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Genome-Wide Expression Profiling of the Response to Azole, Polyene, Echinocandin, and Pyrimidine Antifungal Agents in *Candida albicans*†

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Antifungal agents exert their activity through a variety of mechanisms, some of which are poorly understood. We examined changes in the gene expression profile of *Candida albicans* following exposure to representatives of the four currently available classes of antifungal agents used in the treatment of systemic fungal infections. Ketoconazole exposure increased expression of genes involved in lipid, fatty acid, and sterol metabolism, including *NCP1*, *MCR1*, *CYB5*, *ERG2*, *ERG3*, *ERG10*, *ERG25*, *ERG251*, and that encoding the azole target, *ERG11*. Ketoconazole also increased expression of several genes associated with azole resistance, including *CDR1*, *CDR2*, *IFD4*, *DDR48*, and *RTA3*. Amphotericin B produced changes in the expression of genes involved in small-molecule transport (*ENA21*), and in cell stress (*YHB1*, *CTA1*, *AOX1*, and *SOD2*). Also observed was decreased expression of genes involved in ergosterol biosynthesis, including *ERG3* and *ERG11*. Caspofungin produced changes in expression of genes encoding cell wall maintenance proteins, including the β-1,3-glucan synthase subunit *GSL22*, as well as *PHR1*, *ECM21*, *ECM33*, and *FEN12*. Flucytosine increased the expression of proteins involved in purine and pyrimidine biosynthesis, including *YNK1*, *FUR1*, and that encoding its target, *CDC21*. Real-time reverse transcription-PCR was used to confirm microarray results. Genes responding similarly to two or more drugs were also identified. These data shed new light on the effects of these classes of antifungal agents on *C. albicans*.

Candida albicans is the most common human fungal pathogen and is the fourth leading cause of bloodstream infections in the United States (6, 14, 24). Currently only four antifungal drug classes are available for the management of systemic infections due to *Candida* species. Recently we examined changes in the genome-wide expression profile of *Saccharomyces cerevisiae* in response to representatives of the polyene, pyrimidine, azole, and echinocandin antifungal agents in an effort to identify class-specific and mechanism-independent changes in gene expression (1). In the present study, we extend this analysis to the pathogenic fungus *C. albicans*. By using the same representative drugs and similar growth conditions as in our previous study, we are able to show similarities and differences in the responses to these antifungal agents between *S. cerevisiae* and *C. albicans*. Gene expression profiling experiments revealed drug-specific responses consistent with their mechanisms of action, responses indicative of other pathways that may be affected by these agents, and responses that reflect known and potential mechanisms of resistance to these antifungal drugs.

MATERIALS AND METHODS

Antifungal agents. Ketoconazole (KTZ) and flucytosine (5-FC) were obtained from Sigma (St. Louis, MO). Amphotericin B (AMB) was obtained from ICN Biomedicals (Aurora, OH). The commercially available preparation of caspofungin (CPF) acetate for injection (Cancidas) was used. Stock solutions of various concentrations were made in dimethyl sulfoxide (DMSO; Sigma).

***C. albicans* strains and media.** *C. albicans* strain SC5314, obtained from the American Type Culture Collection (Manassas, VA), was used for microarray experiments. Synthetic dextrose medium, containing 0.67% (wt/vol) yeast nitrogen base without amino acids and 2% (wt/vol) dextrose, was used to grow strain SC5314. The medium was buffered with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), and the pH was adjusted to 7.0 with NaOH.

IC_{50} determinations. To ensure consistent results, 50% inhibitory concentrations (IC_{50}s) were determined under the same environmental conditions used in subsequent microarray analysis, including incubation times, culture volume (100 ml), flask size (250-ml Corning 430281 roller bottle), temperature (30°C), and shaking speed (225 rpm). All measurements of turbidity were read at 600 nm on a Beckman DU530 spectrophotometer. Cultures with an A_{600} exceeding 1.0 were diluted 1:10 in water for measurement. One hundred milliliters of Synthetic dextrose medium was inoculated from a fresh, saturated culture of *C. albicans* to an initial A_{600} of 0.005 and grown at 30°C until the A_{600} reached 1.0 (approximately 12 h). The culture was then diluted to an A_{600} of 0.1 and allowed to recover from stationary phase at 30°C/225 rpm until an A_{600} of 0.2 was reached (approximately 2 h). Antifungal drug was then added according to the desired concentration from a 10-mg/ml drug stock in DMSO, and an equal amount of DMSO was added to the control culture. DMSO concentrations were below 0.05% and did not affect the growth curves of control cultures. The cultures were grown for 6 h, and the A_{600} was measured at both 3 and 6 h. IC_{50}s were calculated at 6 h, in reference to the control culture. Three rounds of experiments were conducted from which the average IC_{50} was calculated. In the first experiment, a broad range of six twofold serial dilutions were tested based on the MIC. A narrow range of three drug concentrations was then tested in the second and third rounds (KTZ, 6.25 to 25 μg/ml; AMB, 0.025 to 0.1 μg/ml; CPF, 0.005 to 0.02 μg/ml; 5-FC, 0.05 to 0.2 μg/ml). Cells used for subsequent microarray analysis were grown at the average IC_{50} to ensure 50% growth inhibition.

Drug exposure for microarray experiments. *C. albicans* was grown as described in the IC_{50} section above. For a single microarray experiment, a total of six 100-ml cultures were prepared, three with an antifungal drug added at a concentration equivalent to the IC_{50} (KTZ, 19.13 μg/ml; AMB, 0.029 μg/ml; CPF, 0.0075 μg/ml; 5-FC, 0.098 μg/ml) and three control cultures treated with an

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appropriate amount of DMSO. Three hours after drug addition, the A_{600} was measured and two control cultures and two drug-treated cultures were harvested by centrifugation at 3,600 × *g* at 4°C for 5 min. The medium was completely removed by aspiration, and the cell pellets were flash frozen on dry ice and stored at −80°C until RNA preparation. The remaining control and drug-treated cultures were allowed to grow for an additional 3 h, at which point the A_{600} was measured and the percent growth calculated.

RNA preparation. RNA was isolated using the hot phenol method (30). Frozen cells were resuspended in 12 ml of AE buffer (50 mM sodium acetate [pH 5.2], 10 mM EDTA) at room temperature, after which 800 μl of 25% sodium dodecyl sulfate and 12 ml of acid phenol (Fisher Scientific, Houston, TX) were added. The cell lysate was then incubated for 10 min at 65°C with vortexing each minute, cooled on ice for 5 min, and subjected to centrifugation for 15 min at 11,952 × *g*. Supernatants were transferred to new tubes containing 15 ml of 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 1 volume of isopropanol and 0.1 volume of 2 M sodium acetate (pH 5.0), mixing well, and subjecting the mixture to centrifugation at 17,211 × *g* for 35 min at 4°C. The supernatants were removed, the pellet was resuspended in 10 ml of 70% ethanol, and the RNA was collected by centrifugation at 17,211 × *g* for 20 min at 4°C. Supernatants were again removed, and the RNA pellet was resuspended in diethyl pyrocarbonate-treated water. Optical densities were measured at 260 and 280 nm, and integrity of RNA was visualized by subjecting 2 to 5 μl of the sample to electrophoresis through a 1% agarose-MOPS gel.

Microarray hybridization. The *C. albicans* microarrays used in this study were manufactured by Eurogentec SA (Ivoz-Ramet, Belgium) in collaboration with the European Galar Fungail Consortium (www.pasteur.fr/recherche/unites/Galar_Fungail/). Two independent sets of RNA from control and drug-treated cells (biological replicates) were used in these studies to prepare two independent cDNA probe sets. Ten micrograms of total RNA sample was added to a mixture of 1 pmol of T20VN and oligo(dT) (18- to 21-mer) primer mix (*C. albicans*-specific primer mix plus) (Eurogentec, Ivoz-Ramet, Belgium); 0.5 mM each dATP, dGTP, and TTP; 20.5 μM dCTP; 37.5 μM Cy3- or Cy5-dCTP (NEN Life Sciences; Boston, MA); and 10 mM dithiothreitol in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. The reaction mixture was denatured at 65°C for 5 min and incubated at 42°C for 5 min, after which 1 μl of RNasin (Promega; Madison, WI) and 200 U of Superscript II reverse transcriptase (Life Technologies/Invitrogen; Carlsbad, CA) were added to the mixture. The reaction proceeded at 42°C for 1 h, after which an additional 200 U of Superscript II reverse transcriptase was added and the reaction mixture was incubated at 42°C for an additional hour. To stop the reaction, EDTA (pH 8.0) and sodium hydroxide were added to final concentrations of 5 mM and 0.4 N, respectively, and the mixture was incubated at 65°C for 20 min. Finally, acetic acid was added to achieve a final concentration of 0.37 M.

The labeled cDNA probes were purified using QIAquick columns (QIAGEN, Valencia, CA) and following the manufacturer's instructions. The cDNA probes were then fluorescently labeled. One set of cDNA probes was labeled using Cy5 for those representing RNA from drug-treated cells and Cy3 for those representing RNA from control cells. The second set was labeled using Cy3 for those representing RNA from drug-treated cells and Cy5 for those representing RNA from control cells. Five microliters each of the Cy3- and Cy5-labeled probes was mixed with 50 μg of heat-denatured salmon sperm DNA, incubated at 95°C for 2 min, and snap-cooled on ice. The mixture was added to 40 μl of hybridization buffer (DIG easy hyb; Roche, Basel, Switzerland) and applied to the array slides under glass coverslips. Hybridization was performed at 37°C overnight in a humidified chamber (Corning Life Sciences; Acton, MA). To wash the slides, the coverslip was removed and the slide was incubated at room temperature in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 5 min with agitation, rinsed at room temperature with 0.2× SSC for 5 min with agitation, and spin dried at 500 rpm for 5 min. Slides were scanned using a ChipReader microarray scanner (Virtek Vision Intl.).

Data analysis. GenePix 1.0 software (Axon Instruments, Inc.) was used for image analysis and data visualization. The local background values were calculated from the area surrounding each spot and subtracted from the total spot signal values. These adjusted values were used to determine differential gene expression (Cy3/Cy5 ratio) for each spot. A normalization factor was applied to account for systematic differences in the probe labels by using the differential gene expression ratio to balance the Cy5 signals. In the present study, only spots with a mean balanced differential expression ratio greater than or equal to 1.5 or less than or equal to 0.67 (−1.5) for both spots representing a given cDNA on the array in two independent experiments were considered to be differentially expressed.

DNA sequences were annotated on the basis of results of BLAST*n* searches using GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), the Stanford sequencing database (Stanford University, Palo Alto, CA; http://www-sequence.stanford.edu/group/candida), and the CandidaDB database (http://www.pasteur.fr/Galar_Fungail/CandidaDB/).

Quantitative real-time RT-PCR. An aliquot of the RNA preparations from untreated and treated samples, used in the microarray experiments, was saved for quantitative real-time reverse transcription (RT)-PCR follow-up studies. First-strand cDNAs were synthesized from 2 μg of total RNA in a 21-μl reaction volume using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Quantitative real-time PCRs were performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and the 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and the 18S rRNA using Primer Express software (Applied Biosystems) and the Oligo Analysis & Plotting Tool (QIAGEN, Valencia, CA) and are shown in Table 1. The PCR conditions consisted of AmpliTaq Gold activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the 7000 Sequence Detection System. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the system software, and the threshold cycle (C_T) above the background for each reaction was calculated. 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 an arbitrary calibrator (e.g., untreated sample) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$.

RESULTS

Gene expression responses to antifungal drug exposure. A total of 82 genes were found to be differentially expressed upon exposure to KTZ. Of these, 60 showed an increase in expression and 22 showed a decrease in expression. The distribution of KTZ-responsive genes and their biological roles are shown in Fig. 1. The category of genes with the largest number of responses was that of unknown function (33%), followed by carbohydrate metabolism (16%); small-molecule transport (16%); lipid, fatty acid, and sterol metabolism (15%); and cell stress (10%).

A total of 256 genes were found to be differentially expressed upon exposure to AMB. Of these, 87 showed an increase in expression and 169 showed a decrease in expression. The distribution of AMB-responsive genes and their biological roles are shown in Fig. 2. The category of genes with the largest number of responses was that of unknown function (20%), followed by protein synthesis (14%), small-molecule transport (8%), cell stress (6%), and lipid, fatty acid, and sterol metabolism (6%).

A total of 480 genes were found to be differentially expressed upon exposure to CPF. Of these, 81 showed an increase in expression and 399 showed a decrease in expression. The distribution of CPF-responsive genes and their biological roles are shown in Fig. 3. The category of genes with the largest number of responses was that of unknown function (26%). Of particular interest, however, were the responses by genes in the categories of small-molecule transport (8%); cell stress (6%); lipid, fatty acid, and sterol metabolism (3%); and cell wall maintenance (3%).

A total of 439 genes were found to be differentially expressed upon exposure to 5-FC. Of these, 383 showed an increase in expression and 56 showed a decrease in expression. The distribution of 5-FC-responsive genes and their biological

TABLE 1. Gene-specific primers used for real-time RT-PCR assays

Target	Sequence[a]	Amplicon size (bp)
18S	F, 5′-CACGACGGAGTTTCACAAGA-3′ R, 5′-CGATGGAAGTTTGAGGCAAT-3′	135
CDC21	F, 5′-TGTTGCTGGCTCTACTGATGCT-3′ R, 5′-GATGAGTCAACCCTAATTTGTCCAA-3′	104
CDR1	F, 5′-ATTCTAAGATGTCGTCGCAAGATG-3′ R, 5′-AGTTCTGGCTAAATTCTGAATGTTTTC-3′	140
CTA1	F, 5′-ATTTCATCCACACCCAAAAGAGA-3′ R, 5′-TTGCTAGTCAAGTAATCCCAAAACA-3′	79
DDR48	F, 5′-TTCGGTAAAGACGACGACAAAGA-3′ R, 5′-GCCAAATGAAGAGGATCCATAAGA-3′	108
ERG16	F, 5′-TTTAGTTTCTCCAGGTTATGCTCAT-3′	
(*ERG11*)	R, 5′-ATTAGCTTTGGCAGCAGCAGTA-3′	100
GSL22	F, 5′-GGTGGAATCATTGCCGTCTT-3′ R, 5′-TTTCGGACACTTCCTGACACAA-3′	81
SKN1.3	F, 5′-AACCAGAATACAAATCGGTCAAGTT-3′ R, 5′-ATCTATCGTACCCTGCTGATGTTG-3′	70
YHB1	F, 5′-AAGTTGCTCCTCCTGCTGGTAAT-3′ R, 5′-TCCTTGTCTGTAGCTGGGTCATAGA-3′	52
YNK1	F, 5′-TCAGAGGTGATTTGCCATTGAT-3′ R, 5′-ATTCTTCTTTCTTGAACCACAAGTCA-3′	106

[a] F, forward; R, reverse.

roles are shown in Fig. 4. The category of genes with the largest number of responses was that of unknown function (26%), followed by protein synthesis (18%). Of particular interest were the responses by genes in the categories of RNA processing/modification/splicing (3%), DNA repair/synthesis (2%), and nucleotide metabolism (2%).

A complete list of all genes found to be differentially expressed in response to at least one drug can be found in the supplemental material (Table S2). There were no genes that exhibited similar changes in expression for all four drugs. Two genes responded similarly to AMB, KTZ, and CPF. These were *DDR48* (up-regulated) and *FET33* (down-regulated). A complete list of genes similarly differentially expressed in response to at least two of the four drugs can be viewed in the supplemental material (Table S3).

Validation of microarray data by real-time RT-PCR. To validate the differential expression of genes identified by microarray analysis, we performed real-time RT-PCR for nine genes of interest. These were selected based on their role in the specific mechanism of action of these drugs (e.g., *ERG11* for KTZ, *CDC21* for 5-FC, and *GSL22* for CPF) but also to verify responses independent of mechanism of action (e.g., *CDR1* for KTZ, *SKN1.3* for CPF, *YNK1* for 5-FC, and *CTA1* and *YHB1* for AMB). In general, there was a good correlation between real-time RT-PCR and microarray data (Fig. 5A). However, for some genes (*SKN1.3*, *YNK1*, and *DDR48*), we observed greater *n*-fold changes in expression by real-time RT-PCR than with microarray analysis. This may be reflective of the greater dynamic range of real-time RT-PCR analysis. Furthermore, in situations where poor or no hybridization signals are generated for one of the samples tested, *n*-fold induction values can be under- or overestimated.

In order to confirm the drug specificity of some of these responses, we examined changes in expression of *CDR1* in response to 5-FC, CPF, and AMB; *SKN1.3* in response to KTZ, 5-FC, and AMB; and *YNK1* in response to KTZ, CPF, and AMB. We also chose to examine expression of *DDR48* as it responded similarly to three of the four antifungal agents used in this study. Results of these assays indicate that the changes in expression of these genes are drug specific (Fig. 5B).

DISCUSSION

Gene expression responses to KTZ. Genes responsive to KTZ included the ergosterol biosynthesis genes *NCP1*, *MCR1*, *CYB5*, *ERG2*, *ERG3*, *POT14* (*ERG10*), *ERG25*, and *ERG251* and the gene encoding the target of the azoles, *ERG11*. This is somewhat similar to that previously observed in *S. cerevisiae*. With the exception of *POT14* (*ERG10*), most of the products of these genes function downstream of lanosterol demethylase, suggesting that their induction is in response to ergosterol depletion. Alternatively, up-regulation of *ERG25* may serve to facilitate the conversion of lanosterol to alternative membrane sterols such as obtusifoliol and 14-methyl fecosterol.

In addition to the ergosterol biosynthesis genes *ERG2*, *ERG3*, *POT14* (*ERG10*), *ERG11*, and *ERG25*, KTZ induced the expression of the multidrug resistance genes *CDR1* and

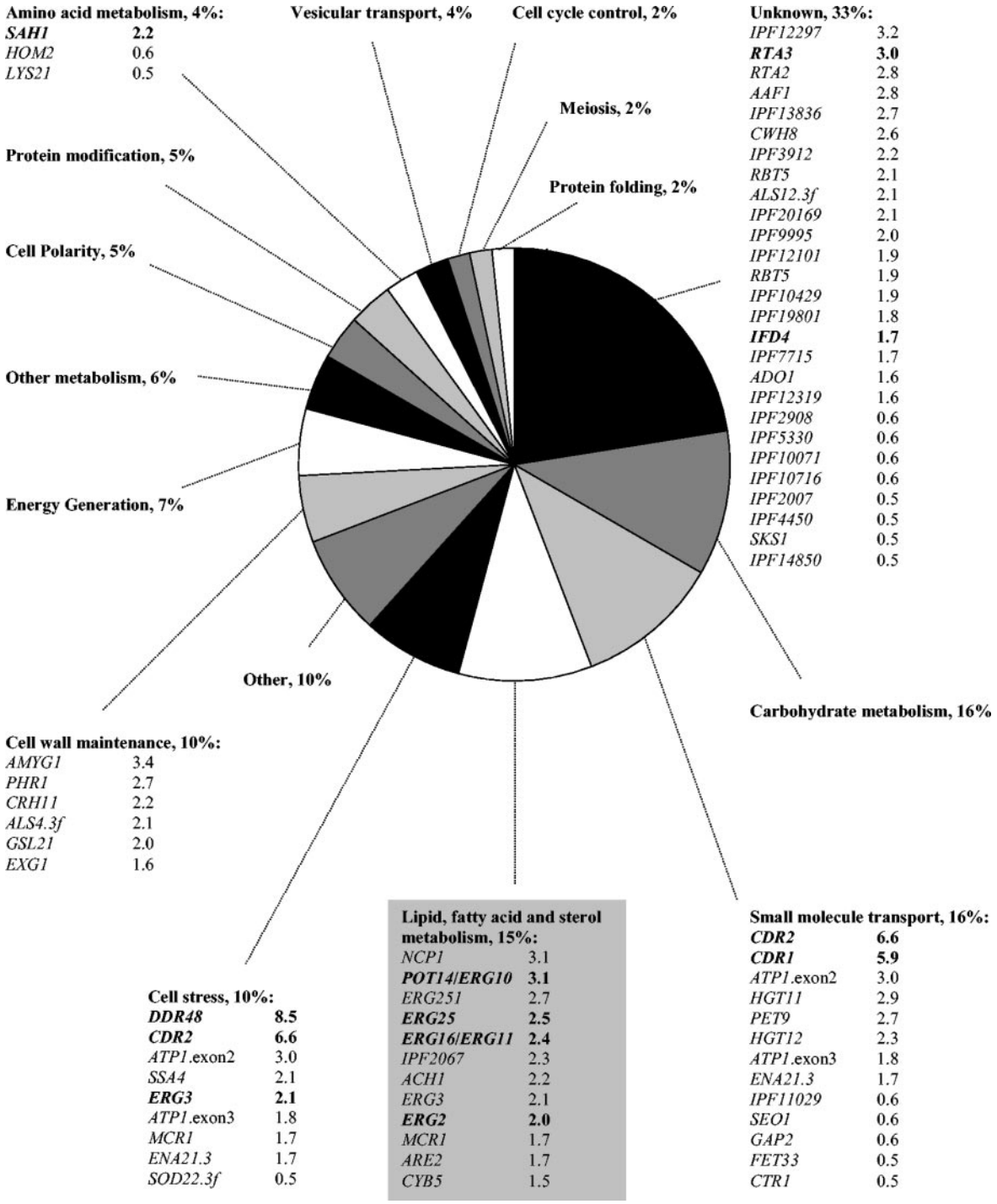

FIG. 1. Distribution of KTZ-responsive genes. Genes were annotated and assigned to functional categories. The average expression ratio from two independent experiments is shown. Genes shaded in gray are indicative of major responses associated with the drug's mechanism of action. Genes in boldface have been previously associated with azole resistance.

CDR2, as well as other genes found to be differentially expressed in azole-resistant isolates such as *RTA3*, *IFD4*, and *DDR48* (15, 27, 28). *RTA3* is a gene of unknown function that is coregulated with *CDR1*, *CDR2*, and *ERG2* in azole-resistant isolates (28). It is homologous to *RTA1* in *S. cerevisiae*, which has been implicated in resistance to the ergosterol biosynthesis inhibitor 7-aminocholoesterol (34). *IFD4* (also known as *CSH1*) is a member of a family of homologs of *YPL088w* in *S. cerevisiae* (31). While its function is unknown, it has been shown to play a key role in cell surface hydrophobicity in *C. albicans*. It should be noted that *YPL088w* was among the KTZ-responsive genes previously identified in *S. cerevisiae* (1).

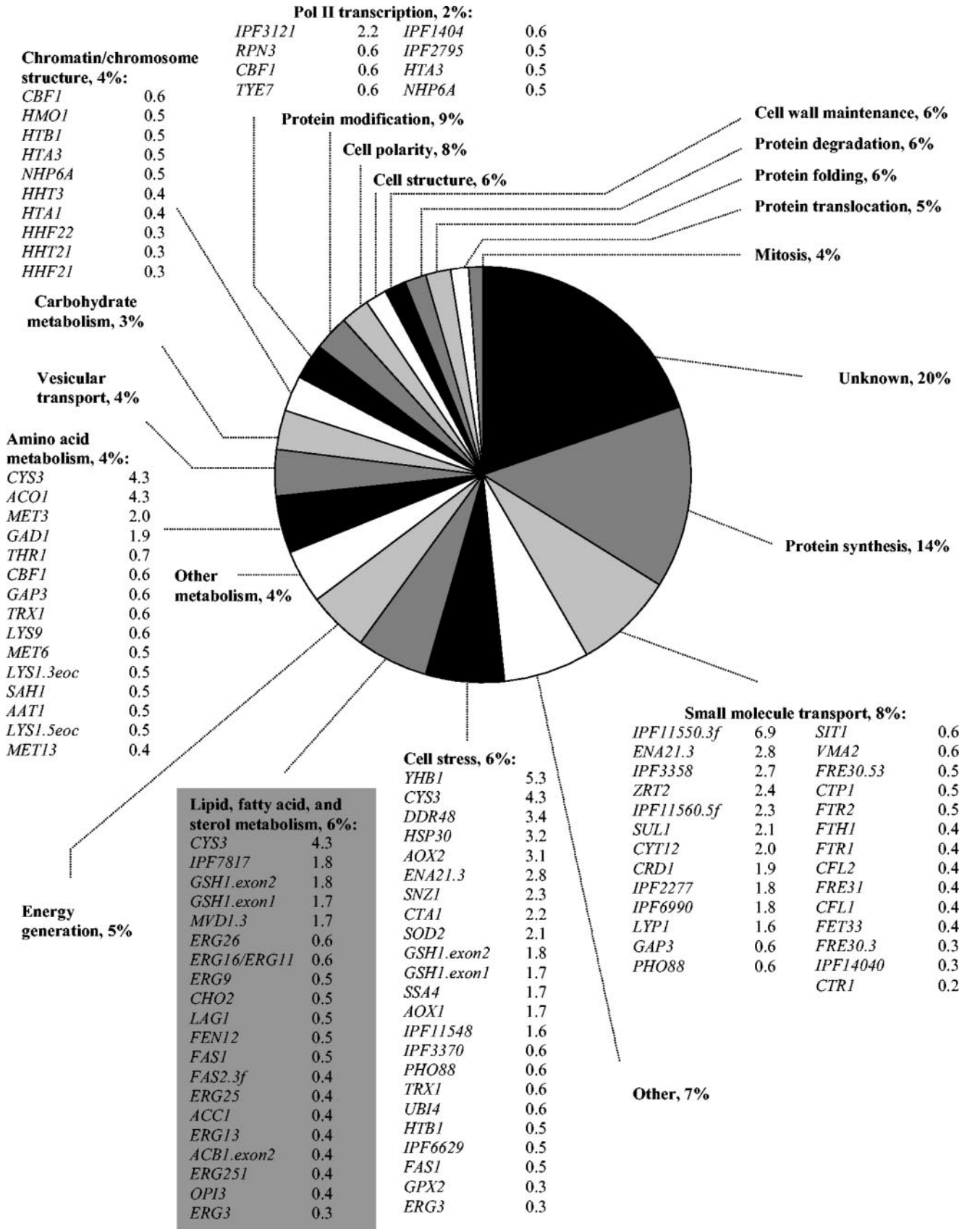

FIG. 2. Distribution of AMB-responsive genes. Gene annotations and expression values are as in Fig. 1. Genes shaded in gray are indicative of major responses associated with the drug's mechanism of action.

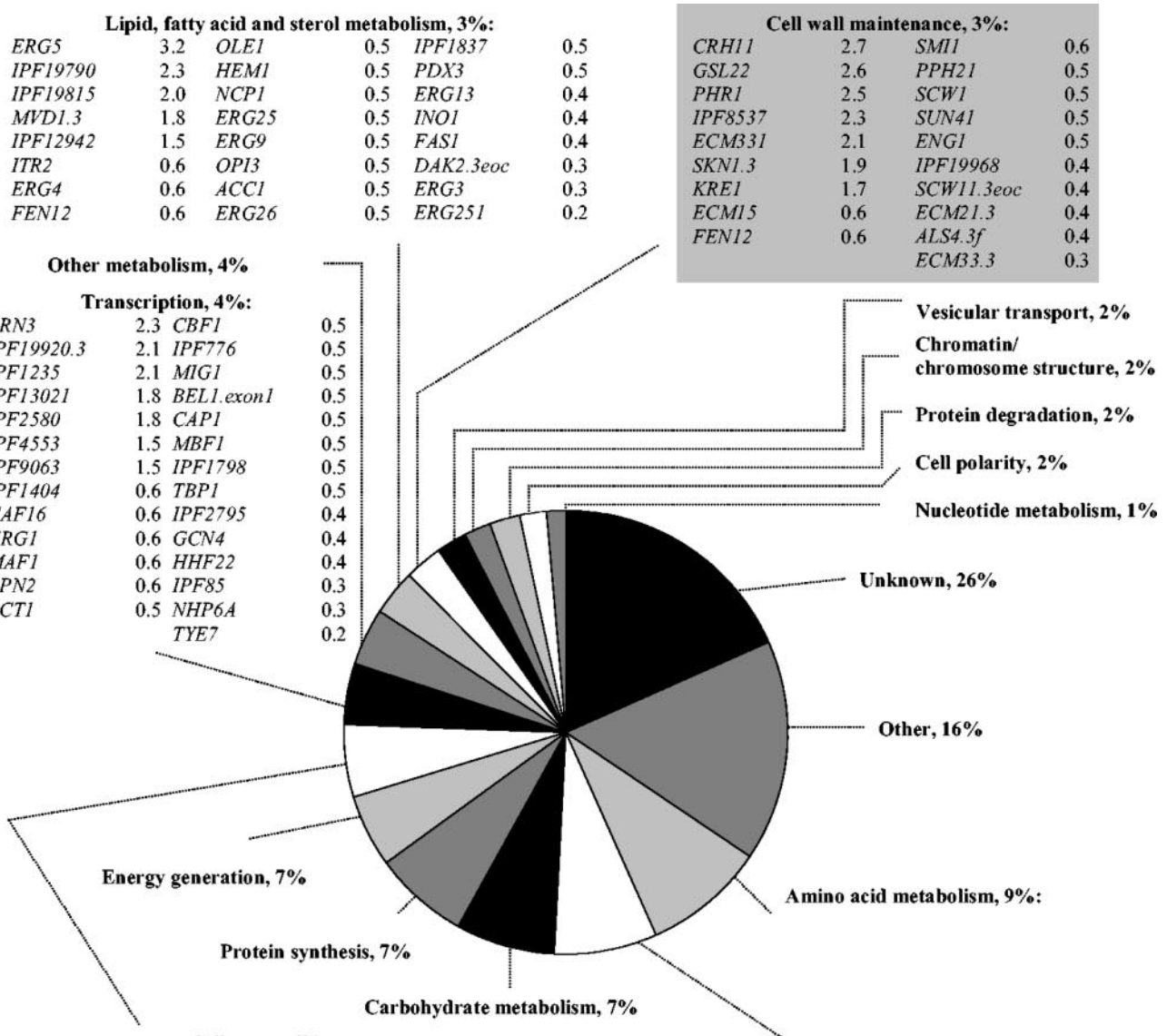

FIG. 3. Distribution of CPF-responsive genes. Gene annotations and expression values are as in Fig. 1. Genes shaded in gray are indicative of major responses associated with the drug's mechanism of action.

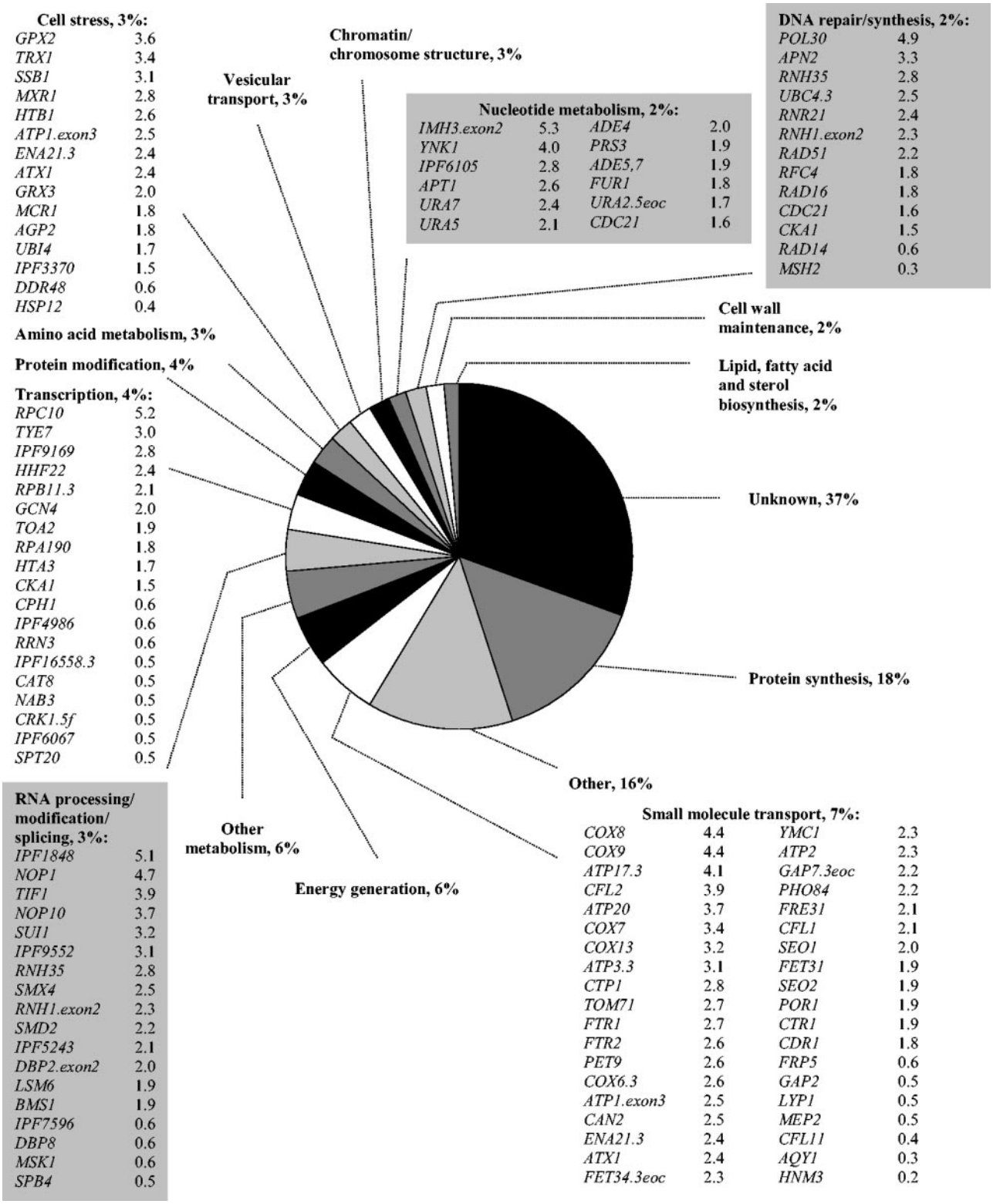

FIG. 4. Distribution of 5-FC-responsive genes. Gene annotations and expression values are as in Fig. 1. Genes shaded in gray are indicative of major responses associated with the drug's mechanism of action.

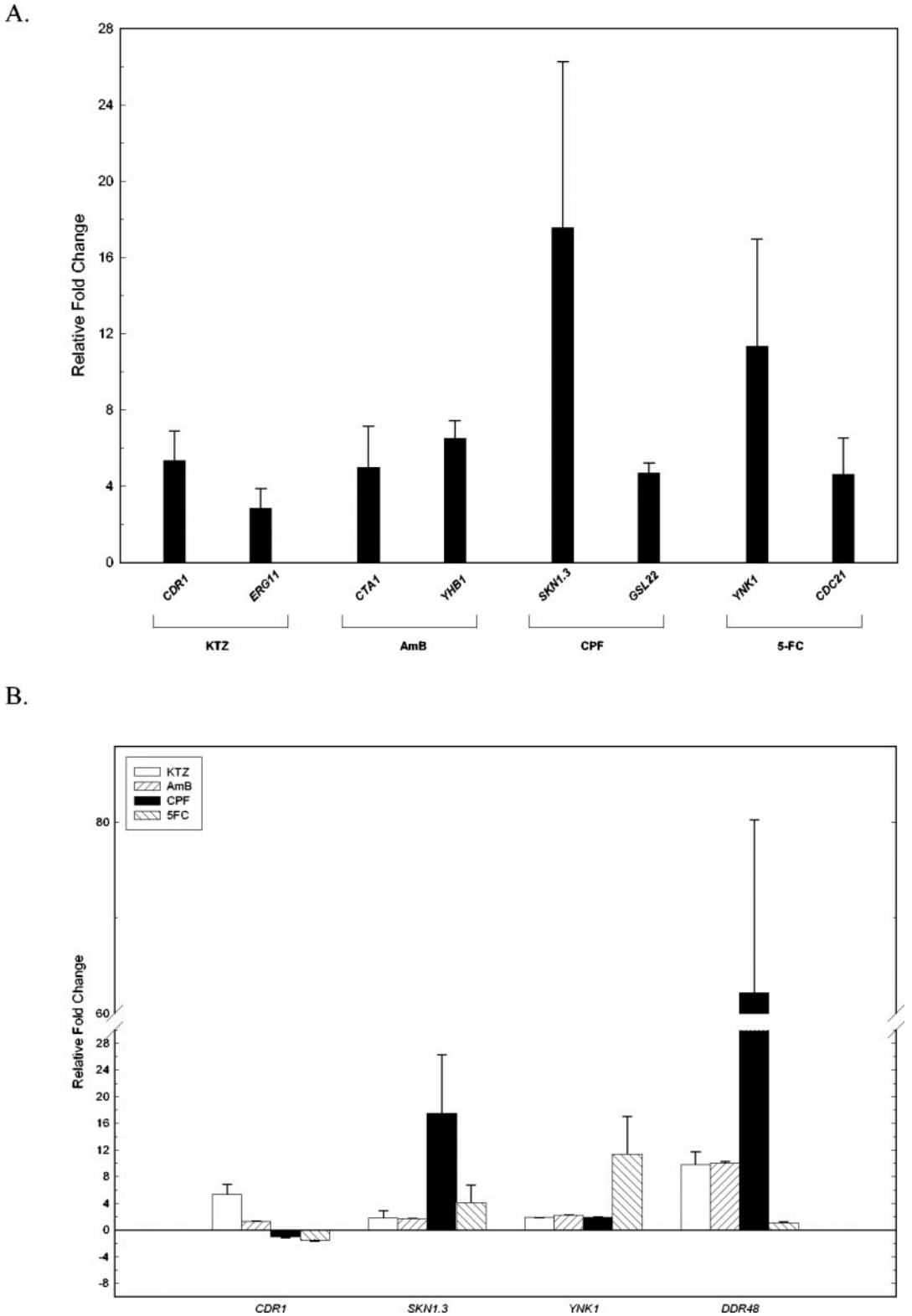

FIG. 5. Quantitative real-time RT-PCR analysis of genes identified as differentially expressed by microarray experiments. Eight genes identified as differentially expressed by microarray analysis were examined by quantitative real-time RT-PCR with gene-specific primers. Data are shown as mean ± S.D. (A) Gene expression changes in 8 genes (2 per drug) detected by real-time RT-PCR. (B) Validation of drug-specific gene expression responses.

DDR48 is homologous to a gene in *S. cerevisiae* that encodes a DNA damage-inducible protein whose expression has been shown to be responsive to cellular stresses (22, 23).

The observation that KTZ induced expression of *UPC2* and associated genes in *S. cerevisiae* was a key finding of our previous study (1). In *S. cerevisiae*, *UPC2* is a transcriptional regulator of sterol uptake under anaerobic conditions (38). Absence of the response of a similar pathway in *C. albicans* is not surprising as *C. albicans* does not appear to take up exogenous sterols (36). Given the fact that *C. albicans* resides primarily in the gastrointestinal tracts of humans, it is possible that this organism has developed alternative strategies for dealing with anaerobic environments, as has recently been suggested by others (13).

Only one other study has examined the effects of azole treatment on the gene expression profile of *C. albicans* (9). Several genes were similarly differentially expressed between that study and the present study, including *ERG2*, *ERG3*, *POT14* (*ERG10*), *ERG11*, *ERG25*, *NCP1*, *CYB5*, *SAH1*, *DDR48*, *CWH8*, and *CTR1*. Differences in the results of these studies can be explained by the differences between the drugs used and the experimental designs employed. In the previous study, *C. albicans* was exposed to itraconazole for 24 h (9). In the present study, we exposed *C. albicans* to a different azole (KTZ) for a shorter time and at a lower effective dose. We would expect changes in gene expression observed in our study to specifically reflect the acute response to azole-induced cell stress.

Gene expression responses to AMB. Responsive genes in the small-molecule category included up-regulation of those involved in calcium (*IPF11550*, *IPF11560*), potassium (*IPF9136*), sodium (*ENA21*), zinc (*ZRT2*), and sulfate (*SUL1*) transport and down-regulation of those involved in phosphate (*PHO88*), hydrogen (*VMA2*), citrate (*CTP1*), copper (*CTR1*), and iron (*SIT1*, *FRE30*, *FRE31*, *FTR1*, *FTR2*, *FTH1*, *CFL1*, *CFL2*, *FET33*) transport. *IPF11550* and *IPF11560* are homologs of the *S. cerevisiae* gene *PMR1*, which encodes a P-type calcium ATPase in the Golgi apparatus involved in regulating cytosolic calcium levels (2, 29). *ENA21* is homologous to the *S. cerevisiae* gene *ENA5*, which appears to encode a sodium transporter (39), whereas *IPF9136* appears to encode a potassium transporter homologous to Hak1p in the yeast *Schwanniomyces occidentalis* (4). Up-regulation of these genes suggests active removal of calcium and sodium from the cytoplasm and is consistent with disruption of the plasma membrane by AMB. Up-regulation of *IPF9136*, as well as the *S. cerevisiae SAT4*/*HAL4* homolog *IPF11548*, is suggestive of the active uptake of extracellular potassium. This is consistent with the ability of AMB to induce the release of potassium in *C. albicans*, which is thought to be the primary mechanism of action of this drug (7). Of interest was the down-regulation of genes involved in iron uptake, suggesting that AMB exposure produces a state of iron overload.

In addition to its deleterious effects on the plasma membrane of *C. albicans*, AMB is also believed to cause oxidative damage to fungal cells (32, 33). It is not surprising that a number of oxidative stress response genes were up-regulated in response to this agent. These included *YHB1*, *AOX1*, *AOX2*, *CTA1*, *SOD2*, and *GSH1*. *YHB1* encodes flavohemoglobin, which in *S. cerevisiae* appears to protect cells against the damage caused by Cu^{2+} and dithiothreitol while sensitizing them to H_2O_2 (8). Recently, *YHB1* in *C. albicans* was shown to be inducible by nitric oxide and to be required for protection against nitric oxide toxicity (37). *AOX1* and presumably *AOX2* encode alternative oxidases which have been associated with the oxidative stress response in some species (16). *SOD2* encodes a mitochondrial manganese superoxide dismutase. Disruption of *SOD2* has been shown to confer hypersensitivity to various stresses, such as redox-cycling agents, heating, ethanol, high concentrations of sodium or potassium, and 99.9% O_2 (17). In *S. cerevisiae*, *GSH1* encodes γ-glutamylcysteine synthetase, which catalyzes a key step in glutathione biosynthesis (12). *CTA1* encodes peroxisomal catalase A, an antioxidant enzyme (40). Interestingly, clinical isolates lacking membrane ergosterol and exhibiting reduced susceptibility to AMB have been shown to have greater levels of catalase activity (32). Collectively, the up-regulation of oxidative stress response genes upon exposure to AMB is consistent with the proposed oxidative damage caused by this antifungal agent.

Since AMB targets the membrane sterol ergosterol, it was significant to observe changes in the expression of genes involved in lipid, fatty acid, and sterol metabolism. These included the up-regulation of *MVD1* (*ERG19*) and the down-regulation of the ergosterol biosynthesis genes *ERG26*, *ERG16* (*ERG11*), *ERG9*, *ERG25*, *ERG13*, *ERG251*, and *ERG3*, the fatty acid biosynthesis genes *FEN12*, *FAS1*, *FAS2*, and *ACB1*, and the phospholipid biosynthesis genes *CHO2* and *OPI3*. Down-regulation of certain ergosterol biosynthesis genes in response to AMB may be reflective of an attempt by the organism to use alternate sterols or sterol intermediates in the cell membrane. We recently examined the gene expression profile of an AMB- and fluconazole-resistant *C. albicans* laboratory-derived isolate (5). This isolate exhibited changes in sterol composition consistent with a defective lanosterol demethylase. Several genes are similarly differentially expressed between AMB exposure in the present study and AMB resistance in the previous study. These include up-regulation of *DDR48*, *CTA1*, *IPF19801*, and *IPF3964* and down-regulation of *TYE7*, *NHP6A*, *UBI4*, *HTB1*, *HFF22*, *HHT21*, *HHF21*, *GPX2*, *IPF11785*, and *IPF11625*. It is tempting to speculate that these changes in gene expression are coordinately regulated in response to reduced ergosterol biosynthesis.

Gene expression responses to CPF. The cell wall, which is primarily responsible for maintaining cell shape and osmotic stability, is composed of mannoproteins and chitin, as well as β-1,3-glucan and β-1,6-glucan. β-1,3-Glucan is the central component to which all other components are cross-linked (19, 20). CPF is thought to inhibit the β-1,3-glucan synthase activity of the *FKS1* and *FKS2* gene products in *S. cerevisiae* (10). The *C. albicans* homologs of these genes are *GSL21* and *GSL23*, respectively. In the present study, CPF induced the expression of several genes involved in cell wall maintenance, including the gene encoding a third β-1,3-glucan synthase subunit, *GSL22*, which is homologous to *S. cerevisiae FKS3*.

Other cell wall maintenance genes that were up-regulated included *CRH11*, *PHR1*, *IPF8537*, *ECM331*, *SKN1.3*, and *KRE1*, and *ECM15*, *FEN12*, *SMI1*, *PPH21*, *SCW1*, *SUN41*, *IPF19968*, *SCW11*, *ECM21*, *ASC4*, and *ECM33* were down-regulated. Several homologs of these genes have recently been

implicated in β-1,3-glucan assembly in *S. cerevisiae*, including the *PHR1* homolog *GAS1*, *KRE1*, *SWI1*, the *FEN12* homolog *FEN1*, and *ECM21* (21). Disruption of *FKS1* in *S. cerevisiae* resulted in up-regulation of the *CRH11* homolog *CRH1* and the *ECM331* homolog *PST1* (35). Furthermore, these genes, as well as *GSC2* (*FKS2*), were up-regulated in response to CPF in *S. cerevisiae* (1). Indeed, changes in the expression of these genes are suggestive of alterations in the cell wall that are consistent with the mechanism of action of this antifungal agent.

Gene expression responses to 5-FC. 5-FC enters the fungal cell via cytosine permease (25), where it is converted to 5-fluoroucil (5FU) by cytosine deaminase. 5FU is then converted to 5-fluorouridine monophosphate by the *FUR1* gene product uracil phosphoribosyltransferase. 5-Fluorouridine monophosphate is subsequently converted to 5-fluorouridine triphosphate, which ultimately interferes with protein synthesis through incorporation into RNA in lieu of UTP (18). 5FU also undergoes conversion to 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthetase, encoded by *CDC21*, by disrupting DNA synthesis (11, 18, 25, 26). It is therefore not surprising that we observed increased expression of a large number of genes involved in protein synthesis, as well as the purine and pyrimidine biosynthesis genes *IMH3*, *YNK1*, *IPF6105*, *URA2*, *URA5*, *URA7*, *ADE4*, *ADE5*, *ADE7*, *PRS3*, *FUR1* and *CDC21*. The changes in gene expression we observed in response to 5-FC exposure are consistent with a need for the cell to increase RNA, DNA, and protein production, as well as uracil phosphoribosyltransferase and thymidylate synthetase production.

Responses common to multiple drugs. As noted above, *DDR48* is homologous to a gene in *S. cerevisiae* that encodes a DNA damage-inducible protein whose expression has been shown to be responsive to cellular stresses (22, 23). *FET33* is homologous to *FET3* in *S. cerevisiae*, which encodes a ferroxidase required for high-affinity iron uptake (3). There were many genes that responded similarly to at least two of the four drugs (Table S3 in the supplemental material). Most striking was the set of genes that were similarly regulated by both AMB and CPF. This included the down-regulation of genes involved in iron regulation (*CFL1*, *CFL2*, *FTR1*, *FTR2*, *CTR1*, and *FTH1*), chromatin/chromosome structure (*HHF21*, *HHT21*, *HHF22*, *HTA1*, *HHT3*, *HTB1*, and *NHP6A*), and lipid, fatty acid, and sterol metabolism (*ERG25*, *ERG251*, *SAH1*, *ERG3*, *OPI3*, *ERG13*, *FAS1*, and *ERG9*). As both AMB and CPF are fungicidal agents, it is tempting to speculate that these commonly differentially expressed genes reflect a response to compromised cellular integrity.

In conclusion, the studies reported here have revealed antifungal drug-specific changes in gene expression consistent with known mechanisms of action and in some cases suggest novel effects of these drugs. We also identify responses shared across multiple classes of antifungal agent. *C. albicans* gene expression profiling is a useful tool for understanding mechanisms of action and potentially mechanisms of resistance for antifungal agents with activity against this pathogenic fungus.

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