Research Article

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Comparative analysis of salt-tolerant gene *HOG1* in a *Zygosaccharomyces rouxii* mutant strain and its parent strain

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Abstract

BACKGROUND: A higher-salt-tolerant mutant strain *Zygosaccharomyces rouxii* 3–2 (strain S3-2) that could be used for improving the flavour of high-salt liquid state soy sauce was previously constructed from parent strain *Z. rouxii* (strain S) by genome shuffling. However, whether the mutations in this strain affect *HOG1* encoding MAPK Hog1p and improve intracellular glycerol production remains to be elucidated.

RESULTS: Although two mutations in the ORF and one in the promoter of the *HOG1* gene sequence of strain S3-2 occurred compared with that of strain S, there was no significant difference in secondary and tertiary structures between S3-2Hog1p and SHog1p. It was found that the expression level of S3-2*HOG1* was higher than that of S*HOG1* in YPDN medium with high salt concentration. Furthermore, overexpression of S3-2*HOG1* in *Saccharomyces cerevisiae* W303-1A could improve the salt tolerance and osmotolerance of engineered yeast compared with that of S*HOG1*.

CONCLUSION: Enhancement of the transcription level of *HOG1* induced by mutation in the promoter region may be one of the main reasons for the improved salt tolerance of strain S3-2 compared with that of strain S. Considering food security, the conservation of S3-2*HOG1* would be beneficial for application of strain S3-2 in the fermentation of soy sauce.

Keywords: salt tolerance; *HOG1*; *Zygosaccharomyces rouxii* mutant strain; osmostress

INTRODUCTION

Soy sauce, which is produced by fermentation of a mixture of wheat, cooked soybean and brine, is a reddish-brown liquid with an ester and alcohol flavour.[1] As one of the traditional fermented seasonings in China and other Asian countries, soy sauce has been consumed for thousands of years, and it is also becoming increasingly popular in both the USA and European countries owing to its special flavour characteristics and high nutritional value. At present, the preparation of soy sauce involves mainly high-salt liquid state fermentation or low-salt solid state fermentation, though the products obtained by the former are more competitive in the international market. One of the most important reasons for this is the flavour improvement due to the addition of yeast to the mash during the high-salt liquid state fermentation of soy sauce. It has been reported that the yeast *Zygosaccharomyces rouxii* can significantly improve the flavour formation of high-salt liquid state soy sauce.[2] However, one problem often encountered in soy sauce fermentation is the 180 g kg^{-1} NaCl used in the mash, which, although limiting the growth of undesirable micro-organisms effectively, can decrease the fermentative activity of the yeast.[3,4] Genome shuffling, as a novel whole genome engineering approach, can improve the cellular phenotype rapidly through recursive protoplast fusion with multiparental strains without genome sequence data or network information.[5] Thus a higher-salt-tolerant mutant strain *Z. rouxii* 3–2 (strain S3-2) was previously obtained by genome shuffling from the wild-type strain *Z. rouxii* (strain S) in our laboratory.[6]

When *Saccharomyces cerevisiae* (yeast) is exposed to hyperosmotic conditions, osmosensors trigger the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway.[7] The MAPK Hog1 phosphorylated through MAPK cascade reaction is translocated into the nucleus and induces up-regulation of the genes required for glycerol biosynthesis, finally resulting in a balance of intracellular and extracellular osmotic pressure.[8,9] The *HOG1* gene encoding MAPK Hog1p plays an essential role in the osmoregulation of yeast. Glycerol was also accumulated intracellularly when *Z. rouxii* was exposed to hyperosmotic conditions. The *ZrHOG1* was equivalent to *HOG1* of *S. cerevisiae* and the ZrHog1p had functions similar to Hog1p of *S. cerevisiae*, suggesting a homologous HOG pathway in *Z. rouxii*.[10]

Since the mutant strain S3-2 exhibits naturally higher starting osmotolerance and salt tolerance than the parent *Z. rouxii* strain,

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this raises the question of whether the *HOG1* gene in the strain (S3-2*HOG1*) is mutated and whether it improves glycerol production intracellularly in S3-2. Moreover, the application security of S3-2 in soy sauce fermentation has not been elucidated. In this study the *HOG1* genes from the genome of S and S3-2 were cloned and the functions of the cloned genes were analysed in *S. cerevisiae* strain W303-1A.

MATERIALS AND METHODS

Strains, media and cultivation conditions

The *Z. rouxii* strains S and S3-2 and the *S. cerevisiae* strain W303-1A (*MATα leu2 ura3 trp1 his3 ade2 can1*) were preserved in our laboratory. Yeast cells were cultivated aerobically in YPD medium (10 g kg^{-1} yeast extract, 20 g kg^{-1} peptone, 20 g kg^{-1} glucose), YPDN medium (NaCl dissolved in YPD substrate to a final concentration of 60–180 g kg^{-1} according to the particular experiment) or CM-URA medium (6.7 g kg^{-1} yeast nitrogen base without amino acids, 20 g kg^{-1} glucose, 1.92 g kg^{-1} CM-URA powder) at 30 °C. High-fidelity *Escherichia coli* Top10 was cultured in LB medium (50 g kg^{-1} yeast extract, 10 g kg^{-1} peptone, 10 g kg^{-1} NaCl, pH 7.2) at 37 °C for plasmid propagation. X-Gal LBA plates (5×10^{-5} g kg^{-1} ampicillin, 0.5 mmol kg^{-1} IPTG, 4×10^{-5} g kg^{-1} X-Gal) were used in blue/white spot screening. The plate cultures were performed with YPD, LB or CM-URA medium solidified with 20 g kg^{-1} agar.

Plasmid and strain construction

Since the complete genome of strains S and S3-2 containing *HOG1* is still unknown, the respective *HOG1* genes were amplified from the genome of S and S3-2 by polymerase chain reaction (PCR). PCR was carried out using *easyPfu* DNA polymerase to clone S3-2*HOG1* and S*HOG1* with the primers *HOG1*-up and *HOG1*-dn, whose respective nucleotide sequences were 5′-GCGAGCTCGTAACACGTCGGAGAACAGA-3′ (*Sac*I) and 5′-GCGGTACCGATACGCCTGAAAATTTATT-3′ (*Kpn*I). The sequencing vector pUC19 and the PCR products of S3-2*HOG1* and S*HOG1* were digested with *Sac*I and *Kpn*I respectively. The digested PCR products were then joined to pUC19 by ligation reaction to obtain the respective plasmids pUC19-S*HOG1* and pUC19-S3-2*HOG1*, which were sequenced in triplicate to obtain reliable results. Analogously, S3-2*HOG1* and S*HOG1* were cloned into the plasmid YEp195 to construct the respective plasmids YEp195-S*HOG1* and YEp195-S3-2*HOG1*. The two recombinant plasmids were then transformed into W303-1A by the lithium acetate method[11] to form the strains W303-YEp195-S*HOG1* (W-S) and W303-YEp195-S3-2*HOG1* (W-S3-2) respectively. Simultaneously, the control strain W303-YEp195 (W-Y) was obtained by transforming YEp195 into W303-1A.

RNA extraction and real-time quantitative PCR

The yeasts S and S3-2 were cultured in YPD at 30 °C. Cells inoculated to 3×10^8 mL^{-1} were collected and subjected to a new medium containing a different concentration of NaCl. After incubation for 60 min, cells were harvested and washed twice with the same NaCl concentration. Total RNA was extracted using Trizol (Beijing TransGen Biotech Co., Ltd, Beijing, China) according to the manufacturer's instructions. The crude RNA obtained was treated with DNaseI to obtain high-purity RNA. Reverse transcription PCR (RT-PCR) was performed to obtain cDNA with an RT kit. Aliquots of 1 mg of total RNA were used in the RT-PCR (42 °C for 30 min, 85 °C for 5 min).

Real-time quantitative PCR (QPCR) was used to investigate the expression level of *HOG1* in S and S3-2 cultivated in YPD and YPDN at 30 °C. The cDNA samples and the primers *HOG1*-R-up and *HOG1*-R-dn were used in the QPCR. The length of the PCR product *HOG1*-R was 409 bp. The nucleotide sequences of the primers used were *HOG1*-R-up: CAGATTTATTGGGCTCACC and *HOG1*-R-dn: GGGACCGTTACTGT TATGC. The internal reference used was 18s (128 bp) and its primers were 18s-up: CCAAGAACATGATGGCTGCT and 18s-dn: CTTGAAGAGCTCCTGGATGG. Annealing temperatures of 58 and 55 °C were used for QPCR of *HOG1* and 18s short product respectively.

Secondary and tertiary structure analyses

The secondary and tertiary structures of proteins encoded by S3-2*HOG1* and S*HOG1* were analysed by the AnthePro 6.0 program and Swiss model (Geneva Biomedical Research Institute, Geneva, Swiss), a fully automated protein structure homology-modelling server, in order to investigate changes in physical and chemical indicators.[12,13]

Tolerance analysis

The biomass of the engineered yeasts in YPDN with 120 g L^{-1} NaCl was evaluated by measuring the optical density of the culture at 660 nm. Dilution experiments were also performed in order to analyse the osmotolerance of the engineered yeasts. Cells were grown for 48 h in YPD liquid medium without salt, then 5 μL aliquots of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were spotted on plates containing 1.5 mol L^{-1} NaCl and 1.8 mol L^{-1} sorbitol respectively. The results were recorded after incubation for 36 h at 30 °C.[6]

For the analysis of intracellular glycerol, the engineered yeasts that had been grown continuously at 30 °C were collected at 5000 × g, resuspended in an equal volume of YPD with various NaCl concentrations and returned to 30 °C for growth. After 1 h the suspensions were centrifuged at 3000 × g for 5 min and the supernatants were discarded. The harvested cells were washed with cold distilled water and recollected, then the samples were crushed as described by Li *et al*.[14] The glycerol content was represented by dry cell weight (DCW). The intracellular glycerol was quantified using an Aminex (California, USA) HPX-87H column and a Shimadzu (Kyoto, Japan) RID-10A refractive index detector. The eluent used was 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.8 mL min^{-1}.

RESULTS AND DISCUSSION

Sequence analyses

Sequence analysis of S3-2*HOG1* and S*HOG1* revealed the whole length of 1860 bp containing the sequences of promoter and terminator and an open reading frame (ORF) of 1296 bp. A similarity of 99.8% between S3-2*HOG1* and S*HOG1* and two mutations in the ORF and one in the promoter region of S3-2*HOG1* compared with S*HOG1* were found through sequence alignment. In the ORF, two cytosines (C) of S were replaced by thymines (T) of S3-2, and in the promoter region, guanine (G), which was 292 bp upstream of the start codon, was mutated into adenine (A) in S3-2 (data not shown).

Structure prediction analyses

Both ORFs encoded a 431-amino-acid protein with a predicted molecular size of 48.657 and 48.629 kDa respectively. Owing to an amino acid mutation from alanine (A) to valine (V) (Fig. 1) induced by DNA mutation in the ORF, the deduced amino acid


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S     1   MATHEEFIRTQVFGTVFEITNRYTDLNPVGMGAFGLVCSATDTLAGQPVAIKKINKFFST  60
S3-2  1   MATHEEFIRTQVFGTVFEITNRYTDLNPVGMGAFGLVCSATDTLVGQPVAIKKINKFFST  60

S     61  AVLAKRTYRELLLKHLRHENLICLQDIFLSPLEDIYFVTELQGTDLHRLLQTRPLEKQF  120
S3-2  61  AVLAKRTYRELLLKHLRHENLICLQDIFLSPLEDIYFVTELQGTDLHRLLQTRPLEKQF  120

S     121 VQYFLYQILRGLKYVHSAGVIHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR  180
S3-2  121 VQYFLYQILRGLKYVHSAGVIHRDLKPSNILINENCDLKICDFGLARIQDPQMTGYVSTR  180

S     181 YYRAPEIMLTWQKYDVEVDIWSAGCIFSEMIEGKPLFPGKDHVHQFSIITDLLGSPFRDV  240
S3-2  181 YYRAPEIMLTWQKYDVEVDIWSAGCIFSEMIEGKPLFPGKDHVHQFSIITDLLGSPFRDV  240

S     241 INTICSENTLKFVTSLPHRDPVPFQERFKTVEPDAVDLLERMLVFDPKKRITAADALVHP  300
S3-2  241 INTICSENTLKFVTSLPHRDPVPFQERFKTVEPDAVDLLERMLVFDPKKRITAADALVHP  300

S     301 YLAPYHDPTDEPTAEAQFDWDFNDADLPVDTWRVMMYSEILDFHKIGGGDGQIDTNAAFD  360
S3-2  301 YLAPYHDPTDEPTAEAQFDWDFNDADLPVDTWRVMMYSEILDFHKIGGGDGQIDTNAAFD  360

S     361 DQVAAATAAAAHAAAMAQHHHQTQQQSSGKHTNPTTSSSAATHNSNGPHSNHDAIANYGN  420
S3-2  361 DQVAAATAAAAHAAAMAQHHHQTQQQSSGKHTNPTTSSSAATHNSNGPHSNHDAIANYGN  420

S     421 QAVHYANEFQQ  431
S3-2  421 QAVHYANEFQQ  431
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Figure 1. Amino acid sequences encoded by S*HOG1* and S3-2*HOG1*. The oval indicates the mutation (in the 45th amino acid) and the rectangle indicates the TGY motif.

sequence of S3-2Hog1p was approximately 99% homologous to that of SHog1. Also, both SHog1p and S3-2Hog1p were highly similar to Hog1p kinases of *S. cerevisiae* (90% identity), which was in accordance with the conclusion that the products encoded by *ZrHOG1* had similar functions to MAPK in *S. cerevisiae* cells.[9] The TGY motif for phosphorylation by MAPK was also found in the SHog1p and S3-2Hog1p sequences (Fig. 1).[10] The secondary structures of SHog1p and S3-2Hog1p were further analysed using AnthePro software in order to investigate S*HOG1* and S3-2*HOG1*. The predicted results showed a slight difference between SHog1p and S3-2Hog1p. As shown in Fig. 2, the secondary structure of S3-2Hog1p was composed of 32% helix, 13% sheet, 3% turn and 52% coil, while that of SHog1p comprised 32% helix, 12% sheet, 3% turn and 53% coil. In addition, SHoglp and S3-2Hoglp had an identical isoelectric point (5.636).

The Swiss tool was used to calculate the model of protein encoded by the ORF. The 3D model of S3-2Hog1p and SHog1p showed an overall structure similar to that of other known MAPKs. Apart from one mutation point (Ala^{45}Val) in S3-2Hog1p, no obvious difference was found on the whole in the spatial structures of S3-2Hog1p and SHog1p (data not shown). The high similarity of secondary and tertiary structures of S3-2Hog1p and SHog1p indicated that the activity of S3-2Hog1p was not affected by conformational changes induced by gene mutation in the ORF. In addition, conservation of the gene would be beneficial for the safety of S3-2 and its security of application in the fermentation of soy sauce.

Expression level of *HOG1*

To clarify the molecular mechanism of improved salt tolerance of S3-2, the RNA of S and S3-2 subjected to osmotic shock in YPD and YPDN containing 60, 120 and 180 g kg^{-1} NaCl was extracted, followed by RT to obtain cDNA. QPCR was performed with 18s rDNA as reference to analyse the transcription levels of S*HOG1* and S3-2*HOG1*. As shown in Fig. 3, the transcription levels of S3-2*HOG1* and S*HOG1* in YPD were close to each other. However, the transcription level of S3-2*HOG1* was higher than that of S*HOG1* in YPDN, indicating that S3-2 was capable of

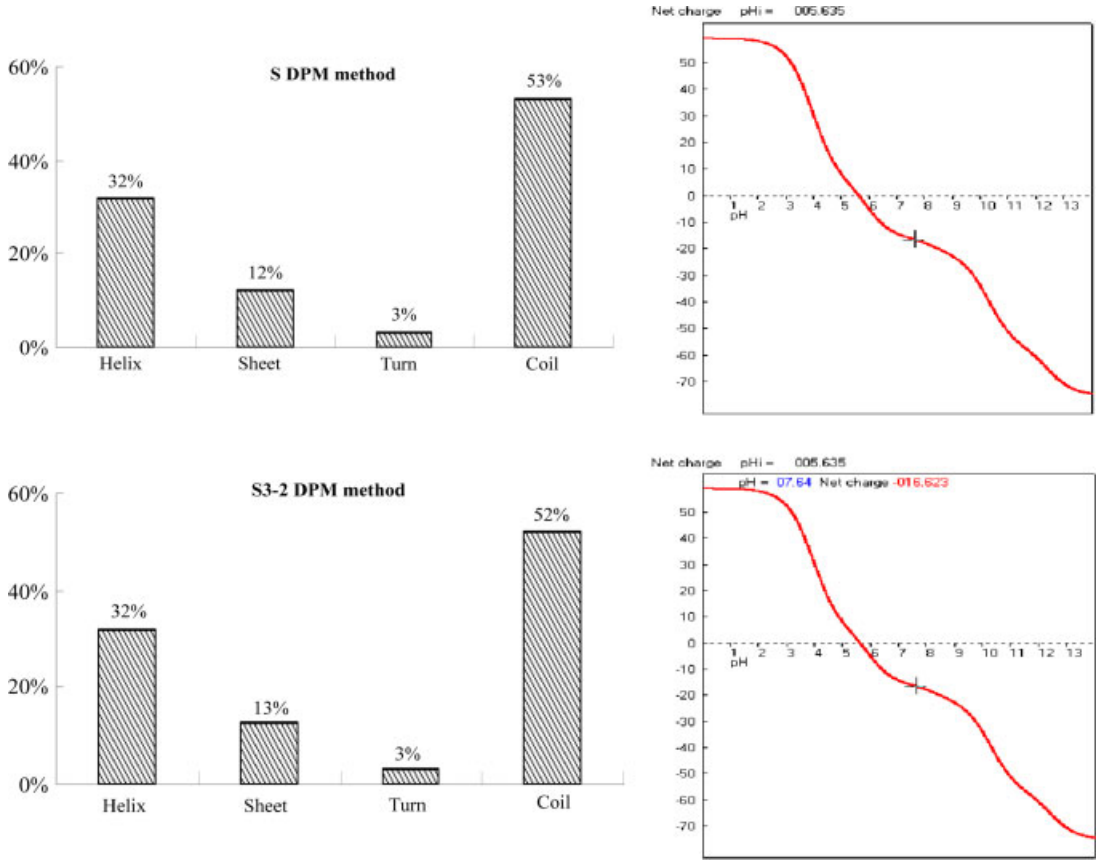

Figure 2. Details of secondary structure and isoelectric point of S*HOG1* and S3-2*HOG1* using DPM method.

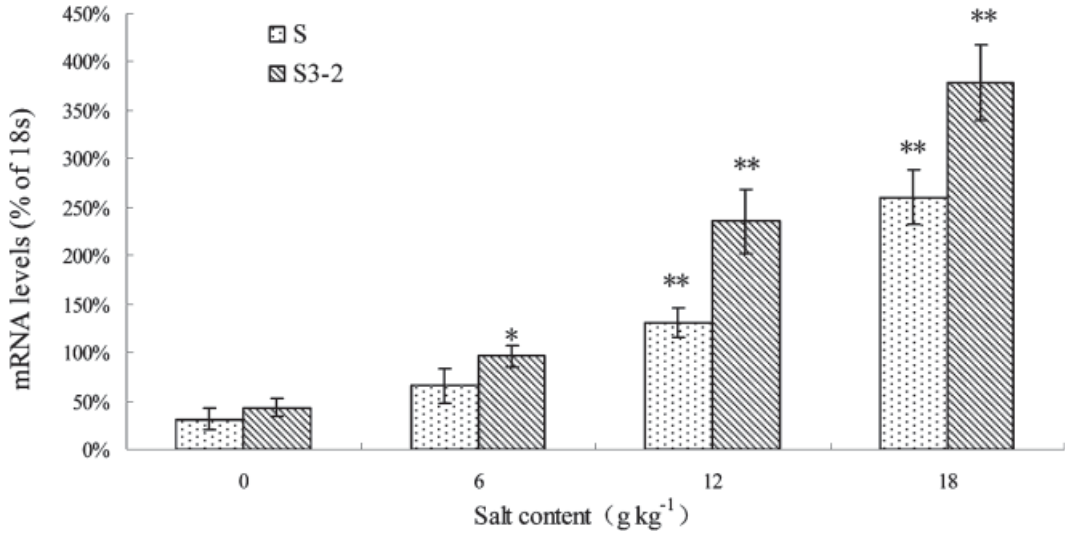

Figure 3. Expression level of *HOG1*. RT-QPCR analysis of total RNA extracted from S*HOG1* and S3-2*HOG1* cells cultivated in presence of 0, 60, 120 and 180 g kg^{-1} NaCl. Each value represents mean ± SD of three independent experiments. Significant differences at $*P < 0.05$ or $**P < 0.01$ are indicated.

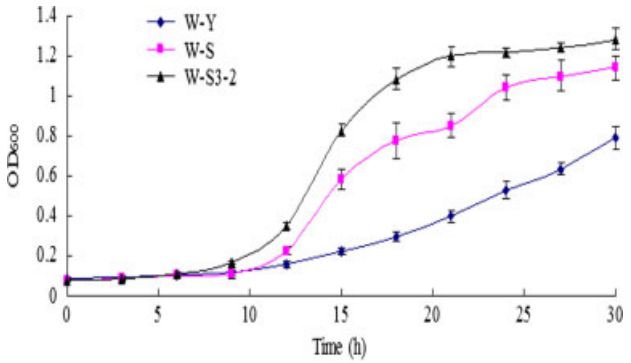

Figure 4. Growth of engineered strains W-Y, W-S and W-S3-2 in presence of 120 g kg^{-1} NaCl. Each value represents mean ± SD of three independent experiments.

producing more glycerol under osmotic shock. The promoter activity could be enhanced by mutation in the promoter region. Thus it was concluded that enhancement of the transcription level of *HOG1* rather than change in the conformation of S3-2Hog1p may be one of the main reasons for the salt tolerance improvement of S3-2.

Salt tolerance and osmotolerance analyses

It was reported that overexpression of the *HOG1* gene in cells could improve the overall tolerance of high osmotic pressure.[15] In this study, engineered strains W-Y, W-S and W-S3-2 were cultivated in YPDN with 120 g kg^{-1} NaCl. Figure 4 shows their growth. The results indicated that the growth rates of W-S and W-S3-2 were higher than that of the control W-Y, while the growth rate of W-S3-2 was higher than that of W-S.

Dilution experiments with engineered strains W-Y, W-S and W-S3-2 were carried out by cultivating cells on solid YPD with high concentrations of NaCl and sorbitol to determine their osmotolerance. The results showed that W-S3-2 was markedly resistant to high concentrations of NaCl and sorbitol compared with other strains (Fig. 5). This confirmed that the S3-2*HOG1* gene could enhance the osmotolerance of yeast.

The intracellular glycerol content of engineered strains under salt stress was also measured quantitatively. When strains W-Y, W-S and W-S3-2 were cultivated in YPD, their glycerol contents did not differ. However, the glycerol contents of W-Y, W-S and W-S3-2 cultured in YPDN with 60 and 120 g kg^{-1} NaCl were significantly higher than those of the controls (Fig. 6). At the same time, the glycerol content of W-S3-2 was higher than that of W-S at both NaCl concentrations, which was consistent with the QPCR and growth rate results. This indicated that strain S3-2 had a higher salt tolerance than strain S.

When yeast cells undergo a hyperosmotic shock, post-transcription processes in the cytosol and gene expression in the nucleus are regulated by activated Hog1p following cell shrinkage.[16] Accordingly, biosynthesis of the osmolyte glycerol is induced by the up-regulated *GPD1* genes. The resulting cytosolic glycerol accumulation leads to increased internal osmolarity and balances the intracellular and extracellular osmotic gradient.[17] Another study in our laboratory demonstrated a positive correlation between expression levels of *GPD1* and *HOG1* (data not shown), implying that more glycerol would be induced by higher expression levels of *HOG1* eventually. Intracellular glycerol is involved not only in osmoadaptation[7] but also in oxidative stress protection[18] and response to heat shock.[19,20] In particular, Hog1p is essential for proliferation under osmostress conditions.[8] Therefore improving the transcription level of S3-2*HOG1* could enhance the tolerance to a succession of detrimental conditions that affect S3-2 viability and fermentation efficiency, such as high temperature,[19] high sugar[21] and accumulation of ethanol.[19]

It was found that increasing NaCl concentration could stimulate the formation by *Z. rouxii* of higher amounts of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, furaneol), which is an important aroma compound of soy sauce.[22] Therefore the utilisation of S3-2 is a potentially valuable strategy for improving the flavour of soy sauce.

In addition, the Na^+/H^+ antiporter (encoded by *NHA1*),[23] aquaglyceroporin Fps1p (encoded by *FPS1*),[24,25] Gpd1 and Gpd2 for glycerol-3-phosphate dehydrogenase and Gpp1 and Gpp2 for glycerol-3-phosphatase (encoded by *GPP1* and *GPP2*)[16,24] also play important roles in response to osmotic stress. Thus further

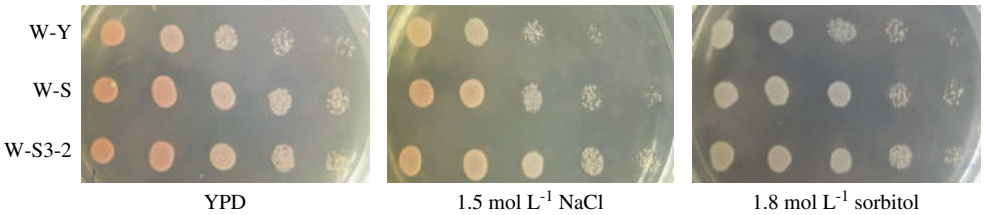

Figure 5. Resistance of W-Y, W-S and W-S3-2 to different stresses within 36 h. Experiments were performed at least in triplicate. One representative result is shown.

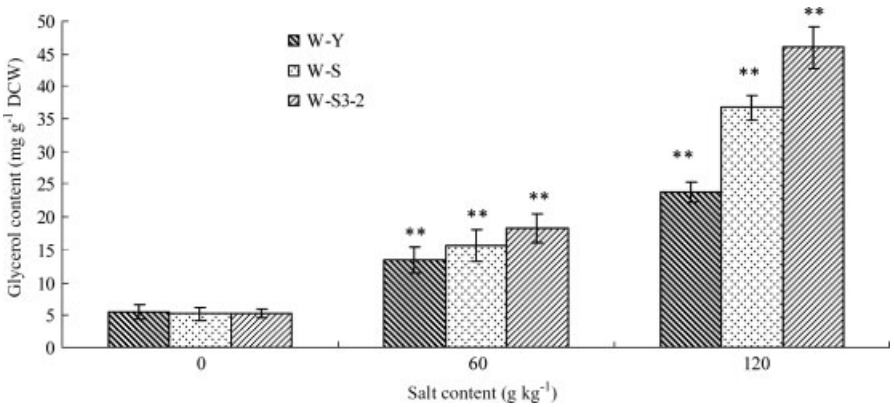

Figure 6. Glycerol content of engineered strains W-Y, W-S and W-S3-2 in presence of 0, 60 and 120 g kg^{-1} NaCl. Each value represents mean ± SD of three independent experiments. Significant differences at $^{**}P < 0.01$ are indicated.

investigation is essential to reveal the effects of other genes such as *FPS1*, *NHA1*[16] and *GPD1* on the salt tolerance of S3-2.

CONCLUSION

The present study revealed that S3-2*HOG1* has higher transcription and expression levels under hypertonic conditions and that a higher intracellular glycerol content of S3-2 in hypertonic medium may be induced by enhancement of the transcription level of S3-2*HOG1*. The higher intracellular glycerol content improved the osmotolerance, which was advantageous for improving the fermentation properties of S3-2 during processing of soy sauce.

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