



# Potent *In Vitro* Synergism of Fluconazole and Osthole against Fluconazole-Resistant *Candida albicans*

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**ABSTRACT** Osthole is a natural coumarin that exhibits wide biological and pharmacological activities such as neuroprotective, osteogenic, immunomodulation, antitumor, and anti-inflammatory effects. In this study, we investigated the antifungal effects of osthole *in vitro*. A checkerboard microdilution assay showed that osthole has significant synergistic effect with fluconazole against fluconazole-resistant *Candida albicans*. Similar results were obtained from a growth curve assay. Meanwhile, XTT reduction assay demonstrated the synergism of fluconazole and osthole against *C. albicans* biofilm formation. Microarray results showed that the expression of genes involved in the oxidation-reduction process, energy metabolism, and transportation changed significantly after the combined treatment with fluconazole and osthole, and further results showed that endogenous reactive oxygen species (ROS) was significantly increased in the combination group. In conclusion, these results demonstrate the synergism of fluconazole and osthole against fluconazole-resistant *C. albicans* and indicate that endogenous ROS augmentation might contribute to the synergism of fluconazole and osthole.

**KEYWORDS** *Candida albicans*, osthole, fluconazole, synergism, reactive oxygen species

*Candida albicans* is one of the most prevalent human fungal pathogens causing superficial and systemic infections (1, 2). *C. albicans* bloodstream infections usually occur in immunocompromised patients, such as organ transplant recipients, AIDS patients, and patients receiving chemotherapy, with an estimated 40% mortality (3, 4). Effective antifungal agents are critical for the treatment of *C. albicans* infection. At present, the antifungal drugs used in clinical practice mainly include azoles, amphotericin B, and echinocandins, with azoles, especially fluconazole (FLC), being the most widely used agents (5, 6). However, the broad utilization of fluconazole has led to rapidly emerging drug-resistant isolates (7, 8). The great threat to human health caused by *C. albicans* infection and the limited number of available antimicrobial classes active against *Candida* spp. underscore the importance of developing new antifungal agents.

The medicinal properties of some traditional Chinese medicines (TCMs) have garnered attention due to easy access and lower toxicity. Many compounds extracted from TCMs, such as berberine, baicalein, and pterostilbene, exhibited significant antifungal activities when used alone or in combination with azoles (9–12). Research on TCMs is known to be one important means of developing new antifungals (13, 14). Osthole (OST; Fig. 1), a natural coumarin extracted from a commonly used TCM *Cnidii fructus*, has received much attention because of its wide biological and pharmacological activities. It was reported that osthole exhibits various bioactivities such as neuroprotective, osteogenic, immunomodulation, anti-tumor and anti-inflammatory effects (15).

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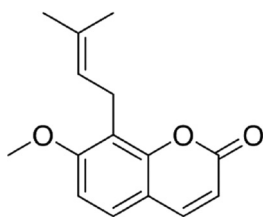
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**FIG 1** Chemical structure of osthole.

Also, osthole was reported to have significant activities against plant-pathogenic fungi such as *Fusarium graminearum* and *Sphaerotheca fuliginea* (16, 17). However, its activity against human fungal pathogens such as *C. albicans* has not yet been investigated.

In this study, we investigated the antifungal effects of osthole against clinical *C. albicans* isolates. Our results demonstrated that osthole has significant synergistic effect with fluconazole against fluconazole-resistant *C. albicans*. Further results indicated that endogenous reactive oxygen species (ROS) augmentation might contribute to the synergism of fluconazole and osthole.

## RESULTS

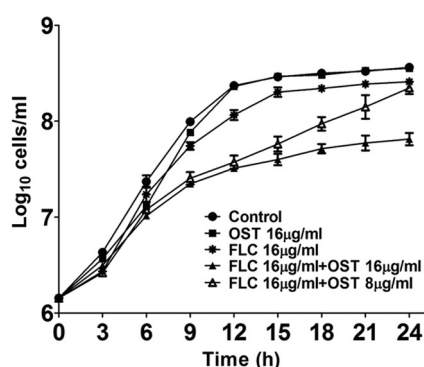
***In vitro* synergism of fluconazole and osthole against fluconazole-resistant *C. albicans*.** Osthole is a natural coumarin first derived from *Cnidium* plant (Fig. 1). In this study, we first evaluated the antifungal effect of osthole on *C. albicans*. A total of 30 clinical fluconazole-resistant *C. albicans* isolates ( $\text{MIC}_{50} \geq 8 \mu\text{g/ml}$ ) with unknown drug resistance mechanisms, and 10 fluconazole-susceptible *C. albicans* isolates ( $\text{MIC}_{50} \leq 1 \mu\text{g/ml}$ ) were used in this study (Table 1). The results showed that osthole did not exhibit any antifungal effect when it was used alone, with an  $\text{MIC}_{50}$  of  $>64 \mu\text{g/ml}$ . Compared to osthole alone, its combination with fluconazole showed a significant synergistic effect against the fluconazole-resistant *C. albicans* by reducing the dose of fluconazole to 1 to 16  $\mu\text{g/ml}$ , the dose of osthole to 4 to 16  $\mu\text{g/ml}$ , and the fractional inhibitory concentration index (FICI) to 0.04 to 0.31 (Table 1). Unlike the results of fluconazole-resistant isolates, fluconazole and osthole did not exhibit synergism on fluconazole-susceptible strains, since the FICI was 0.51 to 2.01 (Table 1). Further, a growth curve assay confirmed the synergism of fluconazole and osthole against fluconazole-resistant *C. albicans* and indicated that the synergistic effect was in a dose-dependent manner with osthole. As shown in Fig. 2, osthole or fluconazole alone at 16  $\mu\text{g/ml}$  had almost no antifungal effect, and the antifungal effect was significantly improved in the combination group. Meanwhile, the antifungal effect was restrained when the dose of osthole was decreased, while the dose of fluconazole remained unchanged (Fig. 2).

In addition, we assessed the *in vitro* synergism of osthole and other azoles such as miconazole, ketoconazole, and itraconazole against fluconazole-resistant *C. albicans*. As shown in Table 2, almost all of these fluconazole-resistant *C. albicans* strains were

**TABLE 1** Interaction of fluconazole and osthole against *C. albicans* as determined by microdilution assay<sup>a</sup>

Parameter	$\text{MIC}_{50}$ ( $\mu\text{g/ml}$ )			
	Fluconazole-resistant strains ( $n = 30$ )		Fluconazole-susceptible strains ( $n = 10$ )	
	Fluconazole	Osthole	Fluconazole	Osthole
Agent alone	8– $>64$	$>64$	0.125–1	$>64$
Agents combination (fluconazole/osthole)	1–16/4–16		0.125–1/1	
FICI	0.04–0.31		0.51–2.01	

<sup>a</sup>Where applicable, the MIC ranges calculated using 50% of growth inhibition as the endpoint are reported. Detailed  $\text{MIC}_{50}$  data are presented in Tables S3 and S4 in the supplemental material. A high off-scale  $\text{MIC}_{50}$  was converted to the next-highest concentration.



**FIG 2** Growth curves of *C. albicans* strain 0304103 treated with different concentrations of fluconazole and osthole. The data represent means  $\pm$  the standard deviations for three independent experiments.

resistant to miconazole, ketoconazole, and itraconazole as well. Meanwhile, the combination of osthole and these azoles showed significant synergistic effects: the FICI values were 0.03 to 0.31 (osthole and miconazole), 0.02 to 0.31 (osthole and ketoconazole), and 0.04 to 0.31 (osthole and itraconazole), respectively (Table 2).

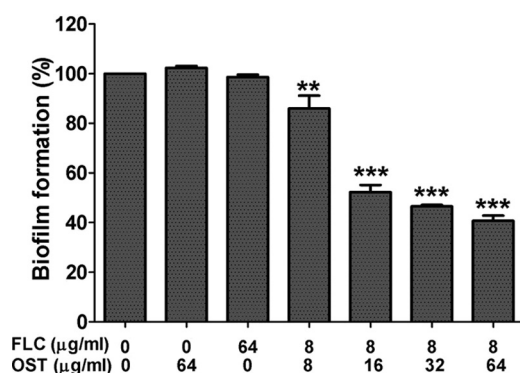
**In vitro synergism of fluconazole and osthole against *C. albicans* biofilm formation.** Moreover, we evaluated the *in vitro* synergism of fluconazole and osthole against *C. albicans* biofilm formation by XTT reduction assay. As shown in Fig. 3, osthole or fluconazole alone at 64  $\mu$ g/ml did not exhibit an antibiofilm effect. However, the combination of fluconazole and osthole had a significant synergistic effect by reducing the biofilm formation  $>50\%$  ( $P < 0.001$ ). As the dose of osthole was increased, the antibiofilm effect became more significant.

**Gene expression changes of *C. albicans* after combined treatment with fluconazole and osthole.** Further, we investigated the synergistic mechanism of fluconazole and osthole against fluconazole-resistant *C. albicans*. Expression profile microarrays were performed to determine whether gene expression changes of *C. albicans* in response to combined treatment with fluconazole at 8  $\mu$ g/ml and osthole at 16  $\mu$ g/ml versus monotreatment with fluconazole at 8  $\mu$ g/ml. Triplicate independent experiments were carried out, and we focused on the genes that displayed a  $>2$ -fold change with a q-value (a random variable to estimate the false discovery rate) of  $\leq 5\%$ . It was found that the expression of 469 genes changed, including 287 upregulated genes and 182 downregulated genes (see Table S2 in the supplemental material). GO-term analysis was performed to evaluate the biological processes related to the altered genes. The results showed that a large number of genes among the upregulated group involved in the oxidation-reduction process (e.g., catalase encoded by *CAT1* and mitochondrial glycosylase encoded by *OGG1*) and carbohydrate catabolic process (e.g., carnitine acetyltransferase encoded by *CTN1* and isocitrate lyase encoded by *ICL1*) (Fig. 4A). Meanwhile, the downregulated genes after combined treatment with fluconazole and osthole were mainly related to transmembrane transportation and drug transportation (e.g., oligopeptide transporter encoded by *PTR2* and multidrug efflux pump encoded by *MDR1*) (Fig. 4B). Many GO-terms overlap, and all of the biological processes

**TABLE 2** Interaction of azoles and osthole against 30 fluconazole-resistant *C. albicans* by microdilution assay<sup>a</sup>

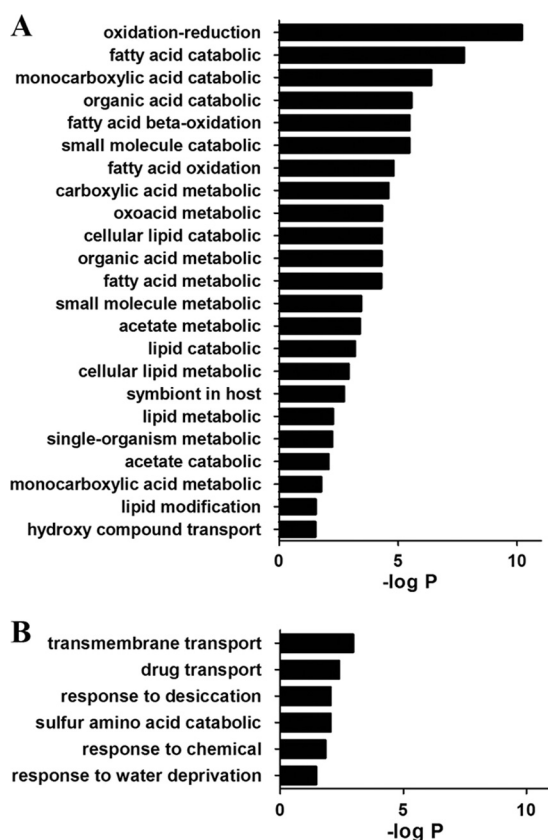
Parameter	MIC <sub>50</sub> ( $\mu$ g/ml) of 30 fluconazole-resistant strains			
	Miconazole	Ketoconazole	Itraconazole	Osthole
Agent alone	2–>16	1–>16	0.5–>16	>64
Agents combination (azoles/osthole)	0.25–4/1–8	0.0625–2/2–8	0.0625–2/2–16	
FICI	0.03–0.31	0.02–0.31	0.04–0.31	

<sup>a</sup>Where applicable, the MIC ranges calculated using 50% of growth inhibition as the endpoint are reported. Detailed MIC<sub>50</sub> data are presented in Table S3 in the supplemental material. A high off-scale MIC<sub>50</sub> was converted to the next-highest concentration.

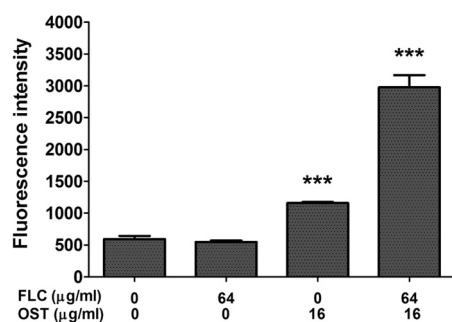


**FIG 3** Effects of different concentrations of fluconazole and osthole on biofilm formation of *C. albicans* SC5314. Biofilm formation was evaluated by an XTT reduction assay, and the results are presented as percentages compared to the control biofilms formed without fluconazole or osthole treatment. Biofilm formation results represent the means  $\pm$  the standard deviations for six independent experiments. \*\*,  $P < 0.01$  compared to the control biofilms; \*\*\*,  $P < 0.001$  compared to the control biofilms.

involved can be generally categorized as either part of the oxidation-reduction process, energy metabolism, or transportation (Fig. 4). Real-time reverse transcription-PCR (RT-PCR) was performed to validate several differentially expressed genes, including *CAS5*, *CAT1*, *OGG1*, *MAS2*, *ECM17*, *PTR2*, *MDR1*, *HGT6*, *NAG3*, and *PDX3* (see Fig. S1 in the supplemental material), and similar gene expression changes were detected.



**FIG 4** GO-term analysis results showing the significant biological process terms for 469 specifically differentially expressed genes in response to combined treatment with 8  $\mu$ g/ml fluconazole and 16  $\mu$ g/ml osthole versus monotreatment with 8  $\mu$ g/ml fluconazole. (A) Upregulated biological processes; (B) downregulated biological processes.



**FIG 5** Intracellular ROS generation in strain 0304103 treated or untreated with fluconazole (64 µg/ml) and/or osthole (16 µg/ml) for 6 h. ROS levels represent the means  $\pm$  the standard deviations for three independent experiments. \*\*\*,  $P < 0.001$  compared to the control group.

**Endogenous ROS augmentation might contribute to the synergism of fluconazole and osthole.** Since the microarray results demonstrated that the most significant differentially expressed genes were oxidation-reduction-related genes ( $-\log P > 10$ ; Fig. 4), endogenous ROS generation in *C. albicans* strain 0304103 after treatment with fluconazole and/or osthole was further measured. As shown in Fig. 5, fluconazole alone did not affect the concentration of endogenous ROS of the strains, and osthole alone caused slightly elevated endogenous ROS (two times that of the control group). Interestingly, combined treatment with fluconazole and osthole caused remarkable augmentation of endogenous ROS (five times that of the control group). These results indicated that the combination of fluconazole and osthole strongly promotes the production of endogenous ROS in fluconazole-resistant strains, which might contribute to the synergism of fluconazole and osthole.

## DISCUSSION

In this study, we found that fluconazole and osthole have significant *in vitro* synergism against fluconazole-resistant *C. albicans*. To study whether the synergistic mechanism was relevant to the drug resistance mechanisms of *C. albicans*, we screened 30 fluconazole-resistant strains without knowing the resistance mechanisms. Fluconazole and osthole exhibited synergism against all of these fluconazole-resistant strains (Table 1). Interestingly, fluconazole and osthole did not exhibit synergism against fluconazole-susceptible strains (Table 1). Thus, it seemed that the synergism is relevant to fluconazole resistance rather than to a specific resistance mechanism, which is similar to the synergism of fluconazole and berberine (18). We assumed that for fluconazole-susceptible strains, a low dosage of fluconazole was effective enough, and the synergism of fluconazole and osthole cannot be displayed. In contrast, for fluconazole-resistant strains, fluconazole monotreatment was not effective, and the synergism was revealed easily.

Osthole is an active O-methylated coumarin mainly derived from *C. fructus*. As a popular traditional Chinese medicinal herb, *C. fructus* has been widely used in China for to treat infectious diseases such as suppurative dermatitis and vaginitis (19, 20). The results in this study demonstrated that osthole is an important antifungal ingredient of *C. fructus*. In addition to osthole, there are other major constituents in *C. fructus*, such as imperatorin, bergapten, and isopimpinellin (21, 22). However, these constituents did not show any anti-*C. albicans* effects either alone or in combination with azoles (data not shown). Hence, we speculated that osthole should be the major bioactive component of *C. fructus* against *C. albicans*. Modern research has indicated that osthole exhibits significant antimicrobial activity against bacteria, viruses, and some plant-pathogenic fungi (15). We revealed in the present study the anti-*Candida* effects of osthole, which expand its antimicrobial spectrum. The synergism of fluconazole and osthole against *C. albicans* biofilms makes osthole suited as a potent antifungal agent for biofilm-related infections. Moreover, the toxicity of osthole was also assessed *in vitro*

and *in vivo*, and no obvious toxic effects were observed (23), which enhances the possibility of using osthole to treat human fungal infections.

The results of an expression profile microarray showed that the expression of genes involved in the oxidation-reduction process, energy metabolism, or transportation changed significantly after the combined treatment with fluconazole and osthole. Actually, gene expression profiles of *C. albicans* under various kinds of stimulation have been previously assessed (12, 18, 24). These studies indicated that alterations in transportation, metabolism, and stress response genes often occur upon interaction with host cells. For example, after terbinafine treatment, the expression of genes encoding membrane transport proteins and stress response are changed (24). In addition, exposure to ketoconazole caused the upregulation of genes involved in lipid, fatty acid, and sterol metabolism (25). In this study, genes that specifically respond to oxidative stress (e.g., *CAT1*, which encodes catalase in *C. albicans* and plays an important role in resistance to oxidative stress [26], and *OGG1*, which repairs oxidative damage to mitochondrial DNA in *C. albicans* [27]) were highly induced after the combined treatment with fluconazole and osthole, leading to significant enrichment of multiple GO terms related to oxidation-reduction process (Fig. 4). These results indicate that combined treatment with fluconazole and osthole appears able to cause oxidative damage to *C. albicans*. These findings are consistent with a recent study reporting that osthole treatment can extensively induce the expression of stress response genes, including oxidative stress-related genes in *Schizosaccharomyces pombe* (28). Given the ROS data showing that the combined treatment with fluconazole and osthole resulted in marked augmentation of endogenous ROS (Fig. 5), we favor the idea that oxidative damage is the major cause of the synergistic fungicidal effects.

In addition to oxidative damage, our results showed that metabolism-related *C. albicans* genes were upregulated in the presence of fluconazole and osthole. For example, genes related to the fatty acid catabolic process and the carboxylic acid catabolic process were upregulated in the combination group (Fig. 4 and see Table S2 in the supplemental material). This was consistent with previous findings that after phagocytosis, glycolytic genes are downregulated, while fatty acid oxidation and glyoxylate cycle genes are upregulated (29, 30). Thus, metabolic alterations might be an important cause of the synergistic effects of fluconazole and osthole. Actually, the effect of osthole on metabolism was also detected with *S. pombe* and *Mycobacterium tuberculosis*. Wang and Shen (28) found that, after osthole treatment, amino acid metabolism-related genes were significantly upregulated in *S. pombe*. Also, in *M. tuberculosis* the central intermediary metabolism processes and energy metabolism processes were greatly affected after osthole exposure (31).

In addition, from the microarray results we found that the expression of multidrug transporter genes showed the opposite trend after combined treatment with fluconazole and osthole. For example, major facilitator superfamily multidrug efflux pump gene *MDR1* was downregulated in the combination group (see Fig. S1 and Table S2 in the supplemental material), while multidrug transporter genes of the ABC superfamily, including *CDR1* and *CDR2*, were upregulated (see Table S2 in the supplemental material). The effect of drug efflux pumps on the synergism of fluconazole and osthole is not yet clear.

Collectively, these results demonstrate the synergism of fluconazole and osthole against fluconazole-resistant *C. albicans in vitro* and indicate that endogenous ROS augmentation might contribute to the synergism of fluconazole and osthole. These results provide new insights into the antimicrobial combinations, and further research is required to determine whether the combination is applicable *in vivo* and in a clinical setting.

## MATERIALS AND METHODS

**Yeast strains, growth conditions, and antifungal agents.** *C. albicans* reference strain SC5314 and



40 clinical *C. albicans* isolates, including 30 fluconazole-resistant strains and 10 fluconazole-susceptible strains, were used in this study. *C. albicans* strain 0304103 is a widely used fluconazole-resistant clinical isolate in our lab (18). All of the clinical isolates were provided by Changhai Hospital Mycology Laboratory, Shanghai, China. All strains were routinely grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) liquid medium at 30°C in a shaking incubator. For susceptibility testing, a standard powder of fluconazole (Sigma) was used and dissolved with water. Miconazole (Sigma), ketoconazole (Sigma), itraconazole (Sigma), and osthole (purity > 99%; National Institutes for Food and Drug Control, Beijing, China) were dissolved with dimethyl sulfoxide.

**Antifungal susceptibility testing.** The assay was carried out according to the guidelines in CLSI document M27-A3 (32). MIC values were determined visually after 24 h of incubation as the lowest concentration of agents that caused  $\geq 50\%$  inhibition of growth relative to that of the control group. Synergy and antagonism were defined by FICIs of  $\leq 0.5$  and  $> 4$ , respectively. An FICI of  $> 0.5$  but  $\leq 4$  was considered indifferent (33).

**Growth curve assay.** Growth curve assay was performed as described previously (10). In brief, *C. albicans* 0304103 in exponential phase were centrifuged and resuspended in YPD medium to  $1.5 \times 10^6$  cells/ml. Different antifungal agents were added, and the strains were cultured at 30°C under 200 rpm with shaking and counted at the designated time points.

**In vitro biofilm formation assay.** Biofilm formation assay was performed in a 96-well tissue culture plate as described previously (34). Briefly, *C. albicans* SC5314 cells ( $10^6$  cells/ml in RPMI 1640 medium) were inoculated in selected cells and incubated statically at 37°C. After a 90-min adhesion, the medium was aspirated, nonadherent cells were removed, and fresh RPMI 1640 with different concentrations of fluconazole and osthole was added. The plate was incubated at 37°C for another 24 h. The formed biofilms was calculated by using an XTT reduction assay (35).

**Gene expression analysis.** Exponentially growing *C. albicans* 0304103 cells were harvested and resuspended in YPD medium at  $1.5 \times 10^6$  cells/ml. Then, 8  $\mu\text{g/ml}$  fluconazole or 8  $\mu\text{g/ml}$  fluconazole plus 16  $\mu\text{g/ml}$  osthole was added because significantly different growth curves were found under these concentrations of the compounds. The samples were incubated at 30°C for 6 h. The medium was centrifuged, and the cells were stored in liquid nitrogen. RNA extraction and *Candida* genome microarray were performed as described previously (CapitalBio Corp., Beijing, China) (36). Briefly, total RNA was isolated by the hot-phenol method. cDNA generated by reverse transcription of the total RNA was purified and vacuum evaporated. The cDNA was mixed with random nanomers, heated to 95°C for 3 min, and snap cooled on ice for 5 min. Then, Klenow buffer, deoxynucleoside triphosphate, Cy5-dCTP/Cy3-dCTP, and Klenow enzyme were added. The reaction was performed at 37°C for 90 min. Labeled cDNA was purified and resuspended in elution buffer. Labeled controls and test samples were quantitatively adjusted and dissolved in 80  $\mu\text{l}$  of hybridization solution containing  $3\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% sodium dodecyl sulfate (SDS),  $5\times$  Denhardt solution, and 25% formamide. DNA in hybridization solution was denatured at 95°C for 3 min prior to loading onto a microarray. Arrays were hybridized in a CapitalBio BioMixer II hybridization station overnight and washed in a CapitalBio slide washer with two consecutive solutions (0.2% SDS- $2\times$  SSC at 42°C for 5 min and  $0.2\times$  SSC for 5 min at room temperature). After background correction and the removal of bad spots, a space- and intensity-dependent normalization based on a LOWESS program was used. The genes were named according to the *C. albicans* genome database (<http://www.candidagenome.org/>) and divided according to their biological functions.

Real-time RT-PCR was performed to confirm microarray results. An aliquot of the RNA preparations used in the microarray experiments was saved for the real-time RT-PCR study. Primers used in the real-time RT-PCR analysis were designed by using Primer Premier 5.0 software (see Table S1 in the supplemental material).

**ROS production measurement.** Endogenous ROS production was measured as described previously (37). In brief, *C. albicans* strain 0304103 ( $10^7$  cells/ml) were incubated with 20  $\mu\text{g}$  of 2,7-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes)/ml at 30°C for 30 min. The cells were then washed with phosphate-buffered saline and treated with different concentrations of fluconazole and osthole for another 6 h. The fluorescence values were detected on a POLARstar Galaxy with an excitation wavelength at 488 nm and an emission wavelength at 525 nm.

**Accession number(s).** All of the transcription data were deposited in the NCBI Gene Expression Omnibus database under accession number [GSE84936](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84936).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00436-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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