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Candida albicans Czf1 and Efg1 Coordinate the Response to Farnesol during Quorum Sensing, White-Opaque Thermal Dimorphism, and Cell Death

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Quorum sensing by farnesol in *Candida albicans* inhibits filamentation and may be directly related to its ability to cause both mucosal and systemic diseases. The Ras1-cyclic AMP signaling pathway is a target for farnesol inhibition. However, a clear understanding of the downstream effectors of the morphological farnesol response has yet to be unraveled. To address this issue, we screened a library for mutants that fail to respond to farnesol. Six mutants were identified, and the *czf1Δ/czf1Δ* mutant was selected for further characterization. Czf1 is a transcription factor that regulates filamentation in embedded agar and also white-to-opaque switching. We found that Czf1 is required for filament inhibition by farnesol under at least three distinct environmental conditions: on agar surfaces, in liquid medium, and when embedded in a semisolid agar matrix. Since Efg1 is a transcription factor of the Ras1-cyclic AMP signaling pathway that interacts with and regulates Czf1, an *efg1Δ/efg1Δ czf1Δ/czf1Δ* mutant was tested for filament inhibition by farnesol. It exhibited an opaque-cell-like temperature-dependent morphology, and it was killed by low farnesol levels that are sublethal to wild-type cells and both *efg1Δ/efg1Δ* and *czf1Δ/czf1Δ* single mutants. These results highlight a new role for Czf1 as a downstream effector of the morphological response to farnesol, and along with Efg1, Czf1 is involved in the control of farnesol-mediated cell death in *C. albicans*.

Candida albicans is a member of the microbial flora in the gastrointestinal and urogenital tracts of many healthy people, but it can also cause both mucosal and disseminated opportunistic infections when host defenses are compromised. Mucosal infections involve the formation of a biofilm at the site of infection. In severely immunocompromised patients, disseminated infections often result in death. *C. albicans* is a polymorphic fungus, and the ability to transition between different morphologies is strongly correlated with its ability to cause both disseminated and mucosal infections (1, 2). *C. albicans* switches between yeast and filamentous forms of growth, and it grows in two distinct yeast forms, white and opaque, named for their colony morphology. Opaque cells are the mating-competent form. *C. albicans* is also able to form chlamydospores; however, the function of chlamydospores is unknown. All of these cell types are affected by the quorum-sensing molecule *E,E*-farnesol (referred to here as farnesol), which emphasizes its influential role in *C. albicans* morphology.

Farnesol has multiple physiological effects. It blocks the transition from yeast to filaments once it accumulates above a threshold level (3). It also has an inhibitory role in biofilm formation (4) and a protective role against oxidative stress (5, 6). In addition, very high levels of farnesol can increase chlamydospore formation (7), while even very low levels of farnesol induce cell death by necrosis in opaque cells (8). White cells can also be killed by farnesol under some environmental conditions; log-phase cells that are energy deprived are particularly sensitive, while stationary-phase cells in growth medium are quite farnesol tolerant (9–11). Given its important role in physiology, it comes as no surprise that farnesol signaling is also relevant during an infection and that it has distinct roles at different sites of infection. For example, farnesol is a virulence factor in a mouse model of disseminated infection (12), yet it protects mice from oral candidiasis (13). These results

highlight the need for a complete understanding of the signaling response induced by farnesol in *C. albicans*.

Factors that play a role in the *C. albicans* farnesol response include Tup1/Nrg1 (14), the Hog1-mediated mitogen-activated protein kinase (MAPK) pathway (15), the Cek1 MAPK pathway (16), and the cyclic AMP-protein kinase A complex (cAMP/PKA) signaling pathway (17). *C. albicans* histidine kinase (Chk1) is also implicated in the response to farnesol (18).

In this paper, we identified Czf1 (*C. albicans* zinc finger 1) as an additional factor that is important for the response to farnesol. The known roles of Czf1 include induction of contact-induced filamentous growth (19) and regulation of both biofilm formation (20) and the switch from white to opaque cell morphology (21, 22). *CZF1* negatively regulates its own mRNA expression (23). Czf1 also has ties to the cAMP/PKA pathway because *CZF1* expression is regulated by Efg1, a transcription factor activated by cAMP/PKA signaling (24). Further, the Efg1 and Czf1 proteins interact in a yeast two-hybrid assay (25, 26). This regulation by Efg1 and the interactions between Efg1 and Czf1 are intriguing because these proteins have opposing roles in the cell with respect to morphogenesis (22, 25, 26). Efg1 is required for filamentation

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TABLE 1 *C. albicans* strains and plasmids used in this study

<i>C. albicans</i> strain or plasmid	Parental strain	Genotype/description or relevant markers	Source or reference
Strains			
SC5314	Clinical isolate	Clinical isolate	47
CAF2	CAI-4	<i>URA</i> ⁺ derivative of CAI-4	48
CAI-4	SC5314	<i>ura3::imm434/ura3::imm434</i>	49
BWP17	RM1000	<i>ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	50
HLC67	CAI-4	<i>ura3::imm434/ura3::imm434 efg1::hisG/efg1::hisG</i>	1
CKY101	CAI-4	<i>ura3::imm434/ura3::imm434 ade2::pDB152</i>	19
CKY230	CAI-4	<i>ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG ade2::pMAL2-URA3</i>	23
CKY116	CAI-4	<i>ura3::imm434/ura3::imm434 CZF1/czf1::hisG-URA3-hisG</i>	Carol Kumamoto
CKY231	CAI-4	<i>ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG ade2::pMAL2-CZF1-URA3</i>	19
CKY283	CAI-4	<i>ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG-URA3-hisG efg1::hisG/efg1::hisG</i>	Carol Kumamoto
AAC2	CKY283	<i>ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG efg1::hisG/efg1::hisG</i>	This study
AAC7	AAC2	<i>ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG efg1::hisG/efg1::hisG ade2::pMAL2-CZF1-URA3</i>	This study
Plasmid pDB212			
		<i>pMAL2-CZF1 URA3 ade2'</i> Amp ^r	19

under aerobic conditions, and it represses filamentation in hypoxia. In contrast, Czf1 is required for filamentation in hypoxia. Further, Efg1 and Czf1 are part of the transcriptional circuit that regulates white-to-opaque switching where Czf1, with Wor1 and Wor2, activates the switch to the opaque state while Efg1 represses Wor2 expression to maintain the white state (20).

In this study, we identified Czf1 by screening a library for mutants that did not respond to farnesol. We found that Czf1 was essential for the response to farnesol and that it functions downstream of Efg1 in this response. Within the context of previous work demonstrating that Czf1 promotes the white-to-opaque switch even though opaque cells are very sensitive to farnesol, we propose that Czf1 functions with Efg1 to coordinate farnesol regulation of two major pathways, yeast-to-mycelium transitions and white-to-opaque switching.

MATERIALS AND METHODS

Strains and media. The *C. albicans* strains and plasmids used in this study are listed in Table 1. Modified glucose phosphate proline (mGPP) medium at pH 6.8 was prepared as described by Kebaara et al. (14). Yeast extract-peptone-dextrose (YPD) medium contained 1% yeast extract, 0.5% peptone, and 2% dextrose, and solid medium included 2% agar. Resting cells were prepared as described by Kebaara et al. (14), with modifications. Single colonies were grown in 25 ml YPD broth at 30°C for 22 to 24 h to the stationary phase, washed three times with 50 mM potassium phosphate buffer (pH 6.8), resuspended in phosphate buffer, and stored at 4°C overnight before use. For liquid farnesol response assays, resting cells were inoculated at 10⁶/ml into mGPP or mSPP (2% sucrose replacing glucose) broth with the indicated farnesol concentrations. Cells were then grown at 37°C while shaking in glass flasks for the indicated times. Farnesol (Sigma, St. Louis, MO) was stored under nitrogen and freshly prepared as a 100 mM stock solution in methanol for each experiment.

Mutant library screening. The mutant libraries were obtained in 96-well plates and plated on mGPP (with 40 μg/ml uridine, arginine, and histidine added) agar plates containing 0, 10, or 50 μM farnesol. Plates were incubated at 37°C for 2 days before colony morphology was assessed and mutants were compared to the parental strain, BWP17, for farnesol response. Colonies with a wrinkled/hairy morphology were considered to be composed primarily of filaments, and colonies with a smooth morphology were considered to be mostly yeast cells. Farnesol-nonresponsive mutants identified by primary screening were restreaked onto individual plates to rule out interference from neighboring mutant colonies and to

confirm the farnesol-nonresponsive phenotype observed. Liquid germ tube assays (3) were used to further confirm that the colony morphology was due to farnesol resistance.

Embedded cell growth. Embedded media were prepared by mixing 10⁴ resting cells/ml in 30 ml GPP (no GlcNAc added) or SPP molten agar (cooled to 50°C) with appropriate concentrations of farnesol and plated. Embedded plates were incubated at 37°C for 12 or 17 h, as indicated. Only colonies beneath the agar surface were examined. Embedded micrographs were taken with a custom MVI TDM400 tetrad dissecting microscope and a Sony Cybershot camera.

Microscopy and cell death determination. Cellular morphology during the germ tube assays was determined with a Zeiss Stemi 2000-C light microscope. Differential interference contrast micrographs were taken with an Olympus BX51 microscope and a Photometrics CoolSnap HQ charge-coupled device camera. Cell death was determined by methylene blue staining as described by Gibson et al. (27).

DNA analysis and transformation. To create strain AAC2, CKY283 was plated on 5-fluoroorotic acid-containing medium to select for *ura*[−] mutants. AAC2 was subsequently transformed with BsgI-digested pDB212 to create and AAC7. Transformations were performed by the lithium acetate method, and transformants were selected on medium lacking uridine (28). Newly created strains were confirmed by PCR and Southern blot analysis (data not shown); restriction digestion and Southern blotting were performed as described in the GeneScreen Plus hybridization transfer and detection protocols (DuPont NEN Research Products, Boston, MA).

Quantitative Northern analysis. Resting cells were inoculated into 75 ml mGPP broth at 5 × 10⁶/ml, and 0, 50, or 100 μM farnesol was added to each flask. Cells were incubated at 37°C and harvested at 40, 60, or 80 min. Cells were harvested by passage of the cultures through glass fiber filters to collect the cells, and then the cells were scraped off the filters. mRNA was extracted with the RiboPure yeast kit (Applied Biosystems, Foster City, CA).

A 10-μg RNA sample was fractionated on 1.0% agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane (NEN Life Science Products, Inc., Boston, MA) with NorthernMax transfer buffer (Applied Biosystems/Ambion, Austin, TX). The membrane was probed with ³²P-labeled DNA probes in NorthernMax prehybridization/hybridization buffer (Applied Biosystems/Ambion, Austin, TX). The template DNAs for probe synthesis were prepared by PCR with primers CZF1 (5'-CGTC AATCACAACCACAACC-3' and 5'-TACCAAACCTCGGCATGTTCA-3') and ACT1 (5'-AGTTATCGATAAGCCTTCTG-3' and 5'-AGATTTC CAGAATTTCACTC-3'). Probes were labeled with [³²P]dCTP (GE Health Sciences, Piscataway, NJ) with a RadPrime DNA Labeling system

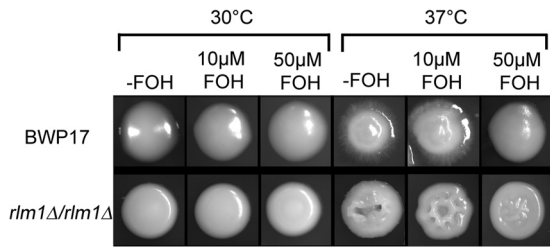


FIG 1 Colony morphological response to farnesol (FOH) at 30 and 37°C. Representative micrographs were taken of the colony morphology of the parental strain (BWP17) capable of filamentation (rough/wrinkled) and response to farnesol (smooth), as well as the farnesol-resistant *rlm1Δ/rlm1Δ* mutant strain (rough/wrinkled colonies instead of smooth at 37°C with farnesol).

(Invitrogen, Carlsbad, CA). Northern blot assays were imaged with a Storm phosphorimager (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and quantified with ImageQuant software (version 5.0; Molecular Dynamics, Sunnyvale, CA). mRNA abundance was normalized to an ACT1 loading control. All of the values reported are averages of three independent experiments.

Characterization of white versus opaque-cell-like morphology.

Resting cells were prepared with single colonies selected from YPD plates grown at 37°C for 48 h. Under these conditions, the cells are yeasts in the white phase. These single colonies were inoculated into 25 ml YPD and grown at 30 or 37°C for 24 h. At 30°C, but not 37°C, a high percentage of the cells (>90%) switched from the white phase to an opaque-cell-like phase between 21 and 24 h of growth. Cells were harvested at the 2- and 24-h time points, and RNA was extracted as will be described elsewhere (J. J. Bunker, S. Ghosh, B. W. Kebaara, K. W. Nickerson, and A. L. Atkin, unpublished data). Contaminating DNA was removed from the RNA extracts with the TURBO DNA-free kit (Ambion, Inc.). The reverse transcription (RT) reaction was performed by the two-step protocol of the RETROscript RT-PCR kit (Ambion, Inc.), with a 20-μl volume and a final total RNA concentration of 1 μg/μl. The same PCR primers used by Miller and Johnson (29) were used to detect *WH11* and *OP4*. The *TUB2* mRNA RT product (5'-CAACTGGTCAATGTGGTAATCA-3' and 5'-CAATGTTTGGGCTAAAGGTCATTAC-3') was used as a positive control. The PCR thermal cycling conditions used were an initial step of 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at the annealing temperature (46°C for *WH11*, 52°C for *OP4*, and 49°C for *TUB2*), and 1.5 min at 72°C and a final step of 72°C for 7 min.

Whole-cell PCR was performed to verify the presence of the *MTLa1* and *MTLα1* genes in CKY283. WO-1 was used as a homozygous *MATα* control, and amplification of *ERG8* was used as a positive control (data not shown). PCR primers unique to *MATa1* and *MTLa1* were previously described (30), and the primers used for amplification of *ERG8* were 5'-

CCTGGAAAAGCATTTCTTGC and 5'-CAAGTCACCTTCTGTTTG CTC. Initial denaturation at 94°C for 3 min was followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min. The final elongation step was 72°C for 3 min. All PCRs were performed in a Hybaid PCR Sprint thermocycler. PCR products were analyzed by agarose gel electrophoresis.

Farnesol production measurements. Extracellular farnesol was extracted from cell-free supernatants of stationary-phase cultures grown in GPP at 30°C and analyzed by gas chromatography-mass spectrometry as described by Hornby et al. (3).

RESULTS

Identification of new genes required for the morphological response to farnesol. The factors already known to play a role in the response to farnesol, Tup1, Nrg1, Ras1, Cyr1, Efg1, Cek1, and Chk1, are also regulators of morphogenesis. We set out to identify additional regulators of the farnesol response in *C. albicans* by screening a library of 507 unique homozygous insertion or deletion mutants defective in only their morphological response to farnesol (31, 32). *C. albicans* normally forms yeast at 30°C and hyphae at 37°C. Thus, the response to farnesol was tested by plating the cells on mGPP medium at 30 or 37°C in the absence or presence of farnesol. Under these conditions, wild-type cells form smooth colonies at 30°C both with and without farnesol, whereas at 37°C they form filamentous colonies in the absence of farnesol and smooth colonies in the presence of farnesol (BWP17 in Fig. 1). Mutants that formed smooth colonies at 30°C and filamentous colonies at 37°C both in the presence and in the absence of farnesol were considered morphologically nonresponsive or resistant to farnesol. The colony phenotype of the *rlm1Δ/rlm1Δ* mutant is representative of the type of morphological response seen in the farnesol-nonresponsive mutants (Fig. 1; Table 2). These farnesol-nonresponsive mutants were of interest. In contrast, mutants that formed smooth colonies at 37°C in the absence of farnesol or that formed filamentous colonies at 30°C were not studied further because they were defective in morphological switching in general and not the specific response to farnesol. Thirteen of the 507 mutants were defective in filamentation, and 6 mutants did not grow or grew more slowly on mGPP medium at 30 and 37°C in both the absence and the presence of farnesol. Both of these types of mutants were not considered further. In the remaining 488 mutants, six farnesol-nonresponsive mutants were identified (Table 2). We found no farnesol-hypersensitive mutants or mutants whose growth was inhibited by farnesol.

Two of the farnesol-nonresponsive strains carried mutations in the genes for Czf1 and Tpk1, two proteins involved in morphogenesis (Table 2). The identification of the *tpk1/tpk1* mutant val-

TABLE 2 Summary of insertion mutants with an impaired farnesol response

Mutant	<i>S. cerevisiae</i> ortholog	Predicted or known biological process(es) (reference[s])	Reference(s)
<i>czf1Δ/czf1Δ</i>	None	Zinc finger transcription factor for filamentation under embedded conditions and positive regulator of white-to-opaque switching; binds Efg1 and expression controlled by Efg1 and Czf1	19, 21–23, 38
<i>tpk1/tpk1</i>	Tpk2	Catalytic subunit of cAMP-dependent protein kinase A, regulator of morphogenesis; Tpk2 isoform; involved in multiple stress responses	51–54
<i>rlm1Δ/rlm1Δ</i>	Rlm1	Transcription factor for genes involved in cell wall organization and biogenesis and various stress responses	55, 56
<i>stp2/stp2</i>	Stp2	Transcription factor for amino acid permease genes	57
<i>yck2/yck2</i>	Yck2	Maintenance of cell polarity, antimicrobial peptide resistance, contributes to epithelial cell damage	58
<i>hap43/hap43</i>	Yap3	Transcription factor, involved in iron limitation response	59

idates the results of our screening. Tpk1 is the catalytic subunit of cAMP-dependent PKA, a component of the cAMP/PKA-dependent pathway. We expected to recover mutants in this pathway because it is known to be important for the response to farnesol (17).

Mutations in *RLM1*, *YCK2*, and *HAP43* also conferred resistance to farnesol. These genes function in diverse stress responses. These mutants are interesting because farnesol treatment leads to increased survival after heat shock (17) and confers protection from oxidative stress (5, 6, 32). Osmotic stress protection is also mediated by the cAMP/PKA pathway (4, 5).

CZF1 is required for a wild-type morphological response to farnesol under both liquid and embedded conditions. *Czf1* was selected for further study because it is a transcription regulator that is critical for morphogenesis processes, including hyphal growth, embedded growth, and white-to-opaque switching frequency (19, 22), suggesting that it could have a role in the response to farnesol. Further, *Czf1* is unique to the *Candida* genus. It is a zinc finger transcription regulator that is most similar to functionally distinct Ume6 in *Saccharomyces cerevisiae*. The *C. albicans* homologs of *UME6* are *UME6* (orf19.1822) and *UME7* (orf19.2745).

We determined that *Czf1* is required for the response to farnesol under two additional growth conditions, liquid assays and embedded assays. To test the role of *Czf1*, we compared five strains (Table 1) in a liquid farnesol response assay (Fig. 2): The *czf1Δ/czf1Δ* null mutant (CKY230) was compared with its parental strain (CKY101), the heterozygous mutant (CKY116), and the null mutant ectopically expressing *CZF1* under the control of the *MAL2* promoter (CKY231), as well as with a wild-type clinical isolate (SC5314).

In both glucose- and sucrose-containing media, the addition of farnesol reduced germ tube formation by SC5314 and CKY101 (Fig. 2). However, in both mGPP and mSPP media, the *czf1Δ/czf1Δ* mutant showed only a minimal reduction in the percentage of cells forming germ tubes, even in the presence of 100 μ M farnesol, while an intermediate, haploinsufficient farnesol response phenotype was observed in the heterozygous mutant (Fig. 2). For SC5314, CKY101, the *czf1Δ/czf1Δ* mutant, and the heterozygous mutant, the results were similar regardless of whether glucose or sucrose was used as the carbon source. However, ectopic complementation of the *czf1Δ/czf1Δ* mutant under *pMAL2*-inducing conditions (Fig. 2B, sucrose-containing media) restored the farnesol response to a level similar to that of the heterozygous mutant, while there was only a minimal farnesol response under noninducing conditions (Fig. 2A, glucose-containing medium). Consistent with the phenotypes, we found that expression of *CZF1* from the *MAL2* promoter was only partially restored relative to the wild-type level, as assessed by real-time quantitative RT-PCR. Thus, *CZF1* is critical to the ability of *C. albicans* to respond to farnesol in liquid medium.

The first described role for *Czf1* was to promote filamentation under embedded conditions (19). As a consequence, we sought to determine whether, under embedded conditions, (i) *C. albicans* can respond to farnesol and (ii) *Czf1* is needed for this response. For consistency with our prior work with farnesol, we used defined GPP and SPP agar plates incubated at 37°C for our embedded condition assays even though these conditions are different from those initially used to study *Czf1*'s role under embedded conditions (19). We observed a strong filamentation response by

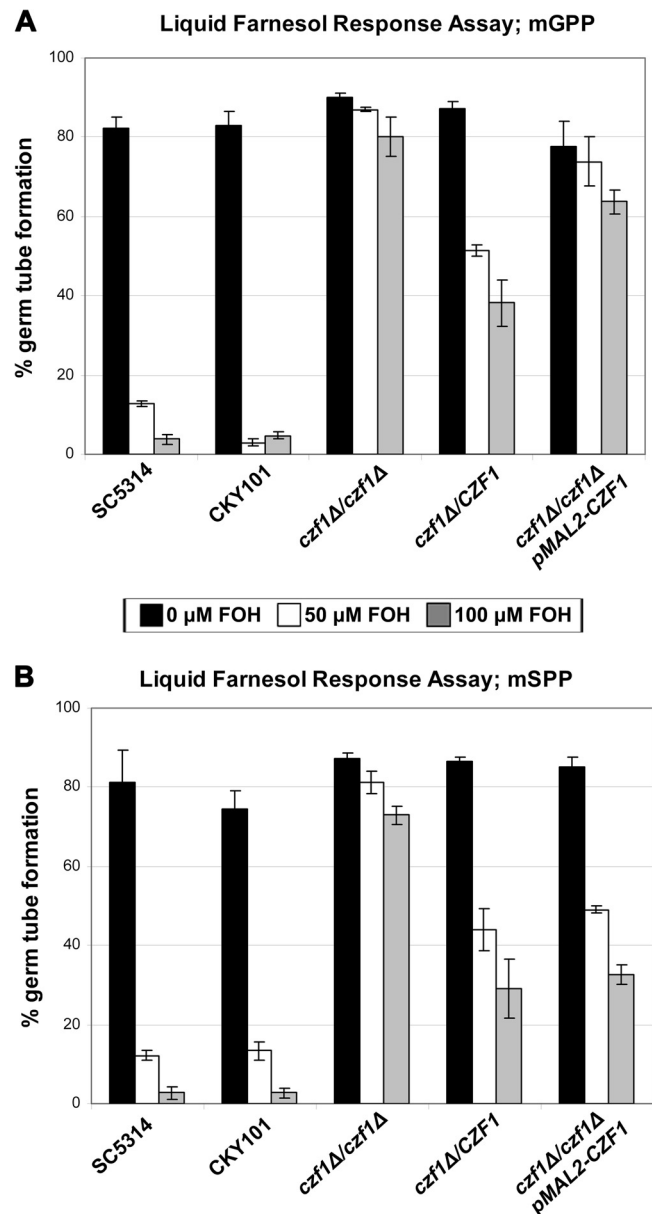


FIG 2 *Czf1* is required for the morphological response to farnesol (FOH) under liquid conditions. Resting *C. albicans* cells were inoculated into glass flasks at 10^6 /ml of either mGPP (A) or mSPP (B) broth, and 0, 50, or 100 μ M farnesol was added. Cultures were incubated for 1 h at 37°C with shaking at 225 rpm, and the percentage of germ tube formation was subsequently determined (*czf1Δ/czf1Δ* = CKY230, *czf1Δ/CZF1* = CKY116, *czf1Δ/czf1Δ pMAL2-CZF1* = CKY231). The data shown are from independent experiments performed in triplicate; these were repeated with similar results on at least two separate occasions.

C. albicans SC5314 and CKY101 cells when they were grown embedded in an agar matrix in that 50 μ M farnesol prevented filamentation by these cells (Fig. 3). As with the liquid germ tube assays, glucose versus sucrose made no difference in either the filamentation or the farnesol response of strains SC5314 and CKY101 (Fig. 3). These results demonstrate that *C. albicans* responds to farnesol in an agar-embedded condition assay.

As expected, the *czf1Δ/czf1Δ* mutant exhibited a defective growth pattern under embedded conditions; filamentation was

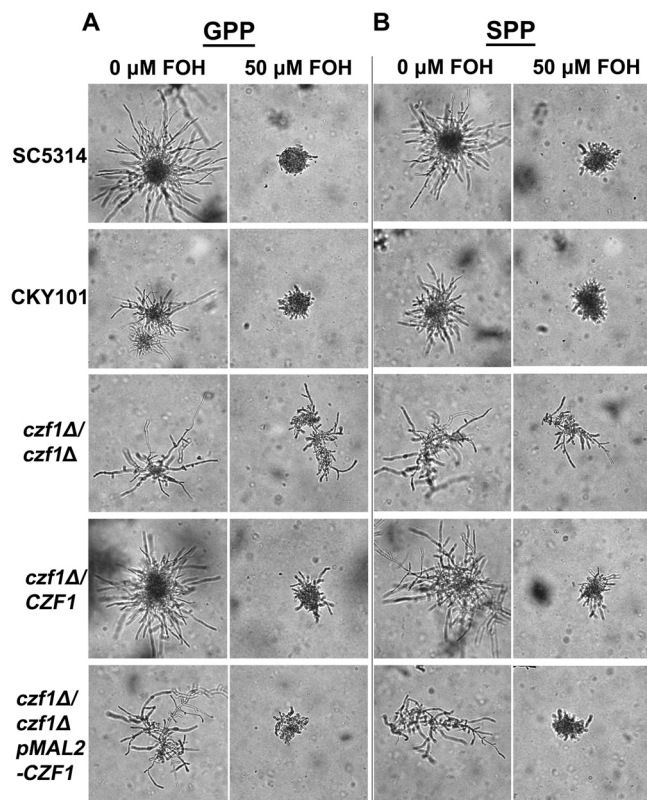


FIG 3 Czf1 is required for the morphological response to farnesol (FOH) under embedded conditions. Resting *C. albicans* cells were mixed with either GPP (A) or SPP (B) molten agar and 0 or 50 μ M farnesol as described in Materials and Methods and incubated at 37°C for 12 h. Independent experiments were repeated in duplicate with similar results.

still observed, but far fewer colonies than those of the wild-type and parental strains were present and colony morphology appeared different as well (Fig. 3). When the *czf1* Δ /*czf1* Δ mutant was grown in agar containing farnesol, only a moderate farnesol response was observed; the filaments appeared shorter than in untreated samples, but the colonies were still filamentous (Fig. 3). As in the liquid farnesol response assays, the *czf1* Δ /*CZF1* heterozygote maintained a haploinsufficient (intermediate) phenotype in both farnesol-treated and untreated samples. On untreated plates, the overall level of growth of the heterozygote was more similar to that of wild-type strains, while on farnesol-treated plates, the colonies were reduced in hyphal formation but they were still more filamentous than wild-type colonies treated with farnesol (Fig. 3). Finally, the *czf1* Δ /*czf1* Δ *pMAL2-CZF1* mutant strain exhibited a response to farnesol similar to that of the heterozygote on both GPP and SPP plates (Fig. 3), conditions that should turn the *MAL2* promoter off and on, respectively (Fig. 2). This similarity on GPP and SPP suggests that under embedded conditions, the *MAL2* promoter is leaky in GPP, resulting in the partial expression of *CZF1* and partial complementation of the farnesol response in both GPP and SPP. Leaky expression of the *MAL2* promoter has been previously reported (33).

In summary, these results indicate that Czf1 is required for farnesol response under at least three distinct environmental conditions, i.e., on agar surfaces, in liquid medium, and when embedded in a semisolid agar matrix. This view is explored more fully by characterizing an *efg1* Δ /*efg1* Δ *czf1* Δ /*czf1* Δ double mutant.

An *efg1* Δ /*efg1* Δ *czf1* Δ /*czf1* Δ double mutant exhibits a temperature-dependent dimorphic morphology. Previous work on the regulation of filamentous growth identified Efg1 as a major transcription regulator and a central control point for the many signaling pathways involved in filamentation (reviewed in reference 34). It is also known that Efg1 and Czf1 directly interact with each other in these morphogenesis processes. Thus, we next tested the genetic interactions between *CZF1* and *EFG1* to determine if they work together in the response to farnesol. In the course of this genetic analysis, we observed a novel temperature-regulated dimorphic phenotype in the *efg1* Δ /*efg1* Δ *czf1* Δ /*czf1* Δ mutant (Fig. 4). In YPD broth at 30°C, this mutant formed small elongated cells that resemble opaque cells (Fig. 4A), while in YPD broth at 37°C, the cells appeared larger and more rounded, reminiscent of white cells (Fig. 4B). This dimorphic phenotype was unexpected because cells of mating type *a*/ α , such as our *efg1* Δ /*efg1* Δ *czf1* Δ /*czf1* Δ mutant, are normally maintained in the white phase by *a1*- α 2 repression of *WOR1* (22) and *czf1* Δ /*czf1* Δ mutant cells are known to have decreased white-to-opaque switching (22), although switching does occur at a very low rate in *MTLa*/*MTL* α clinical isolates under conditions mimicking aspects of the host environment (35).

This morphological switch was observed in greater detail at a constant 30°C (Fig. 4C). Following the inoculation of white-phase cells (grown at 37°C) into YPD broth at 30°C, the conversion to an opaque-cell-like morphology occurred between 21 and 24 h (Fig. 4C), at which time these cells form opaque colonies that stain with phloxine B (data not shown). To test whether turning on opacity-specific genes and turning off white-morphology-specific genes accompanied the morphological switch from white to opaque, we performed RT-PCR analysis of RNA extracted from cells harvested before and after the morphological change, i.e., at 2 and 24 h, respectively. Little change in white-morphology-specific gene expression was observed, but the expression of *OP4*, an opacity-specific gene, increased during the switch from a white to an opaque-cell-like morphology (Fig. 4C) and was maintained following the switch (data not shown). We also confirmed that these strains are still heterozygous for the mating type locus (Fig. 4D).

Next we confirmed the opaque nature of the *efg1* Δ /*efg1* Δ *czf1* Δ /*czf1* Δ mutant grown at 30°C with respect to its farnesol sensitivity. Earlier, we had showed that farnesol kills opaque cells under aerobic conditions (8). Here we show that in liquid assays, *efg1* Δ /*efg1* Δ *czf1* Δ /*czf1* Δ mutant cells pregrown at 30°C (opaque-cell-like morphology) were more sensitive to farnesol killing, as assessed by methylene blue staining, than cells pregrown at 37°C (white cells) (compare Fig. 5A to B and C to D). In contrast, no significant cell death was observed in parental strain CAI4 or the *efg1* Δ /*efg1* Δ single mutant when it was pregrown at 30°C and assayed in either mGPP or mSPP, even in the presence of 100 μ M farnesol (Fig. 5A and C). Further, only a small increase in cell death was observed in the *efg1* Δ /*efg1* Δ single mutant when it was pregrown at 37°C (Fig. 5B and D). We were unable to use methylene blue staining to assess the viability of CAI4 and the *czf1* Δ /*czf1* Δ mutant pregrown at 37°C because they form germ tubes that stain with methylene blue regardless of the presence of farnesol. However, the CAI4 and *czf1* Δ /*czf1* Δ mutant strains are viable in the presence of 50 to 100 μ M farnesol because they grow in liquid, on the surface of agar plates, and under embedded conditions (Fig. 2). Ectopic expression of *CZF1* did not rescue the cell death phenotype in the presence of farnesol because the cells

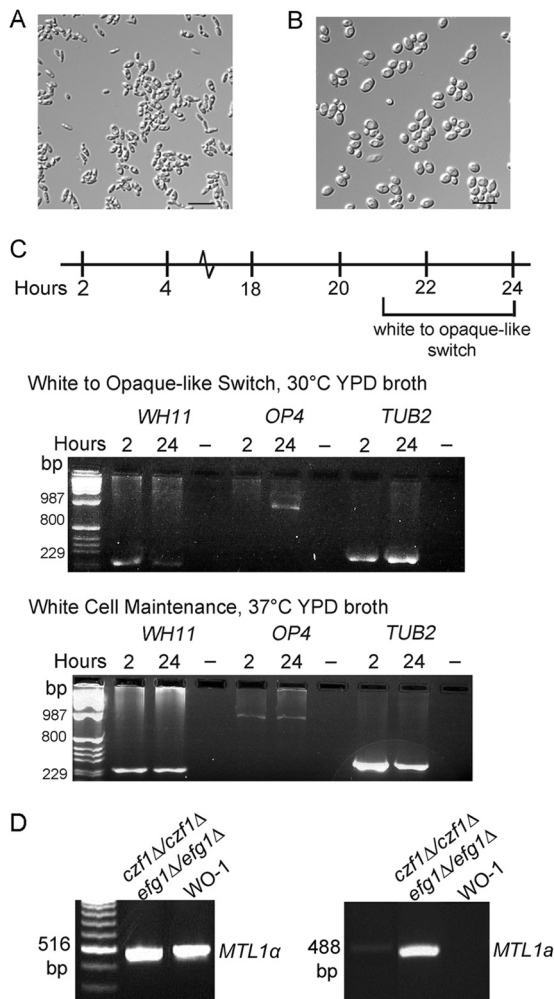


FIG 4 *czf1Δ/efg1Δ* mutant morphogenesis from a white (B) to an opaque-cell-like state (A). Representative micrographs of the different cell morphologies of the *czf1Δ/efg1Δ* double mutant grown in YPD broth at 30°C (A) or 37°C (B) for 24 h (scale bars = 20 μm). (C) Timeline of the morphological switch from a white to an opaque-cell-like state of the *C. albicans czf1Δ/efg1Δ* mutant strain following the inoculation of white-phase cells originally grown on YPD plates at 37°C into YPD broth at 30°C with aeration at 225 rpm. The morphological switch from a white to an opaque-cell-like state was noted between 21 and 24 h postinoculation into 30°C YPD broth. RT-PCR analysis of white-phase- and opaque-phase-specific gene expression (switch from a white to an opaque-cell-like morphology; top gel) by the *czf1Δ/efg1Δ* mutant strain before (2 h) and after (24 h) the morphological change. As a control, RT-PCR analysis of white-phase- and opaque-phase-specific gene expression by cells of the mutant strain pregrown on YPD agar at 37°C, inoculated into 37°C YPD broth, and grown for 24 h was also performed (white cell maintenance; bottom gel). For both gels, total RNA was extracted from cells harvested at 2 and 24 h and the expression of mRNA for the white-phase-specific gene (*WH11*), the opaque-phase-specific gene (*OP4*), and the *TUB2* (β-tubulin) control gene was determined by RT-PCR analysis as described in Materials and Methods. The negative control was no cDNA (-). (D) Products of PCRs showing that the *C. albicans czf1Δ/efg1Δ* double mutant is heterozygous for mating type while the white-to-opaque switching control strain, WO-1, is homozygous for *MTLα*.

were already opaque cell like at the time of farnesol addition (Fig. 5A and C).

We conclude that these cells are opaque cell like; they resemble opaque cells in that they have an elongated cell morphology, stain

with phloxine B, express *OP4*, and are killed by farnesol. However, these cells also differ from opaque cells in that they are smaller than normal opaque cells, still express *WH11*, and are heterozygous for mating type. This combination of features suggests that the genetic network regulating the white-to-opaque switch is misregulated in the *efg1Δ/efg1Δ czf1Δ/efg1Δ* mutant.

Ectopic expression of CZF1 restores filamentation and the farnesol response in an *efg1Δ/efg1Δ czf1Δ/efg1Δ* double mutant under liquid conditions. We next expressed *CZF1* ectopically in the *efg1Δ/efg1Δ czf1Δ/efg1Δ* double mutant to test whether it could restore filamentation and the farnesol response (which blocks filamentation) under liquid conditions. Liquid germ tube formation assays in combination with methylene blue staining were performed with strain CAI4, the *efg1Δ/efg1Δ* mutant, the *efg1Δ/efg1Δ czf1Δ/efg1Δ* double mutant, and the *efg1Δ/efg1Δ czf1Δ/efg1Δ pMAL2-CZF1* mutant (Fig. 6), and only viable cells were counted in these germ tube assays. When the inocula were pregrown at 30°C, only CAI4 was able to produce germ tubes and those germ tubes were inhibited by farnesol in both mGPP and mSPP (Fig. 6A and C). Resting CAI4 cells cannot be prepared at 37°C because under these conditions the cells are filamentous. Inocula of the *efg1Δ/efg1Δ* and *efg1Δ/efg1Δ czf1Δ/efg1Δ* mutant strains pregrown at 37°C were capable of producing low levels of germ tubes with a slight reduction in the presence of farnesol (Fig. 6B and D). Significantly, ectopic expression of *CZF1* in the *efg1Δ/efg1Δ czf1Δ/efg1Δ* double mutant (mSPP) resulted in both an increase in germ tube formation and the inhibition of those germ tubes by farnesol (compare Fig. 6B and D). Thus, ectopic expression of *CZF1* restores both filamentation and the morphological response to farnesol.

Ectopic expression of CZF1 during agar-embedded growth restores filamentation and the response to farnesol. Next we tested whether ectopic expression of *CZF1* could restore filamentation and the response to farnesol in an *efg1Δ/efg1Δ czf1Δ/efg1Δ* double mutant under embedded conditions. As a control, resting CAI4 cells pregrown at 30°C were used for the embedded farnesol response assays. CAI4 produced filamentous colonies when embedded in both GPP and SPP media in the absence of farnesol, and the presence of 50 μM farnesol dramatically decreased filamentation in both media (Fig. 7). The short hyphae seen in the CAI4 colonies treated with farnesol (Fig. 7) appear longer than those of SC5314 and CKY101 (Fig. 3) only because the embedded colonies were photographed at 17 h for Fig. 7 and at 12 h for Fig. 3. No short filaments were observed in the farnesol-treated CAI4 samples at 12 h (data not shown). The 17-h time point was selected for consistency with the other slower-growing mutant strains being tested in Fig. 7.

The *efg1Δ/efg1Δ* mutant was capable of forming filamentous colonies under embedded conditions, and shortened filaments were observed in farnesol-treated samples regardless of whether the inocula had been pregrown at 30°C (Fig. 7) or at 37°C (data not shown). Similarly, the *efg1Δ/efg1Δ czf1Δ/efg1Δ* double mutant produced filamentous colonies in the absence of farnesol (Fig. 6), regardless of the growth temperature used for the inoculum (data not shown). However, both the *efg1Δ/efg1Δ czf1Δ/efg1Δ* and *efg1Δ/efg1Δ czf1Δ/efg1Δ pMAL2-CZF1* strains did not produce any colonies in the presence of 50 μM farnesol when the inocula had been grown at 30°C (data not shown). This absence of colonies under embedded conditions suggests that these cells were killed by farnesol, just as they had been in the liquid assays (Fig. 5A

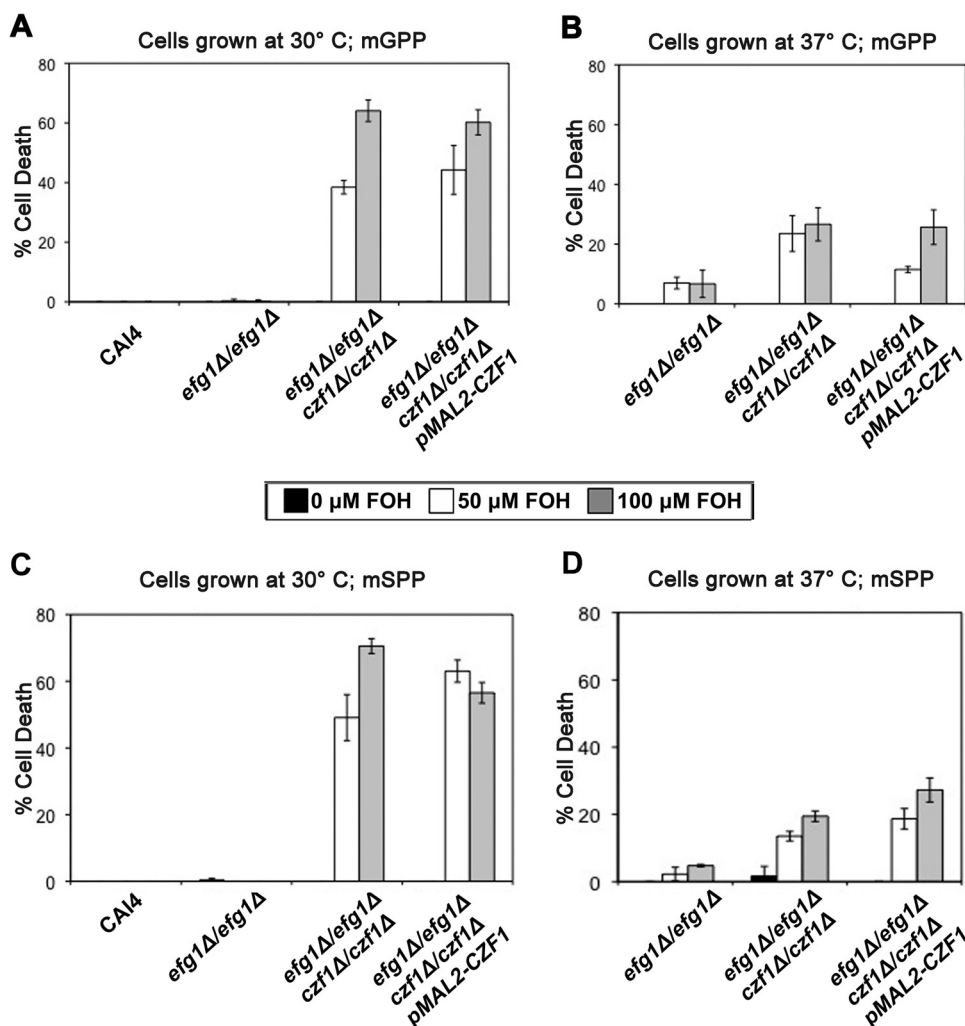


FIG 5 Czf1, Efg1, and temperature play a role in farnesol (FOH) tolerance. Resting cells were prepared from cells grown at either 30°C (A, C) or 37°C (B, D) in YPD broth until stationary phase was reached (24 h, except 48 h for the CAI4 and *efg1Δ/efg1Δ* mutant strains) as described in Materials and Methods. mGPP (A, B) or mSPP (C, D) broth was inoculated with 10^6 resting cells/ml, and 0, 50, or 100 μM farnesol was added. Cultures were incubated at 37°C with shaking at 225 rpm, and cell death was determined by methylene blue staining after 90 min (*efg1Δ/efg1Δ* = HLC67, *czf1Δ/czf1Δ efg1Δ/efg1Δ* = CKY283, *czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1* = AAC7). The data shown are from independent experiments performed in triplicate; these were repeated with similar results on at least two separate occasions.

and C). Both observations are consistent with the double mutant growing as opaque cells at 30°C. With inocula of the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant grown at 37°C, farnesol reduced the number of colonies but the colonies that did grow were filamentous (Fig. 7). The *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant with *CZF1* behaved the same as the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant in noninducing (GPP) medium (Fig. 7A), but an increase in colony size and filamentation was observed in inducing (SPP) medium (Fig. 7B). In the presence of farnesol, these filaments were slightly shorter (Fig. 7B). Thus, ectopic expression of *CZF1* partially restores both filamentation and the morphological response to farnesol under embedded conditions.

To summarize, ectopic expression of *CZF1* in the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant partially restores filamentation and the morphological response to farnesol in both liquid and embedded conditions, with a more prominent restoration occurring under liquid conditions. However, ectopic expression of *CZF1* was

unable to rescue the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant from farnesol-mediated cell death when using an opaque-cell-like inoculum. These results indicate that Czf1 functions downstream of Efg1 in both filamentation and the morphological response to farnesol because Czf1 is sufficient for both responses, regardless of whether Efg1 is present.

Farnesol treatment does not affect *CZF1* expression. Previously, we showed that Tup1 is important for the response to farnesol and that *TUP1* expression levels were increased upon treatment with farnesol. We also showed that this increase in *TUP1* expression corresponds to the commitment point, beyond which added farnesol no longer blocks germ tube formation (14). Since *CZF1* can be regulated at the mRNA level in response to a diverse set of growth conditions, we asked whether *CZF1* mRNA levels are also affected by the presence of farnesol. Thus, a time course experiment was performed to measure *CZF1* expression levels in the presence of 0, 50, and 100 μM farnesol. Samples were taken 40, 60,

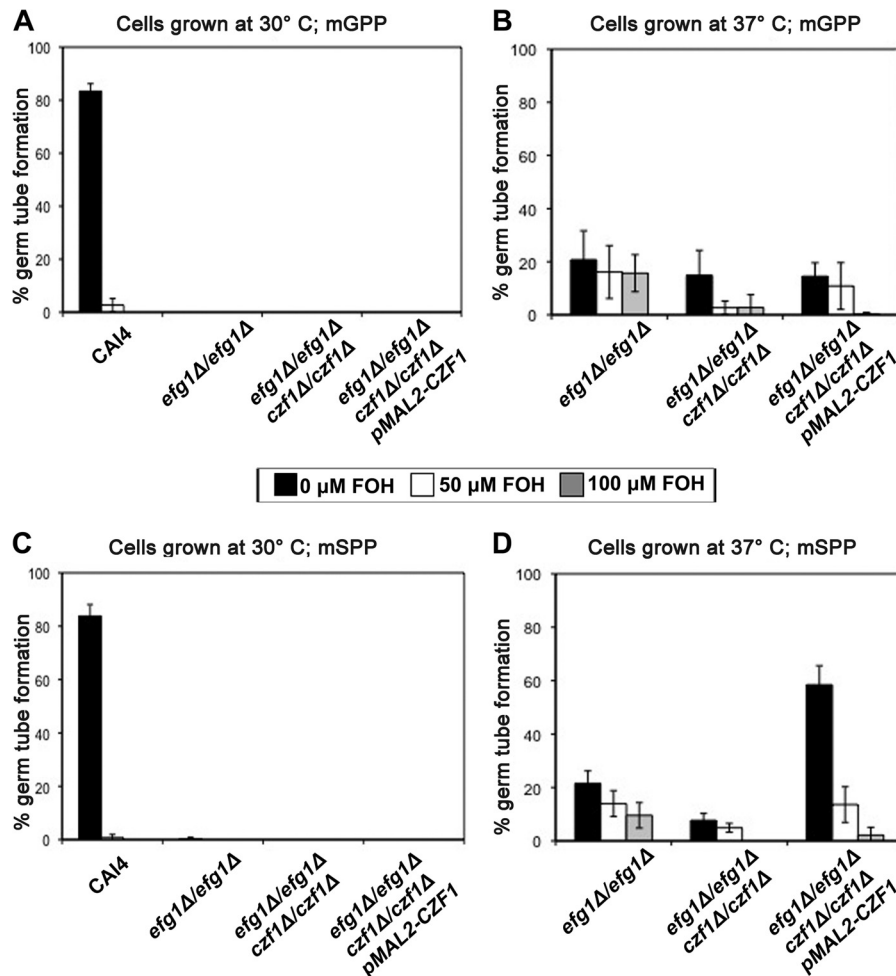


FIG 6 Ectopic expression of *CZF1* in a *czf1Δ/czf1Δ efg1Δ/efg1Δ* double mutant partially restores filamentation and farnesol (FOH) response under liquid conditions. Cells were grown at either 30 or 37°C until stationary phase was reached to prepare resting cells; 10^6 resting cells/ml were inoculated into mGPP (A, B) or mSPP (C, D) broth; and 0, 50, or 100 μM farnesol was added. Cultures were incubated at 37°C with shaking at 225 rpm, and the percentage of germ tube formation was subsequently determined. The data shown are from independent experiments performed in triplicate; these were repeated with similar results on at least two separate occasions.

and 80 min after inoculation on the basis of previous studies showing the importance of this time frame to the farnesol response and the commitment effect (14). As shown by Northern blot analysis (Fig. 8), *CZF1* mRNA levels in wild-type SC5314 showed a slight increase in response to increased farnesol, yet this increase was not statistically significant. In summary, *CZF1* transcript levels are not significantly changed in the presence of farnesol.

Overproduction of farnesol is not a general phenotype of farnesol-resistant mutants. Two additional *C. albicans* transcription regulators that play a role in the farnesol response are Tup1 and Nrg1, and we had previously shown that mutants defective in the production of these regulatory proteins produced 17- and 19-fold higher levels of farnesol, respectively, than did the wild-type and parental strains (14). In order to determine whether farnesol overproduction is a general quality of farnesol-resistant mutants, we tested the farnesol production levels of the *czf1Δ/czf1Δ* mutant (Fig. 9). Farnesol production levels were not significantly altered in the *czf1Δ/czf1Δ* mutant, suggesting a more specific involvement

of Tup1/Nrg1 in farnesol production that does not require the presence of Czf1.

DISCUSSION

In this study, we used genetic screening to identify additional genes that are involved in the *C. albicans* morphological response to farnesol. We showed that Czf1, in particular, plays a prominent role in the morphological response to farnesol under both aerobic (agar surface and liquid) and embedded conditions. The *czf1Δ/czf1Δ* mutant responds poorly, if at all, to added farnesol, but these response capabilities were restored by the ectopic expression of *CZF1*. These observations add a new function for Czf1 in quorum sensing. While the presence of Czf1 is required for filament inhibition by farnesol in an embedded matrix, *CZF1* mRNA levels are not directly regulated by farnesol and Czf1 does not regulate farnesol production levels in the cell. A genetic analysis of *CZF1* in combination with the known morphogenetic transcriptional regulator *EFG1* revealed that *CZF1* works downstream of *EFG1* in its response to farnesol. We used the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double

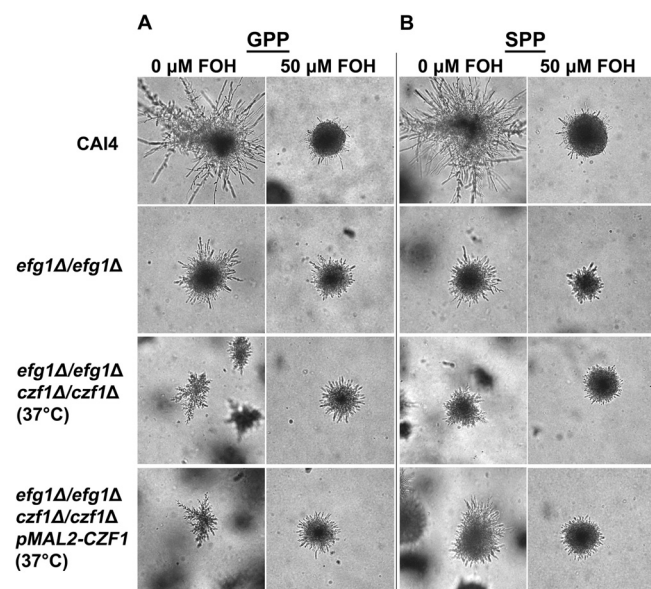


FIG 7 *Czf1* and *Efg1* are both required for a wild-type morphological response and tolerance to farnesol (FOH) in embedded agar. Resting *C. albicans* cells (prepared at 30 or 37°C) were mixed with either GPP (A) or SPP (B) molten agar and 0 or 50 μ M farnesol as described in Materials and Methods and incubated at 37°C for 17 h. The colonies shown were from resting cells pregrown at 30°C unless otherwise noted. Independent experiments were repeated in duplicate with similar results.

mutant coupled with ectopic expression of *CZF1* to show the functional cooperation of *CZF1* and *EFG1* in three aspects of cellular physiology, i.e., the morphological response to farnesol during quorum sensing, sensitivity to killing by farnesol, and white-to-opaque switching. *CZF1* and *EFG1* are the first specific genes identified that control the ability of *C. albicans* to survive in the presence of farnesol, through coordination of the response to farnesol with inhibition of the switch from white to opaque cells.

The cAMP/PKA signaling pathway is proposed to be a direct target for farnesol in *C. albicans* (5, 17), and our study provides additional evidence supporting a primary role for this pathway in farnesol signaling (Fig. 10). Here we showed that *Czf1* and *Tpk1* are required for filament inhibition by farnesol. *Tpk1* is one of two PKA (7) isoforms that function in the cAMP/PKA signaling pathway. A mutant lacking the other PKA isoform, *Tpk2*, could not be identified by farnesol resistance screening, as it was not present in this mutant library. Similarly, adenylyl cyclase (*Cyr1*) could not have been detected even though it is well known to interact with farnesol directly (36). *Czf1* has a tight regulatory relationship with *Efg1*, which is consistent with the cAMP/PKA signaling pathway being a central target of farnesol (Fig. 10). The tight regulatory relationship between *Czf1* and *Efg1* in morphogenesis points toward their pivotal role in intracellular signal integration in response to a myriad of upstream stimuli. Incorporation of the sometimes conflicting signals through the interactions of the transcriptional regulators *Efg1* and *Czf1* gives *C. albicans* the ability to rapidly fine-tune its response to temperature, adhesion to host epithelial matrices, and even fluctuating farnesol concentrations, resulting in the most appropriate morphology for each circumstance. This flexibility is particularly evident in the yeast-to-hyphal transition. *Czf1* strongly contributes to the morphological

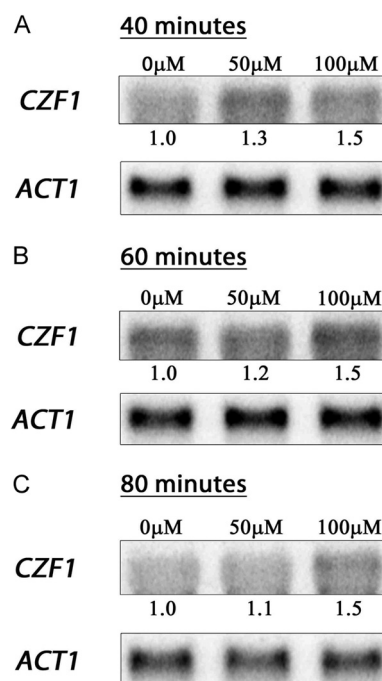


FIG 8 *CZF1* mRNA expression is not significantly altered by the presence of farnesol. Resting SC5314 cells were inoculated into mGPP broth for 40, 60, or 80 min (A to C, respectively) with 0, 50, or 100 μ M farnesol and subsequently harvested for RNA extraction. Quantitative Northern analysis was used to measure relative *CZF1* mRNA levels with *ACT1* as a reference gene. *CZF1* levels in the samples minus farnesol were set at 1, and fold changes in *CZF1* levels in samples with farnesol added are shown below the relevant Northern blot phosphorimages. The data are averages of three replicates.

response to farnesol. In *czf1Δ/czf1Δ* mutant cells, farnesol was unable to suppress filamentation while ectopic complementation of *CZF1* restored the ability of farnesol to block germ tube formation under both liquid and embedded environmental conditions. Further, in *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutants, *CZF1* ecto-

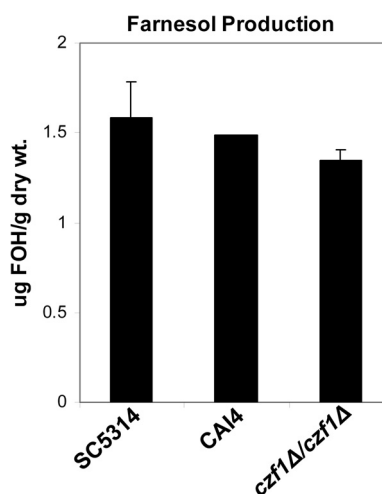


FIG 9 The *czf1Δ/czf1Δ* mutant produces farnesol levels similar to those of the wild-type and parental strains. Cells were grown in GPP broth at 30°C for 48 h prior to farnesol (FOH) extraction and quantification as described in Materials and Methods.

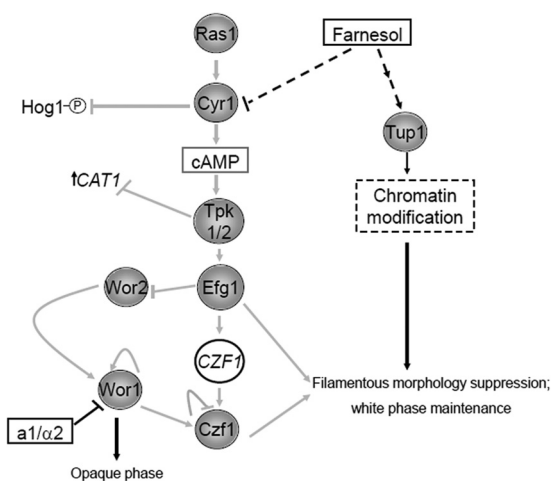


FIG 10 Proposed model of farnesol signaling in *C. albicans*. Dotted lines represent unconfirmed regulatory relationships, black lines represent active regulatory relationships, and gray lines represent regulatory relationships that are inactive because of upstream inhibition.

pic expression partially restored both filamentation and the morphological response to farnesol. These results are consistent with the idea that Czf1 functions downstream of Efg1 during the farnesol response.

Unexpectedly, signal integration between Czf1 and Efg1 is also evident in the temperature-dependent switch from a white to an opaque-cell-like morphology exhibited by the Efg1 and Czf1 double mutant. The double mutant grew as white cells at 37°C and as cells with an opaque-cell-like morphology at 30°C. The 30°C cells were judged to be opaque on the basis of their morphological appearance (Fig. 4A), induction of the opacity-specific gene *OP4* (Fig. 4C), and death on encountering farnesol (Fig. 5A and C). It is known that Efg1 and Czf1 are part of the transcription circuitry that specifies the white and opaque cell types and controls the switching between them (22). Efg1 is a repressor of the opaque state, while Czf1 functions with Wor2 to drive the switch from the white to the opaque state. Without Efg1 and Czf1 within the cell, regulation of the white-to-opaque transition favors Wor1 signaling to promote the formation of an opaque phenotype (22). Disruption of normal interactions between these two transcriptional regulators and their cofactors, even in an α/α mating type background, results in a high frequency of cells with an opaque-cell-like morphology at stationary phase. This unusual morphogenesis may not be coincidental.

Cooperation between Czf1 and Efg1 in that they coordinate the response to farnesol during quorum sensing and the white-to-opaque switch could potentially benefit *C. albicans* by preventing opaque cell formation in the presence of farnesol. Alternatively, white-to-opaque switching during stationary phase—a period typically characterized by nutrient limitation and increasing concentrations of farnesol—could result in a unique altruistic cooperation within the culture where the subpopulation that switches to opaque is killed by farnesol (8), resulting in the release of nutrients into the surrounding medium. Unicellular algal species, such as *Chlamydomonas reinhardtii*, release beneficial nutrients into the surrounding medium during programmed cell death (37), but this type of altruistic behavior has yet to be seen in a *Candida* species. A mechanism for triggering cell death within a

subset of the *Candida* community, for example, within the matrix of a biofilm, could improve survival within a host. The observation that Czf1 and Efg1 play critical roles in both the response to farnesol and white-to-opaque cell switching is probably not a coincidence (21, 22, 38–40), since both are also critical for biofilm formation (20, 41). The hypothesis that intentional dysregulation of Efg1 and Czf1 occurs in biofilms is intriguing, particularly because of its implications for the maintenance of the persistence and virulence of *C. albicans* within the host.

CZF1 mRNA levels do not change in response to farnesol treatment. This is in contrast to previous work demonstrating that *CZF1* transcriptional expression is regulated (23). This result also contrasts with the expression patterns of Tup1, a negative regulator of filamentation, whose mRNA levels are increased upon treatment with farnesol (14). Instead, Czf1 expression is more consistent with the trend seen with the expression of Nrg1 and Efg1, which saw no change in mRNA levels in the presence of farnesol (14). Together, these data suggest that *CZF1* is regulated post-translationally at the protein level, thus allowing rapid signal integration by Efg1 and Czf1 in response to farnesol.

The many effects of farnesol tolerance and signaling in *C. albicans* are unique to this fungus (reviewed in reference 42). Although other *Candida* species can respond to farnesol, specifically, by preventing the yeast-to-pseudohyphal switch in *Candida dubliniensis* (43) and inhibiting biofilm formation by *Candida parapsilosis* (44), these two *Candida* species contrast with *C. albicans* by being highly sensitive to lysis by micromolar levels of farnesol. The finding that Czf1 plays a central role in the unique morphological response of *C. albicans* to farnesol is consistent from an evolutionary point of view because Czf1 is uniquely found within the *Candida* clade (23). As an example, the *CZF1* gene in *C. dubliniensis*, the *Candida* species that produces the second highest level of farnesol, also has the highest homology to the *C. albicans* *CZF1* gene, with 81% identity at the nucleotide level (data not shown). Furthermore, the unusually long 5' untranslated region of *CZF1*, the *CZF1* gene itself, and the neighboring genes at the *CZF1* locus (23) all appear to be conserved to various degrees in other *Candida* species whose genomic sequences have recently become available, i.e., *Candida tropicalis*, *Lodderomyces elongisporus*, *Candida lusitanae*, and *Candida guilliermondii* (data not shown).

In conclusion, we have identified new roles for Czf1 in mediating the *C. albicans* tolerance of farnesol, as well as farnesol-mediated filament inhibition. While the connection of Czf1 to other factors known to play a role in the *C. albicans* farnesol response, such as the cAMP pathway, is apparent, other links remain unclear. For example, cross-regulation among many of the farnesol response pathways was recently summarized (5) but little is known about the regulation of Tup1/Nrg1 and how these factors fit into the farnesol signaling network. Furthermore, other proteins involved in cAMP signaling, such as Ras2, Cap1, and G-actin (36, 45, 46), have not been tested for possible roles in farnesol signaling and may yet prove to be involved. Similarly, the three additional genes identified by the mutant library screening in this study, i.e., *RLM1*, *YCK2*, and *HAP43*, are potentially important for the farnesol response. All three of these genes function in different stress responses, while farnesol, acting through the cAMP/PKA pathway, also confers resistance to oxidative stress. Thus, these mutants would be useful for identifying specific connections between different stress responses and the cAMP/PKA pathway. This idea suggests that farnesol can be an extremely useful tool to dis-

sect the intertwined pathways in *C. albicans*. Although farnesol was initially discovered as a quorum-sensing molecule that regulates morphogenesis, its many other effects produced in the cell may allow a more complete understanding of *C. albicans* signaling as a whole.

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