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Complementary function of mitogen-activated protein kinase Hog1 from *Trichosporonoides megachiliensis* in *Saccharomyces cerevisiae* under hyper-osmotic stress

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A (*TmHog1*) gene encoding a mitogen-activated protein kinase (MAPK) homologous to *Saccharomyces cerevisiae Hog1* (*ScHog1*) involved in hyper-osmotic stress signaling was isolated from *Trichosporonoides megachiliensis* SN-124A, an erythritol-producing yeast. Although *TmHog1*, like other *Hog1* homologs, encoded a kinase catalytic domain containing TGY motif, it was 50–60 amino acid residues shorter than the ScHog1. A *TmHog1* transgene rescued the osmotic sensitivity and glycerol production defect of *S. cerevisiae hog1*Δ, a highly osmo-sensitive strain that does not produce glycerol, a compatible solute, during osmotic stress. Functional analyses of chimeric Hog1 proteins constructed from ScHog1 and TmHog1 sequences indicated that the C-terminal region of TmHog1 is more effective for glycerol biosynthesis than ScHog1 under osmotic stress.

[**Key words:** Osmotic stress; HOG pathway; Chimeric Hog1; *Trichosporonoides megachiliensis*; Compatible solute; Erythritol; Mitogen-activated protein kinase]

Osmo-tolerant microorganisms, especially yeast and fungi, produce a wide variety of polyols, such as glycerol, mannitol, arabitol, and xylitol. *Trichosporonoides megachiliensis* SN-124A (currently renamed as *Moniliella megachiliensis* comb. nov.) (1), a hypomycetous yeast strain isolated from dry fruit, produces 30–40% (w/v) of erythritol when cultivated in a high-glucose medium (2). Erythritol, the sugar alcohol of tetrose, has plain sweetness, low energy value and less hygroscopicity than most commercially available sugars (3). Because of these characteristics, erythritol is currently produced by a large-scale fermentative process and is used commercially in food, medical, and chemical industries.

de Hoog GS et al. described that *T. megachiliensis* is able to survive in 60% glucose solutions due to its extraordinary osmo-tolerance (4). Erythritol in *T. megachiliensis*, like glycerol in *Saccharomyces cerevisiae*, is believed to function as a compatible solute, which are produced as a response to hyper-osmotic stress. Hence, the osmotic stress sensors and signal transduction pathways in *S. cerevisiae* and *T. megachiliensis* may be similar. As well as erythritol, *T. megachiliensis* produces significant amounts of glycerol in hyper-osmotic conditions. Other microorganisms that produce mannitol or arabitol are shown also to produce glycerol in response to osmotic stress (5–7). However, the erythritol biosynthesis pathway is fundamentally different from that of glycerol. Glycerol is mainly synthesized from dihydroxyacetone phosphate, an intermediary metabolite in the glycolytic pathway (Embden-Meyerhof-Parnas pathway, EMP). In contrast, erythritol is probably synthesized from erythrose-4-phosphate through the pentose phosphate pathway (PPP) (8). It remains unclear how the yeast *Trichosporonoides* regulates and harmonizes EMP and PPP, and any other metabolic pathways that may be involved in the osmotic stress response.

The role of the *S. cerevisiae* high osmolarity glycerol (HOG) pathway under hyper-osmotic stress has been studied comprehensively (9). The central signaling cascades of the HOG pathway consist of a mitogen-activated protein kinase (MAPK), Hog1; a MAPK kinase (MAPKK), Pbs2; and three upstream MAPKK kinases (MAPKKKs), which activate Pbs2 via phosphorylation. Phosphorylated Pbs2 then successively phosphorylates Hog1 on Thr174 and Tyr176 in the catalytic domain (10–14), and the phosphorylated Hog1 rapidly translocates to the nucleus (15). The Hog1 that has accumulated in nucleus, together with the transcription factor Hot1, stimulates transcription of target genes involved in osmotic adaptation. Hog1 and Hot1 regulates transcription of the gene encoding glycerol-3-phosphate dehydrogenase (GPD1) via the stress response elements (STRE) located somewhere upstream of

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these genes (16–20). Thus, glycerol accumulates rapidly via the glycolytic pathway and the action of GPD1 on dihydroxyacetone phosphate as a template. This compatible solute plays an important role as an osmoregulator in *S. cerevisiae* cells.

In this study, we cloned the *Trichosporonoides megachiliensis* Hog1 ortholog (*TmHog1*), and investigated the structure and function of it. In particular, osmoregulatory function of TmHog1 was compared with ScHog1 in relation to hyper-osmotic stress response and resulting polyol biosynthesis in *S. cerevisiae hog1*Δ. This study may bring a new insight into the mechanism of osmotic signal transduction in fungi-like yeast, *T. megachiliensis*. Additionally, this may lead to improvements in the production of erythritol, a promising sugar-like substance, via metabolic engineering.

MATERIALS AND METHODS

Strains and cultures *Escherichia coli* DH5α was used for all cloning manipulations and was propagated at 37°C in LB medium (2.0% Bacto Tryptone, 1.0% Bacto Yeast Extract, 2.0% NaCl). *T. megachiliensis* SN-124A (National Food Research Institute, Microbial Gene Bank), *S. cerevisiae* BY4741, and *S. cerevisiae* BY4741 *hog1*Δ were cultured at 30°C in YPD medium (1.0% Bacto Yeast Extract, 2.0% peptone, 2.0% glucose). The recombinant transformants of *S. cerevisiae* were selected on SD (-Leu) medium composed of 0.67% yeast nitrogen base (without amino acids), 2% glucose and amino acids as required. When solid medium was used, 2% agar was added to the culture medium.

Sequence analysis of DNA Nucleotide sequences of DNA fragments were determined using Big Dye Terminator v1.1/3.1 (Applied Biosystems, Tokyo) and an ABI automatic sequencer (PerkinElmer Japan, Tokyo). These sequence data were processed using GENETYX-MAC Version 15.0.7 (GENETYX Corporation, Tokyo)

Cloning of *Hog1* fragment from *T. megachiliensis* SN-124A A fragment of the *Hog1* gene was amplified from *T. megachiliensis* SN-124A genomic DNA using PCR with degenerate primers, Hog1-d-for and Hog1-d-rev (Table 1). These primers were designed based on regions within two different fungal Hog1 amino acid sequences that exhibited high homology to *S. cerevisiae* Hog1 amino acid sequence. PCR was carried out using Amplitaq DNA polymerase kit (Applied Biosystems). Amplified DNA fragments obtained were cloned into pGEM®-T Easy vector (Promega, Tokyo), and the recombinant plasmid was transformed into *E. coli* DH5α using the calcium chloride method (21).

Assembly of a full-length *TmHog1* cDNA The sequence of the 3′ end of the *T. megachiliensis Hog1* (*TmHog1*) transcript was determined by 3′ RACE method (22). The reverse transcription product was amplified with an oligo dT-adapter primer (Table 1), and then PCR was carried out using Hog1-3R-for and adapter primer-rev (Table 1) as primers. The sequence of the 5′ region of the *TmHog1* transcript was determined by analyzing the sequence of a genomic phage clone containing *TmHog1*. A *T. megachiliensis* SN-124A genomic library was constructed in phage using a Lambda FIX® II/*Xho* I Partial Fill-in Vector kit (Stratagene, Tokyo) and Gigapack III Gold Packaging Extract (Stratagene). The clone containing the *TmHog1* gene was selected by direct phage plaque PCR. Using complementary DNA as template, an assembled full-length cDNA was amplified using nested PCR (Fig. 1) with primers designed from 5′ UTR and 3′ UTR sequences, Hog1 ATG-1, and Hog1-STOP, respectively (Table 1). The resulted DNA fragment was cloned into pGEM®-T Easy vector (Promega), and then sequenced.

TABLE 1. Oligonucleotides used in this study.

Primer name	Sequence (from 5′ to 3′)
Hog1-d-for	ATGGGIGCITTYGGIYTIGTTT
Hog1-d-rev	ADATYTTTIARRTCRCARTTYCT
Oligo dT-adapter primer	GTCGAGCTCTACGTACAATTTTTTTTTTTT
Hog1-3R-for	AAGCCCTTCAGTAGCCCGGTTC
Adapter primer-rev	GTCGAGCTCTACGTACAA
Hog1-ATG3-for	CCACCGGCTACCTTGAAG
Hog1-nest-rev	GCGAAGGATTTGGTATAGGA
Hog1 ATG-1	TTTATGGCTGACTTTGTCAA
Hog1-STOP	AAAATTGCAGAACAGTTGA
ScHog1-for	TTTATGACCACTAACGAGGA
ScHog1-rev	TTATTATTACTGTTGGAACTCATTA
ScHog1 Cdel-rev	CTTCCATAAGATTGGTGGCAGTGATTAA
Tm-ScChimera-rev	GGTAAGGAGCAAGGTAAGGATGTGTAAGT
Tm-ScChimera-for	TCCTTACCTTGCTCCTTACCACGATCCAA
Sc-TmChimera-rev	GGTATGGTGCCGAATAAGGATGAGCCAAG
Sc-TmChimera-for	TCCTTACCTTGCTCCTTACCACGATCCAA
pDEST-for	GTTTGCTGTCTTGCTATCA
pDEST-rev	ACCTTGATTGGAGACTTGA

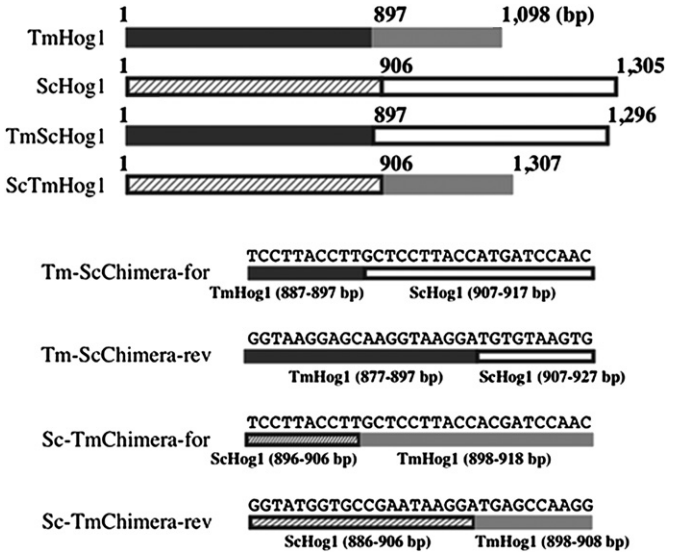

FIG. 1. Construction of chimeric *Hog1* of *ScHog1* and *TmHog1* using PCR and anchor primers. *TmScHog1* (1–897 bp, *TmHog1*; 907–1305 bp, *ScHog1*) and *ScTmHog1* (1–906 bp, *ScHog1*; 898–1098 bp *TmHog1*) were constructed by replacing the non-kinase 3′ region of *TmHog1* and *ScHog1*.

Chimeric *Hog1* constructs Base pairs 36–897 of *TmHog1* were predicted to encode a kinase domain, and base pairs 66–906 of an *S. cerevisiae Hog1* (*ScHog1*) cDNA construct encode the kinase domain. We used PCR to engineer chimeric *Hog1s* constructs containing sequences from *ScHog1* and *TmHog1,* essentially swapping the C-terminal regions lacking the kinase domains (Fig. 2). The N-terminal kinase region of *ScHog1*, (base pairs 66–906) was amplified using Sc-TmChimera-rev and pDEST-for (Table 1) as primers and p*ScHog1* (Table 2) as template; the C-terminal non-kinase region of *TmHog1* was amplified using Sc-TmChimera-for and pDEST-rev (Table 1) as primers and p*TmHog1* (Table 2) as template. These two PCR products were mixed and further amplified using pDEST-for and pDEST-rev (Table 1) as primers. The product, chimeric *ScTmHog1* constructed from *ScHog1* head and *TmHog1* tail, was cloned into pGEM®-T Easy (Promega). The same strategy was employed to construct *TmScHog1* (*TmHog1* head and *ScHog1* tail) using two primers set, Tm-ScChimera-for and pDEST-rev, pDEST-for and Tm-ScChimera-rev (Table 1). Additionally, *ScHog1* and C-terminal region lacking *ScHog1* (1–1050 bp of *ScHog1*), *ScHog1 Cdel*, were amplified by PCR using ScHog1-for; ScHog1-rev and ScHog1-for; ScHog1 Cdel-rev as primers (Table 1) and cDNA of *S. cerevisiae* as template and then, cloned into pGEM®-T Easy.

Yeast transformation To determine whether *TmHog1*, *ScHog1 Cdel*, *ScTmHog1*, and/or *TmScHog1* could complement a *hog1*Δ null allele and rescue the phenotypes associated with this mutation, a yeast centromere plasmid vector was constructed as follows. The multicloning site of pBluescript II SK+ was inserted into the yeast centromere plasmid vector, pDEST32 (Invitrogen), which had been digested with *Hind*III and *Sac*I (pDB05). A *Not*I fragment containing *TmHog1* was isolated from the pGEM®-T Easy plasmid and was ligated into *Not*I digested pDB05 to generate p*TmHog1*, which harbored *TmHog1* (Table 2). *ScHog1*, *ScHog1 Cdel*, *ScTmHog1*, and *TmScHog1* were cloned into pDB05 in the same manner to generate p*ScHog1*, p*ScHog1 Cdel*, p*ScTmHog1*, and p*TmScHog1* (Table 2), respectively. Each pDB05-*Hog1* plasmid, p*TmHog1*, p*ScHog1*, p*ScHog1 Cdel*, p*ScTmHog1*, p*TmScHog1*, and pDB05 was individually transformed to *S. cerevisiae* BY4741 *hog1*Δ cells according to the method of Finlayson et al. (23). Leu+ transformants were selected on SD medium without leucine.

Growth test of yeast cells *S. cerevisiae hog1*Δ transformants were grown up to the early exponential phase in YPD medium and each 5 μl culture was adjusted to 3×10^3 cells just before use. As indicated in Fig. 3, serial, 10-fold dilutions of cell cultures were spotted onto YPD plates supplemented with one of three osmolytes, NaCl, glucose, and sorbitol.

Determination of glycerol content *S. cerevisiae* BY4741 *hog1*Δ, BY4741 transformant harboring p*TmHog1*, p*ScHog1*, p*ScHog1 Cdel*, p*ScTmHog1*, p*TmScHog1*, or pDB05 (a negative control) were cultured aerobically at 30°C in the absence of NaCl. Cells were grown to the early exponential phase (OD_{600} 0.5), and then NaCl (up to 0.5 M) was added to the cultures. After incubation for 1 h at 30°C, cells were harvested and washed with 0.5 M NaCl.

Intracellular glycerol was extracted according to the method of Tanaka-Tsuno et al. (24). To determine intracellular glycerol content, the glycerol was measured using the ICS-3000 (Dionex, Osaka) chromatographic system and a Carbo Pac MA-1 column. Results from three independent experiments were used to calculate the glycerol content values. It was expressed as microgram of glycerol per 1 mg of dry cell weight.