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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 chlamydomospores; however, the function of chlamydomospores 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 chlamydomosome 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.
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.

**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 mutants. 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 10^4 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 assay was determined with a Zeiss Stevi 2000-C light microscope. Differential interference contrast micrographs were taken with an Olympus BX51 microscope and a Photometrix 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 protocol (DuPont NEN Research Products, Boston, MA).

**Quantitative Northern analysis.** Resting cells were inoculated into 75 ml GPP broth at 5 × 10^6/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 AATCACAACACAAAC-3' and 5'-TACGATTAGAATTCC-3') and ACT1 (5'-AGGATCCGATAGTTCTTGTT-3' and 5'-AGGATC CAGAATTCACGTAACCC-3'). Probes were labeled with [32P]dCTP (GE Health Sciences, Piscataway, NJ) with a RadPrime DNA Labeling system.

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

| Strains | Parental strain | Genotype/description or relevant markers | Source or reference |
|---------|----------------|----------------------------------------|-------------------|
| SC5314  | Clinical isolate | Clinical isolate | 47                |
| CAF2    | CAI-4          | URA1 derivative of CAI-4               | 48                |
| CAI-4   | SC5314         | ura3::immm434/ura3::imm434             | 49                |
| BW17    | RM1000         | ura3::immm434/ura3::imm434 arg8G/arg8G his1::hisG/his1::hisG | 50                |
| HLC67   | CAI-4          | ura3::immm434/ura3::immm434 efg1::hisG/efg1::hisG | 1                 |
| CKY101  | CAI-4          | ura3::immm434/ura3::immm434 ade2::pDB152 | 19                |
| CKY230  | CAI-4          | ura3::immm434/ura3::immm434 czf1::hisG/efg1::hisG ade2::pMAL2-URA3 | 23                |
| CKY116  | CAI-4          | ura3::immm434/ura3::immm434 CZF1::czf1::hisG-URA3::hisG | Carol Kumamoto |
| CKY231  | CAI-4          | ura3::immm434/ura3::immm434 czf1::hisG/efg1::hisG ade2::pMAL2-CZF1-URA3 | 19                |
| CKY283  | CAI-4          | ura3::immm434/ura3::immm434 czf1::hisG/EFG1::hisG ade2::pMAL2-URA3 | Carol Kumamoto |
| AAC2    | CKY283         | ura3::immm434/ura3::immm434 czf1::hisG/EFG1::hisG ade2::pMAL2-CZF1-URA3 | This study        |
| AAC7    | AAC2           | ura3::immm434/ura3::immm434 czf1::hisG/EFG1::hisG ade2::pMAL2-CZF1-URA3 | This study        |

Plasmid pDB212

pMAL2-CZF1 URA3 ade2' Amp^r

19
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 rlm1∆/rlm1∆ 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 opaque 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. Kebara, 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 with Miller and Johnson (29) were used to detect WH11 and OP4. The TUB2 mRNA RT product (5′-CAACTGGTCAATGTGGTAATCA-3′ and 3′-C AATGTTTGGGCTAAAGGTCATTAC-5′) 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, and 72°C 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 MTLa1 genes in CKY283. WO-1 was used as a homozygous MATa 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′-CCTGGAAAAAGCATTCTTGGC and 5′-CAAGTCCACCTTCTGTGG TTC. 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 Chl1, 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 rlm1∆/rlm1∆ 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-

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TABLE 2 Summary of insertion mutants with an impaired farnesol response

| Mutant | S. cerevisiae ortholog | Predicted or known biological process(es) (reference[s]) | Reference(s) |
|--------|------------------------|-------------------------------------------------|-------------|
| czf1∆/czf1∆ | 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 |
| tpk1/tpk1 | Tpk2 | Catalytic subunit of cAMP-dependent protein kinase A, regulator of morphogenesis; Tpk2 isoform involved in multiple stress responses | 51–54 |
| rlm1∆/rlm1∆ | Rlm1 | Transcription factor for genes involved in cell wall organization and biogenesis and various stress responses | 55, 56 |
| stp2/stp2 | Stp2 | Transcription factor for amino acid permease genes | 57 |
| yck2/yck2 | Yck2 | Maintenance of cell polarity, antimicrobial peptide resistance, contributes to epithelial cell damage | 58 |
| hap43/hap43 | 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 RLMI, 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* null mutant showed only a minimal reduction in the percentage of cells forming germ tubes, even in the presence of 100 μM 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 *C. albicans* SC5314 and CKY101 cells when they were grown embedded in an agar matrix in that 50 μM 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
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 czf1Δ/czf1Δ mutant was grown in agar containing farnesol, only a moderate farnesol response was observed; the filaments appeared shorter than in un-grown in agar containing farnesol, only a moderate farnesol response was observed; the filaments appeared shorter than in un-grown in agar containing farnesol, only a moderate farnesol response was observed; the filaments appeared shorter than in un-

An efg1Δ/efg1Δ czf1Δ/czf1Δ 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 efg1Δ/efg1Δ czf1Δ/czf1Δ 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 α/α, such as our efg1Δ/efg1Δ czf1Δ/czf1Δ mutant, are normally maintained in the white phase by a1-a2 repression of WOR1 (22) and czf1Δ/czf1Δ mutant cells are known to have decreased white-to-opaque switching (22), although switching does occur at a very low rate in MTLα/MTLα 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 efg1Δ/efg1Δ czf1Δ/czf1Δ 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, efg1Δ/efg1Δ czf1Δ/czf1Δ 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 CA41 or the efg1Δ/efg1Δ single mutant when it was pregrown at 30°C and assayed in either mGPP or mSPP, even in the presence of 100 μM farnesol (Fig. 5A and C). Further, only a small increase in cell death was observed in the efg1Δ/efg1Δ 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 CA41 and the czf1Δ/czf1Δ mutant pregrown at 37°C because they form germ tubes that stain with methylene blue regardless of the presence of farnesol. However, the CA41 and czf1Δ/czf1Δ mutant strains are viable in the presence of 50 to 100 μM 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 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 μM farnesol as described in Materials and Methods and incubated at 37°C for 12 h. Independent experiments were repeated in duplicate with similar results.
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 mis-
regulated in the \(efg1\Delta/\Delta/efg1\Delta/\Delta\) mutant.

**Ectopic expression of CZF1 restores filamentation and the
farnesol response in an \(efg1\Delta/\Delta/efg1\Delta/\Delta\) double mutant under liquid conditions.** We next expressed CZF1 ectopically in the \(efg1\Delta/\Delta/\Delta/\Delta\) 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 \(efg1\Delta/\Delta/\Delta/\Delta\) mutant, the
\(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) double mutant, and the \(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) \(p\)MAL2-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 \(efg1\Delta/\Delta/\Delta\) and \(efg1\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta\) mutant strains pregrown at 37°C were capable of producing lower levels of
tubes with a slight reduction in the presence of farnesol (Fig.
6B and D). Significantly, ectopic expression of CZF1 in the \(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta\) 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 \(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) 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 \(\mu M\) farnesol dramatically decreased filamenta-
tion 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 farnesol-treated CAI4 samples at
12 h (data not shown). The 17-h time point was selected for consist-
ency with the other slower-growing mutant strains being tested
in Fig. 7.

The \(efg1\Delta/\Delta/\Delta\) 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 \(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) 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 \(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) and
\(efg1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) \(p\)MAL2-CZF1 strains did not produce
any colonies in the presence of 50 \(\mu M\) 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
and C).

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

![FIG 4](image-url)

**FIG 4** \(czf1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) mutant morphogenesis from a white (B) to an opaque-cell-like state (A). Representative micrographs of the different cell
morphologies of the \(czf1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) double mutant grown in YPD broth at 30°C (A) or 37°C (B) for 24 h (scale bars = 20 \(\mu m\)). (C) Timeline of the morphological switch from a white to an opaque-cell-like state of the \(C. albicans\) \(czf1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) 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
cDNA (\(OP4\)). (D) Products of PCRs showing that the \(czf1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) double mutant is heterozygous for mating type while the white-
phase cells originally grown on YPD plates at 37°C into YPD broth at
37°C (A) or 37°C (B) for 24 h (scale bars = 20 \(\mu m\)). (C) Timeline of the morphological switch from a white to an opaque-cell-like state of the \(C. albicans\) \(czf1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) 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
cDNA (\(OP4\)). (D) Products of PCRs showing that the \(C. albicans\) \(czf1\Delta/\Delta/\Delta/\Delta\) \(\Delta/\Delta/\Delta/\Delta\) double mutant is heterozygous for mating type while the white-
tube switching control strain, WO-1, is homozygous for \(MTL1\alpha\).

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
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,
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 czf1/H9004/czf1/H9004 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
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 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 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 µM 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.

**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 µM 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.

**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.
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 a/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 posttranslationally 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 lusitaniae, 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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