Two putative MAP kinase genes, *ZrHOG1* and *ZrHOG2*, cloned from the salt-tolerant yeast *Zygosaccharomyces rouxii* are functionally homologous to the *Saccharomyces cerevisiae* *HOG1* gene

Tomoko Iwaki, Youichi Tamai and Yasuo Watanabe

Author for correspondence: Yasuo Watanabe. Tel: +81 89 946 9849. Fax: +81 89 977 4364.
e-mail: watanabe@agr.ehime-u.ac.jp

Laboratory of Biochemistry,
Faculty of Agriculture,
Ehime University, 3-5-7
Tarumi, Matsuyama, Ehime
790-8566, Japan

The salt-tolerant yeast *Zygosaccharomyces rouxii* can adjust its osmotic balance when responding to osmotic shock by accumulating glycerol as the compatible osmolyte. However, the mechanism of glycerol production in *Z. rouxii* cells and its genetic regulation remain to be elucidated. Two putative mitogen-activated protein (MAP) kinase genes, *ZrHOG1* and *ZrHOG2*, were cloned from *Z. rouxii* by their homology with *HOG1* from *Saccharomyces cerevisiae*. The deduced amino acid sequences of ZrHog1p and ZrHog2p indicated close homology to that of Hog1p and contained a TGY motif for phosphorylation by MAP kinase kinase. When *ZrHOG1* or *ZrHOG2* was expressed in an *S. cerevisiae* *hog1*Δ null mutant, the salt tolerance and osmotic tolerance characteristics of wild-type *S. cerevisiae* were restored. In addition, the aberrant cell morphology and low glycerol content of the *hog1*Δ null mutant were corrected, indicating that ZrHog1p and ZrHog2p have functions similar to Hog1p. While the transcription of the glycerol-3-phosphate dehydrogenase gene (*GPD1*) of the *ZrHOG1*-harbouring *S. cerevisiae* mutant was similar to that of wild-type *S. cerevisiae*, the *ZrHOG2*-harbouring strain showed prolonged *GPD1* transcription. Both *Zrhog1*Δ and *Zrhog2*Δ *Z. rouxii* null mutants showed a decrease in salt tolerance compared to the wild-type strain. The present study suggested the presence of a high-osmolarity glycerol response (HOG) pathway in *Z. rouxii* similar to that elucidated in *S. cerevisiae*. Two putative MAP kinase genes in *Z. rouxii* appeared to be significant in either osmotic regulation or ion homeostasis.

Keywords: *Zygosaccharomyces rouxii*, salt tolerance, MAP kinase gene, glycerol production, glycerol-3-phosphate dehydrogenase gene

INTRODUCTION

In *Saccharomyces cerevisiae*, hyperosmotic shock induces the enhanced formation and accumulation of glycerol as a compatible osmolyte (Reed *et al.*, 1987). This protects against osmotic lysis. Recently, the osmotic stress signal transmission pathway of *S. cerevisiae* has been elucidated, and was named the high-osmolarity glycerol response (HOG) pathway (Brewster *et al.*,

1993). It belongs to the mitogen-activated protein (MAP) kinase cascade (Brewster *et al.*, 1993).

In the HOG pathway of *S. cerevisiae*, there are two osmosensors (encoded by *SLN1* and *SHO1* genes) present in the plasma membrane (Ota & Varshavsky, 1993; Maeda *et al.*, 1994). The osmotic stress signal sensed by the *SLN1* product (Sln1p) is, in turn, transmitted from Ypd1p to Ssk1p to Ssk2/22p (MAP kinase kinase kinase) to Pbs2p (MAP kinase kinase) and then finally to Hog1p (MAP kinase) (Posas *et al.*, 1996). On the other hand, Sho1p (Maeda *et al.*, 1995) regulates the action of Hog1p via Ste11p (MAP kinase kinase kinase) and Pbs2p (Posas & Saito, 1997). The activated Hog1p

Abbreviations: GPDH, glycerol-3-phosphate dehydrogenase; HOG, high-osmolarity glycerol response; MAP, mitogen-activated protein.

The GenBank/EMBL/DDBJ accession numbers for the sequences in this paper are AB012146 (*ZrHOG1*) and AB012088 (*ZrHOG2*).

directly or indirectly enhances the activity of a transcriptional regulator which induces transcription of stress protein genes harbouring stress regulatory elements (STRE) in their promoters (Schuller *et al.*, 1994). Since the glycerol-3-phosphate dehydrogenase (GPDH) gene (*GPD1*) harbours four STREs in the 5'-flanking region, the transcription of *GPD1* is enhanced and the enzymic activity increases under hyperosmotic conditions (Albertyn *et al.*, 1994; Akhtar *et al.*, 1997; Norbeck & Blomberg, 1997). As a result, the intracellular concentration of glycerol rises and the osmotic balance is adjusted.

The salt-tolerant yeast *Zygosaccharomyces rouxii* also accumulates glycerol intracellularly as the main compatible osmolyte in response to hyperosmotic shock (Reed *et al.*, 1987). The participation of GPDH in the production of glycerol in *Z. rouxii* cells is not obvious. Moreover, the transmission pathway of the osmotic stress signal has not yet been elucidated. Thus, in the present study, to clarify whether a similar pathway to that elucidated in *S. cerevisiae* is present in *Z. rouxii*, two *Z. rouxii* genes with homology to *HOG1* were cloned and the functions of the cloned genes were analysed in *S. cerevisiae* *hog1Δ* null mutants.

METHODS

Strains and cultures. A salt-tolerant yeast, *Z. rouxii* strain MA11-3 [from Dr Ushio, Higashimaru Shoyu Co. Ltd, Hyogo, Japan; *MATα leu2* (pSR1)], was cultured aerobically at 30 °C in YPAD medium (0.5%, w/v, yeast extract; 2%, w/v, polypeptone; 2%, w/v, glucose; 0.5%, w/v, KH_2PO_4 ; 0.2%, w/v, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 400 µg adenine ml^{-1}) containing 5% (w/v) NaCl. *S. cerevisiae* JBY10 (from Dr Gustin, Rice University, Houston, TX, USA; *hog1Δ* null mutant, *MATα ura3 leu2 his3 trp1 lys2 ade2 hog1-Δ1::TRP1*) and INVSc1 strains (from Invitrogen; *MATα his3-01 leu2 trp1-289 ura3-52*) were cultured aerobically at 30 °C in YEPD medium (1%, w/v, yeast extract; 2%, w/v, polypeptone; 2%, w/v, glucose). The plate cultures were carried out with YPAD or YEPD plates solidified by 2% (w/v) agar and inoculated with 5 µl from serial 10-fold dilutions of stationary-phase cultures. *Escherichia coli* DH-1 and JM109 strains were cultured aerobically at 37 °C in LB medium (1%, w/v, tryptone; 0.5%, w/v, yeast extract; 1%, w/v, NaCl; pH 7.2). The plate cultures were conducted with LB plates solidified by 1.5% (w/v) agar.

Cloning the *Z. rouxii* gene homologous to the *S. cerevisiae* *HOG1* gene. A *HOG1*-harbouring DNA fragment (approx. 950 bp) was obtained by PCR amplification with oligonucleotides 5' GGAACAAAGGGAAACAGGG 3' and 5' AGCCGAATAAGGATGAGCCA 3' as primers and *S. cerevisiae* genomic DNA as the template DNA. After purification by agarose gel electrophoresis, the DNA fragment was labelled with digoxigenin according to the application manual of the Digoxigenin DNA Labelling kit (Boehringer Mannheim) and used as a *HOG1* probe.

Z. rouxii genomic DNA was digested with *EcoRI*, *BamHI*, *HindIII*, *XbaI*, *KpnI* or *SmaI*. The digests were electrophoresed on an agarose gel and DNA fragments in the gel were transferred to a nylon filter sheet with a vacuum blotter (Pharmacia Biotech). After fixing the DNA fragment to the nylon filter with a UV-cross-linker, hybridization was performed as described in our previous report (Watanabe *et al.*,

1995), using a hybridization solution containing the *HOG1* probe (30 ng ml^{-1}). The hybridized filter was first washed with $3 \times \text{SSC}$ ($1 \times \text{SSC}$ contains 150 mM NaCl and 15 mM sodium citrate), 0.2% (w/v) SDS, 5 mM EDTA at room temperature and then filter sets were washed under one of the following conditions: (i) $2 \times \text{SSC}$, 0.2% (w/v) SDS, 5 mM EDTA, 42 °C; (ii) $0.3 \times \text{SSC}$, 0.2% (w/v) SDS, 5 mM EDTA, 55 °C; (iii) $0.2 \times \text{SSC}$, 0.2% (w/v) SDS, 5 mM EDTA, 55 °C; (iv) $0.1 \times \text{SSC}$, 0.2% (w/v) SDS, 5 mM EDTA, 55 °C. Hybridization of the *HOG1* probe was visualized with a Digoxigenin DNA Detection kit (Boehringer Mannheim). Two signals derived from approximately 5 kb and 6 kb *HindIII* DNA fragments were detected under condition (iii).

Z. rouxii genomic DNA was digested with *HindIII*. DNA fragments sized from 3.5 to 7 kb were collected and ligated with *HindIII*-digested charomid 9-36 cosmid DNA. After the *in vitro* packaging of its ligate, it was transfected to *E. coli* DH-1 cells. The prepared genomic DNA library indicated an efficiency of 2.4×10^5 colonies μg^{-1} for the charomid DNA. Five positive clones were obtained from the library (4×10^4 colonies) by colony hybridization described in a previous report (Watanabe *et al.*, 1995) with the *HOG1* probe.

DNA sequencing analysis and transformation of *S. cerevisiae*.

Restriction enzyme maps of *HindIII* DNA fragments were constructed. After subcloning DNA fragments into pUC vectors, nucleotide sequences were determined with a Takara Taq Cycle Sequencing kit (Takara Shuzo) and an auto-sequencer (DSQ1; Shimadzu). *S. cerevisiae* cells were transformed according to the application manual of the Alkali Cation Yeast Transformation kit (Bio 101).

Construction of gene-disruption mutants of *Z. rouxii* *HOG1* homologous genes (*ZrHOG1* and *ZrHOG2*).

Gene disruption was carried out by the one-step gene disruption method (Rothstein, 1983). Briefly, after subcloning the *HindIII* DNA fragment (*ZrHOG1* or *ZrHOG2*, see Fig. 1) into the pUC19 vector, the recombinant DNA (pUC19-*ZrHOG1* or pUC19-*ZrHOG2*) was digested with *EcoRV* (two *EcoRV* sites are present in the *HindIII* DNA fragment). A *LEU2*-harbouring DNA fragment, which was isolated by digesting YEp13 with *BglII* and then filled with Klenow fragment, was inserted into *EcoRV*-digested pUC19-*ZrHOG1* or pUC19-*ZrHOG2*. After digesting pUC19-*ZrHOG1* or pUC19-*ZrHOG2* with *HindIII* and removing pUC vector DNA, *ZrHOG1-ΔLEU2* or *ZrHOG2-ΔLEU2* DNA was used to transform *Z. rouxii* MA11-3 according to the method of Ushio *et al.* (1988). *Leu*⁺ transformants were selected on SC medium without leucine (Rose *et al.*, 1990).

EcoRV-SalI DNA and *EcoRV-PstI* DNA fragments (see Fig. 1) were isolated from *ZrHOG1* and *ZrHOG2* *HindIII* DNA fragments, labelled with digoxigenin as described above, and used as *ZrHOG1* or *ZrHOG2* probes. The gene disruption of *ZrHOG1* or *ZrHOG2* was determined by Southern blot analysis with *ZrHOG1* or *ZrHOG2* probes and genomic DNA prepared from gene-disruption mutants.

Functional expression of *ZrHOG1* or *ZrHOG2* in *S. cerevisiae* *hog1Δ* null mutants (JBY10 strain).

Two *HindIII* DNA fragments harbouring *ZrHOG1* and *ZrHOG2* were blunted and ligated into *NruI*-digested YCp50 vector or *PvuII*-digested YEp13 vector. The recombinant plasmids (YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1* and YEp13-*ZrHOG2*) were used to transform the *S. cerevisiae* JBY10 strain.

Estimation of intracellular glycerol content in various *S. cerevisiae* strains. Various *S. cerevisiae* strains (JBY10, YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1*, YEp13-*ZHO2* and INVSc1 strains) were cultured aerobically

Fig. 1. Restriction map of *ZrHOG1* and *ZrHOG2* and strategy of gene disruptions. Arrows indicate the loci and orientation of their genes.

Sc HOG1	MTNEEFIRT	QIFGTVFEIT	NRYNDLNPVG	MGAFGLVCSA	TDTLTSQVPA	IKKIMKPFST	AVLAKRTYRE	LKLLKHLRHE
Ca HOG1	MSADGEFRT	QIFGTVFEIT	NRYTELNPVG	MGAFGLVCSA	VDRLTGQVPA	VKKVMKPFST	SVLAKRTYRE	LKLLKHLRHE
Zr HOG1	MATHEEFIRT	QVFGTVFEIT	NRYTDLNPVG	MGAFGLVCSA	TDTLAGQVPA	IKKIMKPFST	AVLAKRTYRE	LKLLKHLRHE
Zr HOG2	MATHEEFIRT	QVFGTVFEIT	NRYTDLNPVG	MGAFGLVCSA	TDTLVGQVPA	IKKIMKPFST	AVLAKRTYRE	LKLLKHLRHE
	***	*****	*****	*****	*****	*****	*****	*****
Sc-HOG1	NLICLDIFL	SPLEDIYFVT	ELQGTDLHRL	LQTRPLEKQF	VQYFLYQILR	GLKYVHSAGV	IHRDLKPSNI	LINENCCLKI
Ca-HOG1	NLITLDDIFL	SPLEDIYFVN	ELQGTDLHRL	LNSRPLEKQF	IQYFTYQIMR	GLKYIHSAGV	IHRDLKPSNI	LINENCCLKI
Zr-HOG1	NLICLDIFL	SPLEDIYFVT	ELQGTDLHRL	LQTRPLEKQF	VQYFLYQILR	GLKYVHSAGV	IHRDLKPSNI	LINENCCLKI
Zr-HOG2	NLICLDIFL	SPLEDIYFVT	ELQGTDLHRL	LQTRPLEKQF	VQYFLYQILR	GLKYVHSAGV	IHRDLKPSNI	LINENCCLKI
	***	*****	*****	*****	*****	*****	*****	*****
Sc-HOG1	CDFGLARIQD	PQMTGYVSTR	YYRAPEIMLT	WQKYDVEVDI	WSAGCIFAEM	IEGKPLFPFK	DHVHQFSIIT	DLGSPPKDV
Ca-HOG1	CDFGLARLQD	PQMTGYVSTR	YYRAPEIMLT	WQKYDTEVDL	WSVGCILAEM	IEGKPLFPFK	DHVHQFSIIT	ELLGSPPADV
Zr-HOG1	CDFGLARIQD	PQMTGYVSTR	YYRAPEIMLT	WQKYDVEVDI	WSAGCIFSEM	IEGKPLFPFK	DHVHQFSIIT	DLGSPPRDV
Zr-HOG2	CDFGLARIQD	PQMTGYVSTR	YYRAPEIMLT	WQKYDVEVDI	WSAGCIFSEM	IEGKPLFPFK	DHVHQFSIIT	DLGSPPRDV
	*****	*****	*****	*****	*****	*****	*****	*****
Sc-HOG1	INTICSENTL	KFVTSPLPHR	PIPFSEFRT	---VEPDAVD	LLEKMLVFDP	KKRITAADAL	AHPYSAPYHD	PTDEPVADAK
Ca-HOG1	IDTICSENTL	RFVQSLPHR	PIPFSEFAS	CTHVEPEAID	LLAKLLVFDP	KKRISAVQGL	THPYMEAYHD	PTDEPVACESK
Zr-HOG1	IITICSENTL	KFVTSPLPHR	PVPFQERFKA	---VEPDAVD	LLGRMLVFDP	KKRITAADAL	VHPYLAPYHD	PTDEPIAEAQ
Zr-HOG2	INTICSENTL	KFVTSPLPHR	PVPFQERFKA	---VEPDAVD	LLRRMLVFDP	KKRITAADAL	VHPYLAPYHD	PTDEPTAEAQ
	*****	*****	*****	*****	*****	*****	*****	*****
Sc-HOG1	FDWBFNDADL	PVDTRVMMY	SEILDFHKIG	(+69)				
Ca-HOG1	FDWSFNDADL	PVDTRVMMY	SEILDFHQTV	(+27)				
Zr-HOG1	FDWDFNDADL	PVDTRVMMY	SEILDFHKIG	(+60)				
Zr-HOG2	FDWDFNDADL	PVDTRVMMY	SEILDFHKIG	(+73)				
	*****	*****	*****					

Fig. 2. Homology alignment of amino acid sequences of Hog1ps from *S. cerevisiae* (Sc-HOG1), *C. albicans* (Ca-HOG1) and *Z. rouxii* (Zr-HOG1 and Zr-HOG2). Identical residues and conservative substitutions are shown by asterisks and dots, respectively. The TGY motif is boxed.

at 30 °C in the absence of NaCl. Cells grown to the early exponential phase (OD_{600} 0.5) were collected and subjected to a new medium containing 0.5 M NaCl. After incubation for an appropriate period, cells were harvested and washed twice with 0.5 M NaCl. The cell pellet was then suspended in 5 ml distilled water and disrupted by vortexing for 10 min with 5 g glass beads at 4 °C. The homogenate was centrifuged for 20 min at 10000 *g* at 4 °C. The supernatant was immediately heated for 10 min in boiling water to inactivate enzymes connected to the metabolism of glycerol. After centrifugation for 20 min at 10000 *g* to remove denatured protein, the supernatant was used to measure the intracellular glycerol concentration. The glycerol content was determined according to the application manual of the Glycerol-F kit (Boehringer Mannheim).

Northern blot analysis of *GPD1* mRNA in *S. cerevisiae* *ZrHOG1*- or *ZrHOG2*-harbouring strains. Total RNA was

prepared from YCp50-*ZrHOG1* and YCp50-*ZrHOG2* strains according to a method described previously (Watanabe *et al.*, 1995). The DNA fragment that encodes the *GPD1* of *S. cerevisiae* was prepared by PCR amplification with oligonucleotides 5' ATGTCTGCTGCTGCTGATAG 3' and 5' AGAGCCTCTGAAAAAAGTGGG 3' as primers and genomic DNA as a template. The DNA fragment was labelled with digoxigenin as described above and used as a *GPD1* probe. Northern blot analysis was carried out as described previously (Watanabe *et al.*, 1993).

RESULTS

Cloning of *HOG1* homologues from *Z. rouxii*

A *Z. rouxii* genomic DNA library was constructed as described in Methods. By screening 40000 independent clones with a *HOG1* probe, five positive clones were

Fig. 3. Growth of *Z. rouxii* wild-type (MA11-3) and *Zrhog1Δ* and *Zrhog2Δ* null mutant strains. Serial 10-fold dilutions of saturated cultures were spotted onto YPAD plates supplemented with NaCl as indicated.

Fig. 4. Growth of *S. cerevisiae* *hog1Δ* (JBY10), wild-type (INVSc1) and *ZrHOG1*- or *ZrHOG2*-harbouring (YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1* or YEp13-*ZrHOG2*) strains in the presence of NaCl, KCl or sorbitol. Serial 10-fold dilutions of saturated cultures were spotted onto YEPD plates supplemented with NaCl, KCl or sorbitol as indicated.

obtained. Restriction enzyme maps of *Hind*III DNA fragments present in cosmid DNAs recovered from the clones were constructed. By comparing their maps, the five clones were divided into two groups based on the size of their *Hind*III DNA fragments (Fig. 1). One group (designated *ZrHOG1*) had 6 kb *Hind*III DNA fragments and the other group (*ZrHOG2*) had 5 kb fragments.

The nucleotide sequence of the region hybridized with the HOG1 probe was determined for the two groups. ORFs that lacked introns were found in both groups. The nucleotide sequences of *ZrHOG1* and *ZrHOG2* indicated approximately 70 and 80% homology to that of *S. cerevisiae* HOG1 (Brewster *et al.*, 1993). The deduced amino acid sequences of ZrHog1p and ZrHog2p were approximately 93 and 89% homologous to that of Hog1p (Brewster *et al.*, 1993; Fig. 2). These results indicate that genes homologous to *S. cerevisiae* HOG1 are present in *Z. rouxii* cells and the copy number of HOG1 homologue in *Z. rouxii* is different from that in *S. cerevisiae*. The TGY motif (Brewster *et al.*, 1993) was found in the ZrHog1p and ZrHog2p sequences.

Salt tolerance of *Z. rouxii* *Zrhog1Δ* or *Zrhog2Δ* null mutants

The disruption of *ZrHOG1* or *ZrHOG2* in *Z. rouxii* cells was carried out as described in Methods. Both disruptions were verified by Southern blot analysis of genomic DNA prepared from *Z. rouxii* *Zrhog1Δ* and *Zrhog2Δ* null mutants (data not shown). Both *Zrhog1Δ* and *Zrhog2Δ* null mutants could grow in the presence of NaCl below 2.5 M (Fig. 3). In contrast, the parental

Fig. 5. Changes in intracellular content of glycerol in *S. cerevisiae* *hog1Δ* (JBY10), wild-type (INVSc1) and *ZrHOG1*- or *ZrHOG2*-harbouring (YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1* or YEp13-*ZrHOG2*) strains. Cells were grown in the absence of NaCl and harvested at OD₆₀₀ 0.5. The cells were resuspended in fresh medium containing 0.5 M NaCl at the same cell concentration and incubated at 30 °C for 1 h, and then glycerol content was determined. Results are the means of at least three independent experiments. The standard errors of means are indicated. □, NaCl-free; ■, 0.5 M NaCl.

MA11-3 strain was able to grow in the presence of 3 M NaCl (Fig. 3). This indicates that the salt tolerance of *Z. rouxii* cells decreases when *ZrHOG1* or *ZrHOG2* was

Fig. 6. Morphology of *S. cerevisiae* *hog1* Δ (JBY10) and *ZrHOG1*- and *ZrHOG2*-harbouring (YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1* or YEp13-*ZrHOG2*) strains. Cells were grown in the presence of 0.5 M NaCl.

disrupted, and both ZrHog1p and ZrHog2p are presumed to participate functionally in the transmission pathway of the osmotic signal in *Z. rouxii* cells.

Complementation of an *S. cerevisiae* *hog1* Δ null mutant by *ZrHOG1* and *ZrHOG2*

To elucidate the functions of *ZrHOG1* and *ZrHOG2*, complementation of an *S. cerevisiae* *hog1* Δ null mutant (JBY10 strain) was tested with *ZrHOG1* and *ZrHOG2*. The salt tolerance of the JBY10 strain is lower than that of the wild-type strain, because the enhancement of GPDH activity in response to osmotic stress is low when there is no MAP kinase gene (*HOG1*). If *ZrHOG1* or *ZrHOG2* complemented the deletion of *HOG1* in the *S. cerevisiae* JBY10 strain, the osmotic tolerance of the recombinant strain should increase when compared to the JBY10 strain. This transcomplementation has been used to analyse the function of MAP kinase genes cloned

from mammalian and plant cells (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Kumer *et al.*, 1995; Popping *et al.*, 1996). DNA fragments (*Hind*III fragments, see Fig. 1) which harbour the promoters, ORFs and terminators of *ZrHOG1* and *ZrHOG2* genes were inserted into the yeast centromeric vector YCp50 and 2 μ -circle-based vector YEp13, and the recombinant plasmid DNAs were transformed into the JBY10 strain. The transformed yeast strains (YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1* and YEp13-*ZrHOG2* strains) were plated on plates containing 1 M NaCl, 1 M KCl or 2 M sorbitol (Fig. 4). The *hog1* Δ null mutant (JBY10 strain) could not grow on these plates, but YCp50-*ZrHOG1*, YCp50-*ZrHOG2*, YEp13-*ZrHOG1* and YEp13-*ZrHOG2* strains indicated growth that was comparable to the wild-type strain (INVSc1) under all conditions. These results indicate that both *ZrHOG1* and *ZrHOG2* have functions similar to the *S. cerevisiae* MAP kinase gene (*HOG1*) and that at least

Fig. 7. Northern blot analysis of the *GPD1* transcript in *S. cerevisiae* *hog1Δ* (JBY10), wild-type (INVSc1) and *ZrHOG1*- and *ZrHOG2*-harbouring (YCp50-*ZrHOG1* and YCp50-*ZrHOG2*) strains. Cells subjected to the NaCl treatment indicated in Fig. 5 were cultivated at 30 °C. Samples of cells were withdrawn from cultures at the intervals indicated. Total RNA (approx. 8 µg) was loaded in each lane. An ethidium bromide gel revealing the 26S and 18S rRNA bands is shown to validate equal loading of the lanes and the integrity of the RNAs.

the promoters and terminators of *ZrHOG1* and *ZrHOG2* are functional in *S. cerevisiae* cells.

In *S. cerevisiae* *hog1Δ* null mutants (JBY10 strain), the enhancement in intracellular glycerol content during osmotic stress was much lower (Fig. 5) than in the wild-type strain (INVSc1). When the JBY10 strain, YCp50-*ZrHOG1* and YCp50-*ZrHOG2* strains and YEp13-*ZrHOG1* and YEp13-*ZrHOG2* strains were cultured in the presence of 0.5 M NaCl, YCp50-*ZrHOG1* and YCp50-*ZrHOG2* strains and the YEp13-*ZrHOG1* strain showed a significant increase in glycerol content compared with the JBY10 strain (Fig. 5). The YEp13-*ZrHOG2* strain showed only a slight increase in glycerol content (Fig. 5). However, these increased levels of glycerol were lower than that observed in the INVSc1 strain. This difference may result from the lower promoter activity of *ZrHOG1* or *ZrHOG2* in *S. cerevisiae* cells. Also, no effect of increased *ZrHOG1*

copy number on glycerol content was observed, and increased *ZrHOG2* copy number negatively affected the production of glycerol. The cause of the negative effect of increases in *ZrHOG2* copy number is unclear at the present.

It has been previously reported that *S. cerevisiae* *hog1Δ* null mutants have an aberrant cell morphology under osmotic stress, being large, multinucleated cells with multiple elongated buds (Brewster *et al.*, 1993). The *S. cerevisiae* *hog1Δ* null mutant JBY10 was similar to this, but a *hog1Δ* mutant harbouring YEp13-*ZrHOG1*, YEp13-*ZrHOG2*, YCp50-*ZrHOG1* or YCp50-*ZrHOG2* showed normal cell shape (Fig. 6). This indicates that the abnormal cell shape of *hog1Δ* null mutants can be rescued by the expression of *ZrHOG1* or *ZrHOG2*.

Transcription of *GPD1* in *S. cerevisiae* cells by expressing *ZrHOG1* or *ZrHOG2*

As shown above, the expression of *ZrHOG1* or *ZrHOG2* enhanced the intracellular glycerol accumulation in *S. cerevisiae* *hog1Δ* null mutants. To clarify whether ZrHog1p and ZrHog2p are functionally equivalent to Hog1p derived from *S. cerevisiae* (Albertyn *et al.*, 1994) in regulating *GPD1* transcription in *S. cerevisiae*, Northern blot analysis on *GPD1* was carried out in *S. cerevisiae* YCp50-*ZrHOG1* and YCp50-*ZrHOG2* strains. After NaCl shock the transcription of *GPD1* in *ZrHOG1*- and *ZrHOG2*-harbouring strains was induced markedly, to the same level as the *S. cerevisiae* wild-type INVSc1 strain, but the *S. cerevisiae* *hog1Δ* null mutant (JBY10 strain) failed to enhance the transcription of *GPD1* under the same conditions (Fig. 7). This indicates that ZrHog1p and ZrHog2p have the same function as Hog1p and can regulate the transcription of *GPD1* in *S. cerevisiae*. Only a slight difference between YCp50-*ZrHOG1* and YCp50-*ZrHOG2* strains was observed. This was that the induction of *GPD1* transcription in the YCp50-*ZrHOG1* strain was maximal at 30 min after NaCl shock and then diminished by 60 min, similar to that observed in the wild-type INVSc1 strain (Fig. 7); in contrast, the transcription of *GPD1* in the YCp50-*ZrHOG2* strain remained at the maximum level for 60 min (Fig. 7). This may indicate that the regulation by ZrHog1p or ZrHog2p is different during the induction of transcription of *GPD1* in *S. cerevisiae* cells.

DISCUSSION

Cloning of putative MAP kinase genes (*ZrHOG1* and *ZrHOG2*) from *Z. rouxii*

When *S. cerevisiae* cells are exposed to a high level of osmotic shock, a signal transmission pathway of osmotic stress, called the MAP kinase cascade (HOG pathway), is activated. This leads to elevated production of glycerol by GPDH and intracellular glycerol accumulated. The *HOG1* gene product of *S. cerevisiae* is the key element in

the HOG pathway, and activated Hog1p has been reported to phosphorylate the transcriptional regulator for the GPDH gene (*GPD1*; Albertyn *et al.*, 1994). Genes homologous to *HOG1* have been identified in the yeasts *Candida albicans* (San Jose *et al.*, 1996) and *Schizosaccharomyces pombe* (Degols *et al.*, 1996; Kato *et al.*, 1996). In the salt-tolerant yeast *Z. rouxii*, also, glycerol is the main compatible osmolyte used to adjust osmotic imbalance (Reed *et al.*, 1987). It has been assumed that a pathway similar to the HOG pathway might be present in *Z. rouxii* cells. Thus, in the present study, we cloned genes homologous to *S. cerevisiae* *HOG1* to help characterize the signal transmission pathway of osmotic stress in *Z. rouxii*.

Two genes (*ZrHOG1* and *ZrHOG2*) homologous to *HOG1* were identified in *Z. rouxii*. The deduced amino acid sequences encoded by *ZrHOG1* and *ZrHOG2* were highly homologous to that encoded by *HOG1* (Fig. 2). Moreover, the TGY motif was found in the sequences of *ZrHog1p* and *ZrHog2p* (Fig. 2). This motif was indicated to be phosphorylated by MAP kinase kinase (Pbs2p; Boguslawski, 1992) in *S. cerevisiae* (Brewster *et al.*, 1993). If a kinase similar to Pbs2p is present in *Z. rouxii* cells, both *ZrHog1p* and *ZrHog2p* should be phosphorylated by the kinase.

Functional expression of *ZrHOG1* and *ZrHOG2* in *S. cerevisiae*

When *ZrHOG1* and *ZrHOG2* were introduced with a centromeric vector, YCp50, and 2 μ -circle-based vector, YEpl3, into an *S. cerevisiae* *hog1* Δ null mutant, *ZrHOG1* and *ZrHOG2* were found to complement the phenotypes (salt tolerance, osmotic tolerance, glycerol content and abnormal cell shape) of the mutant (Figs 4, 5 and 6), indicating that the products encoded by *ZrHOG1* and *ZrHOG2* are functional as MAP kinase in *S. cerevisiae* cells. This strongly suggests that there is an equivalent HOG pathway in *Z. rouxii*. The defect in NaCl-induced *GPD1* transcription in an *S. cerevisiae* *hog1* Δ null mutant was corrected by *ZrHOG1* and *ZrHOG2*. The transcription of *GPD1* in the *ZrHOG1*-harbouring mutant was transient, as it was in wild-type strains of *S. cerevisiae*, but the *ZrHOG2*-harbouring mutant showed a prolonged transcription of *GPD1* (Fig. 7). This prolonged transcription of *GPD1* may be significant in relation to glycerol production in *Z. rouxii* cells. However, since GPDH of *Z. rouxii* was not studied here, the regulation by *ZrHog1p* or *ZrHog2p* of the GPDH gene transcription in *Z. rouxii* subjected to osmotic shock remains to be elucidated. The observed differences in *GPD1* transcription in *S. cerevisiae* could be explained by a difference in the actions of *ZrHog1p* and *ZrHog2p* on the transcriptional regulators (e.g. Msn2p or Msn4p; Inoue *et al.*, 1998) of *GPD1*. One possibility is that *ZrHog1p* and *ZrHog2p* regulate different regulators. Since a negative effect of increases in copy number on glycerol production was observed only in the *ZrHOG2*-harbouring strain (Fig. 5), this seems likely.

Decreased salt tolerance of *Z. rouxii* *Zrhog1* Δ and *Zrhog2* Δ null mutants

The possibility that the functions of both the products encoded by *ZrHOG1* and *ZrHOG2* are related to the salt tolerance of *Z. rouxii* cells was indicated by the analysis of phenotypes of *Z. rouxii* *Zrhog1* Δ and *Zrhog2* Δ null mutants (Fig. 3). Because the GPDH gene of *Z. rouxii* has not been cloned, we could not research the functions of *ZrHOG1/2* in detail in *Z. rouxii*. Their functions will be reported elsewhere, including the construction of *ZrHOG1* and *ZrHOG2* double disruption mutants and the determination of glycerol content in *Z. rouxii* *Zrhog1* Δ or *Zrhog2* Δ null mutants. In *S. cerevisiae*, activated Hog1p has been reported to regulate the transcription of genes encoding stress-induced proteins other than GPDH. For example, the expression of *ENA1*, which encodes Na⁺-ATPase and is related mainly to the efflux of Na⁺ in *S. cerevisiae*, is regulated by Hog1p at low NaCl concentrations (0.3 M) and by calcineurin/calmodulin at higher concentrations (0.8 M; Marquez & Serrano, 1996). Also, the transcription of *GLO1* encoding glyoxalase I is regulated by Hog1p in *S. cerevisiae* (Inoue *et al.*, 1998). Therefore, it is likely that *ZrHog1p* and *ZrHog2p* could regulate the expression of genes related to ion homeostasis and other functions, if there is a relationship to the salt tolerance of *Z. rouxii* cells.

The present study revealed that at least two functional putative MAP kinase genes (*ZrHOG1* and *ZrHOG2*) are present and both of their products are functional in *Z. rouxii*. There is only one *HOG1* MAP kinase gene in other yeasts (Brewster *et al.*, 1993; San Jose *et al.*, 1996; Degols *et al.*, 1996; Kato *et al.*, 1996), thus the two genes seem specific to *Z. rouxii*. This may suggest that two *HOG1* homologues play crucial roles in the salt tolerance (osmotic tolerance) of *Z. rouxii* cells.

ACKNOWLEDGEMENTS

We wish to express thanks to M. C. Gustin for his generous gift of the *S. cerevisiae* JBY10 strain and to K. Ushio for his generous gift of the *Z. rouxii* MA11-3 strain.

REFERENCES

- Akhtar, N., Blomberg, A. & Adler, L. (1997). Osmoregulation and protein expression in a *pbs2* Δ mutant of *Saccharomyces cerevisiae* during adaptation to hypersaline stress. *FEBS Lett* **403**, 173–180.
- Albertyn, J., Homann, A., Thevelein, J. M. & Prior, B. A. (1994). *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol Cell Biol* **14**, 4135–4144.
- Boguslawski, G. (1992). *PBS2*, a yeast gene encoding a putative protein kinase, interacts with the *RAS2* pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**, 2425–2432.
- Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science* **259**, 1760–1763.

- Degols, G., Shiozaki, K. & Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. *Mol Cell Biol* **16**, 2870–2877.
- Galcheva-Gargova, Z., Derijard, B., Wu, L.-H. & Davis, R. J. (1994). An osmosensing signal transduction pathway in mammalian cells. *Science* **265**, 806–808.
- Han, J., Lee, J.-D., Bibbs, L. & Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**, 808–811.
- Inoue, Y., Tsujimoto, Y. & Kimura, A. (1998). Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic stress response. *J Biol Chem* **273**, 2977–2983.
- Kato, T., Jr, Okazaki, K., Murakami, H., Stettler, S., Fonyes, P. A. & Okayama, H. (1996). Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast. *FEBS Lett* **378**, 207–212.
- Kumer, S., McLaughlin, M. M., McDonnell, P. C., Lee, J. C., Livi, G. P. & Young, P. R. (1995). Human mitogen-activated protein kinase CSBP1, but not CSBP2, complements a *hog1* deletion in yeast. *J Biol Chem* **270**, 29043–29046.
- Maeda, T., Wurgler-Murphy, S. M. & Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**, 242–245.
- Maeda, T., Takekawa, M. & Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor. *Science* **269**, 554–558.
- Marquez, J. A. & Serrano, R. (1996). Multiple transduction pathways regulate the sodium-extrusion gene *PMR2/ENA1* during salt stress in yeast. *FEBS Lett* **382**, 89–92.
- Norbeck, J. & Blomberg, A. (1997). Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4 M NaCl. *J Biol Chem* **272**, 5544–5554.
- Ota, I. M. & Varshavsky, A. (1993). A yeast protein similar to bacterial two-component regulators. *Science* **262**, 566–569.
- Popping, B., Gibbons, T. & Watson, M. D. (1996). The *Pisum sativum* MAP kinase homologue (PsMAPK) rescues the *Saccharomyces cerevisiae* *hog1* deletion mutant under conditions of high osmotic stress. *Plant Mol Biol* **31**, 355–363.
- Posas, F. & Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* **276**, 1702–1705.
- Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C. & Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 two-component osmosensor. *Cell* **86**, 865–875.
- Reed, R. H., Chudek, J. A., Foster, R. & Gadd, G. M. (1987). Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Appl Environ Microbiol* **53**, 2119–2123.
- Rose, M. D., Winston, F. & Hieter, P. (1990). *Methods in Yeast Genetics: a Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Rothstein, R. J. (1983). One-step gene disruption in yeast. *Methods Enzymol* **101**, 202–211.
- San Jose, C., Alonso Monge, R., Perez-Diaz, R., Pla, J. & Nombela, C. (1996). The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J Bacteriol* **17**, 5850–5852.
- Schuller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C. & Ruis, H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J* **13**, 4382–4389.
- Ushio, K., Tatsumi, H., Araki, H., Toh-e, A. & Oshima, Y. (1988). Construction of a host-vector system in the osmophilic haploid yeast *Zygosaccharomyces rouxii*. *J Ferment Technol* **66**, 481–488.
- Watanabe, Y., Sanemitsu, Y. & Tamai, Y. (1993). Expression of plasma membrane proton-ATPase gene in salt-tolerant yeast *Zygosaccharomyces rouxii* is induced by sodium chloride. *FEMS Microbiol Lett* **114**, 105–108.
- Watanabe, Y., Miwa, S. & Tamai, Y. (1995). Characterization of Na⁺/H⁺-antiporter gene closely related to the salt-tolerance of yeast *Zygosaccharomyces rouxii*. *Yeast* **11**, 829–838.

Received 27 May 1998; revised 11 August 1998; accepted 17 September 1998.