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STB5 Is a Negative Regulator of Azole Resistance in *Candida glabrata*

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The opportunistic yeast pathogen *Candida glabrata* is recognized for its ability to acquire resistance during prolonged treatment with azole antifungals (J. E. Bennett, K. Izumikawa, and K. A. Marr. Antimicrob. Agents Chemother. 48:1773–1777, 2004). Resistance to azoles is largely mediated by the transcription factor *PDR1*, resulting in the upregulation of ATP-binding cassette (ABC) transporter proteins and drug efflux. Studies in the related yeast *Saccharomyces cerevisiae* have shown that Pdr1p forms a heterodimer with another transcription factor, Stb5p. In *C. glabrata*, the open reading frame (ORF) designated CAGL0I02552g has 38.8% amino acid identity with *STB5* (YHR178w) and shares an N-terminal Zn_2Cys_6 binuclear cluster domain and a fungus-specific transcriptional factor domain, prompting us to test for homologous function and a possible role in azole resistance. Complementation of a Δyhr178w (Δ*stb5*) mutant with CAGL0I02552g resolved the increased sensitivity to cold, hydrogen peroxide, and caffeine of the mutant, for which reason we designated CAGl0I02552g *CgSTB5*. Overexpression of *CgSTB5* in *C. glabrata* repressed azole resistance, whereas deletion of *CgSTB5* caused a modest increase in resistance. Expression analysis found that *CgSTB5* shares many transcriptional targets with *CgPDR1* but, unlike the latter, is a negative regulator of pleiotropic drug resistance, including the ABC transporter genes *CDR1*, *PDH1*, and *YOR1*.

The haploid yeast *Candida glabrata* is closely related to *Saccharomyces cerevisiae*. *C. glabrata* is the second-most-common yeast species known to cause fungemia (2, 11). Antifungals used to treat *C. glabrata* infections include amphotericin B, echinocandins, and azoles. Although azoles, particularly fluconazole, are often used to treat candidemia, *C. glabrata* is intrinsically more resistant than most other *Candida* species and develops further resistance during prolonged azole therapy.

Drug efflux, resulting from the increased expression of ATP-binding cassette (ABC) transporter proteins, is the predominant mechanism by which *C. glabrata* mediates resistance to a wide range of azoles and other antifungal compounds. Several ABC transporters, including Cdr1p, Pdh1p, Yor1p, and Snq2p, contribute to xenobiotic drug efflux. The transcription factor CgPdr1p is the principal regulator of ABC transporter gene expression and has been found to be a key component of Pleiotropic Drug Resistance (PDR) (1, 3–5).

In the related yeast *Saccharomyces cerevisiae*, Pdr1p has been shown to form a heterodimer with another transcription factor, *S. cerevisiae* Sin3 Binding Protein 5 (ScStb5p) (6). Studies in *S. cerevisiae* found that ScStb5p is a Zn_2Cys_6 transcription factor (7, 8) which regulates genes involved in the oxidative stress response by increasing the supply of NADPH through the pentose phosphate pathway (9). Deletion of *ScSTB5* resulted in a growth defect and sensitivity to cold (20°C), caffeine, hydrogen peroxide, diamide, benomyl, calcofluor, methyl methane sulfonate, acetaldehyde, and cycloheximide (7, 9, 10). In addition, a Δ*Scstb5* mutant has been reported to require uracil and methionine for growth (12). Although Akache and Turcotte reported that susceptibility to ketoconazole was not affected in a Δ*Scstb5* mutant (13), we postulated a role of *STB5* in azole resistance in *C. glabrata* because they and their colleagues also reported that Pdr1p and Stb5p dimerize and directly bind the promoter of *PDR5* in *S. cerevisiae* (6).

Here, we report that the open reading frame (ORF) CAGL0I02552g is a homologue of *ScSTB5* (*YHR178w*). We observed the effect that *CgSTB5* has on *C. glabrata* azole susceptibility by gene deletion and overexpression. Furthermore, using microarray hybridization for a genome-wide survey of transcript levels, we studied the effects of *CgSTB5* deletion and overexpression. We conclude that *CgSTB5* can complement some but not all of the defects in the Δyhr178w strain and is a transcriptional repressor of several genes implicated in azole resistance in *C. glabrata*.

MATERIALS AND METHODS

Strains and culture conditions. *Saccharomyces cerevisiae* strains were obtained from Open Biosystems (Huntville, AL) (Table 1). Plasmids were maintained in *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA) (Table 2). Host cells were grown in LB with 50 μg/ml ampicillin or 50 μg/ml kanamycin, depending on the plasmids.

C. glabrata and *S. cerevisiae* strains were cultured either on yeast extract-peptone-dextrose (YPD) medium containing 1% Bacto yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto peptone (Difco Laboratories), and 2% glucose or on minimum (MIN) medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Franklin Lakes, NJ) and 2% glucose. Cells were shaken overnight at 30°C and washed in distilled water three times, and cell density was determined by the optical density at 600 nm (OD_{600}) and used as described below.

Drug sensitivity assays. MICs of fluconazole and voriconazole (Etest; AB Biodisk, Solna, Sweden) were determined by plating 1×10^6 cells on MIN agar plates and reading the zone of inhibition at the paper strip after incubation at 30°C for 2 days. The CLSI microdilution method M27-A3 was also used for susceptibility testing with the following modifications: MIN broth, 30°C incubation for 48 h, and an 80% growth inhibition (15).

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TABLE 1 List of *Saccharomyces cerevisiae* and *Candida glabrata* strains

Strain	Genotype	Source or reference
S. cerevisiae		
BY4741	MATa *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	ATCC 4040002
Δyhr178w	MATa *his3-Δ1 leu2-Δ0 met15Δ0 ura3-Δ0* STB5::kanMX	ATCC 4002872
Δyhr178wSTB5	Δyhr178w expressing *CgSTB5* in pH392 vector	This study
Δyhr178wP	Δyhr178w with empty vector pH392	This study
C. glabrata		
NCCLS84 (Cg84)	Wild-type strain 84	ATCC 90030
84u	NCCLS84 *ura3* mutant	3
Δcgstb5	84u *cgstb5*Δ::*ScURA3*	This study
CgSTB5OE	Overexpression of *CgSTB5* in 84u with pGRB2.2	This study
84uP	84u transformed with pGRB2.2 vector	This study
Δpdr1	84u pdr1Δ::*ScURA3*	This study
Δpdr1Δstb5	Δpdr1 stb5Δ::*ScURA3*	This study
Δpdr1STB5	Δpdr1 overexpressing *CgSTB5* in pGRB2.2	This study
Δpdr1P	Δpdr1 transformed with pGRB2.2	This study
CgPDR1OE	Overexpression of *PDR1* in 84u with pCgCgPDR1	This study

MIN agar and broth provided better growth than RPMI 1640 of the slow-growing *stb5* deletant and preserved the plasmid in the *STB5*-overexpressing strains.

Caffeine and cold sensitivity assays of *Saccharomyces cerevisiae* strains. As described by Akache et al. (7), cells were shaken at 30°C overnight in YPD media and diluted in fresh YPD media to a concentration of 1×10^5 cells per 5 μl. Four 1:10 serial dilutions were made, and 5 μl of cells were spotted onto YPD agar plates, with or without the presence of 0.15% caffeine (Sigma-Aldrich). Plates were incubated at either 30°C for 2 days or 20°C for 4 days to assess their cold sensitivity.

Oxidative stress sensitivity assays of *Candida glabrata* strains. Cells were grown in YPD media overnight and resuspended at a concentration of 1×10^5 cells per 5 μl. As described by Larochelle et al. (9), a series of four 1:10 serial dilutions were made using deionized water, and 5-μl spots were placed on YPD agar plates or YPD agar plates with 10 mM hydrogen peroxide. Plates were incubated at 30°C for 2 days.

Cloning of *CgSTB5*. The ORF of CAGL0I02252g was obtained by PCR using the genomic DNA of *C. glabrata* strain 84 as a template and primers

TABLE 2 List of plasmids used in current study

Plasmid	Source or reference
STB5-Topo	This study
pH392	Kind gift of Herman Edskes
pH392-CgSTB5	This study
pGRB2.2	Kind gift of Brendan Cormack
pGRB2.2-CgSTB5	This study
pCgACU	Kind gift of K. Kitada (14)
pCgPDR1	This study

TABLE 3 Primers and probes used in this study (grouped by application)

Primer	Sequence (5′–3′)
Plasmid construction primers	
CgSTB5S	GCATGGATCCATGAGTGGCCCAGATAAGGG
CgSTB5AS	GCATGAATTCTTATCTAAAGCGATCCTCCCA
Deletion cassette primers	
CgSTB5DS1	TCATATGTCATCATAGCCTACTTGACTAGG AGTTATAACAGATATATTAATAGAGTAGG ATTTACAAGGATTTCACTATGTCGAAAGC TACATATAAGGA
CgSTB5DAS2	CACAATTATTGACAAGTTTCAGTTTCATAA TCCGTCTGCATCTTCATTGATATCATCCT GGGGCTTACTTCTTATCTTATTAGTTTTG CTGGCCGCATCT
CgSTB5DS3	CAAGCTAGTGATCAGATAAAACTGGGGAGT TTAATCAACAAGAGTTTTTAGCCCTTTCT TAGTCCCAATCCAACTGAATCATATGTCA TCATAGCCTACT
CgSTB5DAS4	TCTTGTGCTATACGGCAGAAACATAAATAG ATTAAGATAAACTATTAGCACTAATCGGA ATAAAGGGAACTGAATAGCACAATTATTG ACAAGTTTCAGT
CgPDR1D1	TGGAATTAGTGTTTTATTCTGCCTTTTTTTT TAGAATATATTGGTAAAGTCATTCTTTAG CTACGTTATTGAGAGAATATGTCGAAAG CTACATATAAGG
CgPDR1D2	GCAACATAACCACTAACAAATGATTTTTCA GGTTAAATATAAAATTATACAGGCTATGC ACACTGTCTAAATTAATAGCATTAGTTT TGCTGGCCGCATC
CgPDR1D3	TTATTACTTAAACAATTTTTAAGTAACACAT TCAAACTTCCATTACTTCGTACCCCATAT CGTATTGCCATTGTGATATGGAATTAGT GTTTTATTCTGC
CgPDR1D4	ATTAAACTACAATTTTTTAATGAGAGATAT TGTAGTGTTATCGCTAATTTGAGGTAGTC TAAGTCTCATGTAAAATTATGCAACATAA CCACTAACAAAT
qRT-PCR primers	
CgACT1F	TTGGACTCTGGTGACGGTGTTA
CgACT1R	AAAATAGCGTGTGGCAAAGAGAA
CgCDR1F	AGATGTGTTGGTTCTGTCTCAAAGAC
CgCDR1R	CCGGAATACATTGACAAACCAAG
CgPDH1F	AATGGATGTTAGAAGTAGTTGGAGCAG
CgPDH1R	TGTTCGGAATTTCTCCACACCT
CgYOR1F	CGCTGGGAAGGCCAAGA
CgYOR1R	CTCCCCGGACGTCAGAATAG
CgPDR1F	AACGATTATTCAATTGCAACAACG
CgPDR1R	CCTCACAATAAGGAAAGTCTGCG
qRT- PCR probes	
CgACT1	CCACGTTGTTCCAATTTACGCCGG
CgCDR1	TTATCTGCTGCGATGGTTCCTGCTTCC
CgPDH1	CAGGCTCACATGCAAACCAAGACTACCAT
CgYOR1	CTCGCCGGTGCAGGATTACGATCTAGA
CgPDR1	TCGAATATTATGCACCATCATGTCTGTGTT TAGCT

TABLE 4 *C. glabrata* genes upregulated ≥2-fold in response to both *STB5* deletion (cgstb5Δ) in 84 host and *PDR1* overexpression (PDR1OE)[a]

Group	*C. glabrata* designation	*S. cerevisiae* homologue	Description	Fold expression upregulation		
				Δcgstb5	CgPDR1OE	Δpdr1 Δcgstb5
Small-molecule transport	CAGL0B04455g	AVT4	Export of large neutral amino acids from vacuoles	2.21	8.99	3.06
	CAGL0H06017g	FLR1	Multidrug transporter of the major facilitator superfamily	2.28	4.3	5.85
	CAGL0B04433g	FUR4	Uracil and cation symporter activity	2.51	2.74	1.37
	CAGL0M01760g	PDR5	ABC transporter involved in azole/multidrug resistance	8.75	13.07	1.89
	CAGL0B02475g	PHO84	High-affinity inorganic phosphate transporter and low-affinity manganese transporter	4.48	8.34	3.23
	CAGL0K00715g	RTA1	Involved in 7-aminocholesterol resistance	2.75	3.93	0.89
	CAGL0L13354g	THI7	Putative nicotinamide transporter	4.2	5.5	40.35
	CAGL0C03289g	YBT1	ABC transporter involved in bile acid transport	2.98	5.11	1.67
	CAGL0G00242g	YOR1	ABC transporter involved in oligomycin/multidrug efflux	7.04	2.74	0.58
Transcription	CAGL0K04257g	RME1	Zinc finger protein involved in control of meiosis	4.32	8.38	4.20
	CAGL0L05786g	YPR013c	Uncharacterized; potential zinc finger	4.39	3.35	2.61
	CAGL0M01870g	YPR013c	Uncharacterized; potential zinc finger	3.74	2.23	0.77
DNA damage response	CAGL0M02035g	REV3	DNA repair and translesion synthesis	3.85	3.38	1.63
	CAGL0M09713g	YIM1	Implicated in DNA damage response	8.24	9.89	1.81
Biosynthesis	CAGL0H09064g	FUR1	Putative uracil phosphoribosyltransferase	2.57	4.78	1.68
	CAGL0M12881g	URA1	Dihydroorotate dehydrogenase	2.52	3.04	2.43
	CAGL0L05676g	URA2	Bifunctional carbamoylphosphate synthetase	4.08	3.21	1.97
Cell wall	CAGL0I10362g	FLO5	Cell wall adhesin	17.12	15.35	1.68
	CAGL0B00616g	SPS22	Implicated in organization of the beta-glucan layer of the spore wall	3.5	10.72	1.21
Cell organization	CAGL0E01265g	NUM1	Protein required for nuclear migration	3.3	2.74	1.52
Mitochondria	CAGL0M12232g	AIM1	Involved in mitochondrial function or organization	4.15	2.76	1.13
	CAGL0I09394g	FMP21	Uncharacterized	2.78	3.76	1.70
	CAGL0D01496g	ISA2	Protein required for maturation of mitochondrial and cytosolic Fe/S proteins	2.16	4.24	1.03
	CAGL0M12947g	YIL077c (PUP1)	Mitochondrial protein of unknown function	16.17	22.93	1.89
	CAGL0J04004g	YOR228c	Uncharacterized protein of the mitochondrial outer membrane	4.48	3.1	0.94
Other metabolism	CAGL0K05775g	ACN9	Carbon utilization and regulation of gluconeogenesis	2.61	2.52	1.23
	CAGL0M07271g	GRX5	Disulfide oxidoreductase activity and response to oxidative stress	3.41	2.17	0.85
	CAGL0E01705g	MDH2	L-malate dehydrogenase	2.16	3.37	1.20
	CAGL0J00847g	SDH1	Flavoprotein subunit of succinate dehydrogenase	5.29	4.1	1.64
	CAGL0E03850g	SDH2	Iron-sulfur subunit of sucinate dehydrogenase	4.63	2.52	1.21
	CAGL0F05863g	SDH4	Membrane anchor subunit of succinate dehydrogenase	2.89	2.48	1.25
	CAGL0M14091g	No similarity	Putative quinone reductase/NADPH dehydrogenase	5.45	13.68	1.39
Uncharacterized	CAGL0L05434g	NCA3	Uncharacterized	2.46	13.28	2.14
	CAGL0M12210g	YAL044W-A	Uncharacterized	3.37	2.33	1.11

[a] Δpdr1Δstb5 was added for comparison.

CgSTB5S and CgSTB5AS, containing flanking BamHI and EcoRI restriction sites (Table 3). For sequencing, PCR-amplified *CgSTB5* was ligated into the pCR-BluntII-Topo vector (Invitrogen). The amino acid sequence of our strain was identical to that in GenBank (accession no. CR380955.2), encoding a 1,005-amino-acid protein with an N-terminal Zn_2Cys_6 motif (C-X_2-C-X_6-C-X_6-C-X_2-C-X_6-C) at amino acids 32 to 59, a conserved fungus-specific transcription factor domain (PF04082) at amino acids 408 to 657, and a polyglutamine sequence (Q3-I-Q4-I-Q5) at amino acids 242 to 259.

Construction of the Δ*cgstb5* and Δ*pdr1* mutants. Gene deletions were performed as previously described (4). All primer sequences are presented in Table 3. Primer pair CgSTB5DS1 and CgSTB5DAS2 and primer pair CgPDR1D1 and CgPDR1D2 were designed to create deletion cassettes for CAGL0I02552g and *CgPDR1*, respectively. An additional round of PCR using primer pair CgSTB5DS3 and CgSTB5DAS4 and primer pair CgPDR1D3 and CgPDR1D4 extended the 5′ and 3′ ends of the deletion cassettes. The *S. cerevisiae URA3* gene in the deletion cassettes was used to replace the CAGL0I02552g and *CgPDR1* ORFs. Transformants were selected on MIN media, and deletion of the target genes was confirmed by Southern blot analysis (data not shown). To create the Δpdr1 Δstb5 mutant, we plated the Δpdr1 strain on 0.1% 5-fluoroorotic acid (Lancaster, Pelham, NH) in MIN medium and selected for uracil auxotrophy for recycling of the URA3 selection marker, followed by *STB5* gene deletion as described above.

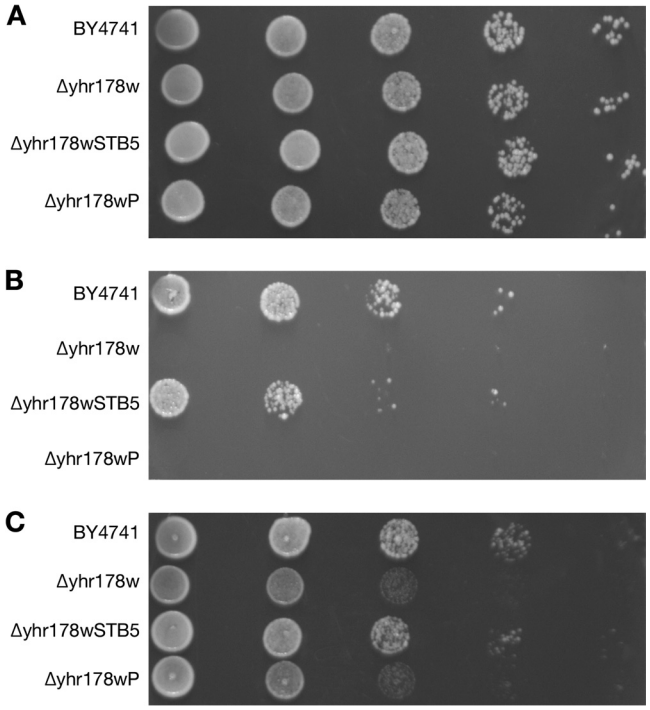

FIG 1 Complementation with *CgSTB5* abrogates the sensitivities to caffeine and cold in the Δyhr178w mutant. (A) Growth on YPD agar plates at 30°C. (B) Growth in the presence of 0.15% caffeine. (C) Growth on YPD agar plates at 20°C. For strains, see Table 1. Cells were diluted to 1×10^5 cells per 5 μl, followed by four 1:10 dilutions, and 5 μl of diluted cell culture was spotted on YPD or YPD with 0.15% caffeine agar plates and incubated at 30°C or 20°C for 48 h.

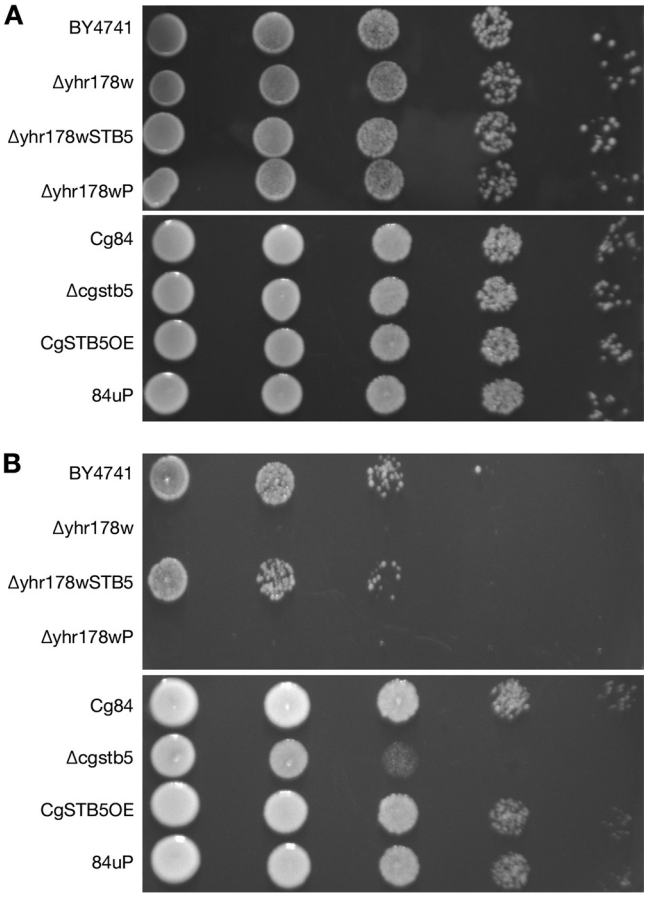

FIG 2 *S. cerevisiae* and *C. glabrata stb5* mutants are sensitive to hydrogen peroxide. Overexpression of *CgSTB5* in 84u had no effect. (A) Growth on YPD agar plates. (B) Growth on YPD agar plates with hydrogen peroxide. Strains were diluted to 1×10^5 cells per 5 μl, followed by four 1:10 dilutions, and 5 μl of diluted cell culture was spotted on medium. *S. cerevisiae* strains were plated on YPD with 5 mM hydrogen peroxide, and *C. glabrata* strains were plated on YPD with 10 mM hydrogen peroxide. All plates were incubated at 30°C for 48 h.

Plasmid construction. *CgSTB5* in the STB5-Topo plasmid was digested with BamHI and EcoRI (both New England BioLabs) and ligated (Rapid DNA Ligation kit; Roche Diagnostics, Germany) into pGRB2.2 (pGRB2.2-STB5) for overexpression under the control of a *PGK1* promoter and transformed into the *C. glabrata* 84u strain (CgSTB5OE).

For expression of *CgSTB5* in *S. cerevisiae*, the STB5-Topo plasmid was digested with EcoRI, and the *CgSTB5* fragment was gel purified. The purified fragment was blunt ended with T4 DNA polymerase, digested with BamHI, and ligated into the pH392 plasmid digested with BamHI and PvuII (New England BioLabs) to create pH392-CgSTB5. This plasmid was used to transform the Δyhr178w strain using uracil prototrophy as a selection marker. The pCgPDR1 plasmid containing the CgPDR1 from strain 38a in pCgACU with the *S. cerevisiae ADH1* promoter was used to overexpress *CgPDR1* in *C. glabrata* (3). The pCgPDR1 was transformed into 84u in this study to create CgPDR1OE for the microarray study.

Growth curve. Yeast cells were grown overnight in MIN media and diluted in MIN media to a final OD_{600} of 0.3. Cells were then placed in a 30°C shaker, and cell concentrations were measured by OD_{600} over a 6-h period.

qRT-PCR. The same sample of total RNA used for cDNA reverse transcription, fluorescent labeling, and microarray hybridization was also DNase treated to remove the minute contamination of genomic DNA. Then, the DNase-treated RNA was reverse transcribed with a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The parallel amplification between *CgACT1* and the gene of interest was confirmed for each probe and primer set. Quantitative real-time PCR (qRT-PCR) was used to determine the expression level of *CgACT1*, *CgPDR1*, *CgCDR1*, *CgPDH1*, *CgSNQ2*, *CgYOR1*, *GND1*, *PGI1*, and *ZWF1* in *C. glabrata*. The sequences of TaqMan probes and forward and reverse primers are listed in Table 3. *CgACT1* was used as an internal control for normalization. The threshold cycle ($2^{-\Delta\Delta CT}$) method was used for calculating the differences in gene expression.

Whole-genome mRNA expression analysis by microarray. Microarray analysis was used to compare the transcription profiles of strains in

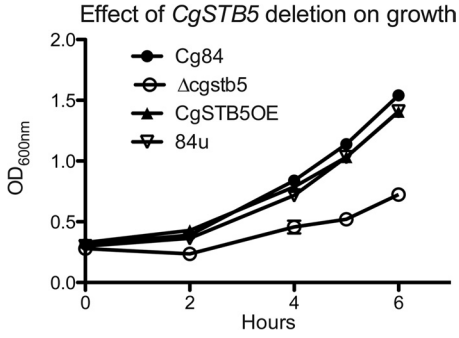

FIG 3 The deletion of *CgSTB5* causes a growth defect. Deletion of *CgSTB5* resulted in poor growth in minimal media. The doubling times for the Cg84, CgSTB5OE, Δcgstb5, and 84uP strains were determined to be 2.3, 2.5, 3.3, and 2.2 h, respectively.

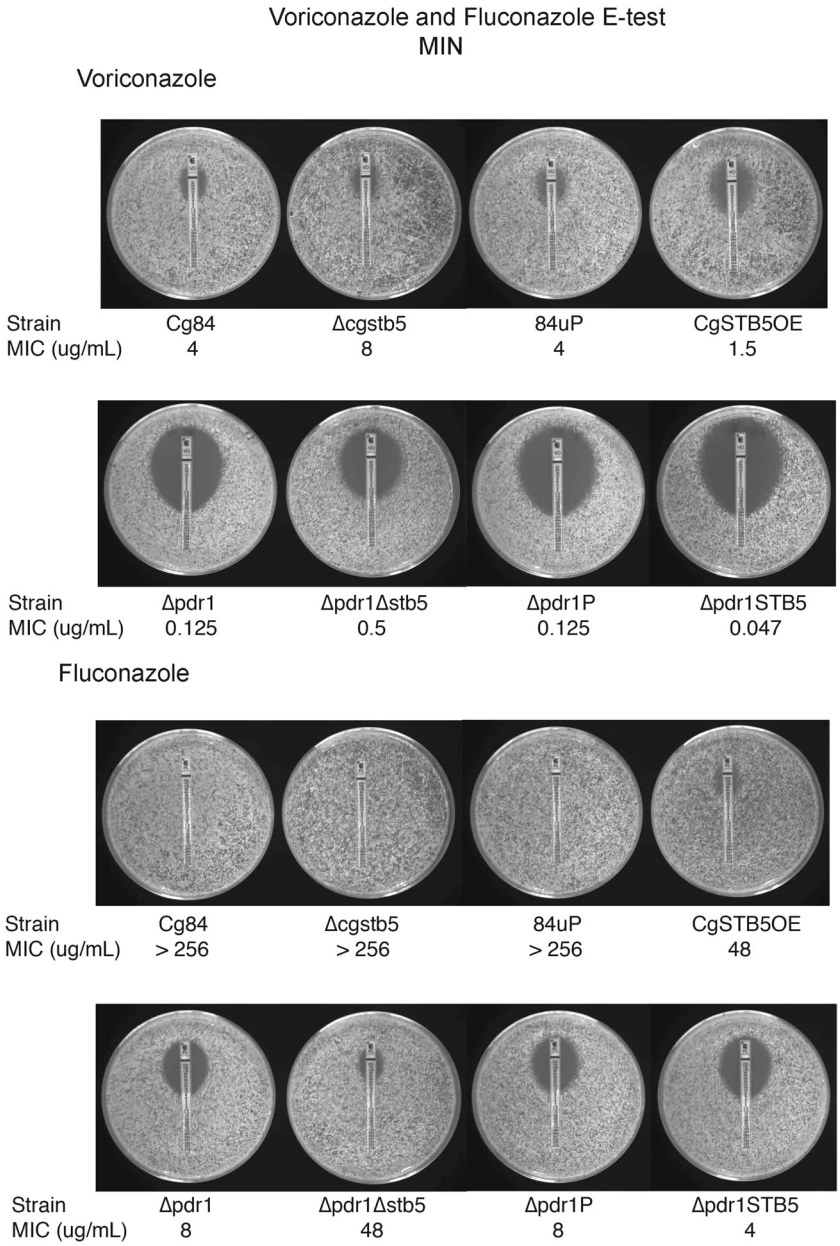

FIG 4 Decreased azole susceptibility of the Δcgstb5 mutant. The deletion of *STB5* resulted in a decrease in susceptibility, and overexpression caused an increase in susceptibility to voriconazole and fluconazole. This finding was also reproducible in a *pdr1* mutant.

which *CgSTB5* had been deleted or overexpressed in a plasmid with a *PGK1* promoter. For comparison, the strain CgPDR1OE, in which *CgPDR1* was overexpressed, was also studied. Total RNA was isolated from log-phase cultures of *C. glabrata* grown in MIN media by using TRIzol (Invitrogen, Carlsbad, CA) and an RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). Ten micrograms of total RNA from strain Cg84 and either the Δcgstb5 or the CgSTB5OE mutant was reverse transcribed to cDNA to incorporate the fluorescent Cy3-dUTP and Cy5-dUTP (GE Health Care, Piscataway, NJ), respectively. The expression arrays used for analysis of *CgPDR1* overexpression were as previously reported (16). This array used 70-mer oligonucleotides spotted on glass arrays by the NIAID Microarray Research Facility (Gene Expression Omnibus [GEO] accession no. GPL8174; see http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL8174). Microarray images were analyzed using GenPix software. For analysis of the *CgSTB5* effect, SurePrint custom arrays (Agilent Technologies, Santa Clara, CA) (GEO accession no. GPL10325; see http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL10325) were used. Agilent Feature Extraction software was used for image analysis. Both sets of arrays used cDNA prepared from Cg84 for the reference channel in two-color hybridization experiments. Analysis of the in-house arrays used 5 microarrays, and analysis of the Agilent arrays used 4 microarrays prepared from 2 to 3 separated RNA preparations, with 1 prepared using reciprocal labeling. Our microarray analysis utilized the mAdb (microArray Database) system provided by the National Institute of Allergy and Infectious Diseases and the Center for Information Technology at the National Institutes of Health, Bethesda, MD (http://madb.niaid.nih.gov/). We applied the Significance Analysis of Mi-

croarrays (SAM) method with a delta of 1.0 and a false-discovery-rate cutoff of 0.1285 for the Δcgstb5 mutant versus the Cg84 strain and a delta of 0.7 and a false-discovery-rate cutoff of 0.0320 for the CgPDR1OE versus Cg84 arrays. After SAM, data were further filtered by including only differences detected in three or more arrays per group with an expression ratio greater than 2.0 or less than 0.5. After the data were filtered, a total of 68 genes remained in the Δcgstb5-versus-Cg84 comparison, and a total of 273 genes remained in the CgPDR1OE-versus-Cg84 comparison. The 34 genes differentially expressed in both microarrays are presented in Table 4, together with microarray results for those same genes in a Δpdr1 Δcgstb5 mutant.

Motif sequence analysis. The 5′ sequences of all 34 open reading frames presented in Table 4 were searched for regulatory protein binding motifs using the MEME Suite Motif-based sequence analysis tool (T. L. Bailey and C. Elkan, presented at the Second International Conference on Intelligent Systems for Molecular Biology, Menlo Park, CA, 1994). We selected the 1,500 bp immediately upstream of the transcription start site for each gene and searched for motifs with widths between 6 and 10 bp that were distributed any number of times per sequence.

***CgSTB5* complementation.** The *Saccharomyces cerevisiae stb5* Δyhr178w mutant strain was transformed with pH392-CgSTB5 using a Yeast Easy Transformation kit (Zymogen) and the transformant, *S. cerevisiae* Δyhr178wSTB5, assessed for susceptibility to fluconazole, voriconazole, hydrogen peroxide, caffeine, and cold temperature. Similarly, 84u was transformed with pGRB2.2-CgSTB5 to create strain CgSTB5OE. Putative transformants were screened by PCR using the primer set CgSTB5S and CgSTB5AS with the following parameters: 95°C for 2 min; 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min; and 72°C for 10 min.

RESULTS

Complementation of the Δyhr178w strain with *CgSTB5*. The susceptibilities of *S. cerevisiae* cells to cold and caffeine were determined by spotting *S. cerevisiae* cells on YPD agar. Overexpression of CAGL0I02552g in the Δyhr178w strain restored its wild-type sensitivity to caffeine (Fig. 1B), cold (Fig. 1C), and hydrogen peroxide (Fig. 2B) but not cycloheximide (data not shown). Both the parent and mutant were methionine and uracil auxotrophs, negating the value of testing these auxotrophies. Other phenotypes of the Δyhr178w strain were not assessed for complementation by *CgSTB5*.

Phenotype of the *Candida glabrata STB5* null mutant. Deletion of CAGL0I02552g (*CgSTB5*) resulted in a mutant (*C. glabrata* Δcgstb5) that grew poorly on MIN agar plates with 2 mM hydrogen peroxide (Fig. 2B) and grew more slowly in MIN media at 30°C than the wild-type strain (Fig. 3). Overexpression of *CgSTB5* in 84u had no effect on hydrogen peroxide susceptibility (Fig. 2B). Unlike the phenotypes reported for the Δyhr178w mutant, the Δcgstb5 mutant did not exhibit altered susceptibility to cycloheximide or caffeine and grew at 20°C. The ΔCgstb5 mutant consistently showed slightly decreased susceptibility to voriconazole by Etest (Fig. 4), though this could not be confirmed by the tube dilution MIC, which was 8 μg/ml in the host and the deletant. This difference also could not be detected with fluconazole (Fig. 4), but the high MIC seen with the host strain limited the value of this observation. Deletion of *CgSTB5* decreased susceptibility to both azoles in the more susceptible Δ*pdr1* background (Fig. 4). Overexpression of *CgSTB5* in the 84u and Δpdr1 backgrounds increased their azole susceptibilities. Microdilution susceptibility testing confirmed the effect of overexpression, though the MICs determined were different from those determined in the Etest. Microdilution testing found a 2-fold increase in fluconazole susceptibility in the *CgSTB5*-overexpressing strains. In the 84 strain, overexpression decreased the fluconazole MIC from 128 to 64 μg/ml. In the Δ*pdr1* background, *CgSTB5* overexpression decreased the fluconazole MIC from 16 to 8 μg/ml.

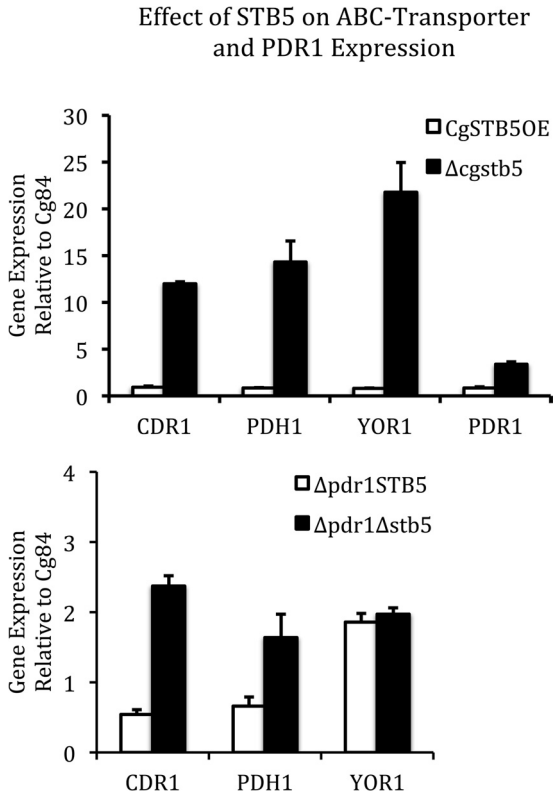

FIG 5 Real-time PCR verification of microarray analysis. The deletion of *CgSTB5* resulted in the upregulation of *CgCDR1*, *CgPDH1*, and *CgYOR1*, all members of the ABC transporter family of proteins, as well as *CgPDR1*, the transcriptional regulator of ABC transporters (upper panel). The same effect was seen in the Δpdr1 background but at a lower magnitude (lower panel). Overexpression of *CgSTB5* did not significantly decrease transcription of these transporters despite increasing azole susceptibility. The standard error of the mean is shown for each gene.

***CgSTB5* functions as a transcriptional repressor of ATP-binding cassette (ABC) transporter genes.** Whole-genome DNA microarray analysis was performed to determine the impact that the *CgSTB5* deletion has on mRNA expression levels. Complete microarray results are available at http://www.ncbi.nlm.nih.gov/geo/ (acquisition no. GSE37071). In the Δcgstb5 strain, 68 genes were upregulated at least 2-fold relative to Cg84. Of the 68 genes upregulated by deletion of *CgSTB5*, 34 were found to be upregulated in the *PDR1*-overexpressing strain, indicating the presence of an overlapping regulon but with opposite transcriptional effects. These 34 genes are listed in Table 4 and grouped by annotated function. For comparison, expression analysis of *PDR1* overexpressed in 84u is also shown. Of the genes upregulated in the Δcgstb5 mutant, nine genes, including the multidrug transporters *PDR5* (*CgCDR1*), *FLR1*, and *YOR1*, have roles in small-molecule transport. Additionally, the 7-aminocholesterol-resistance gene *RTA1* and the bile acid ABC transporter *YBT1* were both upregulated by either *PDR1* overexpression or *STB5* deletion. Other proteins and genes listed in Table 4 that have been previously implicated in azole resistance include the transcription

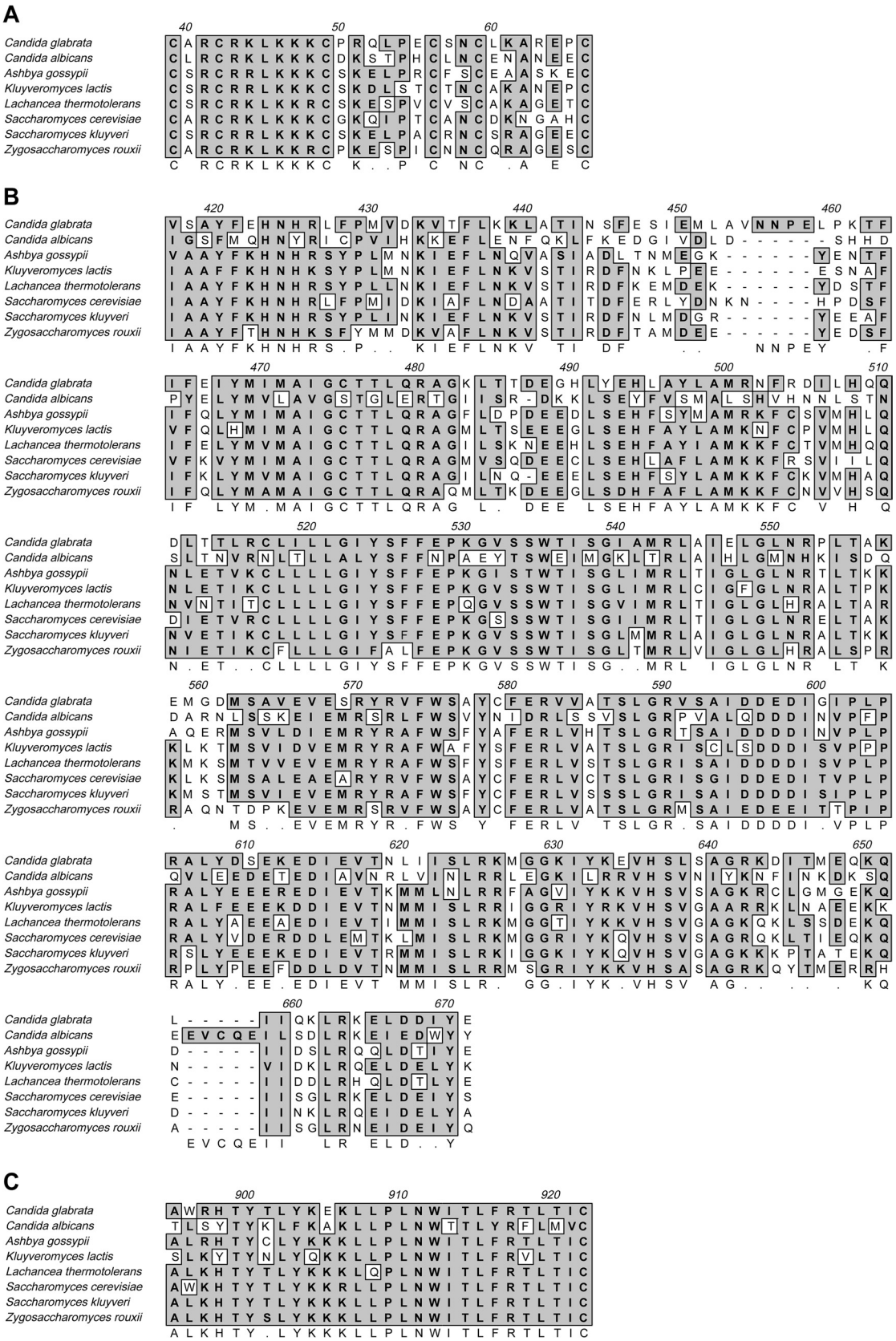

FIG 6 Alignment of encoded amino acids in *CgSTB5* homologues in 7 other yeasts. Numbers shown in the alignment are not identical to their order in CgSTB5p, which are as follows: (A) 32 to 59; (B) 408 to 657; (C) 881 to 908.

factor *YIM1*, the mitochondrial membrane protein encoded by YIL077c (*PUP1*), the L-malate dehydrogenase *MDH2*, and the open reading frame CAGL0M14091g, which encodes a putative quinone reductase/NADPH dehydrogenase (16, 17). Expression analysis by microarray found that deletion of *CgSTB5* in a Δ*pdr1* host had much less of an impact on the same genes (Table 4). The microarray experiments, done in the absence of oxidative stress, did not allow us to assess the relationship between *CgSTB5* and genes in the pentose phosphate pathway which respond to oxidative stress, such as *GND1*, *TKL1*, *ZWF1*, *PGI1*, and *TAL1* (9). We did, however, address the impact of *CgSTB5* deletion on the expression of *GND1*, *PGI1*, and *ZWF1* under conditions of oxidative stress using qRT-PCR. We exposed early-log-phase Cg84 and Δcgstb5 cell cultures to 25 mM hydrogen peroxide for 15 and 30 min before isolating RNA. There was no significant difference between the levels of Cg84 and Δcgstb5 expression of *GND1*, *PGI1*, and *ZWF1* at either time point (data not shown). The upregulation of ABC transporters *CDR1*, *YOR1*, and *PDH1*, as well as *PDR1*, in the Δcgstb5 mutant was confirmed by qRT-PCR in strain 84 (upper panel, Fig. 5). As seen in the lower panel of Fig. 5, two transporters were also upregulated in the Δpdr1 host, though at a lower magnitude (Fig. 5). Overexpression of *CgSTB5* did not result in a substantial decrease in transporter expression, though it did cause increased azole susceptibility. Unlike the results seen with *S. cerevisiae* (13), the membrane transporter *SNQ2* was not affected by deletion of *CgSTB5* (data not shown) The *CDR1*, *YOR1*, *PDH1*, and *PDR1* genes, as well as YIL077c, *YIM1*, and CAGL0M14091g, are thought to be targets of *CgPDR1*. Microarray analysis of CgSTB5OE, in which *CgSTB5* was overexpressed 7-fold, was not informative. Only 1 of 26 genes downregulated 2-fold by overexpression of *CgSTB5* was upregulated 2-fold in the deletant. None of the 25 genes that were upregulated 2-fold by overexpression were downregulated in the deletant. This lack of congruity between overexpression and deletion was interpreted to mean that the suppressive effect of *CgSTB5* could be exposed by deletion but could be not increased by overexpression.

DISCUSSION

A *C. glabrata* ORF, CAGL0I02552g, codes for a protein conserved among hemiascomycete binuclear zinc cluster proteins, indicating a common ancestry and likely an important function in these yeasts (Fig. 6). The N-terminal GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domain (Fig. 6A) is highly conserved (18), as are the fungal transcription factor domain at amino acids 417 to 671 (Fig. 6B) and the C-terminal 200 amino acid residues (Fig. 6C). In addition, synteny is preserved between all eight species with *FMO1* adjacent to the 3′ end, except for in *S. cerevisiae*, which has one intervening ORF. An NADPH dehydrogenase is located 5′ to *STB5* in 4 of the 8 species. Insufficient information is available to evaluate phenotypic similarities among the 8 genes annotated as *STB5*. *CgSTB5* is closely related to *STB5* in *S. cerevisiae* in that they share 50.0% amino acid similarity and 38.8% identity. The ability of *CgSTB5* to complement the Δyhr178w (Δstb5) strain in tolerance to cold, caffeine, and hydrogen peroxide indicates sufficient functional similarity to consider the *C. glabrata* gene a homologue of *STB5*. Although it is not shown in Fig. 6, *Candida albicans* has a gene, *FCR1*, with phenotypic but not genotypic resemblance to *CgSTB5*. *FCR1* is a Zn_2Cys_6 transcription factor with only 7.8% amino acid identity to *CgSTB5*, shares no obvious synteny with it, and has no fungus-specific transcriptional factor. Similar to *CgSTB5* and unlike the other genes in Fig. 6, *FCR1* has a short polyglutamine repeat (Q4-L-Q5). *FCR1* is a negative regulator of drug resistance, decreasing resistance to fluconazole, fluphenazine, cycloheximide, and brefeldin (19). *FCR1* decreases the expression of the *CDR1* ABC transporter and decreases the efflux of rhodamine 6G from the yeast cell (20). *FCR1* and *CgSTB5* are negative regulators of the major azole transporter, *CDR1* in *C. albicans* and *CgCDR1* in *C. glabrata*. The function of these genes is somewhat analogous to those of the negative regulator of drug resistance in *S. cerevisiae*, *RDR1* (cycloheximide) (21), and the transporter *HXT11*, the loss of which confers 4-NQO, cycloheximide, and sulfometuron methyl resistance (22).

As stated previously, Pdr1p has been found to form a heterodimer with Stb5p in *S. cerevisiae.* Our transcriptional analysis indicated a shared regulon between the homologues of these two genes, i.e., *CgPDR1* and *CgSTB5*, in *C. glabrata*. Many of the genes upregulated by overexpression of *CgPDR1* were upregulated by deletion of *CgSTB5* (Table 4). Pearson's correlation analysis showed that the *CgPDR1* overexpression and *CgSTB5* deletion microarray data were correlated, with a P value of <0.0001. The microarray result was confirmed by qRT-PCR, showing the effect of *CgSTB5* deletion and overexpression on the major ABC transporter targets of *PDR1*: *CDR1*, *PDH1*, *YOR1*, and *PDR1* (Fig. 5). The transporters were also upregulated in the Δpdr1 host (Fig. 5). A MEME search to find common elements in the genes coregulated by *CgSTB5* and *CgPDR1* 5′ to the initiation ATG found only the pleiotropic drug response element (PDRE) of *CgPDR1* (TCCAC GGA/G) in 7 genes and no sequences similar to those reported for *STB5* in *S. cerevisiae* [CGGN(c/g)TA] (13, 23). Whether the negative regulation by *CgSTB5* is due to altered promoter occupancy of some transcriptional targets of CgPdr1p is unknown but is suggested by the coregulation and probable overlapping of motifs.

We searched the microarrays of eight paired azole-susceptible and -resistant isolates of *C. glabrata* that we have previously published but found none with a 2-fold difference in Cg*STB5* expression (16). It is possible that the decrease in fitness of the *Cgstb5* mutant (Fig. 1) decreases the likelihood that azole pressure would select for this mutation in the oropharynx.

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