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Original articles

Identification of genes differentially expressed in association with reduced azole susceptibility in *Saccharomyces cerevisiae*

Katherine S. Barker[1], Margaret M. Pearson[2] and P. David Rogers[1,3,4]*

Departments of [1]Pharmacy and [3]Pharmaceutical Sciences, College of Pharmacy; [4]Department of Pediatrics, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163; [2]Department of Pharmacy Practice, School of Pharmacy,University of Mississippi, Jackson, MS 39216, USA

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***Objective*: An isolate of *S. cerevisiae* with reduced susceptibility to fluconazole and itraconazole was developed in the laboratory and used to identify genes that are differentially expressed in association with this phenotype.**

***Methods*: *S. cerevisiae* strain ATCC 9763 was passaged in increasing concentrations of itraconazole. Itraconazole and fluconazole MICs for the initial isolate (9763S) were 2 and 16 mg/L and for the final isolate (9763I) were 16 and ≥64 mg/L, respectively. Duplicate sets of total RNA from 9763S and 9763I were isolated and hybridized to Affymetrix S98 yeast arrays. To validate results, six differentially expressed genes were further examined by RT–PCR.**

***Results*: Of the nearly 6400 open reading frames represented on the array, a total of 116 genes (1.8%) were found to be differentially expressed. Cell wall maintenance genes *TIR4* and *CCW12*, sterol metabolism gene *UPC2*, small molecule transport genes *AUS1* and *YHK8*, and stress response gene *CUP1-1* were expressed at a level at least 2.5-fold higher than the expression level found in 9763S. Eleven energy generation genes, ionic homeostasis genes *FRE1*, *FRE2* and *FRE4*, and sterol metabolism genes *ERG8* and *ERG13* were expressed at least 2.5-fold lower than the expression level found in 9763S.**

***Conclusions*: Several genes found to be differentially expressed in this study have been shown previously to be differentially expressed in the fungal response to azole treatment. In addition, the potential role of *AUS1* and/or *YHK8* as mediators of drug efflux is intriguing and warrants further study.**

Keywords: fungi, microarray, resistance mechanisms

Introduction

The incidence of fungal infections is increasing worldwide.[1,2] Often, these infections, both systemic and superficial, are treated with azoles, a class of antifungal compounds that inhibit lanosterol 14α-demethylase (Erg11p) in the ergosterol biosynthesis pathway. Although azoles are effective in the management of many fungal infections, acquired azole resistance in species such as *Candida albicans*, *Aspergillus* species and *Cryptococcus neoformans*, and intrinsic azole resistance in species such as *Candida glabrata* and *Candida krusei* are frequent occurrences in immunocompromised patients.[3–7] Acquired azole resistance arises from lengthy duration of treatment, repeated courses of therapy and non-compliance with treatment, leaving fungal organisms that are difficult to eradicate. Therefore, recent studies have focused on identifying genes involved in azole resistance in fungal pathogens.

A common theme in azole resistance is over-expression of genes encoding efflux pumps or the azole target lanosterol demethylase, encoded by the *ERG11* gene.[5,8–13] Several groups

*Corresponding author. Tel: +1-901-448-3719; Fax: +1-901-448-6064; E-mail: drogers@utmem.edu

have reported either the up-regulation of *ERG11* or point mutations in this gene in azole-resistant clinical *C. albicans* isolates.[12] Efflux pump genes that have been implicated in azole resistance are the ATP-binding cassette (ABC) transport genes *CDR1* and *CDR2*, as well as the major facilitator gene *CaMDR1* (*BMR1*).[14] Up-regulation of efflux pump genes has been demonstrated in a number of azole-resistant *C. albicans* isolates and is thought to result in extrusion of azoles.

Because the *Saccharomyces cerevisiae* genome has been sequenced and many of the ORFs are well-characterized, it is well suited for studying changes in gene expression in response to antifungal compounds. Previous studies have discovered the pleiotropic drug resistance (PDR) gene family, including the *PDR1* and *PDR3* transcription factors that regulate drug efflux pumps such as the ABC transporter *PDR5*.[15–18] Subsequently, *C. albicans* homologues of *PDR5* were identified as *CDR1* and *CDR2*.[19]

Recent studies have taken advantage of microarray technology to examine the expression of thousands of genes simultaneously in an organism. Bammert & Fostel[20] compared gene expression profiles of wild-type and ergosterol-deficient *S. cerevisiae* strains treated with azoles, finding cell wall function, mitochondrial respiration and oxidative stress genes affected by azole treatment. Comparing expression profiles of wild-type and *PDR* mutant strains of *S. cerevisiae*, DeRisi and colleagues[21] found that several ABC and major facilitator transport, stress response and cell wall function genes were activated in the mutant strains.

Most recently, we have examined *S. cerevisiae* incubated with ketoconazole,[22] and found that ergosterol biosynthesis and small molecule transport genes were primarily up-regulated. De Backer and colleagues[23] studied the response of *C. albicans* to itraconazole where 296 genes were responsive, encompassing many different cellular roles. In a study examining azole resistance in experimentally induced and clinical *C. albicans* isolates, several cell stress, multidrug transport and cell wall maintenance genes were affected.[24] Likewise, in a study using azole-susceptible and -resistant clinical *C. albicans* isolates, we found several of the same genes differentially expressed in azole resistance.[25]

Of all the studies highlighted above, only three examined resistant isolates using microarray techniques.[23–25] The paucity of information on the majority of *C. albicans* ORFs suggests that similar examination of *S. cerevisiae* may yield more information on the specific roles of responsive genes to give greater insight into genes involved in azole resistance.

The current study involves analysis of differential gene expression by microarray analysis in a strain of *S. cerevisiae* in which reduced susceptibility to fluconazole and itraconazole was experimentally induced. Cell wall maintenance, lipid, fatty-acid and sterol metabolism, and small molecule transport genes were among those differentially expressed. This study identifies genes associated with altered azole susceptibility in *S. cerevisiae* whose homologues in fungal pathogens could be exploited as drug targets.

Materials and methods

S. cerevisiae isolate and generation of resistance

Cultures of *S. cerevisiae* isolate ATCC 9763 (ATCC, Manassas, VA, USA) were passed in increasing concentrations of itraconazole (formulated for injection; Ortho Biotech, Raritan, NJ, USA). Aliquots of the parent strain (designated 9763S) and the final strain (9763I) were stored as glycerol stocks. Cultures grown for RNA isolation were initiated by diluting an aliquot of glycerol stock in YPD broth (1% yeast extract, 2% bacto-peptone, 2% glucose; from Sigma Chemical Co.) in the absence of itraconazole and incubating overnight at 30°C in an environmental shaking incubator. Cultures were then diluted to an optical density at 600 nm (OD_{600}) of 0.2 in fresh YPD in the absence of itraconazole and grown as before to mid-logarithmic phase (OD_{600} = 0.5–0.8). At this point, an aliquot of this culture was prepared as a glycerol stock and used for susceptibility testing while RNA was isolated from the remainder of the culture.

MIC determination

The MICs of itraconazole, fluconazole, amphotericin B, flucytosine and caspofungin were determined by broth microdilution as described by the National Committee for Clinical Laboratory Standards (NCCLS).[26] The determinations of the MICs for the isolates were carried out before experimental use and are listed in Table 1.

Total RNA isolation

RNA was isolated from two sets of independently grown cultures of isolates with the hot phenol method, suspended in diethylpyrocarbonate (DEPC)-treated water, and stored at –70°C until use.[27] Absorbances were measured at 260 and

Table 1. MICs (mg/L) for isolates used

Strain name	Amphotericin B	Flucytosine	Fluconazole	Itraconazole	Caspofungin
9763S	1.0	2.0	16	2.0	1.0
9763I	1.0	2.0	≥64	16	1.0

280 nm, and integrity of RNA was visualized by subjecting a portion of the sample to electrophoresis through a 1% agarose–MOPS gel. RNA was isolated from two independent sets of cultures for use in microarray hybridizations, while a third set of RNA was isolated independently for subsequent RT–PCR analysis.

Microarray hybridization

Microarray hybridizations were carried out with the Affymetrix S98 array using protocols as described by Affymetrix, Inc. (Santa Clara, CA, USA). The Affymetrix S98 Yeast Genome array contains ~6400 oligonucleotide probe sets designed from sequence data of *S. cerevisiae* strain S288C and ~600 additional probe sets representing putative ORFs identified by SAGE analysis, mitochondrial proteins, TY proteins, plasmids and a small number of ORFs for strains other than S288C. Probes for hybridization were synthesized from sets of 9763I and 9763S *S. cerevisiae* RNA samples generated from two independent experiments. Ten micrograms of total RNA were subjected to first- and second-strand cRNA synthesis incorporating biotin-labelled nucleotides. Biotinylated product was then fragmented and subsequently hybridized overnight with the microarray chips and the manufacturer's hybridization buffer. Hybridized microarrays were washed according to manufacturer's protocols and subjected to a signal enhancement protocol consisting of an initial incubation with a streptavidin–phycoerythrin conjugate followed by a goat anti-streptavidin biotinylated antibody and a final staining with the streptavidin–phycoerythrin conjugate. The microarrays were then scanned with the GeneArray scanner using an argon ion laser excitation source and emission detected by a photomultiplier tube through a 570 nm long pass filter.

Data analysis

Data were analysed with Affymetrix Microarray Suite 5.0 software. Genes were considered to be differentially expressed if the element was assigned at least one 'present call' for either the 9763S or 9763I hybridization in both experiments, normalized signal ratios were ≥2.5 or ≤0.4 in both experiments, and the change in gene expression was in the same direction in both experiments. Genes were annotated on the basis of results of BLAST*n* searches in GenBank (http://www.ncbi.nlm.nih.gov/entrez/) and the *Saccharomyces* Genome Database (SGD; http://genome-www.stanford.edu/Saccharomyces/).

cDNA synthesis and RT–PCR

Two micrograms of total RNA from each sample was denatured in the presence of 1 μg oligo(dT) primer stock (ResGen/Invitrogen, Carlsbad, CA, USA) at 70°C after which the mixture was chilled on ice, and a master mix containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, dATP, dCTP, dGTP and TTP at 1.25 mM each, and 25 U Superscript II reverse transcriptase (Gibco BRL/Invitrogen, Carlsbad, CA, USA) was added to each tube. The reaction mixture was incubated for 10 min at room temperature, followed by a 60 min incubation at 37°C and a 5 min incubation at 90°C.

PCR was carried out by mixing 1 μL of the appropriate dilution of cDNA (empirically determined for each gene to give product in the linear range), 0.5 μg each forward and reverse primer, 2.5 U *Taq* polymerase, and 0.1% Triton X-100 in EasyStart Micro50 PCR tubes, and subjecting the reaction mixture to the following reaction conditions: one repetition of 94°C for 5 min; 32 repetitions of 94°C for 30 s, gene-specific annealing temperature for 30 s, 72°C for 1 min and one repetition of 72°C for 5 min. Equivalent volumes of PCR product were applied to a 3% agarose gel and separated by gel electrophoresis in 1 × TAE. Primers for RT–PCR are listed in Table 2.

Results and discussion

In the present study, reduced azole susceptibility to fluconazole and itraconazole was experimentally induced by growing the ATCC 9763 strain of *S. cerevisiae* in increasing concentrations of itraconazole. The resulting resistant strain (9763I) and parent strain (9763S) were tested for azole susceptibility (Table 1). MICs of both itraconazole and fluconazole were significantly increased for 9763I.

Table 2. Nucleotide sequence of primers used for RT–PCR

Primer name	Primer sequence	T_m (°C)
AUS1-F	5′-AGGCTGGTGAAATGGTTTTG-3′	55.0
AUS1-R	5′-AGAGCTGTGGCGGAATCTAA-3′	57.2
YHK8-F	5′-CTCCCGAAATGAGATTCGAG-3′	53.6
YHK8-R	5′-CCCAATTCACTCCCATTGAC-3′	54.5
UPC2-F	5′-CAACAGGAACAACTCCAGCA-3′	56.5
UPC2-R	5′-CTGCACGTTCATGATGCTCT-3′	56.6
MET14-F	5′-ACGCAAGGCATTGAGAAAAC-3′	55.5
MET14-R	5′-CACCCTCCCTAGCTTTCTTG-3′	55.8
ERG8-F	5′-ATTCGCATATGCCAGAAAGC-3′	54.9
ERG8-R	5′-TAGCGGTTTGAGCCCTAAGA-3′	56.8
COX5A-F	5′-ATCCAGATGGGAGAACATGC-3′	55.7
COX5A-R	5′-CATCCTGACGACAGCAAAAA-3′	54.6
FRE2-F	5′-GAGCATCACCTGCAAAGACA-3′	56.6
FRE2-R	5′-CCTTTGACATAGGGCGTCAT-3′	55.9
18S-F	5′-GCCAGCGAGTATAAGCCTGG-3′	55.8
18S-R	5′-AGGCCTCACTAAGCCATTCA-3′	62.3

One potential limitation of the present study is that the intravenous formulation of itraconazole was used to generate 9763I. It is therefore possible that some of the changes in gene expression induced by exposure to this formulation were the result of exposure to β-cyclodextrin. However, the use of the intravenous formulation resulted in the generation of a strain with reduced susceptibility to both itraconazole and fluconazole that expressed genes consistent with a response to azole exposure as outlined below.

The *S. cerevisiae* genome was examined with microarrays to compare differences in gene expression between 9763I and 9763S. From both independent experiments, a total of 116 genes (1.8% of all the ORFs) was reproducibly differentially expressed in 9763I, i.e. at least 2.5-fold higher or lower than the expression level found in 9763S. Of the 116 genes, there were 64 up-regulated genes (Table 3) and 52 down-regulated genes (Table 4). Six genes of interest from the array data were further examined by RT–PCR (Figure 1).

Lipid, fatty-acid and sterol metabolism genes

Three lipid, fatty-acid and sterol metabolism genes were differentially expressed, including *ERG8* and *ERG13*, which were down-regulated in 9763I. Whereas several studies have demonstrated the increase in *ERG* gene expression in response to azole treatment in *S. cerevisiae*[20,22] and *C. albicans*[23] or in *C. albicans* azole resistance,[25] none document down-regulation of *ERG* genes. However, there are clinical *C. albicans* isolates that contain a significant fraction of

Table 3. Genes up-regulated in association with reduced azole susceptibility in *S. cerevisiae*

ORF	Gene name	Function	Average fold change in gene expression
Amino acid metabolism			
YKL001C	*MET14*	sulphate assimilation pathway	3.92
YER081W	*SER3*	3-phosphoglycerate dehydrogenase	2.64
Cell wall maintenance			
YOR009W	*TIR4*	seripauperin family of possible cell wall mannoproteins	7.84
YLR110C	*CCW12*	cell wall mannoprotein sim. to Flo1, Fig2, Flo5 and Ykr102	3.87
Carbohydrate metabolism			
YGR192C	*TDH3*	glyceraldehyde-3-phosphate dehydrogenase 3	4.61
YJR009C	*TDH2*	glyceraldehyde-3-phosphate dehydrogenase 2	3.61
YGR254W	*ENO1*	enolase 1	3.16
YCR012W	*PGK1*	phosphoglycerate kinase	2.64
Cell cycle control and mitosis			
YDR068W	*DOS2*	involved in genome stability	3.37
YHR152W	*SPO12*	possible regulator of exit from M-phase in mitosis, meiosis	3.04
Lipid, fatty-acid and sterol metabolism			
YDR213W	*UPC2*	regulatory protein involved in control of sterol uptake	3.47
Other metabolism			
YDR044W	*HEM13*	coproporphyrinogen III oxidase	5.78
YER014W	*HEM14*	protoporphyrinogen oxidase	4.51
Protein synthesis			
YJR047C	*ANB1*	translation initiation factor eIF5A	11.82
YLR167W	*RPS31*	fusion protein comprised of ubiquitin and ribosomal protein S31	3.41
YPR080W	*TEF1*	translation elongation factor EF-1α	3.14
YPL220W	*RPL1A*		2.93
YPR132W	*RPS23B*		2.93
YDL083C	*RPS16B*		2.73

Table 3. (*continued*)

ORF	Gene name	Function	Average fold change in gene expression
YGL189C	*RPS26A*		2.73
YHR021C	*RPS27B*		2.73
Protein targeting, sorting and translocation			
YEL020W-A	*TIM9*		4.55
YBR162W-A	*YSY6*	protein that participates in secretory pathway	2.94
Small molecule transport			
YOR011W	*AUS1*	ABC protein involved in uptake of sterols	6.45
YGR131W	*YG3B*	strong similarity to Nce2	5.03
YBR085W	*AAC3*	ADP/ATP transporter protein	4.30
YHR048W	*YHK8*	similarity to multidrug resistance proteins	2.93
Stress response			
YHR053C	*CUP1-1*	metallothionein (copper chelatin)	7.25
Unknown			
YOR338W			23.4
YCR013C			13.6
YDL228C		similar to *CaRHK1* (mannosyltransferase)	11.0
YLR162W			9.93
YOL101C			8.88
YLR076C		similar to *CaROT2* glucosidase II	8.30
YKL153W	*YKP3*		8.02
YIR043C	*YIW3*		7.91
YOR382W	*FIT2*	possibly involved in iron uptake	7.74
YNL303W	*YN43*		7.22
YPL197C			7.12
YBR089W	*YBT9*		6.56
YPR044C			6.06
YDR524W-A			5.71
YLR264C-A			5.47
YLR062C	*BUD28*	may be involved in bud site selection in diploid cells	5.28
YOR277C			5.00
YCR087W			4.94
YJL188C	*BUD19*	may be involved in bud site selection, bipolar budding	4.61
YGL102C			4.60
YLL044W			4.46
YPL142C			4.44
YLR230W			4.14
YOR006C	*YO06*		3.62
YDR534C	*FIT1*	possibly involved in iron uptake	3.52
YDR417C			3.49
YOR078W	*BUD21*	involved in processing 20S pre-RNA	3.49
YOR169C			3.48
YDR524C-A			3.26
YBL059W	*YBF9*		3.26
YBL049W	*YBE9*		3.25
YNR029C	*YN8H*		3.03
YLL027W	*ISA1*		2.94
YDR133C			2.66
YNR024W	*YN8D*		2.93
YKL056C	*TCTP*		2.83

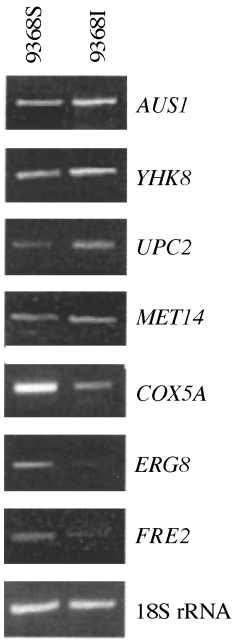

Figure 1. RT–PCR of selected up-regulated and down-regulated genes. cDNA from 9763S and 9763I total RNA was used to amplify the following differentially expressed genes: *AUS1*, *YHK8*, *UPC2*, *MET14*, *COX5A*, *FRE2* and *ERG8*. PCR products were normalized to the relative level of 18S rRNA PCR product amplified from each cDNA sample.

membrane sterols other than ergosterol.[28] It is possible that isolate 9763I contains an increase in alternative sterols in its membrane that may contribute to the increase in MIC and allow for down-regulation of *ERG8* and *ERG13*.

Sterol uptake genes

UPC2, encoding a transcription factor that regulates sterol biosynthesis uptake, was up-regulated in the present study. The role of *UPC2* was examined recently in a microarray analysis comparing wild-type and *upc2-1* mutant strains of *S. cerevisiae*.[29] As a result of the gain-of-function mutation, *UPC2* itself was up-regulated in the mutant strain as were several other genes also found to be differentially expressed in the present study: *TIR4*, *AUS1* (*YOR011W*), *FIT2*, *HEM13*, *HEM14* and *YGR131W*. When *UPC2* was deleted in the *upc2-1* strain, sterol uptake was significantly diminished in anaerobic conditions. Deletion mutants of eight other genes found to be over-expressed in the *upc2-1* strain were constructed, but only the *yor011w*Δ strain (*AUS1* deletion mutant) exhibited a significant decrease in sterol uptake.[29] *AUS1* is an ABC transport gene similar to the *CDR* genes and is up-regulated under aerobic conditions in response to azole exposure.[22] *CDR1* can transport steroid molecules as well as elicit drug efflux;[30] similarly, it may be possible that *AUS1* is a mediator of drug efflux as well as sterol transport. Regardless, *AUS1* is up-regulated both in response to azole treatment and as a result of reduced azole susceptibility in *S. cerevisiae*; therefore, the specific function of *AUS1* in this context should be examined further.

Energy generation, ionic homeostasis and small molecule transport genes

Most of the energy generation gene products down-regulated in this study are mitochondrial electron transport proteins. We speculate that these genes are down-regulated in order to lessen the amount of endogenously generated reactive oxygen species (ROS) in the cell. This would be in response to generation of ROS due to the action of azoles on the cell membrane. Kobayashi and colleagues[31] demonstrated the production of ROS in *C. albicans* by miconazole and fluconazole, and there was a strong inverse correlation between the level of ROS production and the MIC. The authors speculated that isolates may exhibit resistance mechanisms that involve scavenging ROS. In the present study, the metallothionein *CUP1-1* was up-regulated over seven-fold and has been shown to be up-regulated in the ROS response.[32] Three ionic homeostasis genes, *FRE1*, *FRE2* and *FRE4*, and two metal ion transport genes, *SIT1* and *CTR3*, were down-regulated in 9763I. In previous studies, we have found that iron transport genes were down-regulated in fluconazole-resistant clinical *C. albicans* isolates.[27]

Amino acid metabolism genes

Five amino acid metabolism genes were found to be differentially expressed in 9763I compared with 9763S. Found to be up-regulated among these is *ScMET14*, a gene whose product is induced in response to oxidative stress.[33] In microarray analysis carried out previously to examine the *S. cerevisiae* response to cadmium, a potent cell poison that causes oxidative stress, ~50 genes were up-regulated four-fold or more in response to oxidative stress. Most were stress response genes, glutathione synthesis genes and sulphur assimilation genes including *ScMET14*.[33]

Cell wall maintenance genes

CCW12 and *TIR4*, up-regulated in 9763I, both encode cell wall mannoproteins that, although their specific biological functions are unknown, have dramatic effects on the cell if not expressed. In a study demonstrating that the *CCW12* gene product was associated with the cell wall in *S. cerevisiae*, disruption of the gene led to pronounced sensitivity to Calcofluor White and Congo Red as well as decreased mating efficiency and decreased level of agglutination.[34] The gene *TIR4* is one of nine members of the seripauperin family of cell wall mannoproteins, the *DAN/TIR* family. These genes are similarly regulated, and all nine are expressed only during anaero-

Table 4. Genes down-regulated in association with reduced azole susceptibility in *S. cerevisiae*

ORF	Gene name	Function	Average fold change in gene expression
Amino acid metabolism			
YFR055W	*METC*	strong similarity to β-cystathionases	0.27
YCL030C	*HIS4*	catalyses 2nd, 3rd and 10th steps of histidine biosynthesis	0.23
YPL111W	*CAR1*	arginase	0.21
Carbohydrate metabolism			
YIL125W	*KGD1*	α-ketoglutarate dehydrogenase complex E1	0.33
YLR153C	*ACS2*	acetyl-coA synthetase	0.33
Cell cycle control and mitosis			
YNL025C	*SSN8*	C-type cyclin	0.29
Cell rescue, defence, cell death and ageing			
YGR234W	*YHB1*	involved in protection from nitrosative stress	0.38
YBR054W	*YRO2*	homologue to hsp30 heat shock protein Yro1	0.28
YGL021W	*ALK1*	DNA damage-responsive protein	0.25
YLL024C	*SSA2*	cytoplasmic protein chaperone of the hsp70 family	0.22
YBR244W	*GPX2*	glutathione peroxidase	0.13
Energy generation			
YJL166W	*QCR8*	ubiquinol cytochrome *c* reductase subunit 8	0.35
YGR183C	*QCR9*	ubiquinol cytochrome *c* reductase subunit 9	0.32
YPR191W	*QCR2*	ubiquinol cytochrome *c* reductase core protein 2	0.29
YEL024W	*RIP1*	ubiquinol cytochrome *c* reductase iron–sulphur protein	0.24
YHR001W-A	*QCR10*	subunit of ubiquinol–cytochrome *c* oxidoreductase complex	0.23
YMR145C	*NDE1*	mitochondrial NADH dehydrogenase	0.20
YNL052W	*COX5A*	cytochrome *c* oxidase subunit Va	0.20
YMR282C	*AEP2*	protein required for the expression of Atp9	0.17
YGL187C	*COX4*	cytochrome *c* oxidase subunit IV	0.13
YJR048W	*CYC1*	cytochrome *c* isoform 1	0.095
YDL181W	*INH1*	inhibitor of mitochondrial ATPase	0.083
Ionic homeostasis			
YNR060W	*FRE4*	similar to *FRE2*	0.34
YLR214W	*FRE1*	membrane-associated flavocytochrome	0.23
YKL220C	*FRE2*	involved in iron uptake from siderophore rhodotorulic acid	0.23
Lipid, fatty-acid and sterol metabolism			
YMR220W	*ERG8*	phosphomevalonate kinase	0.33
YML126C	*ERG13*	3-hydroxy-3-methylglutaryl coenzyme A synthase	0.27

Table 4. (*continued*)

ORF	Gene name	Function	Average fold change in gene expression
Nucleotide metabolism			
YOR128C	*ADE2*	phosphoribosylaminoimidazole carboxylase	0.33
YMR120C	*ADE17*	5-aminoimidazole-4-carboxamide ribonucleotide transformylase	0.25
YAR015W	*ADE1*	phosphoribosylamidoimidazole-succinocarboxamide synthase	0.18
Other metabolism			
YAL063C	*FLO9*	member of the Flo1 family of flocculation proteins	0.22
YMR282C	*AEP2*	protein required for the expression pf Atp9	0.17
Protein targeting, sorting and translocation			
YAL005C	*SSA1*	cytoplasmic chaperone and heat shock protein of Hsp70 family	0.10
Protein synthesis			
YPL198W	*RPL7B*		0.25
Small molecule transport			
YDR046C	*BAP3*	branched chain amino acid permease	0.35
YLL028W	*TPO1*	polyamine transport protein; MFS-MDR family member	0.33
YEL065W	*SIT1*	ferrioxamine B permease	0.32
YPR138C	*MEP3*	NH_4^+ transporter	0.31
YKR093W	*PTR2*	peptide permease	0.24
YHL036W	*MUP3*	low-affinity methionine amino acid permease	0.23
YCL025C	*AGP1*	amino acid permease with high affinity for asn and gln	0.21
YPR194C	*OPT2*	oligopeptide transporter	0.17
YER411W	*CTR3*	high-affinity copper transporter	0.057
Transcription			
YHR211W	*FLO5*	flocculin	0.31
YHR178W	*STB5*	zinc finger protein	0.20
YML091C	*RPM2*	involved in tRNA maturation	0.16
Unknown			
YJL012C	*VTC4*	involved in vacuolar polyphosphate accumulation	0.37
YER064C			0.20
YAR060C	*YH12*		0.19
YPL014W			0.13
YLR108C			0.11
YAR068W	*YAN8*		0.061

bic conditions. Additionally, when *TIR1*, *TIR3* or *TIR4* is disrupted, cells cannot grow during anaerobis.[35] Since the up-regulation of *TIR4* in the present study does not coincide with the up-regulation of other *DAN/TIR* genes or with the down-regulation of their aerobic-growth counterparts *CWP1* and *CWP2*, it is possible that *TIR4* has additional roles in response to conditions elicited by azoles.

Conclusions

The present study utilizes an experimentally induced isolate of *S. cerevisiae* with reduced susceptibility to fluconazole and itraconazole as a model for finding novel molecules associated with azole resistance. Previous studies have focused on *PDR* and *ERG* genes in *S. cerevisiae* azole resistance. Here we found genes that have previously not been associated with this phenotype. These molecules represent potential new targets to examine in relation to azole resistance.

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