Comparison of Gene Expression Profiles of *Candida albicans* Azole-Resistant Clinical Isolates and Laboratory Strains Exposed to Drugs Inducing Multidrug Transporters

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Azole resistance in *Candida albicans* can be due to upregulation of multidrug transporters belonging to ABC (ATP-binding cassette) transporters (*CDR1* and *CDR2*) or major facilitators (*CaMDR1*). Upregulation of these genes can also be achieved by exposure to fluphenazine, resulting in specific upregulation of *CDR1* and *CDR2* and by exposure to benomyl, resulting in specific *CaMDR1* upregulation. In this study, these two different states of gene upregulation were used to determine coregulated genes that often share similar functions or similar regulatory regions. The transcript profiles of a laboratory strain exposed to these drugs were therefore determined and compared with those of two matched pairs of azole-susceptible and -resistant strains expressing *CDR1* and *CDR2* (CDR strains) or *CaMDR1* (MDR isolates). The results obtained revealed that, among 42 commonly regulated genes (8.6% of all regulated genes) between fluphenazine-exposed cells and CDR isolates, the most upregulated were *CDR1* and *CDR2* as expected, but also *IFU5*, *RTA3* (which encodes putative membrane proteins), *HSP12* (which encodes heat shock protein), and *IPF4065* (which is potentially involved in stress response). Interestingly, all but *HSP12* and *IPF4065* contain a putative *cis*-acting drug responsive element in their promoters. Among the 57 genes (11.5% of all regulated genes) commonly regulated between benomyl-exposed cells and MDR isolates, the most upregulated were *CaMDR1* as expected but also genes with oxido-reductive functions such as *IFD* genes, *IPF5987*, *GRP2* (all belonging to the aldo-keto reductase family), *IPF7817* [NAD(P)H oxido-reductase], and *IPF17186*. Taken together, these results show that in vitro drug-induced gene expression only partially mimics expression profiles observed in azole-resistant clinical strains. Upregulated genes in both drug-exposed conditions and clinical strains are drug resistance genes but also genes that could be activated under cell damage conditions.

Candida albicans is an important opportunistic fungal pathogen in human. It can cause mucosal and systemic infections in immunocompromised patients (20). Among the different antifungal agents available, the class of azoles has been used extensively during the past 20 years. Due to the repeated use of this agent especially in human immunodeficiency virus-positive patients with recurrent oropharyngeal candidiasis, treatment failures were observed to be associated with emergence of azole-resistant *C. albicans* strains (22). We have been interested in understanding the molecular mechanisms of resistance to azole antifungal agents in *C. albicans*. Our work and those of others have already demonstrated the importance of multidrug transporters in this phenomenon, although other mechanisms such as target site alterations were also identified (20, 21, 24, 26–28, 30–32). The upregulation of multidrug transporter genes leads to enhanced efflux of azoles and therefore results in decreased drug accumulation and reduced inhibition of their target encoded by the *ERG11* gene. At least two families of multidrug transporters, the ABC (ATP-binding cassette) transporter family and the major facilitator superfamily (MFS) have been shown to be involved in antifungal resistance. Cdr1p and Cdr2p (*Candida* drug resistance, ABC transport-

ers) and CaMdr1p (*C. albicans* multidrug resistance 1, MFS transporter) were shown to be major mediators of azole resistance. It was previously demonstrated that each gene encoding these proteins can be upregulated in distinct clinical azole-resistant strains (27, 28). Multidrug transporter genes can also be induced transiently by treating cells with different drugs. For example, estradiol and fluphenazine exposure can induce *CDR1* and *CDR2* (10), whereas benomyl and H_2O_2 can induce *CaMDR1* (17). The regulatory elements permitting the upregulation of these genes have been determined only for *CDR1* and *CDR2*. A 22-bp drug-responsive element (DRE) is known to be required for induction of both *CDR1* and *CDR2* (10). Transient upregulation of these genes therefore mimics gene expression levels measured in clinical azole-resistant isolates. However, in vitro exposure of *C. albicans* to these drugs as well as natural history of clinical isolates may result in profound gene expression alterations additional to those already observed.

Several genome-wide studies performed in *Saccharomyces cerevisiae* (1, 3) have established that exposure to drugs can trigger the expression of genes that either belong to pathways affected by these drugs or are involved in resistance to the tested drugs. On the other hand, exposure of *S. cerevisiae* to specific agents can reveal clusters of coordinately regulated genes that possess common regulatory elements recognized by the same transcription factors (11, 33). A few similar genome-wide studies have been undertaken in clinical *C. albicans* iso-

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

Strain name	Genotype or description	MIC (μg/ml) of fluconazole	References(s)
CAF2-1	Δ*ura3*::*imm434*/*URA3*	0.5	16
DSY294	Azole-susceptible clinical strain C43[a]	0.25	4, 28
DSY296	Azole-resistant clinical strain C56[a]	128	4, 28
DSY2285	Azole-susceptible clinical strain 26[a]	0.25	5, 6
DSY2286	Azole-resistant clinical strain 91[a]	16	5, 6

[a] *C. albicans* clinical isolates with original strain numbering.

lates. Rogers et al. and Cowen et al. (8, 23) have analyzed transcript profiles of azole-resistant isolates, while de Backer et al. (9) have determined the effect of azole treatment on gene expression in *C. albicans*. In the present study we aimed to identify genes commonly regulated between cells transiently exposed to drugs and azole-resistant clinical strains. These two different conditions should enable the identification of genes commonly regulated in addition to those already known, such as *CDR1*, *CDR2*, and *CaMDR1*. As mentioned above for *S. cerevisiae*, coordinately regulated genes are helpful in the delimitation of common regulatory elements deduced from the analysis of their promoters. We therefore undertook genome-wide transcript profiling experiments with matched pairs of clinical isolates—one pair with a resistant strain upregulating *CDR* genes and another pair with a resistant strain upregulating *CaMDR1*—and with strains exposed to drugs known to induce multidrug transporter genes, namely, fluphenazine and benomyl.

The results showed that transcripts profiles of the azole-resistant strain upregulating the *CDR* genes revealed more genes regulated in common with profiles of fluphenazine-treated cells than with profiles of benomyl-treated cells. Moreover, transcript profiles of the azole-resistant strain upregulating *CaMDR1* exhibited transcript profiles resembling more closely those found in benomyl-treated cells. As expected, some of the commonly regulated genes observed in both the selected azole-resistant isolate and drug-treated cells possess common regulatory elements. These genes shared functions in drug resistance but also in response to cell-damaging conditions.

MATERIALS AND METHODS

***C. albicans* strains and growth conditions.** Two pairs of *C. albicans* azole-susceptible and azole-resistant clinical isolates and the laboratory strain CAF2-1 were used in this study and are described in Table 1. Each pair of isolates consists of strains with matched Ca3 profiles (4, 5). Strains were cultivated at 30°C under constant shaking in liquid complete medium YEPD consisting of 1% Bacto Peptone (Difco Laboratories, Basel, Switzerland), 0.5% yeast extract (Difco) and 2% glucose (Fluka, Buchs, Switzerland).

Microarray design. Eurogentec (Seraing, Belgium) *C. albicans* cDNA Microarray slides were used in this study. Briefly, 6,039 open reading frames from PCR amplicons 300 bp long were spotted in duplicate onto each aldehyde coated glass slide onto 32 blocks. The microarray covers nearly 98% of the total number of *C. albicans* genes and includes 27 control genes in each block, including, for example, negative controls (intergenic regions), cross hybridization controls (*S. cerevisiae* genes), and dynamic range controls by a serial dilution of *TEF3* gene from 1 to 1/32.

RNA isolation and probe labeling. Each *C. albicans* strain was grown overnight in 5 ml of YEPD medium under constant agitation at 30°C. For RNA extractions, cultures were diluted at a density of 0.75×10^7 cells per ml in 15 ml of fresh YEPD medium and were grown at 30°C with agitation for 2 h to reach a density of 1.5×10^7 cells per ml. At this point cultures of clinical isolates were centrifuged at 4°C, 5 min at $5,500 \times g$ for subsequent RNA isolation. Strain CAF2-1 was exposed either for 20 min to 10 mg liter^{-1} of fluphenazine (Sigma-Aldrich,

Buchs, Switzerland) or for 30 min to 25 mg liter^{-1} of benomyl (Riedel de Hahn, Seelze, Germany) at 30°C under agitation. This strain was cultivated in parallel under the same conditions except that no drugs were added. After drug exposure, cultures were centrifuged at 4°C and $5,500 \times g$ for RNA isolation. Total RNA was extracted using glass beads as described previously (25). A least 200 μg of each RNA was purified according to the supplier (RNeasy kit; QIAGEN Inc., Chatsworth, Calif.). Concentration of purified RNA was measured spectrophotometrically at A_{260} and A_{280} and adjusted to 2.5 mg ml^{-1}. RNA was stored at −80°C until use.

For RNA labeling, 25 μg of total RNA were mixed with 8 μl of 5× First Strand buffer (Invitrogen, Basel, Switzerland), 1 μl of 0.1 μM *C. albicans*-specific primer mix Plus (including T20VN and OligodT18-21) (Eurogentec), 3 μl of 6.67 mM (each) deoxynucleoside triphosphate (Roche, Basel, Switzerland), 1 μl of 1 mM dCTP (Roche), 1.5 μl of 1 mM cyanin 3(Cy3)-dCTP (Amersham Biosciences, Otelfingen, Switzerland) for RNA of non-drug-exposed or azole-susceptible clinical strains or 1.5 μl of 1 mM cyanin 5 (Cy5)-dCTP (Amersham Biosciences) for RNA of drug-exposed or azole-resistant clinical strains, 4 μl of 100 mM dithiothreitol (Invitrogen), 1 μl of RNasin (20 to 40 U/μl; Promega, Wallisellen, Switzerland), and diethyl pyrocarbonate-treated water to a volume of 40 μl. The mixture was heated at 65°C for 5 min and cooled at 42°C for 5 min. The reverse transcription (RT) reaction was performed for 1 h at 42°C with 1 μl of RNasin and 200 U of SuperScript II reverse transcriptase (Invitrogen). The reaction was stopped and RNA degraded by addition of 5 μl of EDTA (50 mM, pH 8.0) and 2 μl of NaOH (10 N) and incubation at 65°C for 20 min. The reaction was neutralized with 4 μl of acetic acid (5 M). The labeled probes (cDNA) were purified according to QiaQuick PCR purification kit protocol (QIAGEN Inc.). The elution step was performed twice with prewarmed H_2O (42°C) and centrifuged at maximum speed for 1 min. The purified labeled probes were concentrated using microcon-30 filter (Amicon, Wallisellen, Switzerland) to a final volume of 5 μl.

Hybridization of the arrays. Cy5- and Cy3-labeled cDNAs synthesized from RNA of cells from which transcription profiles have to be compared were pooled with 5 μl of heat-denatured salmon sperm DNA (10 mg ml^{-1}), boiled at 95°C for 2 min, and chilled on ice. Probes were then mixed with 40 μl of hybridization buffer (DIG easy hyb; Roche), applied to a *C. albicans* microarray slide and covered with a Lifterslips (25 by 44 mm; Erie Scientific Co., Portsmouth, Maine) during all hybridization steps, and loaded onto the hybridization chamber (Corning Inc., Corning, N.Y.) which was kept wet by loading 10 μl of H_2O in each existing well. After overnight hybridization at 42°C, coverslips were removed from slides by dipping in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate. Slides were washed for 5 min at room temperature with occasional agitation and rinsed with 0.2× SSC for 5 min at room temperature with occasional agitation. Slides were then spin dried (500 × g, 5 min) and stored protected from light until scanning.

Quantitative analysis of the microarrays. Hybridized microarray slides were scanned with a ScanArray 4000 (Perkin-Elmer, Schwerzenbach, Switzerland) at a 10-μm resolution. The following wavelengths were used for photoexcitation: 532 nm for Cy3- and 635 nm for Cy5-labeled cDNA. The intensity of each laser light was determined by equalizing the Cy3 and the Cy5 fluorescent signals of the dynamic range controls (*TEF3*) at 3 different blocks for each slide. The resulting 16-bit TIFF files of each signal were quantified and converted into a text file with the ImaGene software (version 4.0; BioDiscovery Inc., El Segundo, Calif.).

Normalization control. Experiments from each hybridization were normalized using a LOWESS analysis for print-tip variability and for slide-to-slide variability using the publicly available software from Lund University (http://www.braju .com/R/com.braju.sma/) run in R project environment (http://cran.r-project .org/). In order to determine a significant threshold, we performed a LOWESS normalization only on the *TEF3* spots, *TEF3* being an housekeeping gene. The significant threshold was considered above the most modulated *TEF3* hybridization signals. A fourfold differential expression limit was therefore chosen in this study in the comparison of pairwise experiments. Normalized data can be downloaded from http://www.hospvd.ch/imul/under the file name Microarray data: array_data_sanglard.xls.

Cluster analysis. Cluster analysis was performed using the CLUSTER program version 2.11 (12). Genes with a twofold differential expression under at least one condition were selected for the clustering of the global analysis (see Fig. 1 and 2). Genes with a fourfold differential expression under at least one condition were selected for clustering of pairwise microarray experiments (see Fig. 3 and 5). Signal intensities of each selected gene were compared between the different conditions using the complete linkage clustering program in the uncentered correlation mode of hierarchical clustering. Results were viewed using TREEVIEW (version 1.6) (12).
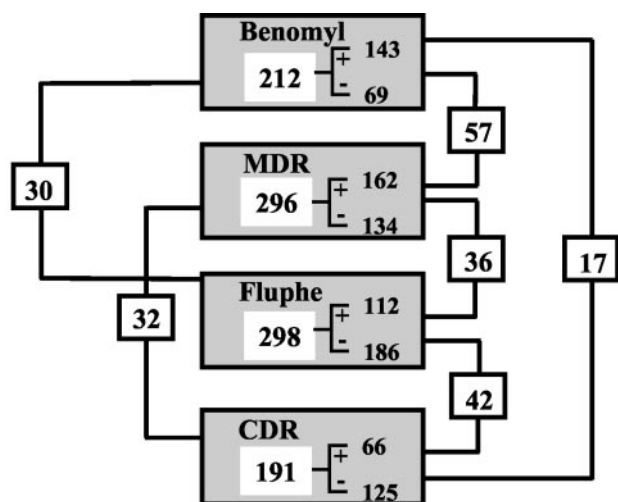
FIG. 1. Numbers of *C. albicans* genes with at least twofold differences in levels of expression. For each experiment, the number of up- or downregulated genes is given. Pairwise comparison between each experiment yielded numbers of commonly regulated genes and these numbers are given linked to each compared experiments.

Northern blot analysis. Northern blotting was carried out as described previously (28). DNA probes used in this study were generated by PCR using pairs of primers described in Table 2. ^{32}P-DNA labeled probes were generated by random priming (14). Quantifications of Northern blot bands were performed by scanning the hybridized membranes in an Instant Imager (Packard Instrument Company, Meriden, Conn.). Signals were integrated by the software supplied by the manufacturer and normalized to the corresponding values of an internal standard. This standard was a *TEF3* probe originating from a 0.7-kb EcoRI-PstI fragment from pDC1 described by Hube et al. (18). The signals obtained for the same gene in two conditions were expressed as a ratio of normalized signal intensities. The ratio obtained for the selected genes (see Fig. 4 and 6) could be thus compared with those calculated in microarray experiments.

RT-PCR analysis. RT was performed on 2 μg of RNA using Superscript II (Invitrogen) with random primers (Invitrogen) following instructions of the manufacturer. PCR were performed by mixing 2 μl of serial dilutions of cDNAs in a total volume of 50 μl with 5 μl of 10× PCR buffer (Roche), 0.5 μl each of forward and reverse primer (100 μM), 5 U of *Taq* DNA polymerase (Roche), and 2 μl of 10 mM deoxynucleoside triphosphates (Roche) in a final volume of 50 μl. PCR was performed under the following conditions: one cycle for 4 min at 94°C; 30 cycles with 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C; and one terminal cycle for 10 min at 72°C. Equivalent PCR product volumes were loaded on a 1% agarose gel and separated by electrophoresis in 1× TAE (40 mM Tris, 10 mM EDTA, 0.1% acetic acid [pH 8.5]). Primer sequences are described in Table 2.

RESULTS

Experimental design and global gene expression results. Previous results showed that multidrug transporter upregulation in *C. albicans* can be observed in several distinct situations, either by drug exposure or as the result of development of azole resistance in clinical isolates (10). It can be expected that other genes will be commonly regulated in these isolates under these conditions. Since these genes often share the same transcriptional activators or utilize similar gene activation pathway, it is therefore of interest to determine groups of commonly regulated genes in conditions known for upregulation of multidrug transporter genes. Transcript profiling with available *C. albicans* microarrays comprising almost the entire genome represents an appropriate tool to address this problem.

In vitro treatment of *C. albicans* with drugs such as fluphen-

azine or benomyl leads to transient upregulation of the ABC transporters *CDR1* and *CDR2* and of the major facilitator gene *CaMDR1*, respectively (10, 17). Therefore, transcript profiles of cells treated with fluphenazine or benomyl were first compared with profiles of cells in nontreated conditions. These experiments are referred to herein as the fluphenazine and benomyl experiments, respectively. Next, transcript profiles of azole-susceptible and azole-resistant *C. albicans* clinical isolates were compared with each other. The azole-susceptible strain DSY294 (MIC of fluconazole, 0.25 μg/ml) was first compared with its matched azole-resistant isolate DSY296 (MIC of fluconazole, 128 μg/ml), which is a strain known to upregulate *CDR1* and *CDR2* (28). This experiment was named CDR. The azole-susceptible isolate DSY2285 (MIC of fluconazole, 0.25 μg/ml) was then compared with its matched azole-resistant isolate DSY2286 (MIC of fluconazole, 16 μg/ml), which is a strain upregulating *CaMDR1* (6). We called this the MDR experiment.

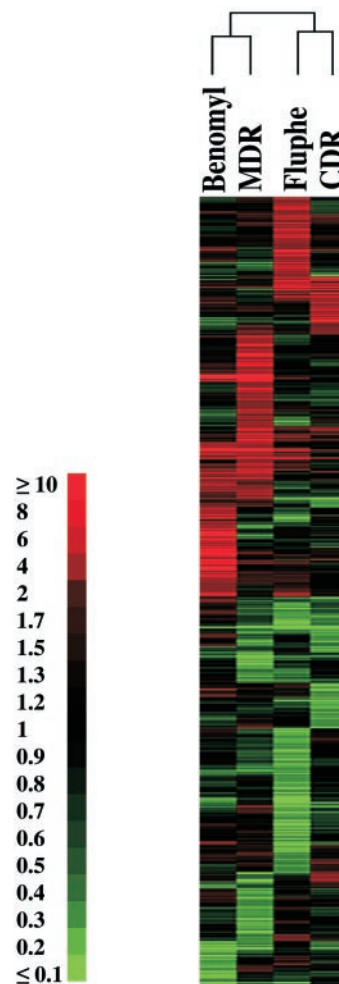Figure 1 summarizes the number of genes differentially ex-

FIG. 2. Cluster analysis of the four experiments undertaken in this study. The threshold value used in the analysis is at least a twofold increase in gene expression. The cluster was constructed as indicated in Materials and Methods. A quantitative color scale of gene expression ratio in expression values (*n*-fold) is given at the left side of the figure.

TABLE 2. Primers used for Northern blot and RT-PCR analysis[a]

Primer name	Sequence	Primer name	Sequence
IPF1514 F	CTGCAAGTGCATTAAGACAT	IFD7 R	CAATAGCTTTGTCATTGTCGTT
IPF1514 R	ATACGAATCGGACATTTTTC	ERG6 F	TATTCCCAATTGACTCATCA
IPF2857 F	F-ATGTTGCGTCAAAGTTTATTC	ERG6 R	TACCATCACCGACTTCAATA
IPF2857 R	CTACATCAAATCTTCTGGTC	GPX1 F	ATGTCTCAATTTTTACGAATTAGC
IPF4065 F	ATGGCTAAAACTTCTCCAC	GPX1 R	CTTCAACAATTCTTCAATCTTGG
IPF4065 R	TTAATTAGCAGCGTCACCATTAC	GRP2 F	AATCCAGTATATGTATTTGGTCC
IPF6629 F	ATGACTGACGGTAAATTCCC	GRP2 R	CTCAATCTTACTTTCAGCTTTCT
IPF6629 R	TTAATTGTGGATTCTCTTTAAAA	HSP12 F	ATTTCTACTAAAATCAACGAAGC
IPF14285 F	F-GATCCACCCTTATCACCTAA	HSP12 R	GACAACTCCACTCACGTATTC
IPF14285 R	AGTAGCAGAATCTGGTGGTT	IFU5 F	AAACCCACCACAAGTTCCTG
CDR1 F	GTTGTTTTGGGGAGACCCGGTGCT	IFU5 R	CTTGGGGCATTAGACCTTGA
CDR1 R	CTTACCAGCACCAGATGCTCCCAT	RTA3 F	TACAGAATGGACTCCTACCT
CDR2 F	ATTCAATTCACGGAAATCGGATA	RTA3 R	GCCGTACGATTTAATCGA
CDR2 R	GTGCACAGTGCACACATTAACC	SAP3 F	CTGATTTATGGGTTCCTGAT
ERG3 F	ATCATCTGGTCTTCTGTAAGATTT	SAP3 R	CACCAAAAATAATTTGTCCA
ERG3 R	AAATTCATTCTTTTCACCGATTGT	SAP5 F	GCTTCTACTGGGCAAATTAT
IPF2186 F	CAACGAGCAAACGTCAGAAA	SAP5 R	CTTCACTTTCACGAATACGA
IPF2186 R	CCCAAACCCAAGCAAATCTA	DDR48 F	TTTCGGTTTCGGTAAAGACG
IPF3264 F	GACAACTCCCCAAGTTACAT	DDR48 R	TGTCAGTGTTTGAGGAGCCA
IPF3264 R	AAATCAGCACCTAAGCTTTC	EBP1 F	GCCTCTAATAAAAGAACCGA
IPF3415 F	AACTTGCTAAACAAGGTGGT	EBP1 R	CAAAGTTTCCTTTCCAAATC
IPF3415 R	GAATCTAACTTCTCGATGGC	EBP4 F	TGGAAGAAAATCAATGATGA
IPF3704 F	GCACACGTTTGTCATGGAAC	EBP4 R	CAATTTTTTCAGCACCAACTA
IPF3704 R	ACTTGTCGCCAAAGCCTAGA	IFR2 F	TCAAGCCACTATCAAATACG
IPF7817 F	TGCTAGAAGAGCTGTTGAAA	IFR2 R	AAGCCCTTGAAAAGTTGTAG
IPF7817 R	TAATCGTTACCACCAGATGA	MRF1 F	GGCGGATATAACGATGCAGT
IPF7863 F	ACCGAATTGCCCACTTGATTC	MRF1 R	ACTTTCGATTGTGGATTGGC
IPF7863 R	TGTCGATAGCACCAGCAAAG	SNZ1 F	CCGGTTTAGCACAAATGCTT
IPF9538 F	CTGGATGCAAATCAAGCAGA	SNZ1 R	CCCATCAATTCACCCAAATC
IPF9538 R	TTTTGTCAAACGGCCATGTA	TRX1 F	TTCACGTTGTCACTGAAGTT
IPF17186 F	GTACTTTTGGATGGGATGAT	TRX1 R	GAGAAGCCAAAGCTTGTTTA
IPF17186 R	TTCAGCAACTTGTTTAATGG	TTR1 F	CAACAATGGTTTCATCTCAA
CaMDR1 F	AAGTCGCACGCGTAAAATGCATTA CAGATTTTTAAGAGAT	TTR1 R	CAGCTTTGATTTTGTCATCT
CaMDR1 R	TCAGCGACGCGTCTAATTAGCATA CTTAGATCTTGCTCT	IFD2 F	GCAACAAGAGATAATTTCCCA
		IFD2 R	CAAGATGTAATAACTTAAAAG
CIP1 F	ATTACAAGAAAAAGAACCGGA	IFD5 F	TGTTGTTGAAAAAGGGTTGACG
CIP1 R	GTCATCAGCATTGATACCAG	IFD5 R	CAATAGTTTTATCAGCTTCACG
IFD1 F	CGTTGTTGAGAAAGGGTGGGCA	TEF3 F	AGAAACCGTCCACTTGTTGG
IFD1 R	CAATAGTATTCTCCATTTTACC	TEF3 R	GCAAGAAAATGGTGGCAAAT
IFD4 F	GGTGCCTCATCTATGAAAACA	DFG5 F	ATAATTGCGGTGGTGGGTTA
IFD4 R	CGTTAATCTTATCTGCGTCTCT	DFG5 R	CACATGTGACTCCATCGGAC
IFD7 F	AATGATGTTGTCGAAAAGGGA	PHR2 F	CTCCAGTTTGGTCTGGTGGT
		PHR2 R	CGGTTCTGACGGTACCAGAT

　　[a] Primers were designed using the primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi) on the ORF sequence. Abbreviations: F, forward primer; R = reverse primer.

pressed in these different experiments. The threshold value for the selected genes was a twofold difference of gene expression between the experiments, which is a value generally accepted in genome-wide expression profiling. These genes were further divided to give the number of genes being either up- or down-regulated in each experiment. Given that approximately 90 to 95% of the genes spotted on the microarrays gave validated data, meaning that reading of spot intensities for individual genes could be made in all hybridized arrays, the numbers given in Fig. 1 indicate that 3 to 4% of the total number of genes in the *C. albicans* genome exhibited transcriptional changes in these conditions. However, only a portion of the differentially expressed genes in these experiments could be commonly regulated between the different experiments. A cluster analysis using these data was performed in order to group these genes according to their expression patterns. The results of this analysis are represented in Fig. 2 and indicate that transcript profiles of the MDR and benomyl experiments

and of the CDR and fluphenazine experiments are grouped together. These results are consistent with previous observations indicating that fluphenazine induces *CDR* genes and that a similar event, i.e., constitutive upregulation of *CDR* genes, can be measured in the azole-resistant isolate DSY296 used in the CDR experiment. A similar relationship can be made for benomyl that induces *CaMDR1* and constitutive upregulation of the same gene existing in the azole-resistant isolate DSY2286 used in the MDR experiment. These results prompted us to compare in details genes differentially expressed between, first, the fluphenazine and CDR experiments and, second, the benomyl and the MDR experiments.

Comparative analysis of both the fluphenazine and the CDR microarray experiments. (i) Classification of expression clusters. Since it appeared from the global analysis of the microarray data that the fluphenazine and CDR experiments were more related to each other than to the other experiments,
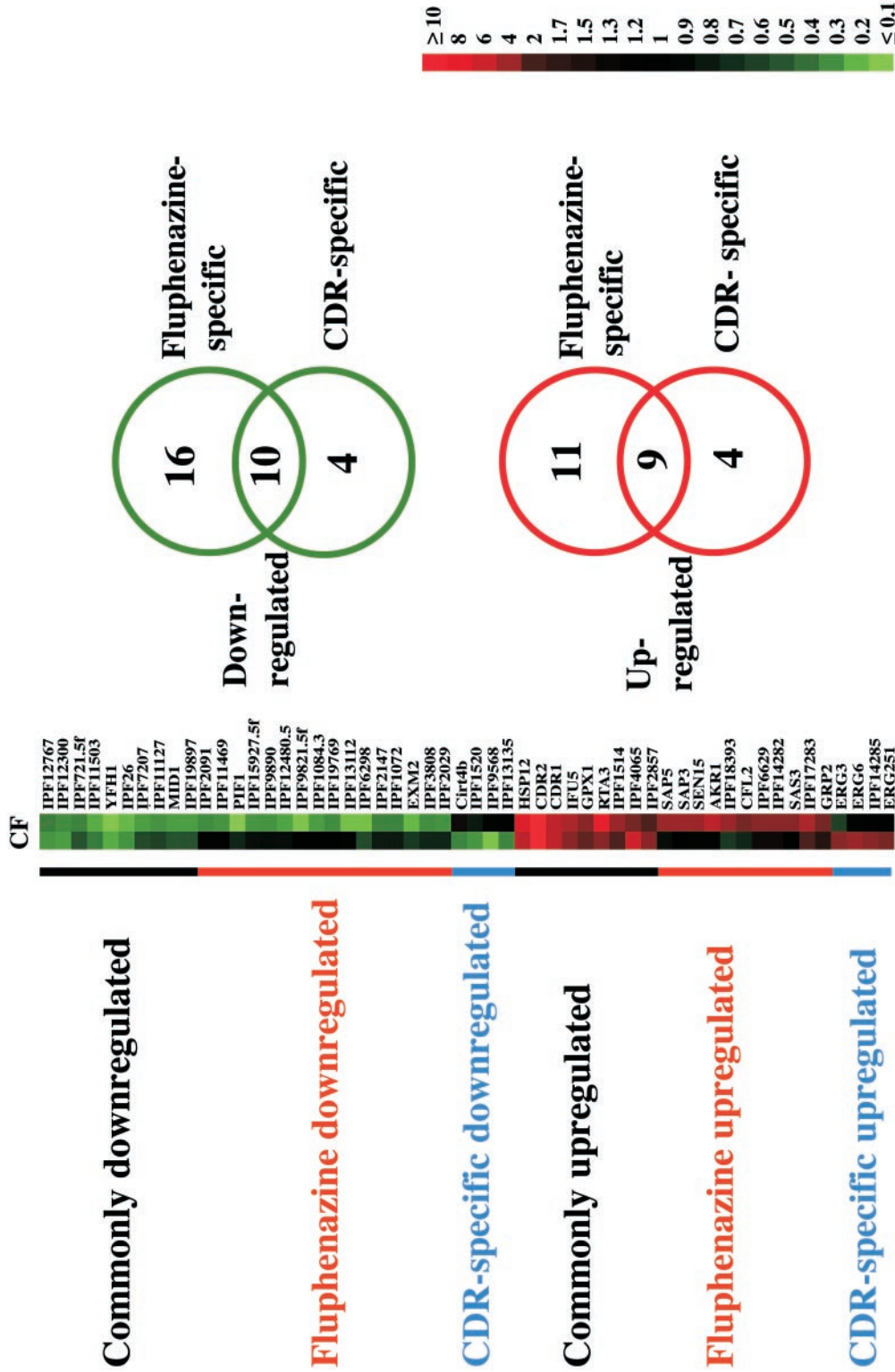
FIG. 3. Cluster analysis of the fluphenazine and CDR experiments. The threshold used in this analysis is a fourfold difference in expression. Genes have been arranged in different categories according to their expression profiles. The number of genes in each category is shown at the right side of the figure. Abbreviations: C, CDR; F, fluphenazine.

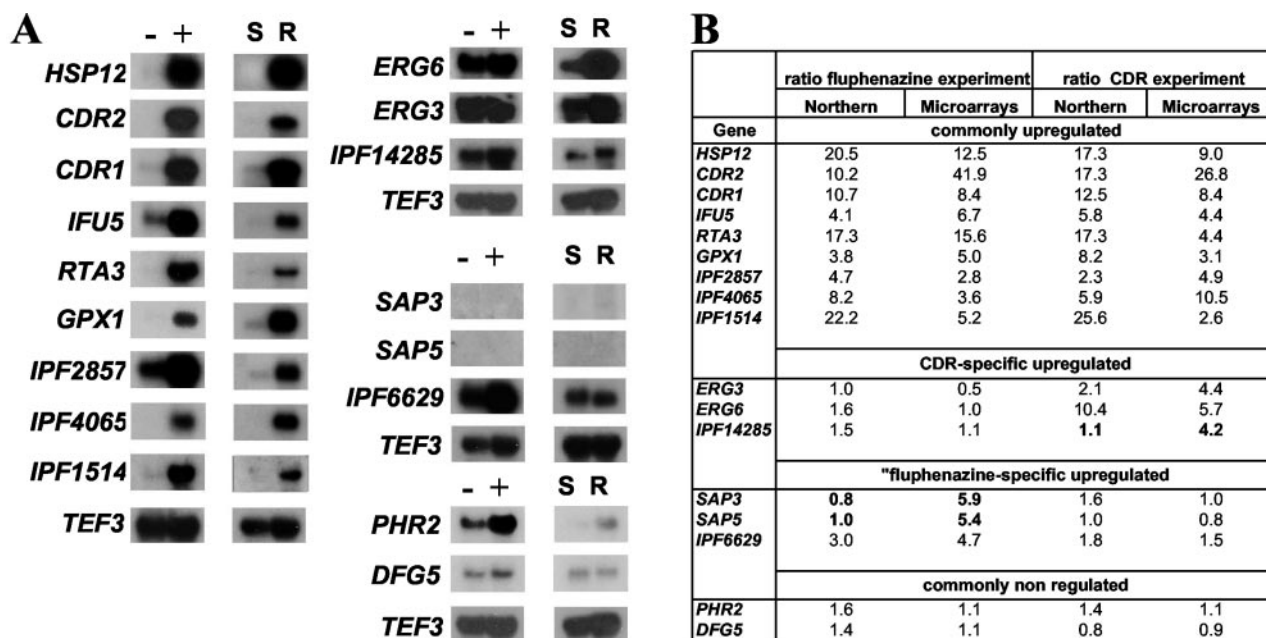|	ratio fluphenazine experiment		ratio CDR experiment	
Gene	Northern	Microarrays	Northern	Microarrays
	commonly upregulated			
HSP12	20.5	12.5	17.3	9.0
CDR2	10.2	41.9	17.3	26.8
CDR1	10.7	8.4	12.5	8.4
IFU5	4.1	6.7	5.8	4.4
RTA3	17.3	15.6	17.3	4.4
GPX1	3.8	5.0	8.2	3.1
IPF2857	4.7	2.8	2.3	4.9
IPF4065	8.2	3.6	5.9	10.5
IPF1514	22.2	5.2	25.6	2.6
	CDR-specific upregulated			
ERG3	1.0	0.5	2.1	4.4
ERG6	1.6	1.0	10.4	5.7
IPF14285	1.5	1.1	**1.1**	**4.2**
	"fluphenazine-specific upregulated"			
SAP3	**0.8**	**5.9**	1.6	1.0
SAP5	**1.0**	**5.4**	1.0	0.8
IPF6629	3.0	4.7	1.8	1.5
	commonly non regulated			
PHR2	1.6	1.1	1.4	1.1
DFG5	1.4	1.1	0.8	0.9

FIG. 4. Verification of differential expression of selected genes upregulated in the fluphenazine and CDR experiments. (A) Northern blot analysis. The *TEF3* gene was used as an RNA loading control. (B) Expression ratio obtained by microarray experiments and by Northern blot analysis. These ratios were obtained as described in Materials and Methods. Numbers in boldface type indicate discrepancies between both analysis. Symbols: (−) and (+), nonexposed or fluphenazine-exposed conditions, respectively; (S), azole-susceptible strain DSY294; (R), azole-resistant strain DSY296.

these two sets of microarray data were compared using a cluster analysis with a threshold of fourfold differential expression in at least one experiment. This threshold value was used since it is situated above the upper limit of *TEF3* expression variation and is therefore yielding genes with more relevant differential expression as outlined in Materials and Methods. Six different groups of differentially expressed genes were observed after cluster analysis among which three contained upregulated genes and the others contained downregulated genes (Fig. 3, left panel). The three clusters of each category were further distributed into commonly regulated genes, fluphenazine-specific or CDR-specific genes. In the group of upregulated genes listed in Fig. 3 (right panel) we identified nine commonly regulated genes, 11 fluphenazine-specific genes and 4 CDR-specific genes. The functions of these genes, grouped in functional categories such as transport, response to stress, ergosterol biosynthesis, or others, are listed in Tables 3 to 5. As summarized in Table 3, *CDR1* and *CDR2* show the largest change in expression (from 8.4- to 41.9-fold in both the CDR and fluphenazine experiments), while other genes such as

TABLE 3. Genes upregulated in the fluphenazine and CDR experiments

Function group and name	Function[a]	DRE sequence[b]	Fold increase (−) in expt	
			CDR	Fluphenazine
Transport				
CDR2	*C. albicans* drug resistance protein 2	CGGAAATCGGATA (−221)	26.8	41.9
CDR1	*C. albicans* drug resistance protein 1	CGGATATCGGATA (−460)	8.4	8.4
Response to stress				
HSP12	Heat shock protein (by homology); similar to *S. cerevisiae HSP12*		9.0	12.6
GPX1	Glutathione peroxidase (by homology); similar to *S. cerevisiae HYR1*		3.1	5.0
IPF4065	Similar to *S. cerevisiae YHR087w* (involved in stress response)		10.5	3.7
Other				
RTA3	Probable transmembrane protein; similar to *S. cerevisiae YOR049c* (involved in lipid transport activity)	CGGAAcTCGGAAA (−595)	4.4	15.7
IPF1514	Unknown		2.6	5.2
IFU5	Unknown; similar to *S. cerevisiae YFL010C*	CGGAAATCGGATA (−248)	4.3	6.7
IPF2857	Unknown		4.9	2.8

[a] Gene functions were obtained using the CandidaDB and MycoPathPD databases available from the Pasteur Institute and from Incyte.
[b] Bases indicated in lowercase type are mismatches compared to the following generic DRE sequence: CGGWWWTCGGWWW. Position of the DRE relative to the start codon is indicated in parentheses.

TABLE 4. Genes specifically induced in the fluphenazine experiment[a]

Function group and name	Function	DRE sequence	Fold increase (−) in expt	
			CDR	Fluphenazine
Virulence factor				
SAP5	Secreted aspartyl proteinase 5; virulence factor		0.8	5.4
SAP3	Secreted aspartyl proteinase 3; virulence factor		1.0	5.9
Response to stress				
CFL2	Similar to *S. cerevisiae YNR060w* (oxidoreductase activity)		0.7	4.1
IPF6629	Similar to *S. cerevisiae YLR109w* (alkyl hydroperoxide reductase)	gGGTATTCGGTTA (−600)	1.5	4.7
SAS3	Silencing protein (by homology); similar to *S. cerevisiae YBL052c* (involved in stress response)		1.2	4.5
IPF17283	Similar to *S. cerevisiae YCR061w* (salinity response, response to heat)		2.4	6.7
GRP2	Reductase (by homology); similar to *S. cerevisiae YOL151w* (induced by osmotic stress)		1.7	4.2
Other				
SEN15	tRNA splicing endonuclease delta subunit (by homology)		0.9	5.4
IPF18393	Similar to *S. cerevisiae YDR469w* (DNA repair activity)		0.6	5.3
IPF14282	Mucin proteins (by homology)		1.4	4.4
AKR1	Ankyrin repeat-containing protein (by homology); similar to *S. cerevisiae YDR264c*		0.9	7.7

[a] See Table 3 footnotes for details.

GPX1 and *IPF1514* are only upregulated (5.0- and 5.2-fold, respectively) above the 4-fold threshold value only in the fluphenazine experiment. These genes, although their increase in upregulation in the CDR experiment is 3.1- and 2.6-fold, respectively, still satisfy their categorization in commonly regulated genes, because of the algorithm used in the cluster analysis. This phenomenon can be observed for a few other genes listed in Tables 3 to 8. Among the downregulated genes (at least fourfold), 10 commonly regulated genes, 16 fluphenazine-specific genes, and 4 CDR-specific genes could be discriminated.

(ii) Validation by Northern analysis. Some of the upregulated genes and especially those commonly regulated or those with low intensity signals (*SAP3* and *SAP5*) were analyzed by Northern blot to confirm the microarray data (Fig. 4A). Radioactive signals obtained by Northern blotting were quantified and compared with fluorescence signals of the microarray analysis (Fig. 4B). Comparison of signal ratios obtained by microarrays or by Northern blot analysis showed a good correlation between the two methods (Fig. 4B). Eight out of the nine genes previously defined as commonly upregulated genes exhibit mRNA signals with ratio above the fourfold cutoff value that was used in the microarray analysis. The remaining gene, *IPF2857*, possessed a signal ratio in clinical strains of only 2.3 by Northern analysis compared to 4.9 obtained by microarray analysis. Given that Northern blot analysis might be less sensitive than microarray analysis, this gene could be therefore classified in the commonly upregulated genes. Three out of 11 genes belonging to the fluphenazine-specific upregulated genes were verified by Northern analysis. *SAP3* and *SAP5* were chosen because they showed low intensity signals in the microarray analysis (Fig. 4B). *IPF6629* was chosen because it contains a DRE-like sequence (see below) in its promoter. *SAP3* and *SAP5* mRNA signals were almost absent under the conditions tested, with ratios of 0.8 and 1.0, respectively. In contrast, *IPF6629* was significantly upregulated in Northern blot with a ratio of 3.0. None of these three genes showed a difference in

gene expression between the azole-susceptible and azole-resistant strains DSY294 and DSY296, which is consistent with the microarray experiments. Three genes out of the four present in the group of CDR-specific upregulated genes were analyzed by Northern blot. *ERG* genes (*ERG3* and *ERG6*) were confirmed as CDR-specific, since their expression ratios in clinical strains were 2.1 and 10.4, respectively. Both genes were not induced by fluphenazine (ratios of 1.0 and 1.6, respectively, by Northern blotting). In contrast *IPF14285* was not confirmed as a CDR-specific upregulated gene, since its expression ratio was only 1.1. In Northern blot this gene does not appear upregulated in fluphenazine nor in CDR experiments (Fig. 4A). The expression of *PHR2* and *DFG5* was taken as a control, since the expression of these genes was changed neither in the fluphenazine nor in the CDR experiments. The absence of regulation of these two genes was confirmed by Northern blot (Fig. 4B). In conclusion, signals revealed by Northern blot analysis were correlating well with those obtained by microarray analysis, except for *SAP3* and *SAP5* that were not upregulated by fluphenazine treatment and for *IPF14285* that was not upregulated in the azole-resistant strain DSY296.

(iii) Functional aspects of differentially expressed genes. Among the nine commonly upregulated genes, four genes (*CDR1*, *CDR2*, *IFU5*, and *RTA3*) contain in their promoter a sequence motif similar to the DRE (CGGWWWTCGGW WW) that was previously described to be important for *CDR2* and *CDR1* upregulation (10) (Table 3). Although the inspection of genome data identified a total of 10 genes with a perfect match to this DRE and 177 with a single mismatch DRE in their promoters, this DRE sequence is not present in the promoters of CDR-specific upregulated genes and only present in one gene (*IPF6629*) among the nine fluphenazine-specific upregulated genes (Tables 4 and 5). We therefore conclude that the DRE is strongly linked to a pathway controlling the expression of genes involved in azole resistance. Since the exact function of *RTA3* and *IFU5* is not yet determined in *C. albicans*, it is not possible to attribute a common functional role to

TABLE 5. Genes specifically induced in the CDR experiment[a]

Function group and name	Function	Fold increase (−) in expt	
		CDR	Fluphenazine
Ergosterol bio-synthesis			
ERG3	C-5,6 sterol desaturase	4.4	0.5
ERG6	C-24 sterol transmethylase	5.7	1.0
ERG251	C-4 sterol methyl oxidase (by homology); similar to *S. cerevisiae ERG25*	4.1	1.1
Other (*IPF14285*)	Unknown	4.3	1.1

[a] See Table 3 footnotes for details.

the four commonly upregulated and DRE-containing genes described above. The product of these four genes share, however, transmembrane segments and thus a membrane localization, therefore suggesting that the common regulation of these genes in the experimental conditions tested in this study has an impact in membrane functions.

Regarding the functions of other genes, we observed that among the nine fluphenazine-specific genes, five were potentially involved in different stress responses such as oxidative stress (*CFL2* and *IPF6629*), osmotic stress (*GRP2* and *IPF17282*), or more general stress responses (*SAS3*) (Table 4). In contrast the CDR-specific genes were belonging to the ergosterol biosynthetic pathway (*ERG3*, *ERG6*, and *ERG251*) (Table 5). Downregulated genes in the two experiments belonged essentially to genes with unknown functions in the three clusters (Fig. 3). Downregulated genes with known functions do not share a common functional category (data not shown). These genes have potential functions in stress response (*IPF2091*) or in transport (*IPF12300*, *IPF9821*, and *MID1*).

Comparative analysis of the benomyl versus MDR microarray experiments. (i) Classification of expression clusters. Microarray data from the related benomyl and MDR experiments were also compared by cluster analysis (Fig. 5). Genes that were differentially expressed by at least a fourfold difference were classified into six clusters. As previously observed for the CDR and fluphenazine experiments up- and downregulated genes were distributed into three clusters containing the commonly regulated genes, the benomyl-specific and the MDR-specific genes. As depicted in the right panel of Fig. 5, among the 22 downregulated genes, 6, 12, and 4 were commonly regulated genes, MDR-specific and benomyl-specific genes, respectively. Similarly, upregulated genes contained 17, 9, and 32 commonly regulated genes, MDR-specific and benomyl-specific genes, respectively. The functions of these genes, grouped in functional categories such as transport, response to stress, cell wall maintenance, or others, are listed in Tables 6 to 8.

(ii) Validation by Northern blot analysis and RT-PCR. To validate the data obtained with microarrays, Northern blot analysis were performed with the most upregulated genes from each group and also with four genes (*IFR2*, *IPF3415*, *TTR1*, and *TRX1*) tested in another published microarray analysis (13). In general, Northern analysis confirmed the microarray data. The transcription of 6 chosen genes among the 17 commonly upregulated ones (*DDR48*, *BMR1/CaMDR1*, *GPX1*, *IPF17186*, *GRP2*, and *IPF7817*) was increased as expected from

the MDR and benomyl microarray experiments. Among 5 genes from the group of 9 MDR-specific upregulated genes (*IPF7863*, *IPF9538*, *IPF2186*, *IPF3704*, and *SNZ1*), all except one (*IPF3704*) appeared effectively only upregulated in the clinical resistant strain DSY2286. As shown in Fig. 6, *IPF3704* had an expression ratio of only 0.8. Nine genes selected from the group of the 32 benomyl upregulated genes were also verified by Northern analysis. *CIP1*, *MRF1*, *EBP1*, *EBP4*, and *IPF3264* were chosen because they corresponded to the most upregulated genes. The four others (*TTR1*, *TRX1*, *IFR2*, and *IPF3415*) were genes already shown as upregulated under oxidative stress conditions as shown by Enjalbert et al. (13). As shown in Fig. 6, all these genes were induced by benomyl treatment. Nevertheless, three of them (*MRF1*, *EBP1*, and *EBP4*) were also upregulated in the clinical resistant strain DSY2286 with expression ratios of 2.2, 3.6, and 6.4 in clinical strains, respectively. Therefore, these three genes could not be considered as benomyl-specific upregulated genes, but rather as commonly regulated genes. As previously mentioned above in the cluster analysis of the fluphenazine and CDR experiments, some genes can be grouped in specific expression categories by the clustering program algorithm, even though their upregulation ratios would group them in other categories. We observed in our microarray analysis that several genes of the *IFD* family (genes coding for putative benzyl alcohol dehydrogenases) were upregulated in both experimental conditions, either by benomyl treatment or in clinical strains. Since these genes are possessing high degree of similarity, it is not possible to distinguish individual members by Northern blot analysis. The expression of these genes was therefore verified by semiquantitative RT-PCR using primers specific for each gene. As shown in Fig. 6B, PCR signals demonstrated that *IFD1*, *IFD4*, *IFD5*, and *IFD7* were upregulated by at least 100-fold after benomyl exposure and in the clinical azole-resistant strain DSY2286, while *IFD2* remained only strongly upregulated in DSY2286. These results were consistent with the microarrays data (in the MDR experiment, *IFD1*, *IFD5*, *IFD7*, *IFD4*, and *IFD2* were upregulated by 683-, 511-, 158-, 129-, and 98-fold, respectively; in the benomyl experiment, *IFD1*, *IFD5*, *IFD7*, and *IFD4* were upregulated by 31-, 18-, 7-, and 8-fold, respectively). Taken together, the results of Northern and RT-PCR analysis correlated well with those obtained with microarray analysis except for four genes (*MRF1*, *IPF3704*, *EBP1*, and *EBP4*).

(iii) Functional aspects of differentially expressed genes. Among the genes upregulated in a specific category, we investigated if their promoters could share putative common regulatory sequence(s). *CaMDR1* belongs to the commonly upregulated genes and its promoter contains one putative Cap1p binding site, TTAG/CTAA (15), localized at bp −532 (with respect to the ATG start codon). Cap1p was shown earlier to be involved in multidrug resistance and oxidative stress response in *C. albicans* (2, 34). The promoters of genes containing this motif were therefore scanned using algorithms available at http://genolist.pasteur.fr/CandidaDB/. This motif is highly distributed in the *C. albicans* genome, since 1,637 open reading frames contain at least one perfect Cap1p binding site sequence in their promoters. Among the 29 benomyl-specific genes, 21 contained this sequence in their promoter (Table 8). Since none of the MDR specific genes and only a few of the commonly upregulated genes contained such a site in their
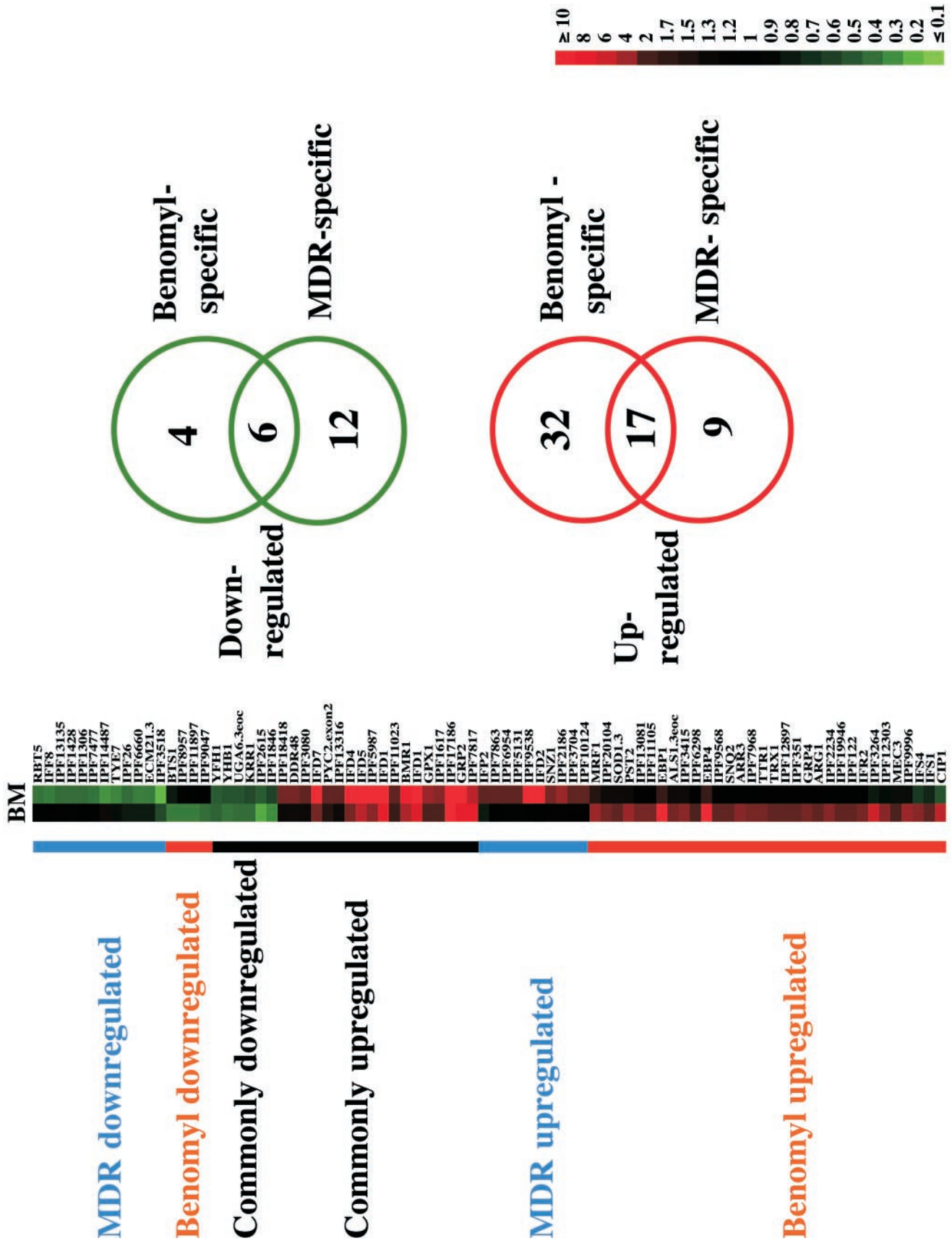
FIG. 5. Cluster analysis of the benomyl and MDR experiments. The threshold used in this analysis is of fourfold difference of expression.

TABLE 6. Genes upregulated in the benomyl and MDR experiments

Function group and name	Function[a]	Cap1p binding site position(s)[b]	Fold increase (−) in expt	
			MDR	Benomyl
Other				
IPF3080	Unknown	−213, −708	4.1	1.6
IPF1617	Unknown	−207	7.3	3.2
IPF11023	Unknown		4.3	2.6
Transport (*CaMDR1*)	Multidrug transporter activity; similar to *S. cerevisiae FLR1*	−532	44.0	12.6
Response to stress				
IPF18418	Unknown; similar to *S. cerevisiae YHR029c* (induced by cell-damaging conditions)		5.4	1.6
IPF13316	Unknown; similar to *S. cerevisiae YNL010w* (induced by cell-damaging conditions)		4.1	1.7
IPF5987	Unknown; similar to *S. cerevisiae YPR127w* (protein with similarity to *S. pombe* pyridoxal reductase, aldo-keto reductase family)		70.2	7.9
IPF17186	Unknown; similar to *S. cerevisiae YDR533c* (induced by cell-damaging conditions)	−720	102.5	56.8
PYC2	Pyruvate carboxylase 2; similar to *S. cerevisiae YBR218c*		5.1	1.9
GPX1	Glutathione peroxidase; similar to *S. cerevisiae HYR1*		9.4	3.6
GRP2	Reductase; similar to *S. cerevisiae YOL151w* (induced by osmotic stress)	−444	52.5	35.1
IPF7817	Putative NADH-dependent flavin oxidoreductase by homology; similar to *S. cerevisiae OYE3*	−743	14.7	37.0
DDR48	Stress protein; similar to *S. cerevisiae YMR173w* (involved in the production of or recovery from mutation)		5.6	1.7
IFD1	Benzyl alcohol dehydrogenase activity (by homology); similar to *S. cerevisiae YPL088w* (aldo-keto reductase family)		748.3	32.6
IFD4	Benzyl alcohol dehydrogenase activity (by homology); similar to *S. cerevisiae YPL088w* (aldo-keto reductase family)		129.6	6.7
IFD5	Benzyl alcohol dehydrogenase activity (by homology); similar to *S. cerevisiae YPL088w* (aldo-keto reductase family)		511.2	16.1
IFD7	Benzyl alcohol dehydrogenase activity (by homology); similar to *S. cerevisiae YPL088w* (aldo-keto reductase family)		158.9	5.4

[a] Gene functions were obtained using the CandidaDB and MycoPathPD databases available from the Pasteur Institute and from Incyte.
[b] The sequence of the Cap1p-binding site considered is TTA(C/G)TAA (15).

promoters (Tables 6 and 7), this element could be rather considered as a benomyl-specific regulatory element. The transcription factor Cap1p is known to be involved in oxidative stress response (34). It is interesting that among the 29 benomyl-specific upregulated genes, 7 (*IPF2897*, *IPF11105*, *PST2*, *IPF3264*, *SOD1*, *TTR1*, and *TRX1*) are potentially implicated in this type of stress response. Five other genes included in this cluster play a role in other stress responses such as osmotic (*GRP4*) or heat shock stress responses (*IFR2*) (Table 8). Among the 17 commonly regulated genes, 9 are also involved essentially in oxidative stress response (*PYC2*, *GPX1*, *GRP2*, *IPF7817*, *IFD1*, *IFD4*, *IFD5*, and *IFD7*), and 1 of them (*DDR48*) corresponds to a protein potentially involved in the production of mutation or recovery from mutation. Interestingly, one of the members of the *IFD* gene family (*IFD4*), the product of which could have benzyl alcohol dehydrogenase activity, has been shown to be identical to the gene *CSH1* coding for a surface hydrophobicity-associated protein (29). *IFD* proteins, if all located at the cell surface, could therefore play an important role at the interface between the cell and its environment.

DISCUSSION

In this work, we compared transcript profiling between clinical strains with known azole resistance mechanisms and laboratory strains exposed to drugs known to induce genes involved in azole resistance. Previous experiments performed by our group and others have established that specific multidrug transporter genes can be induced by distinct agents, thus re-

sulting in similar gene expression patterns than those observed in the azole-resistant isolates. Until now, only a specific set of genes has been analyzed in response to these drugs. Gupta et al. (17) showed that benomyl, methotrexate, and H_2O_2 could enhance the expression of *CaMDR1* in *C. albicans*. On the other hand, fluphenazine, estradiol, or terbinafine can induce the expression of *CDR1* and *CDR2* in the same yeast (10). However, these drugs can induce the expression of additional genes and therefore address the validity of the comparison between transient induction of multidrug transporters and their constitutive upregulation in azole-resistant clinical strains. This study clarifies this question and establishes the number of genes commonly regulated in each situation. A few gene profiling experiments have been carried out with azole-resistant strains until now. Rogers and coworkers (23) have used a well-known series of sequential isolates first analyzed by White (30, 31). In these isolates, an ordered expression of both types of multidrug transporter genes (i.e., *CDR* and *CaMDR1* genes) occurred over time. Another genome-wide study was performed by Cowen et al. (7) with sequential isolates that developed azole resistance by upregulation of *CDR* genes or *CaMDR1*. Although the type of microarray, the gene annotation, and the signal intensities were different between these different studies, we attempted to compare the transcript profiles obtained in these studies with our analysis. We compared first the transcript profiles of the MDR experiment with those obtained by isolate 2-80 (isolate that upregulates *CaMDR1*) versus isolate 2-79 (azole-susceptible isolate) (study published by Rogers et al. [23]) and with those of isolate D12-330 versus an azole-susceptible isolate (study published by Cowen et al. [7]).
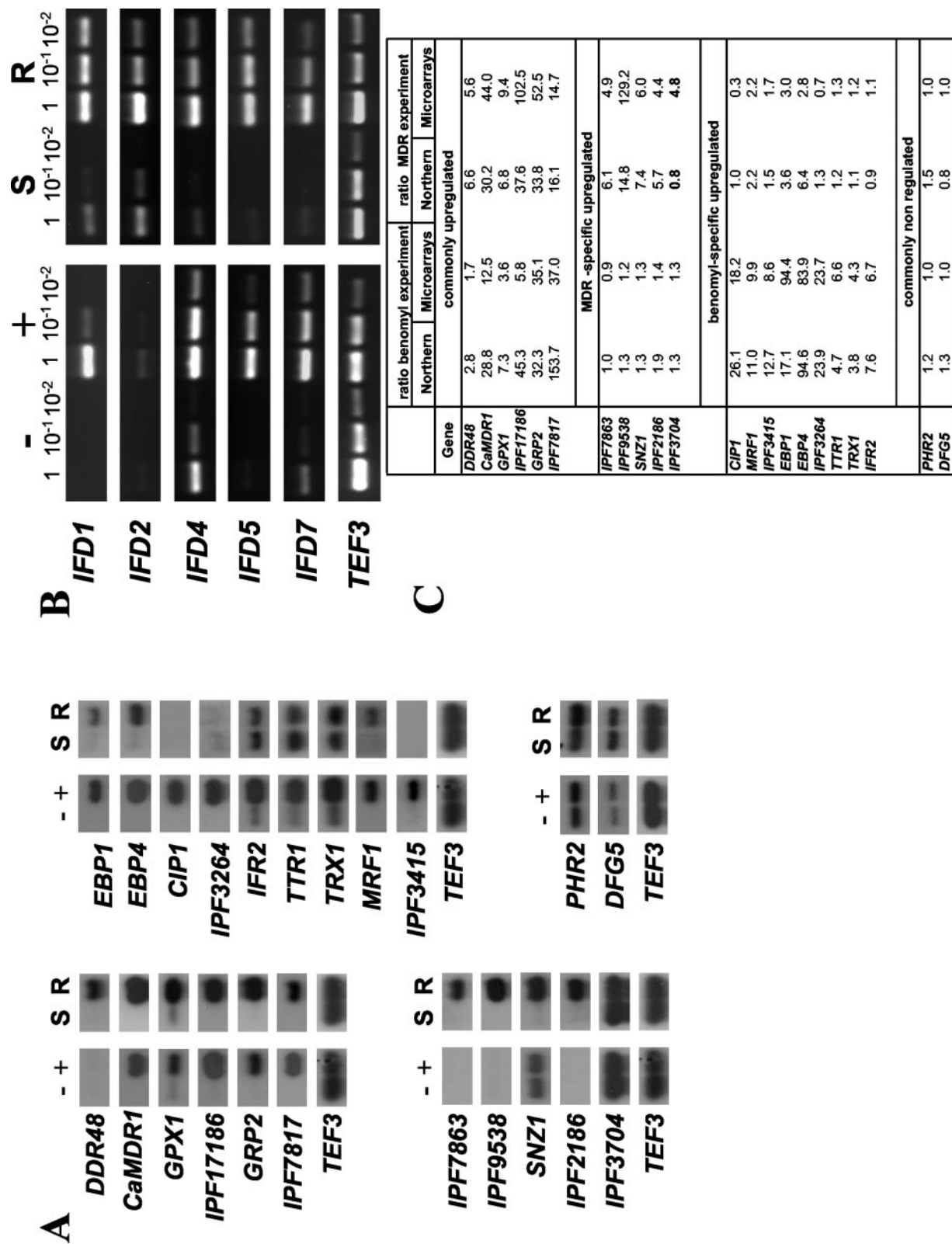
Gene	ratio benomyl experiment		ratio MDR experiment	
	Northern	Microarrays	Northern	Microarrays
commonly upregulated				
DDR48	2.8	1.7	6.6	5.6
CaMDR1	28.8	12.5	30.2	44.0
GPX1	7.3	3.6	6.8	9.4
IPF17186	45.3	5.8	37.6	102.5
GRP2	32.3	35.1	33.8	52.5
IPF7817	153.7	37.0	16.1	14.7
MDR-specific upregulated				
IPF7863	1.0	0.9	6.1	4.9
IPF9538	1.3	1.2	14.8	129.2
SNZ1	1.3	1.3	7.4	6.0
IPF2186	1.9	1.4	5.7	4.4
IPF3704	1.3	1.3	0.8	4.8
benomyl-specific upregulated				
CIP1	26.1	18.2	1.0	0.3
MRF1	11.0	9.9	2.2	2.2
IPF3415	12.7	8.6	1.5	1.7
EBP1	17.1	94.4	3.6	3.0
EBP4	94.6	83.9	6.4	2.8
IPF3264	23.9	23.7	1.3	0.7
TTR1	4.7	6.6	1.2	1.3
TRX1	3.8	4.3	1.1	1.2
IFR2	7.6	6.7	0.9	1.1
commonly non regulated				
PHR2	1.2	1.0	1.5	1.0
DFG5	1.3	1.0	0.8	1.0

FIG. 6. Verification of differential expression of selected genes upregulated in the benomyl and MDR experiments. (A) Northern blot analysis. *TEF3* was used as internal control. (B) Evaluation of differential expression by RT-PCR on the *IFD* genes family upregulated in the benomyl and MDR experiments. (C) Expression ratio obtained by microarray experiments and by Northern blot analysis. These ratios were obtained as described in Materials and Methods. Numbers in boldface type indicate discrepancies between both analysis. Symbols: (−) and (+), nonexposed or benomyl-exposed conditions; (S), azole-sensitive strain DSY2285; (R), azole-resistant strain DSY2286.

TABLE 7. Genes specifically induced in the MDR experiment

Name	Function[a]	Fold increase (−) in expt	
		MDR	Benomyl
IFP2	Unknown	4.6	0.7
IPF6954	Unknown	4.3	0.9
IPF5131	Unknown	4.3	0.9
IPF2186	Unknown; similar to *S. cerevisiae* YGR110w	10.2	1.4
IPF3704	Similar to *S. cerevisiae* YNL218w (DNA-dependent ATPase activity)	4.8	1.3
IPF7863	Similar to *S. cerevisiae* YNL335w (nitrile hydratase activity)	4.9	0.9
IPF9538	Similar to *S. cerevisiae* YNL202w (2,4-dienoyl-coenzyme A reductase NADPH activity)	129.2	1.1
IFD2	Benzyl alcohol dehydrogenase activity (by homology); similar to *S. cerevisiae* YPL088w	95.0	1.1
SNZ1	Stationary-phase protein (by homology); similar to *S. cerevisiae* YMR096w (putative pyridoxine [vitamin B_6] biosynthetic enzyme)	6.0	1.3

[a] See Table 6, footnote *a*, for details.

Only genes upregulated by more than 1.5-fold were taken into consideration. As shown in Fig. 7, the present study yielded a higher number of upregulated genes than measured in the two other studies. These discrepancies reflect not only differences in experimental conditions but also that the origin of isolates in the three studies is different. Figure 7 reveals that only a few genes are commonly regulated in strains upregulating *CDR* genes. Among these commonly regulated genes, we found *CDR1*, *CDR2*, *RTA3*, *IFU5*, and *GPX1*. It is remarkable that these genes are also among those that are also commonly regulated between the fluphenazine and the CDR experiment of this study. Beside being involved in drug resistance (for *CDR1* and *CDR2*), these genes have in common that their *S. cerevisiae* homologues respond to conditions of cell damages that were performed in microarray experiments, as deduced from transcript profile information available at http://www .yeastgenome.org/. This suggests that the common regulation of these genes is not accidental, but constitutes a group of genes necessary for the adaptation of *C. albicans* to drug exposure. One would expect that these genes are controlled

TABLE 8. Genes specifically induced in the benomyl experiment

| Function group and name	Function	Cap1p binding site position(s)	Fold increase (−) in expt	
			MDR	Benomyl
Other				
IPF2234	Unknown	−339	1.1	7.4
IPF122	Unknown	−859, −520	1.1	5.1
IPF13081	Unknown		1.4	5.7
IPF9568	Unknown		1.4	4.6
IPF6298	Unknown		1.4	4.0
IPF351	Unknown; similar to *S. cerevisiae* YMR090w	−78	1.2	4.1
IFS4	Pirin protein (by homology)	−536, −87, −294, −273	0.5	7.2
IFS1	Pirin protein (by homology); similar to *S. cerevisiae* YDR014w	−418, −274	0.6	4.1
CIP1	Cadmium-induced protein c (by homology)	−559	0.3	18.1
IPF19946	Nucleotide-binding protein (by homology); similar to *S. cerevisiae* NBP35		1.1	4.7
ARG1	Argininosuccinate synthetase (by homology); similar to *S. cerevisiae* YOL058w		1.1	4.2
MRF1	Mitochondrial respiratory function protein (by homology); similar to *S. cerevisiae* YBR026c		2.2	9.9
IPF3415	Mitochondrial inner membrane protease (by homology); similar to *S. cerevisiae* YIM1	−168	1.7	8.6
EBP1	NADPH dehydrogenase, estrogen binding protein		3.0	94.4
EBP4	NADPH dehydrogenase, estrogen binding protein		2.8	83.8
IPF20104	Alcohol dehydrogenase (by homology); similar to *S. cerevisiae* ADH6	−179	1.7	5.5
SNQ2	Similar to *S. cerevisiae* YNL334C (transaminase activity, cellular response to starvation)		1.0	5.1
ARR3	Arsenite transporter activity (by homology); similar to *S. cerevisiae* YPR201w	−328	1.0	7.5
Cell wall maintenance				
IPF9996	Similar to *S. cerevisiae* YDR371w (chitinase activity)	−119	0.7	9.3
ALS1	Similar to *S. cerevisiae* YJR151c (cell wall organization and biogenesis)	−424	1.5	5.8
Response to stress				
IPF12897	Putative oxidoreductase (by homology); similar to *S. cerevisiae* YJR096w		1.2	4.2
IPF11105	Probable quinone oxidoreductase; similar to *S. cerevisiae* YBR046c	−580	1.4	7.9
PST2	1,4-Benzoquinone reductase (by homology), similar to *S. cerevisiae* YDR032c	−290	1.5	9.8
IPF3264	Similar to *S. cerevisiae* YML131w (oxidoreductase activity, response to stress)	−306	0.7	23.7
SOD1	Cu,Zn-superoxide dismutase; similar to *S. cerevisiae* YJR104C (oxidative stress response)	−752	1.8	4.1
TTR1	Glutaredoxin (by homology); similar to *S. cerevisiae* YDR513w (response to pH, oxidative stress and heat)	−137	1.3	6.6
TRX1	Thioredoxin (by homology); similar to *S. cerevisiae* YLR043c (response to oxidative stress)	−216, −257, −467, −652, −920	1.2	4.3
GRP4	Putative reductase (by homology); similar to *S. cerevisiae* YOL151w (induced by osmotic stress)	−389	1.2	6.5
IFR2	Similar to *S. cerevisiae* YNL134c (response to heat shock)		1.1	6.7
IPF7968	Similar to *S. cerevisiae* YNL229c (transcription corepressor activity, salinity response)	−161	1.0	6.0
IPF12303	Similar to *S. cerevisiae* YNL229c (transcription corepressor activity, salinity response)	−38, −176	0.8	8.1
MEC3	Similar to *S. cerevisiae* YLR288c (response to stress)		0.8	4.0

[a] See Table 6 footnotes for details.

A

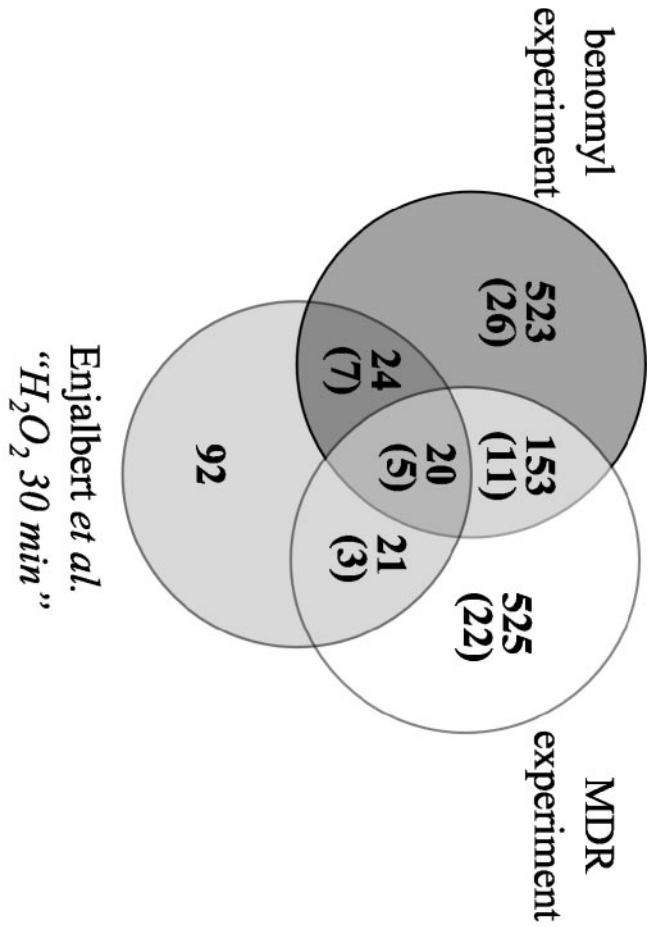### Azole-resistant strains upregulating *CDR* genes

This study — Cowen et al.

321 (13)   3 (1)   25
5
7   1
85
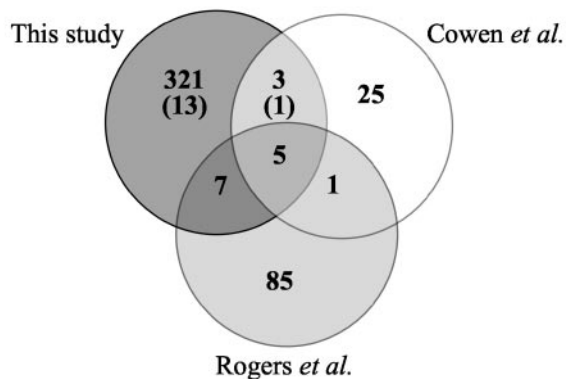
Rogers et al.

Gene	Fold increase (-)		
	This study "CDR"	Rogers et al. 12-99 vs 2-79	Cowen et al. D8-330
CDR1	8.4	9.2	NA
CDR2	26.8	8.4	2.4
GPX1	3.1	3.1	1.5
IFU5	4.4	1.7	1.7
RTA3	4.4	2.5	2.2
ERG25	2.5	1.6	
IPF4999	2.3	2.0	
IPF3251	2.1	1.5	
MIR1	1.7	1.6	
GRP2	1.7	4.1	
INO1	1.7	1.9	
IFA7	1.6	1.5	
HSP12	9.0		1.8
SOU1	2.0		1.5
IPF14743	1.6		1.6
Cirt3	1.8		1.6
IFD5		13.5	1.9

B

Azole-resistant strains upregulating *CaMDR1*

This study — Cowen et al.

483 (12)   28 (5)   64
8 (6)
11 (3)   3
76
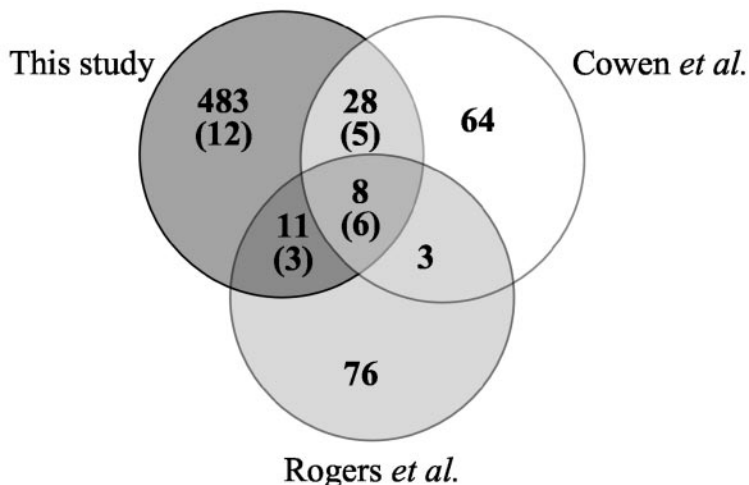
Rogers et al.

Gene	Fold increase (-)		
	This study "MDR"	Rogers et al. 2-80 vs 2-79	Cowen et al. D12-330
IFD1	683.3	17.6	NA
IFD5	511.2	12.5	15.6
IPF5987	70.2	3.3	9.5
GRP2	52.5	2.7	8.6
CaMDR1(BMR1)	44.0	3.7	17.2
SNZ1	6.0	1.6	1.6
GDH3	3.5	1.6	2.1
ARO8	3.2	1.6	2.8
IFD7	158.9	5.4	
IFD4	129.6	11.8	
IFD2	95.0	1.5	
HIS7	2.3	1.5	
HIS4	2.2	1.6	
IPF19892	2.0	2.3	
LYS21	1.9	1.7	
SPT3	1.9	1.5	
IPF9663	1.9	1.7	
HIS1	1.5	1.5	
IPF1583	1.5	1.6	
IPF9538	129.2		24.4
IPF7817	14.7		1.8
GPX1	9.3		4.0
IPF18418	5.4		1.7
IPF7863	4.9		2.9
IPF1266	3.9		2.8
GLK1	3.4		1.5
RPN4	3.1		1.6
IFD3	3.0		2.0
NDH2	2.7		2.6
IPF3964	2.4		1.8
IPF2557	2.3		2.1
URA4	2.1		1.8
MCR1	2.0		1.6
IPF6629	2.0		2.0
SOD2	1.9		1.7
NIT3	1.9		2.0
SPO70.5f	1.8		1.5
IPF8951	1.7		1.9
IPF1264	1.7		2.8
IPF7686	1.7		1.6
IPF1992	1.6		2.6
IPF1835	1.6		1.5
YDC1	1.6		1.7
GPH1	1.5		1.6
IPF4317	1.5		1.5
IPF9450	1.5		1.6
IPF18298.3	1.5		1.5
IPF17024		4.4	1.8
IPF19706		1.8	1.6
HEM13		1.8	1.6

FIG. 7. Comparison of the microarray data of (A) CDR and (B) MDR experiments with previously published microarray analysis performed with other azole-resistant strains (7, 23). Gene expression values obtained from these studies for selected genes with a threshold upregulation of 1.5 are given in the accompanying table and commonly regulated genes can be observed in a Venn diagram. Numbers in boldface type in the accompanying table and in brackets in the Venn diagram indicate the number of commonly regulated genes taking the present data set with a fourfold threshold. NA, not available.

FIG. 8. Comparison of the microarray data of benomyl and MDR experiments with previously published microarray analysis on stress response after 30 min of H_2O_2 exposure (13). Gene expression values obtained from these studies for selected genes with a threshold upregulation of 1.5 are given in the accompanying table and commonly regulated genes can be observed in a Venn diagram. Numbers in boldface type in the accompanying table and in brackets in the Venn diagram indicate the number of commonly regulated genes taking the present data set with a fourfold threshold.

Gene	This study "benomyl"	This study "MDR"	Enjalbert et al. H₂O₂ 30 min
		Fold increase (-)	
IFD1	38.8	626.6	5.0
GRP2	36.8	49.8	3.9
IFD5	18.1	568.8	5.0
IFD4	8.6	133.1	5.0
IFD7	7.0	309.5	5.0
IPF12579	3.4	1.7	1.8
IPF2027	2.6	2.3	2.2
IPF2837	2.5	2.5	2.2
IPF3964	2.5	2.4	2.4
PRC1	2.2	2.8	1.9
IPF16253	1.7	2.8	2.2
LAP41	1.7	2.1	7.5
IPF1912	1.7	2.2	1.9
IPF14871	1.7	1.8	2.3
IPF1835	1.7	1.6	2.7
IPF1384	1.6	2.1	1.6
IPF525	1.6	3.2	3.2
IPF14663	1.6	1.9	1.6
IPF4328	1.5	1.8	2.5
CAN1	1.5	2.0	4.4

Gene	This study "benomyl"	This study "MDR"	Enjalbert et al. H₂O₂ 30 min
		Fold increase (-)	
GRP4	8.7		1.7
IPF12303	8.4		1.5
IFR2	8.3		3.4
TTR1	7.3		1.7
IPF13081	7.2		2.0
PST2	13.6	1.7	6.2
IPF20104	6.7	1.6	5.2
IPF19946	3.1		2.2
SSA4	2.5		3.7
IPF3491	2.4		2.0
SSA1	2.2		3.7
TRR1	2.1		1.6
IPF4256	2.0		1.9
IPF13867	1.9		2.1
HSP104	1.9		1.8
HEM15	1.8		1.6
CTA1	1.8		4.0
IPF1382	1.8		6.5
ARG8	1.7		1.8
IPF14662	1.6		2.6
IFU5	1.6		1.7
IPF614	1.6		1.6
CAN2	1.6		1.6
DD1	1.6		4.4
PRB1	4.7		2.1
GLK1		4.0	2.2
DDR48		6.3	12.2
IPF14899	1.7	3.2	1.7
IPF8814		2.9	1.7
DLD3		2.8	1.7
ECM331		2.1	2.5
IPF6629		2.0	3.1
IPF3484		2.0	4.1
IPF885		2.0	6.9
IPF3094		1.9	2.5
IPF4959		1.7	1.8
ROT2		1.6	1.9
IPF2400		1.6	5.1
GPH1		1.6	1.9
IPF19801		1.6	2.3
IPF4119		1.6	2.4
SOU2		1.5	1.6
UGA2		1.5	1.8
EXG2		1.5	2.0
ATH1		1.5	1.8

by a common regulatory mechanism. Since four of these five commonly regulated genes contained a DRE in their promoter, these results strongly suggest the existence of a common transcriptional pathway important for their regulation. The simplest hypothesis would be that a yet-unidentified transcription factor can bind to the DRE and be activated either after drug treatment or by a mutation(s) existing in azole-resistant strains, thus resulting in transcriptional activation of genes containing the DRE. Current experiments undertaken in our laboratory favor this hypothesis: a transcriptional activator of *CDR1* and *CDR2* has been isolated and alleles of this gene cloned from azole-resistant isolates are able to activate the transcription of *CDR1* and *CDR2* in an azole-susceptible laboratory strain (A. T. Coste, unpublished data).

When comparing the set of genes commonly regulated in isolates upregulating *CaMDR1* by microarray experiments of this study and of those published by Cowen et al. (7) and Rogers et al. (23), only eight genes were upregulated in the three studies. We observed a higher number of genes commonly regulated (28) between the MDR experiment of our study and the set of strains used by Cowen et al. (7) than in the set of strain used by Rogers et al. (23). This probably reflects differences between strains used in these different studies. The genes commonly regulated in the three studies were corresponding to *CaMDR1* (*BMR1*), *GRP2*, *IFD5*, *IPF5987*, and *SNZ1*. Besides the antifungal drug resistance function of *CaMDR1*, the other genes have oxido-reductive functions (*GRP2*, *IFD5*) or are potentially involved in pyridoxine (vitamin B_6) synthesis (*IPF5987* and *SNZ1*). It is, however, interesting that the *S. cerevisiae* homologues of all these five genes respond to cell damage conditions as can be determined from transcript profiling experiments available at http://www.yeastgenome.org/. Therefore, the common regulation of these genes constitute also a necessary process in the development of drug resistance mediated by the "*CaMDR1*" pathway. It is currently difficult to predict the identity of one or several regulatory elements responsible for the upregulation of these genes, since no detailed studies have been carried out to dissect the functional elements of the promoter of these genes. As mentioned above, some of these genes contained in their promoters a putative Cap1p binding site. Therefore, the role of this transcription factor in the regulation of these genes needs to be determined.

Since *CaMDR1* can be also induced by H_2O_2 (17), it was also of interest to compare our results that were obtained using benomyl-exposed cells with another study published by Enjalbert et al. (13) investigating the genome-wide transcriptional response of *C. albicans* to several agents including H_2O_2 (Fig. 8). Interestingly, some of the H_2O_2 upregulated genes in the study of Enjalbert et al. (13) (*IFD1*, *GRP2*, *IFD5*, *IFD4*, *IFD7*, *GRP4*, *IPF12303*, *IFR2*, *TTR1*, *IPF13081*, *PST2*, and *IPF20104*) are those that were found induced also by benomyl in this study. Most of these genes are implicated in response to stress or have oxido-reductive functions (Table 8). Among these, *GRP2*, *GRP4*, *IPF12303*, *TTR1*, *PST2*, and *IPF20104* contained a putative Cap1p binding site in their promoters (Tables 6 to 8). Therefore, a functional linkage involving Cap1p probably exists between benomyl and H_2O_2 exposure. It is possible that this transcription factor could be activated by exposure to these drugs. In *S. cerevisiae*, the functional homologue of Cap1p, Yap1p, is localized in the cytoplasm and it is only after its activation by oxidative stress that this factor migrates to the nucleus and activates the transcription of genes with Yap1p-binding sites in their promoters (19). A similar situation is existing in *C. albicans*, as shown by studies carried out by Zhang et al. (34).

Molecular mechanisms responsible for azole resistance have been investigated in details in *C. albicans*. While the analysis of azole resistance has demonstrated that the major azole resistance mechanisms are involving drug efflux and target alterations, other yet unknown mechanisms are still possibly existing. Transcript profile analysis in clinical isolates may help to identify these alternative pathways. In this work, clinical isolates with resistance mechanisms involving alteration of gene expression have been investigated. As expected, the upregulation of efflux transporter genes could be verified by this type of analysis. Even though the overlap of transcript profiling between drug-exposed cells and azole-resistant isolates help us to determine clusters of genes needed for resistance development, the analysis of additional isolates with still-unknown resistance mechanisms will be an interesting option for the discovery of new resistance mechanisms.

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