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Cap1p plays regulation roles in redox, energy metabolism and substance transport: an investigation on *Candida albicans* under normal culture condition

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1. ABSTRACT

Cap1p, a transcription factor in *Candida albicans*, is believed to be required for tolerance to oxidative stress. However, no information is available concerning its function on basal transcriptional profile. In this study, differentially expressed genes between the *CAP1*-deleted strain and its parental strain under normal culture condition were identified through microarray analysis. Notably, among the 48 down-regulated genes with the deletion of *CAP1*, there were three clusters, functionally related to intracellular redox, energy metabolism and substance transport. *IPF7817*, *IPF11105* and *FDH11*, the three putative Cap1p target genes functionally related to redox, were shown to be activated by oxidative stress in a Cap1p-dependent manner. Furthermore, rhodamine 6G efflux analyses demonstrated that Cap1p contributed to the energy-driven efflux. Taken together, these results reveal that Cap1p plays a significant role in redox status regulation, energy metabolism and substance transport under normal culture condition.

2. INTRODUCTION

Cap1p, encoded by the gene *CAP1*, is a basic region-leucine zipper (bZip) transcription factor in *Candida albicans* and homologous to *Saccharomyces cerevisiae* transcription factor Yap1p (1). It has been demonstrated to be a central regulator in oxidative stress tolerance (2-4) and post-translationally regulated upon oxidant challenge, nuclear localization as the consequence (3). A transcription factor elicits the expression of a variety of genes under various growth conditions to fulfill its essential functions. To date, no information is available concerning its effects on basal transcription profile of *C. albicans* in the absence of environmental stress.

In this study, we attempted to better elucidate the function of Cap1p under the stress-absent condition by comparing the gene expression profile of a *CAP1* deletion strain CJD21 with that of its parental strain CAI4 through microarray analysis. A number of genes were found altered in transcription level with the deletion of *CAP1*, indicating

that Cap1p does play regulation role even under the stress-absent condition. Based on the microarray data, functional studies were further performed to examine the response of some putative Cap1p-responsive genes to oxidative stress as well as to evaluate the difference in efflux ability between the strains with *CAP1* and those without *CAP1*.

3. MATERIALS AND METHODS

3.1. Strains and culture

C. albicans strain CAI4 (*CAP1/CAP1*) and *C. albicans* strain CJD21 (*cap1Δ::hisG/cap1Δ::hisG*, obtained by deleting both copies of the *CAP1* gene in CAI4) were kindly provided by Dr. William A. Fonzi (Department of Microbiology and Immunology, Georgetown University, Washington, U.S.A.) and Dr. Martine Raymond (Institut de recherches cliniques de Montréal, Québec, Canada.) (2).

The strains were cultivated at 30°C under constant shaking (200 rpm) in a liquid complete medium YPD consisting of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose.

3.2. RNA isolation

C. albicans cells collected from YPD cultures in the exponential growth phase (OD_{600}, 0.5) by centrifugation (3,000×g, 5 min, 4°C) were washed with phosphate buffered saline (PBS). Total RNA was isolated by modified one-step method (5). The isolated RNA was resuspended in diethyl pyrocarbonate-treated water. The OD_{260} and OD_{280} were measured, and the integrity of the RNA was visualized by subjecting 2 to 5 μl of the samples to electrophoresis through a 1% agarose-MOPS gel. Poly(A) mRNA was extracted using the Oligotex mRNA kit (Qiagen, Hilden, Germany) and quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA). Four independent experiments were performed to isolate RNA samples for the microarray experiment.

3.3. Gene expression analysis

Microarray preparation, synthetic of fluorescent cDNA probes, hybridization with *C. albicans* 3136 cDNA microarray, and signal analysis were conducted by United Gene Holdings, Ltd. (Shanghai, P. R. China) as described previously (6-8). Four independent experiments were performed to compare the gene expression profile of a *CAP1* deletion strain CJD21 with that of its parental strain CAI4 under the stress-absent experiment condition through microarray analysis. Detailed experiment processes were described as follows:

3.4. Microarray preparation

The *C. albicans* microarray used in our study consisted of full-length and partial cDNA sequences representing the sequences of unknown-in-function, known (including the genes whose functions were inferred based on sequence similarity) and control genes, in which 13 rice genes (AK067859, AK103847, AK102298, NM_193388, AK067901, AK065781, AK121539, AK02163, AK122030, AK102313, AK060202, AK111774, AK067976 in GenBank) were selected as the negative control. In brief, the cDNA inserts were amplified by PCR with universal primers specific for the plasmid vector sequences and then purified by isopropanol precipitation. All PCR products were examined by agarose gel electrophoresis to ensure the quality and the identity of the amplified clones, which were as expected. The amplified PCR products were dissolved in a buffer containing 3 × SSC solution (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate). These solutions were spotted onto sialylated slides (CEL Associates, Houston, Tex.) with a Cartesian PixSys 7500 motion-control robot (Cartesian Technologies, Irvine, Calif.) fitted with ChipMaker Micro-Spotting technology (TeleChem International, Sunnyvale, Calif.). The glass slides spotted with cDNA were then hydrated for 2 h in an atmosphere with 70% humidity, dried for 0.5 h at room temperature, and UV cross-linked (65 mJ/cm^2). They were further processed at room temperature by soaking them in 0.2% SDS for 10 min, distilled water for 10 min, and 0.2% sodium borohydride for 10 min. The slides were dried again, at which time they were ready for use.

3.5. Probe labeling and hybridization

The labeling procedures were conducted as follows: the fluorescent cDNA probes were synthesized from purified mRNA with Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, N.J.) by oligo(dT)-primed polymerization with Superscript II reverse transcriptase (Invitrogen). The reaction buffer mixture contained deoxynucleoside triphosphates (200 μmol of dATP, dCTP, and dGTP per liter, 60 μmol of dTTP per liter, and 60 μmol of Cy3- or Cy5-dUTP per liter), 2 μl of Superscript II reverse transcriptase, and 1× reaction buffer. The reactions were carried out at 42°C for 2 h. Then the RNA was hydrolyzed by the addition of 4 μl of 2.5 mol of NaOH per liter and incubation at 65°C for 10 min, and then the RNA was neutralized with 4 μl of 2.5 mol of HCl per liter.

Dye swap was used to avoid dye-associated effects on cDNA synthesis. That is, four independent hybridization experiments were performed, with RNA from the *CAP1* deletion strain CJD21 labeled with Cy5-dUTP three times and with Cy3-dUTP once. The two color probes were then mixed and diluted to 500 μl with TE (Tris-EDTA), concentrated to 10 μl with a Microcon YM-30 filter (Millipore, Bedford, Mass.), and vacuum dried.

The probes were dissolved in 20 μl of hybridization solution (5 × SSC [0.75 mol of NaCl per liter and 0.075 mol of sodium citrate per liter], 0.4% SDS, 50% formamide). The microarrays were prehybridized with hybridization solution containing 0.5 mg of denatured salmon sperm DNA per milliliter at 42°C for 6 h. The fluorescent probe mixtures were denatured at 95°C for 5 min and were then applied onto the prehybridized chip under a cover glass. The chip was hybridized in a homemade chamber at 42°C for 15 to 17 h. The hybridized chip was then washed at 60°C in solutions of 2 × SSC–0.2% SDS, 0.1 × SSC–0.2% SDS, and 0.1 × SSC for 10 min in each solution and then dried at room temperature.

3.6. Detection and analysis

The chips were scanned with a ScanArray 3000 apparatus (GSI Lumonics, Bellerica, Mass.) at two

wavelengths to detect the emissions from both Cy3 and Cy5. The acquired images were analyzed with ImaGene (version 3.0) software (BioDiscovery, Los Angeles, Calif.). The intensities of each spot at the two wavelengths respectively represent the quantities of Cy3-dUTP and Cy5-dUTP that hybridized to each spot. Quality control and normalization of the data were performed in Microsoft Excel using standardized spreadsheets. The ratios of Cy5 to Cy3 were calculated for each location on each microarray. To minimize artifacts that arise from low expression values, only genes with raw intensity values of >800 counts for both Cy3 and Cy5 were chosen for analysis. Statistical analysis was performed using the available statistical tool (Student's *t* test of replicate samples), false discovery rate (FDR) being less than 5%, and genes with statistical significance ($p < 0.05$) were selected.

DNA sequences were annotated on the basis of the results of BlastN and BlastX searches using the CandidaDB database (http://genolist.pasteur.fr/ CandidaDB/).

3.7. Reintroduction of *CAP1* into *CAP1* deletion strain

The *CAP1* coding region was PCR amplified with Pyrobest DNA polymerase (DR500A, TaKaRa) using primers designed to introduce a BglII site and an XhoI site and CAI4 genome DNA as the template. The primer sequences were 5'-GGAAGATCTATTCGCCTCCTCCCTCCTC-3' (the BglII site is underlined) and 5'-CCGCTCGAGGAGGGAAGGGTCAGTTGAAATAGAT-3' (the XhoI site is underlined). After being purified, the PCR product was digested with BglII and XhoI and then gel purified. The 2.0-kbp *CAP1* coding region was cloned between the BglII and XhoI sites in the *C. albicans* expression vector YPB-ADHpt (kindly provided by Alistair J. P. Brown, Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, U.K.) to generate YPB-ADHpt/CAP1 and then YPB-ADHpt/CAP1 was subjected to sequencing. YPB-ADHpt carries the *C. albicans URA3* marker as well as *C. albicans* and *S. cerevisiae* replication origins (9). The plasmid YPB-ADHpt/CAP1 was transformed into *C. albicans* CJD21 using the previous reported lithium acetate method (10) to generate the strain CJD21[YPB-ADHpt/CAP1], and YPB-ADHpt as a negative control was transformed into *C. albicans* CJD21 to generate the strain CJD21[YPB-ADHpt].

3.8. Protein preparation and Western blot analysis

Total protein extracts were prepared from strains CAI4, CJD21, CJD21[YPB-ADHpt], and CJD21[YPB-ADHpt/CAP1] as follows. *C. albicans* cells collected from YPD cultures in the exponential growth phase (OD_{600}, 0.5) by centrifugation were washed with phosphate buffered saline (PBS). The cells were harvested by centrifugation and resuspended to a final concentration of 1.5 g (wet weight) per ml in the lysis buffer (50 mM Tris [pH 7.5], 1.5 mM EDTA, 1% Triton X 100, 0.4% SDS) in the presence of protease inhibitors (phenylmethylsulfonyl fluoride at 1 mM; leupeptin, pepstatin A, and aprotinin, each at 5 mg/ml). Then 0.5 volume of acid-washed glass beads (425 to 600 mm; Sigma) was added to the cell suspension, and cells were lysed by vigorous vortexing five times in 1-min bursts, interrupted by at least 1 min of cooling on ice. Glass beads and unbroken cells were removed by centrifugation at 1,000×g for 5 min in a microcentrifuge; supernatants were collected and stored at -80°C. Protein concentration was determined by the Bradford method (11), using bovine serum albumin as the standard. Total protein extracts (50 mg) were suspended in Laemmli sample buffer, boiled for 10 min, and separated by electrophoresis on an SDS–12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and blocked. Anti-Cap1p-350 polyclonal antibody (Kindly provided by Dr. Martine Raymond [Institut de recherches cliniques de Montréal, Québec, Canada]) (2) in 5 ml of 5% nonfat milk TBS-T solution (1: 5,000 dilution; 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20) was added to the blot for 2 h at room temperature. Blots were washed in TBS-T, and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Proteins were detected by chemiluminescence detection system under conditions recommended by the manufacturer (Amersham Biosciences).

3.9. Reverse Transcription-PCR (RT-PCR)

With One Step RNA PCR Kit (AMV, DRR024A, TaKaRa) following instructions of the manufacturer, RT-PCR was performed by mixing exactly 1 µg total RNA in a total volume of 50 µl with 5 µl 10×PCR buffer (TaKaRa), 5 mM $MgCl_2$, 1 mM dNTP mixture, forward and reverse primer (50 µM each), 40U RNase Inhibitor, 5U AMV RTase XL, 5U AMV-optimized Taq DNA polymerase (TaKaRa). RT-PCR was performed under the following conditions: one cycle for 30 min at 50°C; one cycle for 2 min at 94°C; 24-30 cycles with 30 sec at 94°C, 30 sec at 50°C, and 1 min at 72°C; and one terminal cycle for 5 min at 72°C. PCR products of equivalent volume were applied to a 1.5% agarose gel and separated by gel electrophoresis in 1×TAE (40 mM Tris, 10 mM EDTA, 0.1% acetic acid [pH 8.5]). Primer sequences used for amplification of specific genes by RT-PCR are shown in Table 1.

3.10. Rhodamine 6G efflux assay

C. albicans cells from YPD cultures in the exponential growth phase (OD_{600}, 0.5) were collected by centrifugation (3,000×g, 5 min, 20°C) and washed three times with phosphate buffered saline (PBS). The cells were subsequently resuspended in PBS (about 5×10^7cells/ml) and incubated 2 h to exhaust the energy. Rhodamine 6G (R6G) was added to the final concentration of 10µM. Cell suspensions were incubated at 30°C with shaking (200 rpm) for 90 min to allow rhodamine accumulation. The cells were washed three times and the final concentration of the cells was kept at 5×10^7cells/ml exactly. Glucose was added to the final concentration of 1 mM in groups with glucose to initiate rhodamine efflux. At specified intervals after the addition of glucose, the cells were removed by centrifugation, and 100-µl volume of the cell supernatants were transferred to the well of 96-well flat-bottom microplates (BMG Microplates, Black 96 well). The rhodamine fluorescence of the samples was measured with FLUOstar/POLARstar Galaxy (BMG labtechnologies, Germany). The excitation wavelength was 515 nm, and the emission wavelength was 555 nm.

Table 1. Names of genes and sequences of primers

Name	Sequence
CAP1-F	TTG CCT CAC CAG CAA ATC TA
CAP1-R	TTG GAT CGG CTT CTG CTT CA
EBP1-F	CAT AAA TAC CAG TAA CAC GAG GTT
EBP1-R	ATG GGC TAG TTT CCA AGG TAT
EBP4-F	ATA AGG AGT CAA AGA AAT CCC ATC A
EBP4-R	GTG CCT CTA ATA AAG GAC TGG ATA AA
ADH4-F	GGC GAT AGT CCG ATT ACG
ADH4-R	CTT TAC AGA GGG CCA GTC
FDH11-F	TAA CAC CAC CAA AGG CAG CAC
FDH11-R	ATG GTG GTT GTG ACT ACA CTT TTG A
IPF7817-F	TTC, TGG, TCA, GCC, CTT, ATT
IPF7817-R	AAC TGC, CAC, CGT, ATT, CA
IPF11105-F	TGG TCC CAT TCA TCA GC
IPF11105-R	CGC TGG TGG TGT TGG T
IPF6600-F	CCA GCC AAG GCA GCA GA
IPF6600-R	TTG CCA CTT TAA GAA TCA CTC A
ATP1-F	AAA CTT AGA AAG AAG CGA CGA A
ATP1-R	TCC CGT GTC GGT TCT GC
MIR1-F	AAA GAG CCA ACG AAG CC
MIR1-R	AGC AAC GGC TGG TGG A
SCJ1-F	CGT AAA GGG GAT TCA GCA
SCJ1-R	CAT AAT GTC TTG GAC CTC GT
SDH12-F	TCT TGG CTG GTG GGA CTG
SDH12-R	TGC ATC TGC TGC TGC TAG
HOL1-F	GAT TTG TGG CTG GGG TAA
HOL1-R	ATG GGT TGG TTG GAT TGC
ARR3-F	AAG TTG GCT ACA TAA GAT ACG GC
ARR3-R	GAT TTA TCA GTC CTT GGG CTT TA
VCX1-F	GTG GAG AAG TAT AAT GAC ATA GGC ACA
VCX1-R	TGG CTT GGT CGT ATT GTT ATT GA
FRE7-F	GCG ACG GCA CTA ACC C
FRE7-R	TAC TGC TTC CGC CTT CA
HEM1-F	TAA CGG TGG TGA TTT CGG
HEM1-R	TGA AGC GTG TTT TAG ACA AGT A
ACT1-F	TTT CCA ACT GGG ACG ATA
ACT1-R	TCT TGG ACA AAT GGT TGG

F: forward, R: reverse

4. RESULTS

4.1. Microarray identification of Cap1p-responsive genes

To reveal the role of Cap1p under the stress-absent condition, we used *C. albicans* cDNA microarrays to identify the difference in gene expression profiles between the *CAP1* deletion strain CJD21 and its parental strain CAI4 strain. Four biological replicates were conducted. To avoid dye-associated effects on cDNA synthesis, RNA from the *CAP1* deletion strain CJD21 labeled with Cy5-dUTP three times and with Cy3-dUTP once. Only the genes with statistical significance (FDR<5%, $p < 0.05$) were selected for further analysis. In this work, 95 genes were found altered in transcription level with the deletion of *CAP1*, with 48 down regulated and 47 up regulated.

Among the 48 down regulated ones, there were clusters of genes functionally related to intracellular redox status, energy metabolism, substance transport, or other biochemical processes (Table 2).

CAP1 deletion resulted in the reduced expression of several genes functionally related to redox, including oxidoreductase genes (e.g. *IPF7817*, *IPF11105* and *IPF6600*) and dehydrogenase genes (e.g. *EBP1*, *EBP4*, *FDH3.3f*, *FDH11*and *ADH4*).

Deletion of *CAP1* also caused the decreased expression of some genes related to energy metabolism and mitochondria. Of the 5 genes, 2 encode F1 subunits of F1F0-ATPase complex (*ATP1* and *ATP2*); the other three encode mitochondrial phosphate transport protein (*MIR1*), mitochondrial and endoplasmic reticulum (ER) import protein (*SCJ1*) and succinate dehydrogenase (*SDH12*).

Moreover, another cluster of genes related to substance transport (e.g. *ATM1*: ATP-binding cassette transporter gene, *HOL1*: multidrug-resistance gene of major facilitator superfamily, *ARR3*: arsenite transporter gene and *ZRT2*: zinc transporter gene) were found to decrease in expression with *CAP1* deletion.

Besides the above three clusters, there were some other down regulated genes, such as those encoding mannosyltransferase (*IPF8746* and *PMT2*), the gene functionally related to ubiquitin-mediated protein degradation (*IPF12963*), and the stearoyl-CoA desaturase gene (*OLE1*).

Among the 47 up regulated ones, there were genes encoding predicted zinc-finger transcription factors (*DAL81* and *IPF15240*), putative phospholipase (*PLB4.5f* and *PLB4.3f*), ribosome-related proteins (*IPF3361* and *RLP7*), putative serine/threonine kinase (*IPF9382.3* and *FUN31*), aquaporin-like water channel proteins (*AQY1*), and proteins of miscellaneous or unknown function (Supplementary Table 1). No gene clusters functionally related to intracellular redox status, energy metabolism and substance transport were found among the up regulated ones.

4.2. Validation of microarray by RT-PCR analysis

To validate our microarray results, the paired mRNA samples were re-prepared and RT-PCR was carried out using the primers described in Table 1. Expression profile of the 16 randomly-selected genes was reproduced well (Figure 1).

4.3. Effects of *CAP1* reintroduction on the gene expression

We constructed a *CAP1*-reintroducted strain CJD21[YPB-ADHpt/CAP1] and its comparable *CAP1* deletion strain CJD21[YPB-ADHpt]. The expression of Cap1p in CJD21[YPB-ADHpt/CAP1] as well as the lack of expression in CJD21[YPB-ADHpt] were verified by Western bolt analysis (Figure 2). Of this pair of strains, RNA was isolated and transcription level of the above 16 genes was examined. RT-PCR results further confirmed the influence of Cap1p on these genes (Figure 1).

4.4. *CAP1*-related genes with putative Cap1p recognition element

Although microarray analysis allowed us to detect *CAP1*-related genes in the given condition, this kind of screening does not distinguish between the direct effects of the transcription factor and an indirect effect via a cascade of biochemical steps. Binding the recognition element sites being necessary for the direct effect of Cap1p, genes with putative Cap1p recognition elements in their

Table 2. Candida albicans down-regulated genes with the deletion of CAP1

Gene name	Entry number[1]	Function	Average ratios (n=4) CJD21 vs CAI4
CAP1	CA0183	Transcriptional activator	0.099
Genes related to redox			
ADH4	CA2333	Probable alcohol dehydrogenase	0.029
EBP1[2]	CA1216	NADPH dehydrogenase	0.071
EBP1[2]	CA1216	NADPH dehydrogenase	0.093
EBP4	CA4030	NADPH dehydrogenase	0.255
FDH3.3f	CA1253	Formate dehydrogenase	0.072
FDH11	CA6000	Glutathione-dependent formaldehyde dehydrogenase	0.305
IPF7817	CA3564	Function putative NADH-dependent flavin oxidoreductase	0.278
IPF11105	CA3578	Probable quinone oxidoreductase	0.279
IPF6600	CA3097	Hypothetical oxidoreductase in RPB5-CDC28 intergenic region	0.493
Genes related to mitochondria and energy metabolism			
SDH12	CA2470	Succinate dehydrogenase	0.331
ATP1	CA4456	F1F0-ATPase complex, F1 alpha subunit	0.350
ATP2	CA4362	F1F0-ATPase complex, F1 beta subunit	0.358
SCJ1	CA4025	Mitochondrial and ER import protein	0.378
MIR1	CA1513	Phosphate transport protein, mitochondrial (MCF)	0.389
Genes related to substance transport			
ATM1	CA0933	ATP-binding cassette transporter (by homology)	0.400
HOL1	CA2820	Member of major facilitator superfamily multidrug-resistance protein subfamily 1 (by homology)	0.420
ARR3	CA4391	Involved in arsenite transport (by homology)	0.409
ZRT2	CA3160	Zinc transport protein (by homology)	0.373
Others			
IPF8746	CA1548	Putative alpha-1,3-mannosyltransferase (by homology)	0.134
IPF12963	CA3886	Ubiquitin-mediated protein degradation	0.170
IPF14968	CA0254	Candida albicans strain ATCC 10261 Tca3 retrotransposon [3]	0.202
OLE1	CA3921	Stearoyl-CoA desaturase (by homology)	0.371
EGD1	CA1565	GAL4 DNA-binding enhancer protein (by homology)	0.382
IPF8245	CA1753	Putative chitinase	0.382
FRE7	CA5621	Ferric reductase transmembrane component	0.383
VCX1	CA1352	Ca^{2+}-transport by homology	0.404
HEM1	CA2266	5-aminolevulinic acid synthase	0.423
IPF2593	CA4301	Amino acid-tRNA ligase homolog (by homology)	0.436
SEC23	CA3453	Component of COPII coat (by homology)	0.462
IPF8576	CA4382	Similar to Saccharomyces cerevisiae Ris1p DNA helicase (by homology)	0.518
PMT2	CA5894	O-D-mannosyltransferase (by homology)	0. 553
IPF14119	CA0689	Unknown function	0.354
IPF14899	CA1986	Unknown function	0.408
IPF3937	CA1203	Unknown function	0.467
IPF6600	CA3097	Unknown function	0.493
IPF17021	CA3662	Unknown function	0.534

[1] Entry numbers refer to those at http://www-sequence.stanford.edu/group/candida/, [2] Different section of *EBP1*,
[3] Identification of this gene was from http://www.ncbi.nlm.nih.gov/blast/

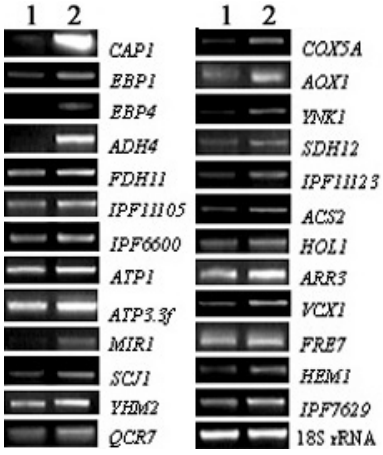

Figure 1. Expression profile of the selected genes in *CAP1* deletion *C. albicans* strains (CJD21 and CJD21[YPB-ADHpt]) and their comparable strains with *CAP1* (CAI4 and CJD21[YPB-ADHpt/CAP1]) by RT-PCR. *ACT1* was amplified as a control.

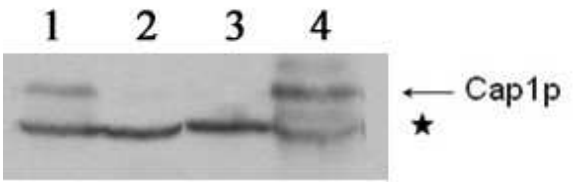

Figure 2. Western blot analysis of Cap1p expression. Total proteins were extracted from CAI4, CJD21, CJD21[YPB-ADHpt], and CJD21[YPB-ADHpt/CAP1] cells. Protein samples (50 mg) were separated by electrophoreses on an SDS–12% polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed with the anti-Cap1p-350 polyclonal antibody. Arrow shows the position of the Cap1p protein. Asterisk points to a nonspecific cross-reacting protein (2).

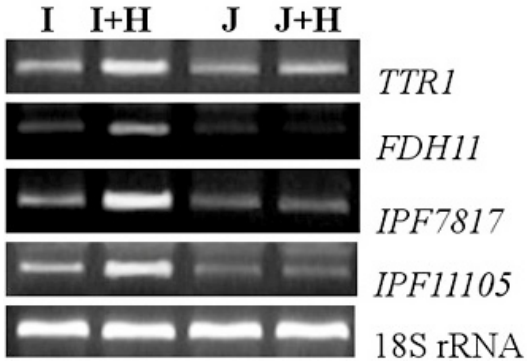

Figure 3. Effects of H_2O_2 treatment on the transcription level of redox-related genes with putative Cap1p recognition elements through RT-PCR analysis. (I) *C. albicans* strain CAI4, (I+H) CAI4 had been exposed to 0.5 mM H_2O_2 for 15 min, (J) *C. albicans CAP1* deletion strain CJD21, (J+H) CJD21 had been exposed to 0.5 mM H_2O_2 for 15 min. *ACT1* was amplified as a control.

promoters are more likely the direct targets of Cap1p. Therefore, sequence analysis was performed on the differential genes to find those containing putative Cap1p recognition elements, TTA(C/G)TAA, TGACTAA and TTAGTCA, in the 5' nucleotide sequences upstream of the ATG transcriptional start codon (12,13) (supplementary Table 2). For the 48 down-regulated genes, twenty were found to contain the putative recognition elements (Table 3), among which, *IPF7817*, *IPF11105* and *FDH11* were redox-related.

4.5. Effects of oxidative stress on the expression of redox-related genes with putative Cap1p recognition elements

Previous studies (2, 3) have strongly argued the effects of Cap1p in oxidative stress. In this work, we were interested in determining whether *IPF7817*, *IPF11105* and *FDH11*, the identified redox-related genes with the putative Cap1p recognition elements, could be transcriptionally activated by H_2O_2 and the influence of *CAP1* deletion in the possible activation process. *C. albicans* strains CAI4 and CJD21 cells were treated with or without 0.5 mM H_2O_2 for 15 min and the transcription levels were evaluated. After treatment, the expressions of *IPF7817*, *IPF11105* and *FDH11* were strikingly increased in CAI4, while no marked increase was observed in CJD21 (Figure 3).

4.6. The role of Cap1p on the energy driven substance efflux in *C. albicans*

Based on the microarray observation that Cap1p was associated with both energy metabolism and substance transport, we further tested whether energy driven substance efflux was mediated by Cap1p in *C. albicans* using rhodamine 6G efflux analyses. Without glucose supply, there was no marked difference of efflux ability either between *CAP1* deletion strain CJD21 and parental strain CAI4 or between *CAP1* deletion strain CJD21[YPB-ADHpt] and *CAP1*-reintroduced strain CJD21[YPB-ADHpt/CAP1]. With the addition of glucose, the function of efflux transporters was enhanced significantly in all strains, while the function of efflux transporters for CJD21 was weaker than that of CAI4 (Figure 4A), and CJD21[YPB-ADHpt] weaker than CJD21[YPB-ADHpt/CAP1] (Figure 4B); the difference was enlarged in both pairs with the lapsing of time (Figure 4).

5. DISCUSSION

In this study, we firstly investigated the roles of bZip transcription factor Cap1p in the basal transcription profile of *C. albicans* through microarray analysis. With the deletion of *CAP1*, 95 genes were identified to be altered in transcription level, indicating a Cap1p-related expression manner under the given growth condition. Interestingly, among the 48 down regulated genes, there are three main clusters functionally related to intracellular redox status, energy metabolism and substance transport. To validate the results of microarrays, RT-PCR analysis was performed on the re-prepared RNA samples and the expression profile of 16 randomly-selected genes was reproduced well. Secondly, we constructed a *CAP1*-reintroducted strain CJD21[YPB-ADHpt/CAP1] and its comparable *CAP1* deletion strain CJD21[YPB-ADHpt]. RT-PCR analysis on this pair of RNA samples further confirmed the influence of Cap1p on these genes. Thirdly, functional studies were performed on *IPF7817*, *IPF11105* and *FDH11*, the three redox-related genes containing putative Cap1p recognition elements in the 5' nucleotide sequences upstream of the ATG transcriptional start codon. They were shown to be activated by oxidative stress in a Cap1p-dependent manner. Finally, rhodamine 6G efflux analysis demonstrated that Cap1p, at least partially, contributed to the energy-driven efflux. These findings add to our understanding of Cap1p function.

Cellular redox homeostasis is required for many aspects of cell function (14, 15). In normal aerobic metabolism process, *C. albicans* cells generate a range of reactive oxygen species (ROS) from the mitochondrial respiratory chain, which can damage many of the cellular components, including DNA, proteins and lipids (16-18). Reasonably, the organisms have therefore evolved a series of regulation mechanisms to maintain their intracellular redox homeostasis (19). In this study, the transcript profiling data

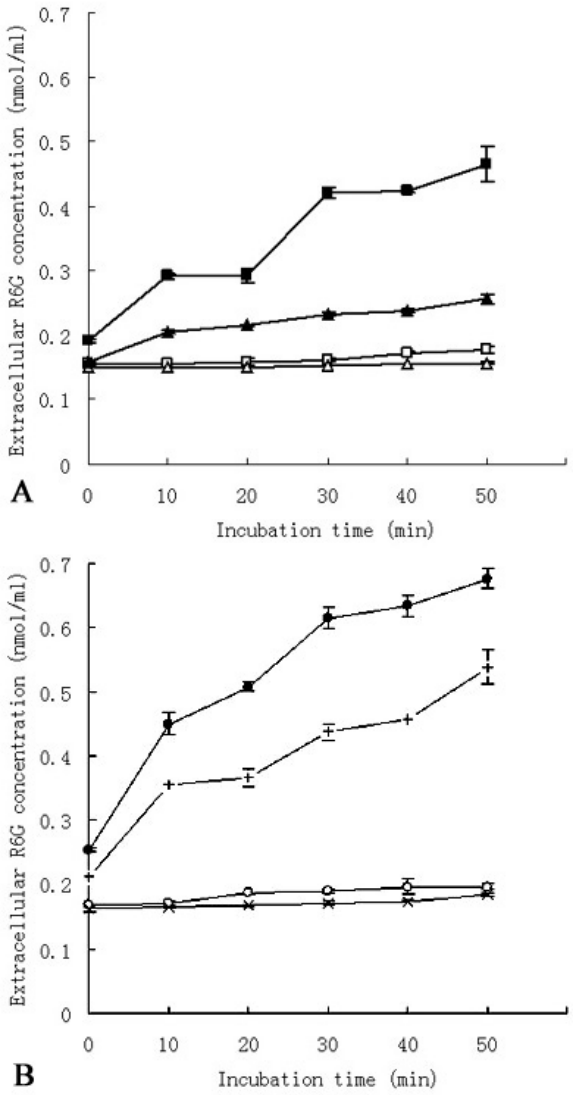

Figure 4. Rhodamine 6G efflux by *C. albicans* strains. Each data point presents the mean±SD of three measurements of the extracellular concentration of rhodamine 6G at the specified time interval. Glucose (1 mM) was added at the starting time point for the groups with glucose supply. (A) (□) CAI4 without glucose, (■) CAI4 with glucose, (Δ) CJD21 without glucose, (▲) CJD21 with glucose. (B) (○) CJD21[YPB-ADHpt/CAP1] without glucose, (●) CJD21[YPB-ADHpt/CAP1] with glucose. (×) CJD21[YPB-ADHpt] without glucose, (＋) CJD21[YPB-ADHpt] with glucose.

appear to support the involvement of Cap1p in intracellular redox even under the condition without environmental oxidative stress. For example, with the deletion of *CAP1*, the NADPH dehydrogenase genes *EBP1* and *EBP4* were decreased. NADPH is a well-known reductive molecule with great importance in both thioredoxin redox cycle and glutathione redox cycle against ROS (20, 21). NADPH dehydrogenase catalyses a series of reactions: NADPH + H^+ + acceptor = $NADP^+$ + reduced acceptor, reducing various substance with the consumption of NADPH. Besides *EBP1* and *EBP4*, redox-related genes such as *IPF7817*, *IPF11105* and *FDH11* were also shown to be Cap1p-related. This cluster of genes indicated that Cap1p participated in redox state regulation under the given stress-absent condition. Moreover, our results further confirmed that Cap1p probably functioned as an important regulator of oxidative stress response in *C. albicans* through the transcriptional control of specific downstream target genes. *IPF7817*, *IPF11105* and *FDH11*, three genes with putative Cap1p recognition elements in the 5' nucleotide sequences upstream of the ATG transcriptional start codon, were strikingly increased in CAI4 after H_2O_2 treatment, while no marked increase was observed in *CAP1*-deleted CJD21. In addition, category and number of Cap1p-realted genes in oxidative stress response identified by our previous microarray analysis (unpublished observation) are more than that under normal culture condition, including not only those with antioxidant cavenging/defense properties (e.g. genes encoding NADPH dehydrogenase, thioredoxin reductase, and glutathione reductase) but also various genes of other kinds (e.g. genes encoding glucose-6-phosphate dehydrogenase, transaldolase, 26S proteasome regulatory subunit, ATP-dependent RNA helicases). Cap1p, just like its homolog Yap1p, has been documented to be post-translationally regulated by oxidants with a nuclear localization mechanism (3). We speculate that, under the condition without environmental oxidative stress, Cap1p could shuttle between the cytoplasmic compartment and the nucleus, eliciting the transcription of some genes and playing roles in biochemical processes such as redox homeostasis, energy metabolism and substance transport; while under the oxidative stress condition, with the change of intracellular biochemical condition, Cap1p accumulated in the nucleus, interacted with other molecules, and regulated the expression of numerous genes to deal with the oxidative challenge. Collectively, our data suggest that Cap1p may regulate specific genes under specific conditions.

A cluster of genes involved in energy metabolism were also found down-regulated with the deletion of *CAP1* under the condition used in this study, which were all functionally related to mitochondrion (e.g. genes encoding F1 alpha or beta subunit of F1F0-ATPase complex, mitochondrial phosphate transport protein, mitochondrial and ER import protein and succinate dehydrogenase). As we all know, mitochondrion is at the core of cellular energy metabolism, being the site of most ATP generation. ATP is synthesized from ADP and inorganic phosphate by ATP synthase, the F1 portion of which functions as a rotary molecular motor in ATP synthesis systems. In this study, a series of genes related to ATP generation or mitochondrial function, from mitochondrial phosphate transport protein to alpha and beta subunits of F1-ATPase, were identified to express in a Cap1p-related pattern, indicating that Cap1p could affect the ATP generation process.

Substance transport is a continual process in living cells, which functions in toxicant eliminating, intracellular substantial homeostasis, electrochemical gradients maintaining etc. In this work, several putative

transporter genes were found down-regulated with the deletion of *CAP1*, including *ATM1* (ATP-binding cassette transporter gene by homology), *HOL1* (member of major facilitator superfamily multidrug-resistance protein subfamily 1 by homology), *ARR3* (arsenite efflux transporter gene by homology) and *ZRT2* (zinc transporter gene by homology). The Cap1p-related expression pattern of these genes suggested that Cap1p could affect some substance transport process in *C. albicans*.

As Cap1p seems to be associated with both energy metabolism and substance transport, we are interested in testing the role of Cap1p on energy driven rhodamine 6G efflux. Our results showed that efflux abilities in all strains were not markedly different without glucose supply, and enhanced greatly with the addition of glucose, indicating the influence of energy status on rhodamine efflux. With glucose supply, the function of efflux transporters for *CAP1*-deleted CJD21 was noticeably weaker than that of CAI4 (Figure 4A), and the reintroduction of *CAP1* gene significantly enhanced the function of efflux transporters (Figure 4B). These findings provide evidence for the role of Cap1p on energy driven efflux.

Cap1p and Yap1p are homologues and their similar characters had been well shown in previous studies, e.g. sharing a common important function in oxidative stress tolerance, directly regulating several common target genes, such as *TRR1*, *GLR1* and *YCF1* (2, 22). Studies in this work further provided evidence for their similar functions. Mannosyltransferase genes *IPF8746* (*MNN1*, alpha-1,3-mannosyltransferase gene with the systematic name of orf19.4279) and *PMT2* (with the systematic name of orf19.6812) were identified to be down regulated with the deletion of *CAP1*, which were consistent with previous findings on Yap1p. Yap1p had been reported to up regulate *MNN1* (alpha-1,3-mannosyltransferase gene), *MNN9* (Golgi mannosyltransferase gene) as well as *OCH1* (alpha-1,6-mannosyltransferase gene) in the absence of environmental stress (23). Therefore, it can be concluded that both Cap1p and Yap1p could regulate the expression of some mannosyltransferase genes and might play roles in the process of protein glycosylation. Nevertheless, difference between Cap1p and Yap1p was also found in this study. For the majority of presented genes in this article, such as *EBP1*, *EBP4*, *IPF7817*, *IPF11105*, *OLE1* and genes of miscellaneous or unknown function, homologues of them have never been reported to be Yap1p-related. In fact, the difference between Cap1p and Yap1p is understandable. The two transcription factors belong to two different microorganisms, Cap1p in pathogenic fungus *C. albicans* and Yap1p in nonpathogenic yeast *S. cerevisiae*. The *candida* lineage appears to have initiated more 150 million years age and *C. albicans* has more opportunity for divergence (24). The different growth environment may also lead to the difference in biochemical processes for these two microorganisms (25).

In summary, the effects of Cap1p on basal transcriptional profile of *C. albicans* revealed that Cap1p is involved in redox status regulation, energy metabolism and substance transport under the growth condition used in this study. It is also shown that energy driven efflux could be mediated by Cap1p.

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