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Translational Effect of Farnesol on TUP1, a Key Regulator of Morphological Differentiation in the Human Fungal Pathogen, *Candida Albicans*

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Abstract

Candida albicans is an opportunistic polymorphic yeast that can cause life threatening systemic infections in immunocompromised individuals. It resides as commensal organism on the skin, in the gastrointestinal tracts and in the genitourinary tracts of mammalian hosts. One key attribute of *C. albicans* that enhances its pathogenicity is its ability to switch morphologies between unicellular yeast cells and filamentous forms, in response to diverse stimuli. *C. albicans* produces farnesol as an extracellular autoregulatory compound and when farnesol accumulates above a threshold level, it inhibits yeast-to-hyphal switch as well as biofilm formation. It was shown that expression of the gene, TUP1 was slightly increased in response to farnesol, particularly at the transcriptional level and strains lacking the gene, did not respond to farnesol. To study the translational effect of farnesol on TUP1, a quantitative reverse transcriptase PCR (qRT-PCR) analysis was used to investigate the effect of farnesol on TUP1 mRNA across the polysome using RNA samples from input and polysome fractions of farnesol treated cells and cells not exposed to farnesol. The qRT-PCR data showed redistribution of the mRNA from the polysomal region to the sub-polysomal region following treatment with 100µM farnesol indicating an inhibition of the translation.

Key Words: Key words: *Candida albicans*, TUP1, Farnesol, Morphological transition.

1. INTRODUCTION

Candida albicans is a polymorphic yeast and a normal part of the human microflora. It is commonly isolated from the skin, the gastrointestinal tracts and the genitourinary tracts of mammalian hosts. It colonizes host tissues and medical devices with ease (Ramage *et al*., 2005), such as dental materials, joint prostheses, urinary catheters, implants and intrauterine devices (IUDs) (Sardi *et al*., 2013; Handorf *et al*., 2019; de

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Oliveira *et al*., 2019). If the normal microflora balance is altered or the immune system is compromised as in cancer, organ transplant or AIDS patients, *Candida albicans*, being an opportunistic pathogen pose a specific and major health problem; as they can quickly become the cause of morbidity. *C. albicans* has been attributed as the fourth leading cause of mortality in nosocomial bloodstream infections of severely immunocompromised patients (Moran et al., 2010). Individuals with healthy immune systems limit Candida growth at mucosal sites but in compromised immune system it often leads to mucocandidiasis, oral thrush or systemic candidiasis (Pfaller and Diekema, 2007).

Pathogenesis of *C. albicans* requires that at each stage of infection process, different virulence factors are expressed. Some of these virulence factors are the ability to exist in various morphologies (filamentous and vegetative forms), expression of surface adhesion molecules to colonise epithelial surfaces, range of proteolytic enzymes such as secreted aspartyl proteinases (SAP) and lipases, that are required for nutrient acquisition and invasion of epithelial surfaces (D'Souza and Heitman 2001; Sudbery *et al*., 2004; Maidan *et al*., 2005; Han *et al*., 2011). *C. albicans* hyphal form is particularly associated with virulence attributes, including passage through host tissues and defence against immune cells. (Fernandez-Arenas *et al*., 2007), whereas the yeast form aids dissemination of the fungus.

Farnesol is an acyclic sesquiterpene alcohol that is produced and secreted predominantly by two species of Candida: *Candida albicans* and *Candida dubliniensis*. Farnesol has been shown to prevent yeast-to-hyphae transition and most likely as a result inhibits the colonization of implanted medical devices such as polystyrene or tissues by *C. albicans* (Ramage *et al*., 2002; Kruppa 2009).

Farnesol is a 15-carbon acyclic sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) produced from 5-carbon isoprene compounds in both plants and animals. In *C. albicans*, farnesol, a metabolite, is produced via the mevalonate/sterol synthesis pathway in the fungi. (Hornby *et al*., 2001; Yoon *et al*., 2018). It has three carbon-carbon double bonds and exists in four isomers but only the enantiomeric excess (EE) isomer has quorum sensing (QS) molecule activity (Shchepin *et al*., 2005; Polke *et al.,* 2018).

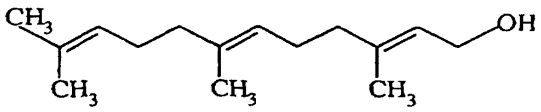


Figure 1: Chemical structure of farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) (Pubchem)

Ramage *et al*., 2002; Martins *et al*., 2007; Araujo *et al*., 2017). Farnesol can also alter the expression of virulence genes in *C. albicans* (Carradori *et al*., 2016).

Transcription factors, cell wall-associated proteins have been found to be play roles in the formation of hyphae and biofilm by *C. albicans* (Pandin *et al*., 2017; Song *et al*., 2020). Gene expression is a two-step process, which involves the formation of mRNA (transcription) and then protein (translation). Translation is an extremely complex process by which the information content of the mRNA is converted to protein. Translation begins with the assembly of several ribosomes connected by a strand of mRNA that runs between the large and small ribosomal subunits referred to as polyribosomes (polysomes). Polysomal profiling is applied to cultured cells and tissues to track the translational status of an identified mRNA (Li *et al*., 2016). The protein product of TUP1, Tup1p in association with either Nrg1p or Rfg1p (transcriptional repressors) negatively regulates the expression of hypha-specific genes (Kadosh and Johnson 2001; Murad *et al*., 2001; Kadosh and Johnson, 2005).

This study investigates the translational effects of farnesol on *C. albicans* by elucidating the effect of farnesol on TUP1 mRNA across the polysome using mRNA samples from input and polysome fractions of farnesol treated and untreated cells. It is hoped that this will provide better insight of the link between farnesol induced translational control and morphological transition in *C. albicans*.

MATERIALS AND METHODS

Strains and growth conditions

The *Candida albicans* CAI4 strain (Fonzi and Irwin 1993), an isogenic derivative of the parental wild-type clinical isolate (SC5314) (Gillum et al. 1984), was used throughout this study. The strain was routinely cultured at 30^{o}C in yeast extract peptone dextrose agar (YPDA) (1% (w/v) Yeast Extract, 2% (w/v) Bacto peptone and 2% (w/v) glucose, 2% agar) to mid-logarithmic phase (~1.0x10^{7} cells/ml or optical density at 600 nm of 0.7).

Morphogenesis assays

Overnight exponential *C. albicans* cultures (~1.0x10^{7} cells/ml or optical density at 600 nm of 0.7) were harvested, washed once in water and resuspended in water to give an OD600 of 1.0. 50µl of the cell suspension was diluted into 5ml YPD containing 10% serum and 150µl of farnesol was added. As controls, cells prepared in the same manner were inoculated into 10% serum medium and into YPD without any treatment. All of the tubes were incubated at 37^{o}C and 200 rpm on an orbital shaker (New Brunswick Scientific). At incubation for 2 hours, cells were mounted onto a microscopy slide and the morphology of the cells was monitored using a Nikon Eclipse E600 with a Nikon x10 and x40 objective and Axiocam MRm camera. Images of the cells were acquired using Axiovision 4.5 software.

To determine colony morphology on solid media, YPD agar supplemented with 150µl farnesol and 10% serum was used. Farnesol was added just before the agar solidified to reduce their volatility. YPD agar with 10%

serum was also prepared to serve as a positive control. Log phase cells washed in water were diluted to give 10^1/ml-10^4/ml of cells. 100µl of the lowest two dilutions were spread on the plates using sterile glass beads. The plates were incubated at 37ºC until colonies appeared. Morphology of the colonies was monitored using a Nikon

Eclipse E600 with a Nikon x10 and x40 objective and Axiocam MRm camera. Images of the cells were acquired using Axiovision 4.5 software.

Preparation of *C. albicans* cell extracts for polysome analysis

C. albicans cultures were grown to an OD_{600} of 0.7 and 100ml aliquots were either maintained as untreated or 150µl of farnesol was added for 10 min. Cultures were transferred to a pre-chilled tube containing 1ml of 50mg/ml cycloheximide (Calbiochem). The cells were harvested by centrifugation at 4,000xg for 5 min at 4ºC in a clinical centrifuge (Sigma). Cells were washed in 25ml pre-chilled lysis buffer (20mM HEPES pH7.4, 2mM Magnesium Acetate, 100mM Potassium acetate, 1mg/ml cycloheximide, 0.5mM dithiothreitol), pelleted by centrifugation at 4,000xg for 5 min and resuspended in 800µl lysis buffer. Cells were pelleted at 10,000xg for 30 sec at 4ºC and resuspended in 200µl lysis buffer. 200µl of 600µm acid-washed glass beads (Sigma-Aldrich) were added and the tubes were mixed vigorously for 6 x 20 sec then cooled for at least 40 sec in iced water between each mixing step. The resulting cell extract was centrifuged twice at 10,000xg for 5 min and 15 min at 4ºC in a microfuge and the OD_{260} was measured using NANODROP-8000 Spectrophotometer (Thermo Scientific). The extract was snap frozen in liquid Nitrogen and then stored at -80ºC.Then one-quarter (1/4) of the polysome extracts (approx. 100µl) was made up to 300µl with the polysome lysis buffer and stored in the -80ºC freezer to serve as the input fraction.300µl of the remaining extract were separated on 15-50% (w/v) sucrose gradients.

Sedimentation of polyribosomes

A volume equivalent to 2.5 OD_{260} units of the *C. albicans* polysome extract was layered onto the top of a 15-50% sucrose gradient in a tube. The gradients were centrifuged in a SW41 rotor (Beckman Instruments) in an L-90K ultracentrifuge (Beckman Instruments) for 2.5 h at 40,000xg. 600µl gradient fractions were collected into tubes and the absorbance was continuously measured at 254 nm to generate polysome profiles using a UV/Vis detector (ISCO UA-6) attached to a pump (Tris TM) and an Optical unit type II (Teledyne ISCO).

Fractions 10-15 harboured the polysomal material and were used to prepare RNA. The polysomal samples were pooled after precipitating with isopropanol.

Extraction of RNA from the input and polysome fractions.

900µl of Trizol (Invitrogen) was added to the input fraction, whereas for the polysome fractions, samples were split into two 300µl amounts and 900µl of Trizol was added to each. The samples were stored in the -20ºC freezer overnight, then thawed on ice, mixed

vigorously for 1 min and left at room temperature for 5 min. 0.2ml of chloroform per 1ml of Trizol was added to the samples and mixed vigorously for 5 min then left at room temperature for 10 min. Samples were then centrifuged at 15,000xg for 15 min at 4ºC in a microfuge to separate the phases. The aqueous layer was transferred to a fresh tube and 1µl of glycoblue was added (glycogen is co-precipitated with the RNA, but does not inhibit first strand synthesis at concentrations ≤ 4mg/ml and does not inhibit PCR). 500µl of 100% isopropanol for 1ml of Trizol reagent used was also added. Samples were incubated at ambient temperature for 10 min and then centrifuged at 15,000 x g for 10 min at 4ºC. The resulting pellet was washed in 1ml of 70% (w/v) ethanol in DEPC treated water. The wash process was repeated, the samples were dried and then resuspended in 10µl of nuclease free water. The RNA suspension was heated to 55ºC for 10 min and the RNA concentration was determined using a NanoDrop 8000 Spectrophotometer (Thermo Scientific). The samples were spiked with Luciferase RNA to serve as a reference.

Quantitative real-time PCR

RNA (6µl of each sample) was reverse transcribed into cDNA using a Protoscript Moloney murine leukemia virus (M-MuLV) RT-PCR kit and random hexamer primer (New England Biolabs). Oligonucleotide primers (Table 1) were designed to TUP1 transcripts and the Luciferase (LUC) RNA using the Primer3 software. Quantification was performed using the CFx Connect Real-Time system with iTaq Universal SYBR Green Supermix (BioRad Laboratories). 10ng of cDNA was added to 1x SYBR green RT-PCR buffer, 30nM of forward and reverse primer, and nuclease-free water up to a volume of 10µl. Reactions were run with initial denaturation of 95ºC for 3 min, 5 sec at 95ºC, 30 sec at 60ºC and 40 cycles of 95-60ºC and finally a melting curve. Luciferase RNA was used as reference for this study. Samples were run in triplicate and for each primer pair, signals were normalized to the untreated sample. Data was analysed manually using the 2-ΔΔCt method (Livak and Schmittgen 2001).

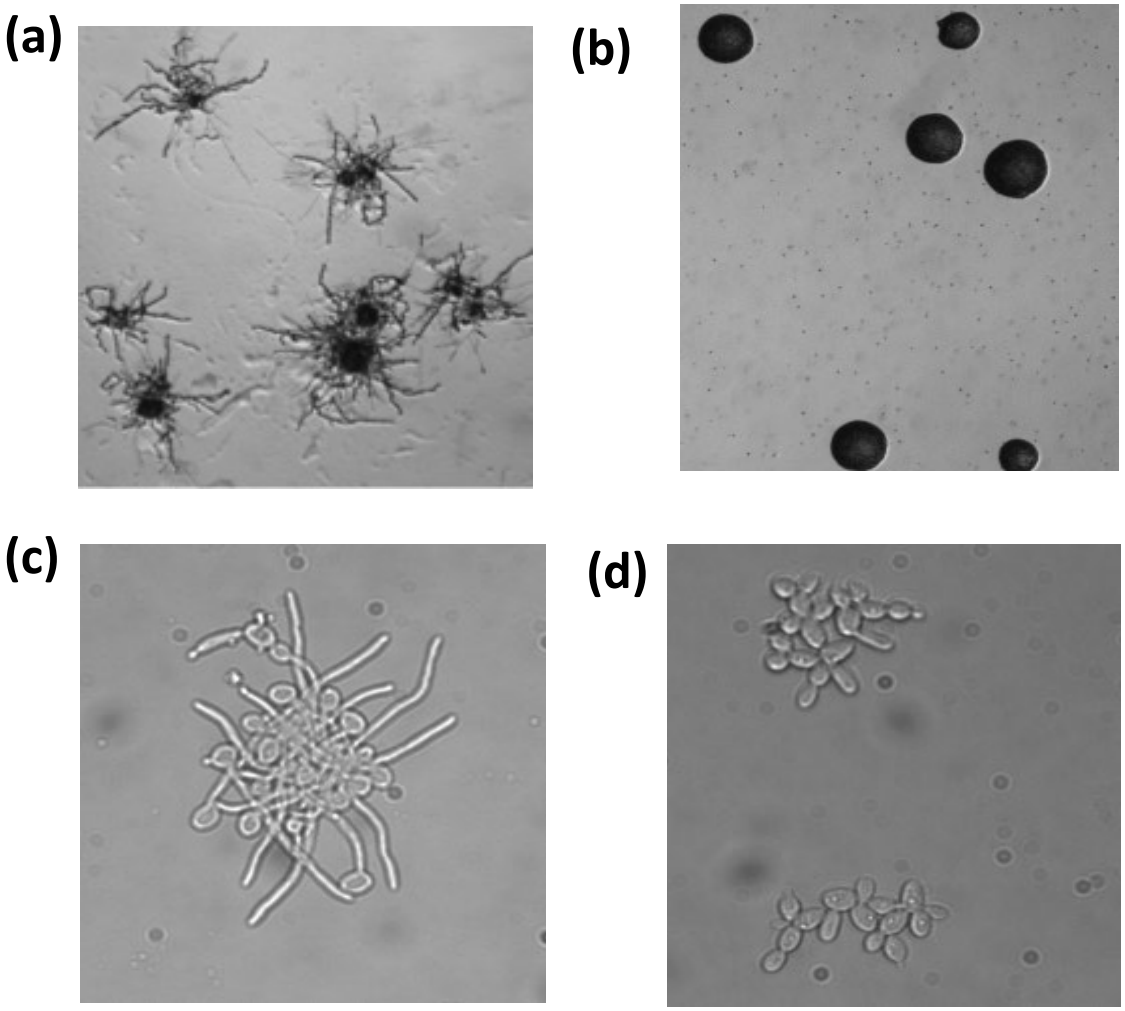
Table1: Oligonucleotides used in this study

Oligonucleotides	Sequence	Use
RT-PCR LUC F	ACGTCTTCCCGACGATGA	RT-PCR FOR LUC
RT-PCR LUC R	GTCTTTCCGTGCTCCAAAAC	RT-PCR For LUC
RT-PCR TUP1 F	CTTGGAGTTGGCCCATAGAA	RT-PCR For TUP
RTPCR-TUP1 R	TGGTGCCACAATCTGTTGTT	RT-PCR For TUP

RESULTS

Farnesol inhibits filamentation in *C. albicans*

Our data showed that farnesol inhibited filamentation and this occurred in the presence of potent inducers like serum. This result was observed both in liquid and solid media as presented in Slide 1.



Slide 1: Addition of farnesol prevents the formation of filament in the presence of serum which is a potent inducer. A and C, cells grown in the presence of serum without farnesol; A, solid media, C, liquid media. B and D, cells grown in the presence of serum and farnesol; B, solid media, D liquid media.

Translational regulation of TUP1 mRNA by farnesol using qRT-PCR.

The qRT-PCR data effect of farnesol on the translation of TUP1 mRNA. The control on Figure 2, shows normal translational profile of TUP1 mRNA with the normal distribution of polysome peaks and shorter 80S peak, however in Figure 3, there is redistribution of the polysomes from the polysomal region to the sub-polysomal region, with increased 80S peak and reduced polysome peaks following treatment with 150µl farnesol. The ratio of polysome to subpolysome in the farnesol treated and untreated cells are shown in Figure 5.

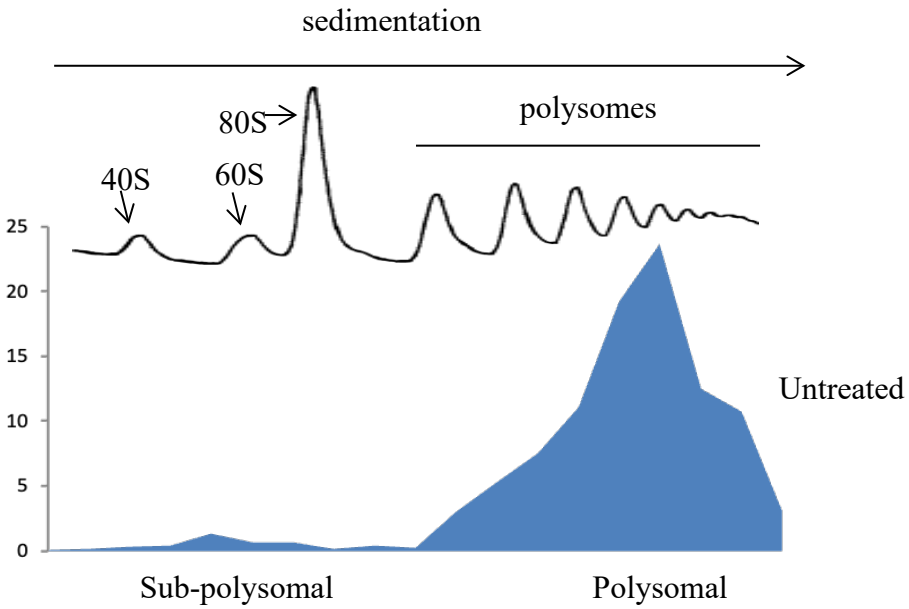


Figure 2: Normal distribution of polyribosomes on *TUP1* mRNA from the *C. albicans* cells not exposed to farnesol

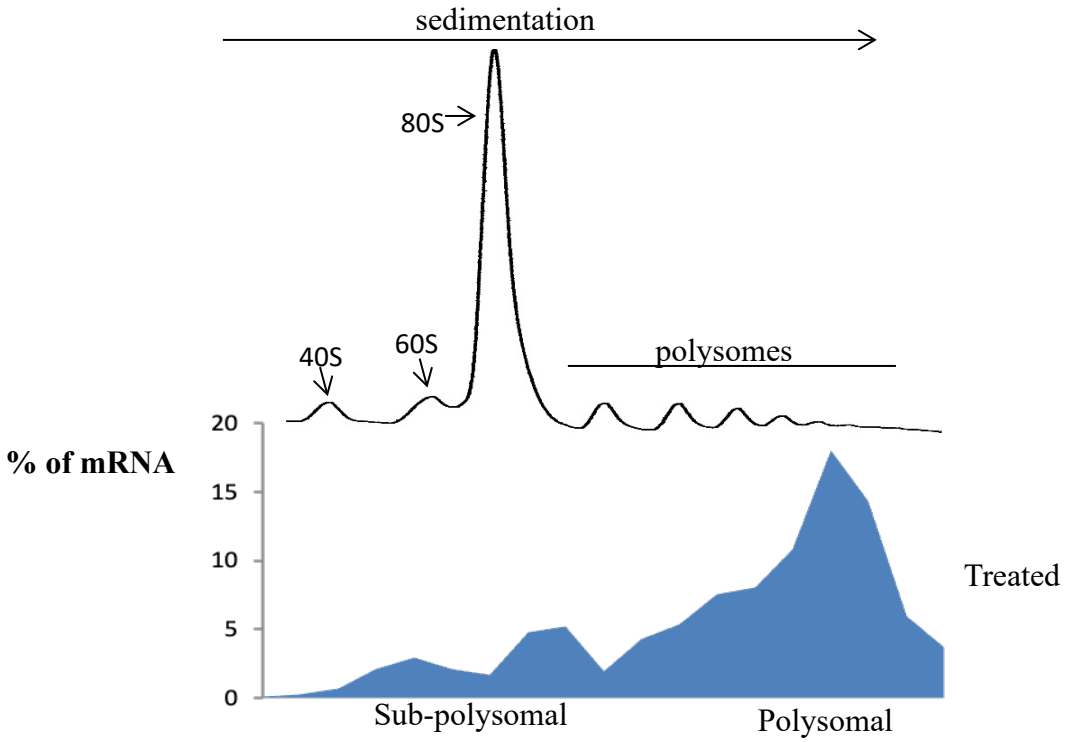


Figure 3: Farnesol causes redistribution of *TUP1* mRNA from the polysome to the submonosomal fraction in farnesol treated *C. albicans* cells.

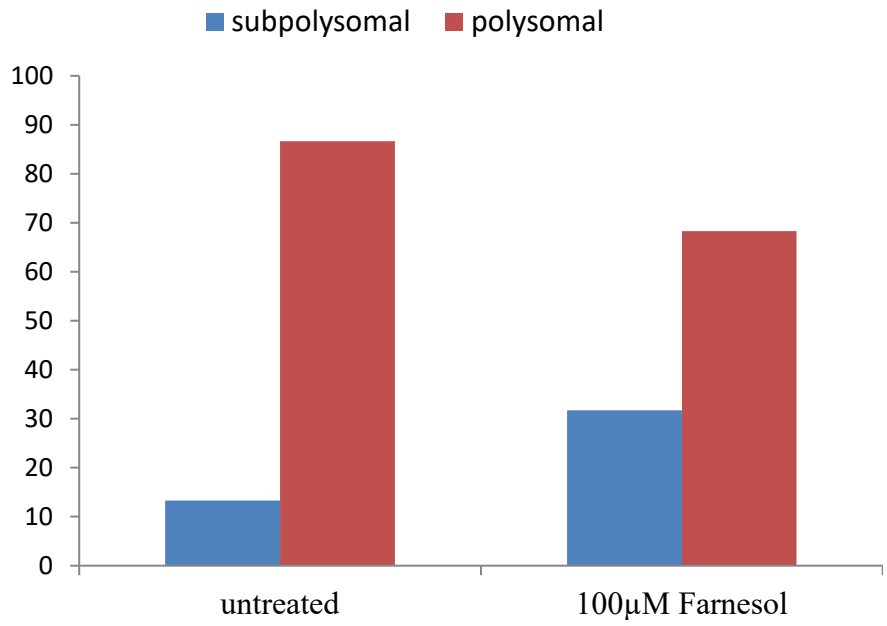


Figure 4: Percentage of mRNA in the monosome versus polysome fractions in cells treated with farnesol and the control

DISCUSSION

Cells respond to changes in the extracellular environment by adjusting the levels of proteins in the cell. Farnesol, a quorum sensing alcohol, has been shown to impact on synthesis of proteins by dramatically inhibiting protein synthesis at the initiation stage. Interestingly, studies presented here and elsewhere have shown that farnesol also inhibits hyphal formation, in *C. albicans* even in the presence of serum which is a potent hyphal inducer (Hornby *et al*., 2001; Ramage *et al*., 2002). A number of suggestions have been previously made, regarding the physiological rationale underlying hyphal formation. The altered growth pattern may allow yeast to forage for nutrients under nutrient limiting conditions (Broach, 2012), it could facilitate escape from accumulating toxic end-point metabolites (Gancedo, 2001), and for *Candida* species, it may also provide a means to evade the immune response of the host.

To study the effect of farnesol on *C. albicans*, RNA sequencing has been applied in the analysis of gene expression in *C. albicans* cells exposed to farnesol (Uppuluri *et al*., 2007; Nobile et al. 2012), this observation had also been made in previous studies using microarrays (Cao *et al*., 2005; Kebaara *et al*., 2008). In their gene expression studies, the authors reported that farnesol upregulated TUP1, a key gene involved in the inhibition of hyphal formation from vegetative cells. The fact that the signaling molecule, farnesol up-regulates this gene which in association with either Nrg1p or Rfg1p (transcriptional repressors) negatively regulates the expression of hypha-specific genes is entirely consistent with the negative impact of farnesol on the transition to filamentous growth in *C. albicans.* However, their studies considered only the transcriptional landscape and not the translational.

Therefore, to investigate the translational regulation of TUP1 in *C. albicans* cells exposed to farnesol, polysome profiling was used. Polysome profiling not only allows the level of protein synthesis to be investigated, but can also pinpoint the step where translation is regulated. RNA extracted from polyribosomal fractions from farnesol treated and untreated cells were subjected to quantitative real time polymerase chain reaction (qRT-PCR). The qRT-PCR data showed redistribution of the mRNA from the polysomal region to the sub-polysomal region following treatment with 100µM farnesol. The increased peaks of the sub-polysomal region and reduced polysome

peaks is characteristic of an inhibition of translation at the initiation stage (Egbe *et al*., 2017). This agrees with the translational down-regulation of TUP1 mRNA. However, as earlier reported, transcription of the TUP1 mRNA increased many folds when *C. albicans* cells are exposed to farnesol. (Uppuluri *et al*., 2007; Nobile et al. 2012). Although Kebaara *et al*. (2008) reported small increase in both TUP1 mRNA and protein levels, it is still unclear how genes are regulated at the translational level in response to farnesol. It is possible that the excess of mRNA in farnesol treated cells is translated less well than the lower levels of mRNA in untreated cells. In this case, the transcriptional response would play a more dominant role in determining the level of Tup1p protein in cells. It could also be that other proteins involved in farnesol respone in *C. albicans* modulate the effect of TUP1 expression. As reported by Pandin *et al*. (2017) and Song *et al*. (2020), transcription factors and cell wall-associated proteins have been found to be involved in hyphae formation and biofilm development by *C. albicans*. Han *et al.* (2012) also reported that farnesol appears to suppress germ tube formation by up-regulating amino acid metabolism, nitrogen metabolism, CoA biosynthesis and nicotinate/nicotinamide metabolism.

CONCLUSION

Farnesol inhibits morphological transition in *C. albicans* through a pathway mediated by TUP1. While the transcriptional upregulation of TUP1 has been established, this study reports that TUP1 mRNA is not effectively translated evidenced by the redistribution of the mRNA from the polysomal region to the sub-polysomal region following treatment with 100µM farnesol. Therefore, given that farnesol could be playing *in vivo* roles in the adaptation, survival and virulence of this opportunistic pathogen, further studies to elucidate mechanisms by which farnesol affect morphological transition at the translational level may help to advance research on the development of novel antifungal agents which may potentially be more effective and less toxic for the treatment of infections caused by Candida species

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