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Bessie W. Kebaara
University of Nebraska - Lincoln

Melanie L. Langford
University of Nebraska - Lincoln

Dhammika H. M. L. P. Navarathna
University of Nebraska - Lincoln

Raluca Dumitru
University of Nebraska - Lincoln

Kenneth W. Nickerson
University of Nebraska - Lincoln, knickerson1@unl.edu

See next page for additional authors

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Candida albicans Tup1 Is Involved in Farnesol-Mediated Inhibition of Filamentous-Growth Induction▽

Bessie W. Kebaara,† Melanie L. Langford,† Dhammika H. M. L. P. Navarathna,‡ Raluca Dumitru,§ Kenneth W. Nickerson, and Audrey L. Atkin*

School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0666

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Candida albicans **is a dimorphic fungus that can interconvert between yeast and filamentous forms. Its ability to regulate morphogenesis is strongly correlated with virulence. Tup1, a transcriptional repressor, and the signaling molecule farnesol are both capable of negatively regulating the yeast to filamentous conversion. Based on this overlap in function, we tested the hypothesis that the cellular response to farnesol involves, in part, the activation of Tup1. Tup1 functions with the DNA binding proteins Nrg1 and Rfg1 as a transcription regulator to repress the expression of hypha-specific genes. The *tup1*/*tup1* and *nrg1*/*nrg1* mutants, but not the *rfg1*/*rfg1* mutant, failed to respond to farnesol. Treatment of *C. albicans* cells with farnesol caused a small but consistent increase in both *TUP1* mRNA and protein levels. Importantly, this increase corresponds with the commitment point, beyond which added farnesol no longer blocks germ tube formation, and it correlates with a strong decrease in the expression of two Tup1-regulated hypha-specific genes, *HWP1* and *RBT1*. Tup1 probably plays a direct role in the response to farnesol because farnesol suppresses the haploinsufficient phenotype of a *TUP1*/*tup1* heterozygote. Farnesol did not affect *EFG1* (a transcription regulator of filament development), *NRG1*, or *RFG1* mRNA levels, demonstrating specific gene regulation in response to farnesol. Furthermore, the *tup1*/*tup1* and *nrg1*/*nrg1* mutants produced 17- and 19-fold more farnesol, respectively, than the parental strain. These levels of excess farnesol are sufficient to block filamentation in a wild-type strain. Our data are consistent with the role of Tup1 as a crucial component of the response to farnesol in *C. albicans*.**

Candida albicans is the opportunistic fungal pathogen most commonly isolated in humans. *C. albicans* is part of the normal flora, and it resides in the gastrointestinal and genitourinary tracts, as well as on the skin. However, *C. albicans* is capable of causing a wide range of diseases, from mild mucosal infections to life-threatening systemic infections termed candidemia (19). Vulnerable patients include those with AIDS and patients undergoing chemotherapy and organ transplantation (19). The annual cost of treating candidiasis in the United States was estimated to be 1 billion dollars, and the mortality rates for patients with candidiasis are 30 to 50%, even with antifungal treatment (28), indicating a need for new antifungal drugs.

The ability of *C. albicans* to cause disease has been strongly linked to its conversion between two distinct morphological forms, yeast and filamentous. Recently, our research has focused on farnesol, the first quorum-sensing molecule discovered in a eukaryote (17). Farnesol is a virulence factor (35) that is excreted continuously by *C. albicans* (17), and when it accumulates beyond a threshold level, it blocks the yeast to filament conversion (17). Stationary-phase cultures of *C. albicans* have accumulated 2 to 4 μM farnesol (17), and the 50% inhibitory concentration value for blocking germ tube formation (GTF) in an *N*-acetylglucosamine-stimulated assay is ca. 1 to 2 μM (*E*,*E*-)farnesol (17, 31, 39), and consequently, these farnesol production levels are physiologically relevant. Other roles described for farnesol include biofilm inhibition (36), protection from oxidative stress (46), and induction of apoptosis in another fungus, *Aspergillus nidulans* (38). While many phenotypic effects produced by farnesol have been described, little is understood about farnesol's mode of action.

In addition to farnesol, *C. albicans* yeast and filamentous growth are controlled by an assortment of signaling pathways (4, 13). The yeast-to-filamentous form conversion is activated by many pathways, including components of the *CEK1* mitogen-activated protein (MAP) kinase pathway, the Ras/cyclic AMP-dependent pathway, the calcium/calmodulin signaling pathway, the Rim101-independent pathway, and the Chk1 two-component signal transduction pathway. Although each pathway has been implicated in filamentation (10, 26, 37), these pathways show some degree of specialization in that they respond to different environmental inducers. The activation and inhibition of filament development are accomplished largely through changes in gene expression mediated by transcription activators and repressors. Efg1 is a major transcription regulator of filamentous growth and is a central control point for many signaling pathways involved in filamentation (14). Efg1 also regulates the expression of multiple genes, including those involved in virulence (6, 29). Mechanisms have also been identified that block filament development, with Tup1 playing a key role in transcriptional repression (5, 6).

Farnesol is able to block filamentous growth induced by environmental signals for most, and possibly for all, of the signaling pathways activating filament development. These sig-

* Corresponding author. Mailing address: School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0666. Phone: (402) 314-5571. Fax: (402) 472-8722. E-mail: aatkin1@unl.edu.
† Bessie W. Kebaara and Melanie L. Langford contributed equally to this work.
‡ Present address: Biomedical Pathology, NIH, Bethesda, MD 20892-1500.
§ Present address: Neuroscience Center, University of North Carolina, Chapel Hill, NC 27599.
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nals include 10% serum, 10 mM L-proline, 2.5 mM *N*-acetylglucosamine, or the combination of *N*-acetylglucosamine and L-proline, all at 37°C (17). Thus, farnesol may individually block each of the morphogenic signaling pathways and/or act at a common control point in morphogenesis. Tup1 repression of filament-specific genes is an attractive candidate for a common control point that may be regulated by farnesol (21).

The *C. albicans* Tup1 protein is a transcription regulator that plays two key roles in the cell: (i) regulation of phase switching, and (ii) inhibition of filamentous growth. Tup1 interacts with the corepressor protein Ssn6 or Tcc1. These complexes function with DNA binding proteins to repress gene expression (23, 42). At least three DNA-binding proteins have been identified that function with Tup1: Nrg1 (homologous to the *Saccharomyces cerevisiae* Nrg1p protein), Rfg1 (homologous to the *S. cerevisiae* Rox1p protein), and Mig1 (homologous to the *S. cerevisiae* Mig1p protein). Homozygous *tup1* mutants are unable to grow as yeast and instead remain locked in the filamentous form, in all media tested (5). Deletion of *TUP1* results in the up-regulation of approximately one-third of *C. albicans* genes (32, 33), and these mutants are also avirulent in a murine model of infection. The activation of Tup1 transcription repressor complexes results in the repression of filament-specific gene expression (5, 6, 32, 33).

Here, we tested the hypothesis that the *C. albicans* response to farnesol involves Tup1. The morphological response to farnesol was tested with wild-type and *tup1*/*tup1*, *tup1*/*TUP1*, *nrg1*/*nrg1*, and *rfg1*/*rfg1* strains to assess the requirement for these genes in the farnesol response. The morphological response and gene expression pattern for *MIG1* were not determined because the Mig1 protein does not play a role in the filamentous growth of *C. albicans* (32). The gene expression patterns of *TUP1*, *NRG1*, *RFG1*, and *EFG1*, as well as genes under their control, were examined in the presence or absence of farnesol by quantitative Northern and Western analyses. Finally, we compared farnesol production levels in *tup1*, *nrg1*, and *rfg1* homozygous mutants relative to that in wild-type cells.

MATERIALS AND METHODS

Strains and media. *Candida albicans* SC5314 is an independent clinical isolate and the reference strain for the *Candida* genome sequence (1). *C. albicans* strains CAF-2 (*ura3*::*imm434*/*URA3*) and CAI-4 (*ura3*::*imm434*/*ura3*::*imm434*) are derived from SC5314 by gene replacement (16). Strains BCa2-9 (*tup1*/*tup1* in CAI-4 [5]), BCa2-10 (*tup1*/*tup1*, frameshift disruption fragment in CAI-4 [5]), DU152 (*nrg1*/*nrg1* in CAI-4 [5]), DU129 (*rfg1*/*rfg1* in CAI-4 [22]), BCa05, which expresses *TUP1* ectopically (*tup1*/*tup1*, *MAL3*::*p455* in CAI-4 [3, 5]), and BCa2-3 (*TUP1*/*tup1* in CAI-4 [5]) were obtained from Alexander Johnson, University of California, San Francisco, CA. Strain MEN was provided by Richard Cannon, University of Otago, Dunedin, New Zealand.

Resting cells were obtained by growing cells in modified glucose salts biotin media (mGSB) overnight, washing them three times with 50 mM phosphate (pH 6.5), resuspending them in 10 ml of 50 mM phosphate, and storing them at 4°C, to be used within a month.

The defined glucose-salts medium GPP (pH 4.8) contained (per liter of distilled water) glucose, 20 g; L-proline, 1.15 g; NaH_2PO_4, 3.2 g; KH_2PO4, 4 g; $MgSO_4 \cdot 7H_2O$, 0.5g; $CuSO_4 \cdot 5H_2O$, 1 mg; $ZnSO_4 \cdot 7H_2O$, 1 mg; $MnCl_2$, 1 mg; $FeSO_4$, 1 mg; biotin, 20 μg; pyridoxine · HCl, 200 μg; thiamine · HCl, 200 μg. The glucose (20% [wt/vol]) and L-proline (100 mM) were autoclaved separately and added aseptically, as were the filter-sterilized vitamins (27). Modified GPP (mGPP) also contained 2.5 mM *N*-acetylglucosamine (17). GPP (pH 6.8) contained 3.2 g/liter Na_2HPO_4 instead of NaH_2PO_4. For maltose phosphate proline (MPP) medium, filter-sterilized maltose replaced the glucose. Cornmeal agar (Difco, Detroit, MI) was also used. Solid medium included 2% (wt/vol) agar. All media for CAI-4 included uridine at 40 μg/ml.

Microscopy. Differential interference contrast images were produced with an Olympus BX51 microscope, and colony morphology photographs were made with an Olympus SZX12 microscope.

Quantitative Northern blotting analysis. To measure mRNA accumulation, SC5314 resting cells were inoculated in mGPP to an optical density at 600 nm of 0.5 to 0.6 and allowed to equilibrate at 37°C for 5 min, whereupon 20 μM farnesol was added to half of the flasks. Cells were grown at 37°C for 0, 20, 40, 60, and 80 min, until the cells were harvested, and total RNA was extracted by the hot phenol method (24). Equal amounts of RNA (15 μg) were resolved on 1.0% agarose-formaldehyde gels, and the RNA was transferred to GeneScreen Plus (NEN Life Science Products, Inc., Boston, MA), using the capillary blot transfer protocol recommended by the manufacturer. The Northern blots were probed with radiolabeled DNA probes. The probe DNA used for synthesis was prepared by PCR using MEN genomic DNA. The probes were labeled with [^{32}P]dCTP (GE Health Sciences, Piscataway, NJ), using an oligo labeling kit, RadPrime DNA labeling system, following the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA). Northern blots were phosphorimaged using a Storm phosphorimager (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and quantified using ImageQuant software (version 5.0; Molecular Dynamics, Sunnyvale, CA). mRNA abundance measurements were done using a minimum of three independent Northern blots.

Western blotting analysis. Western blots were prepared as previously described (2), and Tup1 and Act1 proteins were detected with a Supersignal West Pico chemiluminescent substrate, using the manufacturer's protocol (Pierce, Rockford, IL), except that blocking was done with 5% nonfat dried milk. Rabbit polyclonal antibodies against Tup1 were previously described (18). Mouse monoclonal anti-Act1 antibodies and horseradish peroxidase-labeled ant-rabbit immunoglobulin G antibodies were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Horseradish peroxidase-labeled anti-mouse antibody was from Perkin-Elmer (Boston, MA).

Analysis of farnesol levels. Extracellular farnesol was extracted from cell-free supernatants of cultures grown in mGPP at 30°C and analyzed by gas chromatography-mass spectrometry as described previously (17).

RESULTS

The *tup1*/*tup1* and *nrg1*/*nrg1* mutants lack a morphological response to farnesol, while the *rfg1*/*rfg1* mutant responds to farnesol. The juxtaposition of farnesol's ability to inhibit differentiation and the role of Tup1 as a transcription repressor for filamentation genes suggest that farnesol could function by activating Tup1 and/or one of its coregulators, Nrg1 or Rfg1. Consequently, we examined the effect of farnesol on the morphology of null mutants lacking *TUP1*, *NRG1*, and *RFG1*. As a control, the wild-type *C. albicans* SC5314 in filament-inducing media grew as yeast in the presence of 20 μM farnesol and as filaments in media lacking farnesol, demonstrating a positive response to farnesol (Fig. 1). The *rfg1*/*rfg1* mutant responded to 20 μM farnesol in a manner similar to that of SC5314 (Fig. 1). Unlike SC5314 and *rfg1*/*rfg1*, the *tup1*/*tup1* and *nrg1*/*nrg1* mutants lacked a detectable response to farnesol and remained filamentous in the presence of 20 μM farnesol (Fig. 1). The filamentous-only cell morphology is the phenotype expected for these known mutants (5, 7, 25, 32, 33). However, in this regard, the *tup1*/*tup1* and *nrg1*/*nrg1* mutants differ from the great majority of filamentous-only mutants recovered from a previous study, 96% of which reverted to a smooth (yeast) colony morphology on yeast malt (YM) agar plates with 50 μM farnesol (20). For the *tup1*/*tup1* mutant, the lack of response to farnesol was specific for the loss of Tup1 because we found that ectopic expression of *TUP1* (5) restores the strain's ability to respond to farnesol (data not shown).

***TUP1* mRNA levels increase in the presence of farnesol, while *RFG1* and *NRG1* mRNA levels were not affected by farnesol.** We analyzed the effect of farnesol on *TUP1* mRNA levels over time in *C. albicans* SC5314 cells that had been

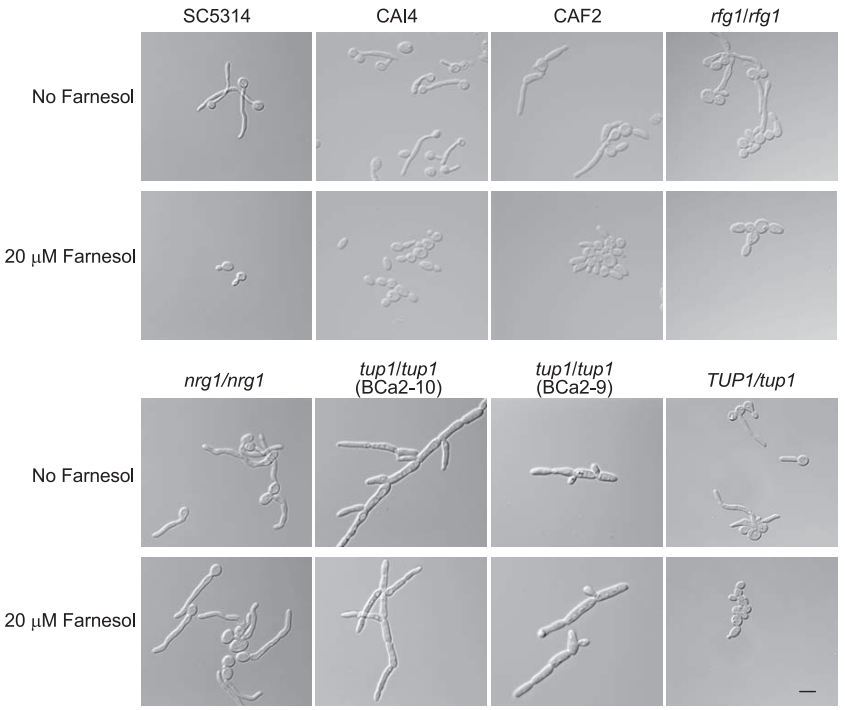

FIG. 1. Response to farnesol by *C. albicans* under conditions that promote GTF and hyphal growth. SC5314, CAI4, CAF2, *rfg1*/*rfg* (DU129), *nrg1*/*nrg1* (DU152), *tup1*/*tup1*(BCa2-9), *tup1*/*tup1* (BCa2-10), and *TUP1*/*tup1* (BCa2-3) resting cells were inoculated into mGPP (pH 4.8) medium at 37°C in the presence or absence of 20 μM farnesol, and their cell morphologies were examined at 4 h. Scale bar = 10 μm.

induced to commence germ tube formation (GTF) by growth at 37°C in mGPP. We previously showed (31) that under these conditions, the first germ tubes appeared at 30 min and the process was complete by 110 min. Furthermore, farnesol no longer blocked GTF when it was added at 60 to 90 min after inoculation (27). Here, our analysis was designed to evaluate changes in *TUP1* mRNA just before GTF, when the cells were still responsive to farnesol. Filamentation was induced by transferring resting cells into mGPP (pH 4.8) at 37°C in the presence and absence of 20 μM farnesol, and mRNA levels were determined at 0, 20, 40, 60, and 80 min following induction. In all experiments, the *TUP1* mRNA levels decreased over the first 20 min and then increased (Fig. 2A). This pattern is consistent with the single-time-point result of Toyoda et al. (45), who showed that *TUP1* mRNA levels increased slightly at 180 min after induction of filamentation. In the presence of farnesol, we found that *TUP1* mRNA consistently increased 2.5-fold ± 0.6-fold ($n = 4$) from 20 to 60 min. Importantly, this is the time period just prior to that at which the cells become committed and are no longer responsive to farnesol (31). In contrast, in the absence of farnesol, there was very little increase (1.4 ± 0.3; $n = 4$) in *TUP1* mRNA levels from 20 to 60 min (Fig. 2A). Thus, farnesol (20 μM) causes a consistent increase in *TUP1* mRNA levels during the precise time period when it blocks GTF. This increase of 2.5-fold in *TUP1* mRNA corresponded to an increase in SC5314 Tup1 protein levels at 60 min following induction (Fig. 3). Tup1 protein in SC5324 was increased in all three replicate experiments by an average of 2.5-fold.

Since Tup1 functions with DNA binding proteins such as Rfg1 and Nrg1, and in *C. albicans* strain JCM9061 the *NRG1* mRNA levels decreased during filamentation (45), we tested the effect of farnesol on the *RFG1* and *NRG1* mRNA levels during differentiation from yeast to filamentous form. Like *TUP1* mRNA, the *RFG1* mRNA levels initially decreased and then increased (data not shown). However, unlike *TUP1* mRNA, the timing and magnitude of the *RFG1* mRNA level changes were similar in the presence and the absence of farnesol (Fig. 4, data not shown). Under the same conditions, the *NRG1* mRNA levels did not change during development, and they too were the same in the presence and absence of farnesol (Fig. 4). Thus, we conclude that farnesol does not affect the *RFG1* or *NRG1* mRNA levels.

Expression of the Tup1-regulated filamentation genes *HWP1* and *RBT1* is inhibited by farnesol. To determine whether the increased *TUP1* expression in the presence of farnesol was biologically significant, we examined the expression of two Tup1-regulated genes, *HWP1* and *RBT1* (Fig. 2B and C [12]). In the absence of farnesol, the *HWP1* and *RBT1* transcripts were undetectable at time zero, but they were strongly expressed from 40 to 80 min (Fig. 2B and C). Farnesol delays and dramatically reduces the magnitude of *HWP1* and *RBT1* mRNA expression (Fig. 2B and C). At 80 min, *HWP1* and *RBT1* levels were 30- and 7.6-fold lower, respectively, in farnesol-treated cells than in untreated cells. Similar results were observed by Davis-Hanna et al. (11) for *HWP1* mRNA at 2 h after treatment with 75 μM farnesol. Thus, there is a strong correlation between elevated *TUP1* expression in response to farnesol and the expression of Tup1-regulated genes.

***EFG1* mRNA levels remain unaffected by farnesol.** Efg1 is a transcription regulator for genes required for filamentation. *EFG1* mRNA levels are downregulated at the initiation of

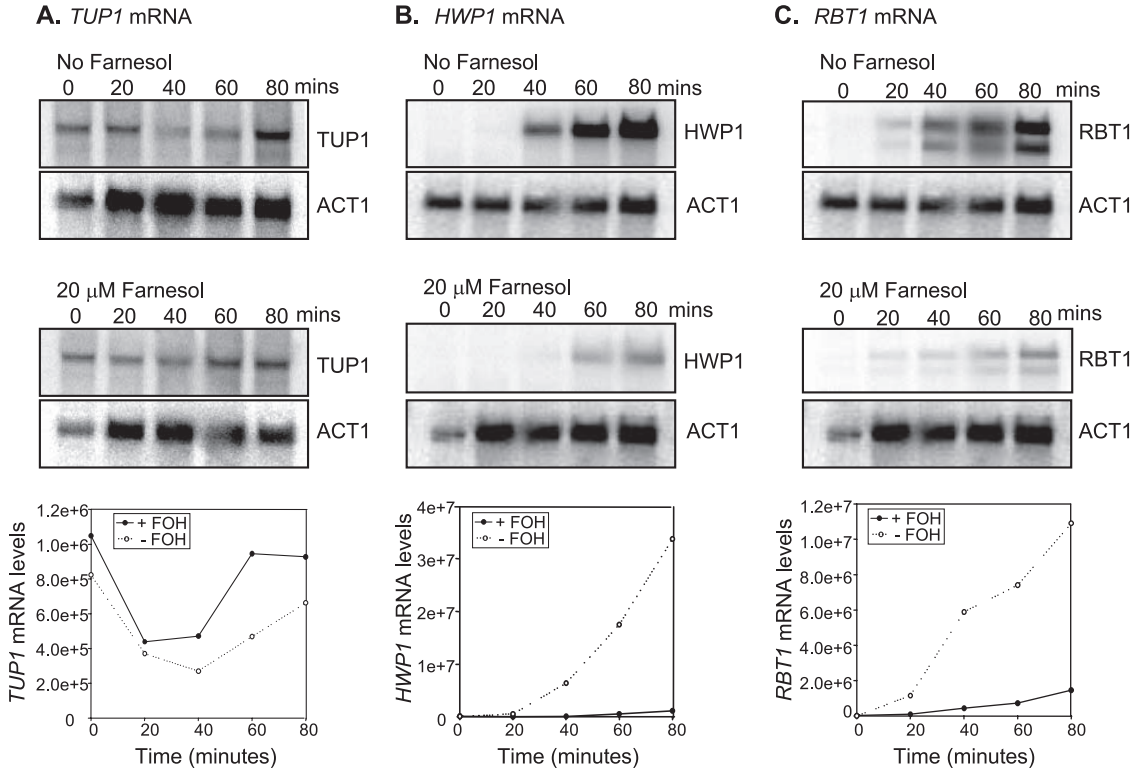

FIG. 2. *TUP1* mRNA levels increased, while two Tup1-regulated genes, *HWP1* and *RBT1*, were downregulated in the presence of farnesol (FOH). *C. albicans* SC5314 resting cells were inoculated into mGPP (pH 4.8) medium in the presence or absence of 20 μM farnesol and incubated at 37°C. Cells were then harvested at 0, 20, 40, 60, and 80 min postinoculation. Northern blots were prepared with total RNA from cells incubated in the presence or absence of farnesol. Shown is a phosphorimage of a representative Northern blot probed with radiolabeled *TUP1* DNA (A), *HWP1* DNA (B), and *RBT1* DNA (C) and a plot of average mRNA levels from a minimum of three independent experiments. *ACT1* mRNA levels were used as a loading control.

filament development and then increase as filament formation progresses (44). *HWP1* and *RBT1* are activated by Efg1 during filamentation (6). Therefore, we determined whether farnesol also affected *EFG1* mRNA levels (Fig. 4). The *EFG1* mRNA levels were high at time zero, decreased to a minimum at 20 min, and then increased steadily throughout the remaining time (data not shown). However, farnesol had no influence on *EFG1* mRNA levels, since the timing and magnitude of the changes were similar in the presence and the absence of farnesol (Fig. 4 and data not shown).

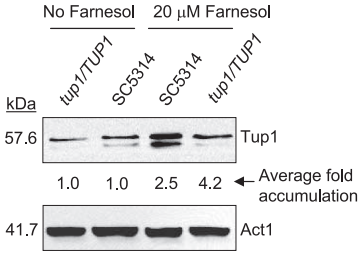

FIG. 3. Tup1 protein levels are higher in the presence of farnesol. Total protein extracts were prepared from SC5314 and *TUP1*/*tup1* (BCa2-3) resting cells inoculated into mGPP (pH 6.8) medium at 37°C in the presence or absence of 20 μM farnesol and incubated at 37°C for 60 min. The average change (fold) in Tup1 protein accumulation for farnesol-treated cells relative to that of untreated cells is shown. Act1 levels were used as a loading control.

Farnesol suppresses the haploinsufficient phenotype of a *TUP1*/*tup1* heterozygote. Braun and Johnson (5) showed that BCa2-3, a *TUP1*/*tup1* heterozygote, is haploinsufficient in that these cells develop a higher proportion of filaments than the

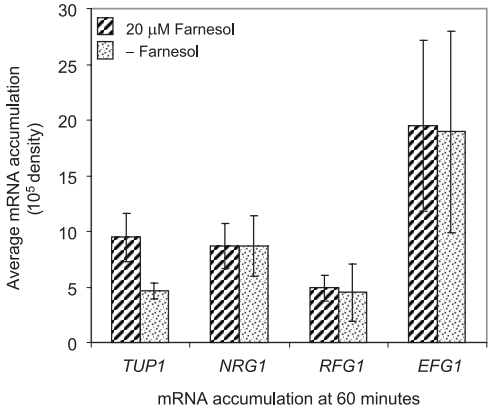

FIG. 4. Farnesol does not affect the expression of *RFG1* or *NRG1*, which encode DNA binding proteins that function with Tup1, or *EFG1*, which encodes a transcription activator of hypha-specific genes. Quantitative Northern blotting analysis was used to measure the *TUP1*, *NRG1*, *RFG1*, and *EFG1* mRNA levels in SC5314 at 60 min after the inoculation of resting cells under conditions that promote GTF in the presence and absence of 20 μM farnesol. The results are averages of three independent experiments.

TABLE 1. The *tup1*/*tup1* and *nrg1*/*nrg1* null mutants do not respond to farnesol but overproduce farnesol

C. albicans strain	Farnesol response[a]	Farnesol production (μg/g dry wt of cells) ± SD[b]	Fold increase in farnesol[c]
CAI-4	Positive	1.6 ± 0.36	
CAF-2	Positive	2.0 ± 1.30	
tup1/*tup1* (BCa2-10)	Negative	30.6 ± 6.40	17
nrg1/*nrg1* (DU152)	Negative	34.5 ± 12.2	19
rfg1/*rfg1* (DU129)	Positive	4.8 ± 2.0	2.6

[a] Farnesol responses on GPP agar with and without 20 μM farnesol, incubated at 37°C for 48 h. A positive response indicates smooth colony morphology (yeast cells) in the presence of farnesol and rough colony morphology (filamentous cells) without added farnesol. A negative response to farnesol indicates rough colony morphology in the presence and absence of farnesol.

[b] Farnesol production values (μg/g dry weight of cells) ± standard deviation (SD) were the averages of three measurements.

[c] Values are based on fold increases over 1.8, the average value for strains CAI-4 and CAF-2.

wild-type cells on most media (5). Presumably, these cells do not make enough Tup1 to compensate for the reduced gene copy number. We hypothesized that farnesol might suppress this phenotype because it increases *TUP1* expression 2.5-fold in SC5314 and 4.2-fold in *TUP1*/*tup1* (Fig. 3). This increase should restore Tup1 to roughly wild-type levels. To test this hypothesis, we examined the effect of farnesol on *C. albicans* BCa2-3 on cornmeal agar plus Tween 80, under a coverslip for 25 h at 25°C. Under these conditions, the *TUP1*/*tup1* mutant was more filamentous than the wild-type colonies but less filamentous than the *tup1*/*tup1* mutant (BCa2-10 [4]; see Table 2). As a control, the *TUP1*/*tup1* mutant was shown to respond to farnesol because, although it forms filamentous cells when grown in mGPP medium, the addition of 20 μM farnesol results in growth as yeast (Fig. 1), and Tup1 protein levels were ca. 4.2-fold higher in the *TUP1*/*tup1* mutant treated with farnesol. In contrast to the haploinsufficient phenotype observed with the absence of farnesol, in the presence of farnesol, the *TUP1*/*tup1* mutant looked identical to the wild-type *C. albicans* (see Table 2). Thus, farnesol suppresses the haploinsufficiency phenotype of the *TUP1*/*tup1* heterozygote.

The *tup1*/*tup1* and *nrg1*/*nrg1* mutants produce excess farnesol. Jensen et al. (20) tested the farnesol production levels for several filament-only mutants. A subset of these mutants produced levels of farnesol significantly higher than those of the wild-type strains. This overproduction suggests that the ability to respond to farnesol may be linked to the regulation of farnesol production. Here, we tested farnesol production levels in the CAI-4, CAF-2, *tup1*/*tup1*, *nrg1*/*nrg1*, and *rfg1*/*rfg1* strains. Farnesol production levels were dramatically increased in the *tup1*/*tup1* and *nrg1*/*nrg1* mutants (Table 1), which were unable to respond to farnesol (Fig. 1). The *tup1*/*tup1* and *nrg1*/*nrg1* mutants produced ca. 17- and 19-fold more farnesol, respectively, than did CAF-2 and CAI-4. In contrast, the farnesol-responsive *rfg1*/*rfg1* mutant produced only ca. 2.6-fold more farnesol than the wild-type strains (Table 1). Thus, the two mutants that are unable to respond to farnesol (*tup1*/*tup1* and *nrg1*/*nrg1*) produced much higher levels of farnesol than did strains that do respond to farnesol.

***tup1*/*tup1* overproduction of farnesol inhibits SC5314 filamentation.** We tested the biological significance of farnesol overproduction by sequentially plating *tup1*/*tup1* and SC5314 next to one another and observing the resultant colony morphologies. When SC5314 was plated and followed 1 day later by another streak with SC5314, a small area of filament inhibition was observed (Fig. 5B). In contrast, when *tup1*/*tup1* was plated first, followed by SC5314, a much larger area of filament inhibition was observed (Fig. 5A). These results are consistent with the *tup1*/*tup1* overproduction of farnesol. As controls, whenever *tup1*/*tup1* was plated second, no filament inhibition was observed (Fig. 5C and D).

DISCUSSION

C. albicans responds to farnesol, in part, by changing gene expression (8, 15). We hypothesize that some of these changes are mediated by changes in the activity of the signaling pathways regulating morphogenesis. Here, we show that the *tup1*/*tup1* and *nrg1*/*nrg1* null mutants are strictly filamentous strains and that the cells remain filamentous in the presence of added

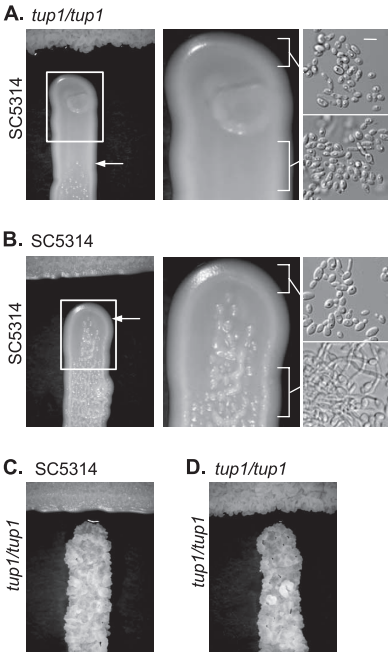

FIG. 5. Overproduction of farnesol by the *tup1*/*tup1* mutant inhibits SC5314 filamentation. Resting cells were grown at 37°C for 24 h on yeast-peptone-dextrose agar plates to allow for farnesol accumulation in the agar (horizontal streak, *C. albicans* strain SC5314 [B and C]; or *tup1*/*tup1*, BCa2-10 [A and D]). Subsequently, either SC5314 (A and B) or *tup1*/*tup1* (C and D) resting cells were plated (vertical streak) and incubated at 37°C for an additional 24 h. The areas above the two arrows (A and B, left panels) are zones of filament inhibition (as evident by smooth morphology) resulting from the farnesol produced by the horizontally streaked strains. Filamentation gives the wrinkled colony morphology seen below the arrows. The pictures in the two white boxes have been magnified ×2.5 so that the colony morphology can be seen more clearly (A and B, center panels). Micrographs of individual cells from the two bracketed regions are shown in the right panels (A and B; scale bar = 10 μm). The cells from the smooth regions are mainly yeast, while there is a much larger proportion of filamentous cells in the wrinkled region.

TABLE 2. Farnesol suppression of the *TUP1*/*tup1* heterozygote[a]

C. albicans strain	Cell morphology at the colony periphery in response to:	
	No farnesol	20 μM farnesol
Wild type (SC5314)	Yeast plus a few filaments	Yeast plus a few filaments
TUP1/*tup1* (BCa2–3)	Yeast plus filaments	Yeast plus a few filaments
tup1/*tup1* (BCa10)	Filamentous form	Filamentous form

[a] Farnesol suppressed the haploinsufficient phenotype of the *TUP1*/*tup1* heterozygote. Cells were plated on a cornmeal agar plus Tween 80 plate, under a coverslip, and grown at 25°C for 25 h. The phenotype for these strains grown on a plate with cornmeal agar plus Tween 80 under a coverslip without farnesol was also previously reported (5).

farnesol (Fig. 1). In these cases, the total farnesol levels are actually much higher than the added farnesol because the mutants themselves produce elevated levels of farnesol (Table 1; see below). Furthermore, Tup1 mRNA and protein levels increased in the presence of farnesol, while mRNA levels of two Tup1-regulated genes, *HWP1* and *RBT1*, decrease (Fig. 2, and 3). Importantly, the timing of this increase (40 to 60 min, Fig. 2) corresponds with the commitment point, beyond which added farnesol no longer blocks GTF (31). Finally, we believe that Tup1 is part of the farnesol response pathway because farnesol suppresses the haploinsufficient phenotype of the *TUP1*/*tup1* strain (Table 2).

Cell synchrony, farnesol concentration, and timing were all important considerations for our experimental design. Previous work examining farnesol-dependent changes in the global transcription profiles of developing biofilms (8, 15) and during resumption of growth following stationary phase (15) were done with mixed cell populations that differed in their ability to respond to farnesol. Furthermore, the effect of adding farnesol on the global gene expression during biofilm formation was determined at a single time point, 24 h after the addition of farnesol (8). This point is significant because such a study could measure only stable long-term farnesol-dependent changes in gene expression. Timing is also important because of the commitment phenomenon. This is the point at which a switch in the environmental stimulus no longer causes the expected switch in morphology (9, 30, 31). It is relevant to farnesol's mode of action because, while farnesol blocks the yeast-to-filament switch, it does not block the elongation of preexisting filaments (31). Thus, for our experiments, we added farnesol at time zero in order to avoid the commitment to filamentous growth, and we harvested cells at 20-min increments to observe changes in transcript levels during the early stages of the farnesol response (31). We also achieved a synchronous cell population by starting with resting cells and inoculating them in mGPP; under these conditions, we routinely got 95 to 100% filamentous cells within 3 to 4 h. Exposing a synchronized cell population to farnesol allowed us to detect subtle and consistent changes in transcript abundance.

Small changes in the expression of a transcription regulator can have profound effects on the genes it regulates. For example, we have shown that nonsense-mediated mRNA decay in *S. cerevisiae* regulates the accumulation of the mRNA for Adr1, a transcription regulator of the genes responsible for making acetyl-coenzyme A and NADH from nonfermentable substrates. In particular, the respiratory impairment seen with nonsense-mediated mRNA decay mutants is due, in part, to the overexpression of Adr1 (43). The change in *ADR1* mRNA levels is small (2.6-fold) but sufficient to affect expression of Adr1-regulated genes. Thus, even though the change in Tup1 expression is relatively small, it can have a profound effect on the expression of the genes it regulates.

Two Tup1 coregulators, encoded by *NRG1* and *RFG1*, were unaffected by farnesol at the mRNA level (Fig. 4). In this regard, it is reasonable that farnesol regulates only one part of the complex, i.e., that farnesol elevates *TUP1* mRNA but not *NRG1* or *RFG1* mRNA. By analogy, for Ca^{2+} and calmodulin, where only the Ca^{2+}-calmodulin complex is active (40), fungi have calmodulin in excess and regulate the activity of the complex by regulating the availability of cytoplasmic Ca^{2+} (34).

The *tup1*/*tup1* and *nrg1*/*nrg1* mutants did not respond to farnesol, suggesting that farnesol acts through a pathway requiring Tup1 and Nrg1. The *rfg1*/*rfg1* mutant responded to farnesol, indicating that the genes regulated by Rfg1 are not required for the response to farnesol. Furthermore, the *tup1*/*tup1* and *nrg1*/*nrg1* mutants overproduced farnesol, while the *rfg1*/*rfg1* mutant produced only slightly elevated levels of farnesol (Table 1). This *tup1*/*tup1* mutant overproduction is biologically significant because the excess farnesol produced by the *tup1*/*tup1* mutant inhibits filamentation of the wild-type *C. albicans* grown on the same plate (Fig. 5). The juxtaposition of farnesol nonresponsive mutants with the overproduction of farnesol implies that a farnesol-Tup1 feedback loop may exist and that Nrg1 may work in concert with Tup1 to negatively regulate farnesol synthesis. This regulation may be direct or indirect. The enzyme responsible for ca. 90% of farnesol synthesis is Dpp3 (35). *DPP3* mRNA levels were not significantly elevated in the whole genome profiles of the *tup1*/*tup1* or *nrg1*/*nrg1* mutants (21); however, *DPP3* does have a putative Nrg1 binding site in its promoter region.

The increased *TUP1* expression we observed for farnesol's blockage of filament development (Fig. 2A) is smaller than that reported for farnesol's blockage of biofilm development, ca. 6.6-fold, as determined with DNA arrays (8). The difference in the response intensity may reflect filament versus biofilm growth conditions, as well as the fact that Cao et al. (8) used one time point 24 h after farnesol addition.

Efg1 is a transcriptional factor that activates hyphal gene expression including that of *HWP1* and *RBT1* (7). *EFG1* mRNA levels are regulated during filamentation, but they were not affected by farnesol, since the timing and magnitude of the changes were similar in the presence and absence of farnesol (Fig. 4). These results are consistent with those of Soto et al. (41), who also found no change in *EFG1* mRNA levels at a single time point with added farnesol (41). Together with our results, this suggests that farnesol does not regulate *EFG1* mRNA levels, but at this time, we cannot exclude the possibility that posttranslational regulation of Efg1 is affected by farnesol.

Two other farnesol-related findings regarding filamentous growth can be accommodated in a Tup1-dependent model because they are downstream from Tup1. Soto et al. (41) suggested that farnesol acts by causing decreased *CPH1* and

HST7 mRNA levels. *CPH1* is a transcription factor that regulates filamentous growth, and *HST7* is a mitogen-activated protein kinase kinase involved in filamentous growth. Both are downregulated by Tup1, and thus, their downregulation by farnesol (41) is consistent with a secondary effect of farnesol on Tup1. Additionally, Chk1, a histidine kinase shown to be required for the farnesol response (26), is also encoded by a Tup1-repressed gene; *CHK1* was elevated 6.5-fold in the *tup1*/*tup1* mutant (21). Taken together, these findings indicate that Tup1 is involved in mediating the *C. albicans* response to farnesol.

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REFERENCES

1. **Arnaud, M. B., M. C. Costanzo, M. S. Skrzypek, G. Binkley, C. Lane, S. R. Miyasato, and G. Sherlock.** 2005. The Candida Genome Database (CGD), a community resource for *Candida albicans* gene and protein information. Nucleic Acids Res. **33:**D358–D363.
2. **Atkin, A. L., N. Altamura, P. Leeds, and M. R. Culbertson.** 1995. The majority of yeast *UPF1* co-localizes with polyribosomes in the cytoplasm. Mol. Biol. Cell **6:**611–625.
3. **Backen, A. C., I. D. Broadbent, R. W. Fetherston, J. D. Rosamond, N. F. Schnell, and M. J. Stark.** 2000. Evaluation of the *CaMAL2* promoter for regulated expression of genes in *Candida albicans*. Yeast **16:**1121–1129.
4. **Berman, J., and P. E. Sudbery.** 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. Nat. Rev. Genet. **3:**918–930.
5. **Braun, B. R., and A. D. Johnson.** 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. Science **277:**105–109.
6. **Braun, B. R., and A. D. Johnson.** 2000. *TUP1*, CPH1 and *EFG1* make independent contributions to filamentation in *Candida albicans*. Genetics **155:**57–67.
7. **Braun, B. R., D. Kadosh, and A. D. Johnson.** 2001. *NRG1*, a repressor of filamentous growth in *Candida albicans*, is down-regulated during filament induction. EMBO J. **20:**4753–4761.
8. **Cao, Y. Y., Y. B. Cao, Z. Xu, K. Ying, Y. Li, Y. Xie, Z. Y. Zhu, W. S. Chen, and Y. Y. Jiang.** 2005. cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. Antimicrob. Agents Chemother. **49:**584–589.
9. **Chaffin, W. L., and D. E. Wheeler.** 1981. Morphological commitment in *Candida albicans*. Can. J. Microbiol. **27:**131–137.
10. **Csank, C., K. Schroppel, E. Leberer, D. Harcus, O. Mohamed, S. Meloche, D. Y. Thomas, and M. Whiteway.** 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect. Immun. **66:**2713–2721.
11. **Davis-Hanna, A., A. E. Piispanen, L. I. Stateva, and D. A. Hogan.** 2008. Farnesol and dodecanol effects on the Candida albicans Ras1-cAMP signalling pathway and the regulation of morphogenesis. Mol. Microbiol. **67:**47–62.
12. **De Groot, P. W., K. J. Hellingwerf, and F. M. Klis.** 2003. Genome-wide identification of fungal GPI proteins. Yeast **20:**781–796.
13. **Dhillon, N. K., S. Sharma, and G. K. Khuller.** 2003. Signaling through protein kinases and transcriptional regulators in *Candida albicans*. Crit. Rev. Microbiol. **29:**259–275.
14. **Eckert, S. E., C. C. Sheth, and F. A. Muhlschlegel.** 2007. Regulation of morphogenesis in *Candida* species, p. 263–291. *In* C. d'Enfert and B. Hube (ed.), *Candida*: comparative and functional genomics, 1st ed. Caister Academic Press, Norfolk, United Kingdom.
15. **Enjalbert, B., and M. Whiteway.** 2005. Release from quorum-sensing molecules triggers hyphal formation during *Candida albicans* resumption of growth. Eukaryot. Cell **4:**1203–1210.
16. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
17. **Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K. W. Nickerson.** 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl. Environ. Microbiol. **67:**2982–2992.
18. **Inglis, D. O., and A. D. Johnson.** 2002. Ash1 protein, an asymmetrically localized transcriptional regulator, controls filamentous growth and virulence of *Candida albicans*. Mol. Cell. Biol. **22:**8669–8680.
19. **Jarvis, W. R., and W. J. Martone.** 1992. Predominant pathogens in hospital infections. J. Antimicrob. Chemother. **29**(Suppl. A)**:**19–24.
20. **Jensen, E. C., J. M. Hornby, N. E. Pagliaccetti, C. M. Wolter, K. W. Nickerson, and A. L. Atkin.** 2006. Farnesol restores wild-type colony morphology to 96% of *Candida albicans* colony morphology variants recovered following treatment with mutagens. Genome **49:**346–353.
21. **Kadosh, D., and A. D. Johnson.** 2005. Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. Mol. Biol. Cell **16:**2903–2912.
22. **Kadosh, D., and A. D. Johnson.** 2001. Rfg1, a protein related to the *Saccharomyces cerevisiae* hypoxic regulator Rox1, controls filamentous growth and virulence in *Candida albicans*. Mol. Cell. Biol. **21:**2496–2505.
23. **Kaneko, A., T. Umeyama, Y. Utena-Abe, S. Yamagoe, M. Niimi, and Y. Uehara.** 2006. Tcc1p, a novel protein containing the tetratricopeptide repeat motif, interacts with Tup1p to regulate morphological transition and virulence in *Candida albicans*. Eukaryot. Cell **5:**1894–1905.
24. **Kebaara, B., T. Nazarenus, R. Taylor, A. Forch, and A. L. Atkin.** 2003. The Upf-dependent decay of wild-type *PPR1* mRNA depends on its 5′-UTR and first 92 ORF nucleotides. Nucleic Acids Res. **13:**3157–3165.
25. **Khalaf, R. A., and R. S. Zitomer.** 2001. The DNA binding protein Rfg1 is a repressor of filamentation in *Candida albicans*. Genetics **157:**1503–1512.
26. **Kruppa, M., B. P. Krom, N. Chauhan, A. V. Bambach, R. L. Cihlar, and R. A. Calderone.** 2004. The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. Eukaryot. Cell **3:**1062–1065.
27. **Kulkarni, R. K., and K. W. Nickerson.** 1981. Nutritional control of dimorphism in *Ceratocystis ulmi*. Exp. Mycol. **5:**148–154.
28. **Kullberg, B. J., and S. G. Filler.** 2002. *Candida* and candidiasis. ASM Press, Washington, DC.
29. **Kumamoto, C. A., and M. D. Vinces.** 2005. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. Cell. Microbiol. **7:**1546–1554.
30. **Mitchell, L. H., and D. R. Soll.** 1979. Commitment to germ tube or bud formation during release from stationary phase in *Candida albicans*. Exp. Cell Res. **120:**167–179.
31. **Mosel, D. D., R. Dumitru, J. M. Hornby, A. L. Atkin, and K. W. Nickerson.** 2005. Farnesol concentrations required to block germ tube formation in *Candida albicans* in the presence and absence of serum. Appl. Environ. Microbiol. **71:**4938–4940.
32. **Murad, A. M., C. d'Enfert, C. Gaillardin, H. Tournu, F. Tekaia, D. Talibi, D. Marechal, V. Marchais, J. Cottin, and A. J. Brown.** 2001. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1. Mol. Microbiol. **42:**981–993.
33. **Murad, A. M., P. Leng, M. Straffon, J. Wishart, S. Macaskill, D. MacCallum, N. Schnell, D. Talibi, D. Marechal, F. Tekaia, C. d'Enfert, C. Gaillardin, F. C. Odds, and A. J. Brown.** 2001. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. EMBO J. **20:**4742–4752.
34. **Muthukumar, G., A. W. Nickerson, and K. W. Nickerson.** 1987. Calmodulin levels in yeasts and filamentous fungi. FEMS Microbiol. Lett. **41:**253–255.
35. **Navarathna, D. H., J. M. Hornby, N. Krishnan, A. Parkhurst, G. E. Duhamel, and K. W. Nickerson.** 2007. Effect of farnesol on a mouse model of systemic candidiasis, determined by use of a *DPP3* knockout mutant of *Candida albicans*. Infect. Immun. **75:**1609–1618.
36. **Ramage, G., S. P. W. Saville, B. L., and J. L. Lopez-Ribot.** 2002. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. Appl. Environ. Microbiol. **68:**5459–5463.
37. **Ramon, A. M., A. Porta, and W. A. Fonzi.** 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *PRR2*. J. Bacteriol. **181:**7524–7530.
38. **Semighini, C. P., J. M. Hornby, R. Dumitru, K. W. Nickerson, and S. D. Harris.** 2006. Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. Mol. Microbiol. **59:**753–764.
39. **Shchepin, R., J. M. Hornby, E. Burger, T. Niessen, P. Dussault, and K. W. Nickerson.** 2003. Quorum sensing in *Candida albicans*: probing farnesol's mode of action with 40 natural and synthetic farnesol analogs. Chem. Biol. **10:**743–750.
40. **Soto, T., Y. Ueno, T. Watanabe, T. Mikami, and T. Matsumoto.** 2004. Role of Ca2+/calmodulin signaling pathway on morphological development of *Candida albicans*. Biol. Pharm. Bull. **27:**1281–1284.
41. **Soto, T., T. Watanabe, T. Mikami, and T. Matsumoto.** 2004. Farnesol, a morphogenetic autoregulatory substance in the dimorphic fungus *Candida albicans*, inhibits hyphae growth through suppression of a mitogen-activated protein kinase cascade. Biol. Pharm. Bull. **27:**751–752.

42. **Sprague, E. R., M. J. Redd, A. D. Johnson, and C. Wolberger.** 2000. Structure of the C-terminal domain of Tup1, a corepressor of transcription in yeast. EMBO J. **19:**3016–3027.
43. **Taylor, R., B. W. Kebaara, T. Nazarenus, A. Jones, R. Yamanaka, R. Uhrenholdt, J. P. Wendler, and A. L. Atkin.** 2005. Gene set coregulated by the *Saccharomyces cerevisiae* nonsense-mediated mRNA decay pathway. Eukaryot. Cell **4:**2066–2077.
44. **Tebarth, B., T. Doedt, S. Krishnamurthy, M. Weide, F. Monterola, A. Dominguez, and J. F. Ernst.** 2003. Adaptation of the Efg1p morphogenetic pathway in *Candida albicans* by negative autoregulation and PKA-dependent repression of the *EFG1* gene. J. Mol. Biol. **329:**949–962.
45. **Toyoda, M., T. Cho, H. Kaminishi, M. Sudoh, and H. Chibana.** 2004. Transcriptional profiling of the early stages of germination in *Candida albicans* by real-time RT-PCR. FEMS Yeast Res. **5:**287–296.
46. **Westwater, C., E. Balish, and D. A. Schofield.** 2005. *Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance. Eukaryot. Cell **4:**1654–1661.