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Characterization of Osh3, an Oxysterol-binding Protein, in Filamentous Growth of *Saccharomyces cerevisiae* and *Candida albicans*

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***OSH3* is one of the seven yeast homologues of the oxysterol binding proteins (OSBPs) which have the major binding affinity to the oxysterols and function as regulator of cholesterol biosynthesis in mammals. Mutational analysis of *OSH3* showed that *OSH3* plays a regulatory role in the yeast-to-hyphal transition through its oxysterol-binding domain in *Saccharomyces cerevisiae*. The *OSH3* gene was also identified in the pathogenic yeast *Candida albicans*. Deletion of *OSH3* caused a defect in the filamentous growth, which is the major cause of the *C. albicans* pathogencity. The filamentation defect of the mutation in the MAPK-associated transcription factor, namely *cph1*Δ was suppressed by overexpression of *OSH3*. These findings suggest the regulatory roles of *OSH3* in the yeast filamentous growth and the functional conservations of *OSH3* in *S. cerevisiae* and *C. albicans*.**

Keywords: oxysterol-binding protein, *OSH3*, filamentous growth, *Candida albicans*, pleckstrin homology domain

Oxysterols are naturally occurring oxygenated derivatives of cholesterol, such as 25-hydroxycholesterol, and are known to regulate lipid metabolism (Ridgway *et al.*, 1992; Xu *et al.*, 2001). A variety of regulatory roles of oxysterols have been reported, which includes transcriptional or translational repression of sterol-biosynthesis genes and activation of ER enzymes (Goldstein and Brown, 1990; Levanon *et al.*, 1990; Ridgway *et al.*, 1992). Oxysterol-binding proteins (OSBP) with the major binding affinity to the oxysterols have been identified from yeast to mammals, and considered to be the candidate transducer for the regulatory functions in cholesterol metabolism (Levanon *et al.*, 1990; Beh *et al.*, 2001). Although much less is understood about the precise cellular functions of OSBP, a family of OSBP homologues with the conserved C-terminus oxysterol-binding domain has been identified in the genomes of all eukaryotes.

The yeast *Saccharomyces cerevisiae* contains seven genes for OSBP homologues (*OSH1-OSH7*) (Jiang *et al.*, 1994; Beh *et al.*, 2001). None of these yeast OSBP genes alone is essential for cell viability but elimination of all seven genes proved lethal. In *S. cerevisiae* and other fungi, the predominant membrane sterol is ergosterol, a close relative of cholesterol. Mutant combinations of the *OSH* genes showed apparently common and cumulative phenotypes in the level of membrane ergosterol (Jiang *et al.*, 1994; Beh *et al.*, 2001). Other cellular functions have been suggested for the *OSH* genes in yeast. *OSH4/KES1* was found to participate in a regulatory pathway for Golgi -derived transport vesicle biogenesis (Fang *et al.*, 1996). Osh3p was shown to bind directly to the ATP-dependent DEAD-box RNA helicase Rok1p and was implicated in microtubule-related functions such as nuclear fusion and mitotic spindle formation (Park *et al.*, 2002; Yano *et al.*, 2004).

In this report, we investigated the functions of *OSH3* in the filamentous growth of *S. cerevisiae* and a pathogenic yeast *Candida albicans*. Upon nitrogen starvation, the cellular morphology of *S. cerevisiae* is switched from the yeast form to the pseudohyphal form (Liu *et al.*, 1993; Roberts and Fink, 1994; Pan and Heitman, 1999). Several signal transduction modules, including the mitogen-activated protein kinase (MAPK) cascade and the cAMP-dependent protein kinase A (PKA) pathway, have been known to participate in this regulation. In *C. albicans*, the yeast-to-hyphal transition in response to various environmental signals has been considered to be the major cause of

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the *C. albicans* pathogenecity (Shepherd *et al.*, 1985; Scherer and Magee, 1990). Previously, it was suggested that *OSH3* overexpression promoted filamentation growth of the ∑1278b wild-type strain and suppressed the filamentation defect of the *ste12* mutation in *S. cerevisiae* (Park *et al.*, 2002). We present here the mutational analysis of *OSH3* in *S. cerevisiae* and showed that *OSH3* plays a regulatory role in the filamentation growth through its oxysterol-binding domain. *C. albicans OSH3* was also identified and its functional conservations were studied.

Materials and Methods

Strains and growth conditions

All of the yeast strains used in this study are listed in Table 1. Standard yeast media were prepared using the established procedure (Adams *et al.*, 1997). To induce pseudohyphal growth, *S. cerevisiae* strains were grown on SLAD media (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate, 2% dextrose, 50 μM ammonium sulfate). Colony morphologies were observed after 5 days of incubation at 30°C (Gimeno *et al.*, 1992). The filamentation phenotype of the *C. albicans* cells was tested with serum-containing media (YEPD + 10% fetal bovine serum) and Spider medium (1% mannitol, 1% nutrient broth, 2% agar, 0.2% K_2HPO_4, pH 7.2) as previously described (Liu *et al.*, 1994). The solid media contained 2% agar. Overnight liquid cultures were diluted in water, plated for single colonies, and incubated at 37°C for 5-7 days.

Domain deletions of OSH3

Deletion plasmids pRS316-OSH3ΔPH and pRS316-OSH3ΔOSBD were constructed by ligating PCR-amplified DNA fragments. Plasmid pRS316-OSH3Δ PH was constructed by inserting the N-terminal DNA fragment of 1.52 kb (primers OSH3-*Kpn*I and OSH-PH1) and the C-terminal DNA fragment of 2.39 kb (primers OSH-PH2 and OSH3-EagI) into *Kpn*I/*Eag*I-digested pRS316 (Sikorski and Hieter, 1989). Plasmid pRS316-OSH3ΔOSBD constructed by inserting the N-terminal DNA fragment of 2.75 kb (primers OSH3-*Kpn*I and OSH3-OSBD1) and the C-terminal DNA fragment of 1.5 kb (primers OSH3-OSBD2 and OSH3 -*Eag*I) into *Kpn*I/*Eag*I-digested pRS316. The primers used in these constructions are as following: OSH3-*Kpn*I, 5'-TTGGGTACC$_{KpnI}$AGGCCAATAGA-3'; OSH3-PH1, 5'-CCCACGCGT$_{MluI}$GTACGATAGGGTTCCATA TCG-3'; OSH3-PH2, 5'-GGGACGCGT$_{MluI}$TGGCATC GTGGGTCCATGCG-3'; OSH3-*Eag*I, 5'-GGGCGGCC G$_{EagI}$ACTGACTGAAAGAGTTCATT-3'; OSH3-OSBD1, 5'-TCCGCTAGC$_{NheI}$GGCTCATTTGAGGTTACTGGC-3'; OSH3-OSBD2, 5'-AGGGCTAGC$_{NheI}$GCCTTCTTT GCAGAGCATCTC-3'.

Cloning of CaOSH3

In the Stanford *C. albicans* genome database, we identified an ORF which is homologous to the *S. cerevisiae OSH3* gene. A 3.6 kb-fragment harboring the *CaOSH3* ORF of 2,535 bp and its 5'- and 3'-regions (698 bp and 357 bp, respectively) was amplified from genomic DNA of the CAI4 strain by using the Expand High Fidelity PCR system (Boeringer-

Table 1. Strains used in this study

Strain	Genotype	Source or Reference
S. cerevisiae		
10560-2B	*MATa ura3-52 his3::hisG leu2::hisG*	G.R. Fink
10560-5B	*MATα ura3-52 trp1::hisG leu2::hisG*	G.R. Fink
JK353	*MATa/MATα ura3-52/ura3-52 his3::hisG/+ trp1::hisG/+ leu2::hisG/leu2::hisG*	This laboratory
JK368	*MATa ura3-52 his3::hisG leu2::hisG osh3::LEU2*	This work
JK369	*MATα ura3-52 trp1::hisG leu2::hisG osh3::LEU2*	This work
JK370	*MATa/MATα ura3-52/ura3-52 his3::hisG/+ trp1::hisG/+ leu2::hisG/leu2::hisG osh3::LEU2/ osh3::LEU2*	This work
C. albicans		
CAI4	*ura3::imm434/ura3::imm43*	G.R. Fink
JKC13	*ura3::imm434/ura3::imm43 OSH3/osh3::hph::URA3*	This work
JKC14	*ura3::imm434/ura3::imm43 osh3::hph/osh3::hisG::URA3*	This work
JKC15	*ura3::imm434/ura3::imm43 osh3::hisG/osh3::hph*	This work
JKC18	*ura3::imm434/ura3::imm43 cph1::hisG/cph1::hisG*	H. Liu

All *S. cerevisiae* strains are derived from ∑1278b strain.

Mannheim). The primers used in this study were as follows: 5'-cgcggatccgatctcccctataaaacc-3'and 5'-gggag aagcagaagaagctttttttcaaattggg-3' (*BamH*I and *Hind*III restriction sites are underlined). The PCR product was then cloned into the *BamH*I and *Hind*III restriction sites of *S. cerevisiae* vector pRS426 (Christianson *et al.*, 1992) to generate the plasmid pRS426-CaOSH3. The nucleotide sequence of the cloned DNA fragment was confirmed by autosequencing. Plsmid pRC18-CaOSH3 was constructed by ligating the *Hind*III fragment of a 3.6 kb *CaOSH3* into the *Hind*III restriction site of the *C. albicans* plasmid, pRC18 (Stoldt *et al.*,

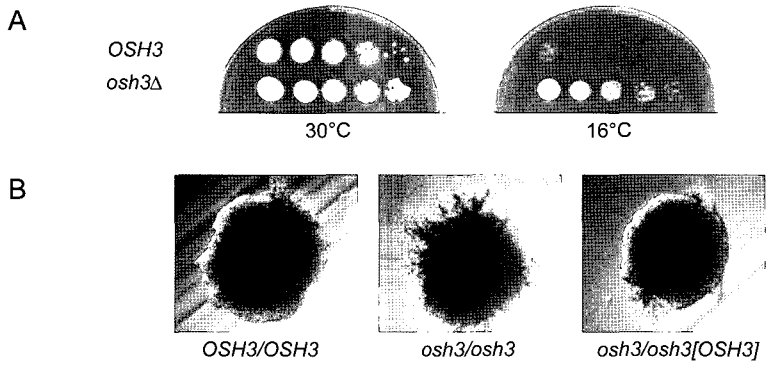

Fig. 1. Phenotype analysis of the *osh3* deletion mutation. (A) Cold-sensitivity test of the wild type (10560-5B) and *osh3*Δ (JK369) mutant strain. Each strain was cultured in YEPD liquid media and the culture was diluted to 10^7, 10^6, 10^5, 10^4 and 10^3 cells/ml. Then each diluted cultures were dropped on YEPD solid media. Plates were incubated at 30°C for 2 days and 16°C for 6 days. (B) Pseudohyphal growth test of the diploid strains. A wild type *OSH3/OSH3* (JK353), *osh3/osh3* (JK370), and *osh3/osh3* carrying plasmid pRS316-OSH3 were streaked on SLAD (50 μM ammonium sulfate) media. After incubation at 30°C for 5 days, the colonies were photographed.

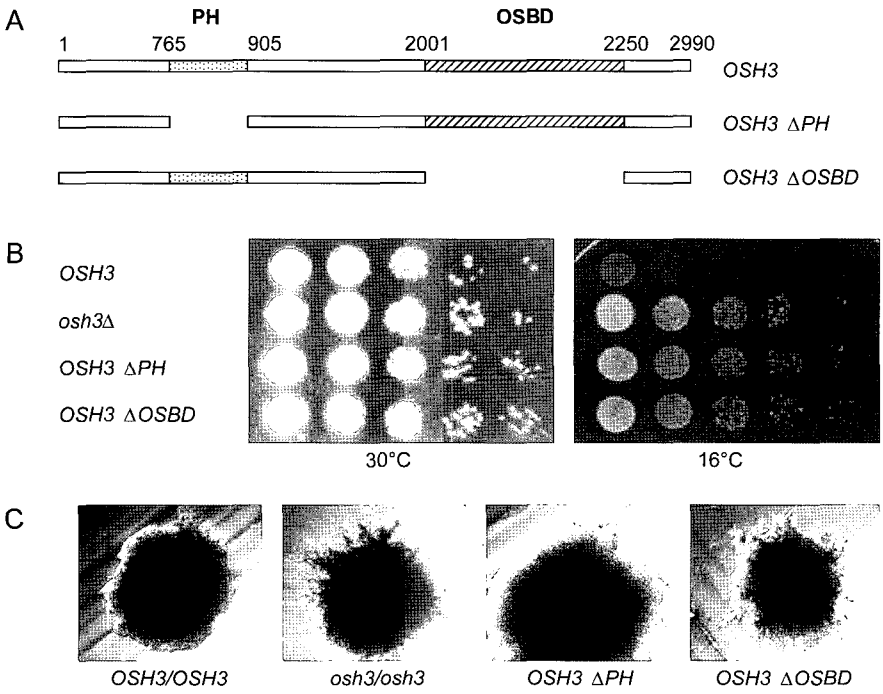

Fig. 2. Phenotype analysis of the domain deletion mutations of the *OSH3* gene. (A) Schematic diagram of the domain deletions of the *OSH3* gene. The Pleckstrin homology domain (PH) and the oxysterol binding domain (OSBD) were indicated. The numbers represent the amino acid residues of the *OSH3* ORF. (B) Cold-sensitivity test of the *osh3* mutant strain (JK369) carrying domain-deletion constructs. Each strain was cultured in YEPD liquid media and the culture was diluted to 10^7, 10^6, 10^5, 10^4, and 10^3 cells/ml. Then each diluted cultures were dropped on YEPD solid media. Plates were incubated at 30°C for 2 days and 16°C for 6 days. (C) Pseudohyphal growth test of the *osh3/osh3* mutant strain (JK370) carrying domain-deletion constructs. Each strain was streaked on SLAD (50 μM ammonium sulfate) media. After incubation at 30°C for 5 days, the colonies were photographed.

1997).

Disruption of CaOSH3

Two different disruption plasmids pDO3Q and pDO3C were constructed (Fig. 4A). Plasmid pRS426-CaOSH3 was digested with *Bgl*II and then was ligated with the *BamH*I fragment of hph-CaURA3-hph cassette isolated from plasmid pQF86 (Feng *et al.*, 1999) or the *BamH*I-*Bgl*II fragment of hisG-CaURA3-hisG cassette isolated from plasmid pCUB-6 (Fonzi and Irwin, 1993) to generate pDO3Q and pDO3C, respectively. To replace the chromosomal copies of *OSH3* with the disruption cassettes, the wild type *C. albicans* strain CAI4 was transformed with the linear *BamH*I fragment, osh3::hph-CaURA3-hph, of pDO3Q. The resulting *CaOSH3/osh3::hph* strain was confirmed by PCR analysis and was transformed again with the linear *BamH*I fragment, osh3::hisG-CaURA3-hisG, of pDO3C. Two subsequent disruptions were confirmed by PCR analysis (Fig. 4B).

Results

osh3 null mutation enhances pseudohyphal growth in S. cerevisiae

In order to investigate the functions of the *OSH3* gene in a pseudohyphal growth, disruption of *OSH3* was carried out in a strain of the ∑1278b genetic background which is commonly used for the study of the filamentation growth. The *osh3* mutant strain showed the cold-resistant phenotype, which confirms the previous results with the S288C strains (Fig. 1A) (Park *et al.*, 2002). The growth of the *osh3/osh3* diploid strain was tested on the pseudohyphal-inducing media (SLAD). The pseudohyphal growth was noticeably enhanced in the *osh3/osh3* mutant colonies as compared with the wild type colonies (Fig. 1B). This enhanced pseudohyphal phenotype of *osh3* was complemented when the wild type *OSH3* plasmid was introduced into the *osh3/osh3* mutant. Previously overexpression of *OSH3* was also shown to induce the pseudohyphal growth (Park *et al.*, 2002). These results suggest that the disturbance of the level of the *OSH3* gene product, either by overexpression or disruption, lead to the enhanced pseudohyphal growth.

Pseudohyphal growth is affected by the oxysterol-binding domain of OSH3

OSH3 contains a PH domain at the amino-terminal end and an oxysterol-binding domain (OSBD) at the carboxyl end (Beh *et al.*, 2001). To determine the specific functions of each domain, we constructed the domain-deletions of *OSH3* and tested their *osh3*-complementating activity. When introduced into the *osh3* mutant strain, the deletion construct, *OSH3ΔPH* or *OSH3ΔOSBD*, did not complement the cold-resistant phenotype of *osh3* (Fig. 2B). These results indicate that both PH and OSBD domains of *OSH3* are required for the normal growth at the cold temperature. Interestingly, when introduced into the *osh3/osh3* mutant strain, the deletion construct, *OSH3ΔPH*, fully restored the wild type phenotype in the pseudohyphal growth test whereas *OSH3ΔOSBD* lost this complementing activity (Fig. 2C). These results indicate that the pseudohyphal growth is regulated by the OSBD domain of *OSH3*.

The mammalian OSBPs bind a range of oxysterols and their interactions were shown to occur through the C-terminal half of OSBP, which contains the OSBD domain (Ridgway *et al.*, 1992). It is possible that the OSBD domain of the yeast Osh3p could also mediate its bindings to oxysterols. Whether the pseudohyphal growth condition alters the intracellular levels of oxysterols is not known. However, Osh3p is a candidate regulatory factor for this regulation.

Identification of OSH3 in C. albicans

Many of the regulatory components of the filamentous growth are conserved in *S. cerevisiae* and the pathogenic yeast *C. albicans*. In order to identify *OSH3* homologs in *C. albicans*, we searched the *C. albicans* databases available at the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida) and the Institute Pasteur (http://genolist.pasteur.fr/CandidaDB). An open reading frame of 844 amino acid residues was revealed, which shows a high homology to the *S. cerevisiae OSH3* (Fig. 3).

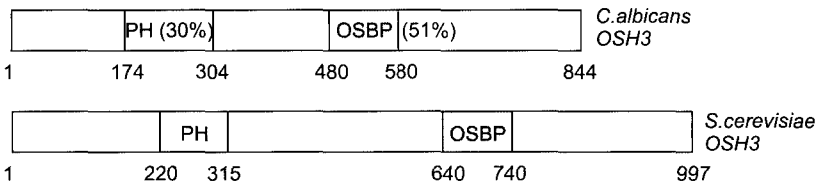

Fig. 3. Amino acid sequence alignment of *C. albicans OSH3* and *S. cerevisiae OSH3* gene products. The Pleckstrin homology motifs (PH) and the oxysterol-binding protein domain (OSBP) are indicated. The percentage of each domain represents the sequence similarities between *OSH3* and *CaOSH3*.

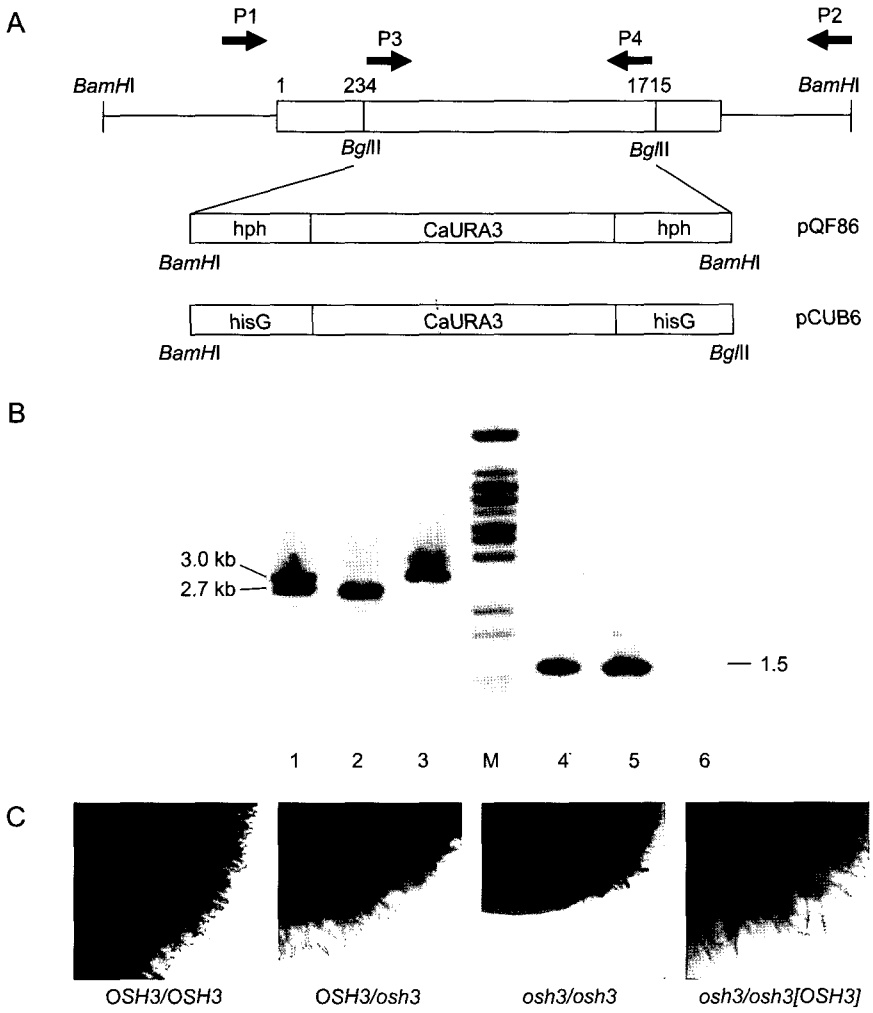

Fig. 4. Disruption of *CaOSH3*. (A) Schematic representations of the disruption cassettes osh3::hph-CaURA3-hph::osh3 and osh3::hisG-CaURA3-hisG::osh3. (B) Identification of *C. albicans osh3* mutants by PCR. Lanel and 5, *osh3::hph/ OSH3* (JKC13); lane 2 and 6 *Caosh3::hph/ Caosh3::hisG* (JKC14); lane 3 and 4, *OSH3/OSH3* (CAI4). Lane 1-3, PCR reactions with primers P1 and P2; lane 4-6, PCR reactions with primers P3 and P4. (C) Colony morphologies of wild type (CAI4), *CaOSH3/caosh3* (JKC13), *caosh3/caosh3* (JKC15), and *caosh3/caosh3* with pRC18-CaOSH3. Strains were streaked on Spider media and plates were incubated at 37°C for 7 days.

OSH3 regulates the filamentation growth in C. albicans

Gene disruption was carried out to investigate the importance of *OSH3* gene for the filamentous growth in *C. albicans*. Two copies of chromosomal *OSH3* gene were sequentially deleted by using osh3::hph-CaURA3-hph::osh3 and osh3::hisG-CaURA3-hisG constructs (Fig. 4A). The heterozygotes, *OSH3/osh3::hph* was isolated and one copy deletion was confirmed by PCR analysis. These strains were used for a second round of gene disruption. The homozygous deletion strain, *osh3::hph/ osh3::hisG* was successfully obtained (Fig. 4B).

To address whether deletion strains of *OSH3* show any defects in filamentation growth, we compared the growth morphology of these strains on various media. In spider medium, *osh3/osh3* mutant strain shows markedly reduced filamentation growth (Fig. 4C). These results suggest that the *OSH3* gene is required for the filamentation growth in *C. albicans*. The filamentation phenotypes of *osh3* deletion mutations were quite opposite in *S. cerevisiae* and *C. albicans*, that is, hyperfilamentation in one and reduced filamentation in the other. We showed that in *S. cerevisiae,* both disruption and overexpression of *OSH3* showed an enhanced filamentation. Again, we speculate that the disturbance of the fine level of the *OSH3* gene product could possibly cause either enhanced or reduced filamentations.

OSH3 overexpression suppresses the filamentation defect of cph1/cph1 mutant strain

In *C. albicans*, the MAP kinase signaling pathway participates in the regulation of the filamentation

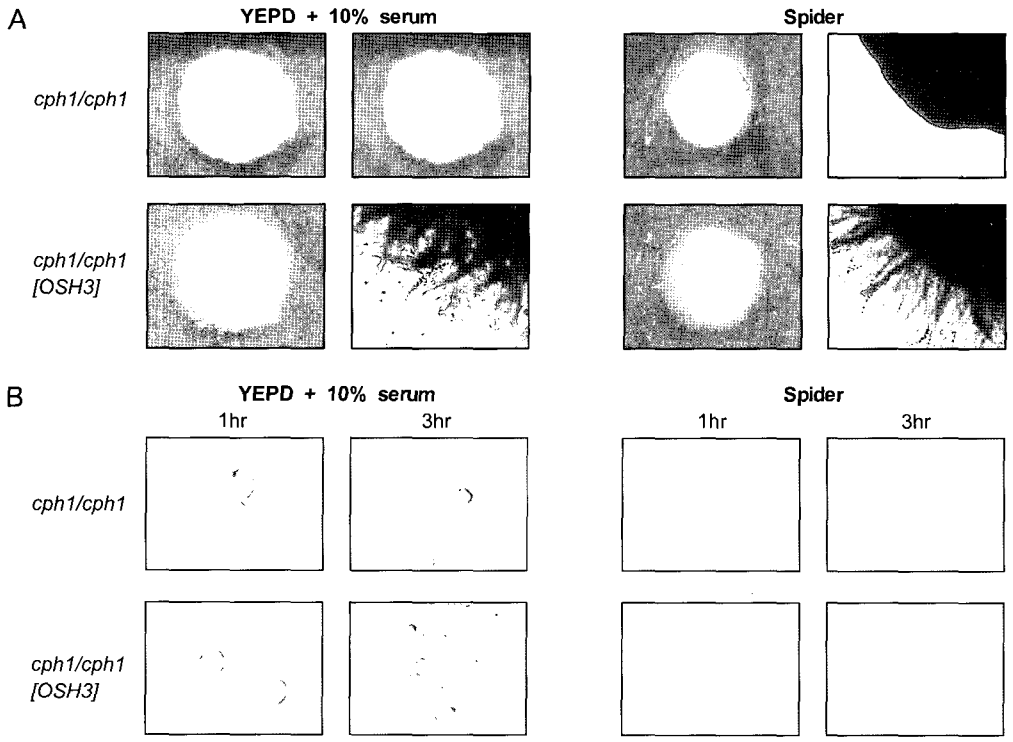

Fig. 5. Suppression of *cph1/cph1* mutant strain by overexpression of *CaOSH3*. (A) Colony morphologies. *C. albicans* strain JKC18 (*cph1/cph1*) carrying pRC18 or pRC18-CaOSH3 is streaked on YEPD with 10% serum. The resulting colonies were photographed. (B) Cellular morphologies of the liquid culture. All strains were incubated in each media at 37°C for 3 h.

growth (Liu *et al.*, 1994). The *CPH1* is the *C. albicans* homolog of *STE12*, a MAPK-associated transcription factor in *S. cerevisiae*, and induces the filamentation-specific genes including *HWP1* in the filamentation-inducing conditions.

To investigate the association of *OSH3* with the filamentation-specific MAPK regulatory cascade, suppression analysis was carried out using the mutation in the *CPH1* gene. The *OSH3* gene was cloned into the pRC18, an autonomously-replicating *C. albicans* vector and introduced this pRC18-CaOSH3 plasmid into the JKC18 (*cph1/cph1*) strain. As shown in Fig. 5A, pRC18-CaOSH3 plasmid restored the filamentation growth phenotype of *cph1/cph1* mutant strain. In liquid culture, *OSH3* transformants showed more increased filamentation than the mutant strain carrying the control vector (Fig. 5B).

These findings suggest that the cellular roles of *OSH3* in the filamentous growth are closely associated with the MAPK cascade, the major signaling pathway in the *C. albicans* filamentation. This work is the first report on the functional analysis of the OSBP homologues in *C. albicans*. Although the yeast *S. cerevisiae* contains seven *OSH* genes, we have identified three ORFs as the OSBP homologues in the *C. albicans* genome data base (data not shown). The *OSH6* gene was previously identified in the sequence analysis of the mating locus (Hull and Johnson, 1999).

Previously, our group reported that the *S. cerevisiae OSH3* gene on the high-copy number vector partially suppressed the haploid invasive growth defect of the *ste12* mutation in *S. cerevisiae* (Park *et al.*, 2002). The invasive growth, observed in haploid cells on rich medium, is a phenotype closely associated with the diploid pseudohyphal growth (Roberts and Fink, 1994). *OSH3* has been suggested to function downstream of or in parallel to the *STE12* transcription factor in the MAPK cascade during the filamentation growth (Park *et al.*, 2002). Our current finding that overexpression of *OSH3* suppressed the filamentation defect of the *C. albicans cph1/cph1* strain leads us to a new understanding of the functional conservations of *OSH3* in *S. cerevisiae* and *C. albicans*.

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