농학박사학위논문

Studies on the Mechanism of Morphogenetic Transition Signaling in *Candida albicans* by Farnesoic acid, a Quorum-sensing Molecule

신호조절물질 **farnesoic acid**에 의한 ***Candida albicans***의 형태변환 조절 메커니즘에 관한 연구

2017년 8월

서울대학교 대학원

농생명공학부 응용생명화학전공

안 찬 홍

A Dissertation for the Degree of Doctor of Philosophy

Studies on the Mechanism of Morphogenetic Transition Signaling in *Candida albicans* by Farnesoic acid, a Quorum-sensing Molecule

August 2017

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Applied Life Chemistry Major

Department of Agricultural Biotechnology

Seoul National University

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지도교수 오 기 봉

이 논문을 농학박사학위논문으로 제출함
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농생명공학부 응용생명화학전공

안 찬 홍

안찬홍의 박사학위논문을 인준함
2017년 7월

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Studies on the Mechanism of Morphogenetic Transition Signaling in *Candida albicans* by Farnesoic acid, a Quorum-sensing Molecule

Advisor: Ki-Bong Oh

A Dissertation Submitted in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

to the Faculty of
Applied Life Chemistry Major,
Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

Chan-Hong Ahn

Date Approved

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ABSTRACT

The ability of *Candida albicans* to switch between yeast and filamentous forms in response to environmental conditions has been postulated to contribute to the virulence of this organism. *C. albicans* hyphal formation is inhibited by a quorum-sensing molecule, farnesoic acid, which accumulates in the medium as the cells proliferate. Many signaling pathways and regulators involved in morphogenetic transition have been identified from intensive investigations, but the molecular networks of farnesoic acid remain poorly understood. It was recently demonstrated that Pho81, a cyclin-dependent protein kinase inhibitor, is for the inhibition of hyphal formation by farnesoic acid. The *pho81* mutant grew exclusively as filaments under the conditions tested. In this study, it was described a newly identified regulator, Hot1, which increases the expression of *PHO81*. By screening a cDNA library with a yeast one-hybrid assay, the *C. albicans* gene *HOT1* was identified, which encodes a protein homologous to the *Saccharomyces cerevisiae* Hot1, an osmostress transcription factor. Sequencing and conceptual translation of HOT1 full-length cDNA revealed an open reading frame (ORF) consisting of 607 amino acid with 19% identity to Hot1 from *S. cerevisiae* over its entire length. The Gcr1_C domain (residues 502–579) was conserved, and the amino acid sequence showed 43% homology with ScHot1. The binding site of Hot1 in the *PHO81* promoter region was investigated by electrophoretic mobility shift assay (EMSA) and DNase I protection assays. The region extending from −479 to −449 relative to the binding site of *HOT1* was identified. To investigate the biological role of *HOT1*

in inhibition of hyphal development by farnesoic acid in *C. albicans*, the *hot1* mutant strain cells were constructed, and the morphological characterization were compared to wild-type cells. The *hot1* mutant strain grew extensively as filaments and was insensitive to farnesoic acid, but not in the wild-type cells. The *hot1* mutant cells expressing *CaHOT1* under control of the *ADH1* promoters restored a wild-type phenotype. From these results, it was concluded that *CaHOT1* had a role in supressing filamentous growth and was a novel molecule for inhibition of the hyphal development by farnesoic acid. Two-hybrid experiments demonstrated that Hot1 interacts with Hog1 mitogen-activated protein kinase (MAPK). Analysis of the expression levels of major signaling pathway components by reverse transcription-polymerase chain reaction (RT-PCR) indicated that farnesoic acid inhibits hyphal formation in *C. albicans* through the coordination of HOG MAPK pathway. These findings suggest that Hot1 is the regulator of *PHO81* transcription and a significant component of filamentation by farnesoic acid in *C. albicans*.

Keywords: *Candida albicans*, hyphal development, quorum-sensing molecule, farnesoic acid, morphogenesis, Hot1, signaling pathway

***Student Number*: 2010-21243**

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LIST OF ABBREVIATIONS

ADH alcohol dehydrogenase

ADHpt promoter of alcohol dehydrogenase

cAMP cyclic adenosine monophosphate

EMSA electrophoretic mobility shift assay

FA farnesoic acid

GS glusoce salt

HOG high osmolarity glycerol

MAPK mitogen-activated protein kinase

PKA protein kinase A

QSM quorum-sensing molecule

RT-PCR reverse transcription-polymerase chain reaction

INTRODUCTION

Candida albicans is the major fungal pathogen in humans, causing common superficial infection of the mucosal epithelia as well as life-threatening disseminated infections via the bloodstream (Biswas *et al*., 2007; Pfaller and Diekema, 2007; Noble *et al*., 2017). *C. albicans* regulates its cellular morphology between yeast and filamentous forms in response to environmental conditions (Klepser, 2006). Its morphogenetic capacity is thought to contribute to the virulence of this organism, as the filamentous form plays key roles in the infection process (Sudbery, 2011). *C. albicans* cells sense the surrounding cell density by a quorum-sensing mechanism. Recently, quorum-sensing molecules such as farnesoic acid, farnesol, and tyrosol were identified in *C. albicans* (Oh *et al*., 2001; Hornby *et al*., 2001; Chen *et al*., 2004). These molecules are secreted into the medium as the cells proliferate and are involved in morphogenesis. Whereas the sesquiterpene compounds, farnesoic acid and farnesol, inhibit hyphal formation, the aromatic alcohol, tyrosol, stimulates *C. albicans* cell growth and hyphal formation (Fig. 1). A yeast-to-hyphae transition assay indicated that farnesoic acid exhibited a weaker inhibitory effect (IC_{50} = 4.56 μg/mL) than farnesol (IC_{50} = 2.82 μg/mL) (Kim *et al*., 2002). Unlike farnesol, farnesoic acid had no detectable effects on yeast cell growth at concentrations below 200 μg/mL. Despite these observations, the morphogenetic regulatory mechanisms of these molecules are not well understood.

In *C. albicans*, hyphal development is mainly regulated by the mitogen-activated protein kinase (MAPK) and cyclic AMP-protein kinase A (cAMP-protein kinase A) pathways, and active Ras1 is required for the regulation of both pathways (Leberer *et al*., 2001; Sudbery, 2011). In addition, three main MAPK pathways have been characterized: cell wall integrity (CWI), high osmolarity glycerol (HOG), and cell morphogenesis pathway (Cek1) MAPK pathways (Navarro-García *et al*., 1995; Smith *et al*., 2004; Chauhan *et al*., 2006; Zucchi *et al*., 2010). *C. albicans* morphogenesis is regulated by a limited number of transcription factors including Cph1, Efg1, Rim101, and Tup1 (Liu *et al*., 1994; Stoldt *et al*., 1997; Braun and Johnson, 1997; Davis *et al*., 2000). The MAPK pathways sense changes in environmental conditions such as osmotic or oxidative stress, pH alterations, temperature, nutrient starvation, and mating pheromones. Some signals, such as serum levels of CO_2, glucose, and amino acids, directly or indirectly regulate the cAMP-PKA pathway through adenylyl cyclase activity (Gow *et al*., 2012). In response to quorum-sensing molecules, biochemical and genetic analyses of signaling pathways suggest that these molecules regulate the network of multiple signaling pathways in *C. albicans* morphogenesis (Fig. 2). For example, treatment of *C. albicans* with farnesol reduces mRNA expression of MAPK cascade components (*HST7* and *CPH1*) (Sato *et al*., 2004). Farnesol also inhibits the Ras1-mediated cAMP-PKA signaling pathway (Hall *et al*., 2011; Davis-Hanna *et al*., 2008), and a recent report showed that it inhibits hyphal initiation mainly by blocking protein degradation of Nrg1 (Lu *et al*., 2014). Much less is known about farnesoic acid with regard to its functions and the mechanisms underlying its ability to function as a quorum-sensing molecule in *C. albicans*.

Recently showed that *PHO81*, which encodes a homolog of *Saccharomyces cerevisiae* Pho81 (a cyclin-dependent protein kinase inhibitor), is essential for the inhibition of hyphal growth by farnesoic acid in *C. albicans* (Chung *et al*., 2010). The *pho81Δ* mutant strain of *C. albicans* grew exclusively as filaments under all of the conditions tested (28℃ and 37℃) and was insensitive to farnesoic acid treatment. Here. it was described another regulator of filamentous growth, the *HOT1* gene, the function of which has been studied in *S. cerevisiae*, where it was shown to regulate the expression of many stress-responsive genes. The *hot1*Δ mutant grew extensively as filaments under favorable yeast growth conditions and was insensitive to farnesoic acid. The protein interaction between Hot1 and MAPK Hog1, which coordinates the transcriptional program required for cell survival upon various stresses, was demonstrated by two-hybrid experiments. Gene expression analysis of signaling pathway components indicated that farnesoic acid regulates the network of multiple signaling pathways in *C. albicans* morphogenesis. This study provides an example of developmental signaling by *C. albicans* in response to a quorum-sensing molecule.

Figure 1. Quorum-sensing molecules (QSMs).

FA and farnesol inhibit the filamentation in *C. albicans*, and tyrosol promotes it.

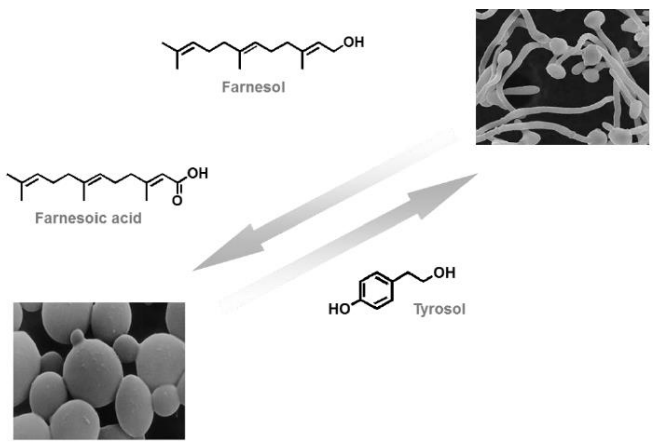
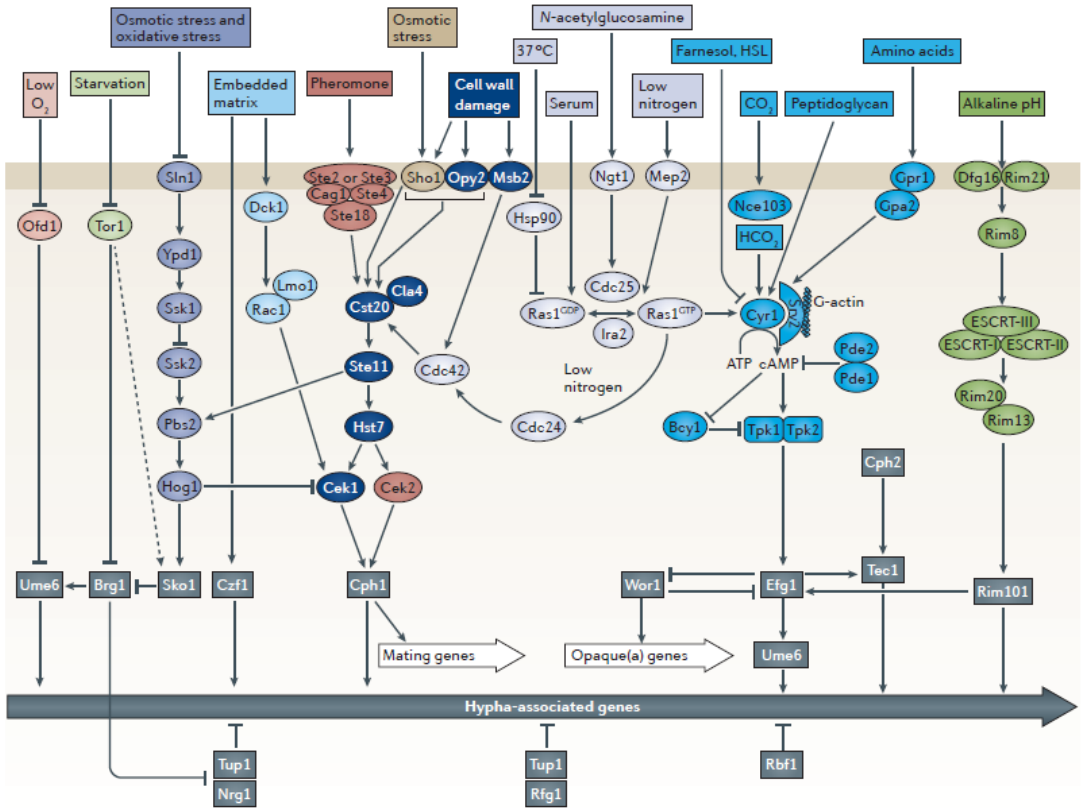

OH
Farnesol
OH
O
Farnesoic acid
OH
HO
Tyrosol

Figure 2. Signaling pathways leading to expression of hypha-specific genes in *C. albicans* (Noble *et al.*, 2017).

Numerous host signals and fungal signalling pathways have been implicated in the regulation of cell shape in *Candida albicans*. The protein kinase A (PKA) pathway (medium blue) incorporates signals through the GTPase Ras-like protein 1 (Ras1;grey) and Ras1-independent inputs that result in the synthesis of cyclic AMP (cAMP) from ATP by the adenylyl cyclase Cyr1 and cAMP-mediated activation of the two catalytic subunits (Tpk1 and Tpk2) of the PKA complex. Signaling through the PKA pathway is inhibited by the quorum-sensing molecules farnesol and homoserine lactone (HSL). The Cek1 mitogen-activated protein kinase pathway (MAPK pathway, dark blue) initiates a kinase signalling cascade in response to embedded growth (light blue), cell wall damage (dark blue), osmotic stress (beige) and low nitrogen (grey), ultimately resulting in the phosphorylation o the transcription factor Cph1 to induce filamentation. The Hog1 MAPK pathway (purple) recognizes osmotic and oxidative stresses through either the Sln1 two-component protein or the Sho1 adaptor protein and leads to phosphorylation of the MAPK Hog1. Activated Hog1 can inhibit both Cek1-mediated and biofilm regulator 1 (Bgr1)-mediated filamentation. The Rim101 pathway (green) senses alkaline pH through two putative receptor (Dfg16 and Rim21) that initiate a proteolytic signalling cascade that results in carboxy-terminal cleavage of the transcription factor Rim101 by the protease Rim13 and the activation of Efg1 and filamentation-specific genes. The Ofd1 pathway (pink) and Tor1 pathway (light green) respond to low oxygen and starvation, respectively, to regulate filamentation through the transcription factors Brg1 and Ume6.


Osmotic stress and oxidative stress
Osmotic stress
N-acetylglucosamine
37°C
Farnesol, HSL
Amino acids
Low O2
Starvation
Embedded matrix
Pheromone
Cell wall damage
Serum
Low nitrogen
CO2
Peptidoglycan
Alkaline pH
Sln1
Ste2 or Ste3
Cag1
Ste4
Ste18
Sho1
Opy2
Msb2
Hsp90
Ngt1
Mep2
Cpr1
Gpa2
Dfg16
Rim21
Ofd1
Tor1
Ypd1
Ssk1
Ssk2
Dck1
Lmo1
Rac1
Cst20
Cla4
Ste11
Hst7
Cek1
Cek2
Pbs2
Hog1
Cdc42
Cdc24
Cdc25
Ras1GDP
Ras1GTP
Ira2
Low nitrogen
Nce103
HCO3
Cyr1
Pka
G-actin
ATP
cAMP
Pde2
Pde1
Bcy1
Tpk1
Tpk2
Rim8
ESCRT-III
ESCRT-I
ESCRT-II
Rim20
Rim13
Cph2
Ume6
Brg1
Sko1
Czf1
Cph1
Wor1
Efg1
Tec1
Rim101
Mating genes
Opaque(a) genes
Ume6
Hypha-associated genes
Tup1
Nrg1
Tup1
Rfg1
Rbf1

1. Dimorphism, an important virulence factor

Candida albicans is the most prevalent opportunistic human fungal pathogen, causing superficial infections as well as life-threatening disseminated and organ infections. Opportunistic fungal pathogens, such as *C. albicans*, are found in the normal gastrointestinal flora and the oral mucosa of most healthy humans. However, in immunocompromised patients, bloodstream infections often cause death, despite the use of antifungal therapies (Biswas *et al.*, 2007).

An important feature of *C. albicans* is dimorphism between a single cell, budding yeast form (blastospore) and a filamentous form (including both pseudohyphae and true hyphae) (Berman, 2006). Typically, *C. albicans* grows as single ellipsoidal cells called blastospores (also called blastoconidia). In the presence of inducing environmental signals, e.g. serum, high temperature (37°C), high ratio of CO_2 to O_2, neutral pH, and nutrient poor media, *C. albicans* can assume filamentous forms in which cells remain attached to each other after dividing and thereby form long branched strings of connected cells. Conversely, low temperature (28°C), a high ratio of O_2 to CO_2, acidic pH (4 to 6), and enriched media promote yeast cell growth. The ability of *C. albicans* to adopt these different morphologies is thought to contribute to colonization and dissemination within host tissues and thereby to promote infection (Lo *et al.*, 1997; Odds, 1994; Saville *et al.*, 2003). Therefore, the regulation of morphogenesis has been intensively investigated in the past years.

2. Signaling pathways and transcriptional regulators required for morphogenetic transition

Many signaling pathways and regulators involved in hyphal development have been identified as a result of the strong molecular conservation between *C. albicans* and *Sacchromyces cerevisiae* in many cellular processes, a *C. albicans* genome sequencing program (http://www-sequence.stanford.edu/group/candida/) and efficient homologous recombination that allows a variety of molecular manipulations to be possible. The Cph1-mediated MAPK (mitogen-activated protein kinase) pathway and the Efg1-mediated cAMP-dependent protein kinase A pathway are two well-characterized signaling pathways in regulation of hyphal development (Brown and Gow, 1999; Ernst, 2000; Whiteway, 2000). Many signaling pathways or regulators have been found to regulate the hyphal development in one or many of the in vitro hypha-inducing conditions.

2.1. Mitogen-activated protein kinase pathway

In *S. cerevisiae*, elements of the pheromone-responsive mitogen-activated protein kinase (MAPK) pathway are involved in pseudohyphal and invasive growth. A MAPK cascade has been shown to be required for morphological change in *C. albicans* (Dhillon *et al.*, 2003). MAPK pathways are cascades of phosphorylation from a MAPK kinase kinase (MAPKKK) to a MAPK and result in the activation of transcription factors (Hall *et al.*, 2009). A MAPK cascade that includes Cst20 (p21-activated kinase; PAK), Hst7 (MAP kinase kinase; MEK) and Cek1 (MAPK) are

also involved in filamentation (Liu, 2001). The transcription factor Cph1 functions downstream of the MAPK cascade. Null mutants of these genes show retarded filamentous growth but no impairment of serum-induced germ tube and hyphae formation (Kohler and Fink, 1996; Kron and Gow, 1995). These observations suggest that a kinase signaling cascade plays a part in stimulating the morphological transition between blastospore and filamentous forms in *C. albicans*.

2.2. cAMP-dependent protein kinase A pathway

Cyclic AMP acts as an intracellular regulator and participates in many cellular processes in both prokaryotic and eukaryotic organisms (Dhillon *et al*., 2003). The cAMP-dependent protein kinase A (PKA) pathway plays a crucial role in filamentous growth in *C. albicans* and other fungi (Lengeler *et al*., 2000). *C. albicans* has a single adenylate cyclase gene (*CDC35*/*CYR1*). The cyclase is not essential for growth in *C. albicans*, but is completely required for hyphal formation. Cyr1 integrates environmental signals from a range of sources and is completely essential for hyphal formation but no yeast-form growth (Dhillon et al., 2003; Whiteway, 2000). Recently, the adenylate- cyclase-associated protein (*CAP1*) has been identified and disrupted in *C. albicans*. The *Δcap1* mutant is defective in germ tube formation and hyphal development in all conditions examined, including serum-containing media. The defects are suppressed by exogenous cAMP or dibutyryl cAMP. *Δcap1* are avirulent in a mouse model for systemic candidiasis (Bahn *et al*., 2003; Bahn and Sundstrom, 2001; Zou *et al*., 2010). There are only two cAMP-dependent PKA catalytic subunits, Tpk1 and Tpk2, in *C. albicans* (Sonneborn *et al*., 2000). Both PKA isoforms are positive regulators of hyphal

morphogenesis. Efg1, a basic helix-loop-helix (bHLH) protein, plays a major role in hyphal morphogenesis in response to serum, neutral pH and *N*-acetyl glucosamine in liquid media, and on solid media such as Spider medium. *Δefg1* null mutant strains do not form hyphae under most hypha-inducing conditions and are defective in the induction of hypha-specific genes. Efg1 is likely to function downstream of the PKAs (Lo et al., 1997; Stoldt et al., 1997). *TPK2* overexpression cannot suppress the *Δefg1* defect in hyphal development, whereas overexpression of *EFG1* can suppress the filamentation defect in *Δtpk2* (Sonneborn *et al*., 2000).

2.3. Ras1-mediated signaling pathway

Ras proteins are members of the small GTPase superfamily that cycle between an inactive GDP-bound and an active GTP-bound form (Bourne *et al*., 1990). The active form of Ras stimulates two main signaling pathways, cAMP-PKA pathway an MAPK pathway (Biswas *et al*., 2007). In *C. albicans*, Ras1 has been identified which is not essential for survival (Feng *et al*., 1999). The *Δras1* deletion mutant cells defect in hyphal growth in response to serum and other conditions. In addition, while a dominant negative Ras1 variant ($Ras1^{G16A}$) caused a defect in filamentation, a dominant active Ras1 variant ($Ras1^{G13V}$) enhanced the formation of hypha (Feng *et al*., 1999). The constitutive hyphal growth induced by overexpression of dominant-active Ras1 variant ($Ras1^{G13V}$) was defected in cells deleted for *HST7*, *CPH1*, *EFG1*, *CYR1*, or both *CPH1* and *EFG1* (Leberer *et al*., 2001). These observations are suggested that Ras1 lies upstream of the MAPK and cAMP-PKA pathways, and required for the regulation of both pathways.

2.4. Transcription repressors

Hyphal development in *C. albicans* is also subject to negative regulation (Dhillon *et al*., 2003). Tup1 encodes a transcriptional repressor that negatively controls filamentous growth in *C. albicans* (Braun and Johnson, 1997). Tup1 is likely to be part of a transcriptional repressor complex that is brought to promoters by sequence-specific DNA-binding proteins. Several genes repressed by Tup1 (*RBTs*) have been identified in *C. albicans*. Many of them are induced during the yeast-to-hypha transition. But, some *RBTs* are not regulated during filamentation, indicating that the regulation of filamentation is not the sole function of Tup1. This also suggests that Tup1 itself is not regulated during filamentous growth; rather, its associated DNA-binding proteins are likely to be regulated (Braun *et al*., 2000). *Δtup1* mutant strains of *C. albicans* form hyphae under conditions that normally promote growth of the yeast form and lead to derepression of hypha-specific genes (Braun and Johnson, 1997; Braun *et al*., 2000). Tup1 appears to act independently of the MAPK and cAMP-PKA pathways to regulate morphogenesis (Braun and Johnson, 2000). Nrg1, another DNA-binding protein, might direct the Tup1 repressor complex to the promoters of hypha-specific genes (Murad *et al*., 2001; Braun *et al*., 2001). *S. cerevisiae* Nrg1 is a sequence-specific zinc-finger DNA-binding protein that directs the Tup1-Ssn6 complex to repress *STA1* transcription in a glucose-dependent manner (Park *et al*., 1999). The *NRG1* transcript is down-regulated during filamentous growth in serum at 37℃ (Braun *et al*., 2001).

3. Morphogenetic quorum-sensing molecules

C. albicans, a polymorphic fungus that is well studied for its importance as an opportunistic human pathogen, was among the first fungi reported to have a quorum-sensing system (Lim *et al*., 2012). It has been documented that *C. albicans* hyphal formation is suppressed at high cell densities and by supernatants from stationary phase *C. albicans* cultures, suggesting that hyphal formation was controlled, at least in part, by a soluble factor (Hogan, 2006).

The dimorphism in *C. albicans* is reportedly under the control of at least three morphogenetic quorum-sensing molecules (QSMs) that accumulate in the medium as the cells proliferate. Farnesoic acid (FA) and farnesol inhibit hypha formation, and tyrosol promotes it. Farnesol is able to block filamentation induced by the environmental signals for most signaling pathways activating hyphae development (Hornby *et al*., 2001). This compound was active against a variety of *C. albicans* strains at concentrations between 1 and 50 μM (Hogan, 2006). However, *C. albicans* strain ATCC10231 produces FA, a compound closely related to farnesol, and that FA is responsible for the inhibition of hyphal growth in dense cultures (Oh *et al*., 2001). But *C. albicans* strain 10231 did not produce farnesol (Hornby and Nickerson, 2004). Farnesol can inhibit hyphae formation at lower concentrations than FA but that FA has decreased toxicity at high concentrations (Kim *et al*., 2001). Recently, farnesol was reported to inhibit a Ras1-mediated cAMP-dependent PKA pathway (Davis-Hanna *et al*., 2008). Another *C. albicans* QSM, tyrosol, was found to promote hyphae development by shortening the lag-

time of cells to begin germinating in hyphae-inducing conditions (Chen *et al*., 2004).

4. HOG MAPK pathway and stress response

4.1. High-osmolarity glycerol MAPK pathway

In *S. cerevisiae*, two independent osmosensors regulate the common high osmolarity glycerol (HOG) response pathway allows adaptation to high-osmolarity conditions and is activated in response to oxidative stress, which induces the Pbs2 MAPKK and Hog1 MAPK (Maeda *et al*., 1994; Maeda *et al*., 1995; Brewster *et al*., 1993) (Fig. 3). This route is triggered in response to high external osmolarity and results in the accumulation of glycerol as an intracellular compatible solute in *S. cerevisiae*. The Sln1-Ypd1-Ssk1 two-component osmosensor uses a multistep phosphorelay mechanism to regulate the redundant MAPKKK Ssk2 (Ota *et al*., 1993; Posas *et al*., 1996). Activated Ssk2 then phosphorylates and activates the Pbs2 MAPKK via Ste11, which is also an integral component of the mating pheromone response pathway (Maeda *et al*., 1995; Posas *et al*., 1997). Under hyperosmotic conditions, *Δhog1 and Δpbs2* mutant strains display different alterations, such as a defective bud repositioning and induction of pseudohyphal growth (Brewster and Gustin, 1994). The second osmosensor, Sho1, contains four transmembrane segments and a COOH-terminal cytoplasmic region with an SRC homology 3 (SH3) domain (Maeda *et al*., 1995). The interaction between an NH2-terminal proline-rich motif in Pbs2 and the Sho1 SH3 domain is essential for the activation of Pbs2 MAPKK. The activation of Pbs2 by Sho1 is also mediated by the Ste11 (Posas and Saito, 1997). The HOG MAPK pathway plays a central role in stress responses in *C. albicans* (Alonso-Monge *et al.*, 1999; Enjalbert *et al.*,

2006). Hog1 is activated by osmotic stress, oxidative stress, and heavy metal stress and is required for the survival of *C. albicans* cells when they encounter the stresses. HOG MAPK pathway participates in a pleiotropic response that enables a correct and rapid adaptation to osmotic stress.

4.2. Control of Hot1 activation by the Hog1

Under high osmolarity conditions, many stress response genes are activated by several unrelated transcription factors that are controlled by the Hog1 kinase. The Hot1 activation is also controlled by Hog1 MAPK. Interaction between Hot1 and Hog1 exists in the transcription of the genes regulated by this transcription factor under osmotic stress conditions. The phosphorylation and activation of Hog1 trigger its rapid and transitory accumulation in the nucleus (Maeda *et al*., 1994; Posas and Saito, 1997). Hog1 regulates several transcription factors, including Hot1, which is responsible for controlling the expression of a subset of osmoresponsive genes (Posas *et al*., 2000; Rep *et al*., 2000). The entry of Hog1 in the nucleus upon stress allows Hot1 to bind, together Hog1, at the *cis*-elements located in the regulatory regions of stress responsive genes, thus targeting the Hog1-RNApol II complex to these genes (Cook and O'Shea, 2012; Nadal-Ribelles *et al*., 2012).

4.3. HOG MAPK pathway controls hyphal development

In *S. cerevisiae*, HOG MAPK pathway controls the hyphal development, which inhibits Cek1 and activates a transcriptional inhibitor of filamentation (Roman *et al*., 2005). The Cek1 MAPK pathway initiates a kinase signaling cascade in

response to embedded growth, cell wall damage, osmotic stress and low nitrogen, ultimately resulting in the phosphorylation of the transcription factor Cph1 to induce filamentation (Noble *et al*., 2017). Activated Hog1 can also inhibit Brg1-mediated filamentation. The GATA family transcription factor Brg1 recruits Hda1 to promoters for sustained hyphal development. Active Hog1 represses the expression of *BRG1* via the transcriptional repressor Sko1 as Sko1 disassociates from the promoter of *BRG1* in the *Δhog1* mutant (Su *et al*., 2013). *Δhog1* null mutant strains are derepressed in the serum-induced hyphal formation and, consistently with this behavior, that *HOG1* overexpression in *S. cerevisiae* represses the pseudodimorphic transition. *Δhog1* strains also resulted in a drastic increase in the mean survival time of systemically infected mice, supporting a role for HOG MAPK pathway in virulence of pathogenic fungi. In *C. albicans*, some elements of the HOG1 MAPK pathway have been identified and characterized. The Hog1 MAP kinase was cloned by its functional homology to *S. cerevisiae* Hog1 and was shown to play a role in osmotic stress and morphogenesis (Alonso-Monge *et al*., 1999).

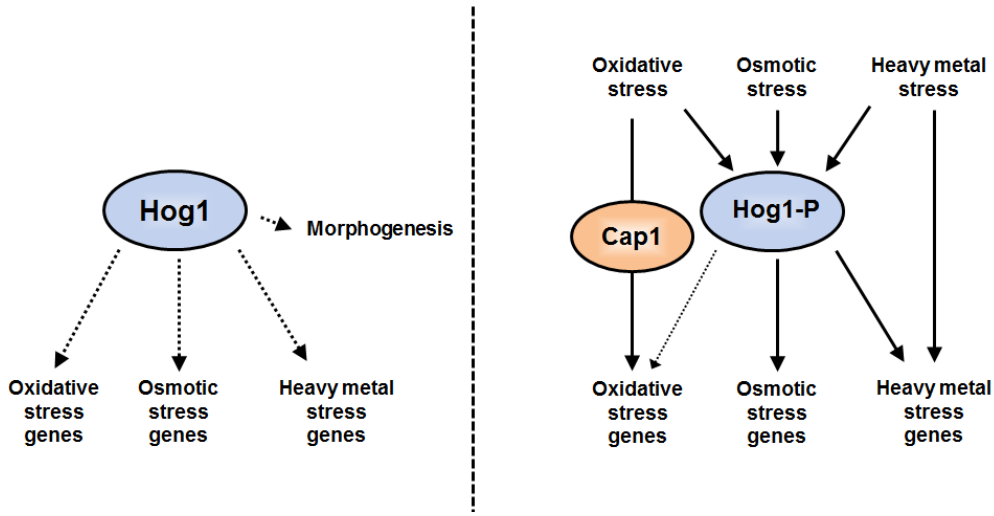
5. Purpose of this study

In previous work, the yeast-to-hyphae transition in *C. albicans* was suppressed by FA, a morphogenetic quorum-sensing molecule that accumulates in the medium as cells proliferate (Oh *et al*., 2001). *C. albicans PHO81*, which encodes a homolog of *S. cerevisiae* Pho81, is essential for the inhibition of hyphal

growth by FA during the yeast-to-hyphae transition (Chung *et al.*, 2010). In this study, several experiments were performed to find the factor that regulates the transcription of *PHO81*. It is showed that *HOT1*, one of genes induced by FA, is required for filamentous growth of *C. albicans*. Hot1 serves as a transcription factor of *PHO81* functions as part of the Ras1-cAMP signaling pathway induced filamentation in *C. albicans*.

Figure 3. Model depicting the role of the Hog1 MAPK in the regulation of gene expression in *C. albicans* (Enjalbert *et al*., 2006).

Deletion of *HOG1* has effects on both basal and stress-induced gene expression. Hyphal-specific genes and stress-related genes are deregulated in *Δhog1* cells under basal conditions. In response to stress, Hog1 plays a central role in the regulation of osmotic- and heavy metal stress-induced gene expression, but a less significant role in the regulation of oxidative stress genes. Instead, other pathways, such as the Cap1 pathway, play key roles in the regulation of oxidative stress genes.


Hog1
Morphogenesis
Oxidative stress genes
Osmotic stress genes
Heavy metal stress genes
Oxidative stress
Osmotic stress
Heavy metal stress
Cap1
Hog1-P
Oxidative stress genes
Osmotic stress genes
Heavy metal stress genes

MATERIALS AND METHODS

1. *C. albicans* strains and culture conditions

The *C. albicans* strains used in this study are listed in Table 1. *C. albicans* strains were routinely cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Strains carrying plasmids and introduced gene disruption cassettes were grown in synthetic defined (SD) medium (0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI, USA), 0.192% yeast synthetic dropout medium (Sigma, St. Louis, MO, USA), and 2% glucose) (Fonzi and Irwin, 1993). GS medium (5 g of glucose, 0.26 g of $Na_2HPO_4 \cdot 12H_2O$, 0.66 g of KH_2PO_4, 0.88 g of $MgSO_4 \cdot 7H_2O$, 0.33 g of NH_4Cl, and 16 μg of biotin per liter) (Oh *et al.*, 2001) was used to cell preparation for cDNA library construction.

2. Construction of the cDNA library

The cDNA library was constructed by reverse transcription (RT)-PCR from the *C. albicans* strain SC5314 (Gillum *et al.*, 1984) according to the manufacturer's instructions. Cells were streaked onto SD plates without uridine and incubated at 28℃ for 3–4 days. A single colony was inoculated in liquid SD medium and cultured for 24 h at 28℃ to early stationary phase. Cells (1×10^7 cells/mL) were harvested and inoculated in GS medium for 40 min at 37℃ with 20

μg/mL farnesoic acid. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolated RNA (1 μg) was treated with DNase I (Invitrogen) for 30 min at 37℃. The reaction was terminated with the addition of 1 μL 25 mM EDTA, and heated for 10 min at 65℃ followed by phenol extraction and ethanol precipitation. cDNA synthesis consisted of three steps: first-strand cDNA synthesis, amplification of the cDNA by long distance (LD)-PCR, and purification of the double-stranded cDNA with a CHROMA SPIN+TE-400 column (Clontech, Palo Alto, CA, USA). For first-strand cDNA synthesis, oligo $[dT]_{20}$ (50 mM) and SMART MMLV RT were used. First-strand cDNA was generated by LD-PCR amplification and the cDNA was purified.

3. Yeast one-hybrid assay

Yeast one-hybrid experiments were performed using a Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech). The *PHO81* PR (promoter) was PCR-amplified (Table 3) from the *C. albicans* strain SC5314 and cloned into the *Kpn*I/*Xho*I sites of the plasmid pAbAi, which encodes the AUR1-C gene (Table 2). The bait plasmids, pPR-AbAi and empty pAbAi, were linearized and integrated into the genome of the yeast strain Y1HGold, and transformants were selected on SD medium without uracil (SD/-Ura) after incubation for 3 d at 30℃. Yeast cells were then transformed with the prey plasmid pGADT7-Rec containing the cDNA library. To assay for prey-bait interaction, a single colony was suspended in 100 μL of sterilised distilled water and 10 μL of cell suspension was

spotted onto SD medium without leucine (SD/-Leu) and supplemented with aureobasidin A (SD/-Leu/AbA). Cells were incubated at 30℃ for 2 d and photographed.

4. β-Galactosidase activity assay

The primers for the β-galactosidase assay were used to amplify various lengths of the *PHO81* promoter (Table 3). The PCR fragments were digested with *Kpn*I and *Xho*I, and subcloned into the similarly digested pENO1LacZ vector (Uhl and Johnson, 2001; Staab *et al*., 2003). The resulting plasmids (pPR595–pPR100) (Table 2) were transformed into the *C. albicans* strain SC5314 and grown in 5 mL of YPD medium. The initial cell density was OD_{600} = 0.05 and cells were allowed to grow for 8 h at 28℃, at which time the cultures were still in the exponential growth phase. The β-galactosidase activity of transformants was determined by quantitative liquid assay using permeabilised cells as described previously (Ausubel *et al*., 1992; Uhl and Johnson, 2001). Briefly, cell suspension (10–100 μL) was added to Z buffer to a final volume of 1 mL and the cells were permeabilised with 30 μL chloroform and 15 μL 0.1% SDS. After equilibration at 37 ℃ for 5 min, the cells were mixed with 0.2 mL *ortho*-nitrophenyl-β-galactoside (4 mg/mL; Sigma) and incubated at 37℃. Reactions were stopped with 1 M Na_2CO_3 (0.5 mL) and centrifuged for 5 min at 10,000 × g. The A_{420} and A_{550} were read and the units of activity were determined by the standard equation (Ausubel *et al*.).

5. EMSA and DNase I protection assay

The *HOT1* gene was fused with the GST coding sequence through the *Bam*HI and *Xho*I sites of the pGEX-5x-1 expression vector (GE Healthcare, Milwaukee, WI, USA) and the GST-tagged Hot1 fusion protein was purified according to the manufacturer's instructions. The upstream region of the *PHO81* promoter, from −495 to −395, was amplified by PCR using [γ-^{32}P]-ATP labeled PR395-R and unlabeled PR495-F primers (Table 3). The DNA-protein binding reactions were performed as follows: the labeled DNA probe (100 bp; 5 nM) was incubated with purified GST-tagged Hot1 for 30 min at 30°C in a 20 μL reaction mixture containing 1× binding buffer [25 mM Tris-Cl (pH 8.0), 50 mM KCl, 1 mM EDTA, 8% (v/v) glycerol, 50 μg/mL bovine serum albumin (Sigma), and 200 ng of poly (dI-dC) (Sigma)] (Lim and Choi, 2014). For competition analysis, an identical but unlabeled DNA fragment was used as a self-competitor DNA. Various amounts of the self-competitor DNA were added to a reaction mixture containing the labeled DNA (5 nM) prior to the addition of Hot1 (0.5 μg).

The upstream region of the *PHO81* promoter, extending from −695 to −280, was amplified by PCR using [γ-^{32}P]-ATP labeled PHO280-R and unlabeled PHO695-F primers (Table 3). The labeled 415 bp DNA probe was used for the DNase I protection assays (Kim *et al.*, 2011). The DNA-protein binding reactions with purified GST-tagged Hot1 were performed as described above and DNase I digestion of the DNA-protein complexes was performed as follows: 20 μL of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ were mixed and added along with 1 μL of DNase I

solution (10 ng/μL) (Invitrogen, Carlsbad, CA, USA). Samples were incubated for 1 min at 25°C, the reactions were stopped with the addition of 80 μL of the stop solution, and the DNA products were purified by ethanol precipitation. The digested DNA products were resolved on a sequencing gel alongside sequencing ladders of pPR695 that were generated using PHO280-R as a primer. The plasmid pPR695 was constructed by cloning the same 415 bp upstream region of *PHO81* into pGEM-T Easy (Promega, Madison, WI, USA). The gels were visualised using a phosphorimager (BAS1500; Fuji Photo Film Co. Ltd., Tokyo, Japan).

6. Gene disruption

To disrupt the first chromosomal *HOT1* allele, pQF183 plasmid (Table 2) was digested with *Hind*III and *Eco*RI, and 5 μg of the linearized *hot1Δ*::-*hph*-URA3-*hph* gene disruption cassette was introduced into the *C. albicans* strain CAI4 using the lithium acetate method (Gietz *et al*., 1995; Lee *et al*., 2014). The 374-bp 5′-*HOT1* region was amplified by PCR using HOT11-1F and HOT11-1R as primers and *C. albicans* SC5314 genomic DNA as the template. The PCR product was cloned into pGF181 digested with *Eco*RI-*Kpn*I resulting in plasmid pGF182. The 364-bp 3′-*HOT1* region was amplified by PCR using primers HOT11-2F and HOT11-2R. The PCR product was cloned into pQF182 digested with *Sal*I-*Hind*III, resulting in plasmid pGF183. (i.e., pQF183 digested with *Eco*RI ad *Hind*III). To disrupt the second chromosomal *HOT1* allele, *C. albicans HOT1/hot1Δ*::*hph* cells (the 4DH strain) were transformed with the linearized *hot1Δ*::*hph*-URA3-*hph* gene

disruption cassette. The 374-bp 5′-*HOT1* region was amplified by PCR using primers HOT12-1F and HOT12-1R and cloned into pGF181 digested with *Hind*III and *Sal*I to obtain pQF184. The 307-bp 3′-*HOT1* region was amplified by PCR using primers HOT12-2F and HOT12-2R, and cloned into pQF184 digested with *Kpn*I and *Eco*RI to obtain pQF185 (i.e., pQF185 digested with *Hind*III and *Eco*RI). Uridine auxotrophs were selected on SD medium containing 1 mg/mL 5-fluoroorotic acid (Sigma). The genotypes of the mutant strains were confirmed by PCR.

7. DNA isolation and Southern blot analysis

HOT1 disruption in heterozygous and homozygous strains was verified by Southern blotting. Genomic DNA from C. albicans was isolated as described previously (Hoffman and Winston, 1987). Genomic DNA was isolated from wild-type (CAI4) and mutant strains, digested with *Bgl*II and *Eco*RI, separated in a 1% agarose gel, transferred onto a nylon membrane, and fixed by UV irradiation. The hybridization probe was amplified by PCR from pQF185 using pQF62 and HOT1probe-R as primers (Table 3). Labeling of the DNA probe and subsequent hybridization was carried out using a random primer DNA labeling kit (Takara, Shiga, Japan) with (α-^{32}P)dCTP (IZOTOP, Budapest, Hungary).

8. Plasmid constructions for morphogenesis

Plasmids used in this study are listed in Table 2. *C. albicans* strains were transformed using the LiAc/SS-DNA/PEG method as described (Bertram *et al*., 1996). The plasmid-borne *HOT1* (pADH-HOT1) gene was generated by PCR amplification using specific primer sets (Table 3) from genomic DNA of *C. albicans* SC5314. PCR products were cloned into the YPB-ADHpt plasmid (Bertram *et al*., 1996).

9. Morphological characterization of the *hot1Δ* mutant

The CAI4 strain (wild-type) and 4DH2 strain (*hot1Δ* mutant) containing the empty YPB-ADHpt vector (Bertram *et al*., 1996) (Table 1) were inoculated onto SD plates without uridine and incubated at 28℃ for 3–4 d. A single colony was selected and grown in YPD liquid medium at 28℃ to early stationary phase. To induce hyphal growth, cells (1×10^7 cells/mL) were incubated in YPD medium for 6 h at 37℃. For the in vitro yeast-to-hypha transition assay, cells (1×10^7 cells/mL) were incubated in GS medium at 37℃ for 6 h with or without 20 μg/mL farnesoic acid.

10. Yeast two-hybrid assay

The primers for the *C. albicans HOT1* and *HOG1* genes are listed in Table S2. The full-length of the *HOT1* gene was amplified by PCR from CAI4 genomic DNA and cloned into the Y2H bait vector pGBKT7 (Clontech) to generate the bait gene construct. The full-length *HOG1* was also amplified by PCR and cloned into the Y2H prey vector pGADT7 AD (Clontech) to generate the prey gene construct. The bait and prey gene constructs were co-transformed into the *S. cerevisiae* strain AH109 and transformants were selected on SD medium lacking leucine and tryptophan (SD/-Leu/-Trp) (Clontech) after incubation for 3 d at 30℃. For the Hot1-Hog1 interaction, a single colony was suspended in 100 μL of sterilised distilled water. The cell suspension (5 μL) was plated on SD medium lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp), incubated at 30℃ for 2 d, and photographed.

11. Gene expression analysis

RNA was isolated from the *C. albicans* strain SC5314 as described above. cDNA synthesis was performed using Superscript III First-Strand Synthesis System (Invitrogen) for RT-PCR, and oligo $[dT]_{20}$ (50 mM) were used to prime the cDNA synthesis reaction. RT-PCR analysis was conducted with gene specific primers (Table 3) for major components of the signalling pathways. The PCR reaction was performed as follows: 98℃ for 10 min, followed by 25 cycles of 98℃ for 30 s,

50℃ for 30 s, and 72℃ for 30s, with a final extension at 72℃ for 5 min. Expression levels were determined by densitometry using Image J software (NIH, Bethesda, MD, USA). The housekeeping gene glycerol-3-phosphate dehydrogenase (*GPD1*) was used as a loading control (Davis-Hanna *et al*., 2008).

Table 1. Strains used in this study.

Strain	Genotype or description	Parent	Reference
C. albicans strains			
SC5314	Prototrophic clinical isolate		Gillum *et al*. (1984)
CAI4	Δ*ura3::imm434*/Δ*ura3::imm434*	CAI4	Fonzi and Irwin (1993)
CAG4711	CAI4 Δ*pho81::hisG*(I-SceI)/Δ*pho81::hisG*(I-SceI)	CAI4	Chung *et al*. (2010)
4DH	CAI4 Δ*hot1::hph*/*HOT1*	CAI4	This study
4DH2	CAI4 Δ*hot1::hph*/Δ*hot1::hph*	CAI4	This study
PR595LacZ	CAI4 with plasmid pPR595 integrated at the *ENO1* locus	CAI4	This study
PR495LacZ	CAI4 with plasmid pPR495 integrated at the *ENO1* locus	CAI4	This study
PR395LacZ	CAI4 with plasmid pPR395 integrated at the *ENO1* locus	CAI4	This study
PR295LacZ	CAI4 with plasmid pPR295 integrated at the *ENO1* locus	CAI4	This study
PR195LacZ	CAI4 with plasmid pPR195 integrated at the *ENO1* locus	CAI4	This study
PR100LacZ	CAI4 with plasmid pPR100 integrated at the *ENO1* locus	CAI4	This study
HWP1LacZ	CAI4 with plasmid pAU95 integrated at the *ENO1* locus	CAI4	This study
ACT1LacZ	CAI4 with plasmid pAU36 integrated at the *ENO1* locus	CAI4	This study
S. cerevisiae strains			
Y1HGold	Yeast one-hybrid strain		Clontech
Y1HGold[PR]	Y1HGold with pPR-AbAi		This study
AH109	Yeast two-hybrid strain		Clontech
E. coli strains			
BL21(DE3)	F^- *ompT hsdS* (rB^-mB^-) *gal* (DE3)		Sigma
GST-Hot1	BL21(DE3) with plasmid pGEX-Hot1		This study

Table 2. Plasmids used in this study.

Plasmid	Description	Reference
pQF181	*Hph*-URA3-*Hph* in pUC18	Lee *et al*. (2014)
pQF182	1-374bp of HOT1 orf in *Eco*RI/*Kpn*I site of pQF181	This study
pQF183	1460-1824bp of HOT1 orf in *SalI*/*Hind*III site of pQF182	This study
pQF184	1-374bp of HOT1 orf in *Hind*III/*Sal*I site of pQF181	This study
pQF185	1154-1460bp of HOT1 orf in *Kpn*I/*Eco*RI site of pGF184	This study
YPB-ADHpt	Promoter and terminator regions of *ADH1* gene in YPB1	Bertram *et al*. (1996)
pADH-HOT1	*C. albicans HOT1* in YPB-ADHpt	This study
pAU13	pBluescript KS(+) with *URA3* gene from *C. albicans*	Uhl and Johnson (2001)
pAU36	pBluescript KS(+) with *ACT1* promoter driving *LacZ* expression	Uhl and Johnson (2001)
pAU95	pBluescript KS(+) with *HWP1* promoter driving *LacZ* expression	Uhl and Johnson (2001)
pENO1LacZ	pBluescript KS(+) with full *ENO1* orf with *LacZ* gene	This study
pGEM-T Easy	PCR product cloning vector	Promega
pPR695	pGEM-T Easy with a 695 bp fragment of the putative promoter region of *PHO81*	This study
pPR595	pENO1LacZ with *PHO81* promoter position -595 - -1	This study
pPR495	pENO1LacZ with *PHO81* promoter position -495 - -1	This study
pPR395	pENO1LacZ with *PHO81* promoter position -395 - -1	This study
pPR295	pENO1LacZ with *PHO81* promoter position -295 - -1	This study
pPR195	pENO1LacZ with *PHO81* promoter position -195 - -1	This study
pPR100	pENO1LacZ with *PHO81* promoter position -100 - -1	This study
pAbAi	Bait plasmid with auerobasidin A resistance	Clontech
pPR-AbAi	*PHO81* Promoter region in *KpnI/ Xho*I site of pAbAi vector	This study
pGEX-5x-1	GST-tag fusion protein expression vector	GE
pGEX-Hot1	pGEX-5x-1 with the *Hot1*	This study
pGADT7-Rec	Prey plasmid for yeast one-hybrid	Clontech
pGADT7-HOT1	*C. albicans HOT1* in pGADT7-Rec	This study
pGADT7 AD	Prey plasmid for yeast two-hybrid	Clontech
pGADT7-HOG1	*C. albicans HOG1* in pGADT7 AD	This study
pGBKT7	Bait plasmid for yeast two-hybrid	Clontech
pGBKT7-HOT1	*C. albicans HOT1* in pGBKT7	This study

Table 3. Primers used in this study.

Primer	Sequence (5′→3′)*	Use
For mutant construction		
HOT11-1F	GAAGAATTCATGAATGAAACTTACAA	
HOT11-1R	TAAGGTACCTTGATAATGAGCAAACT	Construction of
HOT11-2F	GCAGTCGACTGGAAAAATGACAAT	*hot1* 1st mutant
HOT11-2R	GAAAAGCTTTTATGTTGGTGAATTG	
HOT12-1F	GAAAAGCTTATGAATGAAACTTACAA	
HOT12-2R	GCAGTCGACTTGATAATGAGCAAACT	Construction of
HOT12-2F	TAAGGTACCAAACTCATACATTCTT	*hot1* 2nd mutant
HOT12-2R	GAAGAATTCTTCAGATTGTTTCCGT	
For Southern blot analysis		
pQF62	GGATCGATCTATTCCTTTGCCCTCGG	Construction of
HOT1probe-R	GTTGGTGAATTGAACTTTTCAGGCATGTTTG	radiolabeled probe
For β-galactosidase assay		
PR695-F	GCACTGCAGTGAAGCTCAAACTTTAGCTA	
PR595-F	GCACTGCAGATAATCATATACCGTCATTAT	
PR495-F	GCACTGCAGATAATCATATACCGTCATTAT	
PR395-F	GCACTGCAGTAGCTATATAATATGATCTGA	
PR395-R	GCACTGCAGTCAGATCATATTATATAGCTA	Promoter assay
PR295-F	GGCCTGCAGCATCAACTATTATATGTAATAA	
PR195-F	GAACTGCAGAAGAAAGTGGCACACAC	
PR100-F	GAACTGCAGTTCTTTTTTTGGACAACAC	
PR0-R	GGCCTCGAGACCAAATATAAAGTTAAAAG	
For yeast one-hybrid		
CaPR-F	GGCGGTACCATTGAATAATAAATCCTTTGGTA	Construction of
CaPR-R	GGCCTCGAGACCAAATATAAAGTTAAAAG	bait strain
AD-HOT1-F	GAAGAATTCATGAATGAAACTTACAAC	Construction of
AD-HOT1-R	GAACTCGAGTTATGTTGGTGAATTGAA	prey plasmid
For protein expression		
GSTHOT1-F	GGCGAATTCATGAATGAAACTTACAAC	Hot1
GSTHOT1-R	GAACTCGAGTTATGTTGGTGAATTGA	overexpression
For EMSA and DNase I protection assay		
PHO695-F	AGCTATTAGATTTGGTAATAAAGAA	Amplification of
PHO280-R	CCACTTTCTTTTTTTTTCACTATTG	*PHO81*
		upstream region

Table 3. (continued)

Primer	Sequence (5′→3′)*	Use
For RT-PCR		
PHO81RT-F	GGAAGAGGAAGGGGTTCTGG	Expression of
PHO81RT-R	CACCTGATCCGAAGGGAAAA	*PHO81*
HOT1RT-F	TAAAGGACCCACCACTTCAGTAA	Expression of
HOT1RT-R	ACGTTGTAACATAAATTGAGTTGGG	*HOT1*
HOG1RT-F	GGCAAAAAGAACCTACAGAGAAT	Expression of
HOG1RT-R	CAGAATGAATGTATTTTAAACCTCTCA	*HOG1*
CST20RT-F	GTTTGCACCAAGGGGATGATA	Expression of
CST20RT-F	TTGTAAGGACAATGACAAATCTTC	*CST20*
STE11RT-F	GCACTAACAATATAAATGGTAATACC	Expression of
STE11RT-R	GTATCAAATTGAATTTCATCAGGC	*STE11*
CEK1RT-F	TTCAGCCATTCATAAACCATCACA	Expression of
CEK1RT-R	GTAAATCAGTTTCCATCAATTCTTGAA	*CEK1*
CPH1RT-F	TCCTCATGATAAGTTAATGGCAGA	Expression of
CPH1RT-R	TTAAAGTGGTAGCAGTATTAGAAG	*CPH1*
EFG1RT-F	ACAGCCACAACCTCAGCATT	Expression of
EFG1RT-R	GGAAGTGCTCGAGGCGT	*EFG1*
RAS1RT-F	GAAGATGGATTAGCATTGGCTAA	Expression of
RAS1RT-R	TTTGTTGTCCATATTGGTCTTGAC	*RAS1*
GPD1RT-F	AAATGATTATCGTGGTGCCG	Expression of
GPD1RT-R	TATTTTTCAAAGCCCCCGCC	*GPD1*
For overexpression		
ADH-HOT1-F	GGCAGATCTATGAATGAAACTTACAAC	Construction of
ADH-HOT1-R	GAACTCGAGACCAAATATAAAGTTAAAAG	overexpression vector
For yeast two-hybrid		
BD-HOT1-F	GGCGAATTCATGAATGAAACTTACAAC	Construction of
BD-HOT1-R	GAAGTCGACTTATGTTGGTGAATTGAA	bait plasmid
AD-HOG1-F	GAAGAATTCATGTCTGCAGATGGAGA	Construction of
AD-HOG1-R	GAACTCGAGTTAAGCTCCGTTG	prey plasmid

* Regions of primers not complementary to the corresponding genes are underlined.

RESULTS

1. Identification of the *PHO81* transcriptional activator

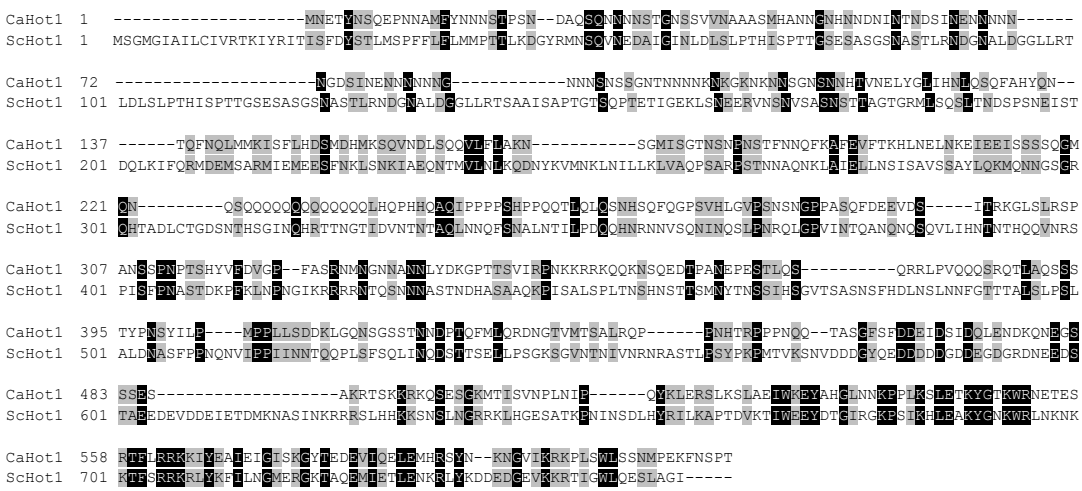
To identify proteins that regulate *PHO81* expression, it was carried out a yeast one-hybrid screen using *PHO81* promoter coding sequences as bait. A search of the Candida genome database (http://candidagenome.org) revealed that the upstream intergenic region of *PHO81* is 600 base pairs (bp). The *PHO81* promoter region (PR) from −595 to −1 bp was cloned into the bait plasmid pAbAi and integrated into the yeast genome (Y1HGold). Yeast cells were then transformed with the prey plasmid pGADT7-Rec containing the *Candida albicans* cDNA library and the prey-bait interaction assay was performed. Using this screening strategy, I identified a positive clone as a DNA-binding element in the *PHO81* promoter. Sequence analysis and conceptual translation of the clone from the genome database revealed an open reading frame (orf19.3328) consisting of 607 amino acids (Fig. 4A). Although this sequence shared only 19% identity with the *Saccharomyces cerevisiae* Hot1, a domain search database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the C-terminus (amino acids 502–579) has 43% conserved amino acid homology with the Gcr1_C domain in *S. cerevisiae* Hot1 (Fig. 4B) (Clifton and Fraenkel, 1981). In *S. cerevisiae*, Hot1 is an osmostress transcription factor and regulates the expression of stress genes primarily involved in glycerol transport and production (Rep *et al*., 1999; Gomar-Alba *et al*., 2015). The DNA-protein interaction was confirmed by one-hybrid assay using pPR (promoter)/AbAi (bait) and pGADT7-Hot1 (prey) (Fig. 4C). The

results suggested that the DNA-binding element in the *PHO81* promoter is for a Hot1-type transcription factor, and was thus named *C. albicans HOT1* (GenBank accession number: KY488487).

Figure 4. Identification of the transcription activator Hot1 that induces expression of *PHO81* in C. *albicans.*

(A) The sequences of Hot1 from *C. albicans* and *Saccharomyces cerevisiae*, as conceptually translated from their DNA sequence, are compared. The alignment was performed using ClustalW; identical sequences (black) and similar sequences (grey) are highlighted. (B) The *HOT1* gene products and the relative arrangement of their C-terminal conserved Gcr1_C domain (filled). (C) Yeast one-hybrid assay. To test DNA-protein interactions, the Y1HGold strain carrying a combination of recombinant pPR/AbAi (bait) and pGADT7-Hot1 (prey) was plated on synthetic defined (SD) medium lacking leucine (SD/-Leu) as a transformation control, or SD/-Leu supplemented with 100 ng/mL aureobasidin A (SD/-Leu/AbA). Plates were incubated at 30℃ for 2 d and photographed. PR: *PHO81* promoter region.

A


CaHot1 1 ---------- MNETYNSQEPNNAMEYNNNSTPSN--DAQSQNNNNSTGNSSVVNAAASMHANNGNHNNDNINTNDSINENNNNNN------
ScHot1 1 MSGMGIAILCIVRTKIYRITISFDYSTLMSPFFLFLMMPITTLKDGYRMNSQVNEDAIGINLDLSLPTHISPTTSSESASGSNASTLRNDGNALDGGLLRT
CaHot1 72 ---------- NGDSINENNNNNNG---------- NNNSNSSGNTNNNNKNKGKNKNNSGNSNNHHVNELYGLIHNLQSQFAHYQN--
ScHot1 101 LDLSLPTHISPTTGSESASGSNASTLRNDGNALDGGLLRTSAAISAPTGTSQPTETIGEKLSNEERVNSNVSASNSTTAGTGRMISQSLTNDSPSNEIST
CaHot1 137 ------TQFNQLMMKISFLHDSMDHMKSQVNDLSQQVLFPAKN---------- SGMISGTNSNENSTFNNQFKQFEVFTKHLNELNKEIEEISSSSQGM
ScHot1 201 DQLKIFQRMDEMSARMIEMEESFNKLSNKIAEQNTMVLNLKQDNYKVMNKLNILLKLVAQPSARESTNNAQNKLAIELLNSISAVSSAYLQKMQNNGSSR
CaHot1 221 QN---------QSQQQQQQQQQQQQQLHQPHHQAQIPPPPSHPPQQTLQLQSNHSQFQGPSVHLGVPSNSNGPPASQFDEEVDS-----IRRKGLSLRSP
ScHot1 301 QHTADLCTGDSNTHSGINQHRTTNGTIDVNTNTAQLNNQFSNALNTILPDQQHNRNNVSQNINQSIPNRQLGPVINTQANQNQSQVLIHNHNTHQQVNRS
CaHot1 307 ANSSPNPTSHYVFDVGP--FASRNMNGNNANNLYDKGPTTSVIRPNKKRRKQQKNSQEDLPANEPPSTLQS----------QRRLPVQQQSRQTLAQSSS
ScHot1 401 PISFPNASTDKPFKLNPNGIKRRRNTQSNNNASTNDHASAAQKPISALSPLTNSHNSTTSMNYTNSSIHSGVTSASNSFHDLNSLNNFGTTTALSLPSL
CaHot1 395 TYPNNSYILP----MPPLLSDDKLGQNSGSSTNNDPTQFMAQRDNGTVMTSALRQP------GNHTRPPPNQQ--TASGFSFDDEIDSIDQLENDKQNEGS
ScHot1 501 ALDNASFPENQNVIPPIINNTQQPLSFSQLINQDSTTSELPSGKSGVNTNIVNRNRASTLGSYPKPMTVKSNVDDDGVQEDDDDDGDDEGDGRDNEEDS
CaHot1 483 SSESS----------AKRTSKKRKQSESGKMTISVNPLNIP------QYKLERSLKSLAEIWKEYAHGLNNKPPLKSLETKYGTKWRNETES
ScHot1 601 TAEEDEVDDEIETDMKNASINKRRRSLHHKKSNSLNGRRKLHGESATKPNINSDLHYRILKAPTDVKTIWEEYDTGIRGKDSIKHLEAKYGNKWRLNKNK
CaHot1 558 RTFLRRKKIYEALEIGISKGYTEDEVHQELEMHRSYN--KNGVIKRKPLSWLSSNMPEKFNSPT
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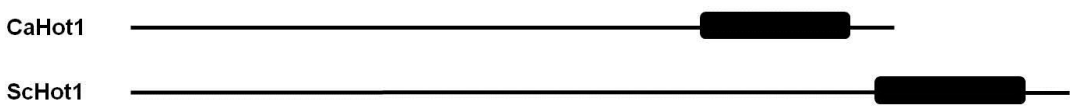
B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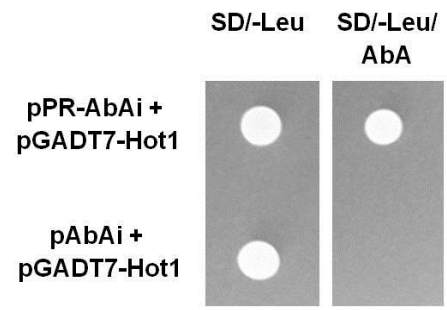
ScHot1

C



2. Determination of the Hot1 binding site in the *PHO81* promoter region

I performed promoter deletion analysis to investigate the region in the *PHO81* promoter that is essential for Hot1 binding. Various lengths of the PCR-generated *PHO81* promoter fragments were cloned into a pENO1LacZ reporter vector (pPR595–pPR100) (Table 2). The resulting vectors were transformed into the *C. albicans* strain CAI4, and cells were grown in yeast extract peptone dextrose (YPD) medium at 28℃ for 8 hours. β-Galactosidase activity was detected by quantitative liquid assay using permeabilized cells (Ausubel *et al*., 1992; Uhl and Johnson, 2001). It was observed the highest level of *lacZ* downregulation in the strain containing the −595 to −1 bp PR (Fig. 5A). While removal of the region from −595 to −495 bp had no significant effect on β-galactosidase activity in cells, deletion of the region from −595 to −395 bp resulted in a severe decrease in expression (62%), indicating that the region from −495 to −395 bp is essential for the highest level of β-galactosidase activity. The DNA-binding property of Hot1 was tested by electrophoretic mobility shift assay (EMSA) using a radioactively labeled *PHO81* promoter element as a target. The PCR-generated *HOT1* gene from the *C. albicans* strain SC5314 was cloned and expressed in *Escherichia coli* BL21(DE3) as previously described (Lee *et al*., 2002). On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the molecular weight of the purified Hot1 was estimated to be 80 kilodaltons (Fig. 5B). A radioactively labeled DNA probe of the *PHO81* PR (from −495 to −395 bp) was incubated with

0.5 μg of purified Hot1 protein and subjected to EMSA. The addition of Hot1 resulted in one retarded band and the unlabeled DNA competed for the binding of Hot1 in a dose-dependent manner (Fig. 5C), indicating that a binding site for Hot1 must be present within this region. To define the Hot1 binding site in the *PHO81* promoter, DNase I protection assays were performed. A DNA fragment of the upstream region of the *PHO81* promoter, extending from −695 to −280, was radioactively labeled and used as a DNA probe. The probe was incubated with increasing amounts of Hot1 and digested with DNase I. As shown in Fig. 6, a region extending from −479 to −449 relative to the transcriptional start site of *PHO81* was protected by Hot1.

Figure 5. Determination of the Hot1 binding site in the upstream region of *PHO81*.

(A) Various lengths of the PCR-generated *PHO81* promoter fragments (pPR595–pPR100) were cloned into a pENO1LacZ reporter vector and transformed into *C. albicans* CAI4. β-Galactosidase activity was detected by quantitative liquid assay using permeabilised cells after grown in YPD medium at 28℃ for 8 h. The activity data were normalised to the expression of pPR595-LacZ, which showed the highest β-galactosidase activity. Each value represents the mean ± standard deviation of three independent replicates. (B) *HOT1* from the *C. albicans* strain SC5314 was cloned and expressed in *E. coli* BL21 (DE3). The purified Hot1 protein was resolved by SDS-PAGE. (C) A DNA fragment of the upstream region of *PHO81* (−495 to −395) was radioactively labeled and used as a probe DNA. The probe DNA (5 nM) was mixed with Hot1 (0.5 μg) and subjected to an EMSA. For competition analysis, the same unlabeled DNA fragment was used as a self-competitor DNA. Various amounts of the self-competitor DNA were added to the reaction mixture containing the 5 nM labeled DNA prior to the addition of 0.5 μg Hot1.

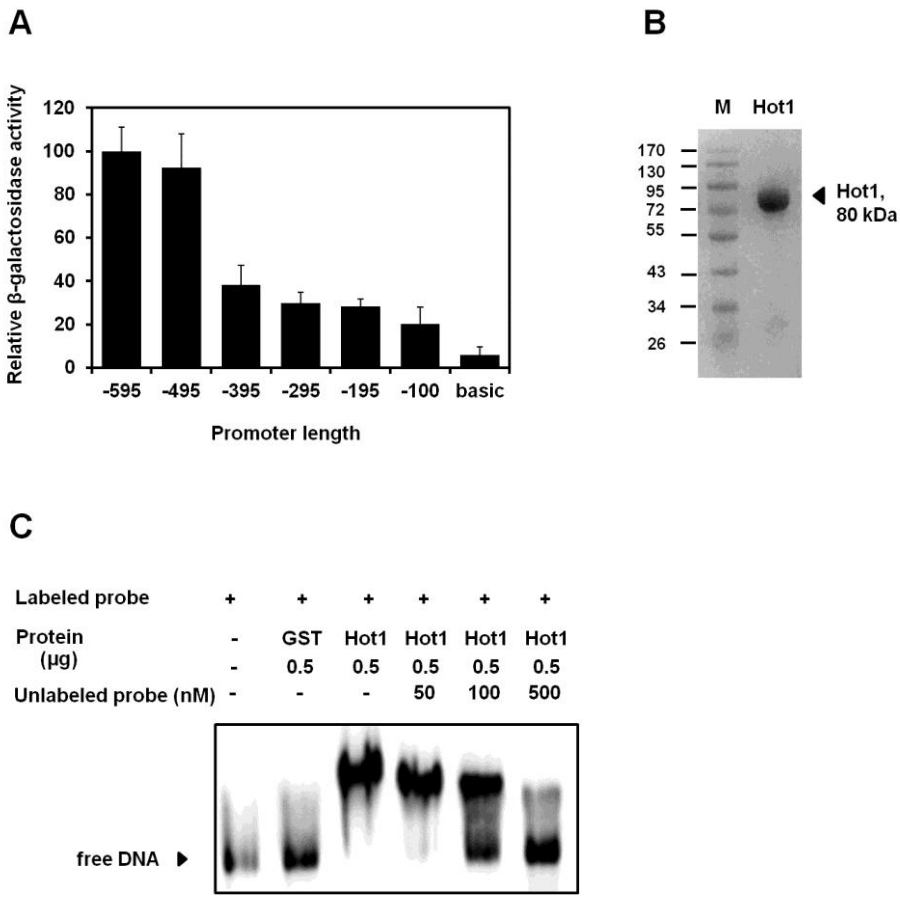
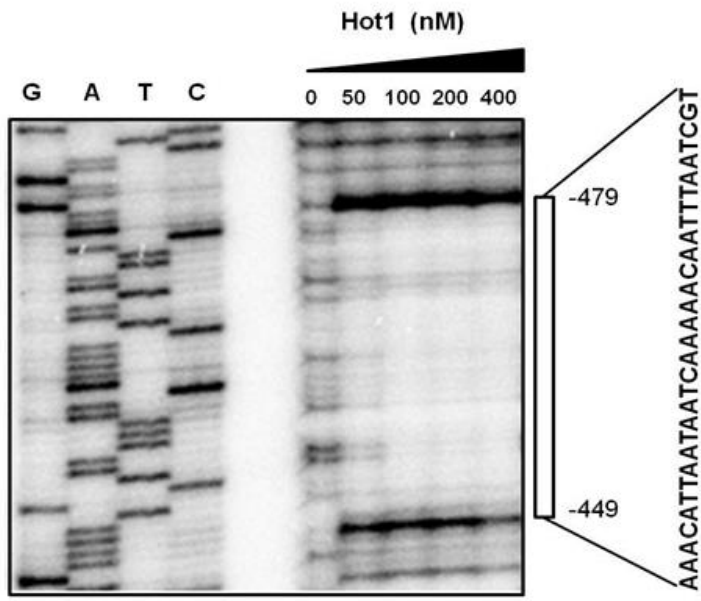

A
Relative β-galactosidase activity
120
100
80
60
40
20
0
-595
-495
-395
-295
-195
-100
basic
Promoter length
B
M
Hot1
170
130
95
72
55
43
34
26
Hot1,
80 kDa
C
Labeled probe + + + + + +
Protein - GST Hot1 Hot1 Hot1 Hot1
(µg) - 0.5 0.5 0.5 0.5 0.5
Unlabeled probe (nM) - - - 50 100 500
free DNA

Figure 6. DNase I footprint analysis of Hot1 DNA complexes.

A 415 bp DNA fragment of the upstream region of *PHO81* was radioactively labeled and then used as a probe DNA. The radiolabeled probe DNA (25 nM) was incubated with increasing amounts of Hot1 and digested with DNase I. The region protected by Hot1 is indicated by the open box. Lanes G, A, T, and C represent the nucleotide sequencing ladders of pPR695. The nucleotide sequences for Hot1 binding are indicated.


Hot1 (nM)
G
A
T
C
0 50 100 200 400
-479
-449
AAACATTAATAATCAAAAACAATTTAATCGT

3. The *hot1Δ* mutant shows constitutive filamentous growth and does not respond to farnesoic acid

To investigate the role of Hot1 in *C. albicans* morphogenesis, it was disrupted both alleles of the gene in two rounds of mutagenesis (Fig. 7). Morphological differences between the wild-type and *hot1Δ* strains were compared under the microscope. Interestingly, the wild-type strain exhibited the expected yeast form, whereas the *hot1Δ* strain was present in the filamentous form under favorable yeast growth conditions (28℃), creating more extended filaments than the wild-type strain at high temperature (37℃) (Fig. 8A). The *hot1Δ* strain expressing wild-type *HOT1* under control of the *ADH1* promoter recovered the wild-type phenotype. Furthermore, the *hot1Δ* mutant existed exclusively as filaments on the nutrient-poor cornmeal agar at 25℃ by microaerobic growth under coverslips (Fig. 8B). The *pho81Δ* cells also grew as filaments, as previously described (Chung *et al*., 2010). Based on these results, I investigated whether Hot1 contributes to the inhibition of hyphal development in *C. albicans* by farnesoic acid (Fig. 9). Wild-type and *hot1Δ* cells were incubated in glucose-salts (GS) medium with or without farnesoic acid (20 μg/mL) at 37℃ for 6 h (Oh *et al*., 2001). As expected, farnesoic acid inhibited hyphal development in wild-type cells. In contrast, *hot1Δ* cells exhibited constitutive filamentous growth and did not respond to farnesoic acid.

Figure 7. Disruption of *C. albicans HOT1*.

(A) The middle line represents the wild-type (SC5314) *HOT1* genomic locus. The bottom line represents the first disruption allele and disruption fragment. The top line represents the second disruption allele and disruption fragment. B: *Bgl*II, E: *Eco*RI, V: *Eco*RV. (B) Southern blot analysis was performed with a ^{32}P-labeled 1.4 kilobase probe amplified by PCR from *HOT1*/*Δhot1* genomic DNA using the primers pQF62 and HOT1probe-R. Genomic DNA samples digested with *Eco*RI and *Bgl*II were prepared from the strains CAI4 (wild-type, *HOT1*/*HOT1*), 4DH (heterozygous, *HOT1*/*Δhot1*), and 4DH2 (homozygous, *Δhot1*/*Δhot1*).

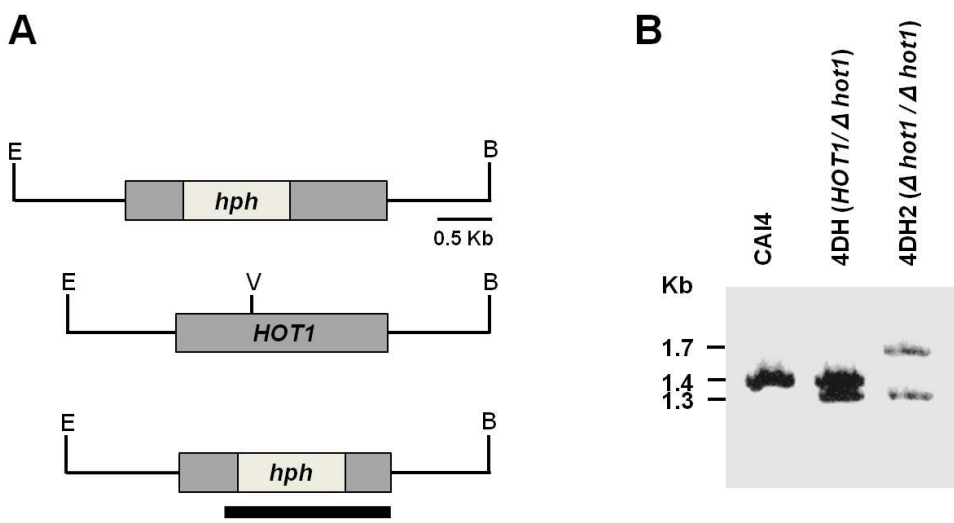

A
E
B
hph
0.5 Kb
E
V
B
HOT1
E
B
hph
B
CAI4
4DH (HOT1/Δ hot1)
4DH2 (Δ hot1 / Δ hot1)
Kb
1.7
1.4
1.3

Figure 8. Morphological characteristics of the *hot1Δ C. albicans*.

(A) Morphological differences between the wild-type (CAI4) and *hot1Δ* mutant (4DH2) were compared under the microscope. Cells were incubated in YPD at 28℃ to early stationary phase, or at 37℃ for 6 h. (B) Cells were placed on cornmeal agar plus Tween-80 (0.33%) plates under a coverslip and incubated at 25℃ for 20 h. Photographs were taken at a magnification of 40× with phase optics. Scale bar = 50 μm.

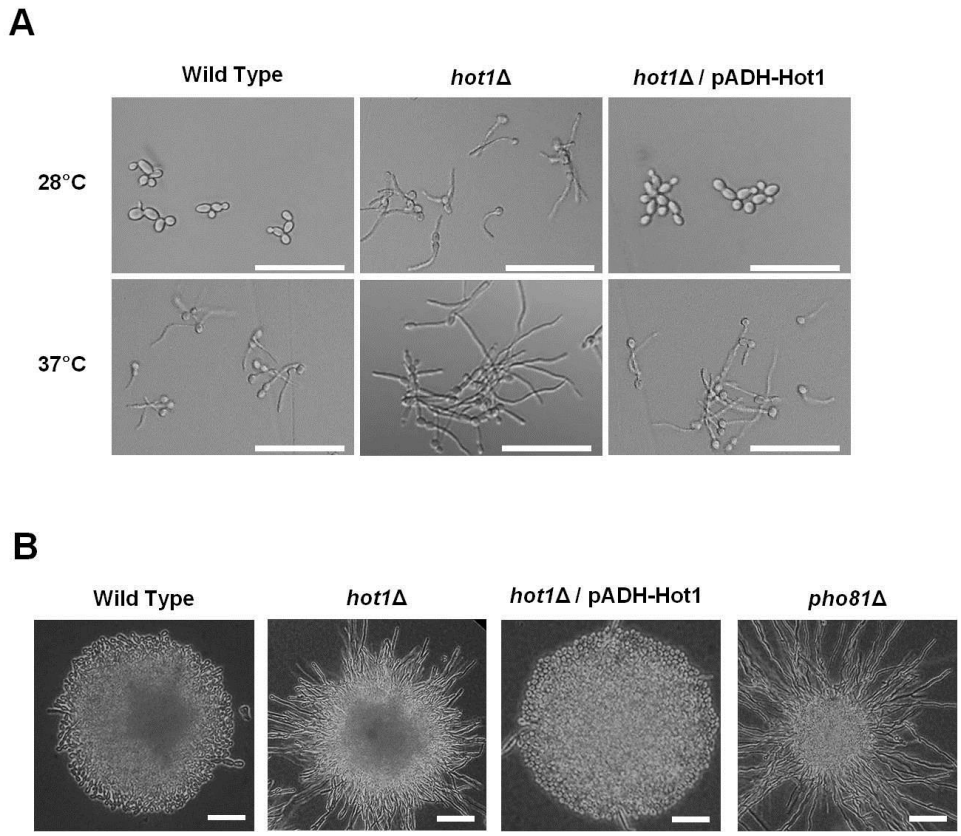
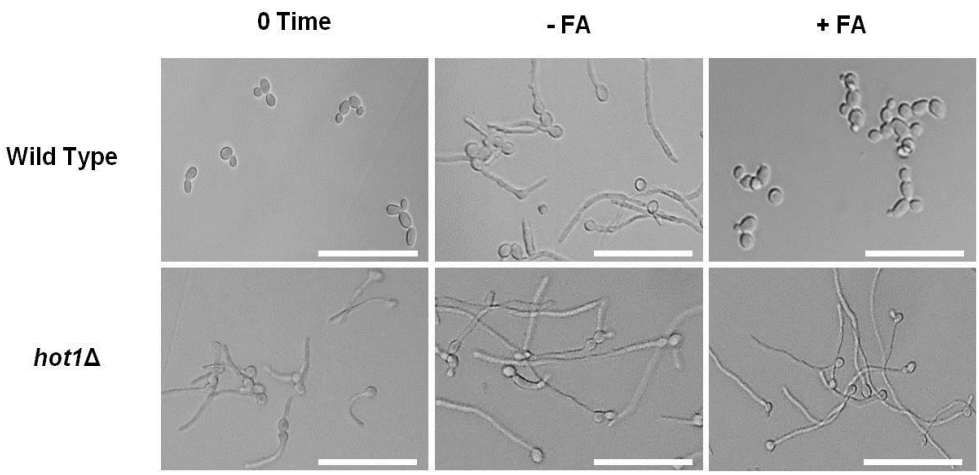

A
Wild Type
hot1Δ
hot1Δ / pADH-Hot1
28°C
37°C
B
Wild Type
hot1Δ
hot1Δ / pADH-Hot1
pho81Δ

Figure 9. Response of the *C. albicans hot1Δ* strain to farnesoic acid.

The wild-type (CAI4) and *hot1Δ* (4DH2) strains containing YPB-ADHpt were incubated in GS medium with 1% dimethyl sulfoxide (-FA) or 20 μg/mL farnesoic acid (+FA) for 6 h at 37℃. Photographs were taken at a magnification of 40× with phase optics. Scale bar = 50 μm.

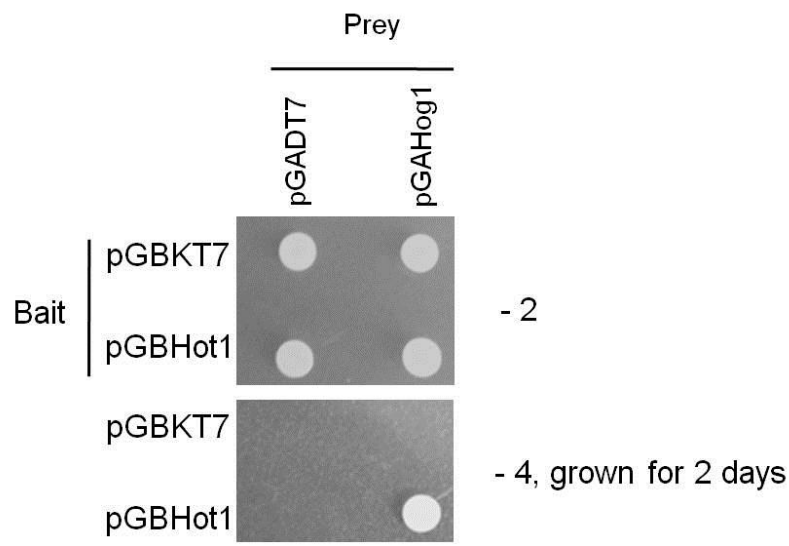

0 Time
- FA
+ FA
Wild Type
hot1Δ

4. Hot1 interacts with *C. albicans* Hog1

In *S. cerevisiae*, the osmotic stress signal is transmitted to the effector Hog1 through a mitogen-activated protein kinase (MAPK) cascade, the HOG MAPK pathway (Hohmann, 2002). Active Hog1 enters the nucleus, allowing Hot1 to bind, together with Hog1, at the regulatory regions of stress responsive genes (Cook and O'Shea, 2012; Gomar-Alba and Del Olmo, 2016). It has also been reported that Hog1 MAP kinase is activated and translocates to the nucleus in response to a diverse range of stress conditions, including oxidative or osmotic stress, in *C. albicans* (Moye-Rowley, 2003; Smith *et al*., 2004; Chauhan *et al*., 2006). Recent work demonstrated that signaling through the HOG MAPK pathway is critical for colonization of the mouse gastrointestinal tract by this organism (Prieto *et al*., 2014). To further explore the relationship between Hot1 and Hog1 in *C. albicans*, it was investigated the Hot1-Hog1 interaction using a yeast two-hybrid assay. The bait (pGBKT7-Hot1) and prey (pGADT7-Hog1) plasmids were constructed and co-transformed into the *S. cerevisiae* strain AH109. From these studies, it was obtained positive evidence for a Hog1-Hot1 interaction (Fig. 10), suggesting that Hog1, together with Hot1, is involved in the regulation of farnesoic acid signaling in *C. albicans*.

Figure 10. Yeast two-hybrid assay of *C. albicans* Hot1-Hog1 protein interaction.

To test the protein interaction, the yeast strain AH109, carrying a combination of the recombinant pGBKT7-Hot1 (bait) and pGADT7-Hog1 (prey), was plated on SD medium lacking leucine and tryptophan (SD/-Leu/-Trp) (-2) as a co-transformation control, or SD medium lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp) (-4). Plates were incubated at 30℃ for 2 d and photographed.


Prey
pGADT7
pGAHog1
Bait
pGBKT7
pGBHot1
- 2
pGBKT7
pGBHot1
- 4, grown for 2 days

5. *PHO81* expression depends on Hot1

Changes in the expression of *HOT1* and *PHO81* were also investigated (Fig. 11A). Total RNA was isolated from *C. albicans* at various times following the addition of 20 μg/mL farnesoic acid at 37℃, and the expression levels were analyzed by reverse transcription (RT)-PCR. Both genes were expressed in the absence of farnesoic acid. However, dramatic increases were observed within 40 minutes of farnesoic acid treatment and continued until at least 240 minutes. *PHO81* expression was activated in farnesoic acid-treated wild-type cells, but not in farnesoic acid-treated *hot1Δ* cells (Fig. 11B), and this defect could be rescued by integration of the wild-type *HOT1* under the control of the *ADH1* promoter. These data indicate that the induction of *PHO81* expression depends on Hot1, suggesting that farnesoic acid inhibits filamentous growth in *C. albicans* through activation of the HOG MAPK pathway, and that Hot1 plays a key role in regulating *PHO81* in the quorum-sensing process.

Figure 11. RT-PCR analysis of *HOT1* and *PHO81* expression.

(A) RT-PCR analysis of *HOT1* and *PHO81* expression in wild-type (CAI4) *C. albicans*. Cells were incubated in GS medium in the presence (+FA) or absence (-FA) of 20 μg/mL farnesoic acid for the indicated times at 37°C and RNA isolation and cDNA synthesis were performed (see Experimental procedures). RT-PCR analysis was conducted with gene-specific primers (Table 3, for RT-PCR). (B) RT-PCR analysis of *PHO81* expression in *hot1Δ* cells. Cells were incubated in GS medium with 20 μg/mL farnesoic acid for the indicated times at 37°C The housekeeping gene glycerol-3-phosphate dehydrogenase (*GPD1*) was used as a loading control.

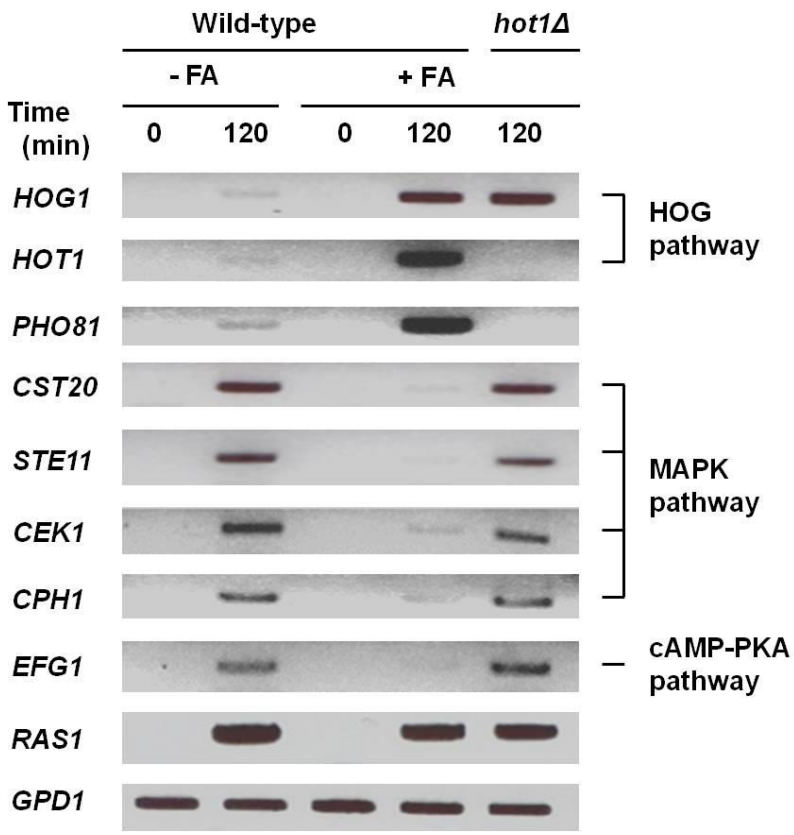

A
- FA
Time (min)
0
20
40
60
120
240
HOT1
PHO81
GPD1
+ FA
Time (min)
0
20
40
60
120
240
HOT1
PHO81
GPD1
B
Wild-type
hot1Δ
hot1Δ / pADH-Hot1
PHO81
GPD1

6. Farnesoic acid inhibits hyphal formation in *C. albicans* through the coordination of different signaling pathways

Based on these studies, it was compared the expression levels of the major signaling pathway components in farnesoic acid-treated wild-type cells with control cells after 2 h incubation in GS medium at 37℃ (Fig. 12). RT-PCR analysis indicated that the expression of *HOG1*, *HOT1*, and *PHO81* was increased in the farnesoic acid-treated wild-type cells, but not in the untreated cells. Interestingly, the expression of the components of the MAPK (*CST20*, *STE11*, *CEK1*, and *CPH1*) and cAMP-PKA (*EFG1*) pathways were repressed in farnesoic acid-treated cells. Expression of these components was restored in the farnesoic acid-treated *hot1Δ* cells. However, similar expression levels of *RAS1* were observed in farnesoic acid-treated wild-type, *hot1Δ*, and farnesoic acid-untreated control cells.

Figure 12. RT-PCR analysis of the expression levels of major signaling pathway components in *C. albicans*.

The wild-type (CAI4) and *hot1Δ* (4DH2) strains were incubated in glucose-salts (GS) medium with (+FA) or without (-FA) 20 μg/mL farnesoic acid for 2 h at 37°C, followed by RNA isolation and cDNA synthesis. RT-PCR analysis was conducted with gene-specific primers (Table 3). Signaling pathway components are coded as follows: *HOG1* and *HOT1*, high osmolarity glycerol (HOG) pathway; *CST20*, *STE11*, *CEK1*, and *CPH1* (transcription factor), mitogen-activated protein kinase (MAPK) pathway; *EFG1* (transcription factor), cAMP-protein kinase A complex (PKA) pathway; *RAS1*, GTPase Ras-like protein 1; *PHO81*, a cyclin-dependent protein kinase inhibitor (whose expression depends on *HOT1*); *GPD1*, glycerol-3-phosphate dehydrogenase (loading control).


Wild-type
hot1Δ
- FA
+ FA
Time (min)
0
120
0
120
120
HOG1
HOT1
PHO81
CST20
STE11
CEK1
CPH1
EFG1
RAS1
GPD1
HOG pathway
MAPK pathway
cAMP-PKA pathway

7. Farnesoic acid and farnesol have different modes of action

It has been reported that *C. albicans* ATCC 10231 produces farnesoic acid while a number of *C. albicans* clinical isolates produce farnesol (Hornby *et al*., 2004). A follow-up question based on our data is whether both quorum-sensing molecules have the same mode of action or not. A yeast-to-hypha transition assay in GS medium indicated that farnesoic acid exhibited a weaker inhibitory activity (IC_{90} = 20.4 μg/mL, 86.3 μM) (Oh *et al*., 2001) than farnesol (IC_{90} = 4.58 μg/mL, 20.6 μM) (Fig. 13). Based on these data, it was investigated whether Hot1 contributes to the inhibition of hyphal development in *C. albicans* by farnesol. Interestingly, at the concentration of 5 μg/mL, farnesol inhibited hyphal development in *hot1Δ* cells (Fig. 14), while farnesoic acid did not (Fig. 9). In addition, RT-PCR analysis indicated that the expressions of *HOT1* and *PHO81* were not increased in the farnesol-treated cells (Fig. 15), indicating that farnesol and farnesoic acid might have different modes of action.

Figure 13. Dose response of *C. albicans* morphology to farnesol.

Data were obtained with our standard assay as inhibition of yeast cells converted to hyphae (see Materials and methods) after 6 h of incubation at 37 ℃ in the GS medium.

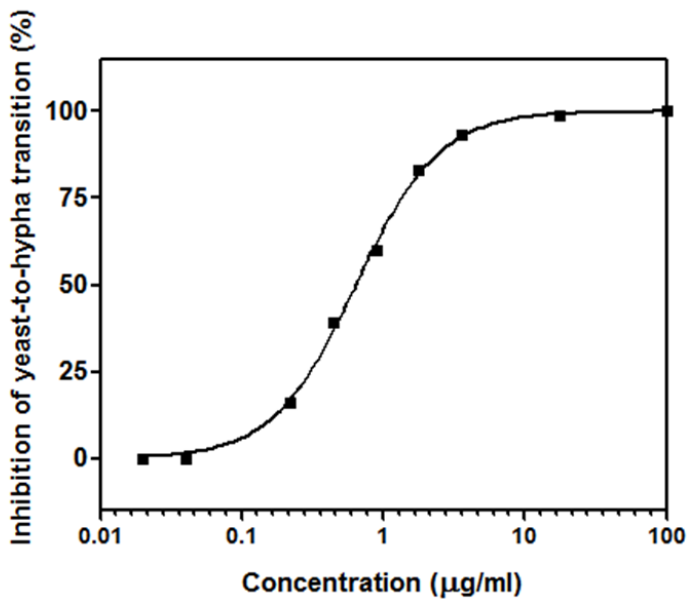

Inhibition of yeast-to-hypha transition (%)
100
75
50
25
0
0.01
0.1
1
10
100
Concentration (μg/ml)

Figure 14. Response of the *hot1Δ C. albicans* to farnesol.

The wild-type (CAI4) and *hot1Δ* (4DH2) strains containing YPB-ADHpt were incubated in GS medium with 1% dimethyl sulfoxide (-FOH) or 5 μg/mL (22.5 μM) (+FOH) for 6 h at 37℃. Photographs were taken at a magnification of 40× with phase optics. Scale bar = 50 μm.

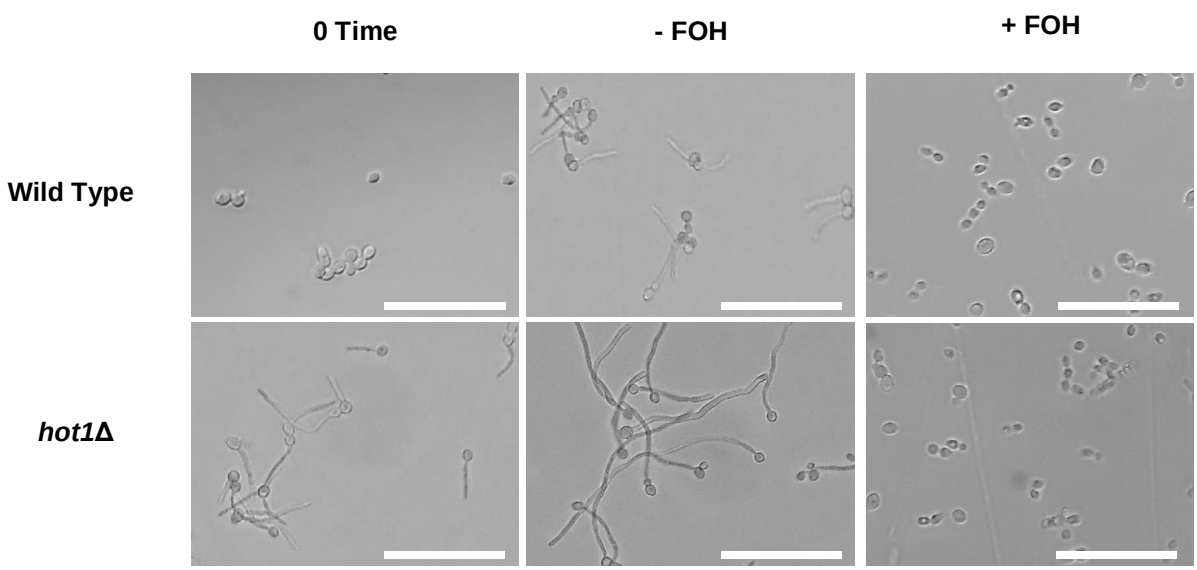
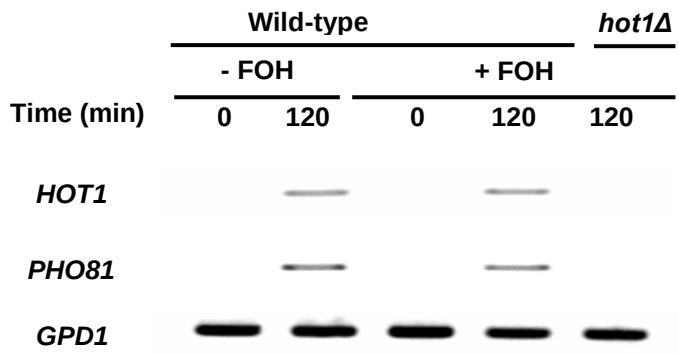

0 Time
- FOH
+ FOH
Wild Type
hot1Δ

Figure 15. RT-PCR analysis of *HOT1* and *PHO81* expression with farnesol.

The wild-type (SC5314) and *hot1Δ* (4DH2) strains were incubated in GS medium with (+FOH) or without (-FOH) 5 μg/mL (22.5 μM) for 2 h at 37℃, followed by RNA isolation and cDNA synthesis. RT-PCR analysis was conducted with gene-specific primers (Table 3). The housekeeping gene glycerol-3-phosphate dehydrogenase (*GPD1*) was used as a loading control.


Wild-type
hot1Δ
- FOH
+ FOH
Time (min)
0
120
0
120
120
HOT1
PHO81
GPD1

DISCUSSION

Farnesoic acid is a natural product that is derived endogenously from isoprene compounds in prokaryotes and eukaryotes including bacteria and fungi. The best known biological functions of farnesoic acid are as a diffusible signal factor that regulates virulence in *Xanthomonas campestris* and as a quorum-sensing molecule that inhibits hyphal formation in *C. albicans* (Oh *et al*., 2001; Wang *et al*., 2004). Farnesoic acid is used by *C. albicans* to communicate with other *Candida* cells, and is secreted into the environment in a density-dependent manner (Oh *et al*., 2001). The formation of hyphae is repressed in cells grown at high densities, whereas cells grown at low densities are able to germinate. Because morphogenetic ability is a key virulence attribute of *C. albicans* and this transition is directly influenced by its environment, the molecular mechanisms involved in farnesoic acid sensing and inhibition of hyphal formation are important for understanding its role in pathogenesis.

It was previously reported that transcriptional upregulation of *PHO81* expression by farnesoic acid is important for the inhibition of hyphal formation (Chung *et al*., 2010). However, upstream effectors responsible for increased *PHO81* expression and sustained hyphal inhibition are unknown. To identify positive regulators, I performed a yeast one-hybrid screen using the *PHO81* promoter coding sequence (600 bp) as bait. I found that Hot1 is a transcription factor required for stimulation of *PHO81* gene expression by farnesoic acid in *C. albicans*. The binding site of Hot1 in the *PHO81* promoter region was identified by

EMSA and DNase I protection assays. These observations indicated that a region extending from −479 to −449 relative to the transcriptional start site is essential for Hot1 binding (Fig. 6). *C. albicans* Hot1 is a functional homolog of *S. cerevisiae* Hot1, which is responsible for activating the core environmental response in this yeast. The *hot1Δ C. albicans* showed constitutive filamentous growth (at 28℃ and 37℃) (Fig. 7) and did not respond to farnesoic acid treatment, confirming the filament-repressing role of *HOT1*.

In response to farnesol, biochemical and genetic analyses of signaling pathways suggest that this molecule regulates the network of multiple signaling pathways in *C. albicans* morphogenesis. For example, the Ras-cAMP-PKA pathway and the general repressor *TUP1* are involved in the regulation of filamentation in *C. albicans* by farnesol (Sato *et al*., 2004; Hall *et al*., 2009; Kebaara *et al*., 2008). Farnesol also inhibits the activity of the Ras-cAMP-Efg1 signaling cascade involved in hyphal formation (Davis-Hanna *et al*., 2008). More recently, Polke et al. reported that farnesol directly binds to and inhibits the function of the *Candida* adenylyl cyclase Cyr1p (Polke *et al*., 2017).

The Hog1 MAPK protein was initially described in *S. cerevisiae*. Additionally, in *S. cerevisiae*, Hot1 was identified in a two-hybrid screen using the *HOG1* coding sequence as bait (Gomar-Alba *et al*., 2015). Under conditions of osmotic stress, Hot1 regulates the expression of some stress genes, mainly those involved in glycerol biosynthesis, and the interaction between Hog1 and Hot1 directs the localization of RNA polymerase II to these promoters (Gomar-Alba *et al*., 2016). The HOG MAPK pathway also plays an important role in stress responses in *C. albicans* (Alonso-Monge *et al*., 1999; Enjalbert *et al*., 2006). Hog1

is activated by oxidative stress, osmotic stress, heavy metal, and the purine analog caffeine in *C. albicans* cells, and is required for survival. A previous study indicated that Hog1 exhibits rapid and sustained activation upon exposure to the quorum-sensing molecule, farnesol, in *C. albicans* (Smith *et al*., 2004). However, the authors noted that *hog1Δ* and wild-type cells grew equally well in the presence of farnesol. In this study, it was demonstrated for the first time that *HOT1* is rapidly expressed when *C. albicans* cells are exposed to farnesoic acid (Fig. 8). The wild-type strain exhibited the yeast form, whereas the *hot1Δ* strain was present in the filamentous form under favorable yeast growth conditions, indicating that this gene is a major component of the farnesoic acid-sensing pathway. To verify the relationship between Hot1 and Hog1 in *C. albicans*, the Hot1-Hog1 protein interaction is under investigation.

Despite the importance of pathways in hyphal development, how *C. albicans* coordinates information from different signaling pathways in regulating the morphological transition remains unclear. In *C. albicans*, positive regulation of hypha-specific gene expression is mainly mediated through the MAPK and cAMP-PKA signaling pathways (Sudbery *et al*., 2011). Ras1 stimulates both pathways (Leberer *et al*., 2001). To investigate the regulatory networks that control morphogenesis of *C. albicans* by farnesoic acid, it was compared the expression levels of these major signaling pathway components in farnesoic acid-treated wild-type cells with control cells. When cells were exposed to farnesoic acid, Hot1 was induced, allowing the expression of Pho81 and subsequent repression of active Ras1 (GTP-Ras1)-mediated MAPK and cAMP-PKA signaling pathways. In the absence of farnesoic acid, Hot1 was not induced, thereby repressing Pho81

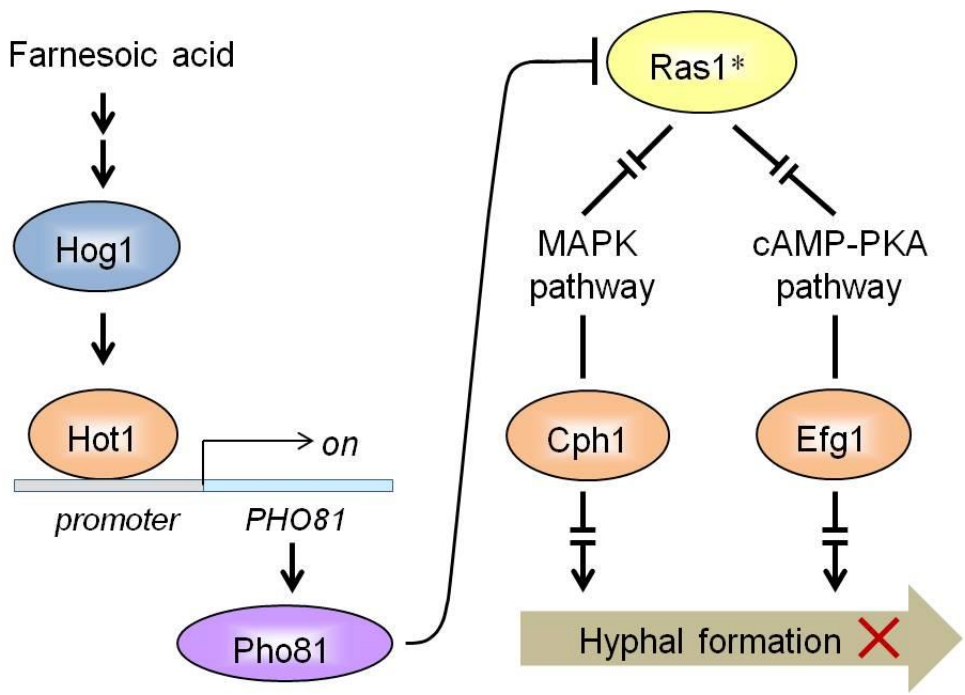
expression; the repression of Hot1 expression, in turn, releases the Ras1 signal to hyphal development signaling pathways (Fig. 11). In a previous study, it was demonstrated that repression of the transcription factors *CPH1* (MAPK pathway) and *EFG1* (cAMP-PKA pathway) was defective in a *pho81*Δ strain, indicating that Pho81 acts upstream of both pathways. A *C. albicans* strain expressing a dominant-active Ras1 ($Ras1^{G13V}$) resulted in hyphal growth in unfavourable hyphal development conditions (Tris-Cl buffer, pH 7.0 at 37℃). Following incubation with 20 μg/mL farnesoic acid under the same conditions, hyphal formation was not inhibited in the *pho81Δ*-$Ras1^{G13V}$ strain. The results of a Ras pull-down assay indicated that GTP-Ras1 (active Ras1) levels in the *pho81Δ* cells were two-fold higher compared with those of the wild-type cells, indicating that transfer of the Ras1 signal to the downstream MAPK and cAMP-PKA pathways is inhibited as a result of the active Ras1. Based on this, and on previous study, I present a simple model to explain the control of hyphal growth in *C. albicans* by farnesoic acid (Fig. 16).

Together with previous report, these results provide insight into the mechanism by which the cell density signal is integrated in the regulation of hyphal formation in *C. albicans*. In this study, it was showed that the Hot1-mediated signaling pathway has evolved in *C. albicans* to control yeast-hypha morphogenesis in response to farnesoic acid. Although the mechanism by which Pho81 regulates Ras1 signaling in response to farnesoic acid is unclear, the data presented in this paper indicate that this quorum-sensing molecule controls hyphal formation in *C. albicans* through the coordination of different signaling pathways. As a previous results, it is likely that Pho81 directly or indirectly inhibit active

Ras1, thereby inhibiting the hyphal formation. Therefore, subsequent experiments should be performed to identify what mechanism Pho81 has to inhibit active Ras1. Further characterization of Pho81 in *C. albicans* will provide greater mechanistic insight into its function and facilitate the development of new strategies for producing antifungal therapies.

Figure 16. Model for control of hyphal growth in *C. albicans* by farnesoic acid.

The quorum-sensing signal, farnesoic acid, is transmitted to the transcription factor Hot1 through the Hog1 MAP kinase. Hot1 binds to the *PHO81* PR and stimulates the expression of *PHO81*, which can then impact Ras1 signaling to inhibit hyphal formation. Further downstream, transfer of the Ras1 signal to the MAPK and cAMP-PKA pathways, major pathways for hyphal development, is inhibited due to the inability of active Ras1 (*), which stimulates both pathways. Cph1 and Efg1 are transcription factors.


Farnesoic acid
Hog1
Hot1
on
promoter
PHO81
Pho81
Ras1*
MAPK
pathway
cAMP-PKA
pathway
Cph1
Efg1
Hyphal formation

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ABSTRACT IN KOREAN

환경 조건에 반응하여 효모와 균사 형태 사이를 전환하는 *Candida albicans*는 이 유기체의 독성에 기여하는 것으로 알려져 왔다. *Candida albicans*의 균사 형성은 세포가 증식함에 따라 배지에 축적되는 quorum-sensing molecule인 farnesoic acid에 의해 저해된다. 형태 형성 변화에 관여하는 많은 신호 전달 경로와 조절 인자가 집중적인 연구에 의해 밝혀졌지만, farnesoic acid에 의한 분자 네트워크는 잘 알려져 있지 않다. 최근 cyclin-dependent protein kinase inhibitor인 Pho81이 farnesoic acid에 의한 균사 형성을 억제한다는 것이 입증되었다. *PHO81* 유전자가 결손된 돌연변이 균주 (*pho81Δ*)는 실험한 조건 아래에서 균사형으로만 성장했다. 이 연구에서는 *PHO81*의 발현을 증가시키는 새로 확인된 조절 인자 Hot1을 설명하려고 한다. Yeast one-hybrid assay로 cDNA library를 screening함으로써, *Candida albicans*의 유전자 *HOT1*이 밝혀졌으며, 이는 osmostress 전사 인자인 *Saccharomyces cerevisiae* Hot1의 상동인 단백질을 코딩 한다. *HOT1*의 전체 cDNA의 서열 분석 및 유사성 검색 결과 *S. cerevisiae*의 Hot1 단백질과 19% 유사성을 갖고 607개의 아미노산으로 구성된 단백질을 암호화 하고 있는 것으로 나타났다. Gcr1_C domain (502–579)이 보존되었고, 아미노산 서열은 ScHot1과 43%의 상동성을 보였다. *PHO81* 프로모터 영역에서 Hot1의 결합 부위는 electrophoretic mobility shift assay (EMSA) 및 DNase1 protection assay에 의해 조사되었다. *HOT1*의 결합 부위에 대하여 -479에서 -449까지의 영역이 확인되었다. *C. albicans*에서 farnesoic acid에 의한 균사 성장 억제에서 *HOT1*의 생물학적 역할을 연구하기 위해 *HOT1* 유전자가 결손 된 돌연변이 균주 (*hot1Δ*)를 제작하고 형태학적 특성을 야생형 세포와 비교하였다. *hot1Δ* 돌연변이 균주는 균사형으로 광범위하게 성장했으며 야생형 세포와는 다르게

farnesoic acid에는 반응하지 않았다. 이러한 *hot1Δ* 돌연변이 균주의 표현형은 *ADH1* 프로모터의 조절 하에 *CaHOT1* 유전자를 과발현 시킴으로써 야생형의 표현형을 회복하였다. 이러한 결과로부터, *CaHOT1*은 균사 성장을 억제하는 역할을 하고 farnesoic acid에 의한 균사 발생의 억제를 위한 새로운 분자라는 결론을 얻었다. Two-hybrid assay 실험은 Hot1이 Hog1 mitrogen-activated protein kinase (MAPK)와 상호 작용함을 보여 주었다. 역전사 중합 효소 연쇄 반응 (reverse transcription-polymerase chain reaction, RT-PCR) 에 의한 주요 신호 전달 경로 구성 요소의 발현 수준 분석은 farnesoic acid가 HOG MAPK 경로의 조정을 통해 *C. albicans*에서 균사 형성을 억제한다는 것을 보여 주었다. 이러한 결과를 종합하였을 때, Hot1이 *C. albicans*에서 *PHO81* 전사인자이며 farnesoic acid에 의한 균사 성장의 억제에 중요한 구성 요소임을 말해준다.

주요어: *Candida albicans*, 균사 발달, 신호조절물질, farnesoic acid, 형태 형성, Hot1, 신호 전달 경로

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