Mild Heat Stress Affects on the Cell Wall Structure in *Candida albicans* Biofilm

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**ABSTRACT**

We previously reported that *Candida albicans* responded to mild heat stress in a range of temperature elevations simulating fever, and concluded that mild heat stress increases susceptibility to antifungal drugs. In this study, we show that mild heat stress causes a morphological change in hyphae during the process of biofilm formation. We found that mild heat stress extended the period of hyphal stage maintenance in *C. albicans* biofilm. Although the rate of hyphal change from yeast form to hyphal form reached the maximum within 3 hr, later, almost every cell quickly reverted to the yeast growth phase within 6 hr at 37°C but not at 39°C, or under mild heat stress. Electron microscopy using a smart specimen preparation technique revealed that mild heat stress significantly increased the thickness of the inner cell wall accompanied by a decrease in density of the outer cell wall in the hyphae of *C. albicans* biofilm. To identify the gene responsible for the morphological changes associated with mild heat stress, we performed microarray gene expression analysis. Eleven genes were upregulated and 17 genes were downregulated under mild heat stress in biofilm cells. The increased *PHR1* gene expression in response to mild heat stress was confirmed in quantitative RT-PCR analysis. The mutant upregulated *PHR1* expression showed the same sensitivity against antifungal drug micafungin as dependent on mild heat stress. Our findings point to possible therapeutic effects of hyperthermia as well as to the effect of fever during infections.

**Key words:** biofilm, *Candida albicans*, cell wall, mild heat stress, *PHR1*

**Introduction**

*Candida albicans* is a commensal of human mucosal surfaces, but it causes serious clinical diseases in immunosuppressed hosts\(^5\). The organism forms biofilms that make antifungal agents on medical implants and intravascular catheters ineffective. On the other hand, treatments against infectious diseases are administered to reduce risks on the host. We have been interested in exogenous mild heat stress (fever-range heat stress) that has been used in physical therapies to treat muscle injuries and malignant tumors\(^6\). Faster pathogen clearance by immunological mechanisms was reported as a beneficial effect of fever in the infected host\(^7\).

Cowen and Lindquist have focused on fungal Hsp90 as a new therapeutic target based on a clinical benefit of fever\(^8\). We reported the effect of mild heat stress on the antifungal susceptibility of biofilm formation in *C. albicans*\(^9\). In particular, mild heat stress at two or four degrees higher than 37°C led to increased susceptibility of *C. albicans* biofilm to antifungal agents such as micafungin, fluconazole, and amphotericin B. The difference in component ratio of yeast and hyphae at an early stage of biofilm formation may be the reason for the increased susceptibility of *C. albicans* biofilm to antifungal agents under conditions of mild heat stress, although the difference was observed only microscopically\(^9\). Moreover, biofilm at over 39°C was observed to somewhat repress further biofilm growth. Pumee sat et al. reported that
the activity of biofilm formation of *C. albicans* at 42°C was lower than at 37°C\(^{10}\). Effectiveness of antifungal agents under mild heat stress was also demonstrated at a late stage of biofilm formation in *Aspergillus fumigatus*\(^{26}\).

In this study, we investigated the alteration of the cell morphology, the ultrastructure of the cell wall, and the gene expression of *C. albicans* exposed to an increase in temperature by two degrees higher than 37°C, as effects of mild heat stress. Using the rapid-freezing method for temperature by two degrees higher than 37ºC, as effects of upregulation of biofilm formation was altered in response to mild heat stress. Moreover, the analysis of gene expression indicated an upregulation of *PHR1* due to mild heat stress. The mutant upregulated *PHR1* expression exhibited high sensitivity against micafungin as observed in conditions of mild heat stress. Our findings point to possible therapeutic effects of hyperthermia as well as to the effect of fever during infections.

### Material and methods

#### Fungal strains

*C. albicans* SC5314\(^{12}\) and *C. albicans* THE1\(^{13}\) were maintained as frozen glycerol stocks at −80°C. *C. albicans* SC5314 was used for biofilm assay, electron microscopy experiment, microarray analysis, and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. *C. albicans* THE1 was used for construction of mutants of *PHR1* gene.

#### Biofilm formation

Biofilms of the organism were formed using a partially modified method of Chandra et al.\(^{44}\). Briefly, cultures of the organism were grown on yeast extract-peptone-dextrose (YPD) agar (1% yeast extract, 2% Bacto peptone, 2% glucose, 1.5% agar) at 37ºC for 24 hr. A cell suspension of approximately \(1 \times 10^{9}\) cells/ml was prepared in phosphate-buffered saline (PBS). Polystyrene, 96-well, flat bottom tissue culture plates were covered with fetal bovine serum (FBS) for 24 hr at 37°C on a rocking platform. The pretreated plates were washed with PBS three times to remove residual FBS. One hundred µl of the cell suspension was inoculated into the FBS-coated plates. The cells were allowed to adhere to the plates for 90 min at 37°C. Wells were gently washed with PBS three times to remove non-adherent cells. Subsequently, yeast nitrogen base (YNB; Difco, Detroit, MI, USA) medium supplemented with 50 mM glucose (YNB-Glc) and with or without antifungal agents was added to the wells and incubated at 37°C and 39°C. The temperatures were controlled exactly.

#### Plasmid construction

Plasmid pSFS2-PHR1 was constructed by cloning the 5' and 3' flanking regions of *PHR1* into the *KpnI/XhoI* site (for the 5' region) and the SacII/SacI site (for the 3' region) in plasmid pSFS2. The 5' region was amplified by PCR with the primers PHR1-KpnI and PHR1-XhoI, while the 3' region was amplified by PCR with the primers PHR1-SacII and PHR1-SacI.

To construct the plasmid p99PHR1, the 5' flanking region (nucleotides –460 to –148) and 5' portion of the coding sequence region (nucleotides – 6 to 308) of *PHR1* were amplified by PCR using the primers PHR1- AF and PHR1-AR or PHR1-BF and PHR1-BR, respectively. The amplified fragments were cloned into the *KpnI/SalI* site (for the 5' flanking region) and the *SpeI/SalI* site (for the 5' portion of coding sequence) in plasmid p99CAU\(^{16}\).

Plasmid p99RP10 was constructed by cloning the 5’ and 3’ flanking regions of *RPS10* into the *KpnI/SalI* site (for the 5’ region) and the *SpeI/NotI* site (for the 3’ region) in plasmid p99CAU. The 5’ region was amplified by PCR with the primers RP10tet-AF and RP10tet-AR, while the 3’ region was amplified by PCR with the primers RP10tet-BF and RP10tet-BR. Primers used in this study are listed in Table 1.

#### Construction of strains

Yeast cells were transformed by a modified version of the lithium acetate method of Umeyama et al.\(^{19}\). The SAT1 flipper was used to disrupt one of the *PHR1* alleles to generate the heterozygous strain\(^{20}\). The *PHR1* deletion cassette (the DNA fragments between the *KpnI* and *SacI* sites) of the plasmid pSFS2-PHR1 was amplified with the primers PHR1-KpnI and PHR1-SacI and the amplified fragments were used to transform into *C. albicans* THE1\(^{13}\). Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 µg/ml of nourseothricin to obtain the *PHR1* heterozygous mutants. For excision of the SAT1 flipper from nourseothricin-resistant transformants by the site-specific recombinase FLP-mediated recombination, the strains were cultivated overnight in YPD medium without selective pressure. In order to replace the endogenous promoters with the tet-regulatable promoter in the remaining *PHR1* allele, the DNA fragment between the *KpnI* and *SacI* sites in plasmid p99PHR1 was amplified with the primers PHR1- AF and PHR1-BR and used to transform the *PHR1* heterozygous mutants. After selection on synthetic dextrose medium lacking uracil, the resulting URA3 transformants were plated on 5-fluoroorotic acid-containing medium to isolate the ura segregants. The resulting strain was designated tetPHR1.

We also constructed a control strain, contPHR1 strain, in which the tet-promoter element was integrated at the *RP10* locus of *C. albicans* genome. The DNA fragment between the *KpnI* and *NotI* sites in plasmid p99RP10 was amplified with the primers RP10tet-AF and RP10tet-BR and used to transform into *C. albicans* THE1 strain. Construction of the strains is described in Fig. S1(a).
Total RNA extraction

Total RNA was extracted from biofilm-forming cells either at 37ºC or 39ºC for 3 hr in YNB-Glc medium using 24-well plates. All cultures collected from the plates with scrapers were quickly filtrated on cellulose acetate membranes in vacuo. They were then quick-frozen and stored at −80ºC. Extraction of total RNA was performed using RNeasy Mini (QIAGEN GmbH, Germany) according to the kit instructions. Cells on the frozen membrane filters were transferred to microtubes containing 0.3 g of zirconia/silica disruption beads and the first solution of the kit, and then shaken vigorously in a Disruptor Genie (Scientific Industries, Inc., NY, USA) for 5 × 45 sec with periodic chilling on ice-water.

Microarray design and gene expression microarray

For the custom microarray slides of C. albicans, we used the C. albicans SC5314 ORF sequence data (https://fungi.ensembl.org/Candida_albicans_sc5314/info/index). Probes were generated from 6251 ORF sequences utilizing the e-array portal hosted by Agilent for array design (https://earray.chem.agilent.com/earray/). Finally, 43718 probes were selected for a 4 × 44K array format. The cRNA was amplified, labeled using a Low Input Quick Amp Labeling Kit (Agilent Technologies), and hybridized to a 4 × 44k Agilent custom array of C. albicans, according to the manufacturer’s instructions. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

Microarray data analysis and filter criteria

The raw signal intensities and flags for each probe were calculated from hybridization intensities and spot information according to the procedures recommended by Agilent Technologies using the Flag criteria in the Gene Spring Software. The raw signal intensities of two samples were log2-transformed and normalized by quantile algorithm with 'preprocessCore' library package on Bioconductor software. To identify up- or downregulated genes, we calculated Z-scores and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe for comparison between control and experimental samples. Then, we established criteria for regulated genes: (upregulated genes) Z-score ≥ 2.0 and ratio ≥ 1.0 - fold, (downregulated genes) Z-score ≤ −2.0 and ratio ≤ 1.0.

Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

To synthesize cDNA, the Advantage RT-for-PCR kit (Clontech, San Jose, CA, USA) was used according to the manufacturer’s protocol. Quantitative PCR was performed and analyzed using the CFX Manager (ver. 3.1) (BIO-RAD). The SSO advanced universal SYBR Green Supermix kit (BIO-RAD) was used for quantitative PCR according to the manufacturer’s protocol. The reference gene used for this experiment was 18S rRNA and ACTI as described previously. Results were analyzed using the 2^{-\Delta\Delta C_T} relative expressions methods. The value given for each gene at 37ºC and 39ºC for 3 hr relative to the expression of each gene

| Primer name | Purpose | A Nucleotide sequence (5’–3’) |
|-------------|---------|-----------------------------|
| PHR1-KpnI   | Construction of plasmid pSF2-PHR1 | GCCGGTGATCCCGTATGACCTTTGTC |
| PHR1-Xhol   | Construction of plasmid p99PHR1   | TTTTGTACAAATGGAAGTTATTTCA |
| PHR1-SacII  | Construction of plasmid p99PR10   | TTTTGTACAAATGGAAGTTATTTCA |
| PHR1-SacI   | Construction of plasmid p99PR10   | TTTTGTACAAATGGAAGTTATTTCA |
| PHR1-AR     | Construction of plasmid p99PR10   | TTTTGTACAAATGGAAGTTATTTCA |
| PHR1-BF     | Construction of plasmid p99PR10   | TTTTGTACAAATGGAAGTTATTTCA |
| PHR1-BR     | Construction of plasmid p99PR10   | TTTTGTACAAATGGAAGTTATTTCA |
| RP10tet-AF  | Construction of plasmid p99RP10   | TTTTGTACAAATGGAAGTTATTTCA |
| RP10tet-AR  | Construction of plasmid p99RP10   | TTTTGTACAAATGGAAGTTATTTCA |
| RP10tet-BF  | Construction of plasmid p99RP10   | TTTTGTACAAATGGAAGTTATTTCA |
| RP10tet-BR  | Construction of plasmid p99RP10   | TTTTGTACAAATGGAAGTTATTTCA |
| P1(PHR1-AF) | Confirmation of the constructed strain shown in Fig. S1(a) | TTTTGTACAAATGGAAGTTATTTCA |
| P2(PHR1-BR) | Confirmation of the constructed strain shown in Fig. S1(a) | TTTTGTACAAATGGAAGTTATTTCA |
| P3          | Confirmation of the constructed strain shown in Fig. S1(a) | TTTTGTACAAATGGAAGTTATTTCA |
| P4          | Confirmation of the constructed strain shown in Fig. S1(a) | TTTTGTACAAATGGAAGTTATTTCA |

* Restriction enzyme sites are underlined.
in biofilm-starting cells that were adhered to wells for 90 min at 37°C was determined by evaluating the expression: 2.464%.

**Microscopy**

Biofilm development in wells was monitored with a Keyence BZ-9000 (KEYENCE, Osaka, Japan).

**Electron microscopy**

Living cells scraped from biofilm culture wells were collected by centrifugation in a microcentrifuge for 10 sec. A small amount of the cell pellet was sandwiched between two copper disks and rapidly frozen by plunging them into melting propane cooled in liquid nitrogen. They were freeze-substituted in acetone containing 2% osmium tetroxide at −80°C for 2 days, embedded in epoxy resin (Quetol 812, Nissin EM Co. Ltd., Tokyo), and polymerized at 60°C. Ultrathin sections were cut to a thickness of 80 nm with a diamond knife (Diatome Co. Ltd., Switzerland) on an Ultracut S ultramicrotome (Leica Microsystems, Vienna) and picked up on 300-mesh grids. They were stained with uranyl acetate and lead citrate in a staining tube, covered with Super Support upon 300-mesh grids. They were stained with uranyl acetate and lead citrate in a staining tube, covered with Super Support Film (Nissin EM Co. Ltd., Tokyo), and observed in a JEM-1400 electron microscope (JEOL, Tokyo) at magnifications of 2,500 to 40,000 at 100 kV.

**Spot assay**

*C. albicans* cells were grown overnight on YPD agar medium at 37°C. The colonies were suspended on YPD liquid medium and adjusted to cell concentration of 1 × 10⁶ cells/ml. Five µl aliquots of 10-fold serial dilutions (from 1 × 10⁵ cells/ml) were spotted onto YPD agar plates supplemented with 0.05 µg/ml micafungin in the presence or absence of 10 µg/ml doxycycline (DOX). Minimum inhibitory concentration (MIC) of micafungin for the contPHR1 strain at 37°C based on E-test was 0.032 µg/ml. Concentrations of micafungin were used based on the MIC value and our previous report [27]. The plates were incubated at 37°C or 39°C for 36 hr.

**Statistical analysis**

Statistical significance was determined by a two-tailed Student’s *t*-test.

**Results**

**Mild heat stress extends the period of hyphal maintenance in *C. albicans* biofilm**

Mild heat stress here means stress by increasing only two degrees from 37°C. This condition simulates fever symptoms in infectious diseases. In our previous report, mild heat stress induced the antifungal susceptibility of *C. albicans* SC5314 biofilm [28]. However, what occurred in the cells due to mild heat stress remained to be solved. We monitored the process of biofilm formation at 37°C and 39°C as shown in Fig. 1(b). Both of the hyphal rates similarly increased in a time-dependent manner until 3 hr. Then, at 4 hr, the culture at 37°C was suddenly induced to switch from hyphal growth phase into yeast growth phase. However, hyphal growth phase at 39°C continued significantly until 6 hr. These results indicated that mild heat stress at 39°C caused a morphological change in the process of biofilm formation.

**Mild heat stress affects the structure of *C. albicans* cell wall**

Fig. 1 shows that the significant difference of the hyphal rate in the process of biofilm formation at 37°C and 39°C appeared between 3 hr and 4 hr, showing dependency on mild heat stress. To understand what occurred in the cells under mild heat stress, we focused on cell surface architecture of hyphae at 3.5 hr following exposure to mild heat stress and observed them in detail through electron microscopy using a smart specimen preparation [27]. The yeast cells that adhered to culture wells at 37°C for 90 min with PBS were set as the 0 hr of biofilm formation. The cells were grown in a hyphal form for 3.5 hr culture at both temperatures. The cells cultured for 3.5 hr in biofilm medium at 37°C and 39°C were scraped from the culture wells, and then were immediately flushed by a microcentrifuge. The living cells were rapidly treated for TEM as mentioned in Materials and methods. Fig. 2(a) showed TEM images of the cell wall from *C. albicans* SC5314 cells. An apparent difference in images of hyphal wall between 37°C and 39°C was observed in the outer cell wall. Electron density of the outer cell wall at 39°C seemed lower compared to 37°C. The thickness of cell wall was measured based on TEM pictures in Fig. 2(b). The total cell wall thickness at 39°C was significantly higher than at 37°C (P < 0.01). Although the outer cell wall thickness did not change between cells grown at 37°C and 39°C, the inner cell wall at 39°C was significantly thicker compared to 37°C (P < 0.01). According to a previous paper, the outer cell wall is the mannan fibrillar layer and the inner cell wall is the chitin and β-glucans layer [29]. Therefore, mild heat stress leads to decrease in electron density of the mannan fibrillar layer and to increase in thickness of the chitin and β-glucans layer.

**PHR1 gene expression in cells that responded to mild heat stress is increased**

Results shown in Fig. 1 and Fig. 2 revealed that the changes in cell wall protein expression in growth cell morphology occurred between 3 hr and 4 hr, and is dependent on the temperature. We first explored whether mild heat stress leads
to changes in expression of genes that affect the cell surface properties by using microarray gene expression analysis. In Fig. 3(a), genome-wide gene expression in biofilm cells of C. albicans SC5314 at 37°C and 39°C for 3-hr cultures was evaluated at the mRNA level. Eleven genes were upregulated in biofilm cells cultured at 39°C for 3 hr compared to 37°C. On the other hand, 17 genes were downregulated at 39°C in biofilm cells. Since the apparent difference was observed in cell wall structure between at 37°C and at 39°C in electron microscopy, we focused on cell surface molecules in further experiments. The upregulated genes related to cell surface molecules were 3 genes; namely, WOR1, TRY6, and PHR1, and the downregulated genes were 5 genes; namely, MNN22, PGA22, YWP1, PGA26, and PGA45. The expression of these eight upregulated or downregulated genes was confirmed in detail using quantitative RT-PCR, as shown in Fig. 3(b and c).

In quantitative RT-PCR analysis, the gene expression level in cells that adhered to culture wells at 37°C for 90 min prior to biofilm culture was used as the basal expression level. The quantitative RT-PCR analysis revealed that PHR1 gene expression was significantly upregulated at 39°C compared to 37°C. These results suggest that PHR1 might have some function in the cell surface architecture of hyphae at 39°C in biofilm cells.

The mutant upregulated PHR1 expression shows the same sensitivity against micafungin as dependent on mild heat stress

To confirm the relationship with PHR1 gene expression and mild heat stress, we constructed a tetPHR1 in THE1 strain where PHR1 gene expression was regulated by a tet-repressive promoter, which is repressed in the presence of the tetracycline analog DOX and is overexpressed in the absence of DOX. The mutant upregulated PHR1 expression shows the same sensitivity against micafungin as dependent on mild heat stress.
Fig. 3. PHR1 gene expression in cells increased in response to mild heat stress.
(a) Relative quantification of gene expression using microarray. The graph represents log2 ratios of gene expression at 39°C versus 37°C for both 3-hr cultures for biofilm formation of C. albicans SC5314. Positive values represent an increase and negative values represent a decrease of gene expression at 39°C compared to 37°C. Black bars represent genes related to cell surface structures.
(b) and (c) Relative quantification of gene expression by quantitative RT-PCR using the comparative C_T analysis. Quantification RT-PCR of eight genes selected by microarray in Fig. 3(a) was performed. Gene expression level in cells that adhered to culture wells at 37°C for 90 minutes prior to biofilm culture was used as the basal expression level. (b) Upregulated genes. (c) Downregulated genes. Statistical significance was assessed using a two-tailed Student’s t-test (**P < 0.01; NS, not significant)
We also constructed the contPHR1 strain in which the tet-promoter element was integrated at the RP10 locus. The integration of the tet-promoter component was confirmed by PCR using appropriate primers as shown in Fig. S1(b). A quantitative RT-PCR analysis revealed that PHR1 gene expression level in the tetPHR1 strain was 17-fold higher compared to the contPHR1 strain in the absence of DOX. Meanwhile, PHR1 gene expression level in the tetPHR1 strain was completely repressed more than 10-fold compared to the contPHR1 strain in the presence of DOX at 37ºC as shown in Fig. 4(a). We found that mild heat stress increased PHR1 gene expression level (Fig. 3(b)). Therefore, since the tetPHR1 strain expressed PHR1 highly without DOX as shown in Fig. 4(a), we assumed that the effect of mild heat stress would be observed more significantly at 37ºC in the tetPHR1 strain compared to the contPHR1 strain.

First, we performed the biofilm formation assay using the contPHR1 strain. However, it was impossible to assess morphological changes because the biofilm of contPHR1 strain showed yeast-dominant formation unlike the SC5314 strain, even though the contPHR1 strain possessed the ability for morphogenesis at 37ºC (data not shown). We next performed spot assay to confirm the effectiveness of antifungal drugs at 37ºC in the tetPHR1 strain with or without DOX. The sensitivity of micafungin under mild heat stress in C. albicans was higher than amphotericin B or fluconazole in our previous report. The tetPHR1 strain was grown at 37ºC without DOX, wherein PHR1 was overexpressed, it showed higher sensitivity to micafungin compared to the contPHR1 strain at 37ºC (Fig. 4(b)). This phenomenon was similar to the sensitivity of micafungin in the contPHR1 strain at 39ºC. The sensitivity to micafungin of the tetPHR1 strain recovered to the control level when grown with DOX at 37ºC. Meanwhile, the high sensitivity of the tetPHR1 strain to amphotericin B or fluconazole at 37ºC was not observed through this assay (Fig. S2). These results suggest that the mild-heat-stress-dependent expression of PHR1 induced sensitivity to micafungin.

**Discussion**

Multiple environmental stresses, including osmotic stress and thermal stress, lead to remodeling of cell wall structure. We reported that C. albicans biofilm responded to mild heat stress (elevation in the temperature from 37ºC to 39ºC and 41ºC) by showing increased susceptibility against antifungal drugs. It was reported that the mass, thickness, and metabolic activity of biofilm produced at 42ºC were lower than those at 37ºC. There have been few reports, however, on the analysis of mild heat stress of about two-degree-increase simulating human fever during infection. Mild heat stress (37ºC to 39ºC) was reported as a useful physical therapy in orthopedic surgery. In this study, we focused on the cell wall structure and gene expression to examine changes in the cell surface in response to mild heat stress by a two-degree temperature elevation.

As shown in Fig. 1, the main form of C. albicans cell morphology occupying biofilm beyond 3 hr at 37ºC changed from hyphae to yeast. On the other hand, maintenance of hyphae continued until 6 hr at 39ºC. It appeared that the
elevation in the temperature by two degrees induces the maintenance of hyphal growth of the cell in biofilm. We examined the structural changes of the cell surface layer of hyphae at 3.5-hr culture under mild heat stress (Fig. 2). The two distinct layers of the cell wall, the inner and outer layers, were clearly visible. The outer wall is composed of the mannann fibrillar layer, and the inner wall is composed of β-glucan and chitin25, 28. Mild heat stress did not affect the thickness of the outer wall, but decreased the electron density of the mannann fibrillar layer. Furthermore, the thickness of the inner wall increased under mild heat stress for 3.5 hr. The increased thickness of the inner wall in response to mild heat stress may be to compensate for the fragility of the outer wall. The fungal cell wall is affected by environmental changes including osmotic shock, carbon source, and oxygen concentration27, 30. In this study, the vulnerability of the mannann layer appeared in response to a two-degree temperature elevation from 37°C in the early stage of biofilm formation in C. albicans as shown in Fig. 2(a). Zeng et al. reported that the sensitivity of antifungal drugs to mild heat stress in Aspergillus fumigatus biofilm was more evident at the late stage of biofilm formation11. A. fumigatus differs from C. albicans in structure and composition of cell walls, and these phenomena in response to mild heat stress may occur in other kinds of fungi as well.

Based on the data in Fig. 1, RNAs were extracted from cells cultured at 37°C and 39°C for 3 hr, and gene expression levels were compared at 37°C and 39°C using microarray analyses as shown in Fig. 3 (a). The gene expression levels were confirmed using quantitative PCR method. The gene expression in cells that adhered at 37°C for 90 min in the wells was used as the basal expression level. Therefore, this difference in comparison criteria for gene expression between microarray analysis and quantitative PCR method may yield differences in results. The β-glucan transglycosylase gene PHR127, 29 was selected as a gene whose expression is suppressed at 37°C and is promoted at 39°C (Fig. 3(b)). This result was similar to the result reported by Heilmann et al. showing that PHR1 gene expression was higher at 42°C compared to 37°C11. In addition, they also reported an increase in other cell surface proteins at 42°C; namely, chitin-related proteins Crhl1 and Utr2 and cell wall maintenance protein Ecm33. Therefore, it is also conceivable from their data that mild heat stress affects genes related to cell surface structure. PHR1 is believed to be involved in the phenomenon resulting to the increase in thickness of the mycelial inner cell wall at 39°C than that at 37°C as shown in Fig. 2. It will be necessary to analyze the cell wall thickness under the electron microscope using the tetPHR1 strain.

Plaine et al. reported that PHR1-deficient strains are resistant to caspofungin31. This result is consistent with our data that PHR1 overexpression in the tetPHR1 strain without DOX at 37°C led to increased sensitivity to micafungin as shown in Fig. 4(b). Our results suggest that the sensitivity phenotypes against micafungin under mild heat stress as we previously reported could be attributed to PHR1 functions. In particular, we believe that the upregulation of PHR1 expression in response to mild heat stress leads to remodeling of cell wall structure via the transglycosylase function of PHR1, which in turn confers the susceptibility to micafungin. The new findings obtained in this study point to a possible application of hyperthermia as a therapeutic method. Further studies including in vivo experiments will be required to substantiate the therapeutic effect of hyperthermia as well as the effect of fever during infections.

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Conflicts of interest

None.

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