

# A Novel Function for Hog1 Stress-Activated Protein Kinase in Controlling White-Opaque Switching and Mating in *Candida albicans*

Shen-Huan Liang, Jen-Hua Cheng, Fu-Sheng Deng, Pei-An Tsai, Ching-Hsuan Lin

Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei, Taiwan

*Candida albicans* is a commensal in healthy people but has the potential to become an opportunistic pathogen and is responsible for half of all clinical infections in immunocompromised patients. Central to understanding *C. albicans* behavior is the white-opaque phenotypic switch, in which cells can undergo an epigenetic transition between the white state and the opaque state. The phenotypic switch regulates multiple properties, including biofilm formation, virulence, mating, and fungus-host interactions. Switching between the white and opaque states is associated with many external stimuli, such as oxidative stress, pH, and *N*-acetylglucosamine, and is directly regulated by the *Wor1* transcriptional circuit. The Hog1 stress-activated protein kinase (SAPK) pathway is recognized as the main pathway for adapting to environmental stress in *C. albicans*. In this work, we first show that loss of the *HOG1* gene in *a/a* and  $\alpha/\alpha$  cells, but not *a/\alpha* cells, results in 100% white-to-opaque switching when cells are grown on synthetic medium, indicating that switching is repressed by the  $a1/\alpha2$  heterodimer that represses *WOR1* gene expression. Indeed, switching in the *hog1* $\Delta$  strain was dependent on the presence of *WOR1*, as a *hog1* $\Delta$  *wor1* $\Delta$  strain did not show switching to the opaque state. Deletion of *PBS2* and *SSK2* also resulted in *C. albicans* cells switching from white to opaque with 100% efficiency, indicating that the entire Hog1 SAPK pathway is involved in regulating this unique phenotypic transition. Interestingly, all Hog1 pathway mutants also caused defects in shmoo formation and mating efficiencies. Overall, this work reveals a novel role for the Hog1 SAPK pathway in regulating white-opaque switching and sexual behavior in *C. albicans*.

*Candida albicans* is a diploid hemiascomycete yeast that is commonly found in the human gastrointestinal tract (1, 2). However, *C. albicans* can become a pathogen that attacks multiple organs in the body, including kidney and brain, resulting in life-threatening systemic infections with high mortality rates (3). Phenotypic transitions represent an important mechanism for the striking ability of *Candida* cells to colonize a wide variety of habitats with diverse properties. For example, under conditions of various environmental signals, the yeast forms of *C. albicans* can switch to filamentous cells (4–8). The reversible yeast-hypha transition regulated by a core set of transcription factors that mediate its developmental program is complex (7, 9–13). Hyphal formation also plays a major role in biofilm formation, which is closely linked with the propensity to cause infection and is associated with antifungal resistance (14–16).

In addition to the yeast-hypha transition, an important morphological change in *C. albicans* is the white-opaque switch (17). This epigenetic transition between white and opaque states regulates many aspects of *C. albicans* biology. In particular, opaque cells are mating competent (18, 19), whereas white cells do not mate but can generate biofilms in response to pheromones (20, 21). The regulation of mating between *C. albicans a/a* and  $\alpha/\alpha$  opaque cells occurs via pheromone signaling between cells of opposite mating types (18, 19, 22, 23). Pheromone responses induce a mitogen-activated protein kinase (MAPK) signal transduction cascade leading to the formation of mating projections (shmoos) and subsequent cell-cell conjugation (21, 24). Furthermore, opaque cells are less susceptible to phagocytosis than white cells due to the lack of secretion of a chemoattractant for leukocytes (25–27), indicating that switching plays a role in host-fungus interactions. Thus, the phenotypic plasticity of *C. albicans* in response to different host niches is important for its ability to colonize and infect the host.

*C. albicans* cells typically exist in the default white state, where

they are round and form bright, shiny colonies, but can stochastically switch to the opaque state, where cells are more elongated and form darker, flatter colonies (17). The white-opaque switch is regulated by the master regulator *Wor1*, which acts in concert with several other transcription factors as part of a transcriptional network (28, 29). This network is also controlled by the  $a1/\alpha2$  complex which is produced in *a/\alpha* heterozygous cells and which represses opaque-cell formation (30), although a recent study showed that some clinical *a/\alpha* cells have the ability to switch to opaque under certain culture conditions (31). Several environmental factors, including CO<sub>2</sub>, temperature, oxidative stress, *N*-acetylglucosamine (NAG), and growth rate, also regulate white-opaque switching (32–34), suggesting that the transition between white and opaque cells is extremely sensitive to external signals and dependent on the expression levels of the master regulator *Wor1*.

In eukaryotic cells, MAP kinases are responsible for transducing a variety of extracellular signals for growth and differentiation (35, 36). One of them, the high-osmolarity glycerol (Hog1) pathway, is not only responsible for the cellular response to osmotic stress but is also required for responses to UV radiation, temperature, and oxidative stress in the budding yeast (37–39). This pathway is known as the Hog1 stress-activated protein kinase (SAPK) pathway or the Hog1 mitogen-activated protein kinase (MAPK) pathway. Osmosensing triggers Hog1 activation through

Received 13 October 2014 Accepted 17 October 2014

Published ahead of print 24 October 2014

Address correspondence to Ching-Hsuan Lin, chinghsuanlin@ntu.edu.tw.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/EC.00235-14

the stimulation of a MAPK kinase kinase (MAPKKK), Ssk2, and a MAPK kinase (MAPKK), Pbs2 (35, 39, 40).

In regard to white-opaque switching, several stresses have been found to promote the switch from white to opaque (32–34). We therefore hypothesized that the Hog1 SAPK pathway of *C. albicans* could be involved in regulating this phenotypic transition. In this study, the results of deletion of *HOG1*, *PBS2*, and *SSK2* genes in *C. albicans* *MTLa/a*, *MTL $\alpha$ / $\alpha$* , and *MTLa/ $\alpha$*  strains were compared. Mutants in all three strain backgrounds showed high sensitivity to osmotic and oxidative stress, indicating that the roles of the Hog1 SAPK pathway are similar in different cell types. Interestingly, homozygous *MTLa/a* and *MTL $\alpha$ / $\alpha$*  Hog1 pathway mutants exhibit 100% switching from white to opaque but also display shorter shmoo and have reduced mating efficiencies. Overall, our data therefore reveal a novel role for the Hog1 SAPK pathway in white-opaque switching and indicate a further route for the integration of environmental signals into the white-opaque regulatory circuit.

## MATERIALS AND METHODS

**Media and reagents.** Yeast extract-peptone-dextrose (YPD), Spider, and synthetic complete dextrose (SCD) media used in experiments were prepared as described previously with slight modifications (38, 41). YPD medium, composed of 2% (wt/vol) peptone, 1% (wt/vol) yeast extract, and 2% (wt/vol) glucose, was used for cell growth experiments. Nourseothricin-resistant strains were selected on YPD medium supplemented with 0.2 mg/ml nourseothricin (Werner BioAgents). SCD consisted of 0.7% (wt/vol) yeast nitrogen base without amino acids, 0.17% complete amino acids powder, and 2% (wt/vol) glucose. Synthetic complete (SC) medium was formulated as SCD medium without the addition of glucose. Preparation of Lee's *N*-acetylglucosamine (Lee's NAG), which contained 1.25% *N*-acetylglucosamine (Alfa Aesar), followed an established protocol (34). SCD, SC, and Lee's NAG media were used for white-opaque phenotypic transition assays. Spider medium (pH 7.2), containing 1% (wt/vol) mannitol, 1% (wt/vol) nutrient broth, and 0.4% (wt/vol) dipotassium phosphate (Showa Chemical Industry Co.), was used for opaque cell maintenance. Leu<sup>-</sup>, Arg<sup>-</sup>, and Arg<sup>-</sup> Leu<sup>-</sup> media were used to perform a quantitative mating assay. Agar was added to reach a final concentration of 2% (wt/vol) to make solid plates. All chemicals were purchased from Sigma-Aldrich Chemical Co., unless otherwise stated.

**Plasmid and strain constructions.** Strains and primers used in this study are listed in Table 1 and Table 2, respectively.

To generate *hog1 $\Delta$*  strains, 5' and 3' flanking regions of *HOG1* were amplified using primer pair CH1 and CH2 and primer pair CH3 and CH4. The 5' and 3' PCR products were digested with *Apa*I and *Xho*I and with *Sac*II and *Sac*I and cloned into plasmid pSFS2A (42) to generate pSFS-HOG1 knockout (KO). This plasmid was linearized with *Apa*I and *Sac*I and then transformed into SC5314 (a/a), RBY717 (a/a), and RBY722 ( $\alpha$ /a) to generate strains YL31, YL34, YL8, YL9, YL26, and YL27. To construct the *HOG1* complementary plasmid, primers 10 and 11 were used to amplify the promoter and open reading frame (ORF) of the *HOG1* gene. The PCR product was digested with *Apa*I and *Xho*I and cloned into pSFS2A to construct pSFS-HOG1 AB. This construct was linearized with *Bsa*BI and transformed into YL31, YL34, YL8, YL9, YL26, and YL27 to create the YL38, YL39, YL11, YL12, YL35, and YL36 strains.

For the *wor1 $\Delta$*  mutant and *hog1 $\Delta$  wor1 $\Delta$*  double mutant, the pSFS-WOR1 KO plasmid (32) was cut with *Kpn*I and *Sac*I and transformed into RBY717 and YL8 to create strains YL135 and YL136.

To create the *ssk2 $\Delta$*  mutants, 5' and 3' flanking regions of the *SSK2* gene were amplified using primer pair 70 and 71 and primer pair 72 and 73. The 5' and 3' PCR products were digested with *Kpn*I and *Apa*I and with *Sac*II and *Sac*I and cloned into pSFS2A to generate pSFS-SSK2 KO. *Kpn*I and *Sac*I were used to linearize these constructs. Linearized pSFS-

SSK2 KO plasmid was transformed into SC5314, RBY717, and RBY722 to generate the YL442, YL443, YL193, YL194, YL402, and YL403 strains.

For *pbs2 $\Delta$*  strains, 5' and 3' flanking regions of the *PBS2* gene were amplified using primer pair 78 and 79 and primer pair 80 and 81. The 5' and 3' PCR products were digested with *Apa*I and *Xho*I and with *Sac*II and *Sac*I. The digested PCR products were cloned into plasmid pSFS2A to generate the pSFS-PBS2 KO plasmid. The constructed plasmid was linearized with *Nhe*I and *Sac*I and transformed into SC5314, RBY717, and RBY722 to generate the YL404, YL405, YL195, YL196, YL400, and YL401 strains.

To generate green fluorescent protein (GFP) reporter strains of *OP4/OP4::OP4p-GFP* in RBY717 and the *hog1 $\Delta$*  mutant, the *OP4* promoter and GFP gene were amplified with primer pair 191 and 192 and primer pair 193 and 194, respectively. The PCR products were mixed and amplified again with primer pair 191 and 194 to generate an *OP4*-GFP gene fragment. The fused fragment was digested with *Apa*I and *Sac*I and cloned into pSFS2A to construct pSFS-OP4-GFP. The construct was digested with *Bgl*I and transformed into RBY717, YL8, and YL9 to create strains YL275, YL276, YL279, and YL280.

To delete the *ARG4* gene in *C. albicans*, the *hog1 $\Delta$* , *hog1 $\Delta$ ::HOG1*, *pbs2 $\Delta$* , and *ssk2 $\Delta$*  mutants, primer pair 114 and 115, and primer pair 116 and 117 were used to amplify the 5' and 3' flanking regions of the *ARG4* gene, respectively. The PCR products were digested with *Apa*I and *Xho*I and *Not*I and *Sac*II and cloned into plasmid pSFS2A to construct pSFS-ARG4 KO. The construct was digested with *Apa*I and *Sac*II and transformed into RBY717, YL26, YL27, YL35, YL36, YL193, YL194, YL195, and YL196 to generate the YL97, YL98, YL304, YL305, YL328, YL329, YL528, YL529, YL524, and YL525 strains.

To delete the *LEU2* gene, the *hog1 $\Delta$* , *hog1 $\Delta$ ::HOG1*, *pbs2 $\Delta$* , and *ssk2 $\Delta$*  mutants, primer pair 122 and 123, and primer pair 124 and 125 were used to amplify the 5' and 3' flanking regions of *LEU2* gene, respectively. The PCR products were digested with *Apa*I and *Xho*I and with *Sac*II and *Sac*I and cloned into plasmid pSFS2A to construct pSFS-LEU2 KO. The construct was digested with *Apa*I and *Sac*I and transformed into RBY722, YL8, YL9, YL11, YL12, YL402, YL403, YL400, and YL401 to generate the YL133, YL134, YL191, YL192, YL226, YL227, YL530, YL531, YL526, and YL527 strains.

Opaque cells of each strain were obtained and purified after treatment with Lee's NAG (Table 1).

**Quantitation of opaque-cell formation.** Overnight cultures of *C. albicans* were plated onto SC, SCD, or Lee's NAG media supplemented with phloxine B and incubated at 25°C for 4 to 7 days. Opaque colonies were stained red. The white-to-opaque switching efficiency was expressed as follows: (number of opaque colonies)/(total number of colonies)  $\times$  100%.

**Sensitivity tests.** Hydrogen peroxide and sodium chloride were purchased from Sigma-Aldrich Co., St. Louis, MO, USA, and MDBio, Inc., Shandong, China, respectively. Determinations of susceptibilities to hydrogen peroxide and sodium chloride were performed by spotting serial 4-fold dilutions of 10<sup>5</sup> cells (total volume of 3  $\mu$ l) from overnight cultures of *C. albicans* onto YPD plates supplemented with 10 mM H<sub>2</sub>O<sub>2</sub> or 1 M NaCl. Plates were incubated at 30°C and examined after 48 h.

**Western blotting.** To examine the level of Hog1 phosphorylation, 200  $\mu$ l of overnight cultures was diluted into 10 ml of YPD broth and grown for 5 h at 30°C. H<sub>2</sub>O<sub>2</sub> was added to each culture to reach a final concentration of 10 mM, and the reaction mixture was incubated for 15 min after the addition. Samples were collected by centrifugation, suspended in 10 mM Tris-HCl (pH 8.0), and then incubated at 100°C for 10 min to lyse cells. Equal amounts of proteins were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). ScHog1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) were used to detect Hog1 and phos-

TABLE 1 Strains used in this study<sup>a</sup>

Strain	MTL type	Genotype	White or opaque	Reference or source
SC5314	a/α	Wild type	White	22
RBY717	a/a	<i>ura3::imm434/URA3 iro1::imm434/IRO1</i>	White	22
RBY722	α/α	<i>ura3::imm434/URA3 iro1::imm434/IRO1</i>	White	22
YL8	a/a	<i>hog1/hog1</i>	White	This study
YL9	a/a	<i>hog1/hog1</i>	White	This study
YL11	a/a	<i>hog1/hog1::HOG1</i>	White	This study
YL12	a/a	<i>hog1/hog1::HOG1</i>	White	This study
YL26	α/α	<i>hog1/hog1</i>	White	This study
YL27	α/α	<i>hog1/hog1</i>	White	This study
YL31	a/α	<i>hog1/hog1</i>	White	This study
YL34	a/α	<i>hog1/hog1</i>	White	This study
YL35	α/α	<i>hog1/hog1::HOG1</i>	White	This study
YL36	α/α	<i>hog1/hog1::HOG1</i>	White	This study
YL38	a/α	<i>hog1/hog1::HOG1</i>	White	This study
YL39	a/α	<i>hog1/hog1::HOG1</i>	White	This study
YL45	a/a	<i>ura3::imm434/URA3 iro1::imm434/IRO1</i>	Opaque	This study
YL46	a/a	<i>ura3::imm434/URA3 iro1::imm434/IRO1</i>	Opaque	This study
YL47	α/α	<i>ura3::imm434/URA3 iro1::imm434/IRO1</i>	Opaque	This study
YL48	α/α	<i>ura3::imm434/URA3 iro1::imm434/IRO1</i>	Opaque	This study
YL97	a/a	<i>arg4/arg4</i>	White	This study
YL98	a/a	<i>arg4/arg4</i>	White	This study
YL133	α/α	<i>leu2/leu2</i>	White	This study
YL134	α/α	<i>leu2/leu2</i>	White	This study
YL135	a/a	<i>wor1/wor1</i>	White	This study
YL136	a/a	<i>hog1/hog1 wor1/wor1</i>	White	This study
YL191	a/a	<i>hog1/hog1 leu2/leu2</i>	White	This study
YL192	a/a	<i>hog1/hog1 leu2/leu2</i>	White	This study
YL193	a/a	<i>ssk2/ssk2</i>	White	This study
YL194	a/a	<i>ssk2/ssk2</i>	White	This study
YL195	a/a	<i>pbs2/pbs2</i>	White	This study
YL196	a/a	<i>pbs2/pbs2</i>	White	This study
YL226	a/a	<i>hog1/hog1::HOG1 leu2/leu2</i>	White	This study
YL227	a/a	<i>hog1/hog1::HOG1 leu2/leu2</i>	White	This study
YL234	a/a	<i>hog1/hog1 leu2/leu2</i>	Opaque	This study
YL235	a/a	<i>hog1/hog1 leu2/leu2</i>	Opaque	This study
YL238	a/a	<i>arg4/arg4</i>	Opaque	This study
YL239	a/a	<i>arg4/arg4</i>	Opaque	This study
YL259	a/a	<i>hog1/hog1</i>	Opaque	This study
YL260	a/a	<i>hog1/hog1</i>	Opaque	This study
YL267	α/α	<i>hog1/hog1</i>	Opaque	This study
YL268	α/α	<i>hog1/hog1</i>	Opaque	This study
YL271	a/a	<i>hog1/hog1::HOG1 leu2/leu2</i>	Opaque	This study
YL272	a/a	<i>hog1/hog1::HOG1 leu2/leu2</i>	Opaque	This study
YL273	α/α	<i>leu2/leu2</i>	Opaque	This study
YL274	α/α	<i>leu2/leu2</i>	Opaque	This study
YL275	a/a	<i>OP4/OP4::OP4p-GFP</i>	White	This study
YL276	a/a	<i>OP4/OP4::OP4p-GFP</i>	White	This study
YL279	a/a	<i>hog1/hog1 OP4/OP4::OP4p-GFP</i>	White	This study
YL280	a/a	<i>hog1/hog1 OP4/OP4::OP4p-GFP</i>	White	This study
YL298	a/a	<i>hog1/hog1 OP4/OP4::OP4p-GFP</i>	Opaque	This study
YL299	a/a	<i>hog1/hog1 OP4/OP4::OP4p-GFP</i>	Opaque	This study
YL304	α/α	<i>hog1/hog1 arg4/arg4</i>	White	This study
YL305	α/α	<i>hog1/hog1 arg4/arg4</i>	White	This study
YL328	α/α	<i>hog1/hog1::HOG1 arg4/arg4</i>	White	This study
YL329	α/α	<i>hog1/hog1::HOG1 arg4/arg4</i>	White	This study
YL330	α/α	<i>hog1/hog1 arg4/arg4</i>	Opaque	This study
YL331	α/α	<i>hog1/hog1 arg4/arg4</i>	Opaque	This study
YL332	α/α	<i>hog1/hog1::HOG1 arg4/arg4</i>	Opaque	This study
YL333	α/α	<i>hog1/hog1::HOG1 arg4/arg4</i>	Opaque	This study
YL400	α/α	<i>pbs2/pbs2</i>	White	This study
YL401	α/α	<i>pbs2/pbs2</i>	White	This study

(Continued on following page)

TABLE 1 (Continued)

Strain	MTL type	Genotype	White or opaque	Reference or source
YL402	$\alpha/\alpha$	<i>ssk2/ssk2</i>	White	This study
YL403	$\alpha/\alpha$	<i>ssk2/ssk2</i>	White	This study
YL404	<b>a/a</b>	<i>pbs2/pbs2</i>	White	This study
YL405	<b>a/a</b>	<i>pbs2/pbs2</i>	White	This study
YL442	<b>a/a</b>	<i>ssk2/ssk2</i>	White	This study
YL443	<b>a/a</b>	<i>ssk2/ssk2</i>	White	This study
YL458	$\alpha/\alpha$	<i>ssk2/ssk2 leu2/leu2</i>	Opaque	This study
YL459	$\alpha/\alpha$	<i>ssk2/ssk2 leu2/leu2</i>	Opaque	This study
YL469	<b>a/a</b>	<i>pbs2/pbs2 arg4/arg4</i>	Opaque	This study
YL470	<b>a/a</b>	<i>pbs2/pbs2 arg4/arg4</i>	Opaque	This study
YL484	$\alpha/\alpha$	<i>pbs2/pbs2 leu2/leu2</i>	Opaque	This study
YL485	$\alpha/\alpha$	<i>pbs2/pbs2 leu2/leu2</i>	Opaque	This study
YL498	<b>a/a</b>	<i>ssk2/ssk2 arg4/arg4</i>	Opaque	This study
YL499	<b>a/a</b>	<i>ssk2/ssk2 arg4/arg4</i>	Opaque	This study
YL524	<b>a/a</b>	<i>pbs2/pbs2 arg4/arg4</i>	White	This study
YL525	<b>a/a</b>	<i>pbs2/pbs2 arg4/arg4</i>	White	This study
YL526	$\alpha/\alpha$	<i>pbs2/pbs2 leu2/leu2</i>	White	This study
YL527	$\alpha/\alpha$	<i>pbs2/pbs2 leu2/leu2</i>	White	This study
YL528	<b>a/a</b>	<i>ssk2/ssk2 arg4/arg4</i>	White	This study
YL529	<b>a/a</b>	<i>ssk2/ssk2 arg4/arg4</i>	White	This study
YL530	$\alpha/\alpha$	<i>ssk2/ssk2 leu2/leu2</i>	White	This study
YL531	$\alpha/\alpha$	<i>ssk2/ssk2 leu2/leu2</i>	White	This study
YL532	<b>a/a</b>	<i>ssk2/ssk2</i>	Opaque	This study
YL533	<b>a/a</b>	<i>ssk2/ssk2</i>	Opaque	This study
YL534	<b>a/a</b>	<i>pbs2/pbs2</i>	Opaque	This study
YL535	<b>a/a</b>	<i>pbs2/pbs2</i>	Opaque	This study
YL536	$\alpha/\alpha$	<i>pbs2/pbs2</i>	Opaque	This study
YL537	$\alpha/\alpha$	<i>pbs2/pbs2</i>	Opaque	This study
YL538	$\alpha/\alpha$	<i>ssk2/ssk2</i>	Opaque	This study
YL539	$\alpha/\alpha$	<i>ssk2/ssk2</i>	Opaque	This study
YL899	<b>a/a</b>	<i>OP4/OP4::OP4p-GFP</i>	Opaque	This study
YL900	<b>a/a</b>	<i>OP4/OP4::OP4p-GFP</i>	Opaque	This study

<sup>a</sup> All of the strains were SC5314 derived.

phorylated Hog1 proteins, respectively. Blots were developed with Clarity Western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Pheromone response assays.** To test formation of mating projections, opaque cells of *MTLa/a* strains were grown in Spider medium at 25°C overnight. Overnight cultures (500  $\mu$ l) were diluted into 3 ml of Spider broth, and  $\alpha$ -pheromone peptide (GFRLTNFGYFEPG; synthesized by GMBiolab Co., Ltd.) was added to reach a final concentration of 10  $\mu$ g/ml as previously described (43). After 8 h of incubation at 25°C, an Eclipse Ti inverted microscope (Nikon Instruments Inc., Melville, NY, USA) was used to examine the mating projections.

**Quantitative mating assays.** To generate auxotrophic strains, *ARG4* and *LEU2* genes were deleted in *MTLa/a* and *MTL $\alpha$ / $\alpha$*  strains, respectively. Opaque cells were grown in Spider medium at 25°C overnight. Cells ( $2 \times 10^7$ ) of *MTLa/a* and *MTL $\alpha$ / $\alpha$*  strains were mixed and placed on Spider plates at 25°C for 48 h. The mixture was collected, diluted, and plated onto selective medium, Arg<sup>-</sup>, Leu<sup>-</sup>, and Arg<sup>-</sup> Leu<sup>-</sup> plates. Mating efficiencies were calculated by the number of tetraploid colonies on Arg<sup>-</sup> Leu<sup>-</sup> plates divided by the total colony number on Arg<sup>-</sup> or Leu<sup>-</sup> plates.

**Fluorescence-activated cell sorter (FACS) analysis.** Tetraploid cells from mating progeny were incubated with 95% ethanol for at least 1 h at 4°C and washed with Tris-EDTA (TE) buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0). Samples were incubated with RNase A solution (2 mg/ml RNase A, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) for at least 2 h at 37°C and then with pepsin solution (5 mg/ml pepsin, 55 mM HCl) for 30 min at 37°C and washed with TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5). Samples were incubated with Sytox solution (1 mM Sytox green, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) for 1 h at 4°C. A BD FACSCanto II

system (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was used to examine the fluorescence of samples.

**Fluorescence microscopy.** White cells and opaque cells of *OP4* promoter-driven GFP reporter strains of the wild-type (WT) strain or *hog1 $\Delta$*  mutants were mixed, and the expression of fluorescence was examined by the use of an Eclipse Ti inverted microscope (Nikon Instruments Inc., Melville, NY, USA).

## RESULTS

**Deletion of the *HOG1* gene promotes opaque-cell formation.** To understand the role of Hog1 in regulation of white-opaque switching, *hog1 $\Delta$*  mutants of *MTLa/a*, *MTL $\alpha$ / $\alpha$* , and *MTLa/ $\alpha$*  white cells were plated on synthetic complete (SC), synthetic complete dextrose (SCD), and *N*-acetylglucosamine (NAG) media supplemented with phloxine B to test their switching frequencies. Figure 1A shows that both **a/a** and  $\alpha/\alpha$  colonies of *hog1 $\Delta$*  mutants switched to the opaque state (100% switching) when grown on SC medium, whereas **a/a** cells remained in the white state. Reintegration of the *HOG1* gene in each mutant restored the WT phenotype on SC medium, with colonies remaining in the white state.

Interestingly, the high rates of switching observed with *hog1* mutants were not observed on medium supplemented with glucose (SCD) (Fig. 1B), suggesting that glucose plays a negative role in opaque-cell formation. Upon NAG treatment, *hog1 $\Delta$*  mutants from both homozygous strain backgrounds also showed 100%

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')
CH1	GGAGCGGGGCCCCGAGCCGAGAACAGACTTTGAAC
CH2	GGAGCGCTCGAGTGTGTGTTATTATTCCACGGTG
CH3	GGAGCGCCCGGTCTTCAAAAATACAAGCTAGCAATTATAG
CH4	GGAGCGGAGCTCTTGCCCAACAAAGTGTGG
2	CCTTCAATTGCATCGTAAGTACC
3	GAAGATGACTCAGGTCATGC
4	GTGGTCAATGGAGCTGATAC
5	ACATGTGGTCGCCCAACTCC
10	GGAGCGGGGCCCCACACATGTTTGATTTCGATTCTTG
11	GGAGCGCTCGAGAGCTAATGTTGCTGGAGACGAC
70	GGAGCGGTACCCCTTCCCTGTTAACTTTG
71	GGAGCGCTCGAGGGGCCCTTTTCCAATTGAAC
72	GGAGCGCCCGGGAGGGAAACCAGAGCTTATTCC
73	GGAGCGGAGCTCAAGACTCCTAAACCGGAATTACAATT
78	GGAGCGGGGCCCTGGTCCAATCTGGTTGGGAG
79	GGAGCGCTCGAGCGCTGTCCCTCTCTCGC
80	GGAGCGCCCGGGCCAGGTTATTGGGCTGTA
81	GGAGCGGAGCTCCTATGCAAATGGAGGTTGGGAA
114	GGAGCGGGGCCCCGACAGGAATCACGTCATCGTTG
115	GGAGCGCTCGAGTGTGGGATGGCCAGCG
116	GGAGCGCGCCGCCCTTCCATCACAACACACAACACG
117	GGAGCGCCCGGGGAATCCATGAACTACTGCTTC
122	GGAGCGGGGCCCTTGCTGGGTTTGGTGAAGC
123	GGAGCGCTCGAGTGAAAAAAGGGCACCCAG
124	GGAGCGCCCGGGCCTGGTAGATGTAATGGTGGC
125	GGAGCGGAGCTCGAACAAACGAACCACCTCGC
191	ATGAGTAAGGGAGAAGAACTTTTCACT
192	GGAGCGCTCGAGTTATTTGTATAGTTCATCCATGCCAT
193	GGAGCGGGGCCGATAATGCAAGAGCTCGCCACCAC
194	AGTGAAAAGTTCTTCTCCCTTACTCATTGTAAATTATTTATATTTGTATGTGTGATAGGA

opaque switching rates, whereas the WT and complemented mutant strains showed intermediate (63% to 85%) levels of opaque-cell formation (Fig. 1C). To test whether opaque-looking cells were expressing opaque state-specific genes, we expressed the *OP4* promoter fused to GFP in both wild-type and *hog1Δ* strains. As shown in Fig. 1D, only the opaque cells exhibited strong GFP expression, whereas white cells showed no GFP expression. Together, these results demonstrate that Hog1 in *C. albicans* possesses a novel role in the regulation of white-opaque switching.

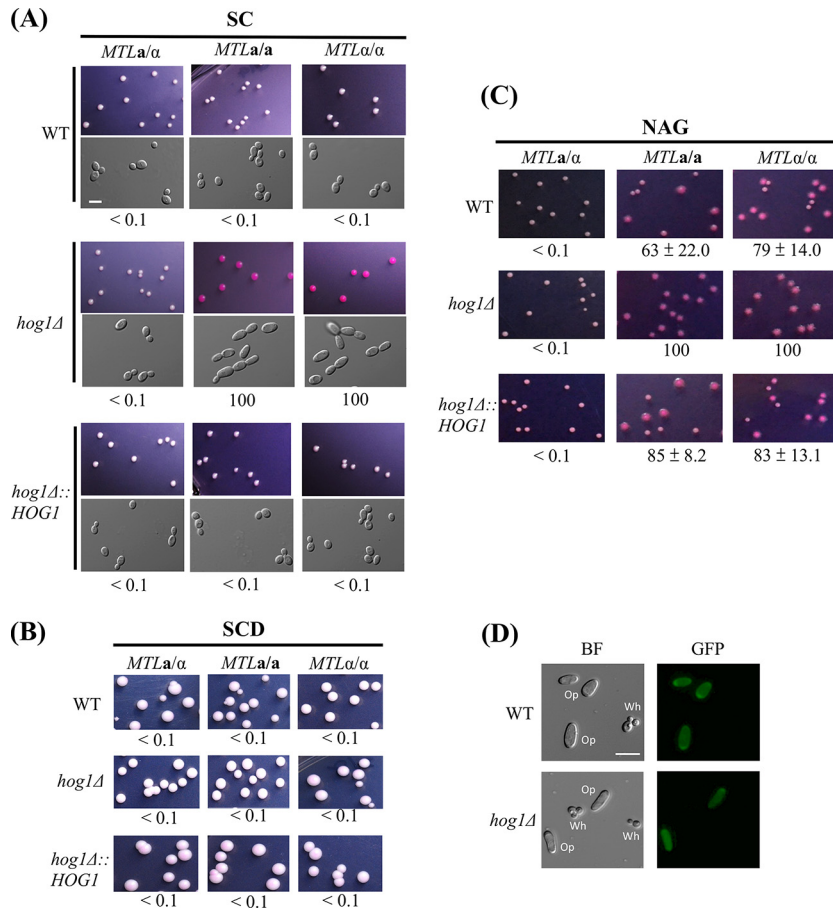
**Induction of opaque-cell formation in *hog1Δ* cells requires *Wor1*.** We observed that *hog1Δ a/α* cells remain locked in the white phenotype (Fig. 1). This is presumably due to the role of the *a1/α2* heterodimer in inhibiting *WOR1* expression (30), thereby impeding opaque-cell formation. To determine whether *WOR1* is necessary for opaque-cell formation in *hog1Δ* mutants, *hog1Δ wor1Δ* double-knockout strains were created. In contrast to the *hog1Δ* mutant, the *hog1Δ wor1Δ* mutant lacked the ability to switch to opaque when plated on SC medium (Fig. 2).

**Both *Pbs2* and *Ssk2* are involved in white-opaque switching.** The SAPK signaling cascade consists of three serine/threonine protein kinases: MAPKKK (*Ssk2*), MAPKK (*Pbs2*), and MAPK (*Hog1*) (39, 40, 44). *Ssk2* phosphorylates *Pbs2*, which in turn phosphorylates and activates *Hog1*. To determine whether *Pbs2* and *Ssk2* also regulate the white-opaque transition, mutants were constructed and plated on SC medium. Similarly to the *hog1Δ* mutant (Fig. 1), the *pbs2Δ* and *ssk2Δ* mutants exhibited 100% opaque-cell formation on SC medium supplemented with phlox-

ine B (Fig. 3). Once again, switching occurred only in *MTL* homozygous strains and not in the *MTL* heterozygous strain.

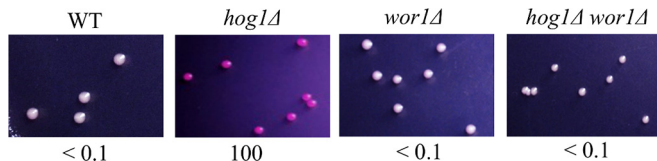
**Functions of the SAPK components are well conserved in both homozygous and heterozygous strains.** The Hog1 SAPK pathway is necessary for resistance to osmotic and oxidative stresses. However, previous studies were performed in *C. albicans a/α* heterozygous cells (45, 46). We therefore compared and tested the roles of SAPK pathway components in both the heterozygous and homozygous strain backgrounds. As shown in Fig. 4A, mutants of each SAPK component were highly sensitive to both osmotic and oxidative stress in *a/α*, *a/a*, and *α/α* cells. Western blotting also revealed that the function of SAPK in *C. albicans* is conserved and that *Hog1* activation is regulated by *Ssk2* and *Pbs2* in *MTL* homozygous strains, as it is in heterozygous strains (Fig. 4B).

**Deletion of *HOG1*, *PBS2*, and *SSK2* genes results in shorter mating projections.** *C. albicans* strains must undergo several developmental steps before having sex (47). Most isolates are diploid *a/α* strains and have to undergo homozygosity to generate *a/a* and *α/α* cells. These homozygous cells must then switch from white to opaque to become mating competent (18, 19). Pheromone treatment of *C. albicans* opaque cells activates mating responses and leads to the formation of mating projections (21–23, 43). We further examined the mating characteristics of opaque cells obtained from *hog1Δ*, *pbs2Δ*, and *ssk2Δ* mutants. Surprisingly, mutants of each SAPK component exhibited marked reductions in the length of their mating projections (10 to 12 μm) relative to the length

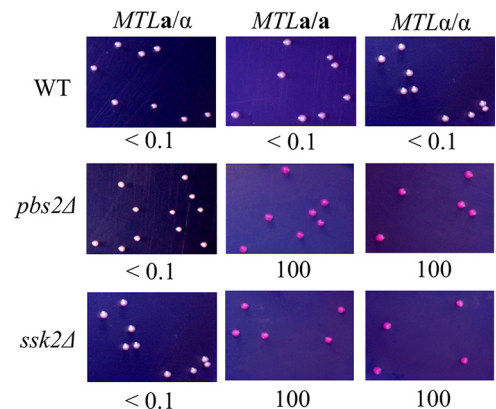


**FIG 1** Deletion of the *HOG1* gene induces white-to-opaque switching in *MTL/a* and *MTL/α* strains of *C. albicans* on SC plates. White cells from YPD medium were plated onto SC, SCD, or Lee's *N*-acetylglucosamine plates containing phloxine B and incubated at 25°C for 4 to 7 days. Opaque cells were stained red. At least 1,000 colonies were examined for each strain. White-to-opaque switching efficiency (percentage of average ± standard deviation [SD]) is indicated below each image. “<” indicates that no opaque-cell formation was observed. (A) *MTL/a* and *MTL/α* of *hog1Δ* mutants were able to undergo white-to-opaque switching on SC plates. (B) *C. albicans* strains were not able to undergo white-to-opaque switching on SCD plates. (C) *N*-acetylglucosamine induced 100% of white-to-opaque switching in homozygous *hog1Δ* mutants. (D) Only opaque cells expressed the GFP fluorescence in strains of the WT strain and the *hog1Δ* mutant. BF, bright field. Scale bar, 20 μm.

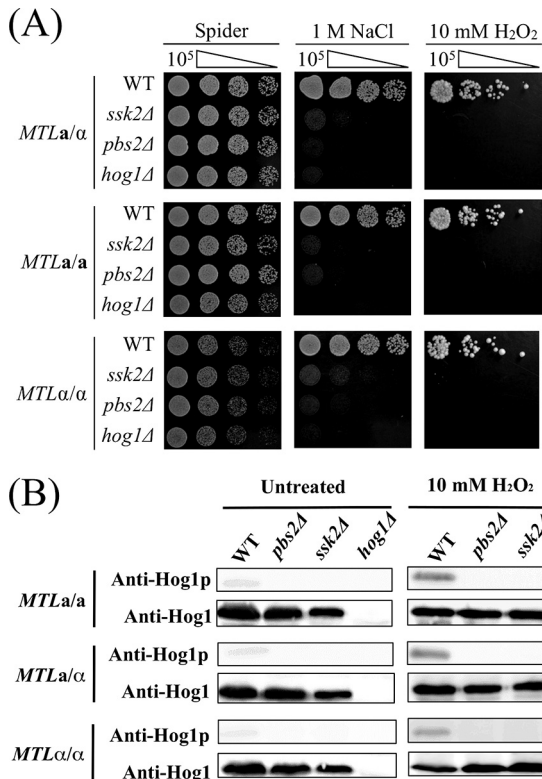
seen with the wild-type strain (24 μm) (Fig. 5). These results indicate that although deletion of *HOG1*, *PBS2*, and *SSK2* promotes opaque-cell formation, these mutants are less capable of forming mating projections of normal lengths.



**FIG 2** The lack of *WOR1* gene strongly inhibits Hog1-mediated white-to-opaque switching. White cells of the *MTL/a* WT strain, *hog1Δ* mutants, *wor1Δ* mutants, and *hog1Δ wor1Δ* double mutants were plated onto SC plates supplemented with phloxine B. Images were taken after a 7-day incubation at 25°C. Opaque cells were stained red. The total numbers of cells examined for each strain were 1,000 to 1,200. Switching frequency was expressed as a percentage of the mean ± SD and is shown below each image. “<” indicated that no opaque or opaque-sectored colony was observed.



**FIG 3** MAPKKK Ssk2 and MAPKK Pbs2 are involved in the regulation of white-to-opaque switching. White cells of each mutant were plated onto the SC medium supplemented with phloxine B and incubated at 25°C for 7 days. White-to-opaque switching efficiencies were expressed as percentages of averages ± standard deviations (SD) and are shown below each image. At least 1,000 colonies were examined. “<” indicates that no opaque-cell formation was observed.

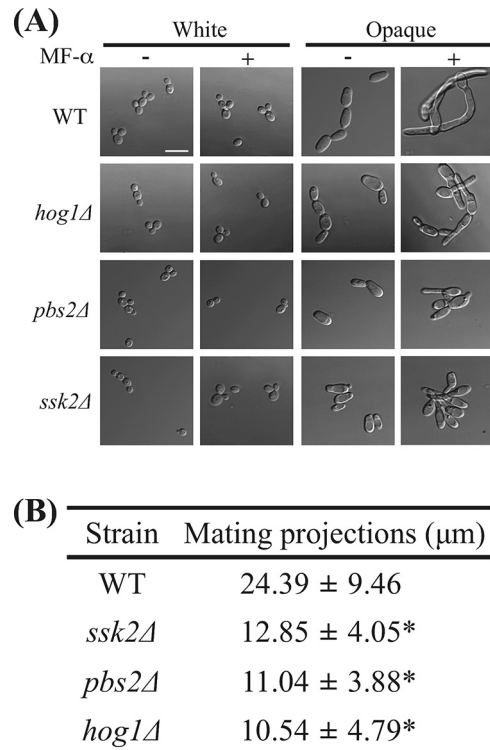


**FIG 4** The roles of *SSK2*, *PBS2*, and *HOG1* in regard to osmotic and oxidative stresses are indistinguishable between homozygous and heterozygous strains of *C. albicans*. (A) Serially diluted cells of *MTLα/a*, *MTLα/α*, and *MTLα/α* strains of the wild-type, *ssk2Δ*, *pbs2Δ*, and *hog1Δ* mutant strains were spotted onto Spider medium supplemented with 1 M NaCl or 10 mM hydrogen peroxide. (B) Phosphorylation levels of Hog1p were analyzed in *MTLα/a*, *MTLα/α*, and *MTLα/α* strains of *ssk2Δ* and *pbs2Δ* mutants.  $H_2O_2$  (10 mM) was added to exponentially growing cultures, and samples were collected after a 15-min treatment. Anti-Hog1 antibody and phospho-p38 (Thr180/Tyr182) antibody were used to detect Hog1 and phospho-Hog1 (Hog1p), respectively.

***ssk2Δ*, *pbs2Δ*, and *hog1Δ* mutants exhibit reduced mating efficiencies.** Given that *ssk2Δ*, *pbs2Δ*, and *hog1Δ* strains exhibited shorter mating projections (Fig. 5), it is possible that these mutants would also have defects in mating. Opaque *MTLα/a* and *MTLα/α* cells containing different selection markers were constructed as described in Materials and Methods. Quantitative mating assays were performed, and the results are shown in Fig. 6A. As expected on the basis of the defective mating responses, the mating efficiencies of *ssk2Δ*, *pbs2Δ*, and *hog1Δ* mutants were 0.6%, 1.6%, and 2.7%, respectively (Fig. 6A). In contrast, the wild-type strain showed a mating efficiency of 16.7% (Fig. 6A). All the mating progeny were confirmed by PCR and FACS analysis to be tetraploid *MTLα/α* cells (Fig. 6B and C).

## DISCUSSION

*C. albicans* is the most important fungal pathogen in humans, where many infections arise and the difficulties for treatment are due to its inherent flexible lifestyle and ability to avoid the immune system. In addition to the yeast-hypha transition, epigenetic variation via white-opaque switching is perhaps the best example of morphological plasticity. In this work, we first demonstrated that the Hog1 SAPK signaling cascade is involved in regulating the



**FIG 5** *ssk2Δ*, *pbs2Δ*, and *hog1Δ* mutants exhibited shorter mating projections. (A) White cells and opaque cells of the *hog1Δ*, *ssk2Δ*, *pbs2Δ*, and WT strains were challenged with or without  $\alpha$ -factor for 8 h at 25°C. The cells were then imaged with a Nikon Eclipse Ti microscope. (B) At least 50 cells were analyzed to measure the projection length after an 8-h pheromone treatment. The mating projection length of each strain is expressed as the mean  $\pm$  SD. MF- $\alpha$ , *C. albicans* pheromone peptide. \*,  $P < 0.001$ .

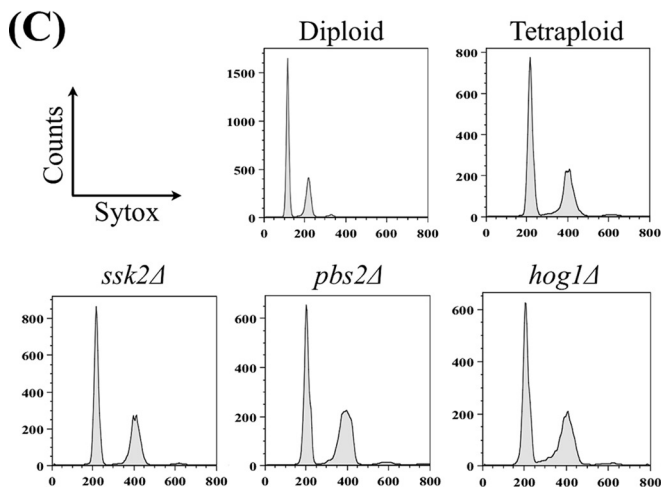
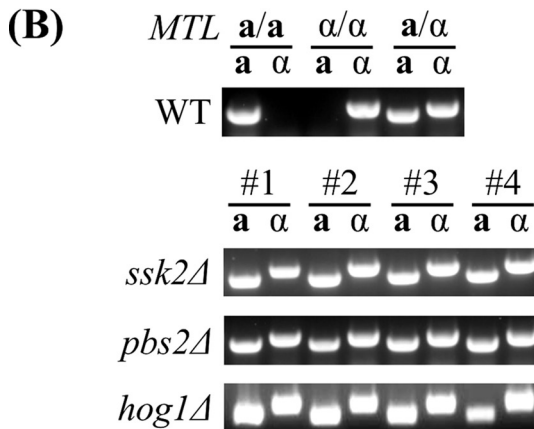
white-opaque epigenetic transition. Inactivation of *SSK2*, *PBS2*, and *HOG1* genes in *MTLα/a* and *MTLα/α* strains led to high sensitivity to osmotic and oxidative stresses and also promoted switching *en masse* to the opaque cell state under certain culture conditions. These results indicate that although the SAPK in *C. albicans* is evolutionarily well conserved among eukaryotes, the biological functions of each component kinase are highly dependent on the lifestyles of the species and their environment.

Given that *HOG1* can impact white/opaque phenotypic states, we were curious whether the postmodification states of Hog1 differ between white and opaque cells. However, cells of the two types showed similar levels of phosphorylation of the Hog1 protein (data not shown). Consistent with previous results, expression levels of Hog1 were also comparable between white and opaque cells (48). Nevertheless, we cannot rule out the possibility that changes in gene expression levels or postmodification states of Hog1 occur during the white-to-opaque switching process.

*C. albicans* Hog1 SAPK is activated by many environmental cues, including osmotic and oxidative stimuli, heavy metal, glucose, and cationic peptides (45, 49–51). Two of them, oxidative-stress levels and glucose concentrations, have been reported to be involved in opaque-cell formation and its stability (32, 34, 52). Our studies highlighted that deletion of *HOG1* led to switching of white cells to the opaque state on SC and NAG media. These observations could be because removal of *HOG1* causes cells to be more sensitive to internal and external oxidative microenviron-

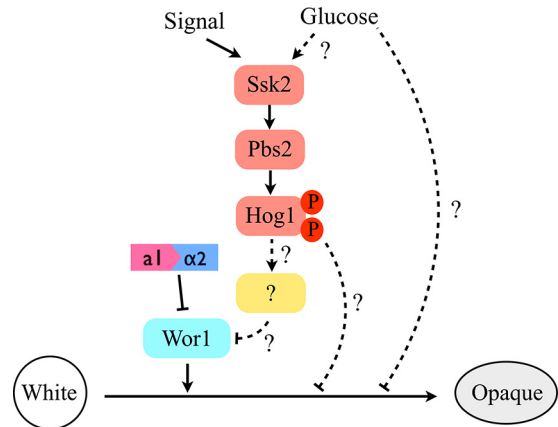
**(A)**

Cross	Mating efficiency (%)
WT $\alpha$ × WT $\alpha$	16.7 ± 5.96
<i>ssk2Δa</i> × <i>ssk2Δa</i>	0.6 ± 0.23*
<i>pbs2Δa</i> × <i>pbs2Δa</i>	1.6 ± 0.37*
<i>hog1Δa</i> × <i>hog1Δa</i>	2.7 ± 2.43*



**FIG 6** Mating efficiencies of *ssk2Δ*, *pbs2Δ*, and *hog1Δ* mutants were significantly reduced. (A) Auxotrophic strains were coincubated on Spider medium for 48 h, and cells were subsequently plated onto selective medium to determine the mating efficiencies. Mating efficiencies of each cross are expressed as means ± SD. Experiments were repeated three times at least. (B) The *OPBa* gene and *OPBα* gene (primer pair 2 and 3 and primer pair 4 and 5) were used to determine the mating type. (C) Progeny ploidies were determined by the use of a BD FACSCanto II system. \*,  $P < 0.05$ .

ments that drive cells to the opaque state. Furthermore, it is possible that a transcription factor regulated by Hog1 impacts *WOR1* expression and switching frequencies (Fig. 7). Indeed, our studies demonstrated that *Wor1* plays a crucial role in the morphological change in *hog1Δ* mutants, as no white-to-opaque switching was observed in *MTLa/α* cells or in *hog1Δ wor1Δ* mutants. Whether genetic control (induction of *Wor1* expression) or environmental control (increased sensitivity to stress conditions) or a combina-



**FIG 7** A proposed model of Hog1 MAPK pathway-mediated white-opaque switching in *C. albicans*. The master regulator of *Wor1* and the  $\alpha 1/\alpha 2$  heterodimer still control the white-opaque switching. The presence of glucose could be a negative factor for opaque-cell formation. The dashed lines represent unknown mechanisms or unidentified regulators for Hog1 SAPK in regulating this phenotypic transition.

tion of these factors induces switching remains an open question. Despite high switching rates of *hog1Δ* mutants on SC and NAG media, we have not observed opaque-colony formation on SCD plates. Similar observations have been reported to show that the presence of glucose (dextrose) inhibited opaque-cell formation and its stability (34, 52). However, the exact mechanism by which glucose or its metabolism influences phenotypic switching or the stability of opaque cells remains obscure (Fig. 7).

The pheromone response for *C. albicans* is mediated by *Cph1*, a downstream target of *Cek1/Cek2* MAP kinases (21). Deletion of the *CPH1* gene resulted in loss of pheromone-stimulated cell adhesion in white cells and mating-projection formation in opaque cells (21). Despite loss of Hog1 SAPK components promoting opaque-cell formation, upon pheromone challenge, *hog1Δ*, *pbs2Δ*, and *ssk2Δ* mutants exhibited projections that were shorter than those of the wild-type strain. Apparently, the pheromone response in these mutant cells is decreased relative to the level in the wild type. These mutants also exhibited a significant decrease in the level of sexual mating. It is clear that *C. albicans* cells have to switch to the opaque state before having sex and that the mechanisms of the switch and mating response are disparate (22, 53). For example, when the pheromone receptor *Ste2* or *Cph1* is removed, cells are still able to switch to the opaque state but cannot respond to pheromones and cannot undergo  $\alpha$ - $\alpha$  mating (21).

Regulatory interactions between the Hog1 and mating pathways in *Saccharomyces cerevisiae* have been reported (54–60). Analyses of the antagonistic manners of the potential interactions of two MAPKs were conducted via examination of the insulation mechanism, a physical separation of the shared components of two cascades (54, 59, 61). Since the Hog1 and mating pathways share several components, including *Cdc2*, *Ste20*, *Ste50*, and *Ste11*, it is therefore possible that Hog1-mediated phosphorylation might cause the conformational changes of shared components to control cell fate (59, 61). The *Ste5* and *Pbs2* scaffold proteins exclusively required for the mating and hyperosmotic responses, respectively, also appear to regulate and maintain the response specificity (61). Additionally, pathway cross-inhibition has been previously addressed (61). When Hog1 is deleted or inhibited, mating is promoted under conditions of hyperosmotic stress (54, 57). A recent study further indicated that activation of Hog1 can (i) phosphorylate *Ste50*, leading to the downregulation



of mating signal, and (ii) repress the Fus3 response shown by Rck2, a protein kinase of the cell cycle checkpoint (60). It was previously noted that *S. cerevisiae* contains two osmosensors (Sho1 and Sln1) for Hog1 activation (39). The shared components of Cdc2, Ste20, Ste50, and Ste11 in *S. cerevisiae* cooperate only with the Sho1-mediated branch (39, 60). Interestingly, although a conserved mechanism by which external signals are sensed and relayed to the SAPK pathway has been observed in *C. albicans*, mechanisms for activation of the SAPK pathways in the budding yeast and *C. albicans* have diverged. Indeed, Hog1 SAPK signaling in *C. albicans* is entirely dependent on a sole osmosensor, Sln1, whereas the Sho1 branch in *C. albicans* plays no role in Hog1 activation (39, 46). These results therefore suggest a more interesting issue: the potential mechanisms by which the Hog1 SAPK pathway impacts mating in *C. albicans*.

Taking the data together, white and opaque cells differ in many aspects, as they exhibit different colony morphologies and different mating efficiencies, colonize different niches in the host, and have different interactions with host immune cells. Our work has identified a novel role for the Hog1 SAPK pathway in mediating white-opaque switching. Whether there is a niche in the human body that inhibits Hog1 signaling, leading to increased opaque-cell formation and potential escape from the immune system, is therefore an interesting issue. Future studies will investigate the roles of several key upstream regulatory factors and downstream targets of the Hog1 SAPK pathway to determine if these factors function in regulating this unique phenotypic transition in the human fungal pathogen *C. albicans*.

## ACKNOWLEDGMENTS

We thank Richard Bennett for stains and plasmids. We also thank Richard Bennett and members of his laboratory for critical reading of and comments on the manuscript. We are grateful to the staff of Technology Commons, College of Life Science, National Taiwan University, for the help with the BD FACSCanto II system.

This research was supported by NSC102-2320-B-002-027-MY3, MOST 103-2628-B-002-003-MY3, and NTU 103R7787 from the National Science Council, the Ministry of Science and Technology, and National Taiwan University, respectively.

## REFERENCES

- Gargas A, Taylor JW. 1995. Phylogeny of discomycetes and early radiations of the apothecial Ascomycotina inferred from SSU rDNA sequence data. *Exp. Mycol.* 19:7–15. <http://dx.doi.org/10.1006/emyc.1995.1002>.
- Hedges SB. 2002. The origin and evolution of model organisms. *Nat. Rev. Genet.* 3:838–849. <http://dx.doi.org/10.1038/nrg929>.
- Ruhnke M, Maschmeyer G. 2002. Management of mycoses in patients with hematologic disease and cancer – review of the literature. *Eur. J. Med. Res.* 7:227–235.
- Buffo J, Herman MA, Soll DR. 1984. A characterization of pH-regulated dimorphism in *Candida albicans*. *Mycopathologia* 85:21–30. <http://dx.doi.org/10.1007/BF00436698>.
- Mardon D, Balish E, Phillips AW. 1969. Control of dimorphism in a biochemical variant of *Candida albicans*. *J. Bacteriol.* 100:701–707.
- Simonetti N, Strippoli V, Cassone A. 1974. Yeast-mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. *Nature* 250:344–346. <http://dx.doi.org/10.1038/250344a0>.
- Sudbery PE. 2011. Growth of *Candida albicans* hyphae. *Nat. Rev. Microbiol.* 9:737–748. <http://dx.doi.org/10.1038/nrmicro2636>.
- Taschdjian CL, Burchall JJ, Kozinn PJ. 1960. Rapid identification of *Candida albicans* by filamentation on serum and serum substitutes. *AMA J. Dis. Child.* 99:212–215. <http://dx.doi.org/10.1001/archpedi.1960.02070030214011>.
- Sudbery P, Gow N, Berman J. 2004. The distinct morphogenic states of *Candida albicans*. *Trends Microbiol.* 12:317–324. <http://dx.doi.org/10.1016/j.tim.2004.05.008>.
- Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* 16:1982–1991. <http://dx.doi.org/10.1093/emboj/16.8.1982>.
- Si H, Hernday AD, Hirakawa MP, Johnson AD, Bennett RJ. 2013. *Candida albicans* white and opaque cells undergo distinct programs of filamentous growth. *PLoS Pathog.* 9:e1003210. <http://dx.doi.org/10.1371/journal.ppat.1003210>.
- Shapiro RS, Uppuluri P, Zaas AK, Collins C, Senn H, Perfect JR, Heitman J, Cowen LE. 2009. Hsp90 orchestrates temperature-dependent *Candida albicans* morphogenesis via Ras1-PKA signaling. *Curr. Biol.* 19:621–629. <http://dx.doi.org/10.1016/j.cub.2009.03.017>.
- Shapiro RS, Robbins N, Cowen LE. 2011. Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol. Mol. Biol. Rev.* 75:213–267. <http://dx.doi.org/10.1128/MMBR.00045-10>.
- Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD. 2012. A recently evolved transcriptional network controls biofilm development in *Candida albicans*. *Cell* 148:126–138. <http://dx.doi.org/10.1016/j.cell.2011.10.048>.
- Nobile CJ, Mitchell AP. 2005. Regulation of cell-surface genes and biofilm formation by the *Candida albicans* transcription factor Bcr1p. *Curr. Biol.* 15:1150–1155. <http://dx.doi.org/10.1016/j.cub.2005.05.047>.
- Nobile CJ, Mitchell AP. 2007. Microbial biofilms: e pluribus unum. *Curr. Biol.* 17:R349–R353. <http://dx.doi.org/10.1016/j.cub.2007.02.035>.
- Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR. 1987. “White-opaque transition”: a second high-frequency switching system in *Candida albicans*. *J. Bacteriol.* 169:189–197.
- Lockhart SR, Pujol C, Daniels KJ, Miller MG, Johnson AD, Pfaller MA, Soll DR. 2002. In *Candida albicans*, white-opaque switchers are homozygous for mating type. *Genetics* 162:737–745.
- Magee BB, Magee PT. 2000. Induction of mating in *Candida albicans* by construction of *MTLa* and *MTLalpha* strains. *Science* 289:310–313. <http://dx.doi.org/10.1126/science.289.5477.310>.
- Daniels KJ, Park YN, Srikantha T, Pujol C, Soll DR. 2013. Impact of environmental conditions on the form and function of *Candida albicans* biofilms. *Eukaryot. Cell* 12:1389–1402. <http://dx.doi.org/10.1128/EC.00127-13>.
- Lin CH, Kabrawala S, Fox EP, Nobile CJ, Johnson AD, Bennett RJ. 2013. Genetic control of conventional and pheromone-stimulated biofilm formation in *Candida albicans*. *PLoS Pathog.* 9:e1003305. <http://dx.doi.org/10.1371/journal.ppat.1003305>.
- Bennett RJ, Johnson AD. 2006. The role of nutrient regulation and the Gpa2 protein in the mating pheromone response of *C. albicans*. *Mol. Microbiol.* 62:100–119. <http://dx.doi.org/10.1111/j.1365-2958.2006.05367.x>.
- Bennett RJ, Uhl MA, Miller MG, Johnson AD. 2003. Identification and characterization of a *Candida albicans* mating pheromone. *Mol. Cell. Biol.* 23:8189–8201. <http://dx.doi.org/10.1128/MCB.23.22.8189-8201.2003>.
- Magee BB, Legrand M, Alarco AM, Raymond M, Magee RT. 2002. Many of the genes required for mating in *Saccharomyces cerevisiae* are also required for mating in *Candida albicans*. *Mol. Microbiol.* 46:1345–1351. <http://dx.doi.org/10.1046/j.1365-2958.2002.03263.x>.
- Geiger J, Wessels D, Lockhart SR, Soll DR. 2004. Release of a potent polymorphonuclear leukocyte chemoattractant is regulated by white-opaque switching in *Candida albicans*. *Infect. Immun.* 72:667–677. <http://dx.doi.org/10.1128/IAI.72.2.667-677.2004>.
- Lohse MB, Johnson AD. 2008. Differential phagocytosis of white versus opaque *Candida albicans* by *Drosophila* and mouse phagocytes. *PLoS One* 3:e1473. <http://dx.doi.org/10.1371/journal.pone.0001473>.
- Sasse C, Hasenberg M, Weyler M, Gunzer M, Morschhauser J. 2013. White-opaque switching of *Candida albicans* allows immune evasion in an environment-dependent fashion. *Eukaryot. Cell* 12:50–58. <http://dx.doi.org/10.1128/EC.00266-12>.
- Morschhäuser J. 2010. Regulation of white-opaque switching in *Candida albicans*. *Med. Microbiol. Immunol.* 199:165–172. <http://dx.doi.org/10.1007/s00430-010-0147-0>.
- Zordan RE, Miller MG, Galgoczy DJ, Tuch BB, Johnson AD. 2007. Interlocking transcriptional feedback loops control white-opaque switching in *Candida albicans*. *PLoS Biol.* 5:e256. <http://dx.doi.org/10.1371/journal.pbio.0050256>.
- Huang G, Wang H, Chou S, Nie X, Chen J, Liu H. 2006. Bistable

- expression of WOR1, a master regulator of white-opaque switching in *Candida albicans*. Proc. Natl. Acad. Sci. U. S. A. 103:12813–12818. <http://dx.doi.org/10.1073/pnas.0605270103>.
31. Xie J, Tao L, Nobile CJ, Tong Y, Guan G, Sun Y, Cao C, Hernday AD, Johnson AD, Zhang L, Bai FY, Huang G. 2013. White-opaque switching in natural *MTLa*α isolates of *Candida albicans*: evolutionary implications for roles in host adaptation, pathogenesis, and sex. PLoS Biol. 11: e1001525. <http://dx.doi.org/10.1371/journal.pbio.1001525>.
  32. Alby K, Bennett RJ. 2009. Stress-induced phenotypic switching in *Candida albicans*. Mol. Biol. Cell 20:3178–3191. <http://dx.doi.org/10.1091/mbc.E09-01-0040>.
  33. Huang G, Srikantha T, Sahni N, Yi S, Soll DR. 2009. CO(2) regulates white-to-opaque switching in *Candida albicans*. Curr. Biol. 19:330–334. <http://dx.doi.org/10.1016/j.cub.2009.01.018>.
  34. Huang G, Yi S, Sahni N, Daniels KJ, Srikantha T, Soll DR. 2010. N-acetylglucosamine induces white to opaque switching, a mating prerequisite in *Candida albicans*. PLoS Pathog. 6:e1000806. <http://dx.doi.org/10.1371/journal.ppat.1000806>.
  35. Correia I, Alonso-Monge R, Pla J. 2010. MAPK cell-cycle regulation in *Saccharomyces cerevisiae* and *Candida albicans*. Future Microbiol. 5:1125–1141. <http://dx.doi.org/10.2217/fmb.10.72>.
  36. Xu JR. 2000. MAP kinases in fungal pathogens. Fungal Genet. Biol. 31: 137–152. <http://dx.doi.org/10.1006/fgbi.2000.1237>.
  37. Folch-Mallol JL, Garay-Arroyo A, Lledias F, Covarrubias Robles AA. 2004. The stress response in the yeast *Saccharomyces cerevisiae*. Rev. Latinoam. Microbiol. 46:24–46.
  38. Liu H, Kohler J, Fink GR. 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science 266:1723–1726. <http://dx.doi.org/10.1126/science.7992058>.
  39. Smith DA, Morgan BA, Quinn J. 2010. Stress signalling to fungal stress-activated protein kinase pathways. FEMS Microbiol. Lett. 306:1–8. <http://dx.doi.org/10.1111/j.1574-6968.2010.01937.x>.
  40. Monge RA, Roman E, Nombela C, Pla J. 2006. The MAP kinase signal transduction network in *Candida albicans*. Microbiology 152:905–912. <http://dx.doi.org/10.1099/mic.0.28616-0>.
  41. Guthrie C, Fink GR (ed). 1991. Methods in enzymology, vol 194. Guide to yeast genetics and molecular biology. Academic Press, Inc, San Diego, CA.
  42. Reuss O, Vik A, Kolter R, Morschhauser J. 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene 341:119–127. <http://dx.doi.org/10.1016/j.gene.2004.06.021>.
  43. Lin CH, Choi A, Bennett RJ. 2011. Defining pheromone-receptor signaling in *Candida albicans* and related asexual *Candida* species. Mol. Biol. Cell 22:4918–4930. <http://dx.doi.org/10.1091/mbc.E11-09-0749>.
  44. de Dios CH, Roman E, Monge RA, Pla J. 2010. The role of MAPK signal transduction pathways in the response to oxidative stress in the fungal pathogen *Candida albicans*: implications in virulence. Curr. Protein Pept. Sci. 11:693–703. <http://dx.doi.org/10.2174/138920310794557655>.
  45. Alonso-Monge R, Navarro-García F, Roman E, Negro AI, Eisman B, Nombela C, Pla J. 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. Eukaryot. Cell 2:351–361. <http://dx.doi.org/10.1128/EC.2.2.351-361.2003>.
  46. Cheetham J, Smith DA, da Silva Dantas A, Doris KS, Patterson MJ, Bruce CR, Quinn J. 2007. A single MAPKKK regulates the Hog1 MAPK pathway in the pathogenic fungus *Candida albicans*. Mol. Biol. Cell 18: 4603–4614. <http://dx.doi.org/10.1091/mbc.E07-06-0581>.
  47. Ni M, Feretzaki M, Sun S, Wang X, Heitman J. 2011. Sex in fungi. Annu. Rev. Genet. 45:405–430. <http://dx.doi.org/10.1146/annurev-genet-110410-132536>.
  48. Tuch BB, Mitrovich QM, Homann OR, Hernday AD, Monighetti CK, De la Vega FM, Johnson AD. 2010. The transcriptomes of two heritable cell types illuminate the circuit governing their differentiation. PLoS Genet. 6:e1001070. <http://dx.doi.org/10.1371/journal.pgen.1001070>.
  49. Rodaki A, Bohovych IM, Enjalbert B, Young T, Odds FC, Gow NA, Brown AJ. 2009. Glucose promotes stress resistance in the fungal pathogen *Candida albicans*. Mol. Biol. Cell 20:4845–4855. <http://dx.doi.org/10.1091/mbc.E09-01-0002>.
  50. Smith DA, Nicholls S, Morgan BA, Brown AJ, Quinn J. 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. Mol. Biol. Cell 15:4179–4190. <http://dx.doi.org/10.1091/mbc.E04-03-0181>.
  51. Vylkova S, Jang WS, Li W, Nayyar N, Edgerton M. 2007. Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. Eukaryot. Cell 6:1876–1888. <http://dx.doi.org/10.1128/EC.00039-07>.
  52. Lohse MB, Hernday AD, Fordyce PM, Noiman L, Sorrells TR, Hanson-Smith V, Nobile CJ, DeRisi JL, Johnson AD. 2013. Identification and characterization of a previously undescribed family of sequence-specific DNA-binding domains. Proc. Natl. Acad. Sci. U. S. A. 110:7660–7665. <http://dx.doi.org/10.1073/pnas.1221734110>.
  53. Alby K, Bennett RJ. 2010. Sexual reproduction in the *Candida* clade: cryptic cycles, diverse mechanisms, and alternative functions. Cell. Mol. Life Sci. 67:3275–3285. <http://dx.doi.org/10.1007/s00018-010-0421-8>.
  54. O'Rourke SM, Herskowitz I. 1998. The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*. Genes Dev. 12:2874–2886. <http://dx.doi.org/10.1101/gad.12.18.2874>.
  55. Harris K, Lamson RE, Nelson B, Hughes TR, Marton MJ, Roberts CJ, Boone C, Pryciak PM. 2001. Role of scaffolds in MAP kinase pathway specificity revealed by custom design of pathway-dedicated signaling proteins. Curr. Biol. 11:1815–1824. [http://dx.doi.org/10.1016/S0960-9822\(01\)00567-X](http://dx.doi.org/10.1016/S0960-9822(01)00567-X).
  56. Nelson B, Parsons AB, Evangelista M, Schaefer K, Kennedy K, Ritchie S, Petryshen TL, Boone C. 2004. Fus1p interacts with components of the Hog1p mitogen-activated protein kinase and Cdc42p morphogenesis signaling pathways to control cell fusion during yeast mating. Genetics 166: 67–77. <http://dx.doi.org/10.1534/genetics.166.1.67>.
  57. Westfall PJ, Thorner J. 2006. Analysis of mitogen-activated protein kinase signaling specificity in response to hyperosmotic stress: use of an analog-sensitive *HOG1* allele. Eukaryot. Cell 5:1215–1228. <http://dx.doi.org/10.1128/EC.00037-06>.
  58. Hao N, Zeng Y, Elston TC, Dohlman HG. 2008. Control of MAPK specificity by feedback phosphorylation of shared adaptor protein Ste50. J. Biol. Chem. 283:33798–33802. <http://dx.doi.org/10.1074/jbc.C800179200>.
  59. Patterson JC, Klimenko ES, Thorner J. 2010. Single-cell analysis reveals that insulation maintains signaling specificity between two yeast MAPK pathways with common components. Sci. Signal. 3:ra75. <http://dx.doi.org/10.1126/scisignal.2001275>.
  60. Nagiec MJ, Dohlman HG. 2012. Checkpoints in a yeast differentiation pathway coordinate signaling during hyperosmotic stress. PLoS Genet. 8:e1002437. <http://dx.doi.org/10.1371/journal.pgen.1002437>.
  61. Schwartz MA, Madhani HD. 2004. Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. Annu. Rev. Genet. 38:725–748. <http://dx.doi.org/10.1146/annurev.genet.39.073003.112634>.