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CaIPF7817 is involved in the regulation of redox homeostasis in *Candida albicans*

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Abstract

CaIPF7817, a functionally unknown gene in *Candida albicans*, was suggested to be involved in the redox system previously, but its exact role is unknown. In this study, *ipf7817* null mutant was generated with the URA-blaster method. After the deletion of *CaIPF7817*, intracellular levels of reactive oxygen species were significantly increased; mitochondrial membrane potential, a direct indicator of mitochondrial function, was elevated; some important redox-related genes, including *GLR1*, *SOD2*, and *TRR1*, were up-regulated; and the GSH/GSSG ratio was raised. These changes indicated that *CaIPF7817* played important roles in the regulation of redox homeostasis in *C. albicans*.

Keywords: *Candida albicans*; *CaIPF7817*; Redox; Gene disruption

Candida albicans, the major human fungal pathogen, causes a range of disorders from mild infections to life-threatening diseases [1,2]. Nowadays, with the widespread use of immunosuppressive therapy, infections by *C. albicans* have become more common and more studies about this pathogen are needed [3].

Like other living cells, *C. albicans* cells generate various oxidative agents, such as reactive oxygen species (ROS), from the mitochondrial respiratory chain in the normal aerobic metabolism process. ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, can damage many of the cellular components [4–6]. Therefore, regulation of the redox homeostasis is important for cellular functions [7,8]. To maintain intracellular redox homeostasis in *C. albicans*, a series of regulation mechanisms are involved [9], which require the participation of numerous genes [10]. However, our knowledge about the redox-related genes in *C. albicans* is limited today.

CaIPF7817, a potential redox-related gene probably encoding a NAD(P)H oxidoreductase, was investigated in this work. This gene was found highly up-regulated during oxidative stress [11,12]. Here, we studied the roles of *CaIPF7817* in cellular redox regulation by constructing *CaIPF7817* deletion mutants through deletion of both copies of the gene using the URA-blaster method and measuring various redox-related properties of these mutants.

Materials and methods

Strains, culture, and growth of C. albicans. *Candida albicans* strains used in this study were listed in Table 1. The strains were cultured at 30 °C in YPD medium (1% Difco yeast extract, 2% Difco peptone, 2% dextrose) or YPD medium supplemented with 0.005% uridine (Sigma, USA). Agar (1.5%) was added to prepare the solid media. The *URA3* transformants were selected on MIN (2% glucose, 0.67% Difco yeast nitrogen base without amino acids) agar plates supplemented with 25 μg/mL histidine. The *ura3* auxotrophs were obtained on MIN agar plates containing 0.1%

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Table 1
Candida albicans strains and plasmids used in this study

	Parent	Genotypes	Reference
Strains			
RM1000	RM100	*ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG*	Negredo et al. (1997)
JH1U	RM1000	*ipf7817Δ::hisG-URA3-hisG/IPF7817 ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG*	This work
JH1	JH1U	*ipf7817Δ::hisG/IPF7817 ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG*	This work
JH2U	JH1	*ipf7817Δ::hisG/ipf7817Δ::hisG-URA3-hisG ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG*	This work
JH2	JH2U	*ipf7817Δ::hisG/ipf7817Δ::hisG ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG*	This work
JHEXP	JH2	*ipf7817Δ::hisG/ipf7817Δ::hisG::IPF7817*	This work
Plasmids			
p5921		Contains hisG-URA3-hisG cassette	Fonzi et al. (1993)
p196		Contains the full-length of *IPF7817* gene	This work
p196U		hisG-URA3-hisG inserted into *IPF7817* gene	This work
pCaEXP		Expression vector	Umeyama et al. [15]
pCaEXP-7817		Expression vector containing *IPF7817* gene	This work

5-fluoroorotic acid (5-FOA; Lancaster, USA) and 50 μg/mL uridine. *Escherichia coli DH5α* strain was grown in LB medium (0.5% Difco yeast extract, 1% Difco Bacto tryptone, 0.5% NaCl) at 37 °C.

The *C. albicans* strains were grown in the YPD medium at 30 °C under constant shaking (200 rpm). OD_{600} measurement at specific time points was used to monitor the growth of *C. albicans* cells.

Disruption and reintroduction of CaIPF7817. The plasmids used in this study were listed in Table 1. The plasmid p196 was derived from the *C. albicans* cDNA library by integrating into the vector pBSK [13]. It contained the whole ORF of *CaIPF7817* gene. To construct the *ipf7817* null mutant of *C. albicans*, *CaIPF7817* was disrupted by the method described by Fonzi and Irwin [14]. The URA-blaster cassette, derived from plasmid p5921 by BglII and BamHI digestion, was inserted into the BglII site of p196 to yield plasmid p196U. The plasmid p196U was propagated in *E. coli DH5α* and linearized by PvuII to yield a 5.34 kb *Δipf7817::hisG::URA3::hisG* fragment, which was used to transform *C. albicans* strain RM1000. To reintroduce *CaIPF7817* into the null strain, the whole *CaIPF7817* gene was PCR amplified with Pyrobest DNA polymerase (TaKaRa) using primer 5′-GGTCGGAT CC*ATG*ACAGTTCCATACCAAGT-3′ and 5′-TCGG CTGCAGCTAATTTACTTCTAAT TTAG-3′. After being purified, the PCR product was cloned into the integrative expression vector pCaEXP [15] to generate the recombinant plasmid pCaEXP-7817. After sequencing, pCaEXP-7817 was linearized and used to transform the null JH2 strain to yield the JHEXP strain. Lithium acetate method was used for the transformation [16,17].

Isolation of C. albicans DNA and Southern hybridization analysis. The small-scale isolation of DNA from *C. albicans* was performed as described previously [16]. Approximately 5 μg genomic DNA was digested with PstI and SphI and then separated on a 1% agarose gel. The separated DNA fragments were transferred onto nylon membranes, and Southern hybridization was performed using DIG labeling and detection kit (Roche). The 408 bp hybridization probe was PCR amplified with Pyrobest polymerase (TaKaRa) using the primers 5′-TCTGGAAGGATGA ACAAGCA-3′ and 5′-CAAAACTGCCACCGTATTCA-3′ from the genomic DNA of RM1000.

Measurement of ROS levels. Intracellular levels of ROS were measured with DCFH-DA (2′,7′-dichlorodihydro-fluorescein diacetate, Molecular Probes, USA) [18]. Briefly, *C. albicans* cells cultured in YPD medium at the exponential growth phase ($OD_{600} = 0.5$) were collected by centrifugation (3000*g*, 5 min, 4 °C) and washed three times with PBS. The cells were subsequently resuspended in PBS ($OD_{600} = 1.0$). DCFH-DA was added to the final concentration of 20 μg/mL. Cell suspensions were incubated at 30 °C with shaking (200 rpm) for 30 min. At specified intervals, 100 μl cell suspensions was transferred to the wells of a flat-bottom microplate (BMG Microplates, 96 well, Black) to measure fluorescence intensity on a FLUOstar fluorometer (BMG labtechnologies, Germany) with excitation at 485 nm and emission at 520 nm. Triplicate experiments were conducted to generate a mean value.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$). Candida albicans cells from YPD cultures ($OD_{600} = 0.5$) were collected by centrifugation (3000*g*, 5 min, 20 °C) and washed three times with PBS. The cells were subsequently resuspended in PBS (3×10^6 cells/mL). After treatment with 10 μg/mL of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide, Molecular Probes, USA) in the dark at 30 °C for 20 min, cells were washed twice with PBS and analyzed on a FLUOstar fluorometer with excitation at 490 nm and emission at 530 and 590 nm, respectively [19]. $\Delta\psi_m$ was determined by the ratio of fluorescence intensity at 590 nm to that at 530 nm.

RNA isolation and real-time RT-PCR. RNA isolation and real-time RT-PCR were performed as described previously [11]. Triplicate experiments were performed with the Chromo4 Real-Time PCR System (Bio-Rad, USA). SYBR Green I (TaKaRa) was used to visualize and monitor the amplified products in real time. Gene-specific primers were designed according to the manufacturer's protocol. Primers for *SOD2* were 5′-CAG CACTATCGGAAGTAAC TC-3′ and 5′-GGCATGTTATCATACTGG AAGG-3′; primers for *GLR1* were 5′-GCTCATCTAAGTCATTGTGA CC-3′ and 5′-GCTGGACCAGAAGAAAAAGTTG-3′; primers for *TRR1* were 5′-ACATTCCAAGGCAGCCATAC-3′ and 5′-AGACCGAC GAAGCTGGTTAC-3′; primers for *18S* rRNA were 5′-TCTTTCTTGA TTTTGTGGGTGG-3′ and 5′-TCGATAGTCCCTCTAAGAAGTG-3′. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the Opticon software (Bio-Rad, USA), and the threshold cycle (C_T) above background for each reaction was calculated. The C_T value of *18S* rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The ΔC_T value of an arbitrary calibrator (i.e. sample of parental strain RM1000) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$.

Colorimetric determination of reduced and oxidized glutathione. Candida albicans cells from YPD cultures ($OD_{600} = 0.5$) were collected by centrifugation (9000*g*, 1 min, 4 °C) and washed once with PBS. The cells were subsequently resuspended in the buffer for lyticase. Lyticase (Sigma, USA)

was added according to the manufacturer's protocol. After incubating at 30 °C for 30 min, the lysate was clarified by centrifugation (9000*g*, 5 min, 4 °C) and the supernatant was used to determine total free glutathione. Total glutathione was determined with the GSH and GSSG Assay Kit (Beyotime, PR China) [20,21]. Colorimetric determination was conducted using a MultiskanMK30 microplate reader (Labsystems, Finland).

Statistical analysis. Student's *t* test was used to determine the statistical significance between experimental groups. Difference was considered significant if the *P* value was less than 0.05.

Results

Generation and characterization of ipf7817 mutants

To investigate the roles of *CaIPF7817* in *C. albicans*, we disrupted the two alleles of *CaIPF7817* sequentially in RM1000 strain using the URA-blaster method and 5-FOA selection, yielding the following strains: the Ura^+ *ipf7817/IPF7817* strain (JH1U), Ura^- *ipf7817/IPF7817* strain (JH1), Ura^+ *ipf7817/ipf7817* strain (JH2U), and Ura^- *ipf7817/ipf7817* strain (JH2) (Table 1). The strategy to disrupt both copies of *CaIPF7817* was depicted in Fig. 1A. Southern blot analysis of DNA from the constructed strains showed that the recombination patterns agreed with our expectation (Fig. 1B).

To further characterize the generated mutants, the growth curves of RM1000, JH1, and JH2 were obtained. They showed identical growth rates. To investigate if other deletions were introduced into the *C. albicans* DNA, *CaIPF7817* was reintroduced into JH2 using the plasmid pCaEXP-7817 to generate the JHEXP strain. PCR analysis confirmed that *CaIPF7817* was integrated into the RP10 locus in JHEXP strain. The growth rates of the RM1000 strain and JHEXP strain were similar, suggesting there was no other gene disruption that affected the growth of *C. albicans*.

Increased intracellular ROS levels in ipf7817 mutants

In order to study the roles of *CaIPF7817* in intracellular redox balance, changes in intracellular levels of ROS after *CaIPF7817* disruption were examined with the fluorescent dye DCFH-DA [22]. The changes of levels of ROS were monitored for 48 h. Striking increases of intracellular ROS level were observed in *ipf7817* null mutants. After 36 h, the levels of ROS in JH2U and JH2 were nearly five times that in the parental strain RM1000. In the heterozygote strain JH1 and JH1U, intracellular ROS levels were also elevated. In the JHEXP strain that *CaIPF7817* was reintroduced, the change of ROS levels was similar with that in the parental strain RM1000 (Fig. 2).

Increased mitochondrial membrane potential in ipf7817 mutants

Mitochondria are the main generator of intracellular ROS. We therefore investigated the effects of *CaIPF7817* on mitochondria function by measuring $\Delta\psi_m$, the direct indicator of mitochondrial function, of *ipf7817* mutant strains. With the deletion of *CaIPF7817* in JH2, $\Delta\psi_m$ was increased significantly. In the heterozygote JH1, slight

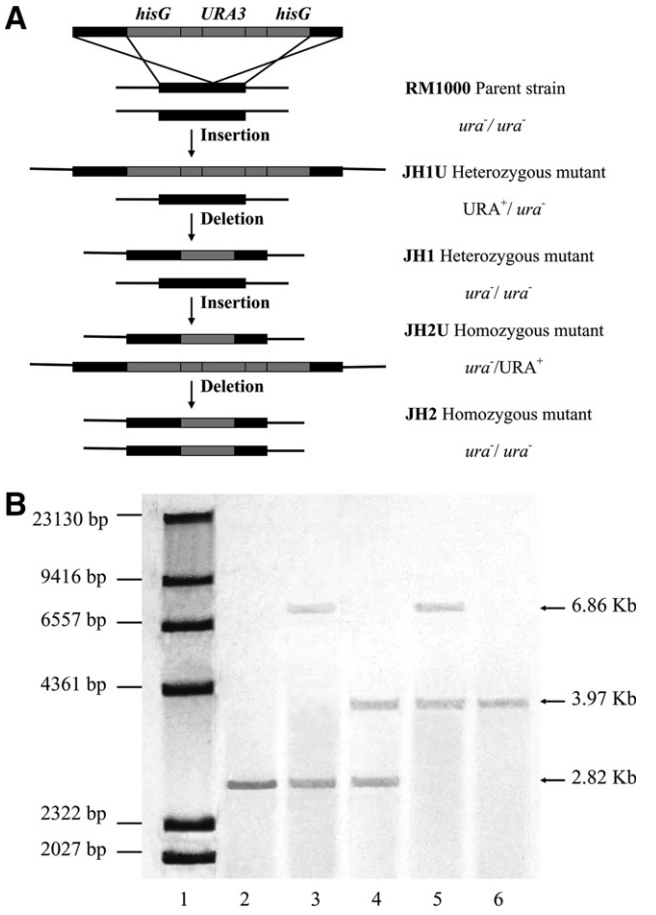

Fig. 1. Deletion of *CaIPF7817* in *C. albicans*. (A) A diagram of the strategy to disrupt both copies of *CaIPF7817*. (B) Southern blot analysis on the genomic DNA digested with PstI and SphI. Lane 1, DNA molecular weight marker II, DIG-labeled (Roche); lane 2, RM1000; lane 3, JH1U; lane 4, JH1; lane 5, JH2U; lane 6, JH2.

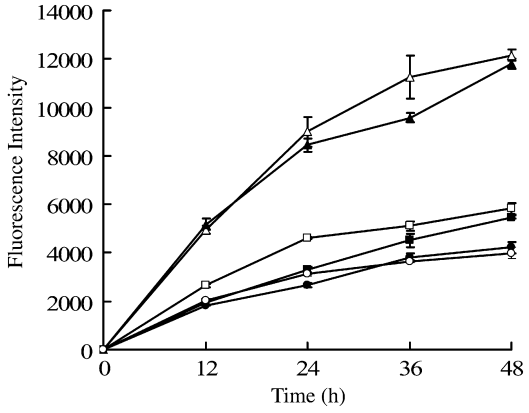

Fig. 2. Changes of the levels of ROS in different strains of *C. albicans* with time. ROS level was measured using DCFH-DA. Filled circles, RM1000; open squares, JH1U; filled squares, JH1; open triangle, JH2U; filled triangle, JH2; open circles, JHEXP. Each datum point represents means ± SD of three independent samples.

increase was observed for $\Delta\psi_m$. No significant difference was found between JHEXP and RM1000 (Fig. 3).

Up-regualtion of some redox-related genes in ipf7817 mutants

Since the deletion of *CaIPF7817* resulted in the dramatic increase of intracellular ROS, we then investigated the expression of some important redox-related genes, including *GLR1* (glutathione reductase gene), *SOD2* (manganese–superoxide dismutase gene) and *TRR1* (thioredoxin reductase gene), through real-time RT-PCR analysis. In the JH2, null mutant of *ipf7817 GLR1* was found to be up-regulated about 7-fold. *SOD2* and *TRR1* were also up-regulated in JH2 (Fig. 4).

Increased [GSH]/[GSSG] ratio in ipf7817 mutant

Given the deletion of *CaIPF7817* resulted in the up-regulation of *GLR1*, we measured the cellular [GSH]/[GSSG] ratio. In *ipf7817* null mutant JH2, GSH level was increased by *ca.* 46% and GSSG level decreased by *ca.* 25%, compared with that of the parental strain. The [GSH]/[GSSG] ratio in JH2 increased by approximately 2-fold compared with that of the parental strain (Table 2).

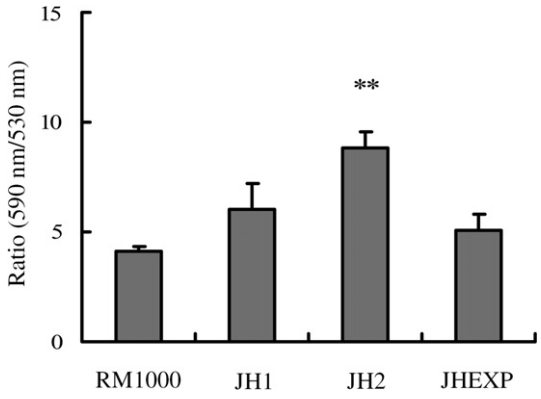

Fig. 3. The mitochondrial membrane potential in different strains of *C. albicans*. Results are shown as means ± SD of three independent experiments. ** indicates $P < 0.01$ compared with RM1000.

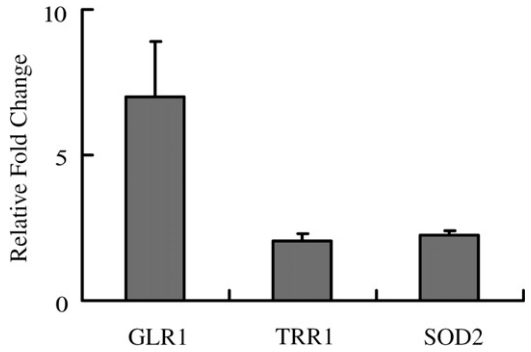

Fig. 4. Changes of expression of some redox-related genes in *ipf7817* null mutant determined by real-time RT-PCR. Gene expression is indicated as the fold increase relative to that of the RM1000 strain. Data are shown as means ± SD of three independent experiments.

Table 2
Comparison of [GSH]/[GSSG] ratio between the mutant and wild-type *C. albicans cells*

	GSH (μM)	GSSG (μM)	GSH/GSSG
RM1000	17.98 ± 1.25	1.42 ± 0.03	12.69 ± 1.12
JH1	14.41 ± 2.74	0.83 ± 0.05	17.53 ± 4.45
JH2	26.29 ± 0.40[a]	1.07 ± 0.08	24.51 ± 2.21[a]
JHEXP	17.01 ± 1.35	1.27 ± 0.13	13.57 ± 2.50

[a] $P < 0.05$ compared with RM1000.

Discussion

In this work, we interrupted both copies of *CaIPF7817* in *C. albicans* and studied the role of *CaIPF7817* in cellular redox regulation. Intracellular ROS level, mitochondrial membrane potential, expressions of some important redox-related genes and GSH/GSSG ratio were measured to reveal changes in cellular redox condition after *CaIPF7817* deletion. Without *CaIPF7817*, the intracellular ROS level was increased; mitochondrial membrane potential was elevated; the expressions of some important redox-related genes were up-regulated; and the GSH/GSSG ratio was raised. These changes indicated that *CaIPF7817* played roles in the regulation of redox homeostasis of *C. albicans*.

Mitochondrial respiratory chain is the main source of intracellular ROS and $\Delta\psi_m$ is an important parameter on the redox status of mitochondria. Sophisticated regulation mechanisms exist between intracellular ROS and $\Delta\psi_m$ [23]. Because levels of intracellular ROS were affected by *CaIPF7817*, we also investigated the effects of *CaIPF7817* on $\Delta\psi_m$. The results indicated that $\Delta\psi_m$ was increased significantly with the deletion of *CaIPF7817*.

The deletion of *CaIPF7817* led to a significant increase of intracellular ROS level, but the growth of the null mutant was not affected, indicating that the *Δipf7817* cells reached a new intracellular redox balance. We postulated that some reductive molecules and/or reductases might be up-regulated in *Δipf7817* cells to counter the increased intracellular ROS. Superoxide dismutases (SODs), which catalyze the direct removal of ROS, play a critical rule in the first line of defense of antioxidants [24,25]. The glutathione and thioredoxin systems are also very important in the redox homeostasis [26,27], in which glutathione reductase (*GLR1*) and thioredoxin reductase (*TRR1*) are the key enzymes [28]. Real-time RT-PCR showed that all these three genes, *SOD2*, *GLR1*, *TRR1*, were significantly up-regulated in *ipf7817* null mutant JH2. The up-regulation of these genes is agreement with our prediction that antioxidation system should be activated in *Δipf7817* cells to balance the increased intracellular levels of ROS. Moreover, the increased GSH/GSSG ratio also supported this prediction. Therefore the increase of ROS production and activa-

tion of the antioxidation system led to a new intracellular redox balance in *Δipf7817* cells.

CaIPF7817 is highly up-regulated during oxidative stress [11,12], implying that it has a role in oxidative stress tolerance. We therefore, examined the sensitivity of the *ipf7817* null mutant to H_2O_2. Contrary to our expectation, no difference was found between the growth of the *ipf7817* null mutant and that of the parental strain in the presence of H_2O_2 (data not shown). It was reported that, *CaIPF7817* was not uniquely up-regulated upon extracellular oxidative damage [11,12]. Our H_2O_2 sensitivity experiment showed that *CaIPF7817* was not essential for the tolerance of extracellular oxidants, suggesting that *CaIPF7817* was mainly involved in the regulation of intracellular redox homeostasis in *C. albicans*, especially during the normal aerobic metabolism of *C. albicans*.

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