DSY1024, 13 genes were up-regulated and 22 genes down-regulated in DSF28. The distribution of those genes and their biological roles are listed in Supplementary Table 3. Among the 35 genes, most were of unknown function (49%), followed by those involved in transcription and RNA processing (14%), calcineurin stress–response pathway (9%), amino acid and carbohydrate metabolism (9%), small molecule transport (9%), energy generation (5%), and those not classified (5%).

To confirm the differential expression result obtained by microarray, sixteen important genes were subjected to quantitative RT-PCR to compare their transcription level. The results are shown in Fig. 2, which were consistent with the microarray data.

Candida albicans rta2 mutant displayed an increased sensitivity to azoles

To determine whether the above up-regulated genes are associated with the enhanced azole resistance in DSY1024, we chose four up-regulated genes: *RTA2* and *MXR1*, *IPF14030*, and *IPF6235* for gene disruptions. Finally, we successfully obtained *rta2* and *ipf14030* heterozygous and homozygous mutants in DSY9u (isolated from DSY1024 on 5-FOA plates) after verification by Southern blot analysis (Fig. 3 and unpublished data).

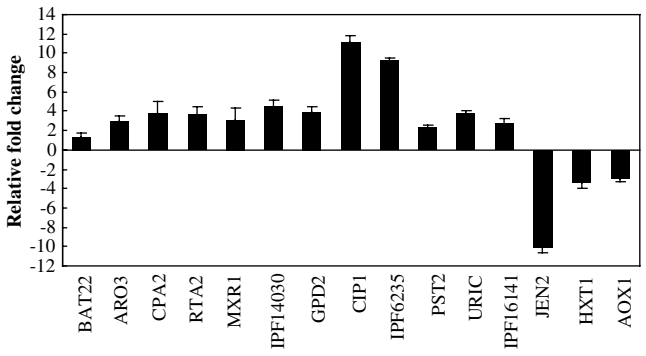

Fig. 2. Quantitative RT-PCR analysis of genes identified as differentially expressed by microarray experiments. Data are shown as means ± standard deviation.

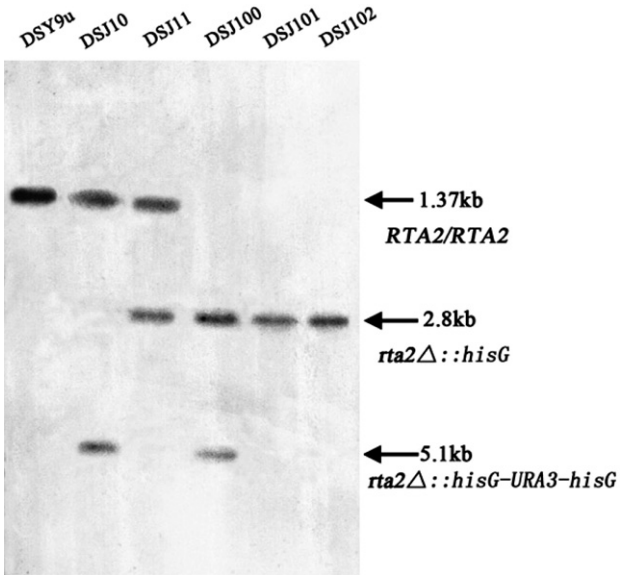

Fig. 3. Southern analysis of the genomic DNA digested with BglII and SalI. The exact size and genotype of the expected hybridizing DNA fragment are indicated on the right.

Drug susceptibility assay demonstrated that the *rta2Δ/Δ* mutants (DSJ101 and DSJ102) from DSY9u with deletions of *CDR1*, *CDR2*, and *CaMDR1* were more susceptible to azoles, including FLC, ketoconazole, econazole, itraconazole, and voriconazole, and were more resistant to brefeldin A (an inhibitor of organelle assembly) (Fig. 4A). The depletion of *RTA2* showed no obvious effect on the susceptibility to other agents, including terbinafine, nystatin, FLP and cerulenin (Fig. 4A). The disruption of *IPF14030* did not influence the sensitivity of DSY9u to azoles (unpublished data).

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 (DSJ102) at the *RP10* locus as determined by PCR (data not shown). Ectopic over-expression of *RTA2* in DSJ102 transformed with over-expressing vector pCaEXP-RTA2 was confirmed by quantitative RT-PCR with its expression in its parental stains (DSY9u) harboring empty vector pCaEXP serving as a control, respectively (Supplementary Fig. 1). The growth of DSJ102 transformed with empty vector pCaEXP was severely impaired on SC plates in the presence of azoles, just like those on YPD plates (Fig. 4B). Drug susceptibility assay also showed that the strains DSJ102 transformed with pCaEXP-RTA2 rendered more resistance to azoles than did their parental strains DSY9u harboring pCaEXP (Fig. 4B). Together, these results demonstrate a critical role of *RTA2* in the development of azole resistance.

Discussion

FLP as a calmodulin antagonist could promote the development of azole resistance in *Saccharomyces cerevisiae* [29]. At low concentrations, FLP also can decrease the sensitivity of *C. albicans* to azoles [30]. In the present study, we exposed DSY1024 to low concentrations of FLC and FLP for 28 passages and obtained a hyper-resistant stain (DSF28) to azoles. Since MDR transporter genes (*CDR1*, *CDR2*, and *CaMDR1*) have been disrupted in DSY1024, there might be novel genes or pathways responsible for the development of azole resistance.

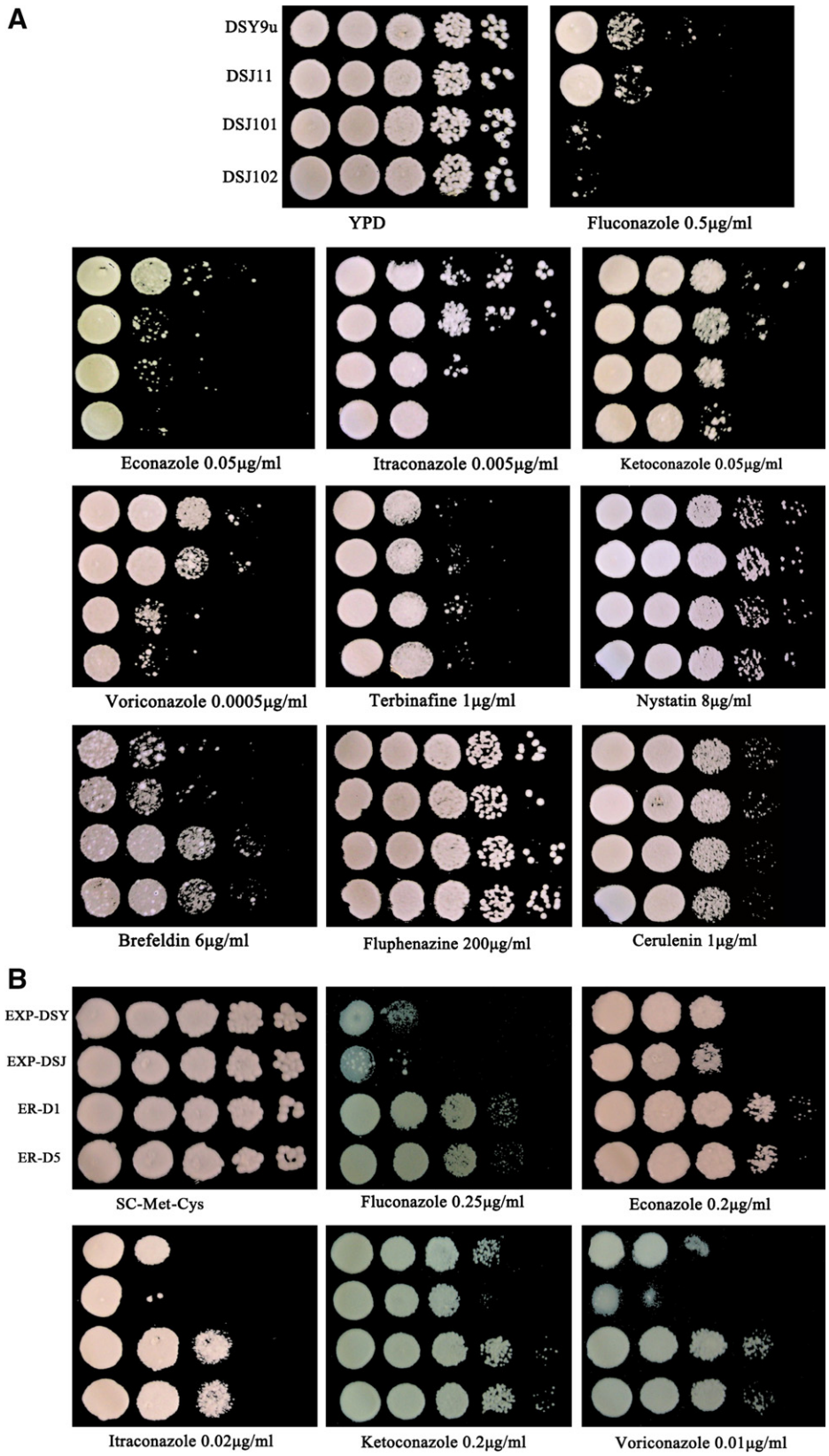

Fig. 4. Drug susceptibility profiles of *C. albicans* strains (Supplementary Table 1) as determined by spot assays. (A) The *rta2* heterozygous and homozygous mutant strains from DSY9u were spotted on YPD agar plates supplemented with 0.0025% uridine with or without different antifungal agents at indicated concentrations. Plates were incubated for 48 h at 30 °C. (B) 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-D1 and ER-D5 (DSJ102 harboring pCaEXP-RTA2), EXP-DSJ (DSJ102 harboring pCaEXP) and EXP-DSY (DSY9u harboring pCaEXP). Plates were incubated for 72 h at 30 °C. The names of strains are indicated on the left.

Microarray analysis showed that 35 genes were differentially expressed in DSF28. Among 13 up-regulated genes, 3 genes (*RTA2*, *MXR1*, and *IPF14030*) contain calcineurin-dependent responsive element (CDRE) sequence in their promoters (Supplementary Table 3) and CDREs are controlled by either calcineurin and/or Crz1p [31]. FLP was recently shown to activate calcineurin-dependent genes in *S. cerevisiae* [29], thus consistent with a calcineurin activating action of FLP in *C. albicans*.

To determine whether the above up-regulated genes are associated with the enhanced azole resistance in DSY1024, we successfully constructed the *rta2* and *ipf14030* null mutants in the strain with deletions of *CDR1*, *CDR2* and *CaMDR1*. Using spot dilution assay, we demonstrated that the disruption of *RTA2* increased the susceptibility of *C. albicans* to azoles while the disruption of *IPF14030* did not influence the sensitivity of *C. albicans* to azoles (Fig. 4 and unpublished data). We employed the *rta2Δ/Δ* mutant with deletions of *CDR1*, *CDR2*, and *CaMDR1* to develop FLC resistance by long time exposure to FLC- FLP combination again. When exposed to FLC-FLP combination, the *rta2Δ/Δ* mutant could not survive from passage 7 (data not shown). We further re-introduced the ORF of *RTA2* into the *rta2Δ/Δ* mutant at the *RP10* locus of *C. albicans*. Using spot dilution assay, we found that ectopic overexpression of *RTA2* increased the resistance of *C. albicans* to azoles (Fig. 4). Previous studies have also showed that *RTA2* was over-expressed in experimentally induced azole-resistant *C. albicans* strains [14,17]and in clinically resistant [19] isolates. Consistently, we also found elevated *RTA2* expression levels in clinical isolates of azole-resistant *C. albicans* (Supplementary Fig. 2). All these findings support the hypothesis that *RTA2* is a novel gene responsible for the emergence of azole resistance in *C. albicans* strain DSY1024. Further analysis is required for elucidation of the precise molecular mechanisms of azole resistance by Rta2p.

A BLAST search of the *C. albicans* genome (available at http://www.genolist.pasteur.fr/CandidaDB/) revealed that **t**he *RTA2* gene encodes a putative polypeptide of 453 amino acids, with a deduced molecular mass of 50.9 kDa. The SOSUI [32] program 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 *S. cerevisiae* genome database (available at 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 had a long C-terminal tail and contains an extended loop between the predicted transmembrane domains 5 and 6. Rsb1p is a translocase that translocates sphingolipid long-chain bases from inside to the outside of the membrane in *S. cerevisiae* [33]. Further studies are needed to verify whether Rta2p is a lipid transporter.

We found that (1) hyper-resistance to azoles in DSY1024 (with the deletion of *CDR1*, *CDR2*, and *CaMDR1*) was obtained by long time exposure to FLC-FLP combination; (2) 13 genes were up-regulated and 22 genes down-regulated in hyper-resistance strain DSF28 compared to its matched hyper-susceptible strain DSY1024 using cDNA microarray analysis; (3) the disruption and ectopic overexpression of *RTA2* in *C. albicans* strains with deletions of *CDR1*, *CDR2*, and *CaMDR1* resulted in increased and decreased susceptibility to azoles, respectively. In conclusion, our findings suggest that *RTA2* is involved in the emergence of azole resistance in *C. albicans.*

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.093.

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