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Farnesol Signaling in *Candida albicans*

By

Melanie L. Langford

A Dissertation

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor Audrey L. Atkin

Lincoln, Nebraska

May, 2010

Farnesol Signaling in *Candida albicans*

Melanie L. Langford, Ph.D.

University of Nebraska, 2010

Advisor: Audrey L. Atkin

Candida albicans is a polymorphic fungus that causes a range of disease in humans, from mucosal infections to systemic disease. Its ability to cause disease is linked to conversion between yeast and filamentous forms of growth, and the first quorum-sensing molecule discovered in an eukaryote, farnesol, blocks this transition. In *C. albicans*, farnesol also kills mating-competent opaque cells, inhibits biofilm formation, protects the cells from oxidative stress, and can be a virulence factor or protective agent in disseminated and mucosal mouse models of infection, respectively. While much emphasis has been placed on determining its effect on *C. albicans* morphology, the molecular response to farnesol is not completely understood. The overall theme for this dissertation was to better understand the *C. albicans* molecular response to farnesol under quorum sensing conditions. Due to the duplicitous nature of the farnesol response in *C. albicans*, i.e., its ability to kill cells or simply alter morphology, we clearly defined the environmental conditions in which farnesol acts as a quorum sensing molecule or as a toxic agent towards *C. albicans*. This clarification enabled a subsequent two-pronged approach to study the molecular response to farnesol during morphological regulation. A direct approach was used to investigate the role of a likely candidate, Tup1, a negative regulator of hyphal development, in farnesol signaling. Secondly, a screening approach was utilized to identify new farnesol resistant mutants that may participate in the farnesol

response. From the mutants identified, Czf1 (*C. albicans* zinc finger) was selected for further characterization and was shown to play a vital role in the morphological response to farnesol as well as farnesol tolerance. Overall, this study identified two new factors involved in farnesol signaling, and highlights the power of farnesol as a tool with which to unravel the complex signaling networks present in *C. albicans*.

ACKNOWLEDGEMENTS

No one ever gets through their Ph.D. program on their own, and I am certainly no exception. I would like to begin by thanking my wonderful advisor, Audrey Atkin. What a happy accident joining your lab turned out to be- I never would have guessed that I would switch from studying bacteriology to fungal biology! It makes an incredible difference to work for someone that not only encourages quality research but is so pleasant on a personal level as well, things that I will miss. During my time spent in your lab, I have learned nearly everything I know about fungi, and I have had the chance to learn several new lab techniques as well, such as working with RNA. I am happy you encouraged me to give several oral and poster presentations, both locally and nationally; what initially seems like more work always pays off in learning experiences. I also think it says a lot about the career development information you provide your lab members when I have heard nearly everything discussed in our Professionalism course this semester from you already over the course of my graduate program. Lastly I always appreciated your support whenever a crisis arose (be it professional, experimental, or personal).

I would also like to extend my thanks to Kenneth Nickerson, my favorite fungal biology neighbor. I will miss having a chemistry expert right next door and your enthusiasm for nearly everything and anything in science. I have truly enjoyed having joint Atkin-Nickerson lab meetings, and I feel they have been critical to the completion of my degree. To both Audrey and Ken, I am always amazed at how well your collaboration works (given your entirely different research styles, personalities, etc), but I am happy that it does (maybe I fall somewhere in the middle of you two?). I think I have

learned about balancing careful experimental design with exploratory experiments moving in new directions, and I have also enjoyed writing papers with both of you and learning about the process of paper submission.

Next I would like to thank all my committee members: Steve Harris, Jack Morris, and Luwen Zhang. You always provided interesting ideas and suggestions during my committee meetings, and I appreciate that you always did so in a very constructive manner. Thanks to Cheryl Bailey for teaching me real-time PCR from scratch- it is easy to find people that will let you borrow their machines, but not so easy to find someone to teach you the method and theory behind it. There are also so many past and current lab members to thank. First, I would like to thank Bessie Kebaara, a former post-doc in the lab that I still miss having around. I could always go to you with technical questions, scientific ideas, or just to complain about the cold weather. Dhammika Navarathna and Raluca Dumitru, former Ph.D. students in the Nickerson lab, both taught me important *Candida* techniques and were great role models for me as a new student. And thanks to Suman Ghosh, another former Ph.D. student, for teaching me farnesol extraction techniques and for being a good friend. Of the current students in our research group, I would first like to extend my gratitude to Sahar Hasim, for being such an excellent collaborator on the farnesol toxicity project. You are such an amiable and reliable person to work with, and even though things took longer to finish than we originally thought (seems they always do), I think we learned some very valuable information over the course of that project. Thanks also to Swetha Tati (my technical support aide prior to my defense), Krista Fager, Jeff Bunker, and Narmin Tahirova for all your helpful comments and suggestions during journal club and lab meetings.

To my family: Mom, Dad, Courtney, Gina, Jim, and all the Langford brothers, I would like to thank you for all of your support during both my M.S. and Ph.D. degrees. Believe it or not, attempting to explain to you what I do in the lab has actually improved my oral presentations to more general scientific audiences! I am very lucky to have such supportive family members on my side.

And lastly, I would like to thank my husband, Gabe. I am certain that I would not have been able to get through graduate school without you, much less have the confidence to even pursue my Ph.D. degree. It has been chaotic but fun to go through the ups and downs of graduate school with you, as you were always the perfect confidant. I am so excited to start the next phase of our life together with our new family.

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INTRODUCTION

Reference:

Langford, M.L., Atkin, A.L., and Nickerson, K.W. (2009) Cellular Interactions of Farnesol, a Quorum Sensing Molecule Produced by *Candida albicans*. *Future Microbiol.* **4:** 1353-62.

INTRODUCTION

The discovery of E,E-farnesol as a quorum sensing molecule in *Candida albicans* (Hornby *et al.*, 2001) demonstrated the existence of quorum sensing in a eukaryotic organism, a process once thought to be confined to bacteria. The earlier work on the discovery and characterization of farnesol was reviewed by Nickerson et al (Nickerson *et al.*, 2006). *C. albicans* is a polymorphic fungus that is capable of growing in yeast, hyphal, pseudohyphal, opaque, and chlamydospore cell morphologies. Farnesol is capable of blocking the yeast to hyphal/pseudohyphal (filaments) switch (Hornby *et al.*, 2001), a conversion that is the focus of intense study in *C. albicans* because of its role in virulence (Lo *et al.*, 1997; Saville *et al.*, 2003). Another quorum sensing molecule (QSM) described in *C. albicans* is farnesoic acid (Oh *et al.*, 2001). Farnesoic acid, like farnesol, acts to block the yeast to filament transition (Oh *et al.*, 2001), but it is far less active than farnesol (Shchepin *et al.*, 2003) and is produced by only one known strain of *C. albicans*, 10231 (Hornby and Nickerson, 2004; Oh *et al.*, 2001). Additional possible quorum sensing molecules are reviewed by Kruppa (Kruppa, 2009). Farnesol also blocks biofilm formation by *C. albicans* (Alem *et al.*, 2006; Cao *et al.*, 2005; Martins *et al.*, 2007; Ramage *et al.*, 2002) in a manner consistent with its inhibition of the yeast to filament conversion. The mating-competent form of *C. albicans*, opaque cells, can be adversely affected by farnesol; in aerobic conditions the cells die by necrosis (Dumitru *et al.*, 2007) but remain unharmed in anaerobic conditions (Dumitru *et al.*, 2004). Lastly, the production of chlamydospores, a *C. albicans* cell morphology with unknown function, is increased in the presence of very high (10 mM) levels of farnesol (Martin *et al.*, 2005). Given that it may affect all five *C. albicans* growth morphologies, it has

become clear that farnesol plays an influential role in *C. albicans* physiology, and the search is underway to elucidate the molecular mechanism(s) of these effects.

The Molecular Response to Farnesol in *C. albicans*

The first described function of farnesol was to block filamentation induced by a variety of environmental signals (Hornby *et al.*, 2001; Mosel *et al.*, 2005), and several factors involved in the filamentation process are known to play a role in the *C. albicans* farnesol response. Ras1 of the cyclic AMP pathway, as discussed later, is a candidate for direct inhibition by farnesol (Davis-Hanna *et al.*, 2008). Repressors of filamentation, Tup1 and Nrg1, also play critical roles in the farnesol response, as *tup1Δ/tup1Δ* and *nrg1Δ/nrg1Δ* mutants are unable to respond to farnesol (Kebaara *et al.*, 2008). The presence of farnesol increases *TUP1* expression and suppresses the haploinsufficient phenotype of a *TUP1/tup1Δ* mutant (Kebaara *et al.*, 2008). A two-component signal transduction pathway histidine kinase, Chk1 (Kruppa *et al.*, 2004), and mitogen activated protein (MAP) kinase pathway components, Cph1 and Hst1 (Sato *et al.*, 2004), may also be involved in the response to farnesol, but Cph1 might play a downstream role since a *cph1Δ/cph1Δ* mutant retains the ability to respond to farnesol (Davis-Hanna *et al.*, 2008). Regulators of filamentation are not the only genes involved in the farnesol response. Microarray analyses have identified other categories of genes affected by farnesol treatment such as heat shock genes, drug resistance genes, cyclin and cell proliferation genes, histone genes, genes expressed at high cell density, phagocytosis response genes, and adhesion genes (Cao *et al.*, 2005; Cho *et al.*, 2007; Enjalbert and Whiteway, 2005; Uppuluri *et al.*, 2007). With all of the different pathways and responses induced by farnesol, it promotes the question: Does farnesol have one or more specific target(s) in

the cell, or does it somehow elicit a more general and nonspecific response with pleomorphic consequences?

Are there farnesol receptors or farnesol binding proteins? The morphological response to farnesol in *C. albicans* appears to be very sensitive to minor changes in the structure of farnesol, providing support for the specific target(s) model, rather than a scenario involving general membrane disruption. For example, there are four isomers of farnesol but only E,E-farnesol is capable of blocking filamentation in *C. albicans* (Shchepin *et al.*, 2003). This conclusion is based on a comparison between pure (96%) E,E-farnesol and two commercial mixed isomers containing 56 and 36% E,E-farnesol. These three farnesol preparations reduced germ tube formation (early stage filaments) to 50% at 1.2, 3.5, and 4.4 μM, respectively (Shchepin *et al.*, 2003). Thus, there was sufficient E,E-farnesol in the two samples with mixed isomers to account for all of their QSM activities. Further, the 12-carbon backbone of farnesol appears to be critical for its ability to block filamentation because altering the chain length generally results in decreased filament inhibitory activity (Hogan *et al.*, 2004; Kim *et al.*, 2002; Shchepin *et al.*, 2003; Shchepin *et al.*, 2005). Other farnesol analogs and related molecules from other organisms, such as 3-oxo-C12-homoserine lactone and dodecanol, are capable of blocking filamentation in *C. albicans*, suggesting some limited flexibility for the inhibitory action of farnesol-related molecules (Davis-Hanna *et al.*, 2008; Hogan *et al.*, 2004; Kim *et al.*, 2002; Shchepin *et al.*, 2003; Shchepin *et al.*, 2005). One, but not the sole explanation for these results is the presence of a farnesol receptor(s) or farnesol binding protein(s) in *C. albicans*. Fluorescent farnesol (Shchepin *et al.*, 2005) and a

farnesol affinity column (Shchepin, 2006) were developed to help isolate farnesol binding proteins, so far with limited success.

Farnesol and Ras1/cAMP Signaling

Ras1 and the adenylyl cyclase-cAMP-protein kinase A (PKA)-Efg1 pathway (Fig. 1) have been proposed as a potential direct target for farnesol inhibition (Davis-Hanna *et al.*, 2008). This idea is very reasonable and it is persuasive that farnesol was able to reverse filamentation in the dominant active (CAI4-Rasl^{G13V}) mutant of *C. albicans* and farnesol inhibition of filament formation was itself reversed by addition of dibutyryl-cAMP (Davis-Hanna *et al.*, 2008, discussed in more detail by Paula Sundstrom and Deborah Hogan in this issue). It may be important that Ras1 is one of the few farnesylated proteins in *C. albicans*, and it has been estimated that the farnesylated, membrane-bound form of Ras1 is ca. 100 X more effective in activating adenylyl cyclase than is the cytoplasmic form (Kuroda *et al.*, 1993). Thus, there are several ways by which farnesol could inhibit the Ras1-cAMP-PKA-Efg1 pathway (Fig. 1). A partial list includes: blocking the farnesylation of Ras1, see review by Hancock et al (Hancock, 2003), releasing farnesylated Ras1 from the membrane (Fig. 1A), binding directly to Ras1 (Fig. 1B), and binding directly to a downstream protein such as adenylyl cyclase (Fig. 1C). It is unlikely that farnesylated Ras1 has the farnesyl tail removed because it is attached by a very stable thioether bond and, where measured, farnesylated Ras1 has a long half life.

Having Ras1 as a direct target for farnesol can provide some unity for many of the filamentation genes involved in the farnesol response since it is a common regulator for the Hst7/Cph1 MAP kinase pathway and the cAMP pathway (Castilla *et al.*, 1998; Feng

et al., 1999; Leberer *et al.*, 2001). While it is unknown what factors regulate Tup1/Nrg1 and Chk1, it is possible that they are also under the control of Ras1. A second possibility for farnesol signaling is the presence of multiple farnesol targets, each affecting different aspects of filamentation in response to different inducing stimuli, while a third possibility is that farnesol interacts with the cell membrane in a very specific manner to induce a pleiotropic signaling response in the rest of the cell. Notice that Figure 1 has been drawn with both intracellular and extracellular farnesol. This was done to emphasize that further research is warranted to determine the exact nature of the molecular response to farnesol during yeast to filament inhibition. It is difficult to say whether farnesol acts from the outside or the inside because it can cross the cytoplasmic membrane by diffusion. On the one occasion when cellular localization was attempted (Navarathna *et al.*, 2005), the values for extracellular, intracellular, and membrane-associated farnesol were 0.115, 0.009, and 0.106 mg/g dry weight of cells, respectively (Navarathna *et al.*, 2005). These values correspond to concentrations of ca. 4 μM extracellular (Nickerson *et al.*, 2006) and 13.5 μM intracellular, with the intracellular calculation based on a yeast cytoplasmic volume of ca. 3 μl/mg dry weight. Similarly, the membrane-associated farnesol would constitute 1-2% of the total extractable membrane lipids based on a value of 8.5 mg extractable lipid/g dry weight (Hitchcock *et al.*, 1986).

Possible Artifacts

We have found several artifacts associated with the cellular response to farnesol. Caveats which must be considered include: 1) some auxotrophic mutants (*his1Δ/his1Δ*, *arg4Δ/arg4Δ*) produce germ tubes faster than their wild type, prototrophic counterparts and 2) as a consequence, when examining a mutant for its response to farnesol, it is

always safer to run a time course with and without farnesol until 90-100% germ tube formation has been achieved in the control lacking farnesol (our unpublished data). This prevents a misinterpretation for lack of farnesol response rather than a faster rate of filamentation. A third potential artifact is derived from farnesol's lipophilic nature. We previously pointed out how ca. 150x more farnesol was needed to block germ tube formation in the presence of 10% serum than in its absence (Mosel *et al.*, 2005). The farnesol concentration would be effectively reduced because of the lipid binding capacity of serum albumins. A similar trap could occur when farnesol's action in blocking germ tube formation or inhibiting growth is reversed by added diacylglycerol (DAG) or 1-oleoyl–2-acetyl-*sn*-glycerol (OAG). Machida et al (Machida *et al.*, 1999), Voziyan et al (Voziyan *et al.*, 1995) and Uppuluri et al (Uppuluri *et al.*, 2007) attributed internal modes of action, i.e. protein kinase C activation (Voziyan *et al.*, 1995) or interference with phosphatidylinositol signaling (Machida *et al.*, 1999; Uppuluri *et al.*, 2007), to this DAG/OAG reversal. However, micelle forming detergents or lipids such as DAG and OAG could incorporate the farnesol and effectively reduce its concentration. Thus, a simple way of distinguishing between reversal by an external "micelle trap" versus a more specific cytoplasmic mechanism is to find out whether equivalent amounts of detergents such as Triton X-100 and NP40 also reverse farnesol's action. This idea was recently tested for farnesol's growth inhibition of *Candida parapsilosis*. OAG and Triton X-100 showed equivalent reversal of farnesol's growth inhibition (T. Rossignol and G. Butler, personal communication), and in this case, no conclusions could be drawn on farnesol's intracellular mode of action.

Farnesol Production by *Candida* Species

Weber et al (Weber *et al.*, 2008) recently compared the farnesol production levels from 56 strains of eight *Candida* species. *C. albicans* had the highest production levels (35 $\pm$ 16 μM) compared to the other *Candida* species tested: *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, and *C. glabrata* (Weber *et al.*, 2008). The closely related species *C. dubliniensis* produced 8.7 $\pm$ 3.8 μM farnesol while the remaining *Candida* species all produced $\leq$ 1 μM farnesol. This value for *C. albicans* (35 μM) is substantially higher than our best estimates (Nickerson *et al.*, 2006) of 2 to 4 μM farnesol at a yeast cell density of 10^8/ml, based on production of ~ 0.13 mg farnesol per g dry weight of fungus (Hornby and Nickerson, 2004). Production levels for two laboratory and four clinical isolates were tightly clustered from 0.11 to 0.14 mg per g dry weight (Hornby and Nickerson, 2004) whereas the seven strains of *C. albicans* studied by Weber et al (Weber *et al.*, 2008) varied from 13 to 58 μM farnesol. This wide variance (Weber *et al.*, 2008) might be resolved by normalizing the data on a per g dry weight basis. Also, despite the different analytical procedures employed, GC/MS by Hornby and Nickerson (Hornby and Nickerson, 2004) and derivatization with 9-anthroyl nitrile followed by HPLC by Weber et al (Weber *et al.*, 2008), the estimates of 35 μM vs. 2-4 μM farnesol may be compatible. If the cell densities achieved following 24 hrs growth in RPMI at 37°C (Weber *et al.*, 2008) were ca. 10^9/ml, compared to only 10^8/ml in GPP at 30°C (Hornby and Nickerson, 2004), then the two data sets would be in remarkably close agreement.

Careful consideration must also be given to the growth medium when measuring farnesol production levels. For example, the addition of 10% fetal calf serum (FCS) lowered farnesol production ca. 18-fold (Weber *et al.*, 2008) but the mechanism behind

this reduction remains unclear. The nonspecific lipid binding capability of serum albumin was evident in the fact that 150-fold more farnesol was needed to block filamentation than in its absence (Mosel *et al.*, 2005). Similarly, industrial stratagems to maximize farnesol production (Muramatsu *et al.*, 2008, 2009) commonly used an external lipid sink such as 5% soybean oil to shift the equilibrium toward production and excretion of farnesol. Thus, 10% FCS should have increased farnesol production rather than decreased it (Weber *et al.*, 2008). Possibly the low levels of farnesol detected with 10% FCS used an extraction protocol which did not fully denature the lipid-binding albumins. The importance of a nearby lipid sink will be revisited in our discussion on the role of farnesol in pathogenicity.

Manipulating the sterol biosynthetic pathway in *C. albicans* and other fungi with inhibitors such as fluconazole, terbinafine, SQAD, or zaragozic acid can result in increased farnesol production levels (Hornby *et al.*, 2003; Hornby and Nickerson, 2004), and three genes have been identified in *C. albicans* thus far that regulate farnesol production: *DPP3*, *TUP1*, and *NRG1* (Kebaara *et al.*, 2008; Navarathna *et al.*, 2007a). *C. albicans* Dpp3 is an ortholog of the *S. cerevisiae* phosphatase Dpp1 (Toke *et al.*, 1998), and it presumably acts as a phosphatase to convert farnesyl pyrophosphate to farnesol (Navarathna *et al.*, 2007a). Deletion of the *C. albicans DPP3* gene results in farnesol production levels that are six times lower than wild type and parental levels (Navarathna *et al.*, 2007a). Conversely, Tup1 and Nrg1 appear to play a negative role in farnesol production; *tup1Δ/tup1Δ* and *nrg1Δ/nrg1Δ* mutant strains produce 17 and 19-fold higher farnesol, respectively, than their parents, but it is unclear whether this is a result of direct or indirect sterol biosynthesis regulation (Kebaara *et al.*, 2008). Further

research is required to fully understand the regulation of farnesol production in both *C. albicans* and other *Candida* species.

Farnesol and Interspecies Communication

A developing branch of research has focused on the effects of farnesol signaling on interspecies communication. A brief summary of the effects of farnesol on other organisms is described in Table 1. Filament and biofilm development are each blocked by farnesol in the closely related *C. dubliniensis,* similar to the *C. albicans* morphological response to farnesol (Henriques *et al.*, 2007; Jabra-Rizk *et al.*, 2006b; Martins *et al.*, 2007). In contrast, *C. parapsilosis*, which normally produces very low levels of farnesol, appears to respond in a different manner; farnesol reduces *C. parapsilosis* biofilm formation independently from filament inhibition (Laffey and Butler, 2005; Rossignol *et al.*, 2007). The use of physiologically relevant farnesol concentrations to avoid unintended cytotoxic effects will determine whether farnesol blocks dimorphism and/or biofilm formation in other *Candida* species. In other cell types, farnesol can be inhibitory or toxic, often accompanied by elevated reactive oxygen species (ROS) production. However, these reports encompass a huge range of farnesol concentrations, many of which are above the ca. 1 mM solubility limit for farnesol, increasing the likelihood that the responses observed are both specific and non-specific, or detergent-like.

Given that the production of extracellular signaling molecules is certainly not unique to *C. albicans*, there has also been some recent work describing the converse situation in which the response of *C. albicans* to bacterial signaling molecules is examined (Table 2). It is important to distinguish between general growth inhibition (an antifungal antibiotic) and specific inhibition of one type of growth, e.g. a QSM blocking

the yeast to filament transition in *C. albicans*. For instance (Table 2), while Burkholderia diffusible signal factor (BDSF) at a 5 μM concentration inhibited 70% of germ tube formation for *C. albicans* cells, that same concentration also strongly inhibited growth (Boon *et al.*, 2008). It is intriguing how a similar molecule produced by *Xanthomonas campestris*, diffusible signal factor (DSF), can inhibit filamentation without any growth inhibition (Boon *et al.*, 2008; Wang *et al.*, 2004), even though the two molecules differ by only one methyl group. Molecules that are not inhibitory for the general growth of *C. albicans*, yet induce a signaling response are legitimate candidates for cross-kingdom signaling molecules (Table 2).

An interesting consideration to make when evaluating the variety of responses to QSMs such as farnesol in different organisms is the likelihood of the cells to interact with one another during the commensal state versus during an infection. Similarly, one must also consider the location of the interaction within the host, as farnesol production by *C. albicans* results in different host responses depending on the site of infection. For instance, it is likely important for *C. albicans* commensal life style in the gastrointestinal tract (Kumamoto and Vinces, 2005b) that farnesol production is turned off during anaerobic growth (Dumitru *et al.*, 2004). Lastly, farnesol must be used carefully and at the appropriate physiologically relevant concentrations in order to avoid artifactual observations, such as unintended cytotoxic effects.

Farnesol-Mediated Cell Death in *C. albicans*

With regard to the possibility of farnesol-mediated cell death, the literature has conflicting reports on the appropriate levels of farnesol with which to treat *C. albicans*. Several groups have used 150-250 μM farnesol with no apparent cell death (Davis-Hanna

et al., 2008; Henriques *et al.*, 2007; Hogan *et al.*, 2004; Kruppa *et al.*, 2004); one study by Jabra-Rizk et al (Jabra-Rizk *et al.*, 2006b) concluded the minimum inhibitory concentration (MIC) for *C. albicans* was >250 μM while another study concluded that 40 μM farnesol induced cell death (Shirtliff *et al.*, 2009). Conflicts have also emerged regarding the concentration of farnesol needed to block filamentation in *C. albicans*, with effective concentration reports ranging from 4 μM (Mosel *et al.*, 2005), up to 250 μM (Kruppa *et al.*, 2004). Another important finding was that of Davis-Hanna et al (Davis-Hanna *et al.*, 2008) who noted that higher concentrations of farnesol were required to block filamentation in plastic microtiter plates compared to borosilicate glass tubes or flasks, suggesting a possible farnesol adsorption effect by plastic that might lower the effective concentration of farnesol. Our summary view is that the appropriate level of farnesol to use for filament inhibition is ≤ 50 μM farnesol when using glassware; this corresponds to physiologically relevant concentrations of farnesol produced by stationary-phase *C. albicans* cultures (Weber *et al.*, 2008). While the plastic versus glassware variable may explain some discrepancies in the literature, additional variables need to be considered when evaluating farnesol-mediated cell death in *C. albicans*. Farnesol is clearly a bioactive molecule. With the caveat that inappropriately high concentrations were often used, the recurrent theme in Table 1 is that farnesol is inhibitory or lethal to a great many other cell types. Thus, a fundamental question is how *C. albicans* has evolved to withstand the farnesol which it produces in great abundance. This question is exactly analogous to an antibiotic producing strain being resistant to that antibiotic. A corollary to this idea is that reports of farnesol mediated cell death be

viewed as circumstances where *C. albicans* has let down its guard, i.e. the molecular defense mechanisms are regulable rather than constant or intrinsic.

Farnesol has been reported to cause growth defects (Uppuluri *et al.*, 2007), necrosis (Dumitru *et al.*, 2007) and apoptosis (Shirtliff *et al.*, 2009) in *C. albicans*, although the precise concentrations required are somewhat unclear, because different conditions were used to grow the cells and different methods were used to assess cell damage. Work is underway in our laboratories to investigate farnesol tolerance by *C. albicans*, taking into account many environmental variables, in an effort to unify and validate existing data in the literature (Langford et al, data not shown). Our conclusions so far are that four factors are significant. First, only white cells of *C. albicans* are resistant. Dumitru et al (Dumitru *et al.*, 2007) observed that the opaque cells were very sensitive to farnesol, being lysed rapidly by ≥ 40 μM. Following this line of thinking, in a saturated aerobically grown culture of predominantly white cells, the farnesol levels could possibly reach levels high enough to kill opaque cells. Second, farnesol resistance is somewhat energy dependent. The farnesol-induced death and apoptosis reported by Shirtliff et al (Shirtliff *et al.*, 2009) was for cells which had been stored for 24h in PBS buffer with no exogenous energy sources. Third, as was first pointed out by Uppuluri et al (Uppuluri *et al.*, 2007), the starting growth phase of the inoculum is critical. At 25°C, *C. albicans* log-phase cells were considerably more sensitive to growth inhibition by farnesol than were stationary-phase cells. Stationary-phase inocula maintained similar growth rates and viability with 0-100 μM farnesol, while log-phase cells grew considerably slower with 40 μM farnesol and viability was only 18% with 100 μM farnesol added (Uppuluri *et al.*, 2007). Fourth, ROS are generated during farnesol

treatment of both white and opaque cells, and evidence supports mitochondrial perturbation when cytotoxic levels of farnesol are present (Dumitru *et al.*, 2007; Shirtliff *et al.*, 2009; Uppuluri *et al.*, 2007). The importance of ROS is also implicit in the findings of Dumitru et al (Dumitru *et al.*, 2007); under anaerobic conditions *C. albicans* tolerated mM levels of farnesol. Careful use of physiologically relevant farnesol concentrations combined with clear indications of the previously mentioned variables will hopefully prevent future confusion on this issue.

Is farnesol-mediated death in *C. albicans* induced in a specific or non-specific manner? Evidence exists supporting both models. Ras1/cyclic AMP signaling again becomes relevant as it is known that an activated Ras-cAMP-PKA pathway and cAMP-stimulatory drugs promoted apoptotic cell death in *C. albicans* (Phillips *et al.*, 2006). However, the connection of farnesol to apoptosis (Shirtliff *et al.*, 2009) is less clear. Based on the findings of Phillips et al (Phillips *et al.*, 2006), one would expect farnesol to lessen apoptosis rather than to promote it. Additionally, the lysis of opaque cells by farnesol was not apoptotic (Dumitru *et al.*, 2007). Using the same procedures as had been used for showing apoptosis in *A. nidulans* (Semighini *et al.*, 2006), including the definitive Annexin V test (Semighini *et al.*, 2006), we were unable to demonstrate apoptosis in farnesol lysed opaque cells (Dumitru and Nickerson, unpublished data). Since farnesol is lipophilic, it has the ability to disrupt cell membranes, and this supports the nonspecific-mediated killing model. Understanding how farnesol regulates *C. albicans* growth morphologies, growth rates, and even cell death, may be essential components for understanding the role of farnesol in the host.

The Role of Farnesol *In Vivo*

Our final topic is the role of farnesol during infection. At the time of its discovery as a QSM, two ideas were put forward regarding its *in vivo* importance (Hornby *et al.*, 2001). One was that, due to its ability to block the yeast to filament transition, farnesol treatment would act as a therapeutic in a mouse model of infection. The other was that farnesol would act as a virulence factor. Research on the topic has shown that the role of farnesol *in vivo* is not so simplistic. Navarathna et al (Navarathna *et al.*, 2005; Navarathna *et al.*, 2007a) showed that farnesol acts as a virulence factor in the mouse tail vein injection assay, leading to the question of just how much farnesol is produced by *C. albicans* in the mouse. A definitive answer to this question will be difficult. The *in vitro* production estimates were for cells grown in glass flasks whereas *in vivo* production would be for cells growing in close proximity to mouse membranes of many types which could easily act as external lipid sinks, thus enhancing farnesol production.

C. albicans is capable of evading part of the host immune system by escaping from macrophages through the production of filaments (Ghosh *et al.*, 2009; Lorenz *et al.*, 2004). While external farnesol reduces the viability of macrophages *in vitro* (Abe *et al.*, 2009), the interesting question remains whether farnesol levels naturally produced by *C. albicans* are high enough to affect macrophage function/viability and thus enable fungal survival and escape from the macrophage. Does farnesol production in the phagolysosome contribute to escape from the macrophage? Experiments which add exogenous farnesol to the macrophage cannot answer this question. Also, an unfortunate obstacle for answering this question directly with the *dpp3Δ/dpp3Δ* mutant currently available is twofold. First, it is knocked out in only one of two genes which convert farnesyl pyrophosphate to farnesol and thus it still produces 15% as much farnesol as its

parent and, second, it is an arginine auxotroph (Navarathna *et al.*, 2007a). Ghosh et al (Ghosh *et al.*, 2009) demonstrated the requirement of arginine biosynthesis by *C. albicans* to effectively penetrate and escape murine macrophages. Briefly (Fig 1), Ghosh et al (Ghosh *et al.*, 2009) showed that the rapid upregulation of arginine biosynthetic genes following macrophage ingestion, originally noted by Lorenz et al (Lorenz *et al.*, 2004), occurred so that the arginine could be degraded to urea and then to CO_2, a known signal for hyphal switching (Bahn and Muhlschlegel, 2006) necessary for *C. albicans* to escape. Therefore, the *dpp3Δ/dpp3Δ* mutant and its parental strain SN152 are unable to escape from macrophages, regardless of the presence of *DPP3*. New prototrophic mutants defective in farnesol production must be constructed before this question can be answered.

Consistent with early hypotheses, Hisajima et al (Hisajima *et al.*, 2008) recently published the finding that farnesol has a protective role against *C. albicans* in an oral model of murine infection. This presents an intriguing conundrum: how does farnesol enhance infection for disseminated candidiasis (Navarathna *et al.*, 2007a) while treating infection for mucosal (oral) candidiasis? A similar conundrum is how farnesol can act externally to protect yeast cells from oxidative stress (Westwater *et al.*, 2005) but also inhibit growth by enhancing ROS production by mitochondria (Machida *et al.*, 1998b). This suggests other factors are at play other than or in addition to the effect of farnesol on *C. albicans*, potentially the host's response to farnesol.

Acknowledgements

We wish to thank Geraldine Butler, Tristan Rossignol, Suman Ghosh, and Raluca Dumitru for allowing us to cite their unpublished data, as well as Deborah Hogan for

helpful reading of the manuscript. This work was supported by a faculty seed grant from the Constance Miriam and Ethel Corrine Syford Memorial Fund (to A.L.A.), the Hammond-Maude Fling Fellowship (to M.L.L.), and the Farnesol and *Candida albicans* Research Fund, University of Nebraska Foundation (to K.W.N.). These authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

Figure legends

Figure 1-1. Farnesol impacts the cyclic AMP pathway in *C. albicans*. Three possible points of inhibition by farnesol.

Figure 1-1

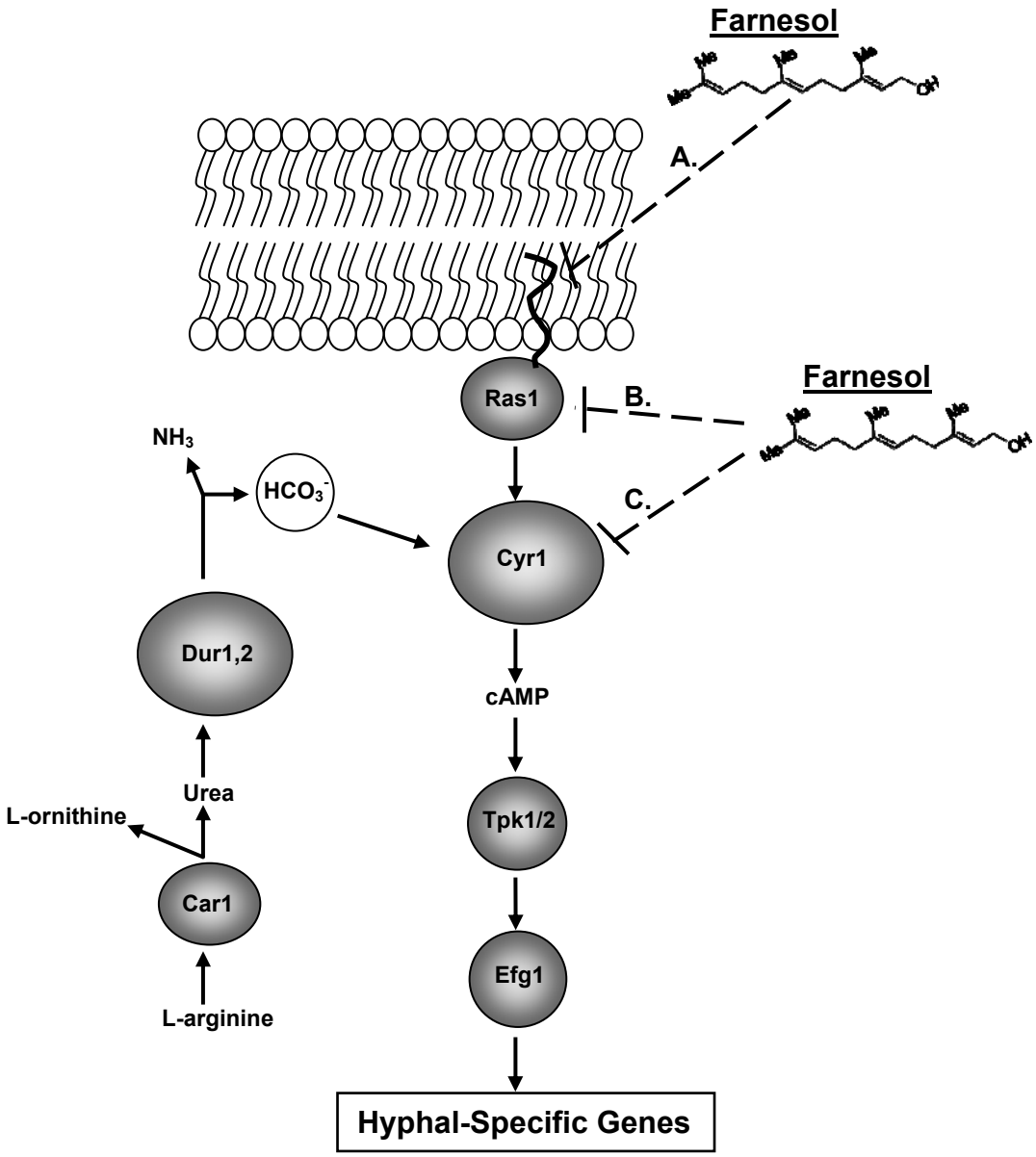
Farnesol
A.
Ras1
B.
Farnesol
C.
NH3
HCO3-
Cyr1
Dur1,2
cAMP
Urea
L-ornithine
Tpk1/2
Car1
Efg1
L-arginine
Hyphal-Specific Genes

Table 1-1. The effects of farnesol treatment in other cell types.

Organism	Farnesol Concentration(s) Used	Summary of Farnesol Response
C. dubliniensis	1.5 nM- 300 μM	Filament inhibition, no effect on growth, (Henriques *et al.*, 2007; Martins *et al.*, 2007), biofilm inhibition, MIC = 200μM, lowered tolerance of fluconazole (Jabra-Rizk *et al.*, 2006b)
C. parapsilosis	1.5 nM-100 μM	Biofilm inhibition (Laffey and Butler, 2005), growth arrest ≥50 μM, lipid metabolism, ribosome biogenesis, and amino acid biosynthesis genes affected, altered lipid polarization (Rossignol *et al.*, 2007)
Acinetobacter baumannii	50 μM- 200 μM	Growth inhibition (Peleg *et al.*, 2008)
Aspergillus fumigatus	10 μM - 250 μM	Growth inhibition, apoptosis (Semighini *et al.*, 2006)
Aspergillus nidulans	1 μM - 250 μM	Apoptosis and ROS production (10 – 250 μM) (Semighini *et al.*, 2006), hyphal inhibition (1-10 μM) (Semighini *et al.*, 2006), autophagy, lipid metabolism, amino acid response, transcription, and translation genes affected (10-100 μM) (Savoldi *et al.*, 2008)
Aspergillus niger	100 μM- 10mM	Inhibition of conidiation (Lorek *et al.*, 2008)
Fusarium graminearum	100 μM - 300 μM	Apoptosis, altered spore germination and lysis (Semighini *et al.*, 2008)
Human gingival cells	10 μM- 300 μM	Reduced proliferation and adhesion (Saidi *et al.*, 2006)
Human oral carcinoma cells	10 μM - 60 μM	Apoptosis (Scheper *et al.*, 2008)
Murine macrophages	56 μM - 112 μM	Apoptosis, ROS production, decreased phagocytic activity (Abe *et al.*, 2009)
Paracoccidiodes brasiliensis	5 μM - 300 μM	Inhibition of yeast to hyphal and hyphal to yeast growth at low concentrations, growth inhibition at high concentrations (Derengowski *et al.*, 2009)
Pseudomonas aeruginosa	30 μM - 300 μM	Inhibition of Pseudomonas quinolone signal (PQS) and pyocyanin production, decreased swarming motility, (Cugini *et al.*, 2007; McAlester *et al.*, 2008)
Saccharomyces cerevisiae	5 μM- 5 mM	Growth inhibition by cell cycle arrest, effect on mitochondria and increased reactive oxygen species (ROS) production (Machida *et al.*, 1998a; Machida *et al.*, 1999), cell death (Fairn *et al.*,

		2007)
Staphylococcus aureus	30 μM - 4.5 mM	Cell death, membrane disruption, biofilm inhibition, lipase inhibition, increased sensitivity to antibiotics (Jabra-Rizk *et al.*, 2006a; Kuroda *et al.*, 2007; Togashi *et al.*, 2008)
Staphylococcus epidermidis	100 μM -200 μM	Cell death (Gomes *et al.*, 2009)
Streptococcus mutans	250 μM - 5 mM	Growth inhibition, biofilm inhibition, prevention of dental caries in rats when in combination with fluoride and apigenin (Koo *et al.*, 2002a; Koo *et al.*, 2002b; Koo *et al.*, 2003; Koo *et al.*, 2005)
Tobacco (N*icotiana tabacum* L. cv Bright Yellow-2)	5 μM -150 μM	Cell death (Hemmerlin and Bach, 2000; Hemmerlin *et al.*, 2006)

Table 1-2. The effects of secreted bacterial molecules on *C. albicans*.

Bacterium	**Secreted Molecule(s)**	**Summary of *C. albicans* Response**
Acinetobacter baumannii	Unknown, found in cell-free supernatant	Filament inhibition, biofilm inhibition (Peleg *et al.*, 2008)
Burkholderia cenocepacia	Cis-2-dodecenoic acid (*Burkholderia* diffusible signal factor, BDSF)	Growth inhibition and filament inhibition (Boon *et al.*, 2008)
P. aeruginosa	3-oxo-C12-homoserine lactone, dodecanol, and C12-acyl homoserine lactone	Filament inhibition and reversion to yeast morphology (Davis-Hanna *et al.*, 2008; Hogan *et al.*, 2004)
P. aeruginosa	Phenazines	Cell death (Gibson *et al.*, 2009)
Salmonella enterica serovar Typhimurium	Unknown, found in cell-free supernatant	Filament inhibition, biofilm inhibition, and reduced viability (Tampakakis *et al.*, 2009)
Streptococcus gordonii	Unknown, found in cell-free supernatant and missing/reduced in *ΔluxS* mutant	Induction of filamentous growth, suppression of farnesol-mediated filament inhibition (Bamford *et al.*, 2009)
Xanthomonas campestris	Cis-11-methyl-2-dodecenoic acid (diffusible signal factor, DSF)	Filament inhibition (Boon *et al.*, 2008; Wang *et al.*, 2004)

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CHAPTER 2

Activity and toxicity of farnesol on *Candida albicans* is dependent on growth conditions

Reference:

Langford, M.L., Hasim, S., Nickerson, K.W., and Atkin, A.L. (2010) Activity and toxicity of farnesol on *Candida albicans* is dependent on growth conditions. *Antimicrob. Agents Chemother.* **54(2):** 940-2.

Abstract

Farnesol interacts with *Candida albicans* as both a quorum sensing molecule and toxic agent, but confusion abounds regarding which conditions promote these distinct responses. Farnesol sensitivity was measured when inoculum cell history and size, temperature, and media were altered. Parameters for farnesol tolerance/sensitivity are defined, validating previous studies and identifying new variables, such as energy sources. This study provides a clear understanding of what farnesol concentrations are lethal to *C. albicans*, based on environmental conditions.

Introduction

Candida albicans is a dimorphic fungus of great medical importance. It is also the model system for studying quorum sensing for fungal dimorphism (Hornby *et al.*, 2001). *C. albicans* excretes a quorum sensing molecule (QSM) called farnesol and when extracellular levels exceed a threshold of 1-5 μM (Mosel *et al.*, 2005), farnesol blocks the yeast-to-filament conversion. One feature of farnesol's action as a QSM was that exogenous farnesol up to 200-300 μM does not alter the growth rate; instead, the cells grow as yeasts rather than as filaments. These results were quickly confirmed by Ramage et al (Ramage *et al.*, 2002), Hogan et al (Hogan *et al.*, 2004), and Kruppa et al (Kruppa *et al.*, 2004). Farnesol also impacts other aspects of *C. albicans* biology: it blocks biofilm formation (Ramage *et al.*, 2002), acts as a virulence factor during systemic infection (Navarathna *et al.*, 2007a), and is a protective factor during mucosal infection (Hisajima *et al.*, 2008). Farnesol production is regulated in that it is turned off in opaque cells (Dumitru *et al.*, 2007) and during anaerobic growth (Dumitru *et al.*, 2004) but elevated in some mutants which are altered in morphology (Jensen *et al.*, 2006) or locked in the filamentous morphology (Kebaara *et al.*, 2008). Farnesol production also increases in the presence of sublethal levels of sterol biosynthesis inhibitors (Hornby and Nickerson, 2004; Navarathna *et al.*, 2005).

Farnesol is a bioactive molecule with mild detergent-like properties. At concentrations in the 20-50 μM range it has been reported to inhibit or induce cell death in *Saccharomyces cerevisiae* (Machida *et al.*, 1998a; Machida *et al.*, 1999), *Aspergillus nidulans* (Semighini *et al.*, 2006), *Fusarium graminearum* (Semighini *et al.*, 2008), *Paracoccidioides brasiliensis* (Derengowski *et al.*, 2009), and *C. albicans* opaque cells

(Dumitru *et al.*, 2007). Similarly, farnesol has been reported to trigger cell death in several mammalian cell lines (Abe *et al.*, 2009; Saidi *et al.*, 2006; Scheper *et al.*, 2008) and bacteria (Gomes *et al.*, 2009; Jabra-Rizk *et al.*, 2006a; Koo *et al.*, 2003; Togashi *et al.*, 2008), and it induces cytokine production, such as IL-6, by macrophages. Thus, the view to this point was that *C. albicans* exhibited exceptional tolerance to farnesol, as a necessary corollary to its production as an antagonistic molecule. This view was recently challenged by Shirtliff et al (Shirtliff *et al.*, 2009) who reported that farnesol, at concentrations as low as 40 μM, killed *C. albicans* by inducing apoptosis. Thus, in the spirit of constructive dialogue, we draw attention to the differences between the growth conditions used in our previous body of work under which farnesol would act as a signaling molecule, innocuous to growth, and the conditions under which Shirtliff et al (Shirtliff *et al.*, 2009) observed cell death.

Previous studies examining *C. albicans* sensitivity to farnesol (Dumitru *et al.*, 2007; Jabra-Rizk *et al.*, 2006b; Shirtliff *et al.*, 2009; Uppuluri *et al.*, 2007) have used vastly different assay conditions (varying temperatures, media, inoculum sizes, and inoculum growth phases), adding to confusion on the matter of farnesol-induced cell death. Critically, Shirtliff et al (Shirtliff *et al.*, 2009) used cells that were grown overnight, washed and resuspended in PBS for farnesol sensitivity assays. Since it is well known that detergent resistance in bacteria is an energy dependent process (Aspedon and Nickerson, 1993; Rajagopal *et al.*, 2003), we were particularly interested whether farnesol resistance in *C. albicans* is similarly energy dependent.

Methods

We examined farnesol sensitivity under a series of different growth conditions in plastic microtiter dishes. Variables included temperature, cell density, growth medium (rich vs. minimal), and inoculum history. We followed cell growth by means of optical density and cell death by methylene blue staining (Gibson *et al.*, 2009). *C. albicans* cells were grown to stationary phase (unbudding cells; cultures were inoculated to an OD_{600} =0.1 and grown at 30°C for 16-18h) or mid-log phase (OD_{600} =0.5), washed three times in PBS, and inoculated at the indicated levels with variable concentrations of farnesol. We used 10 mM and 100 mM stocks of E, E-farnesol in methanol so that the final methanol concentration never exceeded 1%, and this level of methanol had no effect on cell growth or cell death (data not shown).

Results

In rich growth medium (YPD), no cell death was observed at farnesol concentrations up to 300 μM and growth inhibition was only observed with 300 μM farnesol (data not shown). When we switched to a defined glucose-proline (GPP pH6.8) (Kebaara *et al.*, 2008)medium (Fig. 1), very similar growth curves were observed with all concentrations of farnesol up to 300 μM when starting with stationary phase inocula (Fig. 1A). The cell growth experiments (Fig. 1) were simultaneously examined for cell death by staining with methylene blue (Fig. 2B, 2D). Minimal cell death occurred in GPP with a stationary phase inoculum, our standard growth conditions (Hornby *et al.*, 2001; Mosel *et al.*, 2005), and up to 300 μM farnesol (Fig. 2D) consistent with growth curves seen in Fig. 1A. However, when we used inocula of exponentially growing cells, 40 μM farnesol partially inhibited growth and further inhibition correlated with increasing farnesol levels (Fig. 1B). Log phase cells were killed by 100 and 300 μM farnesol in GPP (Fig. 2B), consistent with the delayed growth seen in Fig. 1B. These results support the growth phase-dependent sensitivity described by Uppuluri et al (Uppuluri *et al.*, 2007). Temperature does not play a prominent role in farnesol growth inhibition because we obtained similar growth curves at 25ºC, 30°C, and 37°C (data not shown).

To examine the effects of different media on farnesol sensitivity, cells were compared under both growth (GPP) and storage (PBS) conditions, using both exponential and stationary phase inocula (Fig. 2). For exponential phase cells inoculated in PBS, even low levels of farnesol, i.e. 40 μM, caused cell death (Fig. 2A), consistent with the findings of Shirtliff et al (Shirtliff *et al.*, 2009). The cells in PBS were far more sensitive to farnesol when they had come from an exponential phase inoculum than a stationary

phase inoculum (Fig. 2A, 2C). Interestingly, both exponential and stationary phase cells showed increased tolerance to farnesol when incubated in growth media (GPP or YPD), compared to PBS (Fig. 2). As with growth rates, similar results for cell death were obtained at all 3 temperatures tested, 25°C, 30°C, and 37°C. These observations suggest a role for energy source(s) in *C. albicans* farnesol tolerance.

The previous experiments (Figs. 1 and 2) were conducted in 96 well plates with farnesol added at time zero to washed cells. Because of a possible farnesol adsorption effect with plastic (Davis-Hanna *et al.*, 2008), we confirmed the farnesol sensitivity of exponentially growing cells in glass flasks (Fig. 3). We compared the farnesol sensitivity of *C. albicans* cells in exponential phase (OD_{600} = 0.5, Fig. 3A, 3B) and stationary phase (OD_{600} = 4.0, Fig. 3C, 3D) by adding farnesol directly to unwashed growing cultures. The results with glass flasks (Fig. 3) were consistent with those obtained with plastic 96 well plates.

Discussion

A distinct benefit of this controversy is to focus attention on the conditions in which *C. albicans* tolerates farnesol while so many other cell types are killed by it. Stationary phase *C. albicans* cells exhibit extreme farnesol tolerance, even at 100-300 μM farnesol, and these levels are lethal to most other organisms tested. Throughout, cell death was not accompanied by cell lysis because there was no drop in OD_{600} and the methylene blue positive cells remained intact. Similarly, optical densities of cells in PBS were not significantly affected by the presence of farnesol (data not shown). This lack of cell lysis with white cells of *C. albicans* is in marked contrast with opaque cells where ≥ 40 μM farnesol caused rapid cell lysis (Dumitru *et al.*, 2007). The differences in susceptibility between white and opaque cells (Dumitru *et al.*, 2007), aerobic and anaerobic cells (Dumitru *et al.*, 2004), stationary and exponential cells [(Uppuluri *et al.*, 2007) and Figs. 1-3], and between cells in buffer alone versus minimal media suggest a physiological adaptation to farnesol, depending on environmental conditions. Two places where this selective farnesol tolerance might be localized are the mitochondria, where farnesol stimulates ROS production in *C. albicans* and *S. cerevisiae* (Machida *et al.*, 1998a; Machida *et al.*, 1999; Shirtliff *et al.*, 2009), and the lipid portion of the cytoplasmic membrane. Changes in fatty acid composition, sterol-rich domains (Alvarez *et al.*, 2007), sphingolipids (Brown and London, 2000), and GPI-anchored proteins preferentially associated with lipid rafts (Brown and London, 2000) could be involved, as supported by the lipid domain alteration seen in *C. parapsilosis* after farnesol treatment (Rossignol *et al.*, 2007). Although the mechanisms(s) involved in farnesol tolerance

remain unclear, conditions in which *C. albicans* is either sensitive or resistant to farnesol have now been outlined.

In summary, stationary phase cells inoculated into minimal or rich media are ideal candidates for studying farnesol signaling, as these do not undergo cell death with high farnesol concentrations. This generalization was true for all three commonly used temperatures (25°C, 30°C, 37°C) as well as for experiments with starting cell densities of OD_{600} = 0.05 or 0.10. Conversely, optimal conditions to examine farnesol-mediated cell death include the use of log phase cells in PBS, under energy-starved conditions. Understanding these environmental parameters may unify many previous discrepancies in the literature and provide unambiguous conditions to induce each of farnesol's unique effects on *C. albicans*.

Acknowledgements

This work was supported by a faculty seed grant from the Constance Miriam and Ethel Corrine Syford Memorial Fund (to A.L.A.), the Hammond-Maude Fling Fellowship (to M.L.L.), and the Farnesol and *Candida albicans* Research Fund, University of Nebraska Foundation (to K.W.N.).

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Figure Legends

Fig. 2-1. Effect of farnesol on *C. albicans* cell growth. A) stationary phase inoculum, B) exponential phase inoculum. Cells were grown in duplicate on at least two separate occasions in defined GPP medium with indicated levels of farnesol (see legend in B) at 30°C in 96-well plates, and OD_{600} values were recorded on an automated plate reader (Molecular Devices, Sunnyvale, CA). Note the different y-axis scales in graphs A and B.

Fig. 2-2. Effect of farnesol on *C. albicans* cell death. Percent death was determined by methylene blue staining (Gibson *et al.*, 2009). Cells were incubated in either PBS (A, C) or GPP (B, D) with the indicated levels of farnesol in 96-well plates at 30°C. Incubations were initiated with either exponential (A, B) or stationary phase (C, D) inocula.

Fig. 2-3. Toxicity of farnesol to exponential cultures of *C. albicans*. One culture (A, B) was subdivided (in six) when it had reached exponential phase (OD_{600} = 0.5) and the other (C, D) was subdivided when it had reached stationary phase (OD_{600} = 4.0). Cultures were not washed prior to subdivision and farnesol addition. Cultures containing the indicated levels of farnesol were shaken at 30°C and 250 rpm for 4 hrs and at the indicated times cell growth (A, C) and % dead cells (B, D) were determined. All cultures were in glass flasks in GPP both before and after subdivision.

Figure 2-1

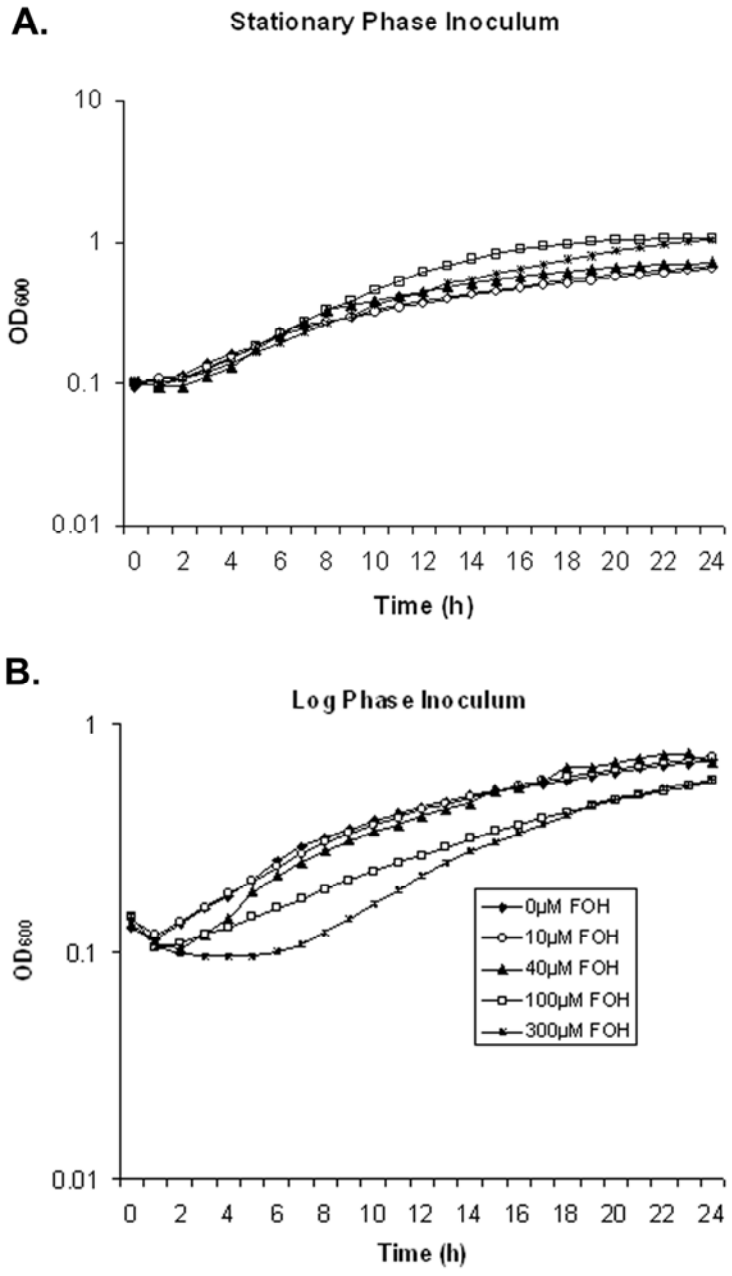

A.
Stationary Phase Inoculum
10
1
0.1
0.01
OD600
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
B.
Log Phase Inoculum
1
0.1
0.01
OD600
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
0μM FOH
10μM FOH
40μM FOH
100μM FOH
300μM FOH

Figure 2-2

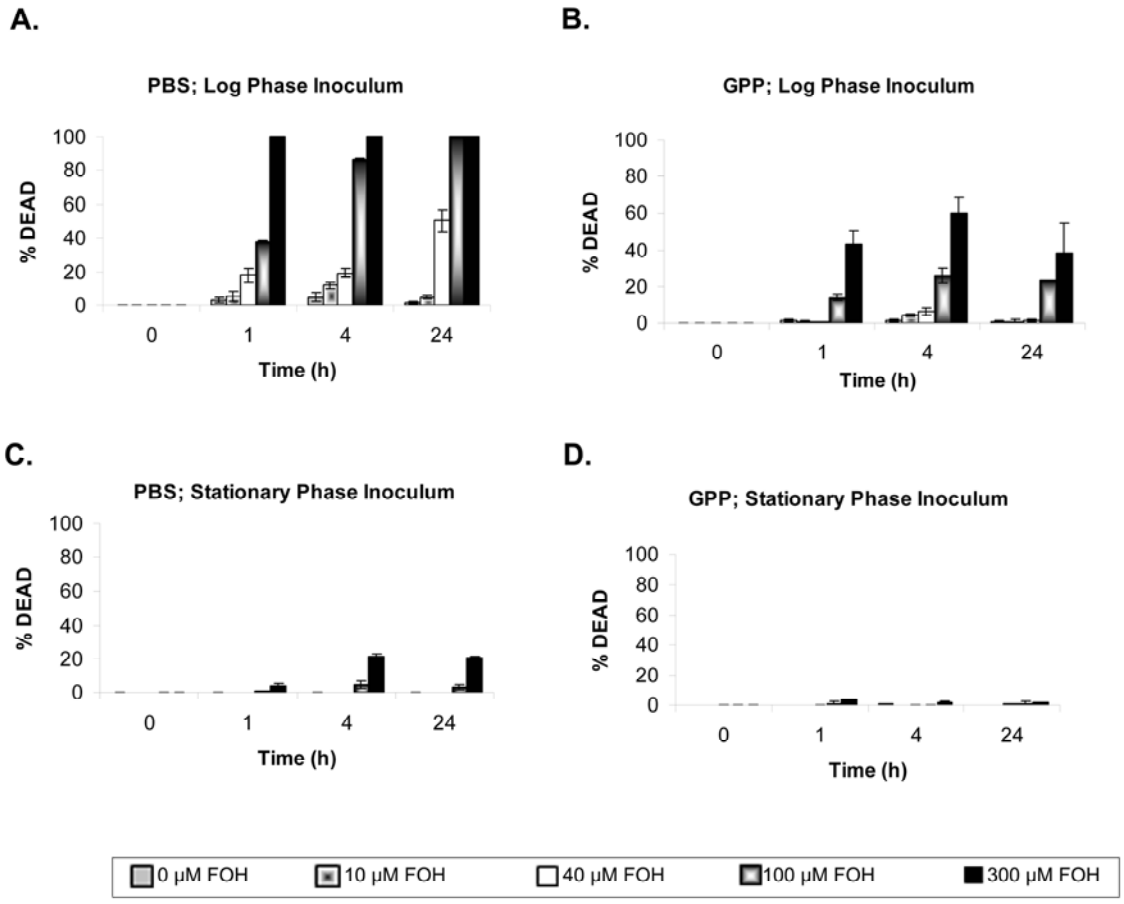
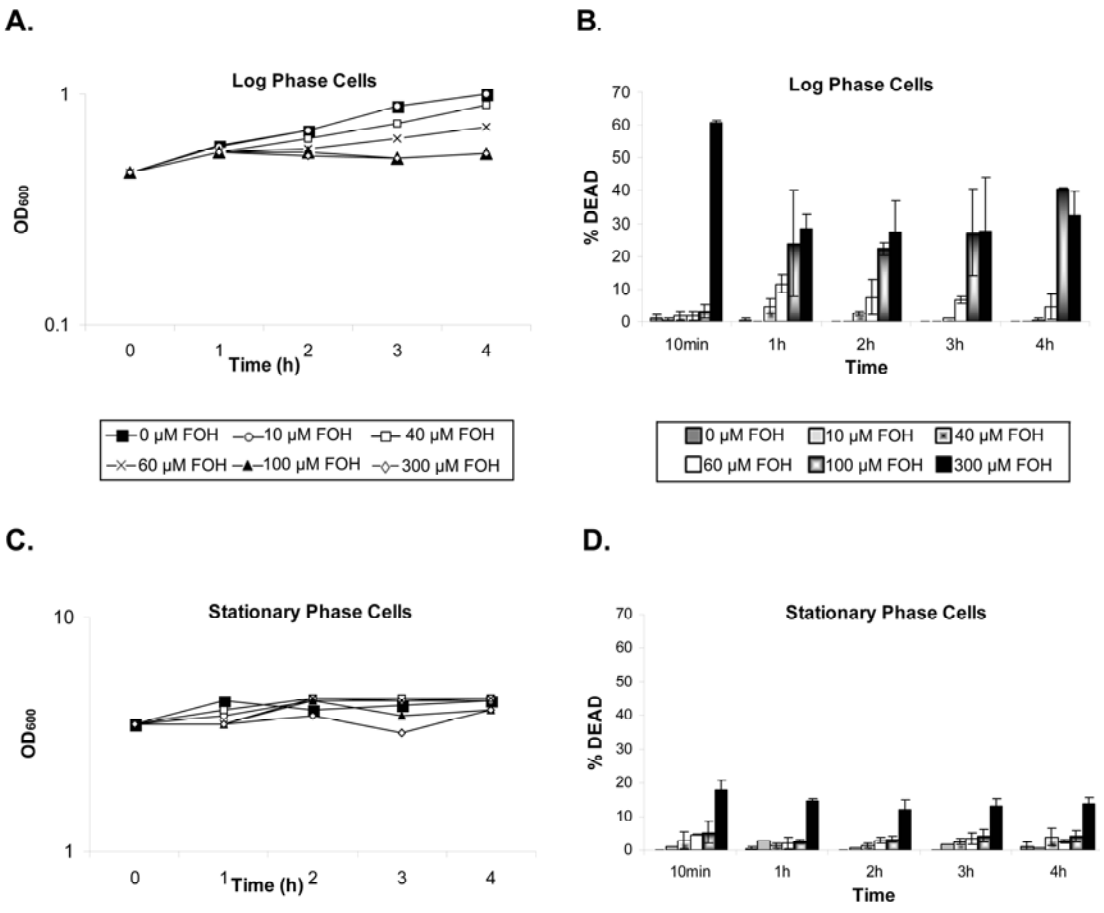

A.
PBS; Log Phase Inoculum
% DEAD
100
80
60
40
20
0
0
1
4
24
Time (h)
B.
GPP; Log Phase Inoculum
% DEAD
100
80
60
40
20
0
0
1
4
24
Time (h)
C.
PBS; Stationary Phase Inoculum
% DEAD
100
80
60
40
20
0
0
1
4
24
Time (h)
D.
GPP; Stationary Phase Inoculum
% DEAD
100
80
60
40
20
0
0
1
4
24
Time (h)
0 μM FOH
10 μM FOH
40 μM FOH
100 μM FOH
300 μM FOH

Figure 2-3


A.
Log Phase Cells
OD600
1
0.1
0
1
2
3
4
Time (h)
0 μM FOH
10 μM FOH
40 μM FOH
60 μM FOH
100 μM FOH
300 μM FOH
B.
Log Phase Cells
% DEAD
70
60
50
40
30
20
10
0
10min
1h
2h
3h
4h
Time
0 μM FOH
10 μM FOH
40 μM FOH
60 μM FOH
100 μM FOH
300 μM FOH
C.
Stationary Phase Cells
OD600
10
1
0
1
2
3
4
Time (h)
D.
Stationary Phase Cells
% DEAD
70
60
50
40
30
20
10
0
10min
1h
2h
3h
4h
Time

CHAPTER 3

Candida albicans Tup1 is involved in farnesol-mediated inhibition of filamentous-growth induction

Reference:

Kebaara, B.W., Langford, M.L., Navarathna, D.H., Dumitru, R., Nickerson, K.W., and Atkin, A.L. (2008) *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous-growth induction. *Eukaryot. Cell.* 7: 980-7.

Abstract

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

Introduction

Candida albicans is the most commonly isolated opportunistic fungal pathogen in humans. *C. albicans* is part of the normal flora, and it resides in the gastrointestinal and genitourinary tracts, as well as on the skin. However, *C. albicans* is capable of causing a wide range of disease, from mild mucosal infections to life-threatening systemic infections termed candidemia (Jarvis and Martone, 1992). Vulnerable patients include AIDS patients, patients undergoing chemotherapy, and organ transplant patients (Jarvis and Martone, 1992). The annual cost of treating candidiasis in the United States was estimated to be one billion dollars, and the mortality rates for patients with candidiasis are 30-50% even with antifungal treatment (Kullberg and Filler, 2002), indicating a need for new antifungal drugs.

The ability of *C. albicans* to cause disease has been strongly linked to its conversion between two distinct morphological forms: yeast and filaments. Recently, our research has focused on farnesol, the first quorum sensing molecule (QSM) discovered in a eukaryote (Hornby *et al.*, 2001). Farnesol is a virulence factor (Navarathna *et al.*, 2007a) that is excreted continuously by *C. albicans* (Hornby *et al.*, 2001), and when it accumulates beyond a threshold level it blocks the yeast to filament conversion (Hornby *et al.*, 2001). Stationary phase cultures of *C. albicans* have accumulated 2-4 μM farnesol (Hornby *et al.*, 2001) and the IC_{50} value for blocking germ tube formation (GTF) in an N-acetylglucosamine stimulated assay is ca. 1-2 μM (*E, E*) farnesol (Hornby *et al.*, 2001; Mosel *et al.*, 2005; Shchepin *et al.*, 2003) and, consequently, these farnesol production levels are physiologically relevant. Other roles described for farnesol include biofilm inhibition (Ramage *et al.*, 2002), protection from

oxidative stress (Deveau *et al.*, 2010; Westwater *et al.*, 2005), and induction of apoptosis in another fungus, *Aspergillus nidulans* (Semighini *et al.*, 2006). While many phenotypic effects produced by farnesol have been described, little is understood about farnesol's mode of action.

In addition to farnesol, *C. albicans* yeast and filamentous growth is controlled by an assortment of signaling pathways (Berman and Sudbery, 2002; Dhillon *et al.*, 2003). The yeast to filamentous conversion is activated by many pathways, including: Components of the *CEK1* MAP kinase pathway, Ras/cAMP-dependent pathway, Calcium/Calmodulin signaling pathway, Rim101-independent pathway, and Chk1 two-component signal transduction pathway. Although each has been implicated in filamentation (Csank *et al.*, 1998; Kruppa *et al.*, 2004; Ramon *et al.*, 1999), these pathways show some degree of specialization in that they respond to different environmental inducers. Activation and inhibition of filament development is largely accomplished through changes in gene expression mediated by transcription activators and repressors. Efg1 is a major transcription regulator of filamentous growth and is a central control point for many signaling pathways involved in filamentation (Eckert *et al.*, 2007). Efg1 also regulates the expression of multiple genes including those involved in virulence (Eckert *et al.*, 2007; Kumamoto and Vinces, 2005a). Mechanisms have also been identified that block filament development with transcriptional repression by Tup1 playing a key role (Braun and Johnson, 1997; Braun and Johnson, 2000).

Farnesol is able to block filamentous growth induced by environmental signals for most, and possibly all of the signaling pathways activating filament development. These signals include: 10% serum, 10 mM L-proline, 2.5 mM N-acetylglucosamine, or the

combination of *N*-acetylglucosamine and L-proline, all at 37^{o}C (Hornby *et al.*, 2001). Thus, farnesol may individually block each of the morphogenic signaling pathways and/or act at a common control point in morphogenesis. Tup1 repression of filament specific genes is an attractive candidate for a common control point that may be regulated by farnesol (Kadosh and Johnson, 2005).

The *C. albicans* Tup1 protein is a transcription regulator that plays two key roles in the cell: 1) regulation of phase switching, and 2) inhibition of filamentous growth. Tup1 interacts with either Ssn6 or Tcc1 corepressor proteins. This complex functions with DNA binding proteins to repress gene expression (Kaneko *et al.*, 2006; Sprague *et al.*, 2000). At least three DNA-binding proteins have been identified that function with Tup1: Nrg1 (homologous to *Saccharomyces cerevisiae* Nrg1p), Rfg1 (homologous to *S. cerevisiae* Rox1p), and Mig1 (homologous to *S. cerevisiae* Mig1p). Homozygous *tup1* mutants are unable to grow as yeast and instead remain locked in the filamentous form in all media tested (Braun and Johnson, 1997). Deletion of *TUP1* results in the up-regulation of approximately one-third of *C. albicans* genes (Murad *et al.*, 2001a; Murad *et al.*, 2001b) and these mutants are also avirulent in a murine model of infection. Activation of Tup1 transcription repressor complexes results in the repression of filament-specific gene expression (Braun and Johnson, 1997; Braun and Johnson, 2000; Murad *et al.*, 2001a; Murad *et al.*, 2001b).

Here, we tested the hypothesis that the *C. albicans* response to farnesol involves Tup1. The morphological response to farnesol was tested in wild-type, *tup1/tup1*, *tup1/TUP1, nrg1/nrg1*, and *rfg1/rfg1* strains to assess the requirement for these genes in the farnesol response. The gene expression pattern for *MIG1* was not determined because

Mig1 protein does not play a role in filamentous growth of *C. albicans* (Murad *et al.*, 2001a). The gene expression patterns of *TUP1*, *NRG1*, *RFG1*, and *EFG1*, as well as genes under their control were examined in the presence or absence of farnesol by quantitative northern and western analyses. Finally, we compared farnesol production levels in *tup1*, *nrg1*, and *rfg1* homozygous mutants relative to wild type cells.

Methods

Strains and media

Candida albicans SC5314 is an independent clinical isolate and the reference strain for the *Candida* genome sequence. *C. albicans* strains CAF-2 (*ura3:imm434/URA3*) and CAI-4 (*ura3::imm434/ura3::imm434*) are derived from SC5314 by gene replacement . Strains BCa2-9 (*tup1/tup1,* in CAI-4; (Braun and Johnson, 1997), BCa2-10 (*tup1/tup1,* frameshift disruption fragment in CAI-4; (Braun and Johnson, 1997), DU152 (*nrg1/nrg1*, in CAI-4; (Braun and Johnson, 1997), DU129 (*rfg1/rfg1*, in CAI-4; (Backen *et al.*, 2000; Kadosh and Johnson, 2001), BCa05 that expresses *TUP1* ectopically (*tup1/tup1, MAL3:p455*, in CAI-4; (Braun and Johnson, 1997) and BCa2-3 (*TUP1*/*tup1,* in CAI-4; (Braun and Johnson, 1997) were obtained from Alexander Johnson, University of California, San Francisco, CA. Strain MEN was provided by Richard Cannon, University of Otago, Dunedin, New Zealand.

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

The defined glucose-salts medium GPP pH 4.8 contained (per liter distilled water): glucose, 20g; L-proline, 1.15g; NaH_2PO_4, 3.2g; KH_2PO4, 4g; $MgSO_4 \cdot 7H_20$, 0.5g; $CuSO_4 \cdot 5H_20$, 1mg; $ZnSO_4 \cdot 7H_20$, 1mg; $MnCl_2$, 1mg; $FeSO_4$, 1 mg; biotin, 20 μg; pyridoxine•HCl, 200 μg; thiamine•HCl, 200 μg,. The glucose (20% w/v) and L-proline (100 mM) were autoclaved separately and added aseptically, as were the filter-sterilized vitamins (Kulkarni and Nickerson, 1981). Modified GPP (mGPP) also contained 2.5 mM N-acetylglucosamine (Hornby *et al.*, 2001). GPP pH 6.8 contained 3.2g/L Na_2HPO_4

instead of NaH_2PO_4. For maltose phosphate proline media (MPP), filter-sterilized maltose replaced the glucose. Cornmeal agar (Difco, Detroit, MI) was also used. Solid media included 1.5% (w/v) agar. All media for CAI-4 included uridine at 40 μg/ml (Backen *et al.*, 2000).

Microscopy

Differential interference contrast (DIC) images were photographed with an Olympus BX51 microscope and colony morphology images were photographed with an Olympus SZX12 microscope.

Quantitative Northern blot analysis

To measure mRNA accumulations, SC5314 resting cells were inoculated in mGPP to an OD_{600} of 0.5-0.6 and allowed to equilibrate at 37°C for 5 minutes whereupon 20 μM farnesol was added to half of the flasks. Cells were grown at 37°C for 0, 20, 40, 60, and 80 minutes until the cells were harvested and total RNA extracted by the hot phenol method (Backen *et al.*, 2000; Kebaara *et al.*, 2003). Equal amounts of RNA (15μg) were resolved on 1.0 % agarose-formaldehyde gels and the RNA was transferred to GeneScreen Plus (NEN™ Life Science Products, Inc., Boston, MA) using the capillary blot transfer protocol recommended by the manufacturer. The Northern blots were probed with radiolabeled DNA probes. The probe DNAs used for synthesis were prepared by PCR using MEN genomic DNA. The probes were labeled with ^{32}P-dCTP (GE Health Sciences, Piscataway, NJ) using an oligolabeling kit, RadPrime DNA labeling system following the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA). Northern blots were PhosphorImaged using a Storm (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and quantified using ImageQuant software (Molecular Dynamics version

5.0, Sunnyvale, CA). mRNA abundance measurements were done using a minimum of three independent Northern blots.

Western blot analysis

Western blots were prepared as previously described (Atkin *et al.*, 1995) and Tup1 and Act1 proteins detected with the Supersignal® West Pico Chemiluminescent Substrate using the manufacturers protocol (Pierce, Rockford, IL) with the exception that blocking was done with 5% nonfat dry milk. Rabbit polyclonal antibodies against Tup1 were previously described (Inglis and Johnson, 2002). Mouse monoclonal Anti-Act1 antibodies and horseradish peroxidase (HRP) labeled ant-rabbit IgG antibodies were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). HRP labeled anti-mouse antibody was from Perkin-Elmer (Boston, MA).

Analysis of farnesol levels

Extracellular farnesol was extracted from cell free-supernatants of cultures grown in mGPP at 30^{o} and analyzed by GC/MS as described (Hornby *et al.*, 2001).

Results

tup1/tup1 and *nrg1/nrg1* mutants lack a morphological response to farnesol while the *rfg1/rfg1* mutant responds to farnesol

The juxtaposition of farnesol's ability to inhibit differentiation, and the role of Tup1 as a transcription repressor for filamentation genes suggests that farnesol could function by activating Tup1 and/or one of its co-regulators, Nrg1 and Rfg1. Consequently, we examined the effect of farnesol on the morphology of null mutants lacking *TUP1*, *NRG1*, and *RFG1*. As a control, the wild type *C. albicans* SC5314 in filament-inducing media grew as yeasts in the presence of 20 μM farnesol and as filaments in media lacking farnesol, demonstrating a positive response to farnesol (Fig. 1). The *rfg1/rfg1* mutant responded to 20 μM farnesol in a similar manner to SC5314 (Fig. 1). Unlike SC5314 and *rfg1/rfg1*, the *tup1/tup1* and *nrg1/nrg1* mutants lacked a detectable response to farnesol and remained filamentous in the presence of 20 μM farnesol (Fig. 1). The filamentous-only cell morphology is the expected phenotype for these known mutants (Braun and Johnson, 1997; Braun *et al.*, 2001; Khalaf and Zitomer, 2001; Murad *et al.*, 2001a; Murad *et al.*, 2001b). However, in this regard, *tup1/tup1* and *nrg1/nrg1* mutants differ from the great majority of filamentous-only mutants recovered from a previous study, 96% of which reverted to a smooth colony (yeast) morphology on YM agar plates with 50 μM farnesol (Jensen *et al.*, 2006). For the *tup1*/*tup1* mutant, the lack of response to farnesol was specific for loss of Tup1 because we found ectopic expression of *TUP1* (Braun and Johnson, 1997) restores the ability to respond to farnesol (data not shown).

TUP1 mRNA levels increase in the presence of farnesol while _RFG1_ and _NRG1_ mRNA levels were not affected by farnesol

We analyzed the effect of farnesol on *TUP1* mRNA levels over time in *C. albicans* SC5314 cells which had been induced to differentiate from yeasts to filaments by growth at 37°C in mGPP. We previously showed (Mosel *et al.*, 2005) that, in these conditions, the first germ tubes appeared at 30 min and the process was complete by 110 min. Further, farnesol no longer blocked germ tube formation when added 60-90 min after inoculation (Kulkarni and Nickerson, 1981). Here our analysis was designed to evaluate changes in *TUP1* mRNA just before germ tube formation when the cells were still responsive to farnesol. Filamentation was induced by transferring resting cells into mGPP (pH 4.8) at 37°C in the presence and absence of 20 μM farnesol and mRNA levels were determined at 0, 20, 40, 60 and 80 min following induction. In all experiments, the *TUP1* mRNA levels decreased over the first 20 min and then increased (Fig. 2A). This pattern is consistent with the single time point results of Toyoda et al. (Toyoda *et al.*, 2004) who showed that *TUP1* mRNA levels increased slightly at 180 minutes after induction of filamentation. In the presence of farnesol, we found that *TUP1* mRNA consistently increased 2.5±0.6 (n=4) fold from 20 to 60 min. Importantly, this is the time period just prior to that at which the cells become committed and are no longer responsive to farnesol (Mosel *et al.*, 2005). In contrast, in the absence of farnesol, there was very little increase (1.4±0.3, n=4) in *TUP1* mRNA levels from 20 to 60 min (Fig 2A). Thus, farnesol (20 μM) causes a consistent increase in *TUP1* mRNA levels during the precise time period when it blocks differentiation from yeasts to filaments. This

increase of 2.5-fold in *TUP1* mRNA corresponded to an increase in SC5314 Tup1 protein levels at 60 min following induction (Fig.3). Tup1 protein in SC5324 was increased in all three replicate experiments by an average of 2.5-fold.

Since Tup1 functions with DNA binding proteins such as Rfg1 or Nrg1, and in *C. albicans* strain JCM9061 *NRG1* mRNA levels decreased during filamentation (Toyoda *et al.*, 2004), we tested the effect of farnesol on *RFG1* and *NRG1* mRNA levels during differentiation from yeasts to filaments. Like *TUP1*, *RFG1* mRNA levels initially decreased and then increased (data not shown). However, unlike *TUP1*, the timing and magnitude of the changes were similar in the presence and absence of farnesol (Fig. 4, data not shown). Under the same conditions, *NRG1* mRNA levels did not change during development and they too were the same in the presence and absence of farnesol (Fig. 4). Thus, we conclude that farnesol does not affect *RFG1* or *NRG1* mRNA levels.

Expression of the Tup1-regulated filamentous genes *HWP1* and *RBT1* is inhibited by farnesol

To test whether the increased *TUP1* expression in the presence of farnesol was biologically significant, we examined the expression of two Tup1-regulated genes, *HWP1* and *RBT1* [Fig. 2B and C, (De Groot *et al.*, 2003)]. In the absence of farnesol, the *HWP1* and *RBT1* transcripts were undetectable at time 0 but they were strongly expressed from 40 to 80 minutes (Fig. 2B and C). Farnesol delays and dramatically reduces the magnitude of *HWP1* and *RBT1* mRNA expression (Fig. 2B and C). At 80 minutes, *HWP1* and *RBT1* levels were 30- and 7.6-fold lower, respectively, in farnesol treated than untreated cells. Similar results were observed by Davis-Hanna et al. (Davis-Hanna *et al.*,

2008) for *HWP1* mRNA 2 hours after treatment with 75 μM farnesol. Thus there is a strong correlation between elevated *TUP1* expression in response to farnesol and the expression of Tup1-regulated genes.

EFG1 mRNA levels remain unaffected by farnesol

Efg1 is a transcription regulator for genes required for filamentation. *EFG1* mRNA levels are down regulated at the initiation of filament development and then increase as filament formation progresses (Tebarth *et al.*, 2003). *HWP1* and *RBT1* are activated by Efg1 during filamentation. Therefore, we tested whether farnesol also affects *EFG1* mRNA levels (Fig. 4). The *EFG1* mRNA levels were high at time 0, decreased to a minimum at 20 minutes, and then increased steadily throughout the remaining time (data not shown). However, farnesol had no influence on *EFG1* mRNA levels since the timing and magnitude of the changes were similar in the presence and absence of farnesol (Fig.4 and data not shown).

Farnesol suppresses the haploinsufficient phenotype of a *TUP1/tup1* heterozygote.

Braun and Johnson (Braun and Johnson, 1997) showed that BCa2-3, a *TUP1/tup1* heterozygote, is haploinsufficient in that these cells develop a higher proportion of filaments compared to wild-type cells on most media (Braun and Johnson, 1997). Presumably these cells do not make enough Tup1 to compensate for the reduced gene copy number. We hypothesized that farnesol might suppress this phenotype because it increases *TUP1* expression 2.5-fold in SC5314 and 4.2 fold in *TUP1/tup1* (Fig. 3). This increase should restore Tup1 to roughly wild type levels. To test this hypothesis, we

examined the effect of farnesol on *C. albicans* BCa2-3 on cornmeal agar plus Tween 80 under a coverslip for 25 hours at 25°C. In these conditions, the *TUP1/tup1* mutant was more filamentous than wild-type colonies but less filamentous than the *tup1/tup1* mutant (BCa2-10; 4 and Table 2). As a control, we showed the *TUP1/tup1* mutant responds to farnesol because, although it forms filamentous cells when grown in mGPP, the addition of 20 μM farnesol results in growth as yeasts (Fig. 1), and Tup1 protein levels were ca. 4.2-fold higher in the *TUP1/tup1* mutant treated with farnesol. In contrast to the haploinsufficient phenotype observed in the absence of farnesol, in the presence of farnesol the *TUP1/tup1* mutant looked identical to wild type *C. albicans* (Table 2). Thus farnesol suppresses the haploinsufficiency phenotype of the *TUP1/tup1* heterozygote.

tup1/tup1 and _nrg1/nrg1_ mutants produce excess farnesol.

Jensen et al (Jensen *et al.*, 2006) tested the farnesol production levels for several filamentous-only mutants. A subset of these mutants produced levels of farnesol significantly higher than wild type strains. This overproduction suggests the ability to respond to farnesol may be linked to regulation of farnesol production. Here we tested farnesol production levels in CAI-4, CAF-2, *tup1/tup1*, *nrg1/nrg1*, and *rfg1/rfg1* strains. Farnesol production levels were dramatically increased in the *tup1/tup1* and *nrg1/nrg1* mutants (Table 1) that were unable to respond to farnesol (Fig. 1). The *tup1/tup1* and *nrg1/nrg1* mutants produced ca. 17- and 19-fold more farnesol, respectively, than did CAF-2 and CAI-4. In contrast, the farnesol responsive *rfg1/rfg1* mutant only produced ca. 2.6-fold more farnesol than the wild-type strains (Table 1). Thus, the two mutants

that are unable to respond to farnesol (*tup1/tup1* and *nrg1/nrg1*) produced much higher levels of farnesol than did strains that do respond to farnesol.

tup1/tup1 overproduction of farnesol inhibits SC5314 filamentation.

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

Discussion

C. albicans responds to farnesol, in part, by changing gene expression (Cao *et al.*, 2005; Enjalbert and Whiteway, 2005). We hypothesize that some of these changes are mediated by changes in the activity of the signaling pathways regulating morphogenesis. Here we show that the *tup1/tup1* and *nrg1/nrg1* null mutants are strictly filamentous and the cells remain filamentous in the presence of added farnesol (Fig. 1). In these cases the total farnesol levels are actually much higher than the added farnesol because the mutants themselves produce elevated levels of farnesol (Table 1, see below). Further, Tup1 mRNA and protein levels increased in the presence of farnesol while two Tup1-regulated genes, *HWP1* and *RBT1* mRNA levels decrease (Fig. 2, and 3). Importantly, the timing of this increase (40-60 min, Fig. 2) corresponds with the commitment point, beyond which added farnesol no longer blocks germ tube formation (Mosel *et al.*, 2005). Finally, we believe that Tup1 is part of the farnesol response pathway because farnesol suppresses the haploinsufficient phenotype of a *TUP1/tup1* strain (Table 2).

Cell synchrony, farnesol concentration, and timing were all important considerations for our experimental design. Previous work examining farnesol-dependent changes in the global transcription profiles of developing biofilms (Cao *et al.*, 2005; Enjalbert and Whiteway, 2005) and during resumption of growth following stationary phase (Enjalbert and Whiteway, 2005) were done with mixed cell populations that differed in their ability to respond to farnesol. Further, the effect of farnesol addition on the global gene expression during biofilm formation was determined at a single time point, 24 hours after addition of farnesol (Cao *et al.*, 2005). This point is significant because such a study could only measure stable long-term farnesol-dependent changes in

gene expression. Timing is also important because of the commitment phenomenon. This is the point at which a switch in the environmental stimulus no longer causes the expected switch in morphology (Chaffin and Wheeler, 1981; Mitchell and Soll, 1979; Mosel *et al.*, 2005). It is relevant to farnesol's mode of action because, while farnesol blocks the yeast to filament switch, it does not block the elongation of preexisting filaments (Mosel *et al.*, 2005). Thus for our experiments, we added farnesol at time zero in order to avoid commitment to filamentous growth, and we harvested cells at 20 minute increments to observe changes in transcript levels during the early stages of the farnesol response (Mosel *et al.*, 2005). We also achieved a synchronous cell population by starting with resting cells and inoculating them in mGPP; in these conditions we routinely get 95-100% filaments within 3-4 hrs. Exposing a synchronized cell population to farnesol allowed us to detect subtle and consistent changes in transcript abundance.

Small changes in expression of a transcription regulator can have profound effects on the genes it regulates. For example, we have shown that nonsense-mediated mRNA decay (NMD) in *S. cerevisiae* regulates accumulation of the mRNA for Adr1, a transcription regulator of the genes responsible for making acetyl CoA and NADH from nonfermentable substrates. In particular, the respiratory impairment seen in NMD mutants is due, in part, to overexpression of Adr1 (Taylor *et al.*, 2005). The change in *ADR1* mRNA levels is small (2.6-fold), but sufficient to affect expression of Adr1-regulated genes. Thus even though the change in Tup1 expression is relatively small it can have a profound effect on expression of the genes it regulates.

Two Tup1 co-regulators, encoded by *NRG1* and *RFG1,* were unaffected by farnesol at the mRNA level (Fig. 4). In this regard, it is reasonable that farnesol regulates

only one part of the complex, i.e. that farnesol elevates *TUP1* mRNA but not *NRG1* or *RFG1* mRNA. By analogy, for Ca^{2+} and calmodulin where only the Ca^{2+}-calmodulin complex is active (Soto *et al.*, 2004a), fungi have the calmodulin in excess and regulate the activity of the complex by regulating the availability of cytoplasmic Ca^{2+} (Muthukumar *et al.*, 1987).

Because the *tup1/tup1* and *nrg1/nrg1* mutants did not respond to farnesol, it suggests that farnesol acts through a pathway requiring Tup1 and Nrg1. The *rfg1/rfg1* mutant responded to farnesol, indicating that the genes regulated by Rfg1 are not required for the response to farnesol. Furthermore, the *tup1/tup1* and *nrg1/nrg1* mutants overproduced farnesol while the *rfg1/rfg1* mutant produced only slightly elevated farnesol (Table 1). This *tup1/tup1* mutant overproduction is biologically significant because the excess farnesol produced by the *tup1/tup1* mutant inhibits filamentation of wild-type *C. albicans* grown on the same plate (Fig. 5). The juxtaposition between farnesol non-responsive mutants and the overproduction of farnesol implies that a farnesol-Tup1 feedback loop may exist, and that Nrg1 may work in concert with Tup1 to negatively regulate farnesol synthesis. This regulation may be direct or indirect. The enzyme responsible for ca. 90% of farnesol synthesis is Dpp3 (Navarathna *et al.*, 2007a). *DPP3* mRNA levels were not significantly elevated in the whole genome profiles of *tup1/tup1* or *nrg1/nrg1* mutants (Kadosh and Johnson, 2005); however, *DPP3* does have a putative Nrg1 binding site in its promoter region.

The increased *TUP1* expression we observed for farnesol blockage of filament development (Fig. 2A) is smaller than that reported for farnesol blockage of biofilm development, ca 6.6-fold as determined with DNA arrays (Cao *et al.*, 2005). The

difference in the response intensity may reflect filamentous vs. biofilm growth conditions as well as the fact that Cao et al. (Cao *et al.*, 2005) used one time point 24 hours after farnesol addition.

Efg1 is a transcriptional factor that activates hyphal gene expression including *HWP1* and *RBT1*. *EFG1* mRNA levels are regulated during filamentation, but they were not affected by farnesol since the timing and magnitude of the changes were similar in the presence and absence of farnesol (Fig. 4). These results are consistent with those of Soto et al (Soto *et al.*, 2004b) who also found no change in *EFG1* mRNA levels at a single time point with added farnesol (Soto *et al.*, 2004b). Together with our results, this suggests that farnesol does not regulate *EFG1* mRNA levels but, at this time we can not exclude the possibility that post translational regulation of Efg1 is affected by farnesol.

Two other farnesol-related findings regarding filamentous growth can be accommodated in a Tup1-dependent model because they are downstream from Tup1. Soto et al (Soto *et al.*, 2004b) suggested that farnesol acts by causing decreased *CPH1* and *HST7* mRNA levels. *CPH1* is a transcription factor that regulates filamentous growth and *HST7* is a MAP kinase kinase involved in filamentous growth. Both are down-regulated by Tup1 and thus their downregulation by farnesol (Soto *et al.*, 2004b) is consistent with a secondary effect of farnesol on Tup1. Additionally, Chk1, a histidine kinase shown to be required for the farnesol response (Kruppa *et al.*, 2004), is also a Tup1 repressed gene; *CHK1* was elevated 6.5-fold in the *tup1/tup1* mutant (Kadosh and Johnson, 2005). Taken together, these findings indicate that Tup1 is involved in mediating the *C. albicans* response to farnesol.

Acknowledgements

We thank Alexander Johnson and Richard Cannon for providing us with *C. albicans* strains and the Tup1 antibody. We also thank Jessica A. Wiles for assisting with the germ tube assays and RNA work.

This work was supported by grants from the National Science Foundation (MCB-0110999), the University of Nebraska Tobacco Settlement Biomedical Research Enhancement Fund, and the Farnesol and *Candida albicans* Research Fund, University of Nebraska Foundation. Any opinions, findings, conclusions, or recommendations expressed in this report are ours, and do not necessarily reflect the views of the National Science Foundation.

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Figure Legends

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

Figure 3-2. *TUP1* mRNA levels increased, while two Tup1-regulated genes, *HWP1* and *RBT1* were downregulated in the presence of farnesol. *C. albicans* SC5314 resting cells were inoculated into mGPP (pH 4.8) in the presence or absence of 20 μM farnesol and incubated at 37°C. Cells were then harvested at 0, 20, 40, 60 and 80 minutes post inoculation. Northern blots were prepared with total RNA from cells incubated both in the presence or absence of farnesol. Shown is a PhosphorImage of a representative Northern blot probed with radiolabeled *TUP1* DNA (**A**), *HWP1* DNA (**B**) and *RBT1* DNA (**C**), and a plot of average mRNA levels from a minimum of three independent experiments. *ACT1* mRNA levels were used as a loading control.

Figure 3-3. Tup1 protein levels are higher in the presence of farnesol. Total protein extracts were prepared from SC5314 and *TUP1/tup1* (BCa2-3) resting cells inoculated to mGPP (pH 4.8) medium at 37°C in the presence or absence of 20 μM farnesol and incubated at 37°C for 60 minutes. The average fold Tup1 protein accumulation for farnesol treated cells relative to untreated cells is shown. Act1 levels were used as a loading control.

Figure 3-4. Farnesol does not affect expression of *RFG1* or *NRG1*, which encode DNA binding proteins that function with Tup1, or *EFG1*, which encodes a transcription activator of hypal-specific genes. Quantitative Northern blot analysis was used to measure *TUP1*, *NRG1*, *RFG1*, and *EFG1* mRNA levels in SC5314 at 60 min after inoculation of resting cells into conditions that promote germ tube formation and hyphal growth in the presence and absence of 20μM farnesol. The results are an average of three independent experiments.

Figure 3-5. Overproduction of farnesol by the *tup1/tup1* mutant inhibits SC5314 filamentation. Resting cells were grown at 37 °C for 24 hours on YPD agar plates to allow for farnesol accumulation in the agar (horizontal streak, *C.albicans* strain SC5314 (B, C), or *tup1/tup1* (BCa2-10, A, D)). Subsequently, either SC5314 (A, B) or *tup1/tup1*(C, D) resting cells was plated (vertical streak) and incubated at 37 °C for an additional 24 hours. Area above the two arrows (left panels A, B) are zones of filament inhibition (as evident by smooth morphology) resulting from the farnesol produced by the horizontally streaked strains. Filamentation gives the wrinkled colony morphology seen below the arrows. The pictures in the two white boxes have been magnified 2.5X so that the colony morphology can be seen more clearly (center panels A, B). Micrographs of individual cells from the two bracketed regions are shown in the right panels (A, B; scale bar = 10μm). The cells from the smooth regions are mainly yeasts, while there is a much larger proportion of filaments in the wrinkled region.

Figure 3-1

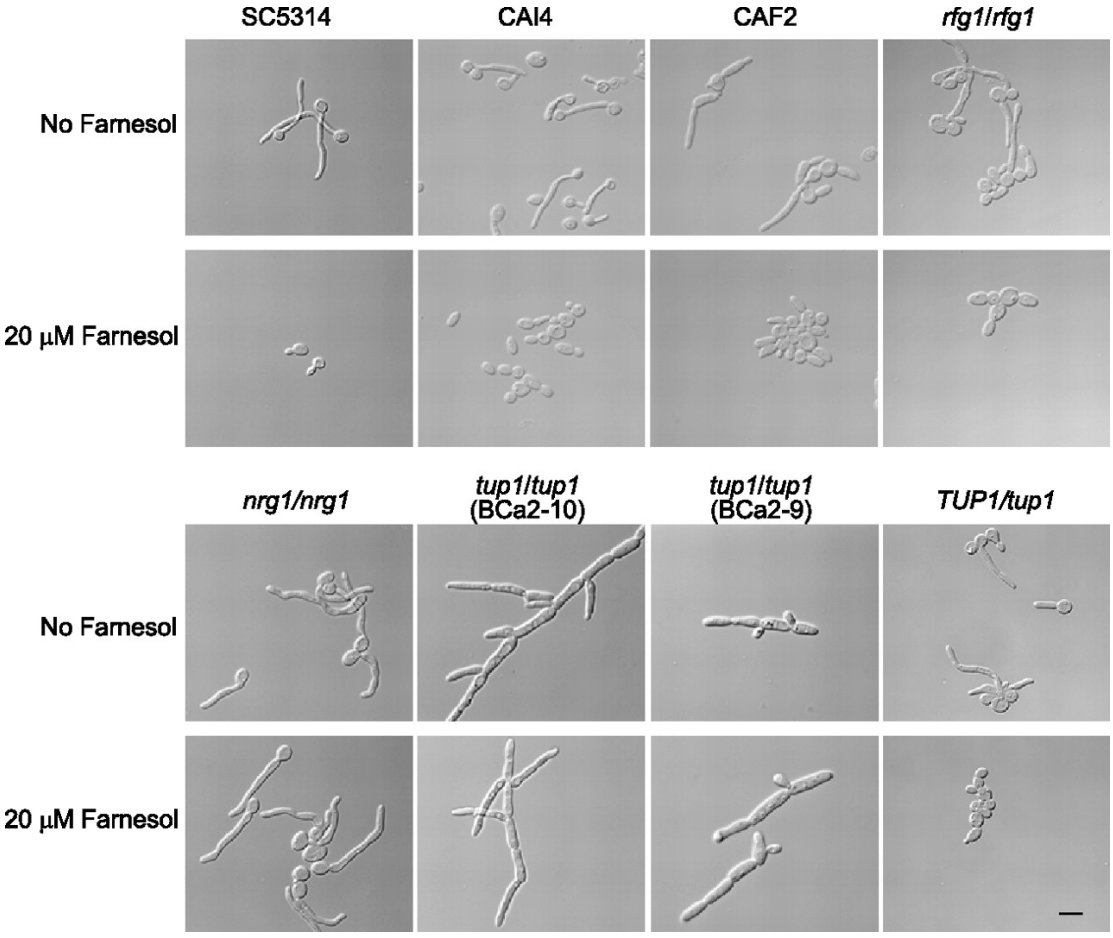
SC5314
CAI4
CAF2
rfg1/rfg1
No Farnesol
20 μM Farnesol
nrg1/nrg1
tup1/tup1 (BCa2-10)
tup1/tup1 (BCa2-9)
TUP1/tup1
No Farnesol
20 μM Farnesol

Figure 3-2

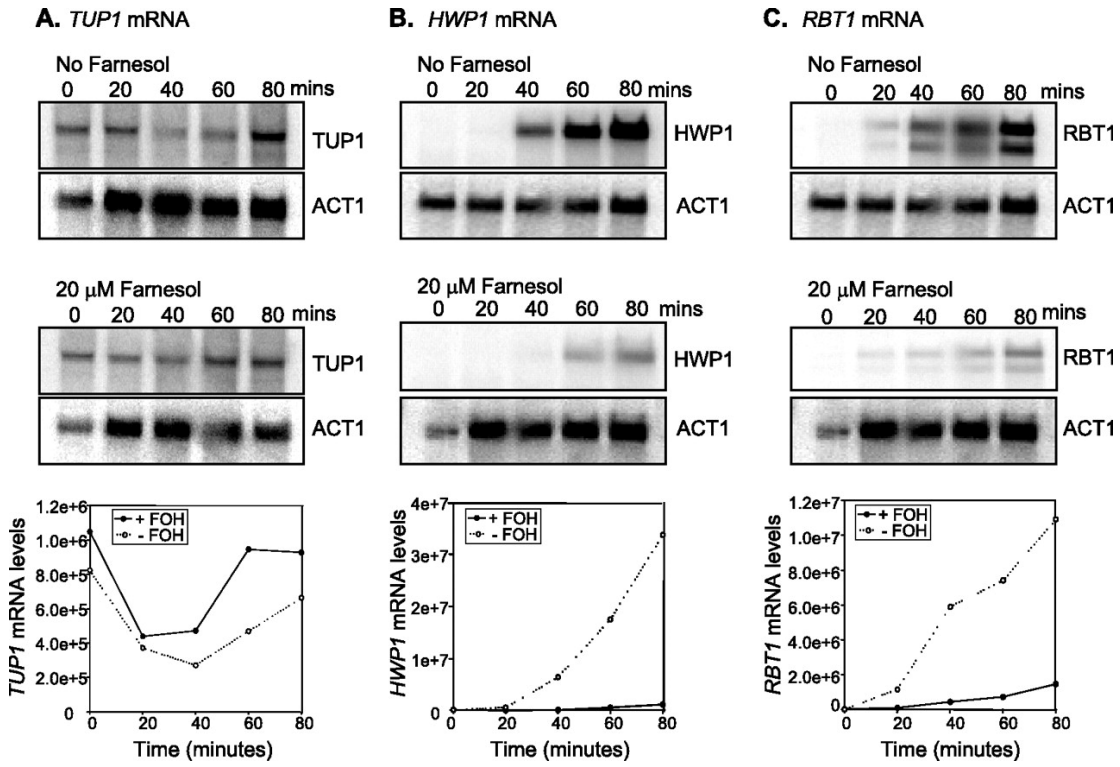
A. TUP1 mRNA
B. HWP1 mRNA
C. RBT1 mRNA
No Farnesol
0 20 40 60 80 mins
TUP1
HWP1
RBT1
ACT1
20 μM Farnesol
+ FOH
- FOH
TUP1 mRNA levels
HWP1 mRNA levels
RBT1 mRNA levels
Time (minutes)

Figure 3-3

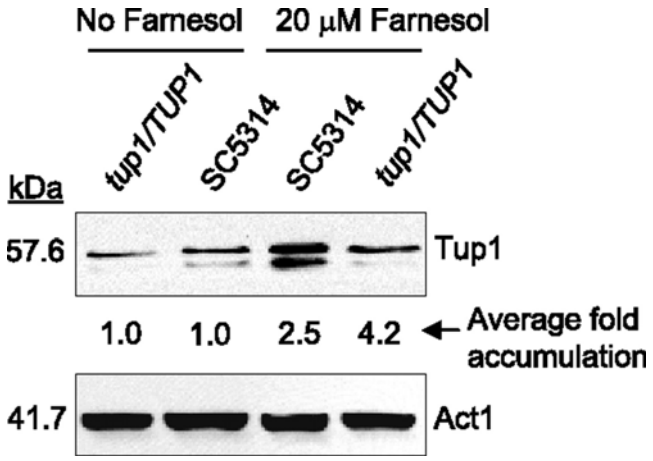
No Farnesol
20 μM Farnesol
tup1/TUP1
SC5314
SC5314
tup1/TUP1
kDa
57.6
Tup1
1.0
1.0
2.5
4.2
Average fold accumulation
41.7
Act1

Figure 3-4

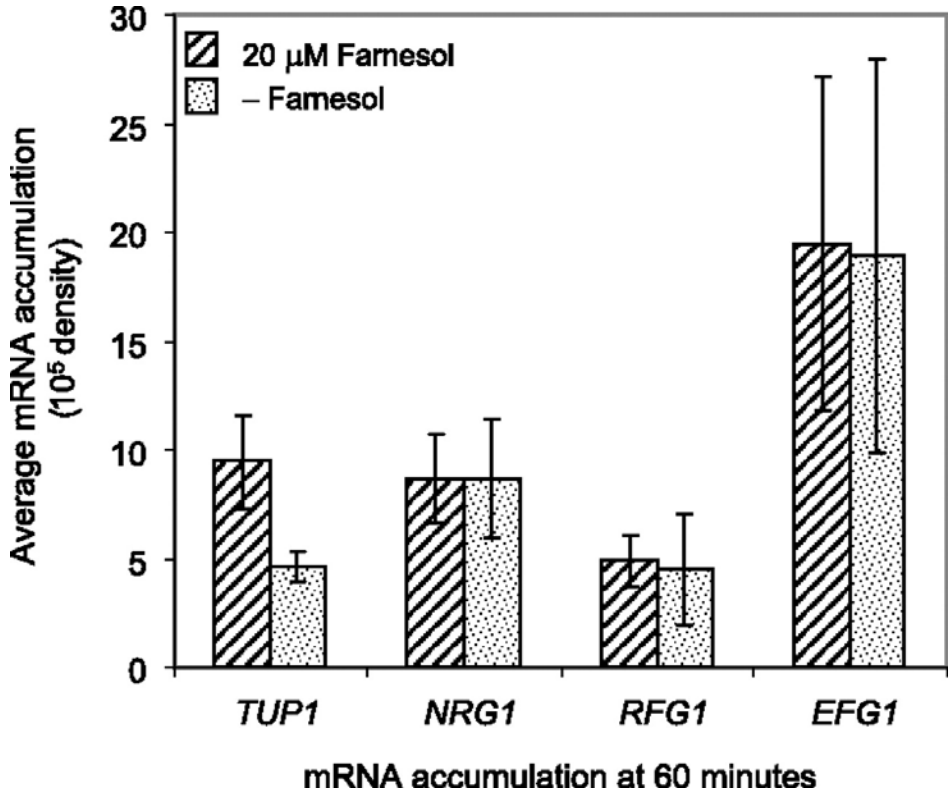
30
25
20
15
10
5
0
20 μM Farnesol
– Farnesol
Average mRNA accumulation
(10^5 density)
TUP1
NRG1
RFG1
EFG1
mRNA accumulation at 60 minutes

Figure 3-5

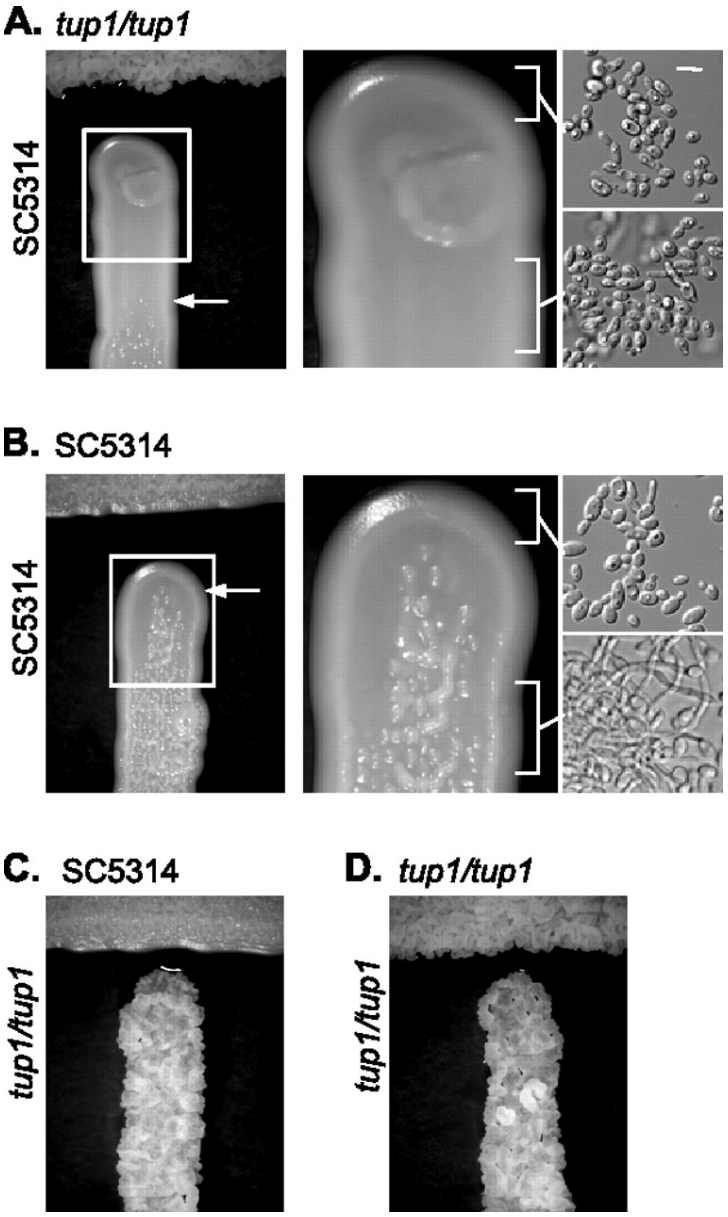
A. tup1/tup1
SC5314
B. SC5314
SC5314
C. SC5314
tup1/tup1
D. tup1/tup1
tup1/tup1

Table 3-1. *tup1/tup1* and *nrg1/nrg1* null mutants do not respond to farnesol but overproduce farnesol.

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

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

[b/] Farnesol production (μg/g dry weight of cells) was the average of three measurements

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

Table 3-2. Farnesol suppresses the haploinsufficient phenotype of a *TUP1*/*tup1* heterozygote. Cells were plated on a cornmeal agar plus Tween 80 plate under a coverslip and grown at 25°C for 25 hours.

C. albicans Strain	Cell morphology at the colony periphery	
	No Farnesol[a/]	20 μM Farnesol
Wild type (SC5314)	Yeasts + few filaments	Yeast + few filaments
TUP1/tup1 (BCa2-3)	Yeasts + filaments	Yeast + few filaments
tup1/tup1 (BCa10)	filaments	filaments

[a/]The phenotype for these strains grown on a cornmeal agar plus Tween 80 plate under a coverslip without farnesol was also previously reported (Braun and Johnson, 1997).

CHAPTER 4

A novel role for Czf1 in farnesol tolerance and the morphological response to farnesol in *Candida albicans*

Abstract

Candida albicans is the primary cause of hospital acquired fungal infections in the United States. The quorum sensing phenomenon in *Candida albicans* is a process that inhibits the transition to filamentous growth and may be directly related to its ability to cause both mucosal and systemic disease. The Ras1-cyclic AMP signal transduction pathway is a proposed target for farnesol inhibition, resulting in filamentous growth obstruction, but a clear understanding of the downstream effectors and signaling networks vital for the morphological farnesol response have yet to be unraveled. To address this issue, we screened a mutant library for farnesol resistant mutants; among the resistant mutants was a *czf1Δ/czf1Δ* mutant. Here, we demonstrate for the first time the capability of *C. albicans* to respond to farnesol in embedded conditions, and in these and liquid assays, Czf1 was required for a wild-type farnesol response. The *CZF1* mRNA transcript levels in response to farnesol, farnesol toxicity, and farnesol production levels were also examined. Finally, *CZF1* ectopic expression restored farnesol response in strains lacking Czf1, highlighting a new role for Czf1 as a critical downstream effector of the morphological response to farnesol in *C. albicans*.

Introduction

Candida albicans is an opportunistic pathogen that is part of the normal flora but can cause mild to severe opportunistic infections in immunocompromised patients. A polymorphic fungus, *C. albicans* can switch between yeast and filamentous forms of growth, and this transition is necessary for causing disease in a mouse model of infection (Lo *et al.*, 1997; Saville *et al.*, 2003). Two additional growth morphologies in *C. albicans* include mating-competent opaque cells and structures with unknown function called chlamydospores. All of these cell types are affected by the quorum sensing molecule, farnesol, highlighting its influential role in *C. albicans* morphology.

Farnesol was the first eukaryotic quorum sensing molecule identified and was initially shown to block the transition from yeast to filaments (Hornby *et al.*, 2001). Subsequent studies identified farnesol as having an inhibitory role in biofilm formation (Ramage *et al.*, 2002) and a protective role against oxidative stress (Deveau *et al.*, 2010; Westwater *et al.*, 2005). In addition, very high levels of farnesol can increase chlamydospore formation (Martin *et al.*, 2005), and low levels of farnesol induces necrosis in opaque cells when oxygen is present, thereby regulating opaque cell stability (Dumitru *et al.*, 2007). White cells can also be killed by farnesol in the right environmental conditions; log phase cells that are energy-deprived are particularly sensitive, while stationary phase cells in growth medium are quite farnesol tolerant (Langford *et al.*, 2010; Shirtliff *et al.*, 2009; Uppuluri *et al.*, 2007). Given its important role in physiology, it comes as no surprise that *in vivo* studies provide physiological relevance for farnesol signaling during infection and suggest farnesol plays distinct roles at different sites of infection. For example, farnesol is a virulence factor in a

disseminated mouse model of infection (Navarathna *et al.*, 2007a), yet it protects mice from oral candidiasis (Hisajima *et al.*, 2008). These results highlight the need for a complete understanding of the signaling response induced by farnesol in *C. albicans*.

Factors identified that play a role in the *C. albicans* farnesol response include Tup1/Nrg1 (Kebaara *et al.*, 2008), Chk1 (Kruppa *et al.*, 2004), the Hog1 MAP kinase pathway (Smith *et al.*, 2004), the Cek1 MAP kinase pathway (Roman *et al.*, 2009), and the Ras1-cyclic AMP (cAMP) signaling pathway (Davis-Hanna *et al.*, 2008). In this paper, we have identified Czf1 (*C. albicans* zinc finger) as an important factor in the response to farnesol by screening a mutant library for mutants with a defective farnesol response. Known roles of Czf1 include induction of contact-induced filamentous growth (Brown *et al.*, 1999), biofilm formation (Stichternoth and Ernst, 2009), and white to opaque cell switching (Vinces and Kumamoto, 2007). It contains an unusually large 5' untranslated region (UTR) of approximately 2 kb (Vinces *et al.*, 2006), and can negatively regulate its own mRNA expression (Vinces *et al.*, 2006). Czf1 also has ties to the cAMP pathway, as a transcription factor downstream of cAMP signaling, Efg1, can bind the promoter of *CZF1* and tightly regulate its expression, since no *CZF1* mRNA was detected in an *efg1Δ/ efg1Δ* background (Vinces *et al.*, 2006). This positive regulation by Efg1 is intriguing because Czf1 and Efg1 appear to play opposing roles in the cell with respect to morphology (Giusani *et al.*, 2002; Noffz *et al.*, 2008). Of note, regulation between Efg1 and Czf1 may also occur at the protein level as well since these two proteins interact during a yeast two hybrid assay (Giusani *et al.*, 2002; Noffz *et al.*, 2008).

Here we have defined a new role for Czf1 in the quorum sensing response of *C. albicans*. We show that Czf1 is not only necessary for a wild type morphological

response to farnesol in a variety of conditions, but in combination with Efg1, is required for farnesol tolerance as well. Our data suggest a vital downstream role for Czf1 in the signaling cascade elicited by farnesol in *C. albicans*.

Methods

Strains and media

Candida albicans strains and plasmids are listed in Table 1. The defined medium, modified glucose phosphate proline (mGPP) pH 6.8 was prepared as described in Kebaara et al (Kebaara *et al.*, 2008) with 2.5 mM N-acetylglucosamine (GlcNAc) and uridine (40 μg/ml). Yeast 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 in Kebaara et al (Kebaara *et al.*, 2008) with modifications: single colonies were grown in 25 mL YPD broth at 30°C (unless otherwise noted) for 22-24h to reach stationary phase, washed three times with 50 mM potassium phosphate buffer (pH6.8), resuspended in potassium phosphate buffer, and stored at 4°C overnight before use. For liquid farnesol response assays, resting cells were inoculated at 10^6 cells/mL in mGPP or mSPP (2% sucrose replacing glucose) broth with indicated farnesol concentrations. Cells were then grown at 37ºC, shaking in glass flasks for the indicated times. Trans-trans 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 screen

Mutants 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 compared to the parental strain, BWP17 for farnesol response. Wrinkled/hairy colony morphologies were considered to be composed primarily of filaments and smooth colony morphologies were considered to be mostly yeast cells. A positive response to farnesol

was viewed as filamentous colonies without farnesol and smooth colonies in the presence of farnesol. Farnesol resistant mutants maintained a filamentous morphology, even in the presence of 50 μM farnesol.

Embedded cell growth

Embedded media were prepared by mixing 10^4 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 hours as indicated. Only colonies beneath the agar surface were examined.

Microscopy and cell death determination

Cellular morphology during the germ tube assays was determined using a Zeiss Stemi 2000-C light microscope. Embedded micrographs were taken using a custom MVI TDM400 tetrad dissecting microscope and Sony Cybershot camera. DIC micrographs were taken using an Olympus BX51 microscope and a Photometrics CoolSnap HQ CCD camera. Cell death was determined by methylene blue staining as described by Gibson et al (Gibson *et al.*, 2009).

DNA analysis and transformation

To create strain AAC2, CKY283 was plated on 5-fluoroorotic acid (5-FOA) containing media to select for ura- mutants. AAC2 was subsequently transformed with BsgI-digested pDB212 to create strain AAC6. Transformations were performed by the lithium acetate method and transformants were selected on media lacking uridine (Gietz and Woods, 2002). 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 real- time PCR

Resting cells were inoculated into 75 mL mGPP broth at 5 x 10^6 cells/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 minutes. For harvesting cells, cultures were passed through glass fiber filters and cells were scraped off the filters to reduce the loss of filamentous cells during centrifugation. Data shown are from a representative independent experiment performed in triplicate. Extraction of mRNA was performed with the RiboPure Yeast Kit (Applied Biosystems, Foster City, CA), and mRNA integrity was assessed by measuring $OD_{260/280}$ ratios and by examining the appearance on agarose gels. Reverse transcription and PCR amplification were performed according to manufacturer's specifications using MultiScribe Reverse Transcriptase and Power SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR reactions were carried out in an ABI Prism 7500 Real-Time PCR machine (Applied Biosystems). *ACT1* mRNA levels were used as controls. Primers used to amplify *CZF1* and *ACT1* and PCR efficiencies were as previously described (Bassilana *et al.*, 2005).

Farnesol Production Measurements

Extracellular farnesol was extracted from cell-free supernatants of stationary phase cultures grown in GPP at 30ºC and analyzed by gas chromatography-mass spectrometry, as described by Hornby et al (Hornby *et al.*, 2001).

Results

Identification of new genes required for the response to farnesol

To identify important regulators of the farnesol response in *C. albicans*, a transposon insertion mutant library of approximately 560 mutants (Supp. Table 1) was screened for mutants defective in the morphological response to farnesol. The majority of mutants in this collection are *Tn7-UAU1* plasmid insertion mutants (Nobile *et al.*, 2006); therefore it is desirable to obtain knockout mutants for further characterization due to the possibility of partial loss-of-function mutations. Mutants that were identified as farnesol resistant in the original screen were re-streaked on individual plates to rule out interference from neighboring mutant colonies and to confirm the farnesol resistant phenotype. Eight mutants were confirmed as farnesol resistant, and they are summarized in Table 1. Mutants that were unable to interconvert between yeast and filaments (at 30° and 37°, respectively) were not examined further in this study to rule out mutants that are merely defective in morphological switching and not the specific response to farnesol. No mutants were identified that were hypersensitive to farnesol, nor were any detected that demonstrated growth inhibition in the presence of farnesol.

CZF1 is required for a wild type morphological response to farnesol in both liquid and embedded conditions

The *czf1/czf1* mutant was identified as farnesol-resistant in the mutant library screen (Table 1) and selected for further characterization based on its known roles in morphogenesis, white/opaque cell switching, and its connection to the cAMP pathway. To test the role of Czf1 in the morphological response to farnesol, a double null

czf1Δ/czf1Δ mutant was compared with its parental strain (CKY101), a wild type clinical isolate (SC5314), the heterozygous mutant, and the double null mutant ectopically expressing *CZF1* under the control of the *MAL2* promoter in a liquid farnesol response assay (Fig. 1). In both glucose and sucrose-containing media, SC5314 and CKY101 displayed the expected reduction of early stage filaments (germ tubes) when in the presence of farnesol. However, the *czf1Δ/czf1Δ* mutant showed only a minimal reduction of germ tubes, even in the presence of 100 μM farnesol, and a haploinsufficient farnesol response phenotype was observed for the heterozygous mutant. Results were similar whether glucose or sucrose was used as a carbon source. Ectopic complementation of the *czf1Δ/czf1Δ* mutant was able to restore the farnesol response similar to that of the heterozygous mutant in *pMAL2*-inducing conditions (Fig. 1B; sucrose-containing media), while there was only a minimal farnesol response in non-inducing conditions, similar to that of the *czf1Δ/czf1Δ* mutant (Fig. 1A; glucose-containing media). These results show that the presence of Czf1 is critical to the ability of *C. albicans* to respond to farnesol in liquid media.

Since the first described role for Czf1 was to promote filamentation in embedded conditions (Brown *et al.*, 1999), we sought to determine whether: 1) *C. albicans* could respond to farnesol under such conditions and 2) Czf1 was needed for farnesol response when embedded in a matrix. For consistency with our prior work studying farnesol signaling and physiological significance (plates were incubated at 37°C), we utilized defined GPP or SPP agar plates (lacking GlcNAc, see experimental procedures) in our embedded assays. Although the growth media and incubation time and temperature used in this study are different from those initially used by Brown et al (Brown *et al.*, 1999),

we still observed a strong filamentation response from *C. albicans* SC5314 and CKY101 cells in response to growth embedded in a matrix (Fig. 2). When SC5314 and CKY101 cells were grown in embedded media containing 50 μM farnesol, filamentous colonies were not observed. As with the liquid germ tube formation assays, using glucose versus sucrose as a carbon source made no difference in filamentation or farnesol response (Fig. 2). This demonstrates the capability of *C. albicans* to respond to farnesol in an embedded assay.

As expected, the *czf1Δ/czf1Δ* mutant exhibited a defective growth pattern in embedded conditions; filamentation was still observed, but far fewer colonies were present compared with the wild type and parental strains and colony morphology appeared different as well (Fig. 2). When the *czf1Δ/czf1Δ* mutant was grown in agar containing farnesol, only a moderate farnesol response was observed in that the hyphae appeared shorter than in untreated samples; however, colonies were still filamentous. As with the liquid farnesol response assays, the *czf1Δ/CZF1* heterozygote maintained a haploinsufficient phenotype in both farnesol treated and untreated samples: in untreated samples, the overall level of growth was more similar to that of wild type strains, and in farnesol-treated samples, colonies were reduced in hyphal formation but still more filamentous than wild type colonies treated with farnesol. Ectopic *CZF1* complementation of the *czf1Δ/czf1Δ* mutant produced positive farnesol responses similar to that of the heterozygote in both GPP and SPP, conditions which should turn off and on, respectively, the *MAL2* promoter (Fig. 2). This suggests unintended leaky promoter activation in GPP media, possibly resulting in the expression of *CZF1* and complementation of the farnesol response in both GPP and SPP embedded media. These

results indicate that Czf1 is required for farnesol response in at least two distinct environmental conditions: liquid, aerobic growth media and embedded in a semi-solid matrix, new conditions in which to test *C. albicans* for a morphological farnesol response.

CZF1 mRNA levels are decreased in the presence of farnesol

Since *CZF1* is regulated at the mRNA level, we asked whether *CZF1* mRNA levels are affected by the presence of farnesol. A time-course experiment was performed to measure *CZF1* expression levels in the presence of 0, 50 or 100 μM farnesol, and 40, 60, and 80 minute time points were selected based on previous studies showing the importance of this time frame to the farnesol response (Kebaara *et al.*, 2008). As with *TUP1* mRNA levels (Kebaara *et al.*, 2008; Mosel *et al.*, 2005), *CZF1* levels were most dramatically affected 60 minutes after treatment with farnesol, showing a greater than 5-fold decrease in expression relative to a sample lacking farnesol (Fig. 3B). At all 3 time points tested, both 50 and 100 μM farnesol reduced *CZF1* levels, relative to samples containing no farnesol (Fig. 3). These data indicate that *CZF1* mRNA levels are decreased in the presence of farnesol, during time points crucial to the farnesol response.

An *efg1Δ/ efg1Δ czf1Δ/ czf1Δ* double mutant exhibits decreased farnesol tolerance that is partially temperature dependent

Upon growth of the *efg1Δ/efg1Δ czf1Δ/czf1Δ* mutant in liquid media for preparation of resting cells, an unusual temperature-regulated phenotype was observed. Mutant cells grown in YPD broth at 30°C appeared small but elongated, reminiscent of

opaque cells (Fig. 4A), and when the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant was grown in YPD broth at 37°C, cells appeared larger and more rounded (Fig. 4B). When these *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant cells (pre-grown at 30°C) were tested for farnesol response in the previously described mGPP/mSPP liquid germ tube formation assays (experimental procedures), we observed what appeared to be cell death. Given the importance of environmental conditions in the ability of *C. albicans* to survive and tolerate farnesol, methylene blue staining was performed during liquid germ tube assays to more accurately assess the level of cell death occurring in these conditions (Fig. 4C-F). For the cell death assays, resting cells were prepared as described in experimental procedures, except that cells were grown at either 30°C or 37°C in YPD broth until stationary phase was reached. The parental strain CAI4 was used as a control in the 30°C resting cell group, and as observed for SC5314 in Langford et al (Langford *et al.*, 2010), no significant cell death was observed in mGPP or in mSPP, even in the presence of 100 μM farnesol. The *czf1Δ/czf1Δ* and *efg1Δ/efg1Δ* mutants (Fig. 4C-F) were similarly resistant to farnesol-mediated killing in the liquid germ tube formation assays. However, the *czf1Δ/czf1Δ* mutant could not be tested accurately with methylene blue in these assays, due to its rapid formation of germ tubes which stain inconsistently with this dye regardless of the strain used or the presence of farnesol. The arguments for the viability of the *czf1Δ/czf1Δ* mutant in the presence of farnesol are twofold: 1) no growth inhibition was observed in the presence of farnesol on agar plates (data not shown) and 2) germ tube formation is a process that requires active protein synthesis (Imanishi *et al.*, 2004) and is unlikely to occur in a dead cell. CAI4 could also not be used as a control at 37°C for similar reasons; cells pre-grown at 37°C start as filaments. On the other hand,

the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant and the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant ectopically expressing *CZF1* (from the 30°C resting cell group) showed extreme sensitivity to farnesol, regardless of the media used to induce *CZF1* expression (Fig 4C, 4E). Conversely, when the *efg1Δ/efg1Δ czf1Δ czf1Δ* double mutant and the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant expressing *CZF1* under the control of the *MAL2* promoter from the 37°C resting cell group were tested for farnesol sensitivity, a general decrease in cell death was observed compared to the 30°C resting cell group (Fig. 4C-F). Based on these results, Czf1, Efg1, and temperature appear to play important roles in farnesol tolerance, and both 30°C and 37°C double mutant resting cells were subsequently utilized for additional experiments in this study.

Ectopically expressing *CZF1* in *an efg1Δ/ efg1Δ czf1Δ/czf1Δ* double mutant partially restores filamentation and farnesol response in liquid and embedded conditions

Can ectopic expression of *CZF1* restore filamentation and response in the *efg1Δ/ efg1Δ czf1Δ/czf1Δ* double mutant? Liquid germ tube formation assays were performed on CAI4, *efg1Δ/efg1Δ,* the *efg1Δ efg1Δ czf1Δ/czf1Δ* double mutant, and the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant ectopically expressing *CZF1* under the control of the *MAL2* promoter, and only cells that did not stain with methylene blue were counted. For the 30°C resting cell group, only CAI4 was able to produce germ tubes and respond to farnesol, in both mGPP and mSPP (Fig. 5A, 5C). For the 37°C resting cell group, the *efg1Δ/efg1Δ* and the *efg1Δ/ efg1Δ czf1Δ/czf1Δ* double mutant were capable of producing very low levels of germ tubes with a slight trend of reduction in the presence of farnesol.

In conditions which induce *CZF1* expression in the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant, an increase in germ tube formation was observed when compared to non-inducing conditions (Fig. 5B, 5D), and farnesol reduced germ tube formation when *CZF1* was expressed.

When CAI4 30°C resting cells were inoculated into the embedded farnesol response assays, results were similar to SC5314 and CKY101 (Fig. 6). CAI4 produced filamentous colonies in the absence of farnesol in both GPP and SPP embedded media and the presence of farnesol resulted in decreased filamentation. Note the smaller hyphae growing out from the CAI4 colonies treated with farnesol (Fig. 6); because farnesol delays filamentation rather than block it entirely, the time points selected for observation are critical. Ideally CAI4 embedded plates should have been checked at 12 h post-inoculation for farnesol response (as in Fig. 2) rather than at 17h as shown (no short filaments are observed in the farnesol-treated CAI4 samples at 12 h, data not shown), but the 17 h time point was selected in order to be consistent with the other slower-growing mutant strains being tested in Fig. 6. The *efg1Δ/efg1Δ* mutant was capable of forming filamentous colonies under embedded conditions, and a very subtle shortening of filaments was observed in farnesol-treated samples. An inoculum using 30°C resting cells for the *efg1Δ/efg1Δ* mutant is shown in Fig. 6, and similar results were obtained using an inoculum of 37°C resting *efg1Δ/efg1Δ* cells. The *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant was capable of producing filamentous colonies in the absence of farnesol, regardless of the temperature used during resting cell preparation (Fig. 6). The *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant and the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant ectopically expressing *CZF1* did not produce any colonies in the presence of farnesol

when 30°C resting cells were used (data not shown), suggesting these cells were killed by farnesol as they were in the liquid assays. When 37°C resting cells were used for the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant, farnesol reduced the number of colonies, but the colonies that grew were filamentous. Unexpectedly, the filamentous colonies from farnesol-treated plates often grew in a different conformation from untreated samples. The *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant expressing *CZF1* under the control of the *MAL2* promoter behaved similarly to the *efg1Δ/efg1Δ czf1Δ/ czf1Δ* double mutant in non-inducing conditions (Fig. 6A), and a subtle increase in colony size and filamentation was observed in inducing media (Fig. 6B). In the presence of farnesol, these filaments were slightly shorter. Taken together, both Czf1 and Efg1 are important for farnesol tolerance in embedded conditions as well, along with temperature. When *CZF1* is ectopically expressed in a strain lacking Czf1 and Efg1, the level of filamentation is partially restored in both liquid and embedded assays, and farnesol partially suppresses filamentation in both conditions.

Overproduction of farnesol is not a general quality of farnesol-resistant mutants

Two of the factors known to play a role in the *C. albicans* morphological farnesol response, Tup1 and Nrg1, both produced 17 and 19-fold higher levels of farnesol than wild type and parental strains (Kebaara *et al.*, 2008). 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. 7). 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 identified new factors involved in the *C. albicans* response to farnesol and have shown that Czf1 in particular plays a prominent role in the morphological response to farnesol in both liquid aerobic conditions and embedded conditions; this adds a new function for Czf1 during the quorum sensing response. Furthermore, *CZF1* mRNA levels are decreased in the presence of farnesol at critical time points during the farnesol response, and unlike another factor involved in farnesol signaling (Tup1), Czf1 does not appear to regulate farnesol production levels in the cell. Surprisingly, *CZF1*, along with *EFG1*, was also shown to play an important part in farnesol tolerance; these are the first specific genes identified that control the ability of *C. albicans* white a/α cells to survive in the presence of farnesol.

The Ras1-Cyr1-cAMP signaling pathway has been proposed to be the only direct target for farnesol in *C. albicans* (Davis-Hanna *et al.*, 2008; Deveau *et al.*, 2010). This study provides additional evidence supporting a primary role of this pathway in farnesol signaling. Here we showed that Czf1 is an important factor in the farnesol response. It has a tight regulatory relationship with Efg1, which is consistent with the Ras1-cAMP signaling pathway as a central target for farnesol. Further evidence for the significant role of cAMP signaling during the farnesol response is provided by the identification of one of the protein kinase A (PKA) isoforms in *C. albicans*, Tpk1, as farnesol resistant. The other PKA isoform, Tpk2, was not identified by the farnesol resistance screen as it was not present in this mutant library collection. A recent study (Deveau *et al.*, 2010) showed that an additional phenotype of the farnesol response in *C. albicans*, reactive oxygen species (ROS) protection, is also mediated by the Ras1-cAMP pathway. The

Hog1 signaling pathway was shown to participate in ROS protection as well, but through an unknown connection between the two pathways (Deveau *et al.*, 2010). Three additional genes were identified that are potentially important for farnesol resistance: *RLM1*, *YCK2*, and *HAP43*, also function in different stress responses. These mutants can possibly be used to fill in some of the gaps between these pathways.

It is intriguing that farnesol can protect the cell from ROS stress, while it can also kill opaque cells and log-phase, energy-deprived white cells. Since farnesol tolerance in *C. albicans* is potentially an active process (Langford *et al.*, 2010), it is of interest to understand the key components that are involved. This study provides a new understanding of farnesol tolerance, by identifying two factors that are required for survival in the presence of farnesol: Czf1 and Efg1. We speculate that it is no coincidence that both of these factors also play critical roles in white/opaque cell switching (Ramirez-Zavala *et al.*, 2008; Sonneborn *et al.*, 1999; Srikantha *et al.*, 2000; Vinces and Kumamoto, 2007; Zordan *et al.*, 2007) since opaque cells are inherently more sensitive to farnesol killing than are white cells (Dumitru *et al.*, 2007). Farnesol killed *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant cells in liquid aerobic conditions, and it appeared to do the same in embedded media due to the lack of growth when using 30°C resting cell inocula. It is unclear why single *efg1Δ/efg1Δ* and *czf1Δ/czf1Δ* mutants are resistant to farnesol killing, while the double mutant is overly sensitive. Czf1 and Efg1 have a synthetic effect on farnesol resistance, as farnesol tolerance was only observed in the single mutants and not the double mutant. The mere presence of *CZF1* in the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant (via expression of *CZF1* by the *MAL2* promoter) was not sufficient, however, to provide protection from farnesol induced cell death,

suggesting *CZF1* may act upstream of *EFG1* in this example. It is also possible that the presence of Czf1's native promoter is required for proper regulation in response to this particular stimulus when Efg1 is absent. Another intriguing question raised by this study is the role of temperature in farnesol tolerance. Why was the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant more tolerant of farnesol when grown at 37°C? The answer may be tied to indirect farnesol production measurements suggesting that *C. albicans* produces slightly higher levels of farnesol at higher temperatures (Hornby *et al.*, 2001). Further, there is likely some additional factor(s) that plays a minor role in farnesol tolerance and is regulated by shifts in temperature. Wor1/2 are potential candidates for such a role, in part due to their regulation of white/opaque switching (Huang *et al.*, 2006; Zordan *et al.*, 2006; Zordan *et al.*, 2007).

While Czf1 was shown to be part of a farnesol-resistance mechanism in *C. albicans*, it also strongly contributes to the morphological response to farnesol. In cells lacking Czf1 (*czf1Δ/czf1Δ* mutants*)*, farnesol was unable to suppress filamentation, and ectopic complementation of *CZF1* in these cells restored the ability of farnesol to block germ tube formation in both liquid and embedded environmental conditions. Furthermore, in *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutants, *CZF1* ectopic expression was able to partially restore filamentation and the morphological response to farnesol. These results are consistent with Czf1 functioning downstream of Efg1 during the morphological farnesol response. Through the course of testing farnesol response in conditions which either turn on or off the *MAL2* promoter, we have shown that the use of glucose versus sucrose as a carbon source had no distinguishable effect on the ability of the cells to respond to farnesol.

The many effects of farnesol on *C. albicans* appear to be relatively unique to the fungus, compared with other bacterial species and fungal genera [reviewed by Langford et al (Langford *et al.*, 2009)]. The finding that Czf1 plays a central role in the unique morphological and tolerance responses in *C. albicans* is consistent with the observation that *Saccharomyces cerevisiae* (which does not respond to farnesol) and other closely related ascomycetes lack a Czf1 homolog (Vinces *et al.*, 2006) while other *Candida* species contain *CZF1* homologs. As an example, the *CZF1* gene in *Candida dubliniensis*, a *Candida* species that produces the second highest farnesol levels, is farnesol tolerant, and morphologically responds to farnesol (Henriques *et al.*, 2007; Weber *et al.*, 2008) has the highest homology to the *C. albicans CZF1* gene with 81% identity at the nucleotide level (data not shown). Furthermore, the unusually large 5' upstream region of *CZF1*, the *CZF1* gene itself, and neighboring genes at the *CZF1* locus (Vinces *et al.*, 2006) appear to be conserved to varying degrees in additional *Candida* species whose sequence information has been recently made available including: *Candida tropicalis*, *Lodderomyces elongisporius*, *Candida lusitaniae*, and *Candida guillermondii* (data not shown).

In conclusion, we have identified new roles for Czf1 in mediating the *C. albicans* tolerance to 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, others links remain unclear. For example, cross-regulation among many of the farnesol response pathways was recently summarized (Deveau *et al.*, 2010), 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 Ras1-Cyr1-cAMP signaling such as Ras2, Cap1, and G-actin (Hall and Muhlschlegel, 2009; Zhu *et al.*, 2009; Zou *et al.*, 2009) have not been tested for possible roles in farnesol signaling and may yet prove to be involved. This points to the fact that farnesol can be an extremely useful tool for studying signal transduction in *C. albicans* by demonstrating the presence of new connections between signaling pathways.

Acknowledgements

We are grateful to Aaron Mitchell, Dominique Sanglard, Carol Kumamoto, Alexander Johnson, and Gerald Fink for kindly providing the strains and plasmids used in this study. We would also like to thank Steve Harris and Gabriel Langford for use of their microscopes and cameras. This work was supported by a faculty seed grant from the Constance Miriam and Ethel Corrine Syford Memorial Fund (to A.L.A.), the Hammond-Maude Fling Fellowship (to M.L.L.), and the Farnesol and *Candida albicans* Research Fund, University of Nebraska Foundation (to K.W.N.).

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Figure Legends

Figure 4-1. Czf1 is required for the morphological response to farnesol in liquid conditions. *C. albicans* resting cells were inoculated into glass flasks at 10^6 cells/mL in either mGPP (**A**) or mSPP (**B**) broth, and 0, 50, or 100 μM farnesol was added. Cultures were incubated for 1 h at 37°C with shaking at 225 rpm and percentage of germ tube formation was subsequently determined (*czf1Δ/ czf1Δ* = CKY230, *czf1Δ/CZF1*= CKY116, *czf1Δ/ czf1Δ pMAL2-CZF1* = CKY231). Data shown are from independent experiments performed in triplicate; these were repeated with similar results on at least two separate occasions.

Figure 4-2. Czf1 is required for the morphological response to farnesol in embedded media. *C. albicans* resting cells were mixed with either GPP (**A**) or SPP (**B**) molten agar and 0 or 50 μM farnesol as described in experimental procedures, and incubated at 37°C for 12 h. Independent experiments were repeated in duplicate with similar results.

Figure 4-3. *CZF1* mRNA expression is decreased in the presence of farnesol. SC5314 resting cells were inoculated into mGPP broth for 40, 60, or 80 minutes (**A-C**, respectively) with 0, 50, or 100 μM farnesol and subsequently harvested for RNA extraction. Quantitative real-time PCR was used to measure relative *CZF1* mRNA levels with *ACT1* used as a reference gene. *CZF1* levels in the minus farnesol samples were set at zero and fold change in *CZF1* levels for samples with farnesol added are shown. Data are averages from three replicates.

Figure 4-4. Czf1, Efg1, and temperature play a role in farnesol tolerance. The *czf1Δ/ czf1Δ* efg*1Δ/ efg1Δ* double mutant was grown in YPD broth at 30°C (**A**) or 37°C (**B**) for 24 h and differences cell morphology was observed. Scale bar = 10 μm. Cells were

grown at either 30°C or 37°C in YPD broth until stationary phase was reached (24 h, except 48 h for CAI4 and efg*1Δ/ efg1Δ* strains) to prepare resting cells as described in experimental procedures. 10^6 cells/mL of these resting cells were inoculated into mGPP (**C,D**) or mSPP (**E,F**) broth and 0, 50, or 100 μM farnesol was added. Cultures were incubated at 37°C with shaking at 225 rpm, and cell death by methylene blue staining was determined after 90 min (*efg1Δ/ efg1Δ* = HLC67; *czf1Δ/czf1Δ* efg*1Δ/efg1Δ* = CKY283; *czf1Δ/czf1Δ* efg*1Δ/efg1Δ pMAL2-CZF1* = AAC6). Data shown are from independent experiments performed in triplicate; these were repeated with similar results on at least two separate occasions.

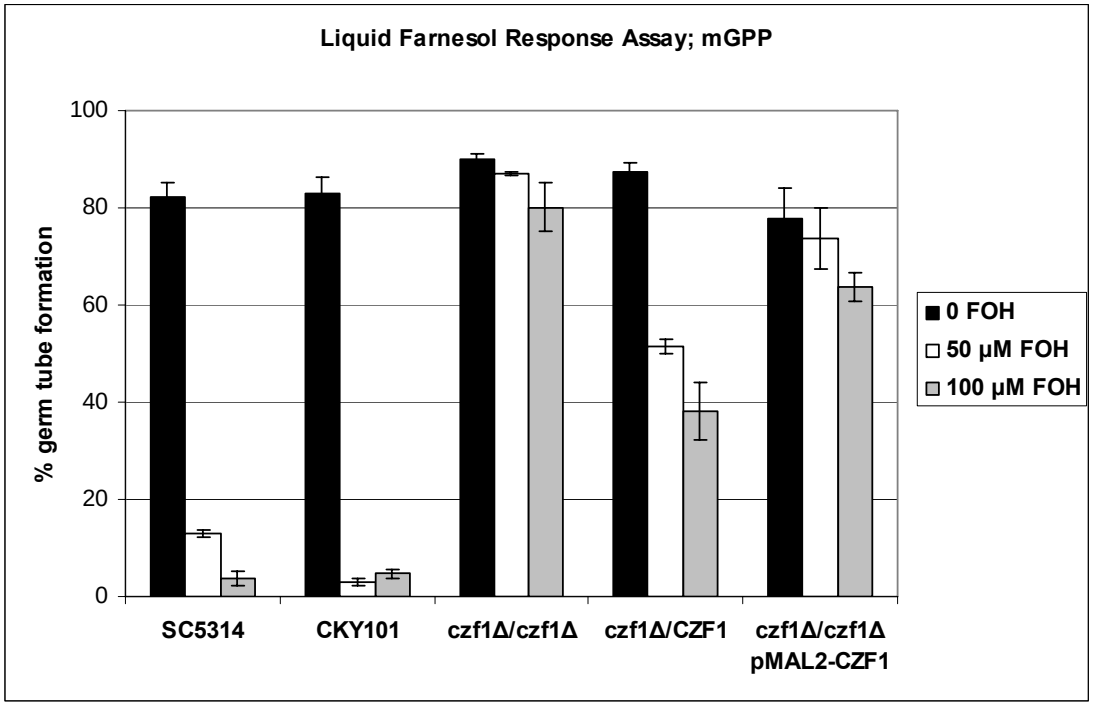
Figure 4-5. Ectopic expression of *CZF1* in a *czf1Δ/czf1Δ* efg*1Δ/efg1Δ* double mutant partially restores filamentation and farnesol response in liquid conditions. Cells were grown at either 30°C or 37°C until stationary phase was reached to prepare resting cells. 10^6 cells/mL of these resting cells 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 percentage of germ tube formation was subsequently determined. Data shown are from independent experiments performed in triplicate; these were repeated with similar results on at least two separate occasions.

Figure 4-6. Czf1 and Efg1 are both required for a wild type morphological response and tolerance to farnesol in embedded agar. *C. albicans* resting cells (prepared at 30°C or 37°C) were mixed with either GPP (**A**) or SPP (**B**) molten agar and 0 or 50 μM farnesol as described in experimental procedures, and incubated at 37°C for 17 h. Colonies in figure were from 30°C resting cells unless otherwise noted. Independent experiments were repeated in duplicate with similar results.

Figure 4-7. A *czf1Δ/czf1Δ* mutant produces farnesol levels similar to that of wild type and parental strains. Cells were grown in GPP broth at 30°C for 48 hours prior to farnesol extraction and quantification, as described in methods.

Figure 4-1.

A.


Liquid Farnesol Response Assay; mGPP
% germ tube formation
100
80
60
40
20
0
SC5314
CKY101
czf1Δ/czf1Δ
czf1Δ/CZF1
czf1Δ/czf1Δ pMAL2-CZF1
0 FOH
50 μM FOH
100 μM FOH

B.

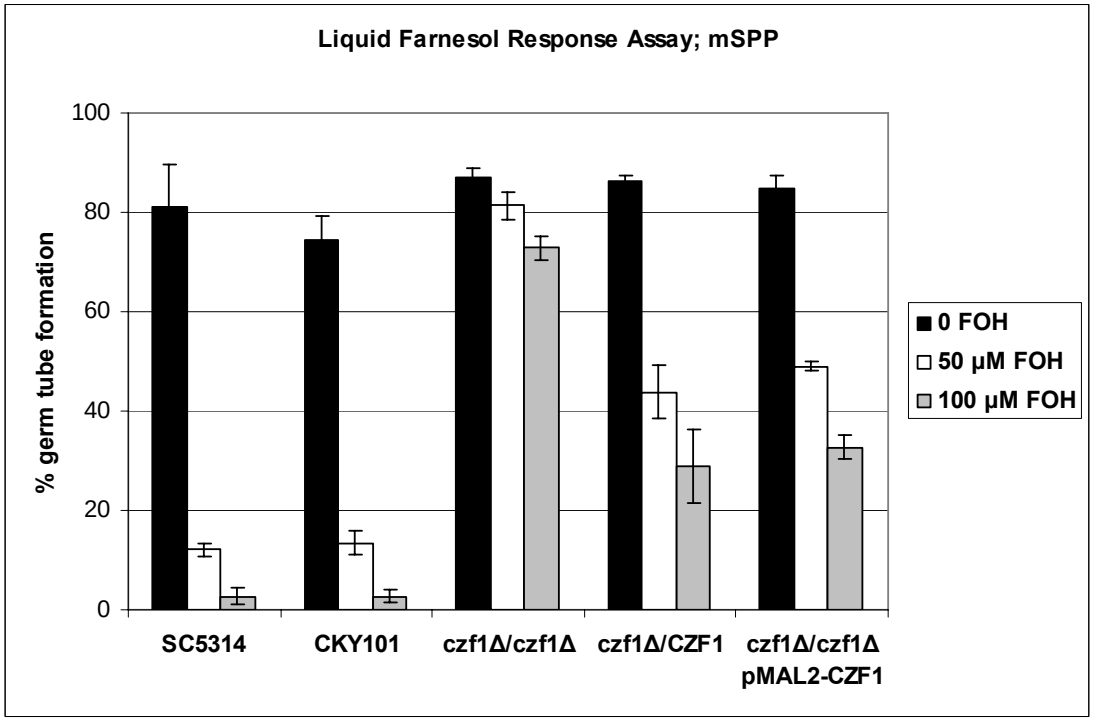

Liquid Farnesol Response Assay; mSPP
% germ tube formation
100
80
60
40
20
0
SC5314
CKY101
czf1Δ/czf1Δ
czf1Δ/CZF1
czf1Δ/czf1Δ pMAL2-CZF1
0 FOH
50 μM FOH
100 μM FOH

Figure 4-2

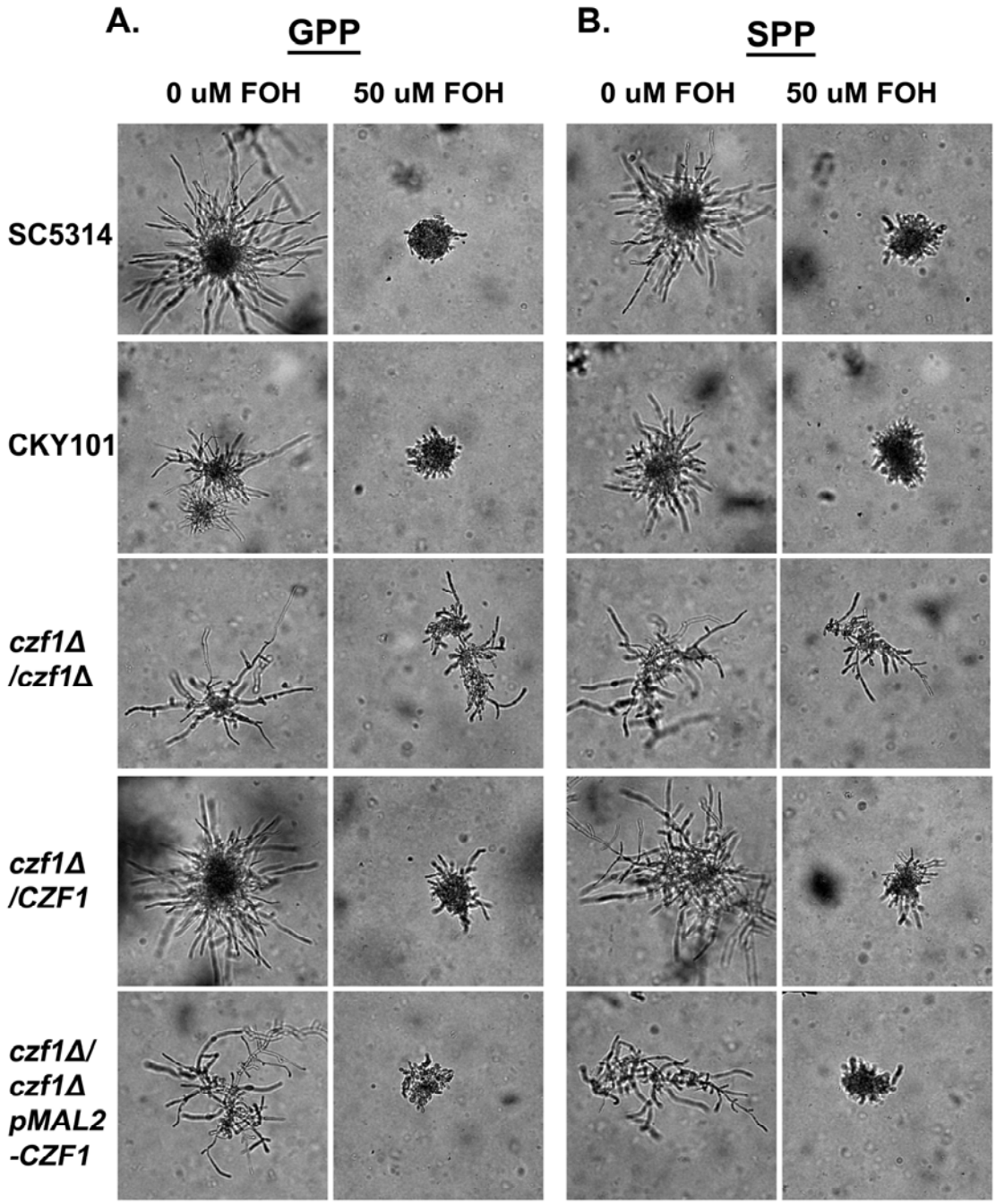
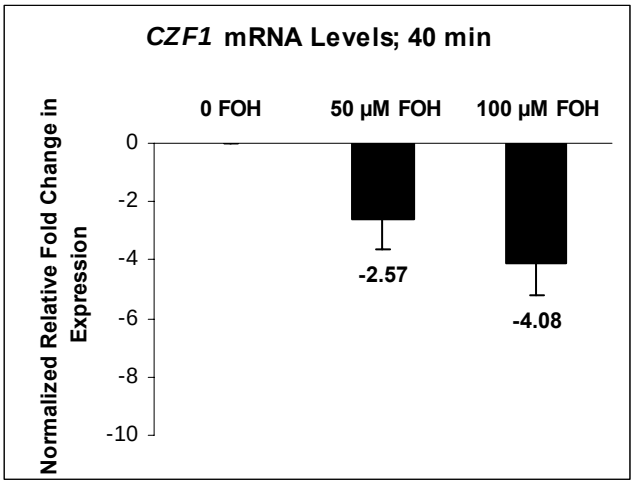
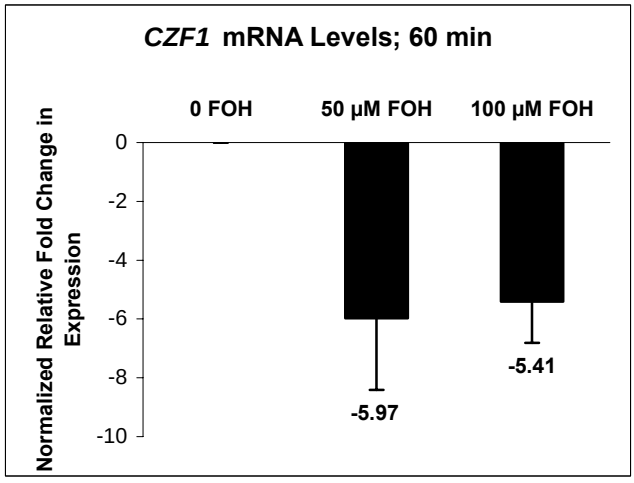

A.
GPP
B.
SPP
0 uM FOH
50 uM FOH
0 uM FOH
50 uM FOH
SC5314
CKY101
czf1Δ /czf1Δ
czf1Δ /CZF1
czf1Δ/ czf1Δ pMAL2 -CZF1

Figure 4-3

A.



B.



C.

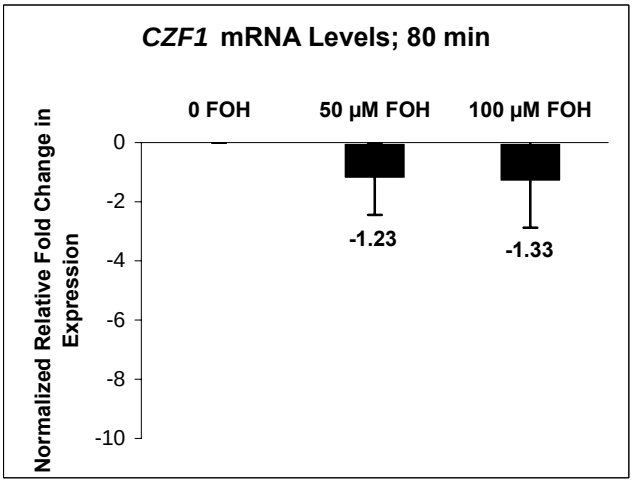
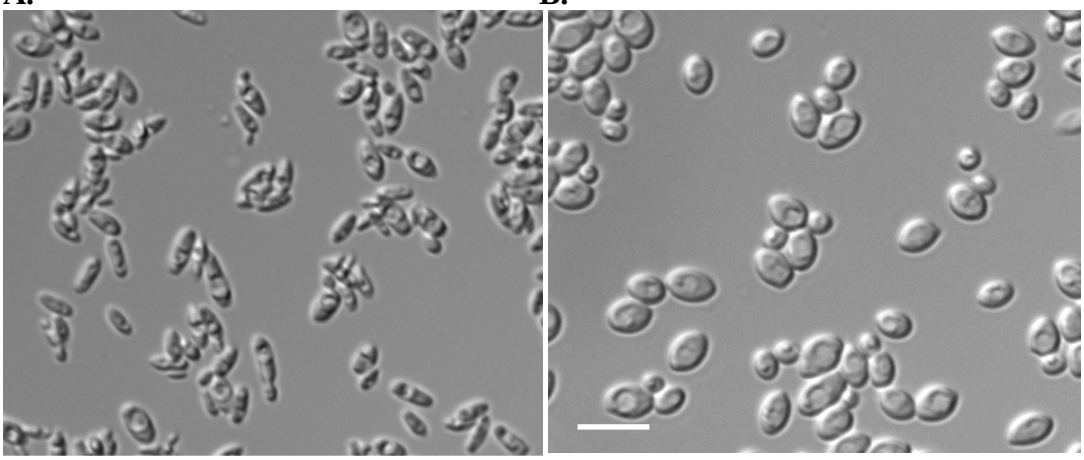
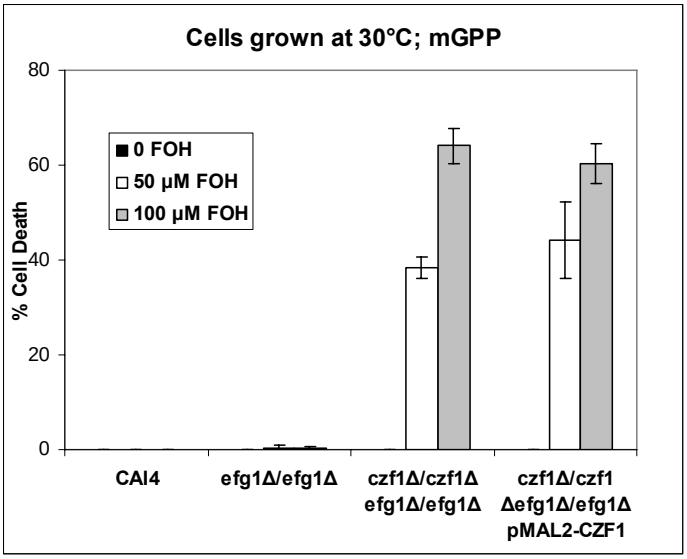


Figure 4-4

A. **B.**



C.



D.

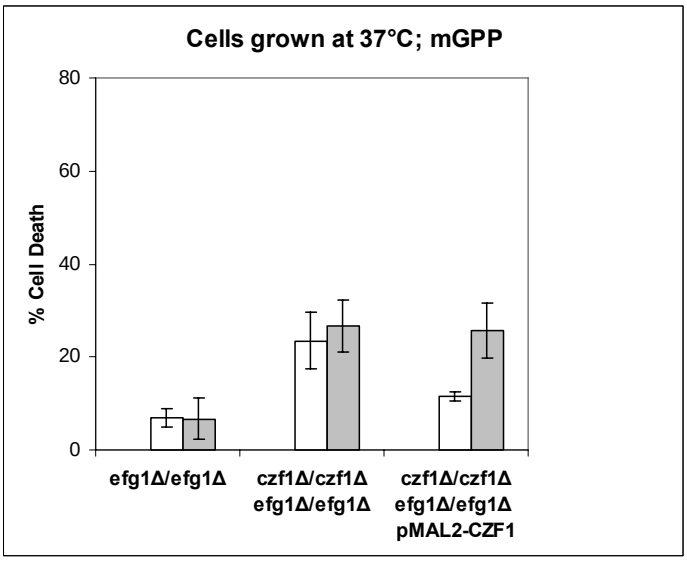
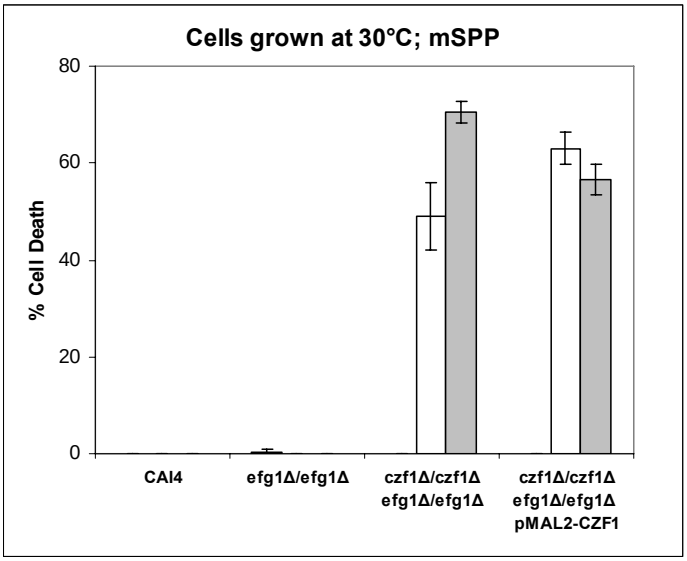


Figure 4-4

E.


Cells grown at 30°C; mSPP
% Cell Death
80
60
40
20
0
CAI4
efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1

F.

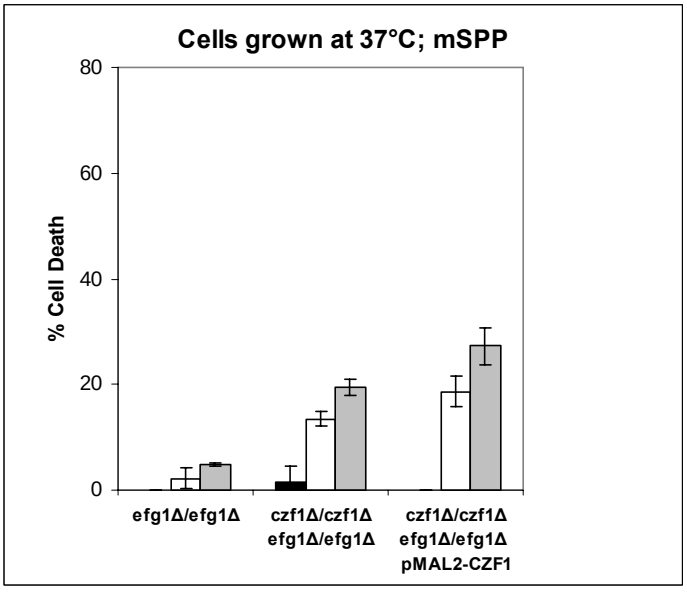

Cells grown at 37°C; mSPP
% Cell Death
80
60
40
20
0
efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1

Figure 4-5

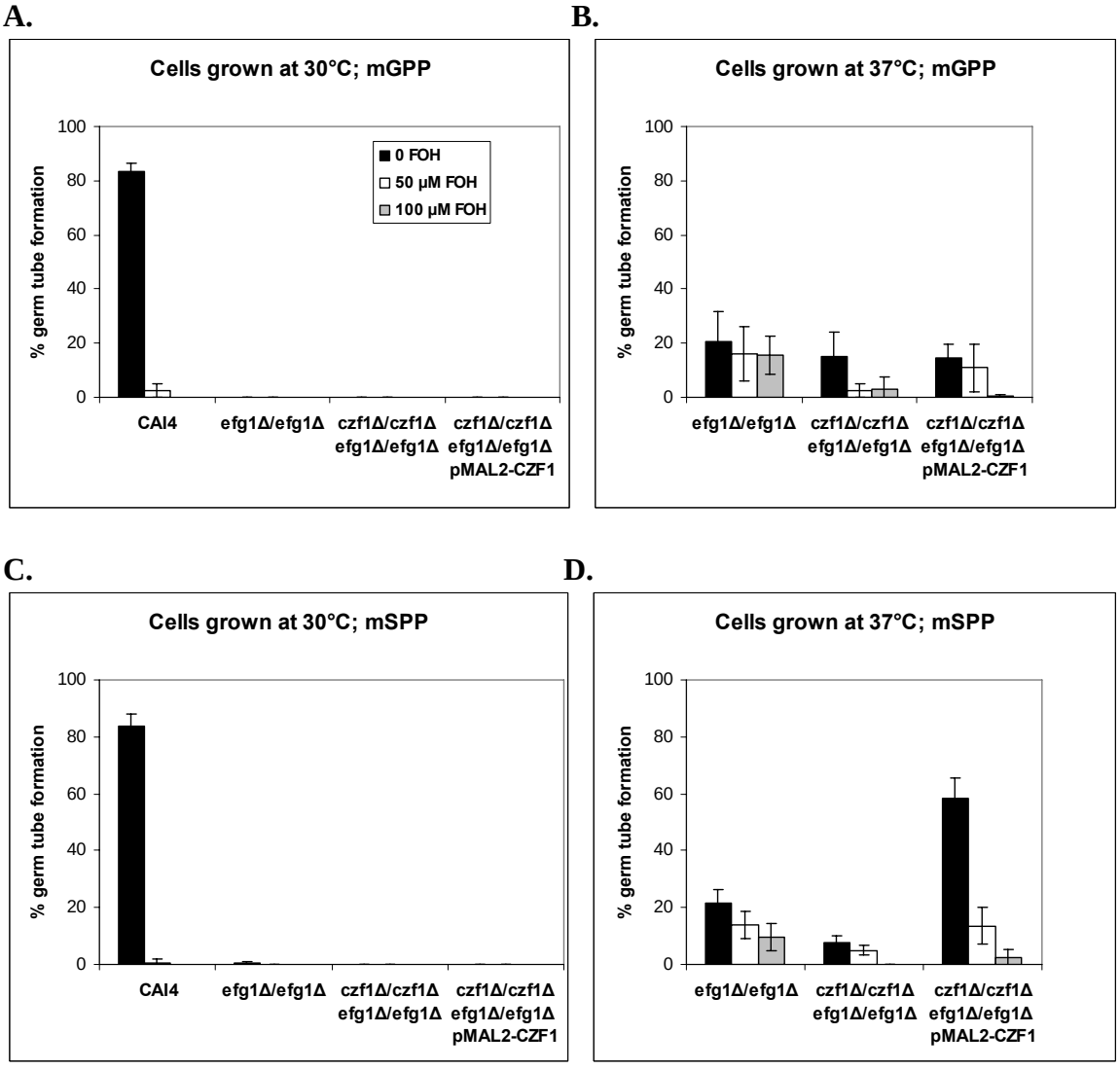

A.
Cells grown at 30°C; mGPP
% germ tube formation
0 FOH
50 µM FOH
100 µM FOH
CAI4
efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1
B.
Cells grown at 37°C; mGPP
% germ tube formation
efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1
C.
Cells grown at 30°C; mSPP
% germ tube formation
CAI4
efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1
D.
Cells grown at 37°C; mSPP
% germ tube formation
efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ
czf1Δ/czf1Δ efg1Δ/efg1Δ pMAL2-CZF1

Figure 4-6

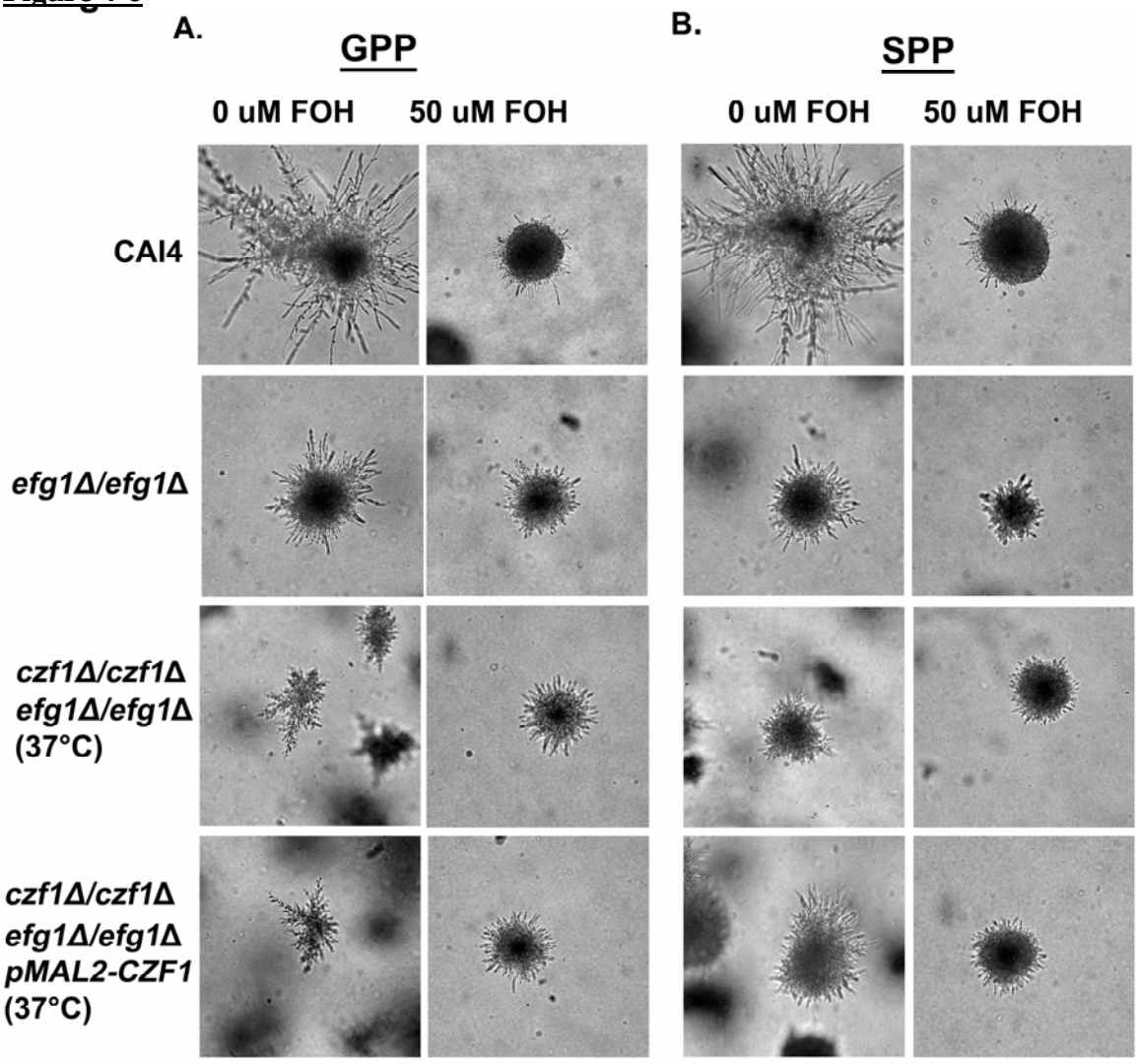

A.
GPP
0 uM FOH
50 uM FOH
B.
SPP
0 uM FOH
50 uM FOH
CAI4
efg1Δ/efg1Δ
czf1Δ/czf1Δ
efg1Δ/efg1Δ
(37°C)
czf1Δ/czf1Δ
efg1Δ/efg1Δ
pMAL2-CZF1
(37°C)

Figure 4-7

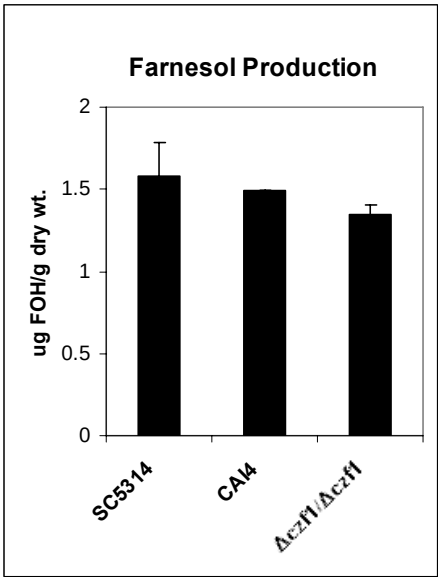

Farnesol Production
ug FOH/g dry wt.
2
1.5
1
0.5
0
SC5314
CAI4
Δczf1/Δczf1

Table 4-1. Summary of insertion mutants with an impaired farnesol response.

Mutant	*S. cerevisiae* ortholog	Predicted or known biological processes
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 (Brown *et al.*, 1999; Hornby *et al.*, 2001; Ramirez-Zavala *et al.*, 2008; Vinces *et al.*, 2006; Vinces and Kumamoto, 2007; Zordan *et al.*, 2007)
tpk1/tpk1	Tpk2	Catalytic subunit of cAMP-dependent protein kinase A, regulator of morphogenesis; Tpk2 isoform; involved in multiple stress responses (Bockmuhl *et al.*, 2001; Cloutier *et al.*, 2003; Giacometti *et al.*, 2009; Maidan *et al.*, 2005)
rlm1/rlm1	Rlm1	Transcription factor for genes involved in cell wall organization and biogenesis and various stress responses (Bruno *et al.*, 2006; Sampaio *et al.*, 2009)
stp2/stp2	Stp2	Transcription factor for amino acid permease genes (Martinez and Ljungdahl, 2005)
hof1/hof1	Hof1	Role in cytokinesis (Li *et al.*, 2006)
yck2/yck2	Yck2	Maintenance of cell polarity, antimicrobial peptide resistance, contributes to epithelial cell damage (Park *et al.*, 2009)
zcf14/zcf14	Hap1	Putative transcription factor
hap43/hap43	Yap3	Transcription factor, involved in iron limitation response (Baek *et al.*, 2008)

Table 4-2. *Candida albicans* strains and plasmids used in this study.

	Parental strain	**Genotype/Description**	**Source**
***C. albicans* strains**			
SC5314	Clinical isolate	Clinical isolate	Alexander Johnson (Gillum *et al.*, 1984)
CAF2	CAI-4	Ura+ derivative of CAI-4	Alexander Johnson (Leberer *et al.*, 2001)
CAI-4	CAF2	*ura3::imm434/ura3::imm434*	Alexander Johnson (Fonzi and Irwin, 1993)
BWP17	RM1000	*ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG*	Aaron Mitchell (Nobile and Mitchell, 2005)
HLC67 (*efg1Δ/ efg1Δ*)	CAI-4	*ura3::imm434/ura3::imm434 efg1::hisG/efg1::hisG*	Gerald Fink (Lo *et al.*, 1997)
CKY101	CAI-4	*ura3::imm434/ura3::imm434 ade2::pDB152*	Carol Kumamoto (Brown *et al.*, 1999)
CKY230 (*czf1Δ/ czf1Δ*)	CAI-4	*ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG ade2::pMAL2-URA3*	Carol Kumamoto (Vinces *et al.*, 2006)
CKY116 (*czf1Δ/CZF1*)	CAI-4	*ura3::imm434/ura3::imm434 CZF1/czf1::hisG -URA3-hisG*	Carol Kumamoto
CKY231 (*czf1Δ/ czf1Δ pMAL2-CZF1*)	CAI-4	*ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG ade2::pMAL2-CZF1-URA3*	Carol Kumamoto (Brown *et al.*, 1999)
CKY283 (*czf1Δ/ czf1Δ* efg*1Δ/ efg1Δ)*	CAI-4	*ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG-URA3-hisG efg1::hisG/efg1::hisG*	Carol Kumamoto (Giusani *et al.*, 2002)
AAC2	CKY283	*ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG efg1::hisG/efg1::hisG*	This study
AAC6 (*czf1Δ/ czf1Δ* efg*1Δ/ efg1Δ pMAL2-CZF1)*	AAC2	*ura3::imm434/ura3::imm434 czf1::hisG/czf1::hisG efg1::hisG/efg1::hisG ade2::pMAL2-CZF1-URA3*	This study
Plasmids			
pDB212		p_{MAL2}-*CZF1 URA3 ade2'* Amp^r	Carol Kumamoto (Brown *et al.*, 1999)

Supplementary Table 4-1. List of mutants screened in this study.

Mutant ORF#
orf19.1795
orf19.7291
orf19.6760
orf19.4669
orf19.5037
orf19.11450
orf19.9364
orf19.6970
orf19.10248
orf19.9791
orf19.6952
orf19.9364
orf19.4966
orf19.7479
orf19.7412
orf19.5328
orf19.1857
orf19.8907
orf19.14178
orf19.4746
orf19.5235
orf19.3995
orf19.8635
orf19.3171
orf19.2723
orf19.768
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CHAPTER 5

Summary and Future Directions

With a brief excerpt from:

Langford, M.L., Atkin, A.L., and Nickerson, K.W. (2009) Cellular Interactions of Farnesol, a Quorum Sensing Molecule Produced by *Candida albicans*. *Future Microbiol.* **4:** 1353-62.

Summary

Farnesol has effects on all eukaryotic cells tested, and for many cell types, a low level of farnesol is sufficient to inhibit growth or induce cell death. In contrast, *C. albicans* has evolved mechanisms to produce and tolerate higher levels of farnesol and use it as a quorum sensing molecule. A current challenge is to develop a unifying model for the mode of action for farnesol in eukaryotes and elucidate the mechanisms used by *C. albicans* to evade the adverse effects of farnesol and respond to farnesol as a signaling molecule. In this study, we established environmental conditions in which *C. albicans* is able to resist farnesol-mediated cell death: stationary phase cells provided with an energy source. Conversely, we have defined conditions that stimulate sensitivity to farnesol in *C. albicans*: energy-deprived, log phase cells (Chapter 2). Using the aforementioned environmental conditions that allow *C. albicans* to tolerate farnesol and use it as a signaling molecule, two novel factors were identified that are involved in the morphological response to farnesol: Tup1 and Czf1 (Chapters 3, 4). It seems that farnesol suppression of filamentation during *C. albicans* quorum sensing works through both positive and negative regulators of filamentation (Czf1 and Tup1, respectively) to achieve the resulting change in morphology. Czf1 also played a dual role in the farnesol response, as it was partially responsible for farnesol tolerance in quorum sensing conditions.

Farnesol Tolerance and Sensitivity in *C. albicans*

The precise mechanism for *C. albicans* white cell farnesol tolerance during quorum sensing remains unclear. Since the effects of farnesol on other organisms have

been studied in seventeen different species (summarized in Chapter 1), this is an important mechanism to understand in the field of interspecies communication. Furthermore, as exogenous farnesol has many effects in the mammalian host during infection such as preventing infection of mucosal surfaces, increasing virulence in a disseminated infection and interference of the host cytokine response [Chapter 1 and (Navarathna *et al.*, 2007)], understanding the farnesol tolerance mechanism in *C. albicans* may prove useful therapeutically.

It is unknown how *C. albicans* white cells are resistant to the toxic effects of farnesol. Do the cells have a type of passive resistance, such as changes in membrane composition, or do they undergo active resistance, such as conversion of farnesol into a non-toxic compound? In order to look for genes that may be involved in farnesol tolerance, a screening approach was used (Chapter 4). However, the screen was unsuccessful in identifying mutants that were growth-inhibited (sensitive) in the presence of farnesol, even at levels as low as 10 μM. Even so, the mutant library collection used in this study is by no means a complete representation of the entire collection of *C. albicans* predicted open reading frames (ORFs); there are 6197 predicted ORFs in the genome, compared with the 560 mutants contained within the mutant library collection used, leaving plenty of room for future identification of farnesol tolerance factors as more mutants become available.

The identification of Czf1 and Efg1 as important players in farnesol survival is intriguing because a farnesol-sensitive phenotype was observed only when both genes were deleted. Ectopic expression of *CZF1* in the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant could not provide complete protection from farnesol killing yet the single *efg1Δ/efg1Δ*

and *czf1Δ/czf1Δ* mutants were unharmed (Chapter 4). Perhaps Czf1 functions upstream of Efg1 in this situation, or the normal regulation of *CZF1* might be especially important in the absence of *EFG1*. In the reverse situation, could ectopic expression of *EFG1* in the double mutant restore partial or full farnesol resistance? Czf1 and Efg1 likely regulate a redundant gene or set of genes for farnesol tolerance because the single mutants do not show a farnesol sensitive phenotype while the double mutant exhibits a synthetic farnesol sensitivity phenotype. While changes in gene expression have been extensively studied in the *efg1Δ/efg1Δ* mutant compared to wild type/parental strains (Braun and Johnson, 2000; Harcus *et al.*, 2004; Sohn *et al.*, 2003), there is no matching data for Czf1 available. Microarray analyses designed to complement existing data on Efg1 may reveal genes that are commonly regulated by Czf1 and Efg1, either directly or indirectly. Genes whose expression was altered in cells lacking Czf1 and Efg1 could also be compared to existing microarray data comparing gene expression between white and opaque cells (Lan *et al.*, 2002; Tsong *et al.*, 2003; Zhao *et al.*, 2005). Genes that are differentially expressed in opaque cells, *efg1Δ/efg1Δ*, and *czf1Δ/czf1Δ* mutants would comprise a list of strong candidates for potential farnesol resistance factors. One further step that may be taken would be to look at other farnesol-sensitive organisms to see if homologs to the candidate farnesol resistance genes are present in those species.

The role of temperature in farnesol tolerance (the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant was more sensitive at 30°C than at 37°C) may be correlated to evidence suggestive of slightly elevated farnesol production levels at higher temperatures (Hornby *et al.*, 2001). It would be informative to examine changes in gene expression and to measure farnesol production in the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant at both 30°C

and 37°C, compared to wild type and parental strains. Differentially regulated genes may be partly responsible for the cellular morphology and farnesol resistance changes observed at these two temperatures. One would also predict that farnesol production may be significantly lowered at 30°C based on the degree of farnesol sensitivity for the double mutant cells grown at this temperature. Analysis of the *efg1Δ/efg1Δ* single mutant would need to be included in this study; initial results using this mutant resulted in high variation between replicates (M.L. Langford, data not shown) and further replicates will need to be conducted to determine whether Efg1 plays a role in farnesol production. Furthermore, a possible role for Tup1 in farnesol resistance should not be ignored. As *tup1Δ/tup1Δ* mutants produced 17-fold more farnesol compared to wild type and parental strains, these cells may have heightened abilities to tolerate farnesol. However, it will be difficult to study higher tolerance to farnesol in these mutant cells since they are locked in the filamentous morphology (unless secondary mutations are introduced), unlike the *efg1Δ/efg1Δ* and *czf1Δ/czf1Δ* mutants. Preliminary experiments must first be conducted to better understand the *tup1Δ/tup1Δ* farnesol resistance, such as a comparison of farnesol tolerance between yeast, pseudohyphal, and hyphal cells. If the loss of Tup1 results in higher farnesol tolerance, would a triple *efg1Δ/czf1Δ/tup1Δ* mutant have restored resistance? What would the farnesol production levels in that mutant be? It is intriguing that all three of these factors play a prominent role in white-opaque switching, farnesol production and/or farnesol tolerance, as well as the morphological response to farnesol.

Signaling During the Morphological Response to Farnesol in *C. albicans*

Because the presence of a single farnesol receptor has still not been identified, it remains a possibility that there may be multiple targets for farnesol in the cell. Two models (network and central processor) have been proposed with regard to *C. albicans* signaling in general (Braun and Johnson, 2000) and are worth considering when examining the farnesol response. That study suggested *C. albicans* follows the network signaling model, whereby many individual connections are formed between the different regulatory pathways in the cell. This conclusion was reached when they observed that several hyphal-specific genes are not regulated in unison in different hyphal inducing conditions but in fact respond as individuals. If network signaling is indeed occurring during the farnesol response, farnesol could help identify many of the different branches in these pathways.

The Ras1-cAMP pathway has been identified as a critical, even a possible direct target for farnesol inhibition during yeast to filament inhibition by farnesol (Davis-Hanna *et al.*, 2008; Deveau *et al.*, 2010). Although a recent study (Davis-Hanna *et al.*, 2008) showed the inability of the *ras1Δ/ras1Δ* mutant to respond to farnesol in aerobic conditions, it remains undetermined if it is capable of responding to farnesol under different environmental conditions, specifically during embedded growth. The *ras1Δ/ras1Δ* mutant is defective in filamentation under aerobic conditions and embedded in YPS (yeast extract, peptone, sucrose) agar (Maidan *et al.*, 2005), but it has not been tested in our GPP embedded media. If filamentation can be achieved in our embedded assay, the morphological response to farnesol can be tested; this would both complement

the existing aerobic data and determine the importance of Ras1 to the farnesol response in other environmental conditions.

A recently characterized protein, Ras2 (Zhu *et al.*, 2009), plays an antagonistic role with Ras1 regarding stationary phase entry, stress resistance, and cellular cAMP levels. Although Ras2 shares poor sequence homology with Ras1 and phylogenetic analyses have suggested it belongs to a divergent group of fungal Ras proteins (Ras2 has a homolog with high sequence similarity to *C. dubliniensis*), it still has functional GTPase activity (Zhu *et al.*, 2009). More importantly, since Ras2 has an antagonistic effect on many of the physiological roles of Ras1, it may somehow be involved in farnesol signaling as well. The *ras2Δ/ras2Δ* mutant maintains the ability to make filaments in aerobic and embedded conditions, making it an ideal candidate for testing the morphological response to farnesol. Might Ras2 play an antagonistic response to Ras1 during farnesol signaling as well?

While one new factor involved in the morphological response to farnesol, Czf1, has a known connection to the Ras1-cAMP pathway in *C. albicans*, it remains unknown how another factor involved, Tup1, fits into this model (Chapters 3, 4). Further studies using farnesol as a tool may decipher whether Tup1 is somehow connected to the cAMP pathway or is independently involved in the farnesol response. Construction of a triple *efg1Δ/czf1Δ/tup1Δ* mutant may prove useful, not only to study farnesol tolerance as mentioned earlier, but to study farnesol signaling as well. If this mutant is viable, what would its morphology be in different growth conditions? Would this mutant be capable of responding to farnesol? Vectors that can be used to delete *EFG1*, *CZF1*, and *TUP1* have already been constructed, as well as ectopic expression constructs for all three genes

(Bockmuhl and Ernst, 2001; Braun and Johnson, 1997; Brown *et al.*, 1999; Lo *et al.*, 1997), a couple of which have already been obtained by the Atkin laboratory. Therefore, all the necessary tools are available to create not only a triple mutant, but the remaining double mutant combinations (i.e. *tup1Δ/czf1Δ, efg1Δ/tup1Δ*) and ectopically express each gene in the desired mutant strains. Construction and analysis of these strains can provide valuable insight into *C. albicans* morphological signaling networks. For example, if *TUP1* was ectopically expressed in the *efg1Δ/efg1Δ czf1Δ/czf1Δ* double mutant background and no differences in farnesol response were observed, Tup1 would most likely be acting upstream of these two factors.

Other ways to test for a possible connection between Tup1 and the Ras1-cAMP pathway would be to determine whether Czf1 and/or Efg1 are responsible for the increase in *TUP1* mRNA levels and/or Tup1 protein levels observed during the morphological response to farnesol. For example, are the *TUP1* mRNA levels in a *czf1Δ/czf1Δ* mutant background still increased during farnesol treatment? If the mRNA levels do not increase in response to farnesol, it would suggest that Czf1 is either directly or indirectly responsible for the regulation of Tup1 during the farnesol response, and this information could potentially provide the first data regarding the regulation of Tup1 since regulatory factors upstream of Tup1 remain unknown.

Regardless of whether Czf1 would specifically regulate *TUP1* expression, follow-up experiments designed to better understand which genes are controlled by Czf1 are desirable for the *C. albicans* research field. While it has been shown to bind its own promoter and negatively regulate its expression, it is unknown what other genes are directly regulated by Czf1. Moreover, because it controls so many processes in *C.*

albicans: morphogenesis, white-opaque switching, farnesol tolerance, and the morphological response to farnesol, it is of considerable interest to identify genes that are directly regulated by this unique transcription factor, both without and in the presence of farnesol. ChIP (chromatin immunoprecipitation)-Seq, would be an ideal procedure to determine direct targets of Czf1. Prior to this experiment, however, Czf1 antibodies would ideally be raised (all protein work concerning Czf1 has utilized an HA-tagged Czf1 protein, which would have the potential to slightly alter the natural function of the protein). The ChIP-Seq method would first cross-link DNA (chromatin)-protein complexes and shear the DNA into smaller pieces. An antibody against Czf1 can be used to immunoprecipitate DNA bound with Czf1, and deep sequencing could subsequently be used to sequence the precipitated DNA. Sequence analyses would finally provide the location of the Czf1 binding sites in the *C. albicans* genome.

In addition to *CZF1*, the mutant screen from Chapter 4 identified 7 other genes that may play a role in the farnesol response. Of note, identification of *STP2* was a bit of a surprise, as it is the only gene identified with no direct ties to morphogenesis or stress responses. Either the acquisition of amino acids plays a more important role during farnesol signaling than is currently appreciated, or Stp2 may participate in farnesol signaling in an undiscovered manner. The *RLM1* and *YCK2* genes are both attractive candidates for further analysis, particularly when examining the farnesol survival mechanism, due to their known roles in stress resistance.

Final Thoughts

One common theme that can be discerned from studying the farnesol response in *C. albicans* is that farnesol affects a variety of regulatory factors within the polymorphic fungus. This unique feature has allowed us to study the interrelatedness of some of the signaling networks in an effort to better understand the genetic pathways present in the opportunistic pathogen. Farnesol can be used as an instrument to dissect the complicated and intertwined pathways present in *C. albicans*, including pathways yet to be fully understood, such as the regulation of Tup1, Hog1 signaling, and stress response signaling. Although farnesol was initially discovered as a quorum sensing molecule that only regulates morphogenesis, the discovery of its many other functions will hopefully allow for a more complete understanding of *C. albicans* signaling as a whole.

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