MAL62 overexpression enhances uridine diphosphoglucose-dependent trehalose synthesis and glycerol metabolism for cryoprotection of baker’s yeast in lean dough

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Research

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**Abstract**

**Background:** In *Saccharomyces cerevisiae*, the alpha-glucosidase (maltase) is a key enzyme in maltose metabolism. Overexpression of the alpha-glucosidase-encoding gene *MAL62* has been shown to increase the freezing tolerance of yeast in lean dough. However, its cryoprotection mechanism is still not clear.

**Results:** RNA sequencing (RNA-seq) revealed that *MAL62* overexpression increased transcription of uridine diphosphoglucose (UDPG)-dependent trehalose synthesis. The changes in transcript abundance were confirmed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and enzyme activity assays. When the UDPG-dependent trehalose synthase activity was abolished, *MAL62* overexpression failed to promote the synthesis of intracellular trehalose.

Moreover, in strains lacking trehalose synthesis, cell viability in the late phase of prefermentation freezing coupled with *MAL62* overexpression was slightly reduced, which can be explained by the increase in intracellular glycerol concentration which was consistent with the elevated transcription of glycerol synthesis pathways.

**Conclusions:** The increased freezing tolerance by *MAL62* overexpression is mainly achieved by the increased trehalose content via the UDPG-dependent pathway, and glycerol plays a secondary role. These findings shed new light to the mechanism of yeast response to freezing in lean bread dough and can help the improvement of industrial yeast strains.

**Background**

The use of frozen dough is now gradually emerging in a multitude of bakery and food chains due to its less time-consuming production after freezing and cheaper bake-off stations [1, 2]. However, freezing often causes oxidative stress and cell death to baker’s yeast [3], which reduces the yeast growth and gas production capacity [4, 5]. A number of protective molecules have been identified in yeast stress tolerance [6–8]. Among them, the compatible osmolyte trehalose has captured wide attention [9]. Yeast trehalose is regulated by two major biosynthetic systems, system I and II. System I is uridine-5′-diphosphoglucose (UDPG) dependent and contains several protein complexes, including one trehalose-6-phosphate synthase (encoded by *TPS1*)[10], one trehalose-6-phosphate phosphatase (encoded by *TPS2*), and one trehalose-synthesis protein complex (encoded by *TSL1*) [11]. The system II trehalose synthetic pathway is adenosine-diphosphoglucose (ADPG)- dependent and uses maltose, a disaccharide, to synthesize trehalose [12, 13].

In baker’s yeast, the *MAL* gene family, which regulates maltose metabolism, consists of five multigene complexes, including *MAL1, MAL2, MAL3, MAL4*, and *MAL6*. Each gene complex encodes a maltose permease, an alpha-glucosidase, and a transacting MAL-activator [14]. We have shown previously that overexpression of *MAL62* enhances the cryotolerance of baker’s yeast [15] and speculated that multiple pathways may be involved in this phenomenon [16]. However, mechanism for the enhanced freezing tolerance is still unknown.
To better understand the role of \textit{MAL62}-overexpression in freezing tolerance of baker's yeast in lean dough, and its possible mechanism, we used transcriptome analysis to characterize a \textit{MAL62}-overexpressing strain and investigated the effects of overexpression of \textit{MAL62}, and deletion of \textit{TPS1} gene, on maltose metabolism, on trehalose and glycerol accumulation, and on the freezing tolerance of baker's yeast in lean dough.

\section*{Materials And Methods}

\subsection*{Strains, plasmids and growth conditions}

The yeast and bacterial strains, as well as the plasmids used in this study, are listed in Table 1. The parent industrial strain BY14 was used to create the high-leavening haploid \textit{BY14a} strain, which was used to create all of the other strains, including the overexpression and deletion strains.

The \textit{Escherichia coli DH5a} and yeast strains were cultured as described previously \cite{15}. Briefly, yeast cells in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C on a rotor with a speed of 180 rpm. 0.08% of G418 (final concentration) (Thermo Fisher, MA, US) was added to YPD plates to select G418-resistant transformants. After growing in YPD for 24 h, 20 mL of cultured cells were inoculated into 200 mL of cane molasses medium (0.5% yeast extract, 0.05% (NH$_4$)$_2$SO$_4$, and 12° Brix cane molasses) at the initial OD$_{600}$ = 0.4. Cells were cultured at 30°C to OD$_{600}$ = 1.8 (about 24 h). Cells were centrifuged at 4°C, 5,000 rpm for 5 min and then washed twice with sterile water. A modified low sugar model liquid dough (LSMLD) medium\cite{17} was used for measurement of trehalose, intracellular glycerol and the cell viability during prefermentation and after prefermentation freezing.

\subsection*{RNA sequencing (RNA-seq)}

RNA-seq based transcriptome analysis was performed to identify the differentially expressed genes after \textit{MAL62} overexpression. Cells constitutively overexpressing \textit{MAL62} (B+MAL62) and its control (BY14a+K) were harvested from cane molasses medium. RNA isolation and cDNA synthesis were performed as previously described \cite{18}. Briefly, total RNA was isolated using the hot acid phenol method, followed by DNase treatment. The RNA concentration was measured using a Qubit fluorometer and a Qubit RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The RNA integrity was assessed using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) and an RNA Nano 6000 Assay Kit.

The RNA-seq libraries were generated from 1 μg of RNA from each sample using a NEBNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA), according to the manufacturer’s instructions. The clustering of samples, which was index coded to attribute individual sample sequences, was performed using a cBot Cluster Generation System (Illumina, San Diego, CA, USA). The RNA libraries were sequenced using an Illumina Hiseq 2500 system (Illumina). Paired-end reads of 125 bp/150 bp were generated and analyzed.
RNA-seq data analysis

The differential expression of two different groups was analyzed using DESeq R software (https://www.bioconductor.org, version 1.18.0). Genes with a false-discovery rate-adjusted $p$-value < 0.05 were considered as differentially expressed.

Volcano plots and hierarchical clustering were used to screen the differentially expressed genes and to analyze the clusters of differentially expressed genes. GOseq[19] was used for gene ontology (GO) term enrichment analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to enrich the pathways. STRING (www.string-db.org/) and Cytoscape (https://cytoscape.org) software programs were used for protein-protein interaction (PPI) network analysis.

Validation of gene expression levels

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was used to detect the expression levels of the target genes. qRT-PCR was conducted using the THUNDERBIRD probe one-step qRT-PCR kit (TOYOBO, Osaka, Japan). The yeast $UBC6$ gene, which encodes a ubiquitin-conjugating enzyme involved in endoplasmic reticulum-resident proteins for degradation, was used as a reference gene[20]. The PCR primers are listed in Table 2. Yeast cDNA was extracted using an RNAiso kit (Takara Biotech, Dalian, China) and a PrimeScript RT reagent kit with gDNA eraser (Perfect Real Time, Takara Biotech). The PCR was conducted using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The reaction conditions were as follows: 95°C for 30 s; 61°C for 20 min; 95°C for 30 s; 43 cycles of 95°C for 5 s, 55°C for 10 s, and 74°C for 15 s; and 72°C for 5 min. Quantitative analysis of the qRT-PCR was conducted using the $2^{-\Delta\Delta CT}$ method.

Measurement of enzymatic activities

Tps1 activity was measured as previously described [21]. One unit of Tps1 activity was defined as the production of 1.0 μM of trehalose-6-phosphate per minute. The final activity was calculated based on cell dry weight (CDW). Data were expressed as mean ± SD from three independent experiments.

For α-glucosidase activity, crude extracts were prepared using the Salema-Oom method[22] and the α-glucosidase activity was measured as previously described [23]. Data were expressed as mean ± SD from three independent experiments.

For the activity of other enzymes, including hexokinase, phosphoglucomutase, UGPase and glycerol-3-phosphate dehydrogenase (G3PDH), cells were grown in cane molasses or LSMLD medium to late-log phase. Cells were centrifuged at 5,000 rpm for 5 min at 4°C, then washed twice with cold sterile water. Activities of hexokinase [24], phosphoglucomutase [25], UGPase [26] and G3PDH [27] were assayed as described. Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, USA) following the manufacturer's instruction. Data were expressed as mean ± SD from three independent experiments.
Measurement of intracellular trehalose contents

A total of 0.1 g of freshly cultured cells were washed twice with water. Trehalose was extracted using 4 mL of 0.5 M trichloroacetic acid then measured as previously described[28]. Data were expressed as mean ± SD from three independent experiments.

Measurement of extracellular maltose

Cultured cells were filtered through a 0.45-µm-pore-size cellulose acetate filter (Millipore, Danvers, MA, USA). The extracellular maltose was measured by high-pressure liquid chromatography (HPLC) analysis using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and an HPLC pump (Waters 515). The column was eluted at 65°C with 5 mM H₂SO₄ at a flow rate of 0.6 ml/min [29]. Maltose was detected with a differential refractometer detector (Waters 410 RI). Data were expressed as mean ± SD from three independent experiments.

Yeast strain construction

Yeast genomic DNA was extracted using a yeast DNA isolation kit (Omega Bio-Tek, Norcross, GA, USA). The \textit{tps1D} (B-T) strain (Table 1) was constructed as follows: the TPS1U fragment containing the \textit{TPS1} upstream homologue sequence and the TPS1D fragment containing the \textit{TPS1} downstream homologue sequence were amplified from the BY14a yeast genome with the primers TU1-F/TU1-R and TD1-F/TD1-R, respectively. The fragment \textit{KanMX} was amplified from the plasmid pUG6 using the primers KAN1-F/KAN1-R [30]. Then, the fragments of TPS1U, TPS1D, and \textit{loxP-KanMX-loxP} were transferred into BY14a using the lithium acetate/polyethylene glycol method [25]. G418 (300 μg/mL) was used to select the positive recombinants, which were further verified by PCR with the primers UUK-F/UUK-R and KDD1-F/KDD1-R. The \textit{tps1D} plus \textit{MAL62}-overexpression (B-T+M) strain (Table 1) was constructed as follows: The \textit{MAL62} gene was amplified from the BY14a genome with the primers TU1-F/TU2-R, TD1-F/TD1-R, and MAL-F/MAL-R. The fragments containing the yeast phosphoglycerate kinase gene promoter (\textit{PGK1p}) and terminator (\textit{PGK1t}) were amplified from the BY14a genome with the primers PGKP-F/PGKP-R and PGKT-F/PGKT-R, respectively. The fragment \textit{loxP-KanMX-loxP} was amplified from the plasmid pUG6 using the primer pair KAN2-F/KAN1-R. Six fragments (TPS1U, \textit{PGK1p}, \textit{MAL62}, \textit{PGK1t}, \textit{KanMX}, and TPS1D) were transferred into the BY14a strain, and the recombinant B-T+M strain was verified via PCR using the primer pairs of UUP-F/UUP-R, PPM-F/PPM-R, MPT-F/MPT-R, PTK-F/PTK-R, and KDD2-F/KDD2-R. B+MAL62, the \textit{MAL62}-overexpression strain, was constructed as described previously[15].

Measurement of intracellular glycerol content

To measure the intracellular glycerol levels, approximately 25 mg (wet weight) of cells was washed, resuspended in 1 mL of deionized water, and boiled twice (30 min, with occasional shaking). The supernatants were then centrifuged for 10 min at 15,000 \times g. The level of glycerol was measured as described previously [31]. Data were expressed as the mean ± standard deviation (SD) from three independent measurements.
Measurement of cell viability and leavening ability

Yeast cells were cultured in the cane molasses medium [5 g/L yeast extract, 0.5 g/L (NH₄)₂SO₄, and 12° Brix cane molasses] for 24 h at 30 °C and then transferred to LSMLD medium. The cells were prefermented for 5, 10, 15, 20, and 25 min, then moved to a −20 °C freezer for 7 days. The cell viability and leavening ability were measured after freezing, as described previously [15]. Data were expressed as the mean ± SD from three independent experiments.

Statistical analysis

Data were represented as mean ± SD from three independent experiments. Differences among the various strains were analyzed using analysis of variance. Differences between the parent and the MAL62-overexpression strains were analyzed using the Student’s t-test. For all analyses, p < 0.05 was considered statistically significant.

Results

**MAL62 overexpression enhances the UDPG-dependent trehalose synthesis**

RNA-seq analysis was performed to identify those differentially expression genes when MAL62 was overexpressed. The results showed that MAL62 overexpression caused significant changes in gene expression (Fig. 1A). Compared to the control (BY14a + K), 1,460 genes were downregulated (Fig. 1B, green) and 1,506 genes were upregulated (Fig. 1B, red). KEGG analysis showed that the upregulated genes were mainly enriched in the metabolism and synthesis of carbohydrates (Fig. 1C). GO analysis of the biological processes (molecular function, cellular component, and biological process) showed that several processes involving trehalose were affected by MAL62 overexpression (Fig. 1D). STRING analysis showed that MAL62 overexpression caused upregulation of TPS1, TPS2, TPS3, and UGP1, which are all key genes of the UDPG pathway (Figs. 2 and S1).

To further examine the possible involvement of the UDPG pathway on trehalose synthesis, the expression levels of trehalose metabolism-related genes (GLK1, EMI2, HXK1, HXK2, PGM2, PRM15, UGP1, GDB1, TPS1, TPS2, and TPS3) were analyzed by qRT-PCR. Our results showed that all of these genes, except for TPS2, had a significantly higher expression in the B + MAL62 strain than in the BY14a + K strain (Fig. 3A). The fold changes were as follows: GDB1, 2.36; TPS1, 2.27; UGP1, 1.90; HXK1, 1.89; EMI2, 1.66; PRM15, 1.69; HXK2, 1.75; PGM2, 1.86; GLK1, 1.41; and TPS3, 1.47. No significant change of TPS2 was observed between the B + MAL62 and BY14a + K strains. The measurement of enzyme activities showed that all of the tested key enzymes related to UDPG pathway, except for hexokinase, were of higher activity in the B + MAL62 strain than in the BY14a + K strain (Table. 3).

Disruption of the TPS1 gene diminishes the UDPG-dependent trehalose synthase activity[13]. To further understand the role of MAL62 overexpression in trehalose synthesis, we constructed a tps1Δ strain (B-T) and MAL62 was overexpressed in a tps1Δ strain (B-T + M) to eliminate the effects of UDPG dependent
trehalose synthesis pathway. Figure 3B showed that the expression levels of TPS1 in the B-T and B-T + M strains were not detectable. Compared with the maltose fermentation (decrease 39.86% concentration of maltose) and alpha-glucosidase activity (3.91 mmol mg\(^{-1}\) min\(^{-1}\), in average) of B + MAL62, B-T and B-T + M had lower consumption of maltose (only decrease 6.07% concentration of maltose, in average) and less activities of alpha-glucosidase (just maintained 1.46 mmol mg\(^{-1}\) min\(^{-1}\), in average) in the first 60 min fermentation (Fig. 4). One possible cause of this is the slower growth rates of the two tps1Δ strains [32]. However, it is worth noting that even when the alpha-glucosidase activities start to rise at the 60 min and reached the peak at nearly 150 min during fermentation, the trehalose levels in the B-T and B-T + M strains were still not detectable. These results suggest that MAL62 overexpression activates the UDPG pathway, which then causes the accumulation of intracellular trehalose and enhanced cryotolerance.

MAL62 overexpression enhances glycerol metabolism

RNA-seq and STRING analyses showed that MAL62 overexpression caused upregulation of GPD1, GPD 2, GPP1, and GPP2 (Figs. 2, S2 and S3). GPD1 and GPD 2 encode the rate-limiting enzymes in high-osmolarity glycerol mitogen-activated protein kinase (HOG-MAPK) pathway, which induces glycerol accumulation [33–36]. We assayed the activities of glycerol-3-phosphate dehydrogenase in BY14a + K and B + MAL62 strains. As shown in Table. 3, the activity of glycerol-3-phosphate dehydrogenase in B + MAL62 was 88.7% higher than that in BY14a + K, suggesting that MAL62 overexpression causes an increase of glycerol content.

To further determine the change of glycerol content and its possible cryopreservation effect in MAL62 overexpressed strain, we used B-T and B-T + M to eliminate the effects of trehalose. As showed in Fig. 5A, The B-T strain exhibits a similar intracellular glycerol synthesis rate compared to the BY14a strain, suggesting that the deletion of TPS1 did not affect glycerol synthesis. However, the intracellular glycerol content in the B-T + M strain is significantly higher than that in the BY14a or B-T strain after prefermentation for 15 min and freezing for 7 d (p < 0.05). After prefermentation for 25 min and freezing for 7 d, the intracellular glycerol content in the B-T + M strain increased by approximately 121.5%, compared with prefermentation for 0 min, while the intracellular glycerol contents in the BY14a and B-T strains increased by only 23.9% and 32.8%, respectively. These results suggest that MAL62 overexpression positively correlates with the accumulation of intracellular glycerol.

Next, we examined whether the increased glycerol level by MAL62 overexpression affects the freezing tolerance by measuring the cell viability. As shown in Fig. 5B, a longer prefermentation duration caused a significant decrease in cell viability after freezing for 7 d in all three strains (BY14a, B-T, and B-T + M). After prefermentation for 25 min, the cell viability of the BY14a and B-T + M strains was similar, but the cell viability of the B-T strain was significantly lower than those of the BY14a and B-T + M strains (p < 0.05). Compared to the BY14a and B-T strains, the cell viability of the B-T + M strain showed a minor decrease after prefermentation for 15 min (Fig. 5B). These findings suggest that in addition to triggering
the accumulation of trehalose, MAL62 overexpression also causes an increment in glycerol content, which can enhance freezing tolerance.

**Increased glycerol content by MAL62 overexpression enhanced the leavening ability after long-term freezing**

The possible effect of the increased glycerol level by MAL62 overexpression on the leavening ability after long-term freezing was determined by measuring CO₂ production. As shown in Fig. 5C, the CO₂ production in all strains decreased as the freezing time increased from 1 to 4 weeks. However, the CO₂ production of the B-T + M strain was significantly higher than either the BY14a or B-T strain before freezing (time = 0) and after freezing for 1 to 4 weeks (p < 0.05). These results suggest that the increased glycerol content by MAL62 overexpression can mitigate the loss of the leavening ability after exposure to the stress induced by long-term freezing.

**Discussion**

Our comparative transcriptome analysis revealed that overexpression of MAL62 causes significant differences in gene expression, as compared to its wild-type control (BY14a+K). Many of these genes are involved in the stress response, especially freezing stress pathways. Several genes involved in starch and sucrose metabolism, glycerophospholipid metabolism, and glycerolipid metabolism are also differentially expressed between these two strains. KEGG analysis further confirmed that many of the pathways are involved in cryotolerance of the B+MAL62 strain. In baker's yeast, trehalose is believed to be the primary compound affecting the viability of yeast in frozen dough[37, 38]. We have previously reported that the enhanced freezing tolerance by MAL62 overexpression is related to the increased activity of Tps1 [16]. Our current study provides further evidence that genes involved in starch and sucrose metabolism, including GLK1, EMI2, HXK1, HXK2, PGM2, PRM15, and UGP1, had a higher expression level in the B+MAL62 strain (Fig. 3A and S1). In addition, PPI network analysis revealed a high score for Tps1, Tps2, and Tps3 (Fig. 2B). Moreover, enzyme activities and fermentation experiments provided further confidence (Table 3 and Figs. 4, 5A and 5B) that the enhanced freezing tolerance by MAL62 overexpression is related to the UDPG-dependent trehalose synthesis pathway.

Microorganisms often accumulate different solutes, such as ions, amino acids, and polyols, to mitigate water loss [39, 40], when they face a water shortage. Baker's yeast responds to freeze stress-induced hyperosmotic stress by activating the HOG-MAPK pathway, which induces glycerol accumulation [33-36]. Our transcriptome analysis revealed that Hog1, Msn2, and Msn4 scored high on the PPI network (Fig. 2B). Genes and rate-limiting enzyme activity involved in glycerol biosynthesis were also upregulated in the B+MAL62 strain (Figs. S2, S3, and Table 3), suggesting that MAL62 overexpression induces the accumulation of glycerol through the HOG pathway.

It was reported that stress-responsive elements (STRE) mediate transcriptional regulation of the trehalose synthase genes TPS1, TPS2, and TPS3 as well as the glycerol 3-phosphate dehydrogenase genes GPD1
and GPD2 [41, 42]. Msn2 and Msn4, which bind specifically to STRE-containing oligonucleotides [43], are controlled by the HOG-MAPK pathway [44]. Hence, we speculated that the accumulation of trehalose and glycerol by MAL62 overexpression may start with the activation of the HOG-MAPK pathway, after which the trehalose synthase genes and glycerol 3-phosphate dehydrogenase genes are upregulated through STRE-mediated transcriptional regulation. This may explain our previous speculation that the enhancement in freezing tolerance by MAL62 overexpression may involve multiple pathways [16]. A possible relationship between maltose metabolism and cryoprotectant synthesis is illustrated in Fig. 6.

Compared with the B-T and B-T+M strains, the BY14a strain has a higher intracellular trehalose content, which results in a higher cell viability at the onset of prefermentation (Figs. 5A and 5B). The B-T strain, which lacks trehalose, had a lower cell viability in the beginning, and the viability decreased rapidly. The B-T+M strain, which had a higher glycerol level, showed a smaller decrease in cell viability in the later stage of prefermentation (Figs. 5A and 5B). This phenomenon suggests that the high glycerol content induced by MAL62 overexpression has some positive effects on the freezing tolerance. Due to the fact that trehalose is the primary compound affecting the freezing tolerance[37], the differences in trehalose accumulation may play an important role in the different cell viabilities exhibited by the three strains after prefermentation and freezing for 7 d. It is possible that the increased level of glycerol, other gene expression changes caused by MAL62 overexpression may also play a role in the enhanced cell viability and leavening ability. Studies are underway in our laboratory to further confirm the roles of glycerol and other related genes in yeast cryoprotection.

Hyperosmotic stress, which causes desiccation and electrolyte release from yeast cells, is a major factor for loss of leavening activity after freezing and thawing[38]. Besides, the formation of ice crystals during freezing causes damage to the cell membrane and subcellular structure [45]. Previous studies have reported that fermentation with high glycerol-producing strains can result in improved cell viability and gas retention in dough [46]. Consistent with the report, our results showed that the B-T+M strain exhibits a higher glycerol content as well as improved gas retention in the dough, especially after the storage of the frozen dough for more than 1 week. We believe that this is partially because the HOG pathway and glycerol synthesis were activated by MAL62 overexpression. It is worth noting that the B-T+M strain shows a high level of CO$_2$ production during the 4 weeks of frozen dough storage. A possible reason is that MAL62 overexpression enhances maltose metabolism, which is vital for dough fermentation [15].

**Conclusion**

Our study indicates that the MAL62 overexpression induced-cryoprotective effects are primarily achieved by the increased trehalose by the UDPG-dependent trehalose synthesis pathway. The increased glycerol content by MAL62 overexpression also plays an important role in the cryoprotection of baker’s yeast, especially in the later stage of pre-fermentation. We believe these findings shed new light to the mechanism of yeast response to freezing in lean bread dough and can help the improvement of industrial yeast strains.
Declarations

Authors’ contributions

XS conceived the study, carried out the experiments, and drafted the manuscript. PX and FL participated in the strain construction. JZ, ZHF, HQL, and WBZ assisted in fermentation experiments. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Acknowledgments

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Consent for publication

All authors give consent to publish the research in Microbial Cell Factories

Competing interests

The authors declare that they have no competing interests.

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Tables
Table 1. Characteristics of the strains used in the present study

<table>
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YCC: Yeast Collection Center of the Tianjin Key Laboratory of Industrial Microbiology.

※BY14a was selected as a high-leavening capacity haploid from 32 clones derived from BY14 (data not shown).

Table 2. Primers used in this study
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<td>KDD1-F</td>
<td>TCGCAGACCGATACCAGGAT</td>
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<tr>
<td>KDD1-R</td>
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<td>UUP-F</td>
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<tr>
<td>UUP-R</td>
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<tr>
<td>PPM-R</td>
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<td>MPT-R</td>
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<tr>
<td>PTK-F</td>
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<tr>
<td>PTK-R</td>
<td>CCGTCAGCCAGTTAGTC</td>
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</table>
KDD2-F  TATGTGAATGCTGGTCGCTAT
KDD2-R  CCGTTGCTACTGCGGTATA

For RT-qPCR

qGDB1-F  AGCCTAACTTCGGCCTCT
qGDB1-R  CACCGTCTCTAATCTCAAATA
qEMI2-F  GGCAAGGATGTCGTGGTT
qEMI2-R  AGCCTGAAGTGTAGCAGTG
qGLK1-F  ATCACGAAGTTGCCACAG
qGLK1-R  TCACCCAAGAACATCCCT
qHXK2-F  TCCGTTTACAACAGATACCC
qHXK2-R  ATAAACAGCGGCAACCAGCA
qHXK1-F  GTGTCAAGACCACCTGCA
qHXK1-R  GGATCTTTGCTTGCGTCACC
qPGM2-F  GAAAAGGAGGCTGTTGGGC
qPGM2-R  GGCTGGGAAGGGCGGATTAA
qPRM15-F  TAAAGCAAGACCGAACCACCCAA
qPRM15-R  CCAATCCCTGAGACGCTTGT
qUGP1-F  CGAGAGCAACACAAACAGCG
qUGP1-R  CCGGGTGGGAGACTTGATC
qTPS1-F  GGGGCAAGGTGTTCTG
qTPS1-R  TCACCGGTTGGACGAGAC
qTPS2-F  CCACCAGTGGCCAAGACAAAT
qTPS2-R  CAGGTGCCTCGTTCTTCT
qTPS3-F  TGCTCAGTCTGCTAGACTTCT
qTPS3-R  GGATCGACATCTGGAACGCT
qUBC6-F[48]  GGACCTGCGGATACTCCTTAC
qUBC6-R[48]  TAATCGTGTGGGCTTGA
Table 3. Activity of enzymes related to UDPG pathway and glycerol metabolism

<table>
<thead>
<tr>
<th></th>
<th>BY14a+K</th>
<th>B+MAL62</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aHexokinase (U/mg pro)</strong></td>
<td>1.19±0.09</td>
<td>1.30±0.11</td>
</tr>
<tr>
<td><strong>aPhosphoglucomutase (U/mg pro)</strong></td>
<td>0.14±0.01</td>
<td>0.31±0.03*</td>
</tr>
<tr>
<td><strong>aUGPase (U/mg pro)</strong></td>
<td>0.43±0.06</td>
<td>1.10±0.08*</td>
</tr>
<tr>
<td><strong>aTps1 (U/g CDW)</strong></td>
<td>0.79±0.07</td>
<td>1.20±0.05*</td>
</tr>
<tr>
<td><strong>bGlycerol-3-phosphate dehydrogenase</strong></td>
<td>1.5±0.07</td>
<td>2.83±0.21*</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SD from three independent experiments.*

*P < 0.05 and **P < 0.01 in comparison with the parent strain.

*a Enzyme activities were measured using cells from cane molasses medium.

*b Activities of glycerol-3-phosphate dehydrogenase were measured using cells from LSMLD medium.

CDW: cell dry weight

**Figures**
Figure 1

Results of RNA-Seq transcriptome analysis. (A) Hierarchical clustering of the significant genes (B indicates BY14a+K, B+M indicates B+MAL62). (B) Volcano plot of all genes. The downregulated genes are shown in green and upregulated genes are shown in red. (C) Kyoto Encyclopedia of Genes and Genomes analyses. (D) Gene ontology functional enrichment analyses. The functions are arranged from deep to shallow according to their relevance.
Figure 2

Interactions between the upregulated proteins. (A) Protein-protein interaction networks. (B) Significant modules in the protein-protein interaction network.
Figure 3

Quantitative RT-PCR analysis of the relative expression levels of genes in the recombinant strain B+MAL62 and the control strain BY14a+K (A) as well as in the recombinant strains B-T and B-T+M and the control strain BY14a (B). Data are expressed as mean ± SD (indicated as error bars) of three independent experiments.
Figure 4

Measurement of alpha-glucosidase (maltase) activity, concentration of residual maltose and the content of intracellular trehalose in four yeast strains cultured in LSMLD medium. (A), (B), (C) and (D) stand for BY14a+K, B+MAL62, B-T and B-T+M, respectively.
Figure 5

Contents of intracellular glycerol and trehalose (A) and cell viability (B) after different fermentation times (0–25 min) and freezing in LSMLD medium for 7 days. Measurement of yeast CO2 production in the lean dough after different periods of freezing (0–4 weeks) (C). Data are expressed as mean ± SD (indicated as error bars) of three independent experiments.
Figure 6

Proposed regulatory mechanism of MAL62 overexpression in yeast. MAL62 overexpression induces the UDPG-dependent trehalose synthesis pathway and the HOG-MAPK-dependent glycerol synthesis pathway.

Supplementary Files

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