Lack of Trehalose Accelerates H2O2-Induced Candida albicans Apoptosis through Regulating Ca2+ Signaling Pathway and Caspase Activity

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Abstract

Trehalose is a non-reducing disaccharide and can be accumulated in response to heat or oxidative stresses in Candida albicans. Here we showed that a C. albicans tps1Δ mutant, which is deficient in trehalose synthesis, exhibited increased apoptosis rate upon H2O2 treatment together with an increase of intracellular Ca2+ level and caspase activity. When the intracellular Ca2+ level was stimulated by adding CaCl2 or A23187, both the apoptosis rate and caspase activity were increased. In contrast, the presence of two calcium chelators, EGTA and BAPTA, could attenuate these effects. Moreover, we investigated the role of Ca2+ pathway in C. albicans apoptosis and found that both calcineurin and the calcineurin-dependent transcription factor, Crz1p, mutants showed decreased apoptosis and caspase activity upon H2O2 treatment compared to the wild-type cells. Expression of CaMCA1, the only gene found encoding a C. albicans metacaspase, in calcineurin-deleted or Crz1p-deleted cells restored the cell sensitivity to H2O2. Our results suggest that Ca2+ and its downstream calcineurin/Crz1p/CaMCA1 pathway are involved in H2O2-induced C. albicans apoptosis. Inhibition of this pathway might be the mechanism for the protective role of trehalose in C. albicans.

Introduction

Candida albicans is the most important human fungal pathogen, causing various diseases from superficial mucosal infections to life-threatening systemic disorders [1–3]. The number of clinical C. albicans infections worldwide has risen considerably in recent years, and the incidence of resistance to traditional antifungal therapies is also rising. Many existing antifungal therapies have unfortunate clinical side effects; therefore, strategies are needed to identify new targets for antifungal therapy.

In the past few years, it became evident that apoptosis might occur not only in multicellular, but also in unicellular organisms, such as fungi. The induction of cell apoptosis is considered as a new and promising strategy for antifungal therapy. It has been reported that Saccharomyces cerevisiae dies in an apoptotic manner in response to weak acid stress, oxidative stress, salt stress, and UV irradiation [4–7]. Ultrastructural and biochemical changes that are characteristic of apoptosis have also been reported in pathogenic fungi. C. albicans can be triggered to undergo an apoptotic cell death response when exposed to environmental stress such as H2O2, amphotericin B (AmB) or intracellular acidification. However, the mechanism of C. albicans apoptosis has not been fully revealed. Ras-cAMP–PKA signaling is essential for the apoptotic response, whereas mutations that stimulated signaling (RAS1val13 and pde2Δ) accelerated the rate of entry into apoptosis [8–10]. We recently found that CaMCA1, a homologue of Saccharomyces cerevisiae metacaspase YCA1, was involved in oxidative stress-induced apoptosis in C. albicans [11].

Trehalose, a non-reducing disaccharide, plays diverse roles, from energy source to stress protectant, and this sugar is found in bacteria, fungi, plants, and invertebrates but not in mammals [12]. In yeast, trehalose acts both as a main reserve of carbohydrates and as a cellular protector against a variety of nutritional and/or environmental stress challenges (oxidative, heat shock, osmotic and/or saline stress, xenobiotics etc.3), increasing cell resistance to such insults [13]. The mechanism of trehalose protection is an active area of research that includes studies of the interaction of sugars with plasma membranes, the effects on cell osmotic responses, and the unique physicochemical properties of trehalose [14]. In yeast, trehalose is synthesized by a large enzyme complex comprising the two catalytic activities of trehalose biosynthesis. Trehalose-6-phosphate (Tre6P) synthase, encoded by TPS1, synthesizes Tre6P from glucose-6-phosphate and UDP-glucose. Tre6P is then hydrolyzed into trehalose by Tre6P phosphatase, encoded by TPS2 [15,16]. In C. albicans, tps1/tps1 mutants are defective not only for Tre6P synthesis but also for growth on glucose or related rapidly fermented sugars and virulence [17,18]. Previous work on C. albicans pointed to a specific role of trehalose.
in cellular protection against oxidative stress. A tps1Δtps1 mutant was shown to be deficient in trehalose synthesis and was extremely sensitive to H_2O_2 exposure [19]. However, the underlying mechanism by which trehalose protects C. albicans from the injuries remains undefined.

Ca^{2+} is an important second messenger in developmental and stress signaling pathways. In fungi, Ca^{2+} is responsible for the regulation of several processes, including cation homeostasis, morphogenesis, virulence traits, and antifungal drug resistance [20–23]. A rise in cytoplasmic Ca^{2+} has been found to be responsible for pheromone-induced S. cerevisiae apoptosis [24]. Fungicidal activity of amiodarone is also tightly coupled to calcium influx [25]. A rise in cytosolic calcium activates the calcium-dependent signaling pathway via the phosphatase, calcineurin (consisting of a catalytic subunit A encoded by CMP1 and a regulatory subunit B encoded by CNB1) and the calcineurin-dependent transcription factor, Crz1p. In C. albicans, Ca^{2+} and its downstream calcineurin/Crz1p pathway are involved in azole resistance, cell morphogenesis and virulence [26–29].

In this study, we show that lack of trehalose can accelerate H_2O_2-induced C. albicans apoptosis. Furthermore, this is linked to an increase of Ca^{2+} concentration and caspase activity. Addition or depletion of Ca^{2+} affected the cell death and caspase activity. Moreover, we investigated the role of Ca^{2+} signaling in C. albicans apoptosis, and found that both calcineurin-deleted and Crz1p-deleted cells showed decreased cell death and caspase activity compared to the wild-type cells. Expression of CaMCA1 in calcineurin-deleted or Crz1p-deleted cells restored the sensitivity to H_2O_2.

Results

Lack of Trehalose Accelerates H_2O_2-induced Apoptosis

In C. albicans, TPS1 encodes trehalose-6-phosphate (Tre6P) synthase that is required for trehalose synthesis. A tps1ΔA mutant is deficient in trehalose accumulation. The impact of TPS1 mutation on trehalose accumulation is shown in Fig. 1A. Trehalase accumulation was increased in wild-type cells after 1 to 3 hours.
mutant when exposed to different concentrations of H$_2$O$_2$. As shown in Fig. 2E, in the absence of H$_2$O$_2$, there was no significant difference between the wild-type cells and the mutants. After treatment with 1 mM H$_2$O$_2$ for 3 hours, the apoptosis rate of the $tps1$ mutant and wild-type cells was 78% (Fig. 2C and 2D), while the apoptosis rate of the $tps1$ mutant showed a higher percentage of cells demonstrating ROS accumulation than the wild-type cells (Table 1).

To ascertain the role of trehalose in $C. albicans$ apoptosis, we compared the apoptosis rate between the wild-type cells and the $tps1$ mutant when exposed to different concentrations of H$_2$O$_2$. As shown in Fig. 1C, upon H$_2$O$_2$ treatment, the apoptosis rate of the $tps1$ mutant was higher than that of the wild-type cells. After 3 hours treatment with 2 mM H$_2$O$_2$, 78% of the $tps1$ mutant were apoptotic, while the apoptosis rate of the wild-type cells was 47%.

**Lack of Trehalose Enhances Ca$^{2+}$ Elevation And Caspase Activity**

In $S. cerevisiae$, elevation of intracellular Ca$^{2+}$ can lead to cell death [25]. We determined the intracellular Ca$^{2+}$ upon H$_2$O$_2$ treatment using a fluorescent calcium indicator Fluo-3/AM. In the absence of H$_2$O$_2$, the intracellular levels of Ca$^{2+}$ in both the $tps1$ mutant and wild-type cells were rather low and almost undetectable. After treatment with 1 mM H$_2$O$_2$ for 3 hours, both of the groups showed obvious elevation of intracellular Ca$^{2+}$, while the $tps1$ mutant showed a much higher level of Ca$^{2+}$ than the wild-type cells (Fig. 2A, 2B).

Since we previously found that the caspase activity was increased in $C. albicans$ apoptosis [11], we investigated the caspase activity by staining the cells with D$_2$R, a nonfluorescent substrate, which is cleaved to green fluorescent monosubstituted rhodamine 110 and free rhodamine [10,11,30]. As shown in Fig. 2C and 2D, after treatment with 1 mM H$_2$O$_2$ for 3 hours, the cell number stainable by D$_2$R in the wild-type cells was 26%, while that in the $tps1$ mutant was 51%. Furthermore, the transcript levels of $CaMCA1$, which is responsible for caspase activity in $C. albicans$, were investigated by real-time RT-PCR. As shown in Fig. 2E, in the absence of H$_2$O$_2$, there was no significant difference in the transcript level of $CaMCA1$ between the $tps1$ mutant and wild-type cells. However, a 4-fold increase of $CaMCA1$ transcript level was recorded in the $tps1$ mutant compared to that in the wild-type cells when exposed to 1 mM H$_2$O$_2$ for 3 hours.

**Adding or Depleting Ca$^{2+}$ Affected Apoptosis and Caspase Activity**

Since the intracellular Ca$^{2+}$ level could be increased by H$_2$O$_2$, especially in the $tps1$ mutant, we hypothesized that Ca$^{2+}$ signaling might regulate $C. albicans$ apoptosis, and the higher sensitivity of the $tps1$ mutant to H$_2$O$_2$ might be due to its higher intracellular Ca$^{2+}$ level. As shown in Fig. 3A, when we stimulated the intracellular Ca$^{2+}$ level by adding CaCl$_2$ (0.3 mM), the apoptosis rate increased in both the $tps1$ mutant and wild-type cells. Similar effects were observed when A23187 (0.5 μM), a calcium ionophore, was added. CaCl$_2$ and A23187 themselves at the concentrations tested had no effects on $C. albicans$ growth. In addition, the presence of both CaCl$_2$ and A23187 resulted in an increased caspase activity in both the $tps1$ mutant and wild-type cells (Fig. 3C).

Furthermore, we tested the effect of depleting Ca$^{2+}$. As shown in Figure 3B, the presence of EGTA (1 mM), an extracellular calcium chelator, attenuated the H$_2$O$_2$-induced apoptosis in both $tps1$ mutant and wild-type cells, accompanied by the decrease of caspase activity (Fig. 3D). Similarly, when BAPTA (1 μM), an intracellular calcium chelator, was added, both the apoptosis rate and caspase activity in the two strains were decreased.

**Deletion of Calcineurin or Crz1p Leads to a Decrease in Apoptosis and Caspase Activity**

In $C. albicans$, calcineurin and Crz1p are two major proteins involved in Ca$^{2+}$ signaling and play an important role in antifungal tolerance, cell morphogenesis and virulence [20,21,26]. So it is possible that the effects of Ca$^{2+}$ on cell death are mediated by calcineurin and its downstream target Crz1p. To test this hypothesis, we examined the viability of calcineurin and Crz1p-deleted cells.

Expression of $CaMCA1$ in Calcineurin-deleted and Crz1p-deleted Cells Restored the Sensitivities to H$_2$O$_2$

Since the caspase activity was decreased in $cmp1$ and $crz1$ mutants upon H$_2$O$_2$ exposure, we introduced $CaMCA1$ into the $cmp1$ and $crz1$ mutants and assessed the phenotype. Upon H$_2$O$_2$ treatment, the apoptosis rates were lower than that in wild-type cells (Fig. 4A). As expected, the caspase activities in both the $cmp1$ and $crz1$ mutants were lower than that in the wild-type cells (Fig. 4B). Consistent with this, the transcription levels of $CaMCA1$ in $cmp1$ and $crz1$ mutants were much lower than that in the wild-type cells (Fig. 4C). The potential role of calcineurin in H$_2$O$_2$-induced apoptosis was further examined using the calcineurin inhibitor cyclosporin A. Upon H$_2$O$_2$ treatment, the wild-type cells showed lower apoptosis rates and caspase activity in the presence of 0.08 μM cyclosporin A as compared to the absence of this compound (Fig. 4A, 4B).

**Discussion**

In yeasts, trehalose acts both as a main reserve of carbohydrates and as a cellular protector against a variety of nutritional and/or environmental stress challenges, increasing cell resistance to such...
injuries. Trehalose accumulation in *C. albicans* has been described as a defense mechanism against oxidative stress. A trehalose-deficient *tps1* mutant is highly sensitive to H$_2$O$_2$ and prone to undergo phagocytic digestion [31]. However, the mechanism by which trehalose protects *C. albicans* from injuries remains unclear.

Since apoptosis is now considered as one of the important ways of *C. albicans* death, we assessed the role of trehalose in H$_2$O$_2$-induced apoptosis using a *tps1* mutant. According to our result, lack of trehalose could accelerate H$_2$O$_2$-induced apoptosis which was accompanied by an increase of ROS, an apoptosis indicator. This result revealed a mechanism for the protective role of trehalose in *C. albicans*. Similar results were reported by other researchers. Liu et al. found that trehalose could inhibit the phagocytosis of refrigerated platelets *in vitro* via preventing apoptosis [32]. Also, trehalose has been found to protect against ocular surface disorders in experimental murine dry eye through suppression of apoptosis [33].

Our detailed studies on the protective effect of trehalose revealed a role of Ca$^{2+}$ signals in *C. albicans* apoptosis. We observed that there was an increase of intracellular Ca$^{2+}$ level in both the *tps1* mutant and wild-type cells upon H$_2$O$_2$ treatment. However, this increase was much stronger in *tps1* mutant, which was consistent with the higher apoptosis rate induced in this strain. When we stimulated the intracellular Ca$^{2+}$ level by adding CaCl$_2$ or A23187, the apoptosis rates in both the *tps1* mutant and wild-type cells were increased. In contrast, when...
Ca\(^{2+}\) was depleted by adding EGTA or BAPTA, the apoptosis rate in both the \(tps1\Delta\) mutant and wild-type cells were decreased. These results indicated that apoptosis could be induced in \(C.\) albicans through increasing intracellular Ca\(^{2+}\) level.

The role of Ca\(^{2+}\) in \(C.\) albicans apoptosis was further examined by the experiments with \(CMP1\) and \(CRZ1\), two genes involved in Ca\(^{2+}\) signaling. We found that \(cmp1\Delta\) and \(crz1\Delta\) mutants showed attenuated apoptosis upon H\(_2\)O\(_2\) treatment, similar to the effect of depleting Ca\(^{2+}\) in wild-type cells. Consistent with this result, addition of cyclosporin A, a calcineurin inhibitor, could also attenuate apoptosis. Taken together, Ca\(^{2+}\) and its downstream calcineurin/Crz1p pathway are involved in H\(_2\)O\(_2\)-induced \(C.\) albicans apoptosis.

In mammals, apoptosis can be directed by the activation caspases, which cleave specific substrates and trigger cell death. In the past few years, it has become evident that caspases might exist not only in multicellular, but also in unicellular organisms, such as fungi. In \(S.\) cerevisiae, \(YCA1\) encodes a single metacaspase, which has caspase activity. \(YCA1\) is involved in the apoptosis of yeast cells exposed to different environmental stresses, such as H\(_2\)O\(_2\), acetic acid, sodium chloride, heat shock, and hyperosmosis [34–36]. In plants, metacaspases have been associated with Norway spruce apoptosis during embryogenesis and tomato plant apoptosis induced by fungal infection [37–39]. Using yeast as a heterologous system for apoptosis evaluation, the metacaspases \(AtMCPlb\) and \(AtMCP2b\) from the plant \(Arabidopsis\) \(thaliana\) were also found to be involved in apoptosis induced by H\(_2\)O\(_2\) [40]. We recently found that H\(_2\)O\(_2\)-induced \(C.\) albicans apoptosis was accompanied with caspase activity, which was encoded by \(CaMCA1\) [11]. In this study, we found that, upon H\(_2\)O\(_2\) treatment, the caspase activities in \(tps1\Delta\) mutant were much higher than those in wild-type cells, similar to the phenomena of intracellular Ca\(^{2+}\) levels. The positive relation between Ca\(^{2+}\) level and caspase activity was proved by adding or depleting Ca\(^{2+}\). Moreover, both calcineurin-deleted and Crz1p-deleted cells showed lower caspase activity compared to the wild-type cells, indicating that \(CaMCA1\) might be a downstream gene which is blocked in calcineurin-deleted or Crz1p-deleted cells (Fig. 5). As expected, when extraneous \(CaMCA1\) was introduced into these cells, the caspase activity and cell sensitivity to H\(_2\)O\(_2\) were resumed. Previous studies showed that \(C.\) albicans \(CaMCA1\) could be activated by Ca\(^{2+}\) and regulated by calcineurin and Crz1p. Moreover, CDRE (calcineurin-dependent responsive element) was found in the promoter of \(CaMCA1\) [26]. Based on these results, we conclude that \(CaMCA1\) is likely to be one of the downstream genes influenced by the Ca\(^{2+}\) signaling and involved with the protective role of trehalose against H\(_2\)O\(_2\)-induced apoptosis.

### Materials and Methods

#### Media and Compounds

Yeast media used were YPD (1% yeast extract, 2% peptone, and 2% glucose) and SD [0.67% (w/v) Difco yeast nitrogen base
without amino acids]. SD medium was supplemented with a complete synthetic mix containing all the amino acids and bases. For prototrophic selection of yeast, the relevant drop-out mixes were used. Because the capacity of the trehalose-deficient mutant tps1Δtps1 to grow on exogenous glucose and fructose as carbon source is seriously compromised, some experiments were carried out in YPgal medium (1% yeast extract, 2% peptone, and 2% galactose) or SDgal [0.67% (w/v) Difco yeast nitrogen base without amino acids, 2% galactose]. *Escherichia coli* DH5α and LB (0.5% yeast extract, 1% peptone, and 1% NaCl) medium were used for transformation and plasmid DNA preparation. Fluo-3/AM, CaCl2, A23187, BAPTA, EGTA, cyclosporin A (Sigma, U.S.A.) were

**Figure 4. Effects of CMP1 deletion, CRZ1 deletion or expression of CaMCA1 on H2O2-induced apoptosis and caspase activity.** The wild-type (CAI4-EXP), cmp1Δ-AEXP, crz1Δ-AEXP, cmp1Δ-CMP1, crz1Δ-CRZ1 camca1Δ, CAI4-CaMCA1, cmp1Δ-CaMCA1 and crz1Δ-CaMCA1 cells were exposed to 2 mM H2O2 for 3 hours. In another experiment, the wild-type cells were exposed to 2 mM H2O2 for 3 hours in the presence of cyclosporin A (0.08 μM). (A) Percentage of cells that were classified as apoptotic by TUNEL assay was shown. (B) The caspase activity was determined by staining the cells with D2R. (C) Transcription level of CaMCA1 in response to 2 mM H2O2 for 3 hours was determined by real time RT-PCR. The mRNA levels were normalized on the basis of their ACT1 levels. Gene expression is indicated as the fold increase of the mutant and CaMCA1-introduced cells relative to that of the wild-type cells. The data are mean values ± S.D. from three independent experiments. * indicates P<0.01 compared with values of CAI4-EXP treated with H2O2 only. ** indicates P<0.01 compared with values of parental cells without CaMCA1.

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to H$_2$O$_2$, the intracellular Ca$^{2+}$ increased expression of CaMCA1.

cyclosporin A can block this pathway. Crz1p might up-regulate the tps1 calcineurin/Crz1p pathway is activated. The calcineurin inhibitor dissolved in either medium or dimethyl sulfoxide (DMSO) and then diluted to the appropriate working concentration.

Plasmids and Strain Construction

The strains (Table 2) were cultivated at 30°C under constant shaking (200 rpm) or incubation. To reintroduce TPS1 to $\psi$1A mutant, the ORF of TPS1 was amplified (using upstream primer 5' ggatatgcgtggaagaggttc' and downstream primer 5' ctgcagctagtccctcaactctttg' with Pyrobest DNA polymerase (TaKaRa Biotechnology, Dalian, P.R. China). After being purified, the BamHI-PstI digested PCR fragment was cloned into the integrative expression vector pCaEXP (Table 3) to generate the recombinant plasmid pCaEXP-TPS1 [41]. After sequencing, pCaEXP-TPS1 was linearized and used to transform $\psi$1A cells, and selected on SD medium lacking uridine, methionine and cysteine. As controls, the empty plasmid pCaEXP was transformed into CAI4 and $\psi$1A cell to produce CAI4-EXP and $\psi$1A-EXP, respectively. The same expression vector and transformation method were used for reintroducing CMP1 (using upstream primer 5' ggatatgcgtggaagaggttc' and downstream primer 5' ctgcagctagtccctcaactctttg' and CRZ1 (using upstream primer 5' ggatatgcgtggaagaggttc' and downstream primer 5' ctgcagctagtccctcaactctttg') to cmp1A and crz1A mutants and CAI4. The expression of TPS1, CMP1, CRZ1 and CaMCA1 in their host cells was confirmed by real time RT-PCR (data not shown).

Cell Treatment and Apoptosis Measurement

Yeast cells grown to early exponential phase at 30°C were exposed to different concentrations of H$_2$O$_2$ for the required time (range 0–3 hours) and then harvested for apoptosis measurement. A terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed in order to confirm the occurrence of the apoptosis process [4]. C. albicans cells were washed twice with PBS and fixed with a solution of 3.6% paraformaldehyde in PBS for 1 hour at 20°C. Cells were rinsed twice with PBS and then incubated with permeabilization solution for 2 minutes on ice. The cells were rinsed in PBS and labeled, using a solution of the label and enzyme solutions from an in situ cell death detection kit, fluorescein (Roche Applied Sciences, Mannheim, Germany), with

**Table 2. C. albicans strains used in this study.**

| Strain | Parent | Genotype | Reference |
|--------|--------|----------|-----------|
| CAI4   | CAI4   | ura3::immm434::HisG/ura3::immm434 | Fonzi et al., 1993 |
| CAI4-EXP | CAI4   | ura3::immm434::HisG::CRZ1 | This study |
| cmp1A (DSY2091) | CAI4   | cna1::HisG::cmp1A::HisG::URA3::HisG | Karababa et al., 2006 |
| cmp1A-CaMCA1 | cna1::HisG::cmp1A::CaMCA1-URA3::HisG | This study |
| cmp1A-CMP1 | cna1::HisG::cmp1A::CMP1-URA3::HisG | This study |
| cmp1A-EXP | cna1::HisG::cmp1A::HisG::URA3::HisG | This study |
| czr1A (DSY2195) | DSY2188 | czr1::HisG::czr1::HisG::URA3::HisG | Karababa et al., 2006 |
| czr1A-CaMCA1 | czr1::HisG::czr1::HisG::CaMCA1-URA3 | This study |
| czr1A-CRZ1 | czr1::HisG::czr1::HisG::CRZ1-URA3 | This study |
| czr1A-EXP | czr1::HisG::czr1::HisG::URA3 | This study |
| camca1A | CAI4   | camca1A::HisG::camca1A::HisG::URA3::HisG | Cao et al., 2009 |
| CAM-CaMCA1 | CAI4   | ura3::immm434::HisG::camca1A::URA3::HisG | This study |
| tps1A | CAI4   | tps1::HisG::tps1::HisG::URA3::HisG | Zaragoza et al., 1998 |
| tps1A-EXP | tps1::HisG::tps1::HisG::URA3::HisG | This study |
| tps1A-TPS1 | tps1::HisG::tps1::HisG::TPS1-URA3::HisG | This study |

**Figure 5. A model for the role of trehalose in the regulation of H$_2$O$_2$-induced apoptosis in C. albicans.** When C. albicans is exposed to H$_2$O$_2$, the intracellular Ca$^{2+}$ is increased and its downstream calcineurin/Crz1p pathway is activated. The calcineurin inhibitor cyclosporin A can block this pathway. Crz1p might up-regulate the expression of CaMCA1 through binding to the CDRE (calcineurin-dependent responsive element) in the promoter of CaMCA1. The increased expression of CaMCA1 results in the increased caspase activity and thus apoptosis occurs. tps1Δ mutation results in the lack of trehalose accumulation thus accelerates C. albicans apoptosis. doi:10.1371/journal.pone.0015808.g005
appropriate controls labeled only with the label solution. The cells were incubated for 1 hour at 37°C in a humidified atmosphere in the dark, rinsed in PBS. The staining of the cells was observed by a fluorescence microscopy. Alternatively, the number of cells determined to be positive by the TUNEL assay was quantified using a BD FACSCalibur flow cytometer with excitation and emission wavelength settings at 488 and 520 nm, respectively.

Assay of the Intracellular Content of Trehalose

For analysis of the intracellular trehalose, the cells grown to early exponential phase at 30°C were exposed to 1 mM H2O2 for 3 hours. At the indicated times, aliquots of cells (about 5×10^6) were taken and immediately centrifuged and washed with cold distilled water. Samples were microwaved (700 W) for 3×60 seconds with 30 seconds intervals between each, 1 ml of distilled water was then used to extract the trehalose for 1 hour. After centrifugation at 15,000×g for 10 minutes, the trehalose in the supernatants was analyzed by HPLC-MS with a detection limit of 1 ng. An HPLC system (Agilent 1100, Wilmington, Germany) equipped with a G1946 mass spectrometer was used in the analysis. The operating conditions were as follows: Extracts were analyzed after separation of an Agilent Zorbax NH2 Column (4.6 mm×250 mm, 5 mm) at a flow rate of 1.0 ml/min. The mobile phase consisted of methanol:water 85:15 (v/v). The HPLC eluant from the DAD detector was monitored at 260 nm with a single fluorescence measurement), melting curve program and the reverse primer 5’-TATAATAGACCTTCTGGAAC-3’ and 2 to 5 μl of the samples to electrophoresis through a 1% agarose-MOPS gel. First-strand cDNAs were synthesized from 3 μg of total RNA in a 60 μl reaction volume using the cDNA synthesis kit for RT-PCR (Enzyme Technology, Dalian, P.R. China) in accordance with the manufacturer’s instructions. Triplicate independent quantitative real-time PCR were performed using the LightCycler System (Roche diagnostics, GmbH Mannheim, Germany). SYBR Green I (TaqKaRa) was used to visualize and monitor the amplified product in real time according to the manufacturer’s protocol. CaMcA1 was amplified with the forward primer 5’-TATAATAGACCTTCTGGAAC-3’ and the reverse primer 5’-TTTGTTGAGCGAGAAATAGT-3’.

The PCR protocol consisted of denaturation program (95°C for 10 seconds), 40 cycles of amplification and quantification program (95°C for 10 seconds, 60°C for 20 seconds, 72°C for 15 seconds with a single fluorescence measurement), melting curve program (60-95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement) and finally a cooling step to 40°C. A standard curve for each primer set was performed with 1:10, 1:25, 1:50, 1:100, 1:250 and 1:500 dilutions of the cDNAs. The slopes of the standard curves were within 10% of 100% efficiency. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the LightCycler system software, and the threshold cycle (Ct) above background for each reaction was calculated. The Ct value of ΔCT (amplified with the forward primer 5’-CAACAGAGCAATACAAATAG-3’ and the reverse primer 5’-GTTGTTGAGCGAGAAATAGT-3’) was subtracted from that of the tested genes to obtain a ΔCt value. The ΔCt value of an arbitrary calibrator was subtracted from the ΔCt value of each sample to obtain a ΔΔCt value. The gene expression level relative to the calibrator was expressed as 2^{-ΔΔCt}.

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Author Contributions

Conceived and designed the experiments: YYG YYJ YFC. Performed the experiments: HL ZYZ LLD XRS. Analyzed the data: ZYZ XMJ LY. Wrote the paper: YYG ZYZ.

Ca2+ Detection

Cells were loaded with 5 μM Fluo-3/AM for 30 minutes at 37°C. Ca2+ levels were determined by a fluorescence microscopy. Alternatively, fluorescence intensity values were determined on the POLARstar Galaxy (BMG, Labtech, Offenburg, Germany) with excitation at 488 nm and emission at 525 nm.

Assessment of Caspase Activity

Caspase activity was detected by staining with D2R (CaspSCREEN Flow Cytometric Apoptosis Detection Kit, BioVision, U.S.A.) [10,11,41]. According to the manufacturer’s instructions, cells were in D2R incubation buffer at 30°C for 45 minutes before viewing and counting under a fluorescence microscope with excitation at 488 nm and emission at 530 nm.

Real-time RT-PCR

RNA isolation and real-time RT-PCR were performed as described previously [42]. The isolated RNA was resuspended in diethyl pyrocarbonate-treated water. The OD260 and OD280 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. First-strand cDNAs were synthesized from 3 μg of total RNA in a 60 μl reaction volume using the cDNA synthesis kit for RT-PCR (TaqKaRa Biotechnology, Dalian, P.R. China) in accordance with the manufacturer’s instructions. Triplicate independent quantitative real-time PCR were performed using the LightCycler System (Roche diagnostics, GmbH Mannheim, Germany). SYBR Green I (TaqKaRa) was used to visualize and monitor the amplified product in real time according to the manufacturer’s protocol. CaMcA1 was amplified with the forward primer 5’-TATAATAGACCTTCTGGAAC-3’ and the reverse primer 5’-TTTGTTGAGCGAGAAATAGT-3’.

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Table 3. Plasmids used in this study.

| plasmid   | Parent       | Genotype             | Reference               |
|-----------|--------------|----------------------|-------------------------|
| pCaEXP    | pCaEXP       | C. albicans expression vector | Care et al., 1999       |
| pCaEXP-MCA1 | pCaEXP       | expression vector containing CaMCA1 | Cao et al., 2009       |
| pCaEXP-CMP1 | pCaEXP       | expression vector containing CMP1 | This study              |
| pCaEXP-CRZ1 | pCaEXP       | expression vector containing CRZ1 | This study              |
| pCaEXP-TPS1 | pCaEXP       | expression vector containing TPS1 | This study              |

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