Research Article

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MAPK/HOG signaling pathway induced stress-responsive damage repair is a mechanism for *Pichia pastoris* to survive from hyperosmotic stress

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Abstract

BACKGROUND: MAPK/HOG signaling pathway plays a key role in the response of yeast to external hyperosmotic stress. Over the past few decades, the regulation mechanism for this pathway in the robust yeast, *Saccharomyces cerevisiae*, has been elucidated. However, the weak ability of the biotechnical workhorse, *Pichia pastoris*, in surviving hyperosmotic stress suggests a unique regulatory mechanism needing further investigation.

RESULT: Here, we identified crucial genes in the MAPK/HOG pathway of *P. pastoris* and investigated their effects on cell growing in osmotically stressed environments by knocking out these genes using a novel CRISPR/Cas9 system. Using real-time polymerase chain reaction (RT-PCR) and yeast two-hybrid assay, transcription factors Hot1, Msn4 and Sko1 were demonstrated to be regulated by Pbs2 and Hog1 either at mRNA or protein level. We also examined the subcellular localization of these transcription factors, reflecting their translocation between cytoplasm and nucleus. The transcriptions of putative osmo-responsive genes were then studied by RT-PCR. We found the induction of glycerol-related genes, such as *GT1* and *GPD1*, was marginal when cells experienced high osmolarity. The ability of *P. pastoris* to increase intracellular glycerol level was determined and found to be much weaker than that in *S. cerevisiae*. By contrast, stress-induced damage repair genes, including *CTT1* and *HSP12*, were dramatically increased.

CONCLUSION: We conclude that *P. pastoris* could barely balance hyperosmotic stress by increasing intracellular glycerol concentrations, and stress-induced damage repair is still an important mechanism for *P. pastoris* survival under hyperosmotic stress. This study demonstrates a description of the MAPK/HOG pathway in *P. pastoris* and provides a trigger for improving its robustness.

Supporting information may be found in the online version of this article.

Keywords: *Pichia pastoris*; MAPK/HOG signaling pathway; osmotic stress response; regulation mechanism

ABBREVIATIONS

Aqy1	AQuaporin from Yeast 1
BLAST	Basic Local Alignment Search Tool
CTT1	CaTalase T 1
DAK1	DihydroxyAcetone Kinase 1
Ena1	Exitus NAtru 1
GPD1	Glycerol-3-Phosphate Dehydrogenase 1
GPP2	Glycerol-3-Phosphate Phosphatase 2
GT1	Glycerol Transporter 1
Hog1	High Osmolarity Glycerol response
Hot1/2	High-Osmolarity-induced Transcription factor 1/2
HSP12	Heat Shock Protein 12
HXT1	HeXose Transporter 1
MAPK/HOG	High Osmolarity Glycerol Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
Msn2/4	Multicopy suppressor of SNF1 mutation 2/4
Nha1	Na+/H+ Antiporter

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Pbs2	Polymyxin B Sensitivity protein 2
Pho89	PHOsphate metabolism 89
Sko1	Suppressor of Kinase Overexpression 1
Ssk2	Suppressor of Sensor Kinase 2
Ssk22	Suppressor of Sensor Kinase 22
STL1	Sugar Transporter-Like protein 1
Trk1	TRansport of potassium (K) 1
sfGFP	super fold Green Fluorescent Protein
OD_{600}	Optical Density at 600 nm

INTRODUCTION

The high-osmolarity glycerol mitogen-activated protein kinase (MAPK/HOG) signaling pathway plays a key role in the adaptation of yeast to external osmotic pressure changes,[1] and this pathway in *Saccharomyces cerevisiae* has been systematically studied for decades. In summary, the external hyperosmotic signals can be transduced to the MAPKKKs Ssk2 and Ssk22 by two branches of osmo-sensors, further activating the MAPKK Pbs2.[2] MAPK Hog1 is then phosphorylated by Pbs2, leading to its accumulation in the nucleus and further activation of the transcription factors including Hot1,[3,4] Msn2/4,[3] and Sko1 by phosphorylation.[5,6] Hot1, Msn2/4 and Sko1 can regulate 88% of Hog1-dependent osmo-responsive genes.[7] Hog1 can also interact with RNA polymerase II, reducing the affinity for non-stress responsive genes by redistributing RNA polymerase II in an osmotic stress environment.[8,9] Among the osmo-responsive genes, key glycerol synthesis genes, *GPD1* and *GPP2*, and glycerol transporter *STL1*, are induced to balance the hyperosmotic stress environment outside by intracellular glycerol accumulation. In addition, stress-induced damage repair genes, including *CTT1* and *HSP12*,[10,11] are activated to protect cells from osmolarity damage.[12]

Although the MAPK/HOG signaling pathway in *S. cerevisiae* is well understood, few studies on this pathway in *Pichia pastoris* have been undertaken. *Pichia pastoris* has a traditional heterologous protein expression system, where the promoter of alcohol oxidase 1 (P_{AOX1}) has been widely used for protein production. The promoter is efficiently induced by methanol and strictly repressed by glycerol and glucose, which limits the application of these carbon sources. Since MAPK/HOG pathway regulates several glycerol related genes, it could represent a potential engineering trigger to improve this system by relieving the glycerol repression on P_{AOX1}.[13–15] Moreover, with the development of systems biology and gene editing tools,[16] *P. pastoris* has been developed as a novel microbial cell factory for production of many valuable chemicals, such as terpenoids and alcohols.[17] Alcohol tolerance, a key compromise for the alcohol productivity,[18,19] has been improved by engineering the MAPK pathway in *Escherichia coli*.[20] Additionally, the components of the MAPK/HOG pathway are critical in xylose assimilation.[21] Therefore, investigating the MAPK/HOG pathway in *P. pastoris* can only provide an opportunity to overcome the carbon source limits of this system; it also benefits to build up a robust *P. pastoris* system. Sequence analysis reveals a high level of homology of MAPKK Pbs2 and MAPK Hog1 in *P. pastoris* to *S. cerevisiae*. However, transcription factors in *P. pastoris*, annotated as Hot1, Msn4 and Sko1, show a low homology to *S. cerevisiae*. Based on these observations, we can assume that the components and regulation mechanism of the pathway in *P. pastoris* might be quite different from that in *S. cerevisiae*, warranting further investigation.

In this study, we present a regulatory approach for the MAPK/HOG signaling pathway in *P. pastoris*, and illustrate a way for *P. pastoris* to survive high osmolarity. Furthermore, this study encourages more investigation and provides an engineering trigger for improving the robustness of *P. pastoris*.

MATERIAL AND METHODS

Strains and media

Yeast strains used in this study are listed in the Supporting Information, Table S1. Media used in this study were YPD medium (2% peptone, 1% yeast extract, 2% glucose) and MD medium (1.34% yeast nitrogen base, 2% glucose). A high-concentration solution of NaCl and KCl was added to the YPD medium to generate a hyperosmotic stress environment.

Plasmids and primers

Plasmids and primers used in this study are listed in the Supporting Information, Tables S2 and S3. For plasmids containing sgRNA sequences constructs, DNA fragments were amplified by polymerase chain reaction (PCR) using the primers harboring designed sgRNA sequences for different genes. PCR products were cloned into plasmid backbones linearized with *Bam*H I to generate the final sgRNA plasmids. In yeast two-hybrid assays, *HOG1* fragment amplified from genome DNA was digested with *Eco*R I and *Bam*H I and ligated to pGADT7 vector. *HOT1/2*, *MSN2*, *SKO1* and *PBS2* were amplified and cloned into pGBKT7 vector with different restriction enzymes (having relevant primers). Codon optimized *sfGFP* DNA fragment was digested with *Bst*B I and *Xho* I and ligated into pGAPZB vector to form pGAPZB-sfGFP. pGAPZB-sfGFP was then digested with *Xho* I and *Not* I, and the resulting product was used as the backbone to generate plasmids harboring *sfGFP* fusion genes, where 30 bp overlapping sequences were added to different genes by relevant primers.

Mutant strains selection and plasmid elimination

The constructed sgRNA plasmids were transformed into GS115-Cas9 strain with electroporation according to the user manual provided by Invitrogen, and transformants were selected in the MD medium. Knockout results were analyzed with the PCR products from genome DNA with Pfx DNA polymerase (Cwbio, China). sgRNA plasmids in mutant strains were eliminated by transferring the cells cultured overnight into fresh MD medium with 0.004% histidine 5–10 times. The cells were then transferred onto the MD plates with 0.004% histidine. The colonies were inoculated into MD and MD with 0.004% histidine liquid media. The clones that could not grow in MD medium were selected for further investigation.

Cell growth on plates and continuous cell growth curves in different osmotic pressure environment

(1) For cell growth on plates, cells cultured overnight were inoculated into YPD medium at an initial OD_{600} = 0.5, and 1 mL of cell suspension was collected when the cell density was approximately OD_{600} = 3.0. The cells were washed twice with phosphate-buffered saline (PBS), and the cell density was accurately calibrated at OD_{600} = 3.0. Next, 2 μL of 10-fold diluted cells were inoculated into plates with (and without) high salt concentrations. The growth of cells on the plates was observed after culturing for 2–4 days. (2) For continuous cell growth curves, overnight culture cells were inoculated into fresh YPD liquid media at an initial OD_{600} = 0.2 in shake flasks. When cells were grown into OD_{600} = 1.0, they were centrifuged, washed and equally divided into fresh YPD media with baffles with different salt

concentrations in multiple deep-well plates, making the initial $OD_{600} = 0.5$. The cell density was detected for 24 h.

Determination of RNA level with real-time PCR

Cells cultured overnight were inoculated into YPD medium at an initial $OD_{600} = 0.2$. When the cell density was approximately $OD_{600} = 1.0$, after 5–6 h culture, the cells were collected. High-salt solution was then added to the medium for osmotic stress treatment and the cells were collected at 10 and 30 min treatment. Total yeast RNA was extracted using an Ultrapure RNA Kit (Cwbio, China). cDNA was synthesized by PrimeScript™ RT reagent Kit (TaKaRa, Beijing, China) following the manufacturer's suggested protocol. TB Green® *Premix Ex Taq*™ kit (TaKaRa) was used for real-time PCR (RT-PCR), and the experiments were performed on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). *ACT1* gene was selected as the reference control to normalize the expression of other genes.

Yeast two-hybrid assay

pGADT7 empty vector was co-transformed with pGBKT7 connected each gene and pGBKT7 was co-transformed with pGADT7 containing *HOG1* gene as control to test their autoactivation and false positive. Then, constructed plasmids containing the activating domain and binding domain were co-transformed into Y2HGold strain, following the transformation guide of TaKaRa. The transformed cells were then cultured both on SD/-Trp-Leu (DDO) and SD/-Trp-Leu-Ade-His (QDO) plates to observe their growth after 3–7 days' culture.

Subcellular localization analysis

Plasmids containing different *sfGFP* fusion genes were transformed into the relevant strains. Transformants were selected on the solid YPD medium with 1 mol L^{-1} sorbitol and 100 μg mL^{-1} zeocin. Cells were then cultivated in YPD medium overnight and transferred to fresh YPD medium at an initial cell density of $OD_{600} = 0.2$. After 8–10 h of culture, cells were collected and observed by confocal fluorescence microscopy to characterize the subcellular localization of proteins of interest. For hyperosmotic pressure treatment, NaCl solution was added to the medium at a final concentration of 0.7 mol L^{-1}. After a 30 min treatment, cells were also collected and observed using the same method.

Determination of the intracellular glycerol level

Gas chromatographic–mass spectrometric (GC-MS) analysis was used to determine the intracellular glycerol concentration in yeasts by benzoyl derivatization.

Standard solution preparation. The glycerol standard solution was diluted in 4 mol L^{-1} NaOH solution; then 1 mL of each sample was placed in the reaction tube; 500 μL n-hexane and 200 μL benzoyl chloride were added and reacted at 40 °C for 4 h.

Sample preparation. Cells were inoculated and treated in different osmotic stress environments consistent with continuous growth curve detection. Cells were collected and optical density was measured after the treatment for 1, 2, 6 and 10 h. After centrifuging the cells in the tubes and discarding the cell supernatant, 1 mL of 4 mol L^{-1} NaOH was added to each tube. The cells were incubated in boiled water for 15 min, and vortexed in a high-throughput oscillator at 6500 W for 2 min after adding glass beads. 500 μL n-hexane and 200 μL benzoyl chloride were added and reacted at 40 °C for 4 h.

Extraction. When the reaction was completed, the tubes were centrifuged at 12 000 × *g* for 2 min and 400 μL supernatant was collected. Reaction tubes were washed with n-hexane three times. All the n-hexane was mixed for GS-MS analysis.

GS-MS analysis. Column: Shimadzu SH-Rtx-5MS (30 m, 0.32 mm ID, 0.25 μm df); flow rate: 1.0 mL min^{-1}; inlet temperature: 300 °C; ion source temperature: 300 °C; interface temperature: 260 °C; injection volume: 1 μL; split ratio: 3:1; temperature program: temperature increased from 180 to 280 °C at a rate of 20 °C min^{-1} and held at 280 °C for 15 min; temperature increased to 300 °C at a rate of 5 °C min^{-1} and held for 1 min.

RESULTS

Identification of MAPK/HOG pathway components in *P. pastoris*

BLAST was used to search for the potential components of MAPK/HOG pathway with high similarity to those in *S. cerevisiae*. Pbs2 and Hog1 were searched in *P. pastoris* first, where proteins XP_002489748.1 (accession number) and XP_002489575.1 were separately scored as the top proteins with the highest percent identity of 66.49% and 84.88%. They were respectively annotated as Pbs2 and Hog1, and they both harbor the protein kinase catalytic domains and ATP binding sites, suggesting they are similar proteins to their homologs in *S. cerevisiae*. Hot1, Msn2/4 and Sko1 in *S. cerevisiae* were then selected as templates to find homologous proteins in *P. pastoris*. In Hot1 searching, proteins XP_002489794.1 and XP_002494036.1 were both identified to have the GCR1_C domain,[22] an important feature of Hot1 in *S. cerevisiae*. In addition, they were listed as Hot1 in different genome annotations. Thus we marked these as Hot1 and Hot2, respectively, for further study to avoid confusion. Hot1 and Hot2 in *P. pastoris* only show 32.18% and 35.53% identity to the template, and their amino acids lengths (402 aa and 345 aa) were much shorter than the 719 aa in *S. cerevisiae*, suggesting they may play different roles in *P. pastoris*. Proteins XP_002491652.1 and XP_002489811.1 are the potential Msn4 and Sko1 homologs in *P. pastoris*, which separately show 50.91% and 55.56% identity to their counterpart in *S. cerevisiae*. There is no Msn2 homolog reported in *P. pastoris*. Msn4 has the characteristics of zinc finger transcription factor, including zinc finger structure and zinc binding sites. Sko1 has similar features to its homolog in *S. cerevisiae*, such as a basic leucine zipper domain of activating transcription factor-2 (ATF-2), an Aft1 osmotic stress response (OSM) domain, an Aft1 HRA domain and DNA binding sites, suggesting it might function as an important transcription factor in the MAPK/HOG pathway. However, the sizes of Msn4 and Sko1 were also much smaller than their homologs, possibly leading to the lack of some functions.

A novel CRISPR/Cas9 system built by our laboratory was used to knock out these putative genes in the MAPK/HOG pathway to further identify these proteins. This system was used to study their effects on cells responding with hyperosmotic stress. For each gene, 1–2 sgRNA plasmids harboring qualified sgRNA sequences were constructed and cloned into GS115-Cas9 strain. After cutting of genomic DNA by Cas9 protein, a non-homologous end-joining (NHEJ) repair system in *P. pastoris* could repair the gap,[23] possibly resulting in a frame-shift mutation. Mutations in genomic DNA were detected by PCR and DNA sequence. The exact base pair changes of mutant strains are shown in the Supporting Information, Figure S1, and were selected for further research after plasmid elimination. GS115, *hog1*Δ, *hot1*Δ, *hot2*Δ, *msn4*Δ and *sko1*Δ strains were treated in normal media (YPD) and hyperosmotic stress media (YPD + 0.7 mol L^{-1} NaCl, YPD + 0.35 mol L^{-1}

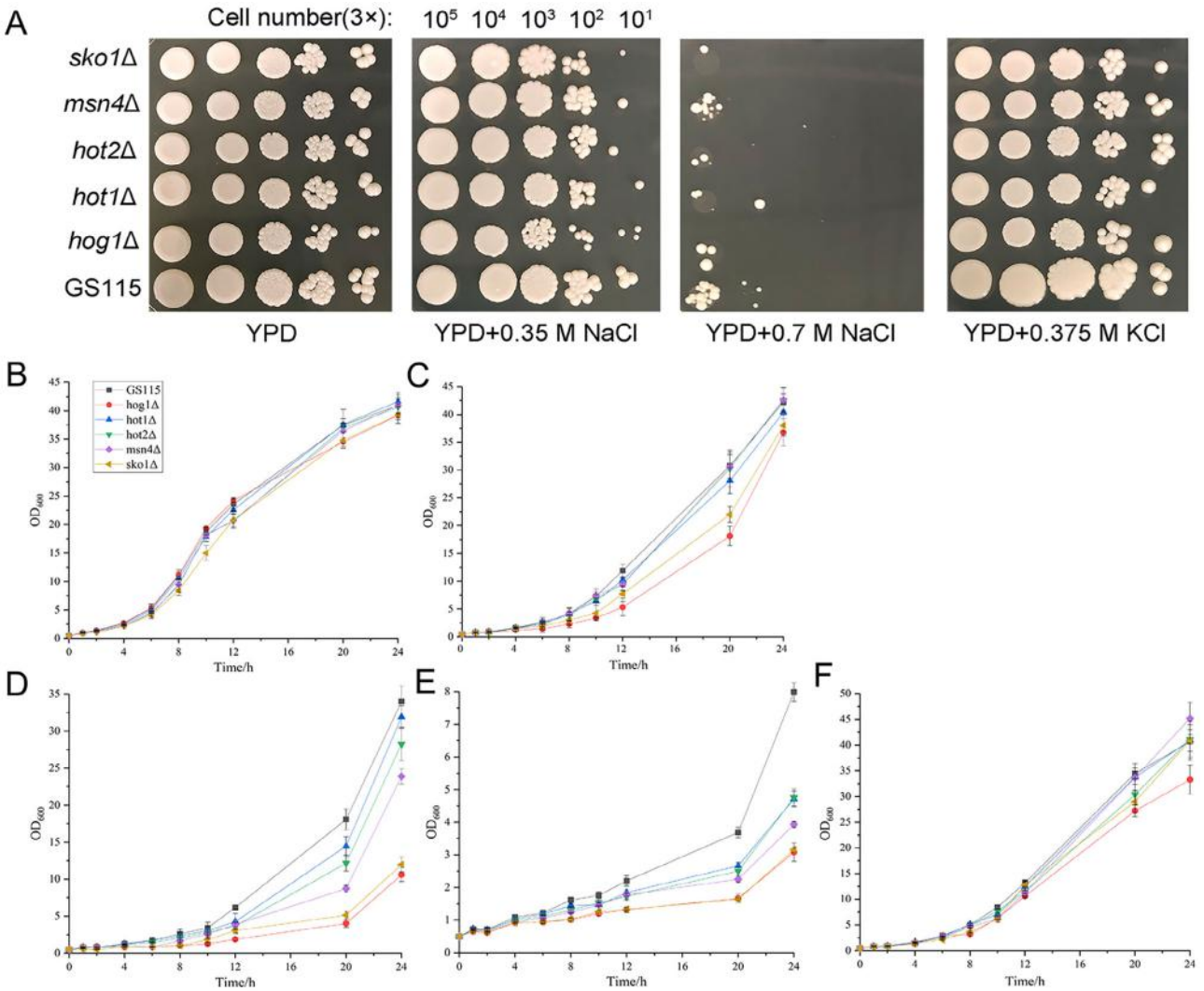

Figure 1. The growth of mutant strains in the media with different salt concentrations. (A) For treatment on a plate, cells were grown to logarithmic phase and diluted to an OD_{600} of 3.0. Tenfold serial dilution series were respectively plated on the YPD medium containing no osmolyte, 0.35 mol L^{-1} NaCl, 0.7 mol L^{-1} NaCl or 0.375 mol L^{-1} KCl. For treatment in liquid media, overnight culture cells were inoculated and grown to OD_{600} = 1.0. Cells were centrifuged, washed and equally transferred to fresh YPD media containing, respectively, no osmolyte (B), 0.35 mol L^{-1} NaCl (C), 0.5 mol L^{-1} NaCl (D), 0.7 mol L^{-1} NaCl (E) or 0.375 mol L^{-1} KCl (F).

NaCl and YPD + 0.375 mol L^{-1} KCl) to study their survival. The growth of GS115 strain in YPD medium was slightly better than other mutant strains, and the growth of *hog1*Δ strain was the worst of the mutant strains (Fig. 1A). The situation was more obvious in hyperosmotic stress media (0.35 mol L^{-1} NaCl and 0.375 mol L^{-1} KCl), where Hog1 and Sko1 had more significant effects on cell growth in a hyperosmotic stress environment. Besides, we detected the growth of mutant strain in liquid salt media (YPD, YPD + 0.35 mol L^{-1} NaCl, YPD + 0.5 mol L^{-1} NaCl, YPD + 0.7 mol L^{-1} NaCl and YPD + 0.375 mol L^{-1} KCl) (Fig. 1B–F). The gap of growing condition in liquid hyperosmotic stress environment between different strains was more obvious. The growth patterns of mutant strains in liquid YPD medium were similar to GS115 strain except that *hog1*Δ and *sko1*Δ strain grew slightly more slowly than GS115 strain (Fig. 1B), which was consistent with the patterns on plates. We suppose that Hog1 and Sko1 may regulate some important genes for cell growth. The growth gap between *hog1*Δ & *sko1*Δ and GS115 strain became gradually larger when salt concentration increased (Fig. 1C–F). In the osmotic treatment of 0.7 mol L^{-1} NaCl, *hog1*Δ and *sko1*Δ could only grow to around OD_{600} = 3.0, while GS115 could reach the biomass at the OD_{600} = 8.0 (Fig. 1E). The growth conditions of *hot1*Δ, *hot2*Δ and *msn4*Δ strains in 0.35 mol L^{-1} NaCl and 0.375 mol L^{-1} KCl were comparable (Fig. 1C,F), but the effect of losing Hot1, Hot2 and Msn4 on cell growth appeared at higher sodium concentrations (Fig. 1D,E). In other words, *hot1*Δ, *hot2*Δ and *msn4*Δ strains grew more slowly than GS115 strain in the treatment of 0.5 mol L^{-1} and 0.7 mol L^{-1} NaCl, and *msn4*Δ strain was the worst of the three mutant strains. Therefore, Hog1 and Sko1 are likely to be important components of the MAPK/HOG pathway and play important roles in the cell response to osmotic stress. It is noteworthy that cells could hardly grow in 0.7 mol L^{-1} NaCl medium, suggesting that *P. pastoris* survival under hyperosmotic stress is much weaker than *S. cerevisiae*, as 1000 *S. cerevisiae* cells could survive in an 0.8 mol L^{-1} NaCl environment.[7] Cell growth in 0.375 mol L^{-1} KCl stress medium was better than that in 0.35 mol L^{-1} NaCl, suggesting that *P. pastoris* could more easily survive KCl stress. These studies further support our hypothesis on the difference of the MAPK/HOG pathway in *P. pastoris*.

Regulation and interaction of transcription factors in the MAPK/HOG pathway

Regulation at both mRNA and protein levels was examined in mutant strains to study the mechanism of osmotic signal transduction by transcription factors. The transcriptional level of the transcription factors, including *HOT1/2*, *MSN4* and *SKO1*, was examined in GS115 and mutant strains by RT-PCR. *HOT1* and *SKO1* were significantly induced by osmotic stress, showing a 6.4- and 25.9-fold increase, respectively, in GS115 strain after a 30 min treatment (Fig. 2A,D). *SKO1* was strongly regulated by Pbs2 and Hog1, as revealed by the dramatic decrease in *pbs2*Δ and *hog1*Δ strains on induction of *SKO1*. Although the final expression of *HOT1* did not show much difference in three strains, the deletion of *PBS2* and *HOG1* postponed the induction after osmotic stress treatment. This expression delay was also observed in the expression of *MSN4* (i.e., *MSN4* declined under osmotic stress) but its decline could only be observed after 30 min of treatment in the *pbs2*Δ and *hog1*Δ strains (Fig. 2C). The decline in *MSN4* was surprising as we had speculated it to be an important transcription activator in the osmotic stress response. Although the mRNA level of *HOT2* did not show significant changes under these conditions (Fig. 2B), the

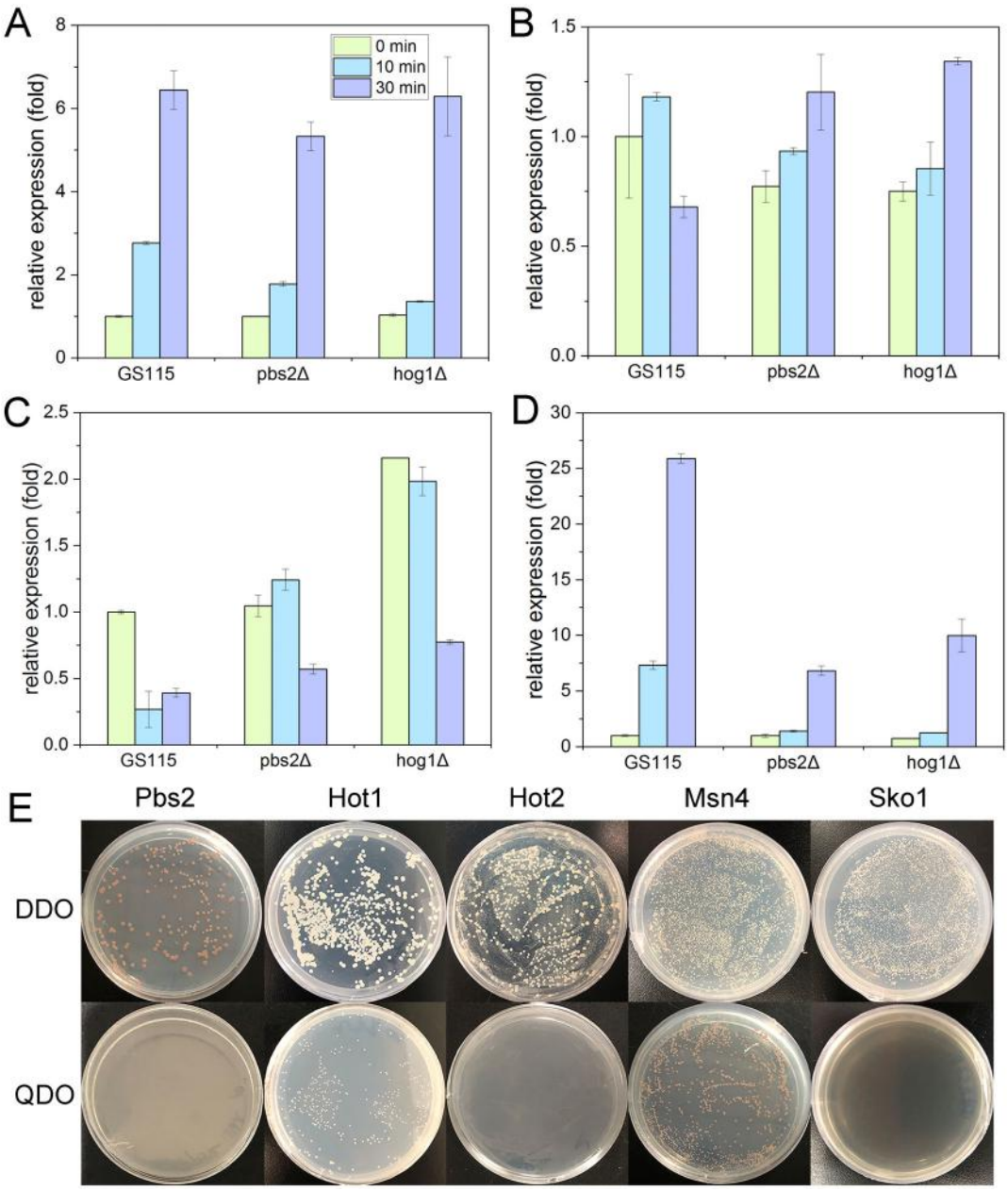

Figure 2. Regulation and interaction between transcription factors. (A–D) The mRNA level of *HOT1*, *HOT2*, *MSN4* and *SKO1* in GS115, *pbs2*Δ and *hog1*Δ strains. Cells were grown to logarithmic phase at OD_{600} = 1.0, and samples were collected with no treatment (0 min). Cells were then exposed to osmotic stress (0.7 mol L^{-1} NaCl) for 10 min and 30 min. The gene expressions at 0 min in GS115 were treated as control and normalized to 1. Yeast two-hybrid assay results (E) shows the growth of co-transformation Y2HGold strains on DDO and QDO plates. Then, plasmids pGADT7 with HOG1Plasmids pGADT7 connected *HOG1* gene were co-transformed into Y2HGold strain respectively with pGBKT7 vectors harboring *PBS2*, *HOT1/2*, *MSN4* or *SKO1* genes. The co-transformation strains were then placed on DDO and QDO plates.

over-expression of *HOG1* led to a decrease in the transcription of *HOT2* (data not shown).

Yeast two-hybrid assay was used in this study to analyze interactions at the protein level in *P. pastoris*, since the osmotic stress signal was mostly dependent on the interactions between the transcription factors in *S. cerevisiae*, leading to an activated status.[2] If the same interactions are present in *P. pastoris*, the introduction of these transcription factors into Y2HGold strain should bring their fusion Gal4 activation domain (AD) and Gal4 binding domain (DNA-BD) into proximity to activate their independent reporter genes (*ADE2* and *HIS3*), making the strains able to grow in QDO medium. First, autoactivation, false positives and toxicity were examined in the experiment. We did not observe any autoactivation and false positives (Supporting Information, Fig. S2), but pGADT7 containing *HOG1* gene was toxic for Y187 strain. Therefore, we selected co-transformation in the more robust Y2HGold strain rather than yeast mating according to the manufacturer's suggestion. What surprised us was that Pbs2 could not interact with Hog1 (Fig. 2E). There were several lines of evidence supporting their interactions. For example, Hog1 has kinase catalytic domains and ATP binding sites. In addition, the expression patterns of many genes were similar in *pbs2*Δ and *hog1*Δ strains, including *HOT1*, *SKO1* (also demonstrated from *CTT1* and *HSP12* discussed later). We speculate that these interactions require the involvement of activated Pbs2 or other components. Hog1 putative target transcription factors, Hot1 and Msn4, can directly interact with Hog1, while Hot2 and Sko1 cannot directly interact with Hog1 based on these results (Fig. 2E).

In summary, Hog1, Hot1/2, Msn4 and Sko1 have all been demonstrated to be important components in the MAPK/HOG signaling pathway. Pbs2 and Hog1 still play a key role in the pathway of *P. pastoris* by regulating their transcription factors either at the mRNA level or protein level.

The subcellular localization of the transcription factors Hog1, Hot1/2, Msn4 and Sko1

Previous studies show that stress-responsive transcription factors can translocate between cytoplasm and nucleus in different osmolarity environments, which is an important mechanism of activating or repressing their target genes. For example, the phosphorylation of Hog1 in *S. cerevisiae* leads to its accumulation in the nucleus,[5] then the activated Hog1 can interact with its transcription factors and DNA polymerase II. Hyperosmotic stress causes Msn2/4 to enter the nucleus from the cytoplasm.[24] In contrast, Sko1 is released from the nucleus when cells are exposed to high osmolarity.[6]

Subcellular localization of the transcription factors was studied by fusing them with sfGfp protein in *P. pastoris* GS115. Strain *pbs2*Δ was used to study the effects of Pbs2 on Hog1 localization, and *hog1*Δ strain was used to investigate the influence on the localization of Hot1/2, Msn4 and Sko1. The fluorescence of Hog1-sfGfp in both the GS115 and the *pbs2*Δ strain was distributed throughout the cytoplasm but with a gathered dot, indicating that Hog1 was localized both in cytoplasm and nucleus independent of whether cells were placed in high osmolarity (Fig. 3A). The translocation of Hog1 under hyperosmotic stress was not observed and Pbs2 seemed not to determine the localization of Hog1. Hot1/2 and Sko1 both localized in the nucleus under all conditions, and Hog1 did not have an impact on this distribution (Fig. 3B). Sko1 could not release from nucleus, demonstrating that the repressor was different from its homolog in *S. cerevisiae*. Translocation was observed in Msn4, where Msn4 was mostly distributed in cytoplasm and a portion of the Msn4 transferred to the nucleus under high-osmolarity environment. The deletion of *HOG1* also did not impact translocation, consistent with observations in *S. cerevisiae*.

Stress-induced damage repair was an important mechanism for *P. pastoris* survival in high osmolarity

Based on the function of the target genes of the MAPK/HOG pathway, *S. cerevisiae* could survive hyperosmotic stress in two important ways: through the increase in intracellular glycerol concentration and through the enhancement of repair from stress-induced damage. Therefore, these target genes were studied in *P. pastoris* to understand how cells adapted to hyperosmotic stress. The genes examined include glycerol synthesis related genes *GPD1*, *HXT1* and *DAK1*, prominent glycerol transporter *GT1* (*STL1* homolog in *P. pastoris*),[25] and general stress-responsive damage repair genes *HSP12* and *CTT1*.[10,11] *GPP2* is not found in *P. pastoris*.

The expression of glycerol-related genes was first examined in GS115 and mutant strains. *GT1* was induced by osmotic pressure (Fig. 4A), but its induction was much weaker than the 30-fold observed in *S. cerevisiae*, and 0.375 mol L^{-1} KCl treatment barely resulted in any increase of *GT1* (data not shown). The expression of *GT1* was no longer determined by Hog1 and Hot1, compared to the abolished expression in *PBS2*, *HOG1* or *HOT1* mutant strains of *S. cerevisiae*. However, the deletion of *PBS2* and *HOG1* delayed the induction as revealed from the expression of *GT1*, which began to climb after a 10 min treatment, except in two of the strains. The mRNA level of *GPD1* and *HXT1* showed a major decline after treatment, in contrast to that observed in *S. cerevisiae* (Fig. 4B,C). *GPD1* was also no longer regulated by these proteins, but the delayed changes in *HXT1* were also observed in the *pbs2*Δ and *hog1*Δ strains. The expression of *DAK1* showed no difference in all tested conditions (Fig. 4D). These results demonstrate that *P. pastoris* barely increased glycerol synthesis genes *GPD1*, *HXT1* and *DAK1*, and transport-related genes *GT1* when exposed to hyperosmotic stress.

The stress-induced repair genes, *HSP12* and *CTT1*, were significantly induced by hyperosmotic stress and strictly inhibited in the *pbs2*Δ and *hog1*Δ strains (Fig. 4E,F). These two genes were also regulated by Sko1. Under general stress conditions, the expressions of *HSP12* and *CTT1* in *sko1*Δ strain were much higher than the expressions in the GS115 strain, which meant that Sko1 depressed their expression. However, compared with the expression in the GS115 strain, the induction of *HSP12* and *CTT1* in *sko1*Δ strain was much lower in the hyperosmotic stress environment (10 min, 30 min), suggesting that Sko1 worked as an activator at this stage. The result of Sko1 reversed from being a repressor to an activator, consistent with the regulation pattern in *S. cerevisiae*. In *S. cerevisiae*, Sko1 acts as a repressor by recruiting the Cyc8-Tup1 corepressor complex to the promoters in the absence of osmotic stress.[26] However, in the presence of osmotic stress, Hog1 is recruited by Sko1 and phosphorylates Sko1, making the Sko1-Cyc8-Tup1 complex recruiting SAGA and SWI/SNF induce the expression of target genes.[27] Since typical stress-induced damage repair genes *CTT1* and *HSP12* are still highly induced by osmotic stress, stress-induced damage repair may still represent an important mechanism for survival in a hyperosmotic stress environment.

Based on the absence of glycerol synthesis gene *GPP2*, we surmise that *P. pastoris* cannot survive high osmolarity through an increase in intracellular glycerol. To further confirm the

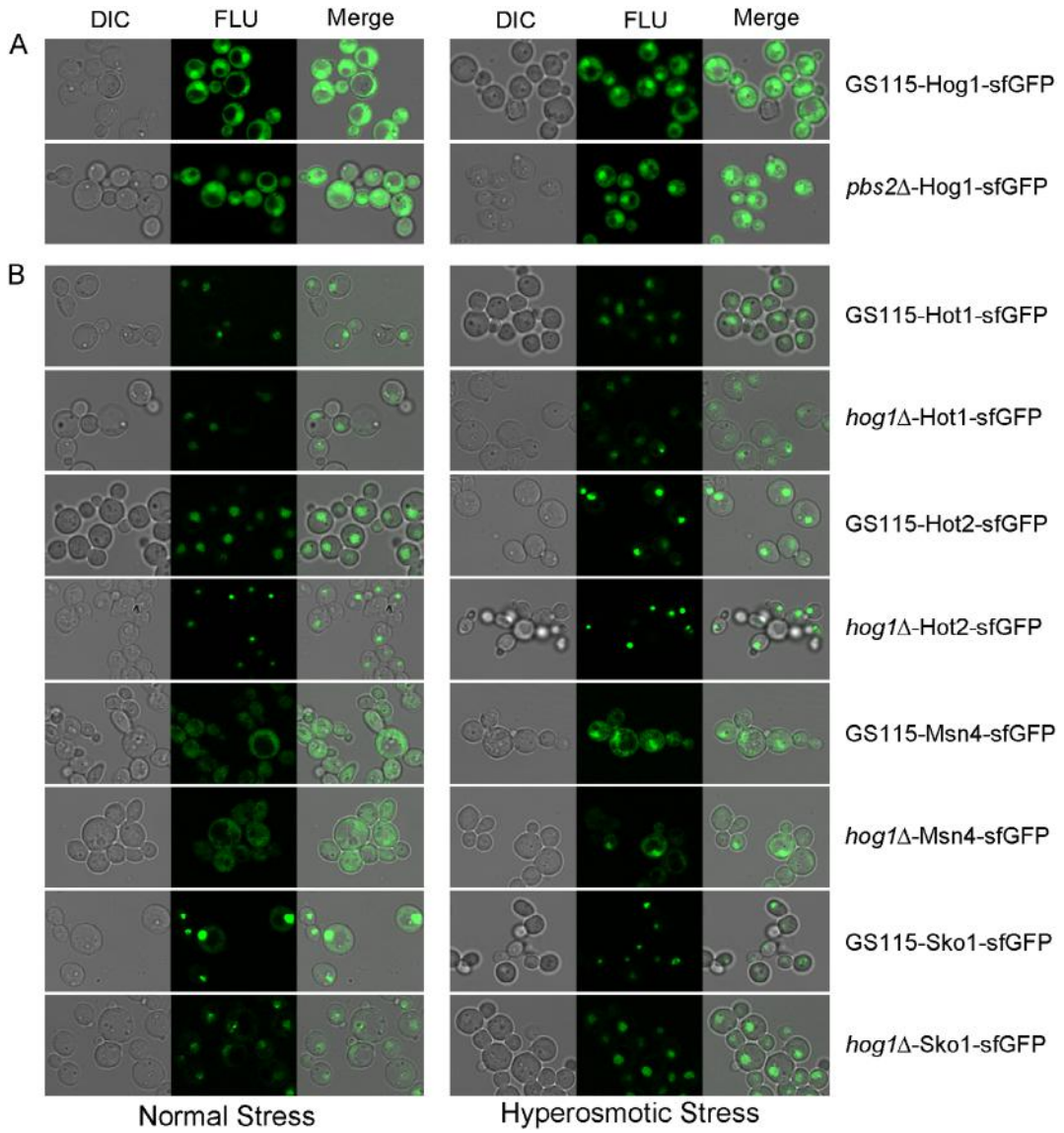

Figure 3. The subcellular localization of the sfGfp fused proteins in GS115 and mutant strains. Logarithmically growing strains transformed with sfGfp fusion proteins were examined by confocal fluorescence microscopy under normal stress or hyperosmotic growth conditions (30 min treatment in media containing 0.7 mol L^{-1} NaCl). DIC, differential interference contrast; FLU, fluorescence of sfGfp; Merge, DIC and FLU merged images. (A) sfGFP fused Hog1 in GS115 and *pbs2*Δ strains; (B) sfGfp fused Hot1, Hot2, Msn4 and Sko1 in GS115 and *hog1*Δ strains.

speculation, we determined the intracellular glycerol level of *P. pastoris* and *S. cerevisiae* under different stress conditions (YPD, YPD + 0.35 mol L^{-1} NaCl, YPD + 0.5 mol L^{-1} NaCl and YPD + 0.7 mol L^{-1} NaCl). The intracellular glycerol concentration in *P. pastoris* did not show a significant increase in different stress environments at all times of treatment. The average of intracellular glycerol level in *P. pastoris* was about 0.4 μg/ODU (Fig. 5A), which was comparable with that in *S. cerevisiae* in the pre-stress environment. However, *S. cerevisiae* could rapidly respond to high osmolarity by the increase in intracellular glycerol level from 0.4 to 4 μg/ODU with 0.5 and 0.7 mol L^{-1} NaCl treatment of 1 h (Fig. 5B), and the intracellular glycerol level held at a high level for at least 2 h (Fig. 5B). In conclusion, *P. pastoris* cannot survive high osmolarity through an increase in intracellular glycerol, and this could be a reason that *P. pastoris* is more sensitive to high osmolarity than *S. cerevisiae*.

Since *P. pastoris* cannot balance the external hyperosmotic stress by intracellular glycerol accumulation, there could be an unrevealed mechanism for osmotic stress balance of cells. Therefore, we examined the water transport and ion transport, which can be beneficial for cells under osmotic stress. *Pichia pastoris* has a single aquaporin Aqy1 regulating the water transport in yeast,[28] and its homologs in yeasts appear to behave as osmosensors. Therefore, we studied the transcription of this gene under different osmotic stress conditions. *AQY1* was only expressed by hyperosmotic stress when Hog1 was present in cells (Fig. 6A). This suggests that Hog1 downregulates the expression of Aqy1 to avoid more water extrusion in high-osmolarity environments. However, the effect on osmoadaptation is limited, because water can still be lost through the lipid bilayer. In the case of ion transport, we studied transcriptional levels of several Na^+ and K^+ transporters, including principal K^+ uptake transporter Trk1,[29]

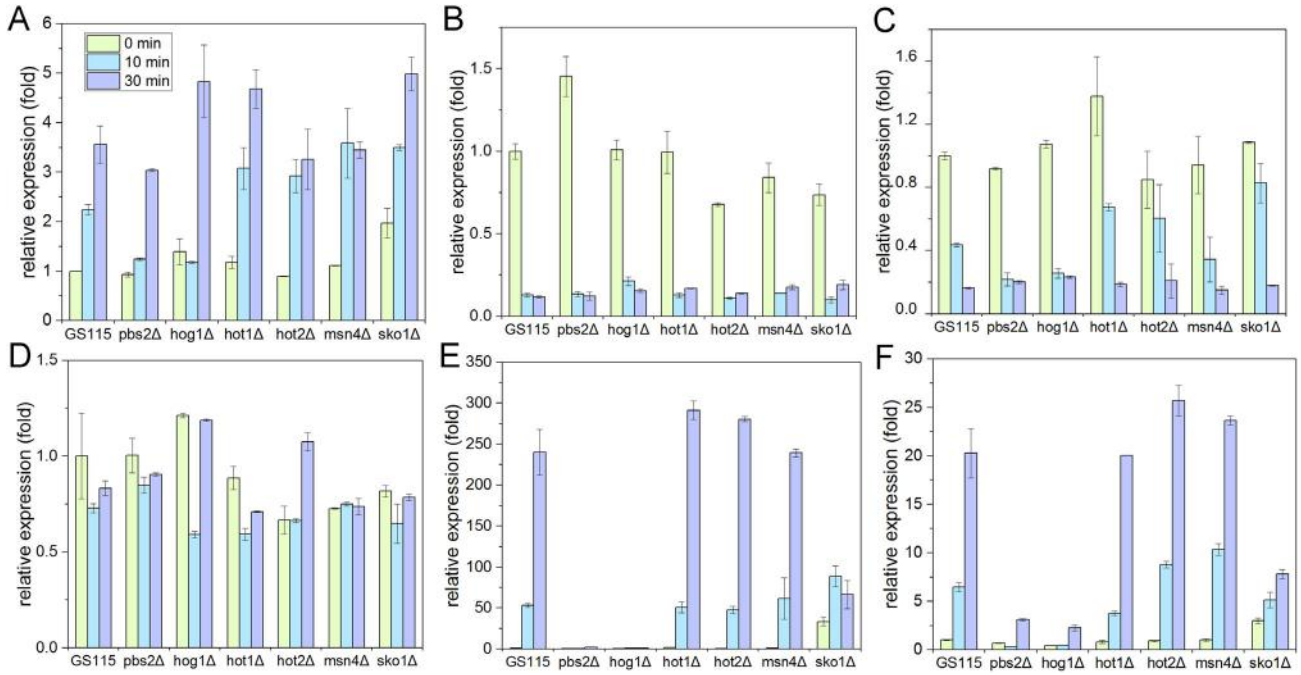

Figure 4. Transcriptional expressions of osmotic-responsive genes. Strain treatment was similar to Fig. 2. The *x*-axis represents different mutant strains. (A) *GT1*; (B) *GPD1*; (C) *HXT1*; (D) *DAK1*; (E) *HSP12*; (F) *CTT1*.

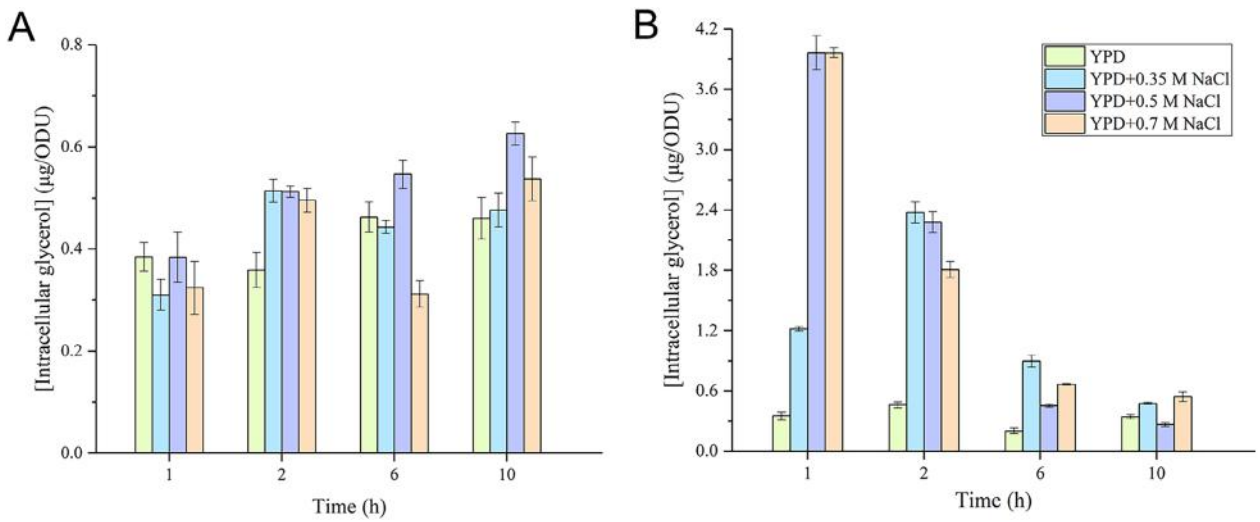

Figure 5. Comparison of the intracellular glycerol level between *P. pastoris* and *S. cerevisiae* under different osmotic stress conditions. Overnight culture cells of *P. pastoris* GS115 and *S. cerevisiae* S288C were inoculated and grown to OD_{600} = 1.0. Cells were centrifuged, washed and equally transferred into fresh YPD media at the initial OD_{600} = 0.5 respectively containing no osmolyte, 0.35 mol L^{-1} NaCl, 0.5 mol L^{-1} NaCl and 0.7 mol L^{-1} NaCl. Cells were collected at the corresponding time point to the intracellular glycerol level. ODU represents unit optical density at A_{600}. (A) intracellular glycerol level of *P. pastoris*; (B) intracellular glycerol level of *S. cerevisiae*.

Na^+ transporter Pho89,[28] Na^+/K^+ antiporter Nha1 and Ena1 (Fig. 6B–E). No increase was observed in Na^+ transport, and the efflux of Na^+, revealed from Nha1[30] and Ena1,[31] was enhanced to avoid intracellular Na^+ accumulation because of Na^+ toxicity. Uptake of K^+ was induced by hyperosmotic stress, indicating that cells may increase the intracellular osmotic stress by potassium. This result can also explain why cells can more easily survive potassium stress. However, since there is limited potassium concentration in media exhibiting sodium stress, this cannot be a key mechanism to balance the external stress. These results demonstrate that water transport and ion transport play a limited role in cell survival under hyperosmotic stress However, the prominent mechanism to balance osmotic stress still requires further investigation.

DISCUSSION

In this study, the components and preliminary regulation mechanism of the MAPK/HOG signaling pathway in *P. pastoris* were investigated. Compared with *S. cerevisiae*, we conclude that the

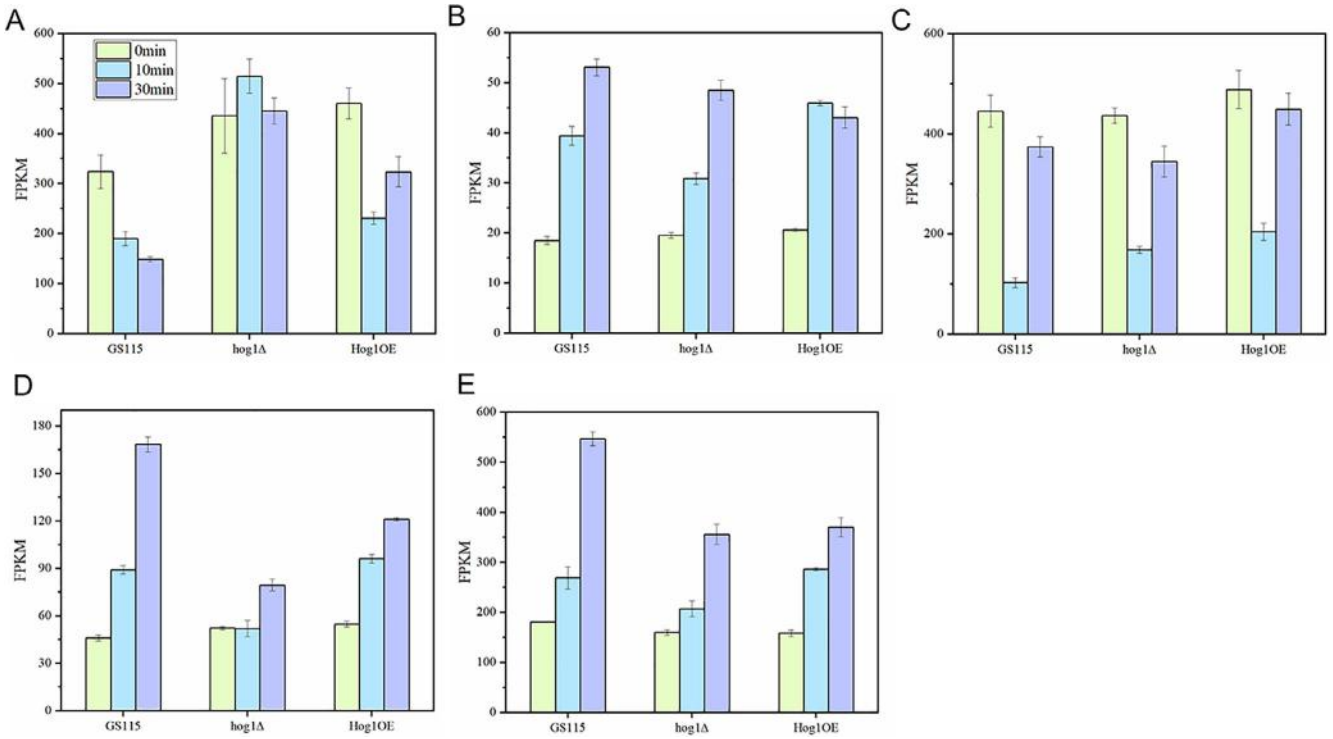

Figure 6. Transcriptome data on the expression of water and ion transport genes. Overnight culture cells were inoculated and grew to logarithmic phase at OD_{600} = 1.0. Samples were collected with no treatment (0 min). Cells were then exposed to osmotic stress (0.7 mol L^{-1} NaCl) for 10 and 30 min. Samples were subjected to high-throughput RNA sequencing. The *y*-axis presents the quantitative expression of genes, where FPKM is fragments per kilobase of transcript per million fragments mapped. The *x*-axis represents different mutant strains, where Hog1OE is hog1 overexpression strain. (A) *AQY1*; (B) *TRK1*; (C) *PHO89*; (D) *NHA1*; (E) *ENA1*.

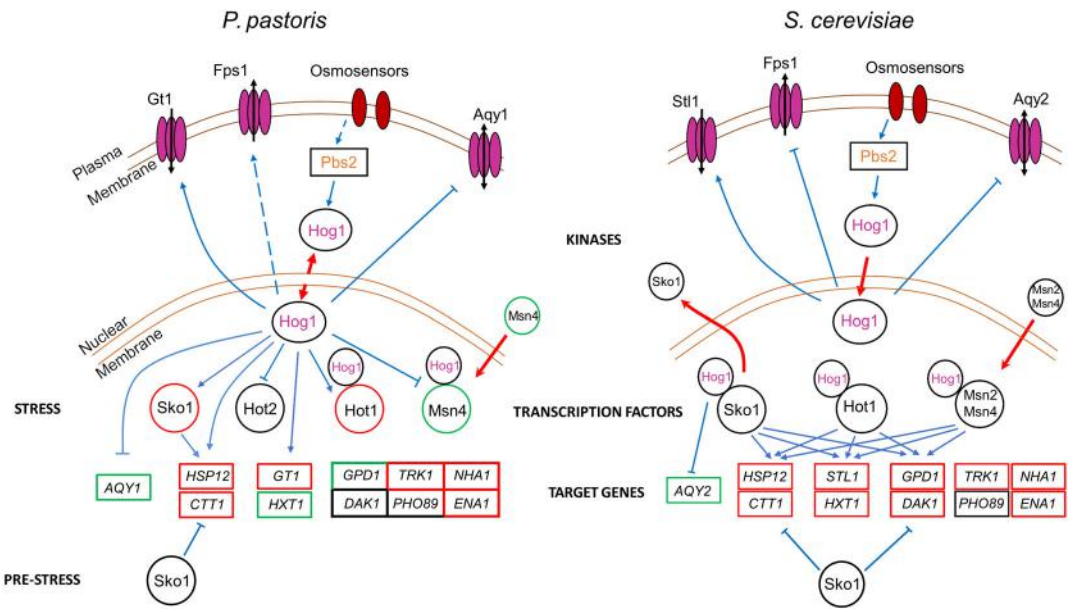

Figure 7. Comparison of MAPK/HOG signaling pathway between *P. pastoris* and *S. cerevisiae*. Blue arrows represent the transcription increase, while blue T shapes indicate the transcription impression. Thick red arrows show the translocation in cells. Solid and dotted lines are respectively experimental and hypothesis. The interactions between Hog1 with the transcription factors are shown by the contacting circles. Red, green and black borders of different shapes separately display the induction, impression and constant expression by hyperosmotic stress.

pathway and mechanism of the cell response to hyperosmotic stress are quite unique in *P. pastoris*, as summarized in Fig. 7.

First, although the important transcription factors, Hot1/2, Msn4 and Sko1, have typical domains consistent with their homologs, their molecular size were all much smaller in *P. pastoris* than in *S. cerevisiae*. This suggests that they may lose some function in *P. pastoris*. We also found that the ability of *P. pastoris* to respond to hyperosmotic stress was considerably less than *S. cerevisiae*. In

S. cerevisiae, the MAPK Hog1 mainly responds to hyperosmotic stress by interacting with transcription factors Hot1, Msn2/4 and Sko1, while Hog1 regulates these transcription factors either at the mRNA or the protein level in *P. pastoris* to further transduce the osmolarity signal. Differences were also detected in Msn4, which translocates between the cytoplasm and the nucleus, while translocation of Hog1, Msn2/4 and Sko1 in *S. cerevisiae* is an important regulation event in activating osmotic stress responsive genes. These differences, made by glycerol-related genes *GT1*, *GPD1*, *DAK1* and *HXT1*, were no longer induced by osmotic stress and regulated by these transcription factors. Based on the different intracellular glycerol level between *P. pastoris* and *S. cerevisiae*, we conclude that *P. pastoris* is unable to balance the high osmolarity by increasing intracellular glycerol. In contrast, typical stress-induced damage repair genes, *CTT1* and *HSP12*, are still highly induced by osmotic stress and are regulated by Pbs2, Hog1 and Sko1. This suggests that general damage repair is still an important mechanism of *P. pastoris* when responding to hyperosmotic stress. Water transport and ion transport, potential mechanisms for the balance of osmotic stress, were also demonstrated to play only a limited role in cell osmoadaptation, and the investigation of other mechanisms should be the subject of future studies. It is noteworthy that no target genes for transcription factors Hot1/2 and Msn4 were found in our study, and Hog1 could regulate many target genes. It seems that Hog1 might directly regulate its downstream target genes without the help of other transcription factors, which is quite different from the mechanism observed in *S. cerevisiae*. The functions of transcription factors regulated by Hog1 should be investigated in further studies.

In conclusion, the preliminary description of the MAPK/HOG pathway in *P. pastoris* presented here suggests the need for more studies to discover how *P. pastoris* survives from hyperosmotic stress, and these could be beneficial in building up a robust microbial cell factory in the future.

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CONFLICTS OF INTEREST

There are no conflicts of interest to report.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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