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Responses of Pathogenic and Nonpathogenic Yeast Species to Steroids Reveal the Functioning and Evolution of Multidrug Resistance Transcriptional Networks▽†

Dibyendu Banerjee,[1,2] Gaelle Lelandais,[3,4] Sudhanshu Shukla,[1] Gauranga Mukhopadhyay,[2] Claude Jacq,[3] Frederic Devaux,[3]*‡ and Rajendra Prasad[1]*‡

School of Life Sciences, JNU, New Delhi, India[1]; Special Centre for Molecular Medicine, JNU, New Delhi, India[2]; and Laboratoire de Genetique Moleculaire, Ecole Normale Superieure/CNRS UMR 8541,[3] and Equipe Bioinformatique Génomique et Moléculaire, INSERM U726/Université Paris 7,[4] Paris 75005, France

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Steroids are known to induce pleiotropic drug resistance states in hemiascomycetes, with tremendous potential consequences for human fungal infections. Our analysis of gene expression in *Saccharomyces cerevisiae* and *Candida albicans* cells subjected to three different concentrations of progesterone revealed that their pleiotropic drug resistance (PDR) networks were strikingly sensitive to steroids. In *S. cerevisiae*, 20 of the Pdr1p/Pdr3p target genes, including *PDR3* itself, were rapidly induced by progesterone, which mimics the effects of *PDR1* gain-of-function alleles. This unique property allowed us to decipher the respective roles of Pdr1p and Pdr3p in PDR induction and to define functional modules among their target genes. Although the expression profiles of the major PDR transporters encoding genes Sc*PDR5* and Ca*CDR1* were similar, the *S. cerevisiae* global PDR response to progesterone was only partly conserved in *C. albicans*. In particular, the role of Tac1p, the main *C. albicans* PDR regulator, in the progesterone response was apparently restricted to five genes. These results suggest that the *C. albicans* and *S. cerevisiae* PDR networks, although sharing a conserved core regarding the regulation of membrane properties, have different structures and properties. Additionally, our data indicate that other as yet undiscovered regulators may second Tac1p in the *C. albicans* drug response.

Candida albicans is a commensal organism that colonizes a large proportion of the population on the mucosal surfaces of the gastrointestinal and urogenital tracts without clinical symptoms. But in immunocompromised patients, *C. albicans* causes a wide spectrum of diseases ranging from mucocutaneous infections like oral thrush to disseminated candidiasis, with a mortality rate of ≈40% despite the use of available antifungal agents (19). The various sites of *Candida* infection in the human body present different challenges, and the conditions within the microenvironments that change the relationship of *C. albicans* with the host from commensal to pathogenic need to be studied in more detail. Recent efforts have revealed that yeast cells can respond to human steroids (15, 27). Some investigators have determined the presence in yeast of steroid binding proteins, such as estradiol binding protein (32), corticosteroid binding protein (33), and progesterone binding protein (12), but their exact role in the steroid response is not known. Early and more recent studies have shown that steroids can induce a pleiotropic drug resistance (PDR) state in both *C. albicans* and *Saccharomyces cerevisiae*. This PDR state was mediated by the overexpression of genes encoding PDR determinants, such as the ABC transporter-encoding genes Ca*CDR1*, Ca*CDR2*, and Sc*PDR5*, which are known to be regulated by the CaTac1p, ScPdr1p, and ScPdr3p transcription factors, respectively (11, 34). It is noteworthy that the Ca*TAC1* gene was itself found responsive to estradiol (9) and progesterone (this study). de Micheli et al. (15) investigated the transcriptional basis of the steroid responsiveness of *Candida* genes by analyzing the *CDR1* promoter and identified a nucleotide stretch spanning −462 to −213 (with respect to ATG) which mediates both drug and steroid responses (15). Although belonging to the same family of Gal4-like zinc finger proteins, Tac1p is not orthologous to Pdr1p and Pdr3p and has less than 20% sequence identity with these proteins. As a consequence, the DNA binding sites of Pdr1p and Pdr3p (named PDRE [5′-TCCGYGGR-3′]) are significantly different from the Tac1p recognition motif (named DRE [5′-CGG AA/TATCGGATA-3′]) (15). Our own analysis of the *CDR1* promoter led to the identification of two hitherto-unknown *cis*-regulatory regions responding specifically to progesterone and β-estradiol and showed that other steroid-responsive genes in the *C. albicans* genome also contain these sequences, indicating a conserved role in mediating the steroid response in *C. albicans* (5, 26). More recently it has been shown by transcriptome analysis that the yeast responses to high doses of progesterone involved a large number of both *C. albicans* and *S. cerevisiae* genes (5, 6, 9).

In the present study, we aimed at deciphering a more accurate evolutionary significance of the steroid response in yeasts

* Corresponding author. Mailing address for Rajendra Prasad: Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India. Phone: 91-11-26704509. Fax: 91-11-26197338. E-mail: rp47@mail.jnu.ac.in. Mailing address for Frederic Devaux: Laboratoire de Genetique Moleculaire, Ecole Normale Superieure/CNRS UMR 8541, Paris 75005, France. Phone: 33 1 44 32 35 72. Fax: 33 1 44 32 39 41. E-mail: devaux@biologie.ens.fr.

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‡ Both authors contributed equally to this work.

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by submitting *S. cerevisiae* and *C. albicans* cells to several doses of progesterone for different time periods. The diploid *C. albicans* evolved and diverged from the haploid *S. cerevisiae* several million years ago. The question of the commonalities and differences between the responses in the two species was particularly intriguing. Indeed, *C. albicans* is a pathogenic fungus which has to adapt to host defense and the relatively stable yet location-specific human-body environments, whereas *S. cerevisiae*, a free-living yeast, has to deal with the very variable chemical and physical features of its environment. These yeast species are also distinct in basal metabolism, which has important implications for the structure and evolution of their transcriptional networks (24).

We show here that the PDR pathway is the most sensitive to progesterone in both species. Our data indicate that progesterone is very close to the optimal chemical signal that triggers a rapid and stable PDR response in both pathogenic and nonpathogenic yeasts. In the present work, we used this property to decipher the respective roles of the wild-type versions of Pdr1p and Pdr3p in the foundation of PDR in *S. cerevisiae*. We showed that in comparison to that of the Pdr1p/Pdr3p pair, the activity of Tac1p in *C. albicans* is much more focused and probably involves other as yet undiscovered transcription factor(s).

MATERIALS AND METHODS

Strains, media, and growth conditions. All *C. albicans* and *S. cerevisiae* strains used in this study were derived from SC5314 and BY4741, respectively. The *pdr1*Δ and *pdr3*Δ strains were obtained from Euroscarf. The *pdr1*Δ *pdr3*Δ strain is described in reference 20. Cells were grown in yeast extract-peptone-dextrose at 30°C till the mid-log phase was reached (optical density at 600 nm [OD_{600}] of 0.8 to 1.0) and then treated with progesterone (stock solution in ethanol; Sigma-Aldrich) for two time points (30 min and 90 min) at three different concentrations (10^{-9} M, 10^{-6} M, and 10^{-4} M). For studying the long-term effects of steroids, progesterone (10^{-9} M, 10^{-6} M, or 10^{-4} M) was added to the medium at the time of inoculation (inoculum OD_{600}, 0.125) and cells were collected after 7 h of treatment (with the final OD_{600} remaining the same). For mock-treated cells, the growth conditions were the same except that only ethanol was added in the medium. The cells were harvested at room temperature in 50 ml centrifuge tubes at 3,000 rpm for 3 min. Thereafter, the cells were washed once with sterile milliQ water and snap-frozen in liquid nitrogen. The cell pellets were stored at −80°C.

RNA isolation, cDNA preparation, and microarray hybridization. Total RNAs were isolated by breaking the cells with glass beads in a Fast-Prep FP120 cell disrupter (Bio 101) at 4°C, followed by RNA purification on columns according to the manufacturer's protocol (RNAeasy minikit; Qiagen). The *C. albicans* microarrays (batch C050G) were obtained from Eurogentec (Seraing, Belgium) and contained cDNA probes, deposited in duplicates, for 98% of the Candida Open Reading Frames (assembly 19). The *S. cerevisiae* microarrays were homemade from the Operon collection of yeast open reading frame (ORF) oligonucleotide probes (version 1.1.2), deposited in duplicate onto Corning ultragap slides, using an Omnigrid II spotter from Biorobotics (Genomic Solutions). Each experiment was done at least three times, with independent biological replicates and using a dye swap. Fifteen micrograms of total RNA was resuspended in 20 μl of RNase-free H_20 along with 1 μl of RNasin RNase inhibitor (Promega) and then used for labeled cDNA synthesis. The direct-labeling protocol provided by Eurogentec was used for *C. albicans* experiments. The *S. cerevisiae* cDNA labeling-and-hybridization protocol can be found at www.transcriptome.ens.fr/sgdb/protocols/labelling_yeast.php.

cDNA microarray data analysis. Slides were read using a Genepix 4000B scanner from Axon. The images were analyzed using the Genepix pro 6.0 software. Data were normalized using the global "lowess" method followed by the print-tip group median method from Goulphar (29). To get a list of statistically significant genes (see Table S1 in the supplemental material), we combined the two methods commonly in use in most microarray articles: we applied sequentially a cutoff based on the gene expression variation (21) and a cutoff based on the reproducibility of the measurement of this variation (38). More precisely, the expression of gene *i* was considered significantly and reproducibly changed when $|Mi|$ was $>3\,Sg$ and $|Mi/Si|$ was >2, where "*Mi*" is the average $\log_2$ value of change measured for gene *i*, "*Si*" is the standard deviation observed for gene *i*, and "*Sg*" is the standard deviation of the global distribution of the *Mi* values for all genes. The *P* value (*t* test) associated with a gene satisfying both criteria is 0.001. The complete microarray data set can be downloaded at www.biologie.ens.fr/lgmgml/publication/albicans/. To address the biological relevance of the genes presented in Table S1 in the supplemental material, we looked for gene functional categories, which were significantly enriched in these lists. Gene ontology searches were performed by submitting the lists of genes in Table S1 in the supplemental material to the Saccharomyces Genome Database and the Candida Genome Database (CGD) GO (gene ontology) term finder tools (3, 4). The promoter analyses were performed by submitting the whole set of microarray results to the t-profiler tool (8), using default parameters. Homology links between *S. cerevisiae* and *C. albicans* were established using the CGD and the Saccharomyces Genome Database. The *S. cerevisiae* protein sequences of interest were compared to the CGD protein sequences using BLAST (3). A homology link was created with the best hit when the corresponding *P* value was less than 10^{-10}. When several *C. albicans* proteins had similar scores, we created multiple homology links (see Table S3 in the supplemental material) (see Fig. 5).

cDNA microarray data verification by Northern blotting and real-time PCR. Twenty micrograms of total RNA was electrophoretically separated on denaturing agarose gels and blotted onto Hybond N^+ nylon membranes (Amersham Biosciences). Probes used for hybridization were obtained by labeling gene fragments obtained by PCR with [α-^{32}P]dATP (Amersham Biosciences), using specific primers on genomic DNA of strain SC5314 (*C. albicans*) or BY4741 (for *S. cerevisiae*). For real-time PCR, *CDR1* and *CDR2* gene-specific primers were designed and used along with the control *ACT1* gene. Table S3 in the supplemental material lists all the primers used in this study. The probe for *PDR5* was prepared by digesting a part of the *PDR5* gene from the plasmid (pDR3.3) containing the *PDR5* clone.

Microarray data accession numbers. The microarrays used in this work are fully described in the ArrayExpress database (www.ebi.ac.uk/miamexpress/; accession numbers A-MEXP-337 [*S. cerevisiae* arrays] and A-MEXP-878 [*C. albicans* arrays]). Raw and normalized data files have been deposited under accession number E-TABM-345.

RESULTS

Quantitative features of the steroid response in *C. albicans* and *S. cerevisiae*. We analyzed the transcriptomes of wild-type cells of both species treated with three different doses of progesterone (two supraphysiological doses, 10^{-4} and 10^{-6} M, and one physiological dose, 10^{-9} M) for three different time periods (30 min, 90 min, or 7 h). None of these doses affected cell growth (data not shown). The corresponding cDNAs were competitively hybridized on DNA microarrays versus cDNAs obtained from mock-treated cells. We observed that for both species, the number of genes whose expression changed significantly and reproducibly was maximal at the 10^{-4} M dose, with only a few genes being induced by a 10^{-6} M treatment (Fig. 1). Few significant and reproducible gene expression changes could be detected for the physiological dose (see Table S1 in the supplemental material). The steroid response was fast and transient, peaking at 30 min for both species (Fig. 1). Only a few genes remained induced or repressed up to 7 h of treatment. The numbers of genes in *C. albicans* and *S. cerevisiae* that changed expression in the presence of progesterone were similar except after 30 min of the 10^{-4} M treatment, when the *C. albicans* response involved about 10 times more genes than that in *S. cerevisiae* (Fig. 1B). A gene ontology analysis indicated that this was mainly due to the induction of genes involved in the oxidative stress response, sulfur and glucose metabolism, and proteasome activity and the repression of genes involved in ribosome biogenesis and in translation (see Table S1 in the supplemental material). This oxidative stress-like

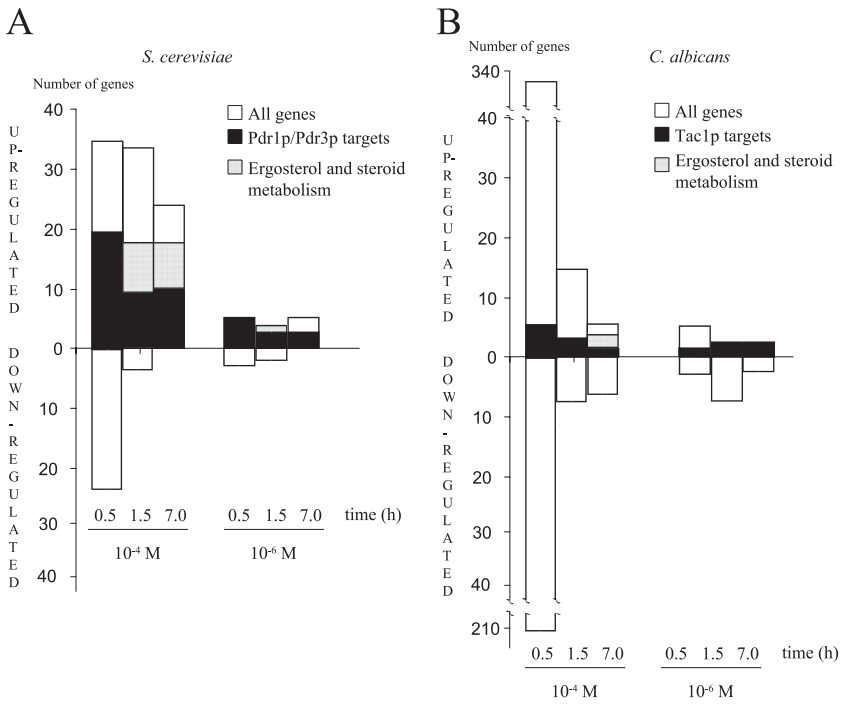

FIG. 1. Genome-wide responses of two yeast species to progesterone. The histograms represent the numbers of genes which were up- and down-regulated in response to progesterone (results from Table S1 in the supplemental material), as a function of the dose of progesterone and time of exposure to the steroid (*x* axis). Panels A and B present results for *S. cerevisiae* and *C. albicans*, respectively. The proportions of *S. cerevisiae* genes referenced as targets of Pdr1p/Pdr3p and of *C. albicans* genes referenced as targets of Tac1p are shown in black. The proportions of genes involved in steroid and ergosterol metabolic pathways are represented as dots.

response in *C. albicans* was fast and transient and was not observed at longer times or lower doses. To get a biologically relevant view of the pathways which are preferentially sensitive to progesterone, we looked for gene groups which were significantly enriched in the lists of significant gene expression variations (see Table S1 in the supplemental material). Note that due to the high number of replicate measurements (six per gene on average) and their high reproducibility, we were able to point out as statistically significant gene expression variations as low as 0.3 ($\log_2$ value). Also, it is important to mention that all the genes discussed in the rest of this article satisfied three criteria: their expression variations were significantly different from the bulk of yeast genes (*Mi* cutoff), this *Mi* value was reproducible over the six measurements which were performed (*S* value criteria), and they belong to a gene category which is significantly enriched in the lists of genes induced by progesterone (see Methods). Moreover, the biological relevance of many of the genes discussed below was supported by Northern blot (see Fig. S2 in the supplemental material) and/or further microarray analyses investigating the transcriptional mechanisms underlying the progesterone response (see Fig. 3 and Table 2). Finally, several of these genes showed changed expression at several time points and doses. For all these reasons, we have a very high level of confidence in the biological and statistical relevance of the results discussed below, even when *Mi* values were unusually low.

Progesterone activates PDR1/3 and ergosterol pathways in *S. cerevisiae*. In *S. cerevisiae*, two pathways are mainly responsible for pleiotropic drug resistance phenotypes: the PDR pathway, controlled by the Pdr1p and Pdr3p transcription factors, and the ergosterol biosynthesis pathway (2). Gene ontology and DNA regulatory motif mining of our microarray data indicated that progesterone specifically and extensively activated these two pathways (see Table S1 in the supplemental material) (Table 1). Four types of PDRE bound by Pdr1 and Pdr3 have been described (16), named A (TCCGCGGA), B (TCCGTGGA), C (TCCGCGCA), and D (TCCGCGGG). PDRE A, B, and D were found to be significantly correlated with progesterone induction at all time points of the supraphysiological-dose treatments except at 7 h with a 10^{-6} M concentration (Table 1). Half of the genes induced at 30 min by 10^{-4} M progesterone are referenced as targets of Pdr1p and Pdr3p (Fig. 1) (see Table S1 in the supplemental material). These genes represent about 80% of the Pdr1p/Pdr3p targets defined previously (1, 16, 17, 18, 20, 28), including genes encoding Pdr3p itself, the ABC transporters Pdr5p, Pdr15p, Pdr10p, Yor1p, and Snq2p, the membrane phospholipid metabolism and transport proteins Rsb1p, Pdr16p, and Rta1p, the oxidoreductase Gre2p, etc.

At 90 min of the treatment with a 10^{-4} M concentration, the PDR response was followed by the induction of genes involved in ergosterol biosynthesis, which represent about 20% of the genes induced at this time point (Fig. 1 and 2B) (see Table S1 in the supplemental material). As a consequence, ergosterol metabolism was one of the main gene ontology categories to be significantly enriched in the list of genes induced at 90 min and 7 h of the 10^{-4} M concentration treatment (see Table S1 in the supplemental material). These genes (*ERG25*, *ERG11*, *ERG5*,

TABLE 1. Known motifs overrepresented in genes up-regulated by progesterone and identified by using t-profiler with default parameters (8)

Organism	Time point, dose, and motif	Name of motif (types)	*t* value	No. of ORFs
C. albicans	30 min, 10^{-6} M			
	TWCCCM	MIG1	3.9	629
	90 min, 10^{-4} M			
	TCTCC	ADR1	3.71	1,600
	CCCCAC	Energy	3.58	370
	7 h, 10^{-4} M			
	TATAWAW	TBP	3.9	2,786
S. cerevisiae	30 min, 10^{-4} M			
	TCCGYGGA	PDRE (A, B)	13.03	52
	TCCGYGGR	PDRE (A, B, D)	11.45	68
	TCCGCGG	PDRE (A, D)	7.82	60
	TGACTCA	GCN4	4.17	94
	GGTGGCRA	RPN4	4.12	84
	30 min, 10^{-6} M			
	TCCGYGGA	PDRE (A, B)	4.93	52
	TCCGYGGR	PDRE (A, B, D)	4.11	68
	90 min, 10^{-4} M			
	TCCGYGGA	PDRE (A, B)	6.83	52
	TCCGYGGR	PDRE (A, B, D)	6.2	68
	TCGTTTA	UPC2	5.72	208
	TCCGCGG	PDRE (A, D)	4.73	60
	CGATGAG	PAC	4.51	198
	90 min, 10^{-6} M			
	CGATGAG	PAC	4.9	198
	TGACTCA	GCN4	4.02	94
	TCCGYGGA	PDRE (A, B)	3.62	52
	7 h, 10^{-4} M			
	TGACTCA	GCN4	8.82	93
	AAAATTT	rRPE	5.39	1097
	TCCGYGGA	PDRE (A or B)	5.06	52
	TCCGYGGR	PDRE (A, B, D)	4.7	68
	CCSGTANCGG	Leu3	3.82	12
	7 h, 10^{-6} M			
	TGACTCA	GCN4	5.83	94

ERG4, *ERG3*, and *ERG28*) encode enzymes from the ERG pathway. Interestingly, the *UPC2* gene, encoding a transcriptional activator of the ERG genes, although not reaching our confidence limit, exhibited an expression profile similar to that of the *ERG* genes (Fig. 2B). Moreover, the Upc2p DNA binding motif was significantly correlated with progesterone induction at 90 min of the 10^{-4} M treatment (Table 1). The PDR and ERG pathways were very stably induced by progesterone, since some of these genes were still induced after 7 h of treatment, although their levels of induction tended to decrease with time (Fig. 2A and B; Table 1).

Transcriptional regulation of the progesterone-induced PDR response in *S. cerevisiae*. Considering the large set of PDR genes which were activated by progesterone, we used microarrays to analyze the effects of deletion of *PDR1* and *PDR3* on the steroid-induced expression of genes belonging to the PDR network (Fig. 3). The progesterone induction experiments were done with the *pdr1*Δ, *pdr3*Δ, or *pdr1*Δ *pdr3*Δ strain at time 30 min and with a dose of progesterone of 10^{-4} M, which corresponded to the maximum PDR response in the wild-type cells (Fig. 2A). We observed three different types of gene expression profiles (Fig. 3A). Some genes (e.g., *GRE2*, *ICT1*, *YOR1*, YPL088w, YLL056c, and *PDR10*) were exclusively Pdr1p dependent (group I), whereas for other genes (*PDR16*, *RSB1*, YGR035c, and YLR346c), the absence of *PDR1* could be partially complemented by Pdr3p (group II). Finally, group III included genes (*PDR5*, *PDR15*, *SNQ2*, *TPO1*, and YAL061w) which are sensitive only to the double deletion and for which the absence of Pdr1p is fully complemented by Pdr3p. Interestingly, we observed that as far as steroid stimulation is concerned, the *PDR3* induction is insensitive to the *PDR1* deletion (Fig. 3A). Even though the number of genes in each group was low, we observed biases in the type and number of PDRE present in each group, which may reflect some functional differences in the binding affinities of Pdr1p and Pdr3p (Fig. 3B). Indeed, the genes depending exclusively on Pdr1p for steroid regulation contained a lower number of PDRE (only one in most cases), which are only of the B type, in their promoters. On the contrary, the promoters at which Pdr3p can fully complement Pdr1p are among the richest in PDRE (2.4 per promoter on average) and almost always contain both the A and B types (Fig. 3B). We verified some of these results by using Northern blotting. The control genes have been chosen so as to include genes from all three groups in Fig. 3. For

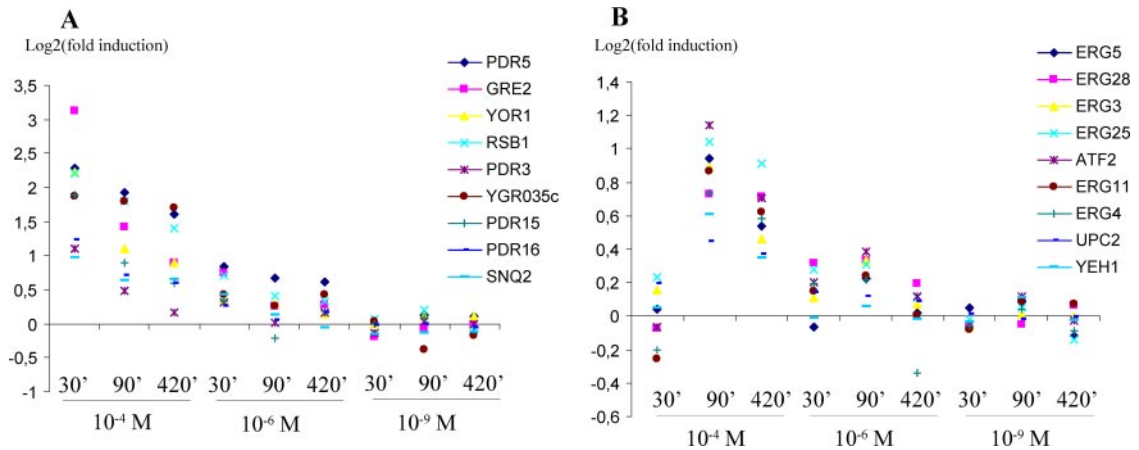

FIG. 2. Gene expression profiles of PDR (A) or ERG (B) *S. cerevisiae* genes in response to progesterone. The $\log_2$ values of *S. cerevisiae* gene induction by progesterone are represented as a function of the dose and time of exposure to the steroid. The genes represented here are a selection of PDR genes (A) or of genes involved in ergosterol biosynthesis (B), responding to progesterone. The gene expression profiles of *ICT1*, *PDR16*, *PDR5*, and YAL061w were confirmed by Northern blot analyses.

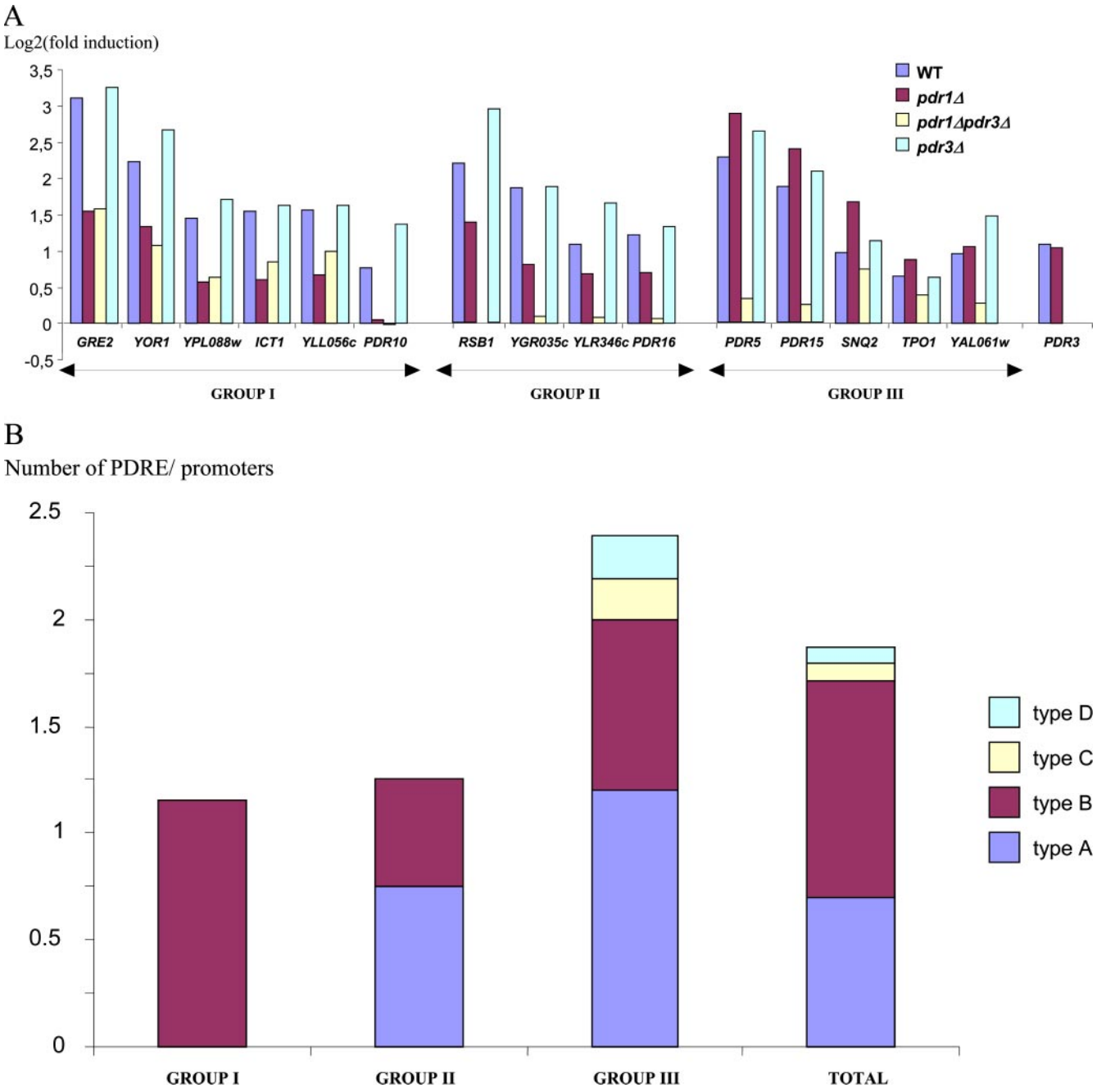

FIG. 3. Analysis of the respective roles of Pdr1 and Pdr3 in the *S. cerevisiae* progesterone-induced PDR response. (A) Progesterone induction of PDR genes in different genetic backgrounds. Microarrays were used to analyze the progesterone induction of genes in strains deleted for *PDR1* and/or *PDR3*. The histograms represent the $\log_2$ values of the expression ratios observed between treated and untreated cells. The time and dose of treatment were 30 min and 0.1 mM, respectively. Three clusters of genes (named groups I to III) were distinguished, depending on their gene expression patterns. (B) Type and number of PDRE in the three groups of Pdr1/Pdr3 target genes. Four types of PDRE have been described (16) and named A (TCCGCGGA), B (TCCGTGGA), C (TCCGCGCA), and D (TCCGCGGG). The histograms represent the number/promoter and types of PDRE in each of the three groups of PDR genes defined from panel A.

example, *ICT1* from group I, *PDR16* from group II, and *PDR5* and YAL061w from group III (see Fig. S2A and B in the supplemental material).

The PDR response to steroids is only partly conserved in *C. albicans*. Except for the 30-min time point with the 10^{-4} M dose (see above), the *C. albicans* response to progesterone was restricted to fewer than 60 different genes involved in arginine metabolism, drug export, lipid and ergosterol metabolism, biofilm formation (up-regulated), and iron and glucose transport (down-regulated) see Table S1 in the supplemental material). As observed for *S. cerevisiae*, PDR transporter-encoding genes (namely, *CDR1* and *CDR2*) were among the most highly and

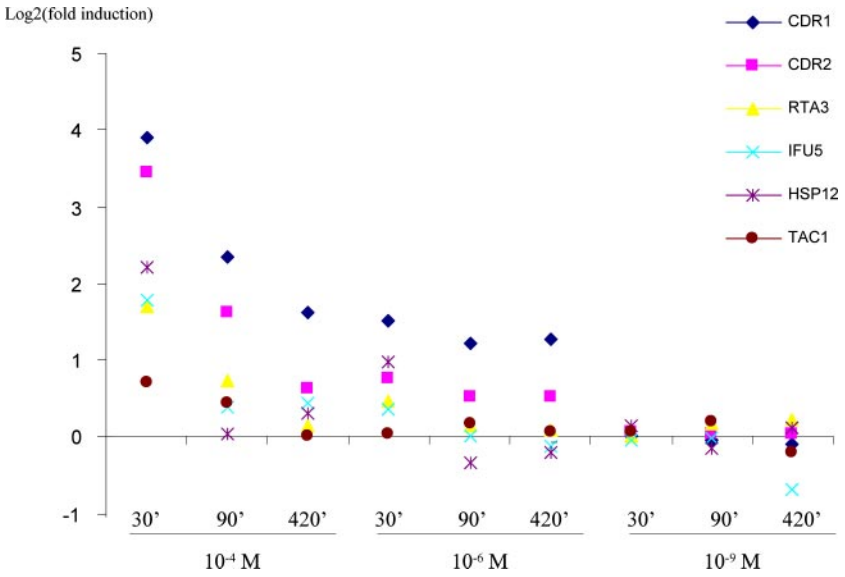

FIG. 4. Expression profiles of *C. albicans* potential targets of the Tac1p transcription factor. The $\log_2$ values of gene induction by progesterone are represented as a function of the dose and time of exposure to progesterone. The genes represented here are the genes which have been defined previously as potential Tac1p targets (11). The genes *RTA3*, *TAC1*, *GDH3*, and *PDR16* were confirmed by Northern blotting, while *CDR1* and *CDR2* were confirmed by quantitative real-time PCR.

stably steroid-induced genes (Fig. 4) (see Fig. S1 in the supplemental material). The Ca*CDR1* gene expression profile was remarkably similar to the Sc*PDR5* expression profile, indicating that the mechanisms involved in progesterone induction of these genes may be conserved from *S. cerevisiae* to *C. albicans*. This is in spite of the fact that the promoters of the two genes harbor different regulatory elements (discussed below). To get a global qualitative and quantitative estimation of the conservation of the PDR and ERG responses to progesterone in both a nonpathogenic and pathogenic yeast species, we made two independent hierarchical clusterings of the PDR and ERG genes induced by progesterone in *S. cerevisiae* and of their closest homologues in *C. albicans*. We then directly compared the gene expression profiles of the homologous gene pairs using Pearson correlation distances (Fig. 5) (see Table S2 in the supplemental material). We observed various situations depending on the gene categories. We found no conservation of the ERG response, identified in *S. cerevisiae* and in *C. albicans*. No conservation was observed for the expression profiles of most of the ERG genes (*UPC2*, *ERG1*, *ERG5*, *ERG10*, *ERG11*, *ERG12*, *ERG25*, and *ERG16*), which were even repressed at 30 min of a 10^{-4} M progesterone treatment (see Table S1 in the supplemental material) (Fig. 5). In contrast, we found full conservation of the induction of the oxidoreductases (Gre2p-like)-encoding and putative flippase (Rsb1p-like)-encoding genes involved in the PDR response, with two homologous genes being similarly induced in both species (Fig. 5). In the case of the ABC transporter family, we found a partial conservation, with only two *Candida* genes (*CDR1* and *CDR2*) being sensitive to progesterone when four of their homologues (*PDR5*, *PDR15*, *PDR10*, and *SNQ2*) were induced in *S. cerevisiae*.

Although about 20 Pdr1/Pdr3 target genes were induced in *S. cerevisiae*, we could find only 9 homologues of these genes being similarly regulated in *C. albicans* (see Table S1 in the supplemental material) (Fig. 5). One simple explanation would be that the PDR pathways in *C. albicans* involve genes which are not clear homologues of the *S. cerevisiae* PDR genes but which play similar functional roles. We found that this is the case for the *C. albicans* PDR transcriptional regulator Tac1p, which is not strictly homologous to *S. cerevisiae* Pdr3p but which belongs to the same family of Gal4p-like transcription factors and exhibited a similar expression profile in response to progesterone (Fig. 2A and 4). Almost all known putative Tac1p targets were induced by progesterone (Fig. 4). This includes *CDR1*, *CDR2* (encoding homologues of Pdr5p), *RTA3* (encoding an orthologue of the putative *S. cerevisiae* flippase Rsb1p) and *IFU5* (Fig. 4). To get a more complete view of the Tac1p role and of the *C. albicans* PDR network involved in the steroid response, we compared the transcriptome of *tac1*Δ cells treated for 30 min with 10^{-4} M progesterone to the transcriptome of either parental cells or the revertant of the *TAC1* knockout (11) with the same treatment. We observed a very limited effect of *TAC1* deletion on the progesterone response. Only *CDR1*, *CDR2*, *RTA3*, *HSP70*, and *MET15* exhibited significantly reduced expression in the mutant strain (Table 2). The involvement of Tac1p in the steroid-induced expression of *CDR1* and *RTA3* was confirmed by Northern blotting experiments (see Fig. S2C in the supplemental material).

DISCUSSION

Progesterone specifically induces a long-term PDR response in pathogenic and nonpathogenic yeasts. Although it has been established for a long time that human steroids affect yeast growth, morphogenesis, and drug resistance, their molecular action and signaling in fungi remain unresolved. Previous microarray experiments showed that an elevated dose (10^{-3} M) of progesterone rapidly (30 min) and transiently induced a large stress response in both *S. cerevisiae* and *C. albicans* (5, 6).

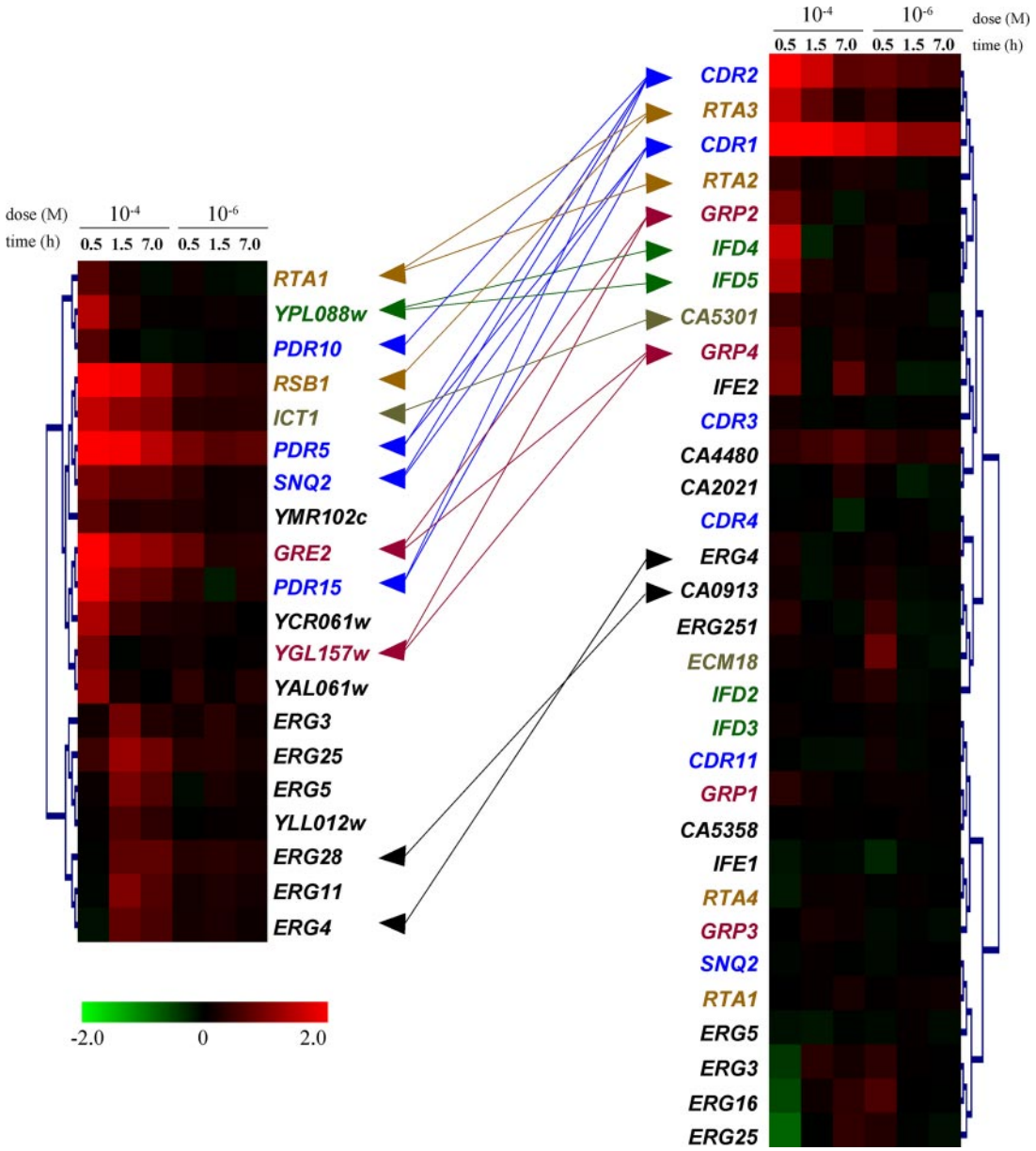

FIG. 5. Comparative functional genomics of the PDR and ERG responses to progesterone. The ERG and PDR genes of *S. cerevisiae* were clustered according to their expression profiles following progesterone treatment. A similar clustering was done for the *C. albicans* orthologues or closest homologues of these genes. The homology links can be found in Table S2 in the supplemental material. Color coding indicates the main PDR gene families in both species: blue, ABC transporters; red, oxidoreductases from the Gre2 family; brown, putative flippases from the RTA family; gray, *ICT1*; green, putative aryl alcohol dehydrogenases; black, others. An arrow connects two homologues when the Pearson correlation distance between their expression profiles is significantly low ($d < 0.4$).

The response included an overexpression of genes involved in the PDR phenomenon. In the present study, we analyzed the *C. albicans* and *S. cerevisiae* responses to lower doses of progesterone (10^{-4} M to 10^{-9} M) for extended time periods (30 min to 7 h) and compared the transcriptional regulation of PDR responses in these two species. We found that the steroid response is more prominent at supraphysiological concentrations and at shorter time points. In other words, the steroid response is transient in nature, the yeast cells probably adapt to the presence of steroids after continuous exposure at low doses, and gene expression probably reaches an equilibrium that is difficult to measure with physiological doses for relatively short time periods. We observed that the PDR pathways and especially the genes encoding the multidrug ABC transporters Pdr5p in *S. cerevisiae* and Cdr1p in *C. albicans* were the most sensitive to and stably induced by progesterone. This was especially impressive with *S. cerevisiae*, in which almost 100% of the genes induced by a 1 μM, 7-h progesterone treatment belong to the PDR pathway. The *S. cerevisiae* PDR network is composed of at least 25 genes regulated by the Pdr1p and Pdr3p transcription factors (34). Our experiments showed that more than 80% of these genes responded to progesterone. For

TABLE 2. Effect of *TAC1* knockout on progesterone response in *C. albicans*

ORF	Name	Ratio of responses to progesterone[a]		
		Progesterone/ mock, WT	WT/Tac1KO, progesterone	Tac1Rev/Tac1KO, progesterone
CA6066	*CDR1*	3.85	1.68	1.98
CA6099	*CDR2*	3.31	2.28	2.26
CA2565	*MET15*	2.45	1.84	0.85
CA1230	*HSP70*	2.17	1.03	0.49
CA3606	*RTA3*	1.53	0.89	0.58

[a] The response of *tac1*Δ (Tac1KO) cells to a 30-min treatment with 0.1 mM progesterone was compared to the responses of the parental (WT) (second column) and Tac1-revertant (Tac1Rev) (third column) strains by using microarrays. The numbers are $\log_2$ values of expression ratios. Gene induction by progesterone in the parental strain is indicated (first column). Only genes which are involved in the progesterone response and which were significantly and reproducibly differentially expressed in the *tac1*Δ strain are represented here.

comparison, only 40% of these genes are up-regulated by fluphenazine, which is known to induce PDR in both *S. cerevisiae* and *C. albicans* (20, 23). This makes progesterone the most efficient inducer of the PDR pathway in *S. cerevisiae* (see Fig. S1 in the supplemental material).

For *C. albicans*, our knowledge of PDR regulation is more limited than for *S. cerevisiae*. A major mechanism of PDR is the up-regulation of multidrug transporters belonging to the ABC (ATP-binding cassette) transporter family (e.g., *CDR1* and *CDR2*), which is the equivalent of the PDR pathway of budding yeast, or of the major facilitator family (e.g., *MDR1*), which is the equivalent of the *YAP1*/*FLR1* pathway in budding yeast (11). Remarkably, progesterone induced almost all the genes known to be coregulated with *CDR1*/*CDR2* in *C. albicans*. This included *IFU5*, *RTA3*, *HSP70*, *HSP90*, and *TAC1*. This striking sensitivity of the PDR network to progesterone in both species suggests that steroids act directly on the physiological parameters which trigger the PDR response. In *S. cerevisiae*, the progesterone treatment strikingly mimicked the gene expression profiles obtained from cells expressing "mini-Pdr1p" or gain-of-function versions of Pdr1p in which the whole Gal4-like inhibitory domain has been deleted or mutated (17). One reasonable hypothesis would be that progesterone indirectly triggers an efficient deregulation of Pdr1p through its inhibitory domains. Progesterone could act on the PDR networks by directly modifying the activity and properties of some PDR ABC transporters. Although it was shown that progesterone is a good substrate of Pdr5p and Cdr1p (27), it is unlikely that progesterone acts just by competing "natural" Pdr5p or Cdr1p substrates for transport, for the following reasons: (i) other good substrates of Pdr5p/Cdr1p, like fluconazole, are poor inducers of the PDR response (27), and (ii) low (1 μM) doses of progesterone are enough to trigger an efficient *PDR5*/*CDR1* induction. Recently estradiol derivatives were shown to efficiently inhibit the drug transport and ATPase activities of Pdr5p and Cdr1p (10). This suggests that human steroids act directly on the activity of the PDR transporters through a mechanism which is yet to be determined but which has important and long-term implications for the activity of the PDR transcriptional networks and thus the PDR status of yeast strains. Noteworthy here is our finding that the steroid response is more prominent at supraphysiological concentrations and at shorter time periods. In other words, the steroid response is transient in nature, the yeast cells probably adapt to the presence of steroids after continuous exposure at low doses, and gene expression probably reaches an equilibrium that is hard to measure at low doses for short time periods.

It should be pointed out that in addition to PDR genes, we found that the ergosterol biosynthetic pathway genes (*ERG* genes) were also induced by progesterone. Of note, ERG genes have often been found to be coregulated along with *CDR1* and *CDR2* in both experimentally induced and azole-resistant clinical isolates of *C. albicans*, and overexpression of *ERG* genes, especially *ERG11*, is one of the predominant mechanisms of multidrug resistance (MDR) in *Candida* (7, 22, 30, 39). Hence, the observed up-regulation of *ERG* genes along with MDR genes in the presence of progesterone is perhaps an indication of the adaptation of the cells to a drug resistance phenotype. *UPC2* is a transcription factor involved in regulation of ergosterol biosynthesis genes and a regulator of sterol uptake across the plasma membrane (37), which was also upregulated by progesterone (present results). Additionally, it is pertinent to mention that the efflux pump proteins, such as Cdr1p, are selectively localized in ergosterol-rich membrane microdomains (36), and an overexpression of Cdr1p may necessitate up-regulation of *ERG* genes as a compensatory sterol homeostasis mechanism (35).

New insights into functioning of yeast PDR transcriptional networks. In *S. cerevisiae*, Pdr1p and Pdr3p share a very similar set of target genes and recognize the same DNA consensus sequence (PDRE) in their promoters (14, 16). The *PDR3* gene itself has been shown to be regulated by Pdr3p and Pdr1p (13) and was sensitive to progesterone in our experiments. In this study, we found that most of the PDR genes were insensitive to progesterone in a *pdr1*Δ *pdr3*Δ strain. Remarkably, Pdr1p was dispensable for the regulation of *PDR3* under these conditions, although it is constitutively bound to its promoter (20), suggesting that the progesterone induction of *PDR3* occurs through autoregulation. We propose that progesterone can be used as a tool to investigate the respective roles of the wild-type versions of Pdr1p and Pdr3p in the PDR response. We confirmed that *PDR3* has a dispensable role in the response to drugs. Based on their *PDR3* dependency in a *pdr1*Δ background, we were able to distinguish between three different groups of PDR targets. We found a correlation between these groups and the nature and number of PDRE present in the promoters of the corresponding genes, suggesting that the DNA binding affinities of Pdr1p and Pdr3p may be different. Interestingly, these groups also present biases in the function of the proteins encoded by the corresponding genes. Pdr1p and Pdr3p have identical activities on the genes encoding most of the main transporters involved in pleiotropic drug export (*PDR5*, *PDR15*, *SNQ2*, and *TPO1*). They have an overlapping effect, with a dominant effect of Pdr1p on genes encoding actors of lipid metabolism and some coregulated proteins of unknown function (*RSB1*, *PDR16*, YGR035c, and YLR346c genes), and Pdr1p specifically regulates genes which, in most cases, are sensitive to other stress response pathways (*GRE2*, YPL088w, YLL056c, *ICT1*, *YOR1*, and *PDR10* genes). The differential regulation of the *PDR1*/*3* genes may reflect differences in their physiological roles and the functional associations of the corresponding proteins.

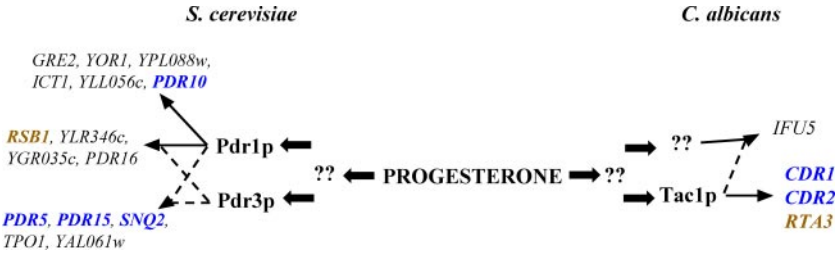

FIG. 6. Comparison of the progesterone responses of the PDR networks in *C. albicans* and *S. cerevisiae*. Regulatory interactions are represented by plain arrows. Dashed arrows symbolize situations in which a transcription factor is known to control the expression of a gene but was dispensable for its progesterone induction in our experiments. The colors indicate the homology links, as in Fig. 5.

In *C. albicans*, Tac1p is the only clear regulator of the PDR response identified to date (11). Tac1p belongs to the same family of Gal4p-like transcription factors as Pdr1p and Pdr3p, and we found that the progesterone induction profile of *TAC1* was very similar to that of *PDR3*, although no evidence exists for autoregulation of *TAC1*. Only six Tac1p target genes (*CDR1*, *CDR2*, *RTA3*, *IFU5*, *PDR16*, and *HSP12*) have been identified, and Tac1p has been shown to recognize the DRE present in their promoters, which is different from the PDRE of *S. cerevisiae* (11, 15). A deletion of *TAC1* is enough to decrease the basal level of expression of *CDR1* and *CDR2*, which is different from the situation for *S. cerevisiae*, in which the double deletion of *PDR1* and *PDR3* is needed to observe the same effect (11, 14). All these observations suggested that progesterone would be a perfect model for exploring the Tac1p-related transcriptional pathway. Surprisingly, only five genes (*CDR1*, *CDR2*, *RTA3*, *MET15*, and *HSP70*) were dependent on Tac1p for their progesterone induction. These results, together with the very limited number of Tac1p targets identified from *TAC1* gain-of-function alleles (11), strongly suggest that the Tac1p effect is much more dedicated and focused than that of the Pdr1p/Pdr3p network. This hypothesis is supported by a recent genome-wide study of Tac1p genomic location and activity, which identified about 37 genomic loci bound by Tac1p, only 8 of which displayed Tac1p dependence for their gene expression (31). It is noteworthy that the induction of the Tac1p target *IFU5* was insensitive to the deletion of *TAC1*. This suggests that another, yet-unknown transcription factor(s) may second Tac1p in the steroid response. Three suppressors of the drug hypersensitivity of *S. cerevisiae pdr1*Δ *pdr3*Δ cells, named *SHY1* to *-3*, have been identified in *C. albicans*, with apparently no role in the regulation of *CDR1* and *CDR2* (11) but which are obvious candidates to be tested for their role in steroid response. Additionally, *ZNC1*, which encodes a Tac1p homologue and is located close to the *TAC1* gene in the *C. albicans* genome, is overexpressed by progesterone and could have a role in drug response, although it has no apparent role in *CDR1*/*CDR2* basal expression (11).

Conserved and divergent features of PDR network in yeasts. The high sensitivity of the PDR genes to progesterone in *S. cerevisiae* and *C. albicans* led us to examine in detail the conservation of the transcriptional networks which drive the drug response in these two hemiascomycetes species, which diverged several million years ago and have very distinct ecological niches. In several cases (Ca*RTA3*/Sc*RSB1*, Ca*CDR1*/Sc*PDR5*, and Ca*CDR2*/Sc*PDR15*), we observed a remarkable similarity of expression profiles between the two yeast species. The most impressive case was that of Ca*CDR1*/Sc*PDR5*, which exhibited almost identical patterns. Of note, the *cis* elements known to regulate the drug response of *CDR1* (DRE) and *PDR5* (PDRE) are quite different (11, 15, 16). It is also worth mentioning here that within the steroid-responsive region found by Karnani et al. (26) in the *CDR1* promoter, which responds to both progesterone and β-estradiol, three consensus 5-bp stretches of nucleotides (AAGAA, CCGAA, and ATTGG) exist, which has also been found in the *PDR5 promoter* (5, 26). This may account for the commonality in expression patterns of *CDR1* and *PDR5*.

On closer inspection, several differences also emerged between the responses of the two yeasts to progesterone. For instance, the expression profiles of four ABC transporter genes, *PDR5*, *SNQ2*, *PDR10*, and *PDR15*, in *S. cerevisiae* correlate with the pattern of only two homologous genes, *CDR1* and *CDR2*, in *C. albicans*, while *CDR3* and *CDR4* are apparently insensitive to progesterone stimulation. In other cases, the *S. cerevisiae* PDR genes just do not have clear homologues in *C. albicans*. This is the case, for instance, for YGR035c and YLR346c. Similarly, *WWM1*, the *S. cerevisiae* homologue of Ca*IFU5* (one of the Tac1p targets responding to steroids), is insensitive to progesterone and has no role in PDR.

Comparative genomics has been used in the past to identify meaningful, conserved DNA or protein motifs from sequence comparisons, and the interspecies comparison of transcriptome data can be very informative in identifying conserved transcriptional modules of genes likely to have common essential functions (25). In the case of yeast PDR, our comparison of the Pdr1p/Pdr3p and Tac1p transcriptional networks suggests that although the DNA binding motifs and the protein sequences of these regulators have significantly diverged, there must have been a strong selection to conserve the pattern of regulation of at least two ABC transporters (Ca*CDR1*/Sc*PDR5* and Ca*CDR2*/Sc*PDR15*) and one putative flippase (Ca*RTA3*/Sc*RSB1*) (Fig. 6). This conserved module, which functions in pleiotropic drug export and is also related to membrane phospholipid translocation, may thus constitute a central, original core of MDR in pathological and nonpathological hemiascomycetes species.

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