DNA microarray analysis of fluconazole resistance in a laboratory Candida albicans strain

Lan Yan1, Jundong Zhang1, Miaohai Li2, Yongbing Cao1, Zheng Xu1, Yingying Cao1, Pinghui Gao1, Yan Wang1, and Yuanying Jiang1*

1 Department of Pharmacology, College of Pharmacy, Second Military Medical University, Shanghai 200433, China
2 Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China

Several mechanisms are responsible for the acquired fluconazole (FLC) resistance in Candida albicans. In this study, we developed a FLC-resistant C. albicans strain through serial cultures of a FLC-susceptible C. albicans strain with inhibitory concentrations of FLC. Complementary DNA microarray analysis and real-time reverse transcription-polymerase chain reaction were used to investigate gene expression changes during the acquisition of azole resistance in the susceptible parental strain and the resistant daughter strain. The differentially expressed genes represented functions as diverse as transporters (e.g. CDR1, PDR17), ergosterol biosynthesis (e.g. ERG2, ERG9), sterol metabolism (e.g. ARE2, IPF6464), energy metabolism (e.g. ADH3, AOX2) and transcription factors (e.g. FCR1, ECM22). Functional analysis revealed that energy-dependent efflux activity of membrane transporters increased and that ergosterol content decreased with the accumulation of sterol intermediates in the resistant strain as compared with the susceptible strain. We found that a point mutation (N977K) in transcription factor TAC1 that resulted in hyperactivity of Tac1 could be the reason for overexpression of CDR1, CDR2, and PDR17 in the resistant strain. Furthermore, a single amino acid difference (D19E) in ERG3 that led to the inactivation of Erg3 could account for both sterol precursor accumulation and the changes in the expression of ergosterol biosynthesis genes in this resistant strain. These findings expand the understanding of potential novel molecular targets of FLC resistance in clinical C. albicans isolates.

Keywords Candida albicans; fluconazole; resistance; microarray

Candida albicans is one of the most common opportunistic fungal pathogens in humans. Certain strains are able to cause systemic infections in immunocompromised patients. Azole antifungal agents, especially fluconazole (FLC), are usually used to treat these infections. However, the overuse of and long-term treatment with FLC have resulted in the emergence and rapid expansion of FLC-resistant C. albicans, which has become a severe clinical and social problem in treating Candida infections [1].

Several mechanisms of FLC resistance in C. albicans have been identified: defective Erg3 function with altered sterol content [2], overexpression of the drug target enzyme lanosterol demethylase gene ERG11 [3], point mutations in ERG11 that result in reduced affinity of Erg11 to azoles [4,5], reduced intracellular accumulation of drugs correlated with the increased expression of ATP-binding cassette (ABC) transporters (CDR1 and CDR2) and/or the major facilitators superfamily efflux pump (MDR1) [6–8], and the formation of biofilms [9]. Many investigations have revealed that FLC resistance in C. albicans results from a combination of several of these mechanisms. Indeed, overexpression of ERG11 has recently been shown to be controlled by the gain-of-function mutation in the transcription factor gene UPC2 [10]. Overexpression of CDR1 and CDR2 is due to the similar mutations in both TAC1 alleles [11,12], while overexpression of MDR1 is due to similar mutations in MRR1 [13].

Recently, genome-wide gene expression studies by DNA
microarray have shown that many genes, such as those involved in sterol mechanism (ERG10, ERG25) [14–16], oxidative stress response (CRD2, GPX1, IFD5, IPF4065, IPF7817) [14,15,17], and carbohydrate mechanism (ADH4, YPL88) [18], are associated with FLC resistance in clinical C. albicans isolates. Moreover, proteomic analyses have also identified several differentially expressed proteins such as Grp2, Ifd1, and Ifd4 in clinical FLC-resistant C. albicans isolates and Pgk1, Qcr7, and Adh1 in a laboratory resistant strain [19–22]. These results have identified differential genes already known to be involved in drug resistance, genes whose expression are coordinately up-regulated or down-regulated with the up-regulation of known resistant genes (CDR1, CDR2, and MDR1).

The present study was designed to explore the potential mechanisms of FLC resistance in a laboratory C. albicans strain. We developed an in vitro model of FLC-resistant C. albicans strain y0109 that resulted from FLC-resistant C. albicans isolates. Moreover, proteomic analyses have also identified several differentially expressed proteins such as Grp2, Ifd1, and Ifd4 in clinical FLC-resistant C. albicans isolates and Pgk1, Qcr7, and Adh1 in a laboratory resistant strain [19–22]. These results have identified differential genes already known to be involved in drug resistance, genes whose expression are coordinately up-regulated or down-regulated with the up-regulation of known resistant genes (CDR1, CDR2, and MDR1).

Drug resistance was induced as described in previous study [22]. Briefly, a single colony of C. albicans y0109 was inoculated into 10 ml YNB broth and incubated overnight at 30 ºC with shaking (200 rpm). An aliquot of this culture containing 10^6 cells was then transferred to 10 ml YNB broth containing twice their most recently measured FLC minimal inhibitory concentration (MIC) and incubated at 30 ºC with shaking. When the cultures reached a density of about 10^8 cells/ml, aliquots containing 10^6 cells were transferred into fresh YNB broth also containing twice their most recently measured FLC MIC and incubated overnight at 30 ºC with shaking (200 rpm). At each passage, an antifungal susceptibility test was performed [23]. All the strains were stored in 1.5 ml 50% glycerol at –80 ºC.

RNA preparation
A pair of FLC-susceptible and resistant C. albicans strains were grown on sabouraud dextrose agar, and a single colony of cells was transferred to 100 ml YPD broth in a 500-ml Erlenmeyer flask. After 16 h incubation at 30 ºC with constant shaking (200 rpm), the suspension (300 µl) was subcultured for 6 h in 100 ml YPD broth to obtain early logarithmic phase cultures. Then, cells were collected by centrifugation at 1000 g for 5 min, flash-frozen, and stored in liquid nitrogen. Total RNA was isolated using the hot phenol method [24]. Frozen cells were resuspended in 12 ml AE buffer [50 mM sodium acetate (pH 5.2), 10 mM EDTA] at room temperature, after which 800 µl 2 M sodium acetate (pH 5.0), 10 mM EDTA at room temperature, after which 800 µl 25% sodium dodecyl sulphate (SDS) and 12 ml acid phenol were added. The cell lysate was then incubated for 10 min at 65 ºC with vortexing each minute, cooled on ice for 5 min, and subjected to centrifugation at 12,000 g for 15 min. Supernatants were transferred to new tubes containing 15 ml chloroform, mixed, and subjected to centrifugation at 200 g for 10 min. RNA was precipitated from the resulting aqueous layer by transferring that portion to new tubes containing one volume isopropanol and 0.1 volume 2 M sodium acetate (pH 5.0), and mixed well. The mixture was centrifuged at 18,000 g for 35 min at 4 ºC. The pellet was resuspended in 10 ml 70% ethanol and centrifuged at 18,000 g for 20 min at 4 ºC to collect RNA. RNA integrity was visualized by subjecting the sample to electrophoresis through 1% agarose gel. Poly(A) mRNA was extracted using Oligotex mRNA kit (Qiagen, Hilden, Germany) and quantitated using the Ribogreen RNA Quantitation kit (Invitrogen, Eugene, USA).

Gene expression analysis
Microarray preparation, synthesis of fluorescent cDNA

Materials and Methods

Strains and media
C. albicans strain y0109 (ATCC76615) was obtained from Changzheng Hospital Strains Collection Center (Shanghai, China). Media used in this study included yeast nitrogen base (YNB) broth (Difco, Detroit, USA) supplemented with 2% glucose, yeast extract pentose dextrose (YPD; Difco), sabouraud dextrose agar (Difco), and RPMI-1640 with 0.165 M morpholinepropanesulfonic acid (Sigma, St. Louis, USA) adjusted to pH 7.0.

Antifungal agents
Standard antifungal powders of FLC, ketoconazole (KTC), itraconazole (ITC), amphotericin B (AMB), and nystatin were obtained from Sigma. Stock solutions were prepared in distilled water (FLC, AMB) or dimethyl sulfoxide (KTC, ITC), filter sterilized and then stored at –70 ºC.
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probes, hybridization with C. albicans 3136 cDNA microarray, and signal analysis were strictly conducted as reported previously [25–28]. In brief, the microarrays consisted of full-length and partial cDNA sequences representative of the sequences of expressed genes in C. albicans, including unknown-in-function, known, and control genes. The fluorescent cDNA probes were synthesized from purified mRNA with Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, USA) by oligo (dT)-primed polymerization using Superscript II reverse transcriptase (Invitrogen). The reaction buffer mixture contained dNTPs (200 µM dATP, dCTP, dGTP, 60 µM dTTP, and 60 µM Cy3- or Cy5-dUTP), 2 µl Superscript II reverse transcriptase, and 1× reaction buffer. Reactions were carried out at 42 °C for 2 h. To stop the reaction, 4 µl 2.5 M NaOH was added, the mixture was incubated at 65 °C for 10 min, and was neutralized with 4 µl 2.5 M HCl. To avoid dye-associated effects on cDNA synthesis, two independent hybridization experiments were performed with RNA from the FLC-susceptible strain labeled by Cy5-dUTP and by Cy3-dUTP separately, and so did RNA from the FLC-resistant strain.

The probes were dissolved in 20 µl hybridization solution [5×sodium chloride sodium citrate (SSC) (0.75 M NaCl and 0.075 M sodium citrate), 0.4% SDS, 50% formamide]. Microarrays were pre-hybridized with hybridization solution containing 0.5 mg heat-denatured salmon sperm DNA at 42 °C for 6 h. The fluorescent probe mixtures were denatured at 95 °C for 5 min, and then were applied to the pre-hybridized chip under a cover glass. Hybridization was performed at 42 °C overnight in a homemade chamber. The slides were then washed for 10 min each at 60 °C in solutions of 2× SSC and 0.2% SDS, 0.1× SSC and 0.2% SDS, and 0.1× SSC. They were then dried at room temperature.

The slides were scanned using a ScanArray 3000 (GSI Lumonics, Bellerica, USA) to detect emissions from both Cy3 and Cy5. The acquired images were analyzed with ImaGene software (version 3.0; BioDiscovery, Los Angeles, USA). The intensities of each spot at the two wavelengths respectively represented the quantities of Cy3-dUTP and Cy5-dUTP that hybridized to each spot. The ratios of Cy5 to Cy3 were calculated for each location on each microarray. To minimize artifacts that arise from low expression values, only genes with raw intensity values >800 counts for both Cy3 and Cy5 were chosen for analysis.

In this study, duplicate independent experiments were carried out, and RNA samples of each strain were labeled and hybridized repeatedly. Only features with a mean balanced differential expression ratio ≥2.0 or ≤0.5 for each spot were considered to be statistically significant (P<0.05). DNA sequences were annotated on the basis of results of BLASTN searches using GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), the sequencing database of the Candida Genome Database (http://www.candidagenome.org/), and the CandidaDB database (http://genolist.pasteur.fr/CandidaDB/).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)
C. albicans strains were cultured in the same conditions as they were prior to microarray analysis. Briefly, the suspension (300 µl) of a 16 h culture was subcultured for 6 h in 100 ml YPD broth to obtain early logarithmic phase cultures. Three independent RNA samples from the FLC-susceptible and resistant strains were isolated for three separate experiments. Genomic DNA contamination was removed, first-strand cDNA was synthesized, and real-time PCR reactions were performed [22]. In brief, RNA samples were treated with 10 U DNase I (Takara, Dalian, China) per 50 µl RNA at 37 °C for 1 h to remove genomic DNA contamination. First-strand cDNA were synthesized from 1 µg total RNA in a 20 µl reaction volume using a cDNA synthesis kit (Takara) according to the manufacturer’s instructions. Real-time PCR reactions were performed in triplicate with cDNA from independent RNA isolations, using SYBR Green I (Takara) on the LightCycler Real-Time PCR system (Roche Molecular Biochemical, Indianapolis, USA). Gene-specific primers were designed using Discovery Studio Gene software (Accelrys Inc, San Diego, USA) (Table 1). The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The ΔC_T value of the FLC-susceptible strain was subtracted from the ΔC_T value of the FLC-resistant strain to obtain a ΔΔC_T value. The gene expression level of the resistant strain relative to that of the susceptible strain was expressed as 2−ΔΔC_T. Triplicate independent real-time RT-PCR analyses were performed for each sample.

Glucose-induced R6G efflux assay
The glucose-induced efflux of R6G (Sigma) from C. albicans strains was investigated [29]. Cells in the middle logarithmic phase were collected by centrifugation at 3000 g for 5 min and washed three times with phosphate buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 140 mM sodium chloride, pH 7.4) buffer. Then cells were resuspended at a concentration of 1×10^7 cells/ml in PBS buffer containing 5 mM 2-deoxy-D-glucose and
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10 µM R6G. Cell suspensions were incubated at 30 ºC with shaking (200 rpm) for 90 min to allow R6G accumulation under glucose starvation conditions. The starved cells were washed twice in PBS buffer, and 1 ml portions were incubated at 30 ºC for 5 min before the addition of 1 mM glucose to initiate R6G efflux. At specified intervals after the addition of glucose, the cells were removed by centrifugation. Triplicate 100 µl volumes of the cell supernatants were transferred to 96-well flat-bottom microtiter plates on a Polarstar Optima instrument (BMG Labtech, Offenburg, Germany). The fluorescence of the samples was measured with an excitation wavelength at 515 nm and an emission wavelength at 555 nm.

Sterol analysis

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Sterol analysis

<table>
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<th>Gene</th>
<th>Primer pairs (5′→3′)</th>
<th>Amplicon size (bp)</th>
</tr>
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<td>CDR1</td>
<td>CCAAACATACAAGCCACGATCTCC (F)/AATCGACGGATGTCACCTTTCTCATAACG (R)</td>
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<tr>
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<td>PDR6</td>
<td>GGAAGACAGGGAGATGATGACGACG (F)/GTTCACATCCTCCTGGCCTGAC (R)</td>
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<td>IPF4065</td>
<td>ACAAAGGAGCATCTCAAATCC (F)/ACCTTCAACCAAGCTGTCG (R)</td>
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<td>EFG1</td>
<td>GCTGCTACTACTACTGCTACTG (F)/CAGCACATACGCTACCAACCCCATAG (R)</td>
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<tr>
<td>ECM22</td>
<td>AGCAACCTCAATAATTCTGTCC (F)/CTTGGTGGTGAGGAACACTCTTTG (R)</td>
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<td>ADH4</td>
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<td>MCR1</td>
<td>ACTGATAGCAATGATGTTG (F)/GTCAACAAACATGAAAGATG (R)</td>
<td>135</td>
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<td>ZCF20</td>
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<td>89</td>
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<tr>
<td>MDR1</td>
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<td>90</td>
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<tr>
<td>CDR2</td>
<td>CGAGGTGGACAGATTTTTTTC (F)/AGCGAATGACGACGATG (R)</td>
<td>141</td>
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<tr>
<td>TAC1</td>
<td>TTTAATCCCGGGAACACCTGAC (F)/TTTGGTTGGGCAACAGGAC (R)</td>
<td>145</td>
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<tr>
<td>18S</td>
<td>GTGCCAGCAGCGCGCAGTGA (F)/TGGACCGGCGACAAGGC (R)</td>
<td>99</td>
</tr>
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</table>

F, forward; R, reverse.
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GC-MS analyses were performed with a Finnigan Voyager instrument (Finnigan, San Jose, USA). The gas chromatograph was equipped with a phenyl-methylpolysiloxane column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm). The settings were as follows: source, positive electron ionization; scan, 50 to 650 Da in 0.9 s; interscan delay, 0.1 s; multiplier voltage, 450 V; solvent delay, 2.0 min; gas chromatograph temperature ramp, 0 min at 150 ºC, 10 min at 290 ºC (15 ºC/min); detector temperature, 280 ºC; sample injection temperature, 250 ºC; carrier gas, helium gas, 1 ml/min. Sterols were identified from their retention times and specific mass spectrometric patterns.

PCR amplification and DNA sequencing
Genomic DNA from the C. albicans strains was extracted with the YeastStar Genomic DNA kit (Zymo Research, Orange, USA) and was used as templates for amplification of ERG3, ERG11, and TAC1 genes, respectively. Primer pairs were chosen to amplify regions spanning the open reading frame of the C. albicans ERG3 gene (5'-CCCATT-TCTTTCCCTATTGTGC-3'; 5'-AGACGACCTTTCAAG-ATTGTCC-3'), ERG11 gene (5'-GTCAATTGGAGAAC-GAGAACG-3'; 5'-CTGAATCGAAAGAAAGTTGCC-3'), and TAC1 gene (5'-AGAGCCTTTCTCTTCTCTTC-3'; 5'-CATCGCTTTCACCAATTACAAC-3'). PCR reactions were carried out with high-fidelity Pyrobest DNA polymerase (Takara). Amplifications were purified with PCR product purification kit (ZhongDing Biotech Company, Ningbo, China), and their nucleotide sequences were determined by primer elongation with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, USA) with Taq dye-primer and dye-terminator chemistries (Applied Biosystems).

Statistical analysis
Experiments were performed at least three times. Data are presented as mean±SD and analyzed using the Student’s t-test where indicated.

Results
Generation of FLC-resistant C. albicans strain
To obtain a FLC-resistant C. albicans strain in the laboratory, the susceptible C. albicans strain y0109⁰ (FLC MIC, 0.5 µg/ml) was serially cultured in inhibitory concentrations of FLC, and developed FLC resistance in a stepwise fashion (Fig. 1). The MIC rose from 0.5 to 8 µg/ml after 20 passages, and this value remained stable for 15 passages. Further increases of FLC MIC were measured: from 8 to 32 µg/ml after 40 passages and from 32 to 64 µg/ml, after 60 passages. The resultant organism showed FLC MIC of 64 µg/ml stable for up to 20 passages in drug-containing medium. Meanwhile, the increase of both ITC and KTC MIC paralleled that of FLC MIC, indicating that the FLC-resistant strain was cross-resistant to both ITC and KTC (Fig. 1). The strain at passage 75 (FLC MIC, 64 µg/ml) was named y0109⁰. Interestingly, the strain y0109⁰ was also resistant to AMB (MIC, 32 µg/ml) and nystatin (MIC, 6.24 µg/ml).

Furthermore, the resistant strain y0109⁰ was serially cultured in FLC-free broth medium. The FLC MIC of strain y0109⁰ decreased to 32 µg/ml after 8 passages and to 16 µg/ml after 18 passages. KTC and ITC MIC similarly decreased (Fig. 2). After 40 drug-free passages, the strain reverted to the FLC-susceptible dose-dependent (16 µg/ml), KTC-susceptible (0.0625 µg/ml), ITC-susceptible (0.125 µg/ml), AMB-susceptible (0.5 µg/ml), and nystatin-susceptible (0.78 µg/ml) phenotype.

Overview of differentially expressed genes
In order to get insight into the development of FLC resistance in this laboratory strain, we performed cDNA microarrays to identify changes in the gene expression profiles of y0109⁰ and y0109⁰. In this study, two biological replicates were performed, and RNA from the FLC-resistant strain labeled by Cy5-dUTP and by Cy3-dUTP separately. In each hybridization process, duplicate spots were measured. Out of 3136 cDNA sequences examined, 278
were shown to have more than 2-fold difference in expression level between the pair of strains. Among these, 130 were up-regulated and 148 were down-regulated in the strain y0109\textsuperscript{R} compared with those in y0109\textsuperscript{S}. According to biological function, they were classified into membrane transporters, ergosterol biosynthesis, sterol metabolism, carbohydrate metabolism, lipid, fatty acid metabolism, morphogenetic transition, and unknown biological process. Differentially expressed genes are shown in Table 2.

Validation of microarray results by real-time RT-PCR analysis
To validate the differential expression of genes identified by microarray analysis, we performed real-time RT-PCR analysis. In general, for the representative genes in each category in the strain y0109\textsuperscript{R}, the \( n \)-fold changes were almost equal between microarray and real-time RT-PCR.

### Table 2 Typically differentially expressed genes between Candida albicans strains y0109\textsuperscript{S} and y0109\textsuperscript{R} were identified by complimentary DNA microarray analysis

<table>
<thead>
<tr>
<th>Gene name( † )</th>
<th>Function description</th>
<th>Accession No.( † )</th>
<th>( y0109^S : y0109^R )</th>
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<tr>
<td><strong>Transporters</strong></td>
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<tr>
<td>\textit{CDR1}</td>
<td>Multidrug transporter of ABC superfamily</td>
<td>CA6066</td>
<td>19.20</td>
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<tr>
<td>\textit{PDR17}</td>
<td>Lipid biosynthesis and multidrug resistance</td>
<td>CA6183</td>
<td>3.01</td>
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<td>\textit{PDR6}</td>
<td>Pleiotropic drug resistance regulatory protein</td>
<td>CA4807</td>
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<tr>
<td>\textit{HXT62}</td>
<td>Sugar transporter</td>
<td>CA1067</td>
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<td>Sugar transporter</td>
<td>CA1069</td>
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<td>\textit{HGT11}</td>
<td>Hexose transporter</td>
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<td>Sugar transporter</td>
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<td>\textit{IPF3277}</td>
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<td>\textit{ERG2}</td>
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<td>CA2154</td>
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<td>\textit{ERG9}</td>
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<td>CA1676</td>
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(To be continued)
Microarray analysis of FLC resistance in a laboratory C. albicans strain

<table>
<thead>
<tr>
<th>Gene name†</th>
<th>Function description</th>
<th>Accession No.†</th>
<th>y0109b:y0109a ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PFK1</em></td>
<td>6-phosphofructokinase, α subunit</td>
<td>CA1834</td>
<td>2.68</td>
</tr>
<tr>
<td><em>PGK1</em></td>
<td>Phosphoglycerate kinase</td>
<td>CA1691</td>
<td>4.24</td>
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<tr>
<td><em>QCR7</em></td>
<td>Ubiquinol-cytochrome-c reductase subunit 7</td>
<td>CA2764</td>
<td>2.41</td>
</tr>
<tr>
<td><em>AOX2</em></td>
<td>Alternative oxidase</td>
<td>CA2189</td>
<td>5.24</td>
</tr>
<tr>
<td><em>IPF7817</em></td>
<td>NADH-dependent flavin oxidoreductase</td>
<td>CA3564</td>
<td>2.24</td>
</tr>
<tr>
<td><em>IPF1732</em></td>
<td>Intramitochondrial protein sorting</td>
<td>CA2009</td>
<td>2.60</td>
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<tr>
<td><em>MRF1</em></td>
<td>Mitochondrial respiratory proteins</td>
<td>CA1333</td>
<td>3.19</td>
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**Morphogenesis**

<table>
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<tr>
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<th>Accession No.†</th>
<th>y0109b:y0109a ‡</th>
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<tbody>
<tr>
<td><em>IFF3</em></td>
<td>Putative GPI-anchored protein</td>
<td>CA2037</td>
<td>2.95</td>
</tr>
<tr>
<td><em>STE7</em></td>
<td>Mitogen-activated protein kinase</td>
<td>CA1623</td>
<td>3.38</td>
</tr>
<tr>
<td><em>SNF1</em></td>
<td>Serine/threonine protein kinase</td>
<td>CA3361</td>
<td>2.19</td>
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<tr>
<td><em>EFG1</em></td>
<td>Enhanced filamentous growth factor</td>
<td>CA2787</td>
<td>3.93</td>
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<tr>
<td><em>NAG2</em></td>
<td>N-acetyl-glucosamine-6-phosphate deacetylase</td>
<td>CA1131</td>
<td>4.98</td>
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<tr>
<td><em>TPS2</em></td>
<td>Trehalose-6-phosphate phosphatase</td>
<td>CA5066</td>
<td>2.89</td>
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<tr>
<td><em>PGA4</em></td>
<td>Putative GPI-anchored protein</td>
<td>CA4800</td>
<td>0.48</td>
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<tr>
<td><em>PGA62</em></td>
<td>Putative GPI-anchored protein</td>
<td>CA4125</td>
<td>0.26</td>
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<tr>
<td><em>PGA52</em></td>
<td>Putative GPI-anchored protein</td>
<td>CA0188</td>
<td>0.31</td>
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<td><em>PGA16</em></td>
<td>Putative GPI-anchored protein</td>
<td>CA2015</td>
<td>0.41</td>
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<tr>
<td><em>PSA1</em></td>
<td>GDP-mannose pyrophosphorylase</td>
<td>CA3208</td>
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<tr>
<td><em>PMT2</em></td>
<td>O-D-mannosyltransferase</td>
<td>CA5894</td>
<td>0.43</td>
</tr>
<tr>
<td><em>DFG5</em></td>
<td>N-linked mannanprotein</td>
<td>CA4822</td>
<td>0.39</td>
</tr>
<tr>
<td><em>CDC3</em></td>
<td>Cell division control protein</td>
<td>CA0844</td>
<td>0.34</td>
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<tr>
<td><em>CDC10</em></td>
<td>Cell division control protein</td>
<td>CA4259</td>
<td>0.18</td>
</tr>
<tr>
<td><em>IPF7841</em></td>
<td>Nuclear envelope protein</td>
<td>CA2931</td>
<td>4.23</td>
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<tr>
<td><em>IPF9864</em></td>
<td>Mitotic spindle protein</td>
<td>CA4648</td>
<td>0.38</td>
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<tr>
<td><em>PHR1</em></td>
<td>pH responsive glycosyl transferase</td>
<td>CA4857</td>
<td>0.06</td>
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<tr>
<td><em>PHR2</em></td>
<td>pH-regulated protein 2</td>
<td>CA3867</td>
<td>0.35</td>
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**Lipid, fatty acid metabolism**

<table>
<thead>
<tr>
<th>Gene name†</th>
<th>Function description</th>
<th>Accession No.†</th>
<th>y0109b:y0109a ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FAA21</em></td>
<td>Long-chain-fatty-acid-CoA ligase</td>
<td>CA1596</td>
<td>2.82</td>
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<tr>
<td><em>FAA23</em></td>
<td>Long-chain-fatty-acid-CoA ligase</td>
<td>CA4311</td>
<td>2.33</td>
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<tr>
<td><em>TES15</em></td>
<td>Thioesterase</td>
<td>CA6220</td>
<td>3.00</td>
</tr>
<tr>
<td><em>CHO1</em></td>
<td>Phosphatidylerine synthase</td>
<td>CA4113</td>
<td>0.30</td>
</tr>
<tr>
<td><em>OPI3</em></td>
<td>Methylene-fatty-acyl-phospholipid synthase</td>
<td>CA5736</td>
<td>0.38</td>
</tr>
<tr>
<td><em>LAB5</em></td>
<td>Lipoic acid synthase</td>
<td>CA4117</td>
<td>0.46</td>
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**Amino acid metabolism**

<table>
<thead>
<tr>
<th>Gene name†</th>
<th>Function description</th>
<th>Accession No.†</th>
<th>y0109b:y0109a ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>BAT22</em></td>
<td>Branched chain amino acid aminotransferase</td>
<td>CA5040</td>
<td>3.10</td>
</tr>
<tr>
<td><em>LEU1</em></td>
<td>3-isopropylmalate dehydratase</td>
<td>CA5842</td>
<td>3.67</td>
</tr>
<tr>
<td><em>ODC1</em></td>
<td>Ornithine decarboxylase</td>
<td>CA1586</td>
<td>2.44</td>
</tr>
<tr>
<td><em>CAR2</em></td>
<td>Ornithine aminotransferase</td>
<td>CA2561</td>
<td>0.45</td>
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</table>

**Macromolecular synthesis**

<table>
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<tr>
<th>Gene name†</th>
<th>Function description</th>
<th>Accession No.†</th>
<th>y0109b:y0109a ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DBP3</em></td>
<td>ATP-dependent RNA helicase</td>
<td>CA1202</td>
<td>3.55</td>
</tr>
<tr>
<td><em>RRP6</em></td>
<td>Involved in 5.8S rRNA processing</td>
<td>CA2593</td>
<td>2.34</td>
</tr>
<tr>
<td><em>NOG1</em></td>
<td>Nucleolar G-protein</td>
<td>CA5682</td>
<td>3.06</td>
</tr>
<tr>
<td><em>MDN1</em></td>
<td>Midasin</td>
<td>CA0283</td>
<td>2.75</td>
</tr>
<tr>
<td><em>IPF2172</em></td>
<td>Nuclear transport factor</td>
<td>CA5307</td>
<td>4.16</td>
</tr>
<tr>
<td><em>MSW1</em></td>
<td>Tryptophanyl-tRNA synthetase</td>
<td>CA2636</td>
<td>2.83</td>
</tr>
</tbody>
</table>

(To be continued)
### Microarray analysis of FLC resistance in a laboratory *C. albicans* strain

**IMH3**  
Inosine-5’-monophosphate dehydrogenase  
CA1246  
3.42

**LEU1**  
3-isopropylmalate dehydratase  
CA5842  
3.67

**PHO13**  
4-nitrophenylphosphatase  
CA3722  
0.30

**HOM6**  
Homoserine dehydrogenase  
CA4181  
0.43

**ILV3**  
Dihydroxyacid dehydratase  
CA4802  
0.33

**CHO1**  
Phosphatidylserine synthase  
CA4133  
0.30

**PHO13**  
4-nitrophenylphosphatase  
CA3722  
0.31

**TRP5**  
Tryptophan synthase  
CA1580  
0.29

### Cell stress

**SSE1**  
Heat shock protein 70  
CA1911  
3.59

**EBP1**  
NADPH dehydrogenase  
CA1216  
0.39

**GRP2**  
Reductase  
CA2644  
0.37

**IPF2857**  
Regulated by cAMP and by osmotic stress  
CA5526  
22.70

**IPF4065**  
Possible stress protein  
CA0386  
3.74

### Transcription

**FCR1**  
Zinc cluster transcription factor  
CA5890  
0.30

**CTA4**  
Transcription factor  
CA5671  
2.67

**IPF928**  
Zinc-finger transcription factor  
CA5976  
3.33

**ZCF20**  
zinc-finger protein of transcription factor  
CA3088  
7.80

**ECM22**  
Putative transcription factor  
CA4071  
0.07

**IPF6235**  
CaTca2 retrotransposon  
CA2216  
12.56

**IPF4292**  
Bleomycin Hydrolase  
CA4250  
5.60

**IPF4905**  
GTPase regulators  
CA0899  
21.21

**IPF29**  
Zinc-finger transcription factor  
CA6084  
3.46

**TBPI**  
TATA-binding protein  
CA2369  
0.41

### Mitosis

**MIF2**  
Kinetochore protein  
CA2284  
0.46

**CBF1**  
Putative centromere binding factor 1  
CA2473  
0.41

**IPF4489**  
Mitotic spindle organization  
CA4744  
2.26

### Chromatin and chromosome structures

**RNR1**  
Ribonucleoside-diphosphate reductase  
CA2805  
0.15

**RNR21**  
Ribonucleoside-diphosphate reductase  
CA4155  
2.39

**HTA1**  
Histone H2A  
CA4696  
0.21

**IPF1055**  
Chromatin binding  
CA5752  
3.39

### DNA replication and DNA repair

**RFC1**  
DNA replication factor C  
CA4578  
0.47

**RFC5**  
DNA replication factor C  
CA0124  
0.33

**RAFI**  
DNA replication factor A  
CA4808  
0.34

**RAD16**  
Nucleotide excision repair protein  
CA0917  
4.78

**SGS1**  
ATP-dependent DNA helicase  
CA5495  
0.34

**IPF8423**  
Exonuclease  
CA1384  
0.32

**IPF9378**  
Subunit of the RNA polymerase II mediator  
CA3968  
0.33

### Others

66 genes of unknown function and others  
>2.00

112 genes of unknown function and others  
<0.50

---

*Gene names and accession numbers according to the *Candida albicans* genomic database (CandidaDB). †Data>2 means genes were up-regulated in y0109r compared to y0109s; data <0.5 means genes were down-regulated in y0109r compared y0109s. ABC, ATP-binding cassette; rRNA, transfer RNA; GDP, guanosine diphosphate.; GPI, glycosylphosphatidylinositol.
data (Fig. 3). As with the microarray data, there was no fold change in \( ERG11 \) and \( MDR1 \) in the strain \( y0109^R \) by real-time RT-PCR compared with the strain \( y0109^S \). Nevertheless, we observed less \( n \)-fold change for \( CDR1 \) in \( y0109^R \) with real-time RT-PCR than with microarray analysis. This may reflect the greater dynamic range of real-time RT-PCR analysis. In addition, since \( CDR2 \) has been shown to be co-regulated with \( CDR1 \) and both genes are regulated by trans-acting factor Tac1 [11], we performed real-time RT-PCR for \( CDR2 \) and \( TAC1 \). As expected, \( CDR2 \) and \( TAC1 \) were observed to be up-regulated 4.8-fold and 2.8-fold, respectively, in \( y0109^R \) in comparison with \( y0109^S \) (Fig. 3).

**Glucose-induced R6G efflux**

Since \( CDR1 \) was observed by microarray analysis to be up-regulated 19-fold and \( CDR2 \) was observed by real-time RT-PCR analysis to be up-regulated 4.8-fold, we then examined the energy-dependent efflux of R6G in both strains. Fig. 4 clearly shows that \( y0109^R \) effluxed more R6G than \( y0109^S \) in the presence of glucose. Efflux from deenergized (by incubation with 2-deoxy-D-glucose), R6G-preloaded cells of \( y0109^R \) required the presence of glucose; within 10 min of adding glucose, the extracellular R6G concentration had increased more than 6-fold. In contrast, R6G efflux from \( y0109^S \) in the presence of glucose was only 1.5-fold greater than that in the absence of glucose. These results provided evidence that overexpression of ABC transporters Cdr1 and Cdr2 in the strain \( y0109^R \) leads to increased energy-dependent extrusion of their substrates.

**Sterol profile analysis**

In light of the changes to ergosterol biosynthetic and sterol metabolic genes in the FLC-resistant strain, sterol components were extracted from both strains with or without FLC treatment and then subjected to GC-MS analysis. As summarized in Table 3, ergosterol was the major sterol in the strain \( y0109^R \), which accounted for 98.9% of the total sterols extracted from the untreated culture. However, FLC induced an increase in the lanosterol fraction at the expense of the ergosterol fraction, consistent with the FLC activity (inhibition of Erg11) in the ergosterol biosynthetic pathway. Particularly, the augment of 24-methylene-lanosterol, the direct substrate of target enzyme of FLC in \( C. albicans \), was higher than that of lanosterol.
Table 3 Sterol compositions of *Candida albicans* strains y0109<sup>S</sup> and y0109<sup>R</sup> with or without fluconazole treatment analyzed by gas chromatography-mass spectrometry

<table>
<thead>
<tr>
<th>Sterol identified</th>
<th>Sterol composition (%) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y0109&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lanosterol</td>
<td>ND</td>
</tr>
<tr>
<td>24-methylene-lanosterol</td>
<td>ND</td>
</tr>
<tr>
<td>4,4-dimethyl-zymosterol</td>
<td>ND</td>
</tr>
<tr>
<td>4-methyl-zymosterol</td>
<td>ND</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>ND</td>
</tr>
<tr>
<td>Ergosta-7,22-dienol</td>
<td>1.10±0.23</td>
</tr>
<tr>
<td>4-methylergoster-7,24(28)-dienol</td>
<td>ND</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>98.9±5.77</td>
</tr>
</tbody>
</table>

The results are the mean±SD for three independent experiments. †F0.25, 0.25 µg/ml fluconazole. ND, not detected.

In contrast, the types of sterol and the sterol composition in y0109<sup>R</sup> significantly differed from those in y0109<sup>S</sup>. Ergosterol accounted for only 60.5% of the total sterols extracted from the untreated culture in y0109<sup>R</sup>, while lanosterol was increased to 2.78%. Moreover, in y0109<sup>R</sup>, sterol intermediates, 4,4-dimethyl-zymosterol, 4-methyl-zymosterol and zymosterol, were identified. Interestingly, ergosta-7,22-dienol was increased to 13.5% of the total sterols. Furthermore, with the treatment of FLC, lanosterol and 24-methylene-lanosterol increased, while 4,4-dimethyl-zymosterol, 4-methyl-zymosterol, zymosterol, and ergosta-7,22-dienol all decreased. However, the degree of the ergosterol decrease was significantly lower than that of the lanosterol increase and the sterol intermediates decrease in the strain y0109<sup>R</sup>. In general, the sterol profile of this pair of strains was largely distinct, and the effect of FLC in sterol composition appeared to be dose dependent and proportional to the susceptibility of the individual strain.

Mutations in *ERG3* and *TAC1*

To screen for potential mutations that may have resulted in decreased FLC susceptibility in y0109<sup>R</sup>, *ERG3*, *ERG11*, and *TAC1* genes in both y0109<sup>S</sup> and y0109<sup>R</sup> were sequenced and compared to the *ERG3*, *ERG11*, and *TAC1* sequences in the *Candida* GenBank Database. Single fragments of the expected length were obtained in each strain. For these two strains, the only one missense mutation in *ERG3* gene was the single amino acid substitution D19E (Table 4). Meanwhile, both strains harbored one nucleotide change at base position 304 (ACC) in *ERG3* which did not affect the amino acid sequence. There were no mutations in *TAC1* in the strain y0109<sup>S</sup>. In contrast, the *TAC1* gene from y0109<sup>R</sup> had a total of two missense mutations (L47K and N977K) (Table 4). In addition, there was no amino acid difference in the two strains.

Discussion

Using *in vitro* conditions simulating chronic FLC exposure, we established the FLC-resistant *C. albicans* strain for the purpose of examining the potential drug resistance mechanisms in this FLC-resistant model. High-density oligonucleotide microarray analysis, confirmed by real-time RT-PCR, was performed with the susceptible parental strain and the resistant daughter strain to explore alteration on gene expression profile during the acquisition of FLC resistance. The differentially regulated genes were found to be involved in multiple biochemical functions. Our particular interest was the overexpression of ABC transporter genes, ergosterol biosynthetic genes, and sterol metabolic genes. Functional analysis was carried out to measure R6G efflux and sterol components. One single point mutation (D19E) in *ERG3* and two point mutations (L47K and N977K) in *TAC1* were identified in the strain y0109<sup>R</sup>, which could contribute to its FLC-resistant phenotype.

Particularly remarkable transcriptional expression change was the up-regulation of the membrane transporter *CDR1* by greater than 19 folds and *CDR2* by 4.8 folds in the strain y0109<sup>R</sup>, while no *MDR1* expression change was detected. With the glucose-induced R6G efflux assay, we further demonstrated that the activity of energy-dependent efflux pump was greater in the resistant y0109<sup>R</sup> than that in the susceptible y0109<sup>S</sup>. Recent studies have reported that *CDR1*, *CDR2*, and *PDR17* contain a common drug-
responsive element in their promoters that belong to the same Tac1 trans-acting regulation network [11,12,30]. In the present study, we showed that the up-regulation of CDR1, CDR2, and PDR17 suggested that the FLC-resistant strain y0109R had an activation of Tac1. Actually, TAC1 was observed to be up-regulated in y0109R, and was identified as having two missense mutations. Among them, the known L47K mutation is not associated with azole resistance [11]. The other mutation in TAC1 changed asparagine (N) to lysine (K) in codon 977. Indeed, the N977D mutation has been confirmed its importance in conferring hyperactivity to Tac1 [11]. Therefore, data in this study strongly suggest that TAC1 can be hyperactive through the N977K mutation and thereby up-regulates the expression of CDR1, CDR2, and PDR17 in y0109R.

In contrast to previous studies that have investigated the changes in response to short-term azole drugs exposure in C. albicans [16,31], there were no genes involved in the ergosterol biosynthesis pathway other than ERG2 and ERG9, which were found to be up-regulated in y0109R. Accordingly, the current data further highlight the importance of differences in constitutive changes in gene expression that result in FLC resistance and transient changes in the organism induced in order to respond to the presence of drug.

The sterol profile of the FLC-susceptible strain y0109S was highest in enriched ergosterol and contained only minor amounts of intermediate ergosta-7,22-dienol. In contrast, the sterol content in the resistant strain y0109R suggested that resistance to FLC, AMB and nystatin was the result of accumulation of sterol intermediates. The strain y0109R contained lanosterol, 4,4-dimethyl-zymosterol, 4-methyl-zymosterol and zymosterol, which were not detected in y0109S, as well as ergosta-7,22-dienol; ergosterol was the predominant sterol. Among these, 4,4-dimethyl-zymosterol, 4-methyl-zymosterol, and zymosterol were sterol intermediates upstream of fecosterol and episterol. As shown in Table 2, ERG2 was overexpressed in y0109R, possibly allowing for increased conversion of fecosterol to episterol. Furthermore, in y0109R, the conversion of toxic episterol to ergosta-7,22-dienol was enhanced at the expense of ergosta-5,7,24(28)-tri-enol and, ultimately, ergosterol. Still, FLC induced the increase in lanosterol fraction at the expense of sterol intermediates instead of ergosterol. Indeed, production of these precursor sterols has been demonstrated in erg3/erg3 mutant [32]. Likewise, our sterol analysis of the FLC-susceptible and the FLC-resistant strains showed a similar sterol profile, suggesting reduced activity of sterol C-5 desaturase (ERG3) in the strain y0109R. The analysis of the ERG3 sequence from these strains identified one missense mutation which changed the aspartic acid in codon 19 to glutamic acid in y0109R. These results strongly imply a relationship between the presence of the D19E mutation and the inactivation of Erg3 function.

In Saccharomyces cerevisiae, the induction of ergosterol biosynthesis genes encoding the enzymes that catalyzed late steps in ergosterol biosynthesis, such as ERG2, could be mediated by either Ecm22 or Upc2 [33]. Recent studies have shown that both Ecm22 levels and the amount of Ecm22 bound to promoters decreased upon ergosterol depletion, whereas Ecm22 is still able to increase the expression levels of its target genes in a Hap-dependent activator of ERG2 manner [34,35]. In the current study, ZCF20, homologous to ScHAP1, was up-regulated 7.8-fold, while the ECM22 was down-regulated. It was pos-
sible that overexpression of \( \text{ERG2} \) was regulated by Ecm22 and Zct20 in response to decreased ergosterol component in the FLC-resistant strain y0109\(^{e} \). Nevertheless, it was more likely that \( \text{ERG2} \) was transcriptionally regulated by Tac1 in y0109\(^{e} \).

Of the genes differentially expressed, a large majority, including \( \text{ADH3}, \text{KGD2}, \text{MDH12}, \text{PFK1}, \text{PGK1}, \) and \( \text{QCR7} \), were involved in energy metabolism. Ergosterol plays an important role in mitochondrial respiration and oxidative phosphorylation [36]. Therefore, the reduction in ergosterol levels in the resistant strain could affect the activities of several metabolic pathways. In fact, as we reported previously [22], increased expression of enzymes involving glycolysis and glyoxylate cycle in the FLC-resistant strain might compensate for the mitochondrial respiration deficiency; this contributes to the FLC tolerance partly resulting from the reduction in the intracellular reactive oxygen species generation after FLC treatment. Furthermore, alternative oxidase is induced as a response to conventional respiration deficiency [37]. Up-regulation of \( \text{AOX2} \) in this resistant strain actually supported this viewpoint.

Other changed genes in the present study included: small-molecular transporters genes \( \text{HGT11}, \text{HXT62}, \) and \( \text{SNF3} \); lipid, fatty acid metabolic genes \( \text{FAA21} \) and \( \text{FAA23} \); protein synthesis genes \( \text{RRP6}, \text{NOG1}, \) and \( \text{MSW1} \); DNA replication and repair genes \( \text{RFC1}, \text{RFA1}, \) and \( \text{RAD16} \); cell wall and cytoskeleton genes \( \text{STE7}, \text{SNF1}, \) and \( \text{EFG1} \). The role of these genes inazole resistance remains unclear. We suggested such alterations were collateral. We previously reported that y0109\(^{e} \) had a great ability to form hyphal from yeast cells, especially relative to y0109\(^{e} \) [38]; this may be partly explained by y0109\(^{e} \)’s overexpression of \( \text{HST7}, \text{SNF1} \) and \( \text{EFG1} \). The signal through Cst20p to the Cst1lp-Hst7p-Cek1p mitogen-activated protein kinase cascade and the signal through serine/threonine protein kinase to Efg1p cascade are two transcriptional regulation pathways involved in \( \text{C. albicans} \) budded-to-hyphal-form transition [39].

Additionally, we identified several genes of unknown and unclassified functions that were differentially expressed between the pair of strains. These genes might be associated with FLC resistance in \( \text{C. albicans} \), and hence, their putative functions require further investigation.

**Conclusion**

In the present study, a FLC-resistant \( \text{C. albicans} \) strain was obtained by serial cultures of a FLC-susceptible strain in inhibitory concentrations of FLC. Using cDNA microarray to profile transcriptional expression of this matched pair of \( \text{C. albicans} \) strains, we were able to identify genes whose expression changed during the acquisition of resistance to azoles. We showed that a point mutation, the replacement of Asn by Lys at position 977 of the transcription factor gene \( \text{TAC1} \), could modify the activity of Tac1 into a hyperactive state, thereby resulting in the up-regulation of \( \text{CDR1} \) and \( \text{CDR2} \). We also discovered that a single amino acid difference (D19E) in \( \text{ERG3} \) could inactivate the Erg3 function, leading to decreased ergosterol fraction content as the sterol intermediates accumulate in the resistant strain. This study also characterized the transcriptional alterations that accompany the development of resistant phenotype. Our results demonstrated that the laboratory FLC-resistant \( \text{C. albicans} \) strain harbored known resistance mechanisms which were observed in the clinical isolates. Findings based on this model could expand our understanding of potential new molecular mechanisms of FLC resistance in \( \text{C. albicans} \).

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