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Farnesol Induces Hydrogen Peroxide Resistance in *Candida albicans* Yeast by Inhibiting the Ras-Cyclic AMP Signaling Pathway[▽]

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Farnesol, a *Candida albicans* cell-cell signaling molecule that participates in the control of morphology, has an additional role in protection of the fungus against oxidative stress. In this report, we show that although farnesol induces the accumulation of intracellular reactive oxygen species (ROS), ROS generation is not necessary for the induction of catalase (Cat1)-mediated oxidative-stress resistance. Two antioxidants, α-tocopherol and, to a lesser extent, ascorbic acid effectively reduced intracellular ROS generation by farnesol but did not alter farnesol-induced oxidative-stress resistance. Farnesol inhibits the Ras1-adenylate cyclase (Cyr1) signaling pathway to achieve its effects on morphology under hypha-inducing conditions, and we demonstrate that farnesol induces oxidative-stress resistance by a similar mechanism. Strains lacking either Ras1 or Cyr1 no longer exhibited increased protection against hydrogen peroxide upon preincubation with farnesol. While we also observed the previously reported increase in the phosphorylation level of Hog1, a known regulator of oxidative-stress resistance, in the presence of farnesol, the *hog1*/*hog1* mutant did not differ from wild-type strains in terms of farnesol-induced oxidative-stress resistance. Analysis of Hog1 levels and its phosphorylation states in different mutant backgrounds indicated that mutation of the components of the Ras1-adenylate cyclase pathway was sufficient to cause an increase of Hog1 phosphorylation even in the absence of farnesol or other exogenous sources of oxidative stress. This finding indicates the presence of unknown links between these signaling pathways. Our results suggest that farnesol effects on the Ras-adenylate cyclase cascade are responsible for many of the observed activities of this fungal signaling molecule.

Candida albicans is the most common fungal pathogen involved in life-threatening systemic infections (32). In the United States, candidemia is the fourth most common type of nosocomial bloodstream infection (5). Once *C. albicans* reaches the bloodstream, the immune system plays an important role in limiting candidiasis (51). Macrophages and neutrophils kill pathogenic cells using a combination of factors, including high levels of reactive oxygen species (ROS) (46). However, *C. albicans* has means by which it can resist being killed by phagocytic cells. If a yeast cell survives within a macrophage for a sufficient period of time, it can differentiate into a hypha that can pierce and kill the host cell, allowing the fungus to escape being killed (36). *C. albicans* resistance to oxidative (OX) stress is critical for survival within macrophages, and cells impaired in oxidative-stress defense show severely reduced infection capabilities (19).

C. albicans also frequently encounters OX stress during its commensal life. A number of the microorganisms that inhabit the same niches as *C. albicans*, such as lactobacilli (18, 60) and streptococci (20, 52), release large quantities of H_2O_2. *Streptococcus* sp. culture supernatants can have H_2O_2 concentrations approaching 10 mM (52). Its interactions both with the host immune system and with microbes within the human microflora have likely led *C. albicans* to acquire the ability to prepare for and survive OX stress. Recent investigations have demonstrated that *C. albicans* production of a small secreted signaling molecule, farnesol, may be one way that the fungus regulates factors necessary for survival in the presence of ROS (10, 65).

C. albicans-produced farnesol was first described as regulating, in a concentration-dependent manner, the morphological shifts between the yeast and hyphal forms of the fungus (26). However, farnesol has additional effects on *C. albicans* physiology (10, 11, 16, 45, 49, 57, 65). Westwater et al. (65) demonstrated that the pretreatment of *C. albicans* yeast cells with either *C. albicans* culture supernatants containing farnesol or exogenous farnesol led to increased survival of OX stress generated by H_2O_2, menadione, and plumbagin. The enhanced survival induced by farnesol was correlated with increased expression of genes involved in OX stress resistance, such as catalase and superoxide dismutase genes, but the mechanism for this protection was not described.

Farnesol has been reported to impinge on at least three central regulatory pathways that are directly or indirectly related to OX stress resistance (10, 30, 61). We previously reported that farnesol inhibits the Ras-cyclic AMP (cAMP)-protein kinase A (PKA) cascade, thereby inhibiting hyphal growth and inducing the expression of the catalase-encoding gene *CAT1* (also called *CTA1*) (10). Although repression of the transcription of genes involved in stress response by the cAMP signaling pathway has been extensively documented (1, 24, 66), the mechanism for this repression is not yet well understood. In addition to farnesol effects on Ras1-Cyr1 signaling, Smith et al. (61) showed the Hog1 mitogen-activated protein (MAP) kinase, which is involved in the response to high levels of OX stress by activating the transcription of stress-related genes, was phosphorylated in the presence of farnesol (8, 39). Lastly, the absence of the histidine kinase Chk1, a regulator of cell wall synthesis and filamentation (35, 49), ren-

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TABLE 1. Strains used in this study

Strain	Genotype	Laboratory no.	Reference
SC5314	Prototrophic clinical isolate	DH35	22
CAI4	Ura$^-$ derivative of SC5314 *ura3*::λ*imm434*/*ura3*::λ*imm434*	DH332	33
CAF2	Ura$^+$ derivative of CAI4	DH331	33
AH81	Ura$^-$ derivative of CDH107 selected on 5-fluoroorotic acid *ura3*::λ*imm434*/*ura3*::λ*imm434 ras1*Δ::*hisG*/*ras1*Δ::*hisG*	DH482	This study
CDH107 (*ras1*/*ras1*)	*ura3*::λ*imm434*/*ura3*::λ*imm434 ras1*Δ::*hisG-URA3 hisG*/*ras1*Δ::*hisG*	DH483	33
CaAP16 (*ras1*/*ras1*/*RAS1)*	*ura3*::λ*imm434*/*ura3*::λ*imm434 ras1*Δ::*hisG*/*ras1*Δ::*hisG*::*FLAG-RAS1-URA3*	DH1383	This study
CR216 (*cyr1*/*cyr1*)	*ura3*::λ*imm434*/*ura3*::λ*imm434 cyr1*Δ::*hisG-URA3-hisG*/*cyr1*Δ::*hisG*	DH346	48
RM100	*ura3*::λ*imm434*/*ura3*::λ*imm434*	DH1270	43
CNC13 (*hog1*/*hog1*)	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*Δ::*hisG*/*his1*Δ::*hisG hog1*Δ::*hisG-URA3-hisG*/*hog1*Δ::*hisG*	DH1269	53
CU2 (*URA3*/*ura3*)	Ura$^+$ derivative of CAI4	DH1209	40
1F54 (*cat1*/*cat1*)	*ura3*::λ*imm434*/*ura3*::λ*imm434 cat1*Δ::*hisG*/*cat1*Δ::*hisG-URA3*	DH1213	40
CHK21 (*chk1*/*chk1*)	*ura3*::λ*imm434*/*ura3*::λ*imm434 chk1*Δ::*hisG-URA3-hisG*/*chk1*Δ::*hisG*	DH177	6
CAT1-GFP	CAI4 with plasmid pCAT1-GFP-*URA3* integrated at the *RPS1* locus[a]	DH939	13

[a] Although the catalase-encoding gene (orf19.6229) has been referred to as *CTA1* by Enjalbert et al. (13) and Davis-Hanna et al. (10), we refer to it as *CAT1* in accordance with the CGD nomenclature.

ders strains more sensitive to OX stress than wild-type (WT) cells (7) and resistant to inhibition of filamentation by farnesol (30). The relationships between these pathways and their roles in farnesol-mediated protection against OX stress have not yet been described.

In addition to its effect on *C. albicans* signaling pathways, farnesol can induce ROS accumulation within *C. albicans* cells (57), which may protect against subsequent OX stress. The cause of ROS generation in response to farnesol is poorly understood. While farnesol is generally nontoxic to *C. albicans* (10, 26, 45), under certain conditions it can inhibit cell growth (31, 63) and induce cell death (31, 57). Although ROS are toxic at high concentrations, more and more reports indicate that they participate in intracellular signaling at lower concentrations (9). Subtoxic concentrations of H_2O_2 stimulate hyphal differentiation of *C. albicans* (42). Furthermore, pretreatment with a low level of ROS can protect against further OX stress in *C. albicans* (28).

Here, we test hypotheses regarding the mechanism by which farnesol protects against oxidative stress. While we observed that farnesol can induce ROS in *C. albicans* yeast from exponential-phase cultures, we show that the accumulation of ROS induced by farnesol is not necessary for protection against OX stress. We report data indicating that farnesol-mediated induction of catalase expression and ROS resistance in yeast occurs mainly by repression of the Ras1-cAMP pathway. Strains defective in this pathway did not show increased resistance to oxidative stress upon the addition of farnesol. In contrast, *hog1*/*hog1* and *chk1*/*chk1* mutants still exhibited increased resistance to H_2O_2 upon incubation with farnesol. While Hog1 was not necessary for farnesol-mediated protection against ROS, Hog1 phosphorylation increased in the presence of farnesol, as has been shown previously (61). We show that the disruption of Ras1 or Cyr1 signaling led to a marked increase in Hog1 phosphorylation even in the absence of farnesol, indicating the presence of undescribed links between the Ras1-cAMP and Hog1 MAP kinase pathways. Farnesol repression of the central Ras1-cAMP signaling pathway enables *C. albicans* to simultaneously control multiple traits relevant to virulence.

MATERIALS AND METHODS

Strains and growth conditions. For a list of all strains used in these studies, refer to Table 1. Although the catalase-encoding gene (orf19.6229) has been referred to as *CTA1* by Enjalbert et al. (13) and Davis-Hanna et al. (10), we refer to it here as *CAT1* in accordance with the *Candida* Genome Database (CGD) nomenclature. Strains were streaked from freezer stocks stored at −80°C onto YPD (2% peptone, 1% yeast extract, and 2% glucose) plates every 8 days. Overnight cultures were grown in 5 ml of YPD at 30°C in a roller drum for 14 to 16 h. The cells were then centrifuged for 5 min and washed once with YPD.

Chemicals. Acidified ethyl acetate (0.01% glacial acetic acid) was used to make 50 mM stock solutions of *trans*,*trans*-farnesol (Sigma-Aldrich). α-Tocopherol (α-TOH) (Sigma-Aldrich) and ascorbic acid (Sigma-Aldrich) were dissolved in ethanol and Milli-Q water to obtain stock solutions of 50 mM and 1 M, respectively. All the stock solutions were made fresh before each experiment and added to appropriate tubes at final concentrations of 50 and 100 μM (farnesol and α-tocopherol) or 50 mM (ascorbic acid).

Molecular biology procedures and plasmid constructions. Standard molecular biology methods were used for genetic constructions. Strain CaAP16 was constructed by transforming strain DH482 (*ras1*::*hisG*/*ras1*::*hisG* [33]) by electroporation with the PacI-digested vector pAP16. To construct pAP16, a 1,000-base region upstream of the *RAS1* open reading frame (ORF) was amplified from strain SC5314 genomic DNA with primers KpnIpRAS1 F (5′-AAGGAAGGTA CCCGTAAAAGGTTTTTGTC-3′) and XhoIpRAS1R (5′-AGGAAGCTCGA GGGTATGTATATGTGTGG-3′) and ligated into KpnI/XhoI-digested pAU34 (62), resulting in pAP1. Next, an 800-base region downstream of the *RAS1* stop codon was amplified from strain SC5314 genomic DNA with primers BamHItRAS1F (5′-CTCTCGGGATCCGCTAACTAAAAAGTTCTCG-3′) and XbaItRAS1R (5′-CCGGGCCGTCTAGACCACTTCTTCTTCCTCC-3′) and ligated into BamHI/XbaI-digested pAP1, resulting in plasmid pAP13. The *RAS1* ORF was amplified from pYPB1-ADHpL-*CaRAS1* (48) with primers XhoIFLRAS1F (5′-CTCGAGATGGATTATAAAGATGATGATGATAA AGCGGCGATGTTGAGAGAATAT-3′) and BamHIRAS1R (5′-CTCGGATC CTCAAACAATAACACAACATCCATT-3′), introducing a single FLAG sequence upstream of the *RAS1* ORF. Following digestion with XhoI and BamHI, the FLAG-RAS1 fragment was ligated into similarly digested pAP13, resulting in plasmid pAP16.

Flow cytometry assays. Washed cells from overnight cultures were resuspended in YPD at a density of 10^5 ml^{-1} and incubated at 30°C in a roller drum for 2 h. The cells were then treated with 50 μM farnesol or acidified ethyl acetate (negative control) and incubated at 30°C for another 2 h. The cells were spun down and resuspended in 250 μl of phosphate-buffered saline (PBS) and then kept on ice until they were sorted. Three populations of cells were sorted,

depending on the intensity of the fluorescence of the cells, using a Becton Dickinson FACStar Plus high-speed sorting cytometer. Five thousand cells of each sorted population were resuspended in YPD containing 10 mM H_2O_2 and incubated for 90 min in a roller drum at 30°C. Following incubation, the cells were plated on YPD and incubated at 30°C for 24 to 36 h. The number of CFU per plate was then determined. Duplicate experiments were performed for each subpopulation, and the experiments were performed twice independently.

Effect of farnesol on survival after H_2O_2 treatments. Washed cells from overnight cultures were resuspended in YPD at a density of 10^5 cells ml^{-1} and incubated at 30°C in a roller drum for 2 h. The cells were then treated with 50 μM farnesol and/or 50 μM α-tocopherol or acidified ethyl acetate (vehicle control) and incubated at 30°C for two more hours. The cells were then harvested by centrifugation to remove farnesol and resuspended in 5 ml of fresh YPD. H_2O_2 was added to a final concentration of 10 mM unless otherwise specified, and the cells were incubated at 30°C for 90 min. Following incubation with H_2O_2, the cells were plated on YPD, and the resultant colonies were counted after 24 to 36 h. Duplicates were included for each treatment, and the entire experiment was repeated independently at least three times.

ROS accumulation in *C. albicans* cells. Intracellular ROS accumulation was examined using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (5 mM in dimethyl sulfoxide [DMSO]; Molecular Probes). Washed cells from overnight cultures were resuspended to a concentration of 10^6 cells ml^{-1} in 5 ml of YPD. The cells were grown at 30°C in a roller drum for 1 h and then treated with either 50 or 100 μM farnesol, 10 mM H_2O_2, 50 mM ascorbic acid, 50 μM α-tocopherol, or the appropriate vehicle control. The cells were incubated at 30°C in a roller drum for 30 min, and then a 1-ml aliquot was harvested, centrifuged, and washed once with YPD. The cells were resuspended in 500 μl of YPD, and 5 μl of DCFH-DA was added. The cells were incubated in the dark for 30 min under 180-rpm agitation. Then, the cells were collected by centrifugation, washed once with 1 ml PBS, and resuspended in 50 μl PBS. Fluorescence was examined by epifluorescence microscopy with a fixed exposure time, using a Zeiss Axiovert inverted microscope equipped with a 63× objective and Axiovision software. For each replicate and treatment, over 200 cells were examined in at least four different randomly chosen fields. The quantification of ROS was performed by scoring the number of green fluorescent cells (ROS) relative to all cells. Each experimental condition was tested in triplicate on different days.

Reverse transcription (RT)-PCR analysis of *C. albicans* transcripts. Cells were grown as described for the H_2O_2 challenge experiments, except that the cells were then treated with 50 μM farnesol and/or 50 μM α-tocopherol or acidified ethyl acetate (vehicle control) prior to the second 2-h incubation or with 10 mM H_2O_2 followed by incubation at 30°C for 30 and 60 min. The cells were then harvested by centrifugation for 5 min at 5,000 rpm and immediately snap-frozen. The cells were lysed by mechanical disruption of the frozen pellet using 0.5-mm silica beads, and total RNA was isolated using the RNeasy Mini Kit (Qiagen) and DNase treated with DNA-free (Ambion). For each reaction, 400 ng of RNA was used in cDNA synthesis. cDNA synthesis and PCRs were performed as previously described (10). The complete experiments were repeated three times independently. Transcripts were quantified using ImageJ.

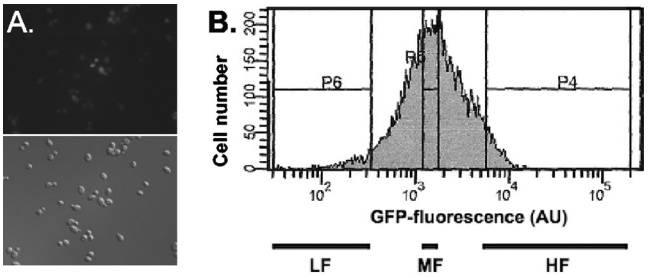
Hog1 phosphorylation assay. Cells were resuspended in YPD at a density of 10^6 ml^{-1} and incubated at 30°C in a roller drum for 2 h. The cells were treated with 10 mM H_2O_2, 50 μM farnesol, or acidified ethyl acetate (vehicle control) and incubated at 30°C for 30 min. The cells were then harvested by centrifugation for 5 min at 5,000 rpm and immediately snap-frozen. Protein extraction was performed as previously described (59). To equalize the amounts of protein loaded, the proteins were quantified using the Bradford assay following the manufacturer's recommendations (Bio-Rad). Protein separation was performed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE). Then, the proteins were then transferred onto nitrocellulose membranes. The phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) antibody (Cell Signaling technology) was used to determine the level of phosphorylation of Hog1 (61). The Hog1 protein level was determined by probing blots with the ScHog1 γ-215 polyclonal antibody (Santa Cruz Biotechnology) (4). Western blots were developed according to the manufacturer's conditions using the SuperSignal West Pico Goat IgG Detection kit (ThermoScientific). The Hog1 protein amount and Hog1 phosphorylation level were quantified using Vision work LS software (UVP, CA). For normalization, the intensities of three individualized protein bands from Coomassie-stained SDS-PAGE gels were measured. The average of the intensities of the three bands was then calculated and used for normalization of the amount of Hog1 protein and the level of phosphorylation of Hog1.

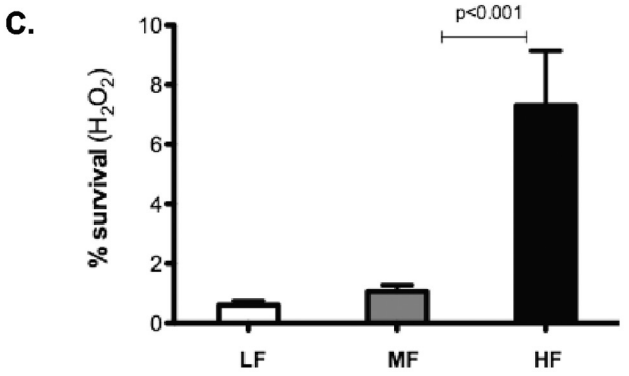
Statistical analyses. One-factor analysis of variance (ANOVA) and *t* tests were performed using Prism 5.0. (GraphPad Software).

RESULTS

***CAT1* induction is essential for farnesol-mediated protection against H_2O_2 killing.** We and others have previously shown that farnesol leads to dose-dependent increases in levels of the *CAT1* transcript, which encodes catalase (10, 16, 65). Because deletion of *C. albicans CAT1* leads to increased sensitivity to OX stress (41, 67) and *CAT1* transcription is activated in response to OX stress (13, 14), it is very likely that the enhancement of *CAT1* transcription by farnesol is predictive of protection against H_2O_2. To assess whether the induction of *CAT1* in the presence of farnesol is correlated with protection against H_2O_2 in yeast cells, we exploited the fact that the induction of *CAT1* expression is heterogeneous within the population at moderate farnesol concentrations (10) (Fig. 1A). Using a *C. albicans* strain with two wild-type copies of *CAT1* and a *CAT1* promoter fusion to *GFP* at the RPS1 locus (13), we sorted yeast cells grown in the presence of farnesol into populations with low, intermediate, and high levels of green fluorescent protein (GFP) fluorescence and analyzed their susceptibility to H_2O_2 (Fig. 1B). The three subpopulations corresponded to the 5% of the whole population with the lowest fluorescence, 10% around the median, and the 5% with the highest fluorescence. A strong correlation between the level of expression of *CAT1* and the resistance to H_2O_2 was observed (Fig. 1C), suggesting that the increase of resistance to OX stress in the presence of farnesol is correlated with an induction of catalase and that *CAT1* transcript levels are a good marker of OX stress resistance. When control populations of *CAT1-GFP* cells grown without exogenous farnesol were analyzed, the proportion of cells producing high levels of *CAT1* transcripts was lower than in farnesol-treated populations, but cells with higher levels of GFP still showed increased survival after H_2O_2 challenge in comparison to cells with intermediate or low levels of GFP (data not shown). As a control for potential effects of farnesol on GFP fluorescence or stability, a population of cells containing an *ACT1* promoter fused to the GFP gene was also sorted, and the H_2O_2 susceptibilities of cells with low, intermediate, and high levels of fluorescence were analyzed (13). No correlation between fluorescence intensity and H_2O_2 resistance was observed with the strain bearing the *ACT1-GFP* fusion (data not shown).

As an alternative approach to determine if farnesol-mediated protection against OX stress is due to induction of *CAT1* expression, we measured the survival of WT and *cat1*/*cat1* cells after H_2O_2 exposure in cells pregrown with farnesol and in untreated cells. Consistent with data previously reported by Westwater et al. (65), WT cells pretreated with 50 μM farnesol had 6- to 8-fold-higher survival of harsh OX stress generated by H_2O_2 (10 mM) than control cultures (Fig. 1D). In contrast, farnesol did not protect *cat1*/*cat1* cells against H_2O_2 stress, indicating that catalase is required for farnesol-mediated protection against H_2O_2. Farnesol pretreatment in fact decreased the subsequent survival of the *cat1*/*cat1* mutant upon H_2O_2 exposure (5.1% survival) relative to control cultures (13% survival). As discussed in more detail below, farnesol can induce ROS accumulation (31, 57), and the inability of *cat1*/*cat1* mutants to detoxify these ROS may have led to increased killing during the H_2O_2 challenge. Lower levels of H_2O_2 were used to assess changes in the sensitivity of the *cat1*/*cat1* mutants upon





D.

	% survival (H_2O_2)		Ratio (Farnesol/control)
	Control	Farnesol	
CU2 (10mM H_2O_2)	0.3 ± 0.2	2.0 ± 1.2	7.4 ± 3
cat1/cat1 (0.5mM H_2O_2)	13.1 ± 4.6	5.1 ± 4.4	0.3 ± 0.3

FIG. 1. Role of *CAT1* in farnesol-induced H_2O_2 survival. (A) Epifluorescence and differential interference contrast (DIC) microscopic views of *CAT1-GFP* cells in exponential phase treated with 50 μM farnesol for 2 h. (B) Histogram of fluorescence intensities of *CAT1* in a farnesol-treated population of *C. albicans* cells with *CAT1-GFP* promoter fusion during early exponential-phase growth in liquid culture as revealed by laser scanning cytometry. Three subpopulations visualized on the histogram by the P4, P5, and P6 bars were sorted and challenged with 10 mM H_2O_2. LF, low fluorescence intensity (P6 subpopulation); MF, medium fluorescence (P5); HF, high fluorescence (P4). AU, arbitrary units. (C) Survival of the three subpopulations of cells shown in panel A under 10 mM H_2O_2 treatment. The data are expressed as the mean value (plus standard deviation [SD]) of duplicate samples. (D) Effect of pretreatment with 50 μM farnesol on the survival of *cat1*/*cat1* and WT cells in H_2O_2. Following 2 h of incubation with 50 μM farnesol in YPD at 30°C, cells were harvested and challenged for 90 min with 0.5 mM (Δ*cat1*/*cat1*) or 10 mM (WT) H_2O_2. Survival was assessed by dilution plating. The fold survival is expressed as the ratio between the survival of farnesol-treated cells and untreated cells. The data are expressed as the mean value (±SD) of three independent cultures. Survival after H_2O_2 exposure and pretreatment with farnesol was significantly higher in CU2 (WT), but not in the *cat1*/*cat1* mutant (*t* test; $P < 0.05$).

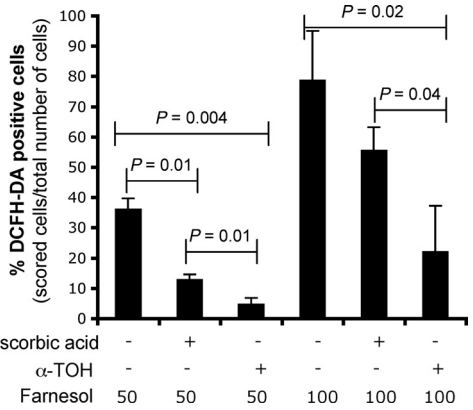


FIG. 2. Effects of farnesol, ascorbic acid, and α-tocopherol on ROS accumulation in *cat1*/*cat1* cells as revealed by DCFH-DA staining. The cells were incubated in YPD at 30°C for 30 min with either 50 or 100 μM farnesol, 50 mM ascorbic acid, 50 μM α-tocopherol, or the appropriate vehicle control. The cells were then harvested, washed, and incubated with DCFH-DA for 30 min. Fluorescence was examined by epifluorescence microscopy with a fixed exposure time, and the quantification of cells accumulating ROS was performed by scoring the number of green fluorescent cells relative to all cells. The data are expressed as the mean value (plus SD) of triplicate samples.

farnesol exposure, as 10 mM H_2O_2 led to complete killing of the *cat1*/*cat1* population.

Farnesol-induced OX stress is not required for protection against subsequent OX stress. Under some conditions, farnesol can be toxic (31, 63), and it has been shown to induce intracellular accumulation of ROS in *C. albicans* (57). To determine if farnesol induced ROS under our growth conditions, we treated cells with farnesol in the presence or absence of ROS scavengers. The accumulation of ROS was measured with the fluorescent dye DCFH-DA (57), and the results are shown here in the *cat1*/*cat1* background to allow easier detection of the generation of lower levels of ROS. As a positive control, cells were challenged with H_2O_2, which led to 97.6% ± 1.0% of the cells being DCFH-DA positive. Consistent with the findings of Shirtliff et al. (57), farnesol induced the accumulation of ROS in a concentration-dependent manner, with 36% of *cat1*/*cat1* cells in exponential growth containing detectable levels of ROS after a 30-min exposure to 50 μM farnesol (Fig. 2A). To determine if the ROS generated by farnesol could be scavenged by known antioxidants that localize either to the cytosol or to membranes, ROS accumulation was measured in farnesol-treated cultures amended with ascorbic acid or α-TOH. As predicted, ascorbic acid suppressed detectable intracellular ROS when added with H_2O_2 (1.3% ± 2.0% of cells were DCFH-DA positive), while the membrane-localized α-TOH was not protective against intracellular ROS generated by H_2O_2 (96.4% ± 2.0% of cells were DCFH-DA positive). α-TOH effectively reduced ROS generated by farnesol when supplied at a concentration of 50 μM (Fig. 2), and 100 μM α-TOH completely suppressed farnesol-induced ROS accumulation, as detected by DCFH-DA (data not shown). While ascorbic acid also reduced the accumulation of ROS upon farnesol exposure (Fig. 2), it never completely suppressed ROS, even at high concentrations (100 mM) (data not shown). Neither ascorbic acid nor α-TOH induced DCFH-DA in cells when added alone (data not shown). These findings suggest that farnesol-mediated induction of ROS may occur in or near plasma or intracellular membranes. When similar experiments were performed in *C. albicans* WT SC5314, ROS were detected in 5% of cells incubated with farnesol, but not in cells treated with 50 μM farnesol and α-TOH (data not shown).

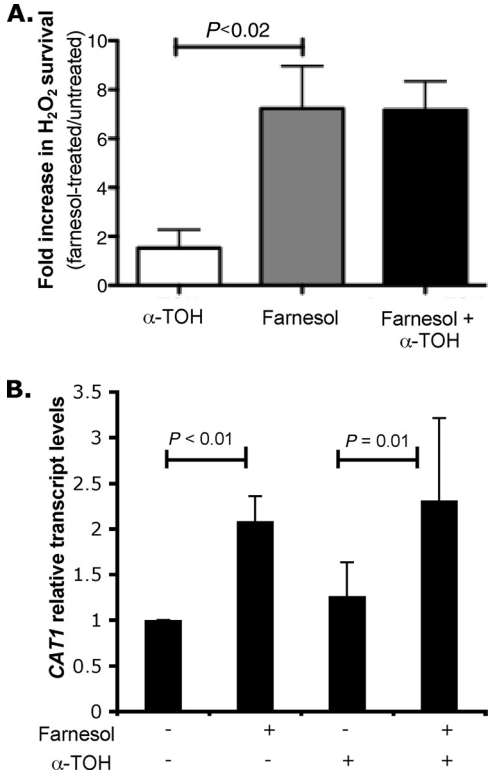


FIG. 3. (A) Effect of α-tocopherol on farnesol protection against H_2O_2. α-Tocopherol, farnesol, or both (50 μM) were added simultaneously to exponential-phase *C. albicans* SC5314 cultures, followed by incubation for 2 h. The cells were then challenged or not with 10 mM H_2O_2 for 90 min, and survival was measured by counting CFU. The data are expressed as the mean value (plus SD) of three independent cultures. (B) Quantification of *CAT1* transcripts in *C. albicans* SC5314 culture. α-Tocopherol, farnesol, or both (50 μM) were added simultaneously to exponential-phase *C. albicans* SC5314 cultures, followed by incubation for 2 h. The transcript levels were normalized to *GPD1* control transcript. The data are expressed as the mean value (plus SD) of three independent cultures.

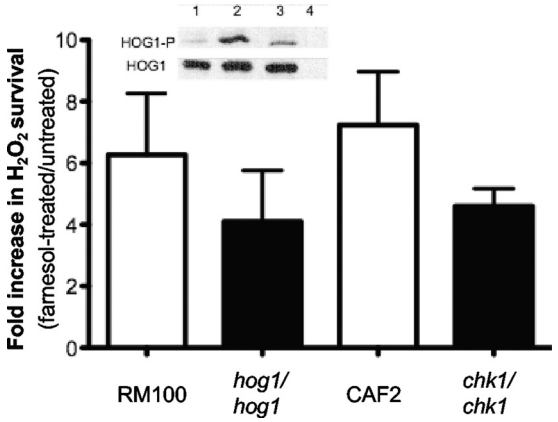


FIG. 4. Hog1 and Chk1 are not required for farnesol-mediated protection against H_2O_2 stress. Following 2 h of incubation with 50 μM farnesol in YPD at 30°C, cells were harvested and challenged for 90 min with 10 mM H_2O_2. Survival was assessed by dilution plating. The fold survival is expressed as the ratio between the survival of farnesol-treated cells and untreated cells. The parental *hog1*/*hog1* and *chk1*/*chk1* strains RM100 and CAF2 were used as WT controls. The data are expressed as the mean value (plus SD) of three independent cultures. (Inset) The phosphorylation of Hog1 in SC5314 cells was assessed by Western blotting after 30 min of incubation without any treatment (lane 1), with 10 mM H_2O_2 (lane 2), or with 50 μM farnesol (lane 3). The *hog1*/*hog1* strain is shown as a control (lane 4).

To determine if the suppression of ROS generated by farnesol would decrease the protective effect of farnesol against subsequent challenge with H_2O_2, WT cells were pretreated with α-TOH, farnesol, or both for 2 h, washed, and then exposed to H_2O_2. The numbers of CFU were determined in H_2O_2- and mock-treated cultures. Pretreatment with α-TOH alone did not alter OX stress resistance, and the combination of farnesol and α-TOH was as effective as farnesol alone in terms of inducing protection against H_2O_2 stress (Fig. 3A), suggesting that ROS generation is not the primary mechanism by which increased oxidative stress resistance occurs.

To confirm that *CAT1* induction by farnesol still occurs in the presence of the antioxidant α-TOH, we followed the transcript levels of *CAT1* in response to α-TOH, farnesol, or both. As expected, farnesol induced increased levels of *CAT1* transcript, and this induction was not suppressed by cotreatment with α-TOH (Fig. 3B) ($P \leq 0.01$; *t* test). This reinforces our hypothesis that ROS generation is not essential for farnesol-mediated induction of *CAT1* expression and oxidative-stress resistance.

Hog1 MAP kinase and Chk1 histidine kinase are not necessary for farnesol protection against H_2O_2 killing. Several signaling pathways have been described as regulating *CAT1* expression. The Hog1 MAP kinase pathway plays an important role in the adaptive response to harsh OX stress in *C. albicans* (8, 39). OX stress is sensed by the membrane-associated protein Sln1, which activates a MAP kinase cascade leading to the phosphorylation of Hog1 (Hog1-P). Once phosphorylated, Hog1-P is translocated into the nucleus, where it activates OX stress response genes, including *CAT1*. Farnesol has been reported to induce the phosphorylation of Hog1, though the cause and the consequences of this increased phosphorylation have not been determined (61). We repeated this result (Fig. 4, inset), and farnesol effects on Hog1 phosphorylation are discussed further below. Since *CAT1* expression is correlated with increased survival against H_2O_2 (Fig. 1B), we tested the hypothesis that Hog1 is necessary for farnesol-induced OX stress resistance by measuring the effects of farnesol pretreatment on survival after H_2O_2 exposure in *hog1*/*hog1* and WT strains. Like the WT reference strain, the *hog1*/*hog1* strain was more resistant to H_2O_2 after pretreatment with farnesol (Fig. 4). The level of protection in the *hog1*/*hog1* mutant was slightly lower than in the WT, although the decrease was not statistically significant (*t* test; $P > 0.05$).

Because deletion of the two-component histidine kinase Chk1 leads to sensitivity to OX stress and filamentation is no longer inhibited by farnesol in the *chk1*/*chk1* mutant (30, 34), we also tested the involvement of Chk1 in farnesol-mediated resistance to H_2O_2. Like the *hog1*/*hog1* mutant, the *chk1*/*chk1* mutant was still protected from H_2O_2-mediated killing by farnesol. Only a small, nonsignificant decrease in farnesol-induced protection against OX stress was observed (*t* test; $P > 0.05$) (Fig. 4). Thus, neither the Hog1 MAP kinase nor the

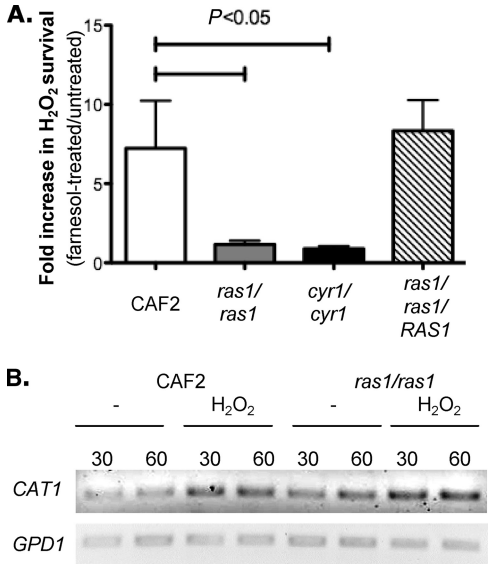


FIG. 5. (A) *ras1*/*ras1* and *cyr1*/*cyr1* mutants lack farnesol-mediated protection against H_2O_2 stress. Survival was assessed as described in the legend to Fig. 4. The fold survival is expressed as the ratio between the survival of farnesol-treated cells and untreated cells. The data are expressed as the mean value (plus SD) of three independent cultures. (B) Expression of *CAT1* and *GPD1* in *C. albicans* CAF2 and *ras1*/*ras1* cultures in response to H_2O_2. Cells were challenged with 10 mM H_2O_2 for 30 or 60 min. The experiments were performed independently three times, and the RT-PCR is representative of the results obtained each time.

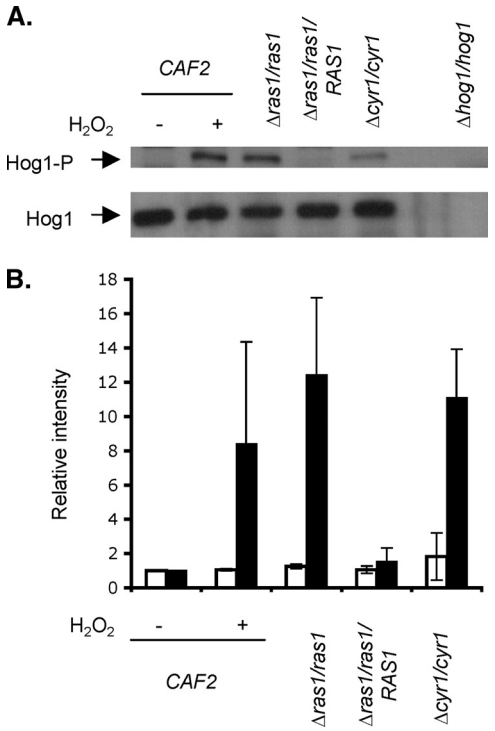


FIG. 6. The Ras-cAMP cascade inhibits Hog1 phosphorylation. (A) Western blot of Hog1 and Hog1-P. Washed cells from overnight cultures of the wild-type CAF2, Δ*ras1*/*ras1*, Δ*cyr1*/*cyr1* Δ*ras1*/*ras1*/*RAS1*, and Δ*hog1*/*hog1* strains were resuspended in YPD and incubated for 2 h at 30°C. The CAF2 cells were then treated with 10 mM H_2O_2 (+) or water (−), and all strains were incubated for another 30 min. Cells were collected and processed for Western blot analyses of Hog1 and Hog1-P levels. Coomassie staining was used to visualize and normalize the total amounts of proteins. The experiments were performed independently at least three times, and the Western blot is representative of the results obtained each time. (B) Quantification of Hog1 protein (white bars) and Hog1 phosphorylation (black bars) levels in the different genetic backgrounds. The levels are expressed relative to the intensity measured in the control treatment. All data were normalized to Coomassie-stained SDS-PAGE protein levels. The data are expressed as the mean value (plus SD) of two independent experiments.

Chk1 histidine kinase signaling pathway is required for farnesol-mediated protection against OX stress.

The Ras-cAMP pathway is necessary for farnesol-mediated protection against H_2O_2. We have previously reported that farnesol inhibits Ras1-adenylate cyclase signaling, leading to the inhibition of hyphal growth (10). As the Ras-cAMP cascade represses the expression of OX stress response genes (1, 24, 66), we sought to test the hypothesis that farnesol-mediated inhibition of this pathway leads to the increased transcription of *CAT1* and protection against ROS in yeast. To do this, we measured the effects of farnesol on *ras1*/*ras1* and *cyr1*/*cyr1* mutant survival after treatment with H_2O_2. Consistent with data reported by Bahn et al. (1), both *ras1*/*ras1* and *cyr1*/*cyr1* were 10 times less sensitive to H_2O_2 than the WT (data not shown). Contrary to what is observed with the wild type, *ras1*/*ras1* and *cyr1*/*cyr1* mutants did not gain increased ROS protection upon incubation with farnesol (Fig. 5A) ($P < 0.05$; *t* test). The *ras1*/*ras1* strain complemented with one copy of the *RAS1* gene was protected by farnesol as well as the wild type (Fig. 5A). Ras-cAMP mutants are thought to be more resistant to OX stress because of increased expression of OX stress response genes (1, 24). Therefore, *ras1*/*ras1* and *cyr1*/*cyr1* cells may not respond to farnesol because *CAT1* is maximally derepressed. To test this hypothesis, we measured the level of expression of *CAT1* in response to 10 mM H_2O_2 in WT and *ras1*/*ras1* cells. Consistent with previous data (1, 24), the level of *CAT1* transcripts was higher in the *ras1*/*ras1* than in the WT cells (Fig. 5B). However, treatment with H_2O_2 induced *CAT1* transcript levels in both backgrounds, indicating that *ras1*/*ras1* cells are still able to respond to OX stress transcriptionally. We previously reported quantitative RT-PCR data that showed that farnesol leads to large induction in *CAT1* transcript levels in WT cells, but not in *ras1*/*ras1* or *cyr1*/*cyr1* mutants (10). Altogether, these data are consistent with the inhibition of Ras-cAMP signaling as an important mechanism for farnesol protection against OX stress.

The existence of cross talk between the Ras-cAMP pathway and the Hog1 MAP kinase cascade has been suggested in *Saccharomyces cerevisiae* (3, 55, 64). In light of our data showing that farnesol acts via the Ras-cAMP pathway to enhance OX stress resistance and that Hog1 phosphorylation is increased in the presence of exogenous farnesol, we determined if there was also a potential connection between these two pathways in *C. albicans*. We studied the amounts of Hog1 and the level of Hog1 phosphorylation in the *ras1*/*ras1* and *cyr1*/*cyr1* backgrounds. We did not observe any difference in the concentrations of Hog1 protein in the different backgrounds (Fig. 6). In contrast, Hog1 phosphorylation was significantly increased in *ras1*/*ras1* and *cyr1*/*cyr1* strains (Fig. 6). The comple-

mentation of *ras1*/*ras1* with one allele of *RAS1* partially complemented the increased phosphorylation of Hog1 in the *ras1*/*ras1* background. This suggests that the Ras1-cAMP cascade inhibits the phosphorylation of Hog1. Therefore, the phosphorylation of Hog1 in response to farnesol is likely to be at least partially a consequence of the inhibition of the Ras1-cAMP pathway by farnesol.

DISCUSSION

Farnesol induces increased resistance to oxidative stress (Fig. 1) (65). We have shown that the accumulation of ROS in the presence of farnesol was not necessary for subsequent protection against OX stress (Fig. 3A). Instead, our data suggest that farnesol-mediated induction of catalase expression and ROS resistance in yeast occurred mainly by inhibition of the Ras1-cAMP pathway (Fig. 5A), though Hog1 or Chk1 regulators may also participate in the response. While Hog1 was not necessary for farnesol-mediated protection against H_2O_2, Hog1 phosphorylation increased in the presence of farnesol, as has been shown previously (61). We demonstrated that the disruption of the Ras1-cAMP cascade led to a marked increase in Hog1 phosphorylation even in the absence of farnesol or other sources of oxidative stress, indicating the presence of undescribed links between the Ras1-cAMP and Hog1 MAP kinase pathways.

It is interesting that *C. albicans* employs a molecule that can generate ROS for the purpose of cell-cell signaling. Farnesol has been previously shown to be poisonous to bacteria, fungi, and mammalian cells (4, 27, 37, 47, 54, 56). In contrast, farnesol toxicity for *C. albicans* is conditional (31), and several studies report a complete absence of lethality (10, 26, 45). Work by Machida et al. (37) suggests that ROS accumulation in the presence of farnesol occurs by perturbation of the mitochondrial electron transport chain in *S. cerevisiae*. Consistent with farnesol acting in a membrane environment, farnesol-induced ROS were only partially suppressed by ascorbic acid, a hydrophilic antioxidant that acts in the cytosol and at the membrane interface (38), and were more fully suppressed by α-TOH, which inserts within membranes (23). The isoprenoid structure of farnesol suggests that it does not directly generate ROS. *C. albicans* seems to be protected against the ROS generated by endogenous farnesol under normal culture conditions (10, 26, 45). This may be due to the signaling roles of farnesol that induce OX stress response genes, the fact that farnesol is at high concentrations when *C. albicans* cultures are in stationary phase, or the presence of other regulated resistance mechanisms, such as efflux pumps. Understanding how *C. albicans* can resist the potential toxicity of farnesol may provide interesting and important insights into *C. albicans* physiology.

The finding that farnesol protects against H_2O_2 killing via inhibition of the Ras1-Cyr1-PKA signaling pathway indicates that farnesol acts in the same way under hypha-inducing (10) and noninducing conditions but leads to different phenotypes. The increase of cellular levels of cAMP activates the Tpk2 subunit of protein kinase A which, mediates the inhibition of the transcription of OX stress-related genes, such as catalase and superoxide dismutase genes (Fig. 7A) (10, 21). The repression of the Ras1 cascade by farnesol would thus relieve the inhibition maintained on catalase transcription by Tpk2. Our

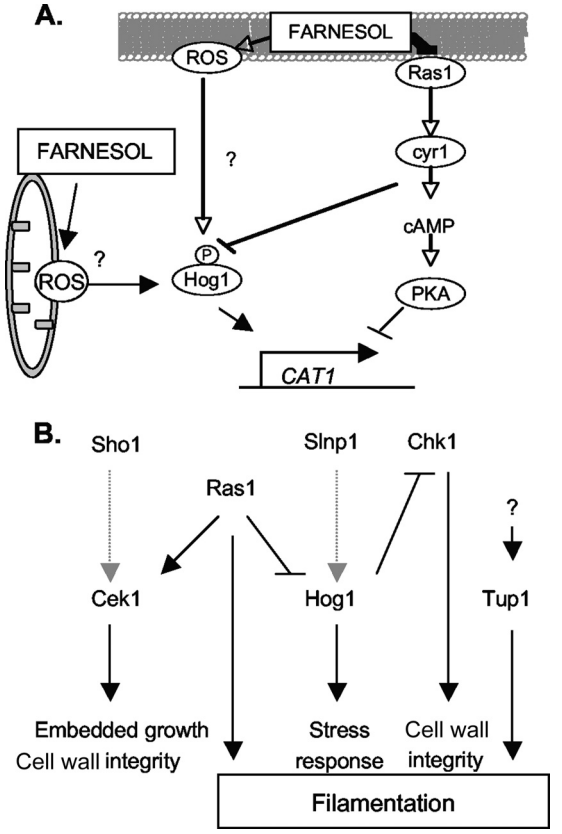


FIG. 7. (A) Proposed model for the mechanism of farnesol protection against OX stress. (B) Interaction between farnesol-associated signaling pathways (2, 12, 33–35, 49, 50).

data indicate that Hog1 phosphorylation is negatively regulated by the Ras1-cAMP cascade via an unknown intermediate (Fig. 6) but that the contribution of Hog1 to the farnesol-mediated protection against OX stress was minor (Fig. 4). It is possible that the contribution of Hog1 has been partially masked by the strong repression of *CAT1* by PKA. It remains to be determined how much ROS production and Ras1 inhibition contributed to Hog1 phosphorylation in the presence of farnesol. Treatment of cells with farnesol and α-TOH did not provide answers, because α-TOH induced Hog1 phosphorylation (data not shown). Moreover, the role of Hog1 may have been hidden by the activity of the transcription factor Cap1, which controls oxidative-stress resistance gene expression (15) in direct response to intracellular ROS. Oxidant conditions within cells inhibit the export of Cap1 from the nucleus and allow Cap1 to activate the transcription of OX stress-related genes (25). Farnesol-mediated ROS production likely activates Cap1, but since suppression of farnesol-induced ROS by antioxidants did not suppress farnesol protection against OX stress (Fig. 3A), Cap1 is not likely a major player in farnesol-induced ROS resistance.

While it has been well established that downregulation of the cAMP signaling pathway increases resistance to stresses (1, 24, 66), perhaps aiding in *C. albicans* survival of host defenses, it is not known how Ras1-cAMP inhibits the expression of stress response genes. Our finding that the Ras1-cAMP cas-

cade negatively regulates Hog1 phosphorylation (Fig. 6), through a mechanism yet to be described, could partially explain this increased resistance. Partial epistasis between Hog1 and Ras-PKA signaling has been also established in *S. cerevisiae*, although its mechanism is unknown, as well (3, 55, 64). PKA could maintain the phosphorylation of Slnp1 or Ssk1 upstream of Hog1 (8), but an intermediate kinase would be necessary, as PKA specifically phosphorylates serine and threonine residues while regulation of the activities of members of the Hog1 MAP kinase pathway involves histidine or aspartic acid phosphorylation. In mammalian cells, cAMP inhibits the phosphorylation of p38, the homolog of Hog1, via the CREB pathway (17, 68). Evidence for the existence of a functional CREB-like protein in *C. albicans* (cAMP response element binding protein) has recently been reported, and such a regulator may mediate Ras1 control of *CAT1* and other stress response genes (58).

The finding of cross talk between the Ras1-cAMP cascade and the Hog1 MAP kinase pathway provides new insight into the understanding of the mechanism of action of farnesol. So far, farnesol has been shown to affect three different signaling pathways activated by multiple environmental cues—the Ras-cAMP pathway (10), the Hog1 MAP kinase pathway (61), and the Cek1 MAP kinase pathway (49)—and two other regulators, Chk1 (30) and the transcription factor Tup1 (29), are resistant to the effects of farnesol. The mechanism by which farnesol modulates the activities of these pathways is still unknown, as no receptor or target has been reported. Farnesol could act independently on each pathway or have a unique target that connects all of the pathways. Cross-regulation between all of these pathways via Ras1 and Hog1 has been established (Fig. 7B), except for Tup1. Because Ras1 associates with membranes and because the lipophilic nature of farnesol strongly predicts its association with membranes, Ras1 appears to be a good candidate for the master receptor of farnesol effect. The environmental conditions also influence which of these pathways are active or dominant, as they are not all necessarily active at the same time. An alternative hypothesis could be that farnesol may disturb, by altering membrane structure, the sensors Sho1, Chk1, and Ras1, which are associated with the membranes and sit on top of each pathway.

The data we have presented in this study were obtained by the addition of exogenous farnesol. Evidence indicates that endogenously produced farnesol also protects against killing by H_2O_2 (65). Farnesol is constantly produced by *C. albicans* cells and reaches its maximal concentration at the entrance to stationary phase, according to Hornby et al. (26). Interestingly, the expression of *CAT1* and the resistance to OX stress are much higher in stationary-phase cells than in exponential-phase cells (65). Farnesol is also thought to accumulate within biofilms and to be responsible for the dispersal of biofilms (44, 45). From our results and those of Westwater et al. (65) linking farnesol and OX stress resistance, we expect that cells released from biofilm in response to farnesol would be highly resistant to OX stress. Cells liberated from *Candida* biofilms grown on implanted biomaterials, such as catheters or heart valves, directly reach the bloodstream, where they encounter macrophages and cells from the innate immune system, which uses OX stress to kill pathogens. Following this model, cells released from biofilms in response to farnesol would be strongly resistant to killing by the primary immune system. Thus, the role of farnesol in *C. albicans* pathogenicity may not be limited to the regulation of morphogenesis but may also include resistance to the host immune response. Further analyses of the links between biofilm dispersal, farnesol production, and resistance to OX stress should provide new and valuable insights into *Candida* pathogenesis that may lead to new strategies for the development of antifungal drugs.

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