Enhancement of sphingolipids synthesis improves osmotic tolerance of *Saccharomyces cerevisiae*

Running title: Complex sphingolipids regulate osmotic tolerance of *Saccharomyces cerevisiae*

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ABSTRACT

To enhance the growth performance of _Saccharomyces cerevisiae_ under osmotic stress, mutant XCG001, which tolerates up to 1.5 M NaCl, was isolated through adaptive laboratory evolution (ALE). Comparisons of the transcriptome data of mutant XCG001 and the wild-type strain identified _ELO2_ as being associated with osmotic tolerance. In the _ELO2_ overexpression strain (XCG010), the contents of inositol phosphorylceramide (IPC, t18:0/26:0), mannosylinositol phosphorylceramide (MIPC, t18:0/22:0(2OH)), MIPC (d18:0/22:0), MIPC (d20:0/24:0), mannosyldiinositol phosphorylceramide (M(IP)_2C, d20:0/26:0), M(IP)_2C (t18:0/26:0(2OH)), and M(IP)_2C (d20:0/26:0(2OH)) increased by 88.3 times, 167 times, 63.3 times, 23.9 times, 27.9 times, 114 times, and 208 times at 1.0 M NaCl, respectively, compared with the corresponding values of the control strain XCG002. As a result, the membrane integrity, cell growth, and cell survival rate of strain XCG010 increased by 24.4% ± 1.0%, 21.9% ± 1.5%, and 22.1% ± 1.1% at 1.0 M NaCl, respectively, compared with the corresponding values of the control strain XCG002 (wild-type strain with a control plasmid). These findings provided a novel strategy for engineering complex sphingolipids to enhance osmotic tolerance.

IMPORTANCE

This study demonstrated a novel strategy for manipulation membrane complex sphingolipids to enhance _S. cerevisiae_ tolerance to osmotic stress. _Elo2_, a sphingolipid acyl chain elongase, was
related to osmotic tolerance, through transcriptome analysis of the wild-type strain and an osmotic tolerant strain generated from ALE. Overexpression of *ELO2* increased the content of complex sphingolipid with longer acyl chain, thus membrane integrity and osmotic tolerance improved.

**KEYWORDS:** Adaptive laboratory evolution; osmotic tolerance; membrane engineering; complex sphingolipid; membrane integrity

**INTRODUCTION**

The growth performance (cell density and growth rate) of industrial strains is a key factor affecting the efficiency of the fermentation process (1). During industrial fermentation, the cell density and growth rate declines when industrial strains are subjected to harsh environmental conditions, including osmotic, pH, and oxidation stresses, which can cause an adverse biological and physiological response to the industrial strains (2, 3). The cell membrane is a natural barrier separating the extracellular environment from the intracellular components (4). Therefore, improving membrane function is a potential strategy to enhance the growth performance of industrial strains under harsh industrial conditions (5-7).

The manipulation of membrane lipids, such as phospholipids, sphingolipids, and sterols, is a primary and efficient strategy to enhance membrane function (8). Based on the structure of the phospholipids, one engineering strategy is to modulate the phospholipid head groups by altering
the expression of the key phospholipid biosynthesis enzymes (9). For instance, when pssA, a phosphatidylserine synthase, was overexpressed, PE content increased and membrane integrity enhanced, as a result, bio-renewable fuel tolerance and titer were improved (9). Another strategy is to regulate the phospholipid fatty acid tails by changing the fatty acid length, increasing the ratio of saturated to unsaturated fatty acids, and producing trans unsaturated fatty acids (tufa) (10-12). For example, through the expression of cis-trans isomerase (Cti) from Pseudomonas aeruginosa, the tufa was incorporated into the E. coli membrane, decreasing membrane fluidity, as a result, robustness and the bio-renewable fuel titer were improved (13). The content and composition of sterols can be changed by engineering the key enzymes associated with sterol biosynthesis or by changing the transcription level of the sterol biosynthesis enzymes, which are affected by global transcription factors, such as Upc2 and Ecm22 (14). For example, the expression of a key sterol C-5 desaturase FvC5SD from an edible mushroom in fission yeast improved the contents of ergosterol and oleic acid, which resulted in enhanced tolerance to ethanol and high temperature (15). Sphingolipids, which are signaling molecules, modulate cellular functions and fate, including cell division, cell death, lifespan, and autophagy (11, 16). Also, sphingolipids in the plasma membrane can help cells tolerate stress by manipulating the target of Rapamycin Complex1 (TORC1), the sphingosine backbone, and the acyl chain (17, 18). For example, when mouse sphingomyelin synthase 1 (Sms1) was expressed in yeast, endogenous sphingolipids got accumulated, as a result of which tolerance of the strain to oxidation, osmotic,
and temperature stresses improved (17). Some attempts had been made to change sphingolipid content by metabolic engineering or the simulation of molecular dynamics (18-20). An increase in sphingolipids with very long fatty acyl chains in Zygosaccharomyces bailii made the membrane thicker and denser, which increased the free energy barrier for the permeation of acetic acid through the membrane and improved acetic acid resistance (18). These findings highlight the importance of developing novel strategies to improve stress resistance by engineering complex sphingolipid.

In this study, a mutant, XCG001, was obtained through adaptive laboratory evolution (ALE), and RNA-seq analysis suggested that the mRNA level of ELO2, which is involved in the biosynthesis of very long fatty acids, was differentially up-regulated in mutant XCG001. Then, ELO2 was overexpressed through metabolic engineering and changed contents of fatty acids, phospholipids, and complex sphingolipids, leading to the improvement of cell membrane integrity, as a result of which, the osmotic tolerance increased.

RESULTS

Global transcriptome analysis of the mutant XCG001 and the wild-type strain at 0 M and 1.5 M NaCl

To understand how S. cerevisiae adapts to higher osmotic stress, ALE was utilized to generate osmosis-tolerant mutants. As shown in Fig. 1A, the concentration of NaCl was increased with
time in a stepwise fashion, to reach 1.5 M. After 300 generations of ALE, a clone (mutant XCG001) was isolated from the evolved population. The osmotic sensitivity of the wild-type strain and mutant XCG001 was tested, the IC$_{50}$ value of the wild-type strain and mutant XCG001 were 0.9 M and 1.4 M NaCl, respectively (Fig. 1B). At 0 M NaCl, the final biomass of mutant XCG001 was similar to that of the wild-type strain, whereas at 1.5 M NaCl, the final biomass of mutant XCG001 increased by 37.3% ± 1.5%, compared with that of the wild-type strain (Fig. 1C and D).

To identify the differentially regulated genes contributing to osmotic tolerance in mutant XCG001, transcriptome sequencing (RNA-seq) was conducted to compare global gene expression in mutant XCG001 and the wild-type strain at 0 M and 1.5 M NaCl. The restrictive thresholds [log$_2$(fold change) ≥1.5; false-discovery rate (FDR), <0.05] of significantly expressed genes were used to screen the genes. First, the differentially expressed genes were analyzed at 1.5 M NaCl relative to 0 M NaCl in both the wild-type strain and mutant XCG001 (Fig. 1F). Transcriptional profiling analysis revealed that the expression levels of 1169 genes were significantly changed in the wild-type strain, where 664 genes were up-regulated and 505 genes were down-regulated. In mutant XCG001, the expression levels of 609 genes displayed differential expression, where 416 genes were up-regulated and 193 genes were down-regulated.

Additionally, 180 up-regulated and 68 down-regulated genes were common to both strains. Gene Ontology (GO) analysis indicated that the commonly up-regulated genes were involved in
glycolysis/gluconeogenesis, pyruvate metabolism, lipid metabolism, signaling transduction, fructose and mannose metabolism. On the other hand, 68 down-regulated genes were involved in the ribosome and amino acid metabolism (supplemental Data Sets S1 and S2).

Then, the significantly expressed genes in mutant XCG001, relative to those in the wild-type strain, were analyzed at both 0 M and 1.5 M NaCl (Fig. 1G). At 0 M NaCl, the expression levels of 212 genes were up-regulated and 271 genes were down-regulated. At 1.5 M NaCl, 131 genes were up-regulated and 235 genes were down-regulated. These 131 up-regulated genes include 13 genes that were commonly up-regulated at 0 M and 1.5 M NaCl, and 118 genes were only significantly up-regulated at 1.5 M NaCl. Based on the GO analysis, these 118 genes were involved in the steroid biosynthesis process, pentose-phosphate shunt, translation, regulation of transcription, phosphate ion transport and response to stress. Moreover, 13 commonly up-regulated genes were involved in transport, pyrimidine metabolism and lipid metabolism, whereas 28 commonly down-regulated genes were involved in pyruvate metabolism and transport (Supplemental Data Sets S3 and S4). These results suggested that mutant XCG001 strengthened transport, pyrimidine metabolism and lipid metabolism, which contribute to osmotic tolerance.

Overexpression of ELO2 enhanced osmotic tolerance

The mRNA levels of the 13 commonly up-regulated genes were tested at 0 M, 1.0 M, and 1.5 M
NaCl using quantitative reverse transcription-PCR (qRT-PCR) analysis (Table S1). At 0 M NaCl, mRNA levels of FET4, ADH6, PHO89, EGT2, SAH1, ELO2, HXT4, SKG6, URA1, HXK2, YBL111C, RNR1, and SRL1 increased by 4.8-, 2.7-, 2.6-, 2.2-, 1.9-, 1.8-, 1.9-, 1.7-, 1.6-, 6.0-, 1.5-, and 1.5-fold, respectively, compared with the corresponding values of the wild-type strain. At 1.0 M NaCl, mRNA levels of FET4, ADH6, PHO89, EGT2, SAH1, ELO2, HXT4, SKG6, URA1, HXK2, and SRL1 increased by 2.6-, 1.6-, 3.5-, 1.1-, 1.9-, 2.1-, 1.7-, 1.8-, 1.3-, 2.3-, and 1.6-fold, respectively, whereas YBL111C and RNR1 decreased by 1.2- and 1.0-fold, respectively. Down-regulated YBL111C and RNR1 may decrease DNA replication for a high level of transcription and translation as a response to osmotic stress (21). At 1.5 M NaCl, mRNA levels of FET4, ADH6, PHO89, EGT2, SAH1, ELO2, HXT4, SKG6, URA1, HXK2, YBL111C, RNR1, and SRL1 increased by 3.6-, 1.5-, 3.0-, 2.3-, 1.8-, 1.8-, 2.6-, 1.5-, 2.9-, 1.6-, 1.6-, 3.1-, and 2.5-fold, respectively. Furthermore, these genes were overexpressed in each strain, and consequence on resistance to osmotic stress was evaluated (Fig. S2). Interestingly, only the overexpression of ELO2 conferred resistance to osmotic stress. To confirm whether the expression of ELO2 was positively correlated with osmotic tolerance, ELO2 was overexpressed with two other constitutive promoters, P_{TDH3} and P_{ADH1} (promoter activity of P_{TDH3} is weaker than that of P_{TEF1}, but stronger than that of P_{ADH1} (22)). The spot results showed no obvious difference among the P_{ADH1–ELO2} (XCG016) strain, P_{TDH3–ELO2} (XCG017) strain, and P_{TEF1–ELO2} (XCG010) strain at 1.0 M NaCl (Fig. 2A), and the IC_{50} values of strains XCG016, XCG017, and XCG010 were...
also equal (Fig. 2B). The growth curves of these four strains were different (Fig. 2C and D): at 0 M NaCl, the final biomass of strains XCG016, XCG017, and XCG010 were similar to that of the control strain XCG002 (wild-type strain with a control plasmid pY13), whereas at 1.0 M NaCl, the final biomass of strains XCG016, XCG017, and XCG010 improved by 19.1% ± 0.7%, 20.8% ± 1.3%, and 21.9% ± 1.5%, respectively, compared with the corresponding value of the control strain XCG002 (Fig. 2C and D). In addition, the survival rates were generated for the four strains over a broad concentration range of NaCl (Fig. 2E). At 1.0 M NaCl, the survival rate of the control strain XCG002 showed 59.5% ± 1.1%, while the survival rates of strains XCG016, XCG017, and XCG010 were 70.2% ± 1.2%, 71.9% ± 1.5%, and 72.6% ± 1.8%, indicating approximate increases of 18.0% ± 0.9%, 20.8% ± 0.6%, and 22.1% ± 1.1%, respectively. These results suggested that the overexpression of ELO2 enhanced the osmotic tolerance of S. cerevisiae.

**Overexpression of ELO2 enhanced very long fatty acid contents**

The fatty acid contents of the strains XCG016, XCG017, XCG010, and XCG002 were analyzed using gas chromatography. It was found that the contents of membrane fatty acids in strains XCG016, XCG017, and XCG010 were altered, especially C22:0 (Fig. 3A and B). At 0 M NaCl, the contents of C20:0, C22:0, and C24:0 in strain XCG010, increased by 52.3%, 94.1%, and 14.4%, respectively, compared with the corresponding values of the control strain XCG002.
whereas the contents of C16:0, C16:1, C18:0, and C18:1 remained the same. At 1.0 M NaCl, the contents of C20:0, C22:0, and C24:0 in strain XCG010 increased by 33.1%, 106.4%, and 31.5%, respectively, while the contents of C16:0, C16:1, C18:0, and C18:1 remained unchanged. All the fatty acid contents in strains XCG016 and XCG017 were similar to those in strain XCG010 at 0 M or 1.0 M NaCl (Fig. 3A and B).

The average fatty acid length in strains XCG016, XCG017, and XCG010 was equal to that of the control strain XCG002 at 0 M or 1.0 M NaCl, suggesting that the membrane “thickness” was not affected (Fig. 3C). The reason for unchanged membrane “thickness” may be that the proportion of C20:0 and C22:0 contents to the total fatty acid contents was only approximately 0.5%. In addition, the fatty acid unsaturation/saturation ratio did not increase in strains XCG016, XCG017, or XCG010 at 0 M or 1.0 M NaCl (Fig. 3D).

**Overexpression of ELO2 altered complex sphingolipid contents**

The effect of overexpression of ELO2 on the contents of phospholipids and complex sphingolipids in strain XCG010 was analyzed (Fig. 4A-G). It was found that overexpression of ELO2 can change contents of the phospholipid (Fig. 4A-F) and complex sphingolipid (Fig. 4G). At 0 M NaCl, the content of phosphatidic acid (PA) decreased by 15.2%, but the contents of phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphocholine (PC), phosphatidylserine (PS), and phosphatidylglycerol (PG) in strain XCG010 remained unchanged.
At 1.0 M NaCl, the contents of PE and PS in strain XCG010 increased by 18.9% and 15.0%, respectively, but the contents of PI, PA, and PC remained unchanged, and the content of PG decreased by 40.0%, compared with that of the control strain XCG002. At 0 M NaCl, the contents of IPC (t18:0/26:0), MIPC (t18:0/22:0(2OH)), MIPC (t20:0/22:0(2OH)), MIPC (d18:0/22:0), and MIPC (d18:0/26:0) in strain XCG010 increased 4868.9%, 4552.7%, 3111.2%, 5500.4%, and 4079.1%, respectively, whereas the contents of MIPC (t20:0/26:0), MIPC (t18:0/20:0(2OH)), MIPC (d18:0/24:0), and M(IP)2C (d18:0/22:0(2OH)) in strain XCG010 decreased by 96.5%, 99.1%, 97.3%, and 99.7%, respectively, compared with the corresponding values of the control strain XCG002. At 1.0 M NaCl, the contents of IPC (t18:0/26:0), MIPC (t18:0/22:0(2OH)), MIPC (d18:0/22:0), MIPC (d20:0/24:0), M(IP)2C (d18:0/26:0(2OH)), M(IP)2C (t18:0/26:0(2OH)), and M(IP)2C (d20:0/26:0(2OH)) in strain XCG010 increased by 8833.4%, 16689.4%, 6329.2%, 2391.1%, 2792.8%, 11376.4%, and 20806.3%, respectively, whereas the contents of IPC (d18:1/22:0), MIPC (t16:0/18:0), MIPC (t16:0/18:0(2OH)), MIPC (t16:0/20:0(2OH)), MIPC (t18:0/20:0(2OH)), and MIPC (d20:0/26:0) in strain XCG010 decreased by 96.7%, 96.3%, 99.7%, 95.7%, 99.3%, and 88.0%, respectively, compared with the corresponding values of the control strain XCG002. These results suggested that a high level of complex sphingolipids with longer acyl chains may enhance osmotic tolerance.
To validate whether the increase in complex sphingolipid contents enhanced osmotic tolerance, the genetic details of strain XCG010 were investigated. The mRNA expression level of the complex sphingolipid biosynthesis genes in strains XCG010 and XCG002 was compared at 0 M and 1.0 M NaCl. At 0 M NaCl, the mRNA levels of AUR1, CSG2, IPT1, LAG1, and LAC1 in strain XCG010 increased by 1.4 ± 0.12-, 1.7 ± 0.13-, 1.3 ± 0.08-, 1.5 ± 0.15-, and 1.8 ± 0.17-fold, respectively, compared to the corresponding values of the control strain XCG002 (Fig. 5A). At 1.0 M NaCl, the mRNA levels of AUR1, CSG2, IPT1, LAG1, and LAC1 in strain XCG010 increased by 1.5 ± 0.12-, 2.8 ± 0.13-, 1.5 ± 0.11-, 2.1 ± 0.10-, and 2.5 ± 0.24-fold, respectively, compared to the corresponding values of the control strain XCG002 (Fig. 5B). These results are consistent with the high content of complex sphingolipids in strain XCG010. However, the mRNA level of the complex sphingolipid biosynthesis genes in strain XCG010 was different from the corresponding values in mutant XCG001 (Table S2). The reason for this may be that the comparison objects (strain XCG010 to XCG002; mutant XCG001 to the wild-type strain) and conditions (under 1.0 M NaCl and 1.5 M NaCl) were different.

To evaluate whether the inhibition of complex sphingolipid biosynthesis affects the growth of strain XCG010, LAC1, which is involved in the synthesis of ceramide, was deleted to generate strain XCG018. The content of the complex sphingolipid in strain XCG018 was tested. At 0 M NaCl, the contents of IPC (t18:0/26:0), MIPC (t18:0/22:0(2OH)), MIPC (t20:0/22:0(2OH)),

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MIPC (d18:0/22:0), and MIPC (d18:0/26:0) in strain XCG018 increased by 3921.3%, 4029.7%, 2392.7%, 3512.4%, and 4411.3%, respectively, compared with the corresponding values of the control strain XCG002. At 1.0 M NaCl, the contents of IPC (t18:0/26:0), MIPC (t18:0/22:0(2OH)), MIPC (d18:0/22:0), M(IP)_2C (d20:0/26:0), M(IP)_2C (t18:0/26:0(2OH)), and M(IP)_2C (d20:0/26:0(2OH)) in strain XCG018 increased by 4758.7%, 8854.8%, 3811.2%, 2598.4%, 1284.3%, and 8745.1%, respectively, compared with the corresponding values of the control strain XCG002 (Fig. 5C). The spot results indicated that at 1.0 M NaCl, the growth of strain XCG018 was better than that of control strain XCG002, but worse than that of strain XCG010 (Fig. 5D). Moreover, at 1.0 M NaCl the survival rate of strain XCG018 (65.6% ± 2.1%) increased by 10.2% ± 0.2%, compared with that of the control strain XCG002 (59.5% ± 1.1%) (Fig. 5E). These results suggested that an increase in complex sphingolipids is crucial for \textit{S. cerevisiae} osmotic tolerance.

Increased content of complex sphingolipid improved membrane integrity

To investigate the effect of complex sphingolipids on membrane integrity, strains XCG002, XCG010, and XCG018 were treated with 0 M or 1.0 M NaCl for 4 hours and subjected to SYTOX green and FM4-64 uptake analysis. As illustrated in Fig. 6, at 0 M NaCl, fluorescent microscope showed that almost all the cells of strains XCG002, XCG010, and XCG018 exhibited an integral membrane (Fig. 6A), whereas at 1.0 M NaCl, the number of cells with an
integral membrane for strain XCG018 was more than that for the control strain XCG002, but less than that for strain XCG010 (Fig. 6B). These cells of strains XCG002, XCG010, and XCG018 were further analyzed using flow cytometry. At 0 M NaCl, the percentage of cells with an integral membrane of strains XCG010 and XCG018 was similar to that of the control strain XCG002, whereas at 1.0 M NaCl, the percentage of cells with an integral membrane for strain XCG010 (85.7% ± 3.2%) and XCG018 (76.6% ± 2.9%) increased by 24.4% ± 1.0% and 11.2% ± 0.4%, respectively, compared with that of the control strain XCG002 (68.9% ± 2.9%). These results suggested that an increase in complex sphingolipid content improved membrane integrity.

**DISCUSSION**

*S. cerevisiae* is a well-established microorganism that is widely used for the industrial production of fine chemicals, such as organic acids and amino acids, which cause the low pH of the fermentation broth (1). To modulate a suitable pH for a medium, some alkaline reagents need to be added, which leads to osmotic stress (23). To elucidate the physiological mechanism of the cell membrane in osmotic resistance, RNA-seq analysis of an osmosis-tolerant mutant XCG001 that was obtained through ALE, found *ELO2* was associated with osmotic tolerance. Overexpression of *ELO2* enhanced the content of complex sphingolipids. The increased lipid content, as mentioned above, contributed to an improvement of membrane integrity, and as a result, osmotic resistance increased.
ALE is a very efficient way to improve the phenotype of industrial strain (24). For example, ALE has been used to increase the specific growth rate for the deletion of some genes in *S. cerevisiae* or genome-reduced *E. coli* with glucose as the energy source (25, 26), or enhance *Schizochytrium* sp. tolerance to high salinity stress (27). After securing the ALE strains, an important objective was to further identify the targets for genetic modification. Three omics tools were applied for this purpose, as follows: genomics, transcriptome, and metabolomics (25, 27, 28). For example, the growth of *S. cerevisiae* on glycerol was increased via ALE, and the transcriptome data revealed that the genes that were related to the tricarboxylic acid cycle and oxidative phosphorylation, contributed to the increased growth (28). In this study, the evolutionary effect on the expression of the osmotic stress related genes was divided into two aspects: (i) up-regulated pathways: glycerol metabolism and ion transport were up-regulated in mutant XCG001 1.5 M NaCl, such as, the expression levels of encoding glycerol-3-phosphate dehydrogenase (*GPD1*) and Na⁺-exporting P-type ATPase (*ENA1*) were up-regulated 1.3- and 0.9-fold in mutant XCG001, respectively, compared with the corresponding values of the wild-type strain at 1.5 M NaCl; (ii) without influence pathway: trehalose metabolism was no influence in mutant XCG001 1.5 M NaCl, such as, the expression level of encoding trehalose-phosphatase (*TPS2*) was up-regulated 0.1-fold (Table S3). Glycerol as a major osmolyte can improve intracellular osmotic pressure, as a result, osmotic tolerance is improved (29). And, Na⁺-exporting P-type ATPase is one of the most important ion transports that could
pump Na⁺ out of the cell to maintain osmotic pressure (30). However, trehalose synthesis was also unchanged in a yeast-like fungus under osmotic stress (31). Therefore, up-regulated glycerol metabolism and ion transport may account for part of the increased osmotic resistance of mutant XCG001. Moreover, RNA-seq analysis of mutant XCG001 and the wild-type strain suggested that ELO2 of 13 commonly up-regulated genes was associated with osmotic tolerance. Elo2 is a fatty acid elongase that catalyzes C16-carbon fatty acids to C22, and its mutations have regulatory effects on 1,3-beta-glucan synthase, vacuolar ATPase, and the secretory pathway (32). ELO2 overexpression may be caused by the changes of the sequence of ELO2 promoter and transcription factors (TFs), due to the evolution under osmotic condition. ELO2 overexpression could be controlled by down-regulated negative TFs or up-regulated positive TFs. For example, when the expression of YY1 (a negative TF of ELO2) decreased due to the change of YY1 sequence, less YY1 can bind to the promoter of ELO2, which could lead to ELO2 overexpression (33).

The overexpression of ELO2 changed the lipid composition, including that of fatty acids, phospholipids, and complex sphingolipids. Sphingolipids play an important role in physiological functions by regulating cell growth and responding to environmental stress (34). The effect of sphingolipid synthesis on environmental stress can be divided into two aspects: (i) the overexpression or knockout of the sphingolipid synthesis gene (17, 35, 36), for example, when SUR1 was deleted in S. cerevisiae, mutant sur1 was sensitive to Ca²⁺ (35); (ii) the addition of a
certain sphingolipid, for instance, the addition of phytosphingosine or glucosylceramide or sphingolipid long-chain bases enhanced tolerance to environmental stress (34, 37, 38). Furthermore, lipid composition and content may undergo changes because of the metabolic pathway genes, harsh environmental conditions, and transcription factors (11, 39). Manipulation of lipid biosynthesis genes can change lipid content. ELO3, an ELO2 paralog, is related with biosynthesis of sphingolipid with C26 acyl chain (32). Environmental or chemical stresses affect lipid metabolism, which plays a role in maintaining membrane homeostasis and cell growth, a case in point is the membrane unsaturated fatty acids to saturated fatty acids ratio being increased under high-pressure homogenization stress, which enables the strain to avoid damage (39). Transcription factors, such as Mga2 that enable changes in the expression of lipid biosynthesis genes may change the lipid composition indirectly (40).

In this study, enhancement of the complex sphingolipid content increased the membrane integrity and osmotic tolerance of S. cerevisiae. Membrane integrity could be enhanced by engineering membrane components including: (i) transporter proteins: for example, when sugar and ion transporter, OmpF, was deleted, and the long chain fatty acids transporter, FadL, was overexpressed in E. coli, the membrane integrity was enhanced and the fatty acid titer improved (41); (ii) phospholipids: membrane integrity can also be altered by modifying the distribution of phospholipid head groups, by adjusting phospholipid saturation, and by altering the phospholipid acyl chain length (11); (iii) sterols: sterols can modulate the membrane integrity to resist stress.
sphingolipids: when the sphingolipid biosynthesis genes were deleted in *S. cerevisiae*, the resultant strains exhibited resistance to amphipathic peptidomimetic, which decreased membrane integrity (43). The increased complex sphingolipids may change the raft structure to help osmotic tolerance in three ways: lipid–lipid interaction, lipid–protein interaction, and membrane fusion. Lipid–lipid interaction requires sterols and sphingolipids acting as functional pairs to help nanodomain formation on the membrane, and as a result, the membrane stability increases (11). Lipid–protein interaction could help enhance the function and stability of GPI-anchored and transmembrane domains of proteins (44). Due to an intrinsic property of the very long acyl chain of sphingolipids, membrane fusion may get stimulated (45). Furthermore, Na⁺ may bind to sphingolipids to enhance osmotic tolerance through the calcium channel being activated directly or indirectly. The Ca²⁺ influx channel is directly activated by Na⁺ binding to sphingolipids, which activates Ca²⁺-binding proteins and upregulates the Na⁺/H⁺ antiporter to pump Na⁺ out of the cell (46). The calcium channel is activated indirectly through Na⁺ binding to sphingolipids, to drive the formation of a microdomain on the membrane, which triggers the calcium signals and leads to osmosis-tolerant responses (47).

In summary, ALE was used to obtain an osmotic tolerant strain, XCG001, where RNA-seq analysis of mutant XCG001 and the wild-type strain was used to identify a key gene, *ELO2*, associated with osmotic tolerance. Furthermore, overexpression of *ELO2* increased the content of complex sphingolipids with longer acyl chains. As a result, membrane integrity increased, and
the osmotic resistance enhanced. This study provides a novel strategy to manipulate membrane complex sphingolipids to increase membrane integrity and osmotic tolerance.

MATERIALS and METHODS

Strains and Media

All S. cerevisiae strains and plasmids used in this study are listed in Table 1. Plasmids pY131 and pY132 were constructed by replacing the promoter P_{TEF} of pY13 plasmid with P_{ADH1} and P_{TDH3}, respectively. Overexpression strains were constructed using pY13, pY131 and pY132 plasmids carrying the target genes. All plasmids were transformed into yeast cells using the lithium acetate transformation method (48). Homologous recombination was used for gene lac1 deletion. The LEU2 marker, the upstream and downstream regions of the target gene open reading frame were fused by fusion-PCR, and the PCR products were transformed into yeast cells using the lithium acetate transformation method. All primers used in this study are listed in Table 2. Yeast was cultivated in yeast extract peptone dextrose (YPD) medium and yeast nitrogen base (YNB) medium at 30°C with shaking at 200 rpm.

Adaptive laboratory evolution

S. cerevisiae BY4741 was cultivated in 25 ml of YNB medium with histidine, leucine, methionine uracil and the increasing salt concentrations in a 100-ml flask (0.5 M NaCl, 0.75 M NaCl, 1.0 M NaCl, 1.5 M NaCl).
When optical density at 600 (OD\(_{600}\)) reached around 4, the strain was transferred to a new salt medium with an initial OD\(_{600}\) of approximately 0.1. The concentration of salt was increased when the maximum specific growth rate reached around 0.3.

**Spot assay**

Yeast cells were cultivated in the logarithmic (log) phase and diluted to OD\(_{600}\) of 1.0. Aliquots (4 μl) of 10-fold serial dilutions were spotted onto YNB agar plates with or without the indicated concentration of NaCl. Growth was assessed after incubation for 2 to 4 days at 30°C.

**IC\(_{50}\), Growth curve and Survival rate**

Maximum exponential growth rates of yeast in YNB supplemented with increasing salt concentrations. The half-maximal inhibitory concentration (IC\(_{50}\)) was calculated by fitting a Hill-type model to the data. Data points and error bars represent mean and s.d. of three biological replicates. To test the growth curve of *S. cerevisiae* at different concentrations of NaCl, cells were cultivated in the log phase and diluted to a fresh YNB medium with OD\(_{600}\) of 0.1 at different concentrations of NaCl. The OD\(_{600}\) values were recorded through taking curves at regular time intervals. Cells survival rates were assessed by log-phase cells were treated with various concentrations of NaCl for 1 h at 30°C with shaking at 200 rpm. Next, cells were diluted and plated on YNB agar plates with various concentrations of NaCl. After incubation for 2 to 4
days at 30°C, the surviving colonies were counted. The survival rates are expressed relative to that of untreated cells of the corresponding strain. The treatment level of NaCl was chosen according to the standard that the cell density of strain growing to the stationary phase is similar to that of wild-type strain at 0 NaCl. At 1.5 M NaCl, mutant XCG001 can grow well and its cell density of stationary phase was close to that of wild-type strain at 0 NaCl (Fig. 1D). At 1.0 M NaCl, mutant XCG010 can grow well and its cell density of stationary phase was close to that of wild-type strain at 0 M NaCl (Fig. 2D). At 1.5 M NaCl, the cell density of the stationary phase in mutant XCG010 decreased by 12.7%, compared with that of the wild-type strain at 0 M NaCl (Fig. S3). Therefore, mutant XCG001 was treated with 1.5 M NaCl, mutants XCG010 was treated with 1.0 M NaCl.

**Transcriptome analysis**

The wild type strain and mutant XCG001 were cultured in the log phase at 0 M and 1.5 M NaCl. The collected strains were frozen at -80°C and sent to the Genewiz Institute for RNA extraction and global gene analysis.

**qRT-PCR analysis.**

Total RNA was extracted using MiniBEST universal RNA extraction kit and 1μg was taken to synthesize cDNA using the PrimeScript II 1st-strand cDNA synthesis kit (TaKaRa, Japan). The cDNA mixture was diluted to about 100 ng/μl and used as the template for the gene expression
level analysis by qRT-PCR. qRT-PCR was performed with TB Green Premix Ex Taq (TaKaRa Bio) using an iQ5 continuous fluorescence detector system (Bio-Rad, Hercules, CA). Data were normalized to that of β-actin gene ACT1. The primer sequences for qRT-PCR are listed in Table 2.

**Fatty acids analysis**

Fatty acids of yeast were extracted using NaOH-methanol-distilled water solution (3:10:10, wt/vol/vol) and freeze-dried. Then the dried sample was treated with 2 ml boron trifluoride (BF₃)-methanol (12:88, vol/vol) and produce fatty acid methyl esters, as described previously (49). Last, samples were analyzed by gas chromatography (GC) with a polyethylene glycol capillary column eluted at a flow rate of 29.6 ml/min and the column pressure of 63.4 kPa. Data analysis was based on the following standard Supelco37 (sigam, USA, CAS:47885-U). Fatty acid was ensured by the order of Supelco 37 retention time and the weight percentage of each fatty acid accounts for Supelco 37 (Fig. S4).

**Phospholipid measure**

Phospholipids were extracted from the freeze-dried samples using chloroform-methanol as described previously (50). Dried phospholipids were obtained under a nitrogen stream and reconstituted in chloroform-methanol (1:1, vol/vol). Samples were analyzed by ultrahigh-performance liquid chromatography-tandem mass spectrometry (UPLC-MS; Waters,
USA) and a CORTECS UPLC hydrophilic interaction liquid chromatography (HILIC) column (2.1 by 150 mm; inner diameter, 1.6-μm) with gradient elution at 45°C and a rate of infusion of 0.3 ml·min⁻¹.

**Complex sphingolipid measure**

Strains were cultured in YNB medium with or without 1.0 M NaCl for 6-8 h and washed with PBS. The cell pellets were lysed in PBS by bead-beating mechanical disruption at 4°C. The supernatants were then extracted with chloroform-methanol (2:1, vol/vol) at a final ratio of 20% (v/v). Centrifuge using a refrigerated centrifuge at 4°C to obtain the supernatant. The extracts were evaporated to dryness under nitrogen at room temperature and stored at −80°C. The dried samples were sent to the Profleader Institute for complex sphingolipids analysis and solubilized in dichloromethane-methanol (2:1, vol/vol) before analysis by UHPLC-QTOF-MS (Agilent) analysis (Fig. S5).

**Cell membrane integrity analysis**

Cell membrane integrity was analyzed by microscope and flow cytometry. For microscope analysis of cell membrane integrity, the log phase cells were treated with 0 M and 1.0 M NaCl for 4 h and washed with PBS two twice. Then, samples were subjected to SYTOX green and FM4-64 uptake for 20 mins and placed on a microscope slide, covered with a coverslip (51-53). Images were acquired using a Nikon ECLIPSE 80i microscope equipped with a Nikon DS-Ri1.
camera. For flow cytometry of cell membrane integrity, 10,000 counts of stained cells were recorded using a 0.5 mL S⁻¹ flow rate. All data were exported in FCS3 format and processed using FlowJo software (FlowJo, LLC).

Statistical analysis.

Experimental data are shown as the means ± standard errors of the means (SEM). All quantitative data were analyzed using Student’s t-test or one-way analysis of variance (ANOVA). Each experiment was repeated at least three times.

Accession number(s).

The RNA-seq raw reads were submitted to NCBI under BioProject number PRJNA568205, and the Sequence Read Archive (SRA) entries are SRR10150286, SRR10150285, SRR10150284, SRR10150283. The RNA-Seq data are available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA568205.

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G.Z, L.L and J.W designed the research; G.Z and N.Y performed the research; G.Z, Q.L, J.L, X.C and J.W analyzed the research; and G.Z and L.L wrote the paper.

We declare no competing financial interests.

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Table 1. Strains used in this study

| Strain        | Relevant characteristic | Ref               |
|---------------|-------------------------|-------------------|
| BY4741        | Mata hisΔ1 leu2Δ0 met15Δ0 uraΔ0 | This study       |
| XCG001        | ALE mutant from BY4741 tolerates 1.5 M NaCl | This study       |
| XCG002        | BY4741 harboring pY13 | This study       |
| XCG003        | BY4741 harboring pY13-FET4 | This study       |
| XCG004        | BY4741 harboring pY13-ADH6 | This study       |
| XCG005        | BY4741 harboring pY13-PHO89 | This study       |
| XCG006        | BY4741 harboring pY13-EGT2 | This study       |
| XCG007        | BY4741 harboring pY13-SAH1 | This study       |
| XCG008        | BY4741 harboring pY13-HXT4 | This study       |
| XCG009        | BY4741 harboring pY13-SKG6 | This study       |
| XCG010        | BY4741 harboring pY13-ELO2 | This study       |
| XCG011        | BY4741 harboring pY13-URA1 | This study       |
| XCG012        | BY4741 harboring pY13-HXK2 | This study       |
| XCG013        | BY4741 harboring pY13-YBL111C | This study       |
| XCG014        | BY4741 harboring pY13-RNR1 | This study       |
| XCG015        | BY4741 harboring pY13-SRL1 | This study       |
| XCG017        | BY4741 harboring pY131-ELO2 | This study       |
| XCG017        | BY4741 harboring pY132-ELO2 | This study       |
| XCG018        | gene lac1 deleted in strain XCG010 | This study       |
| pY13          | 2 μm, Amp, HIS1, PTEF   | Lab storage      |
| pY13-FET4     | 2 μm, Amp, HIS1, PTEF-FET4 | This study       |
| pY13-ADH6     | 2 μm, Amp, HIS1, PTEF-ADH6 | This study       |
| pY13-PHO89    | 2 μm, Amp, HIS1, PTEF-PHO89 | This study       |
| pY13-EGT2     | 2 μm, Amp, HIS1, PTEF-EGT2 | This study       |
| pY13-SAH1     | 2 μm, Amp, HIS1, PTEF-SAH1 | This study       |
| pY13-HXT4     | 2 μm, Amp, HIS1, PTEF-HXT4 | This study       |
| pY13-SKG6     | 2 μm, Amp, HIS1, PTEF-SKG6 | This study       |
| pY13-ELO2     | 2 μm, Amp, HIS1, PTEF-ELO2 | This study       |
| pY13-URA1     | 2 μm, Amp, HIS1, PTEF-URA1 | This study       |
| pY13-HXK2     | 2 μm, Amp, HIS1, PTEF-HXK2 | This study       |
| Construct         | Description                                      | Notes       |
|-------------------|--------------------------------------------------|-------------|
| pY13-YBL11C       | 2 μm, *Amp, HIS1*, $P_{TEF}$-YBL11C              | This study  |
| pY13-RNR1         | 2 μm, *Amp, HIS1*, $P_{TEF}$-RNR1                | This study  |
| pY13-SRL1         | 2 μm, *Amp, HIS1*, $P_{TEF}$-SRL1                | This study  |
| pY131             | Replace pY13 promoter $P_{TEF}$ with promoter $P_{ADH1}$ | This study  |
| pY132             | Replace pY13 promoter $P_{TEF}$ with promoter $P_{TDH3}$ | This study  |
| pY131-ELO2        | 2 μm, *Amp, HIS1*, $P_{ADH1}$-ELO2               | This study  |
| pY132-ELO2        | 2 μm, *Amp, HIS1*, $P_{TDH3}$-ELO2               | This study  |
| Primer function and name | Sequence (5’–3’) |
|-------------------------|------------------|
| **Overexpression**     |                  |
| FET4-F1                 | TAGTGGATCCATGGTAAAATGCA |
| FET4-F2                 | ATGACTCGAGCTATTGCTCCACATCATA |
| ADH6-F1                 | CTAGTGGATCCATGTTTTCCTGAGAA |
| ADH6-F2                 | ATGACTCGAGCTATTGCTAGAAAATCTC |
| PHO89-F1                | TAGTGGATCCATGGCTTTATCATA |
| PHO89-F2                | ATGACTCGAGCTATTGCTAGAAAATGGA |
| ADH6-F1                 | CTAGTGGATCCATGGCTTTATCATA |
| ADH6-F2                 | ATGACTCGAGCTATTGCTAGAAAATGGA |
| PHO89-F1                | TAGTGGATCCATGGCTTTATCATA |
| PHO89-F2                | ATGACTCGAGCTATTGCTAGAAAATGGA |
| **Gene deletion**       |                  |
| L-lac1-F1               | GAATGAAAAATAGTTGGAAAGGAAACA |
| L-lac1-F2               | GAATGAAAAATAGTTGGAAAGGAAACA |
| **Replace promoter**    |                  |
| P\textsubscript{ADH1}-F1 | GCTGGAGCTCCTCTCTTTGGTTTGGT |
| P\textsubscript{ADH1}-F2 | GGATCCAGCGGGAGTTGATGAGG |
| P\textsubscript{TDH3}-F1 | GCTGGAGCTCCTCTCTTTGGTTTGG |
| P\textsubscript{TDH3}-F2 | CTAGTTCTAGATTTGTGGTTTTATGTTGTTTATTCC |
L-lac1-F2  AGCTCTTGGTTATTGATACGTGTC
Leu2 (lac1)-F1  AGTATCAATAAAACAAGAGCTATGTCTGCCCTAAGAA
Leu2 (lac1)-F2  CTAAAAACACCGTTTTTCTTTAAAGCAAGGATTTTCTT
AACTTCTTGG
R-lac1-F1  AGGAAAACGGTGTTTTTAAGATGA
R-lac1-F2  CATATTTAGTTTGACTGACGGAGAA
**RT-PCR**
LAC1-F1  TTCATTCTGGAACACTA
LAC1-F2  CTAATAGCGAACGGGTCTA
LAG1-F1  CTTGACTGGTGACTCTAA
LAG1-F2  TATGATATGGCTACGAACA
AUR1-F1  ATGGTCATACACTTCAAT
AUR1-F2  GGTTCATCAGCATATAAG
CSG2-F1  CAAGTGTAATAGGCTACG
CSG2-F2  AAGGTCAGATAGAAGTGA
IPT1-F1  CATCTTATTCACCGCAT
IPT1-F2  TTATGGCATTGCTGTAAA
FIGURE LEGENDS

FIG. 1. Globe-transcriptome analysis of the adaptive laboratory evolution (ALE) mutant XCG001 and the wild-type strain. (A) Cell growth trajectory showing changes in fitness during the ALE in the YNB medium with different concentrations of NaCl. The concentration of NaCl was stepwise improved from 0.5 to 1.5 M over time (orange line). (B) Maximum specific exponential growth rates of the wild-type strain and mutant XCG001 in YNB medium supplemented with increasing concentrations of salt. The half-maximal inhibitory concentration (IC50) was calculated by the fitting curve to the data. (C) Growth profiles of mutant XCG001 and the wild-type strain in YNB medium under 0 M NaCl condition. (D) Growth profiles of mutant XCG001 and the wild-type strain in YNB medium under 1.5 M NaCl condition. (F) Venn diagrams depicting the numbers of up-regulated and down-regulated genes in the wild-type strain and mutant XCG001 under 1.5 M NaCl condition compared with those genes expression level in the corresponding strains under the 0 M NaCl condition. (G) Numbers of up-regulated and down-regulated genes in mutant XCG001 relative to their expression in the wild-type strain under 0 M and 1.5 M NaCl conditions.

FIG. 2. Overexpression of ELO2 enhanced osmotic tolerance. (A) Control strains XCG002 (wild-type strain with a control plasmid pY13), PADH1–ELO2 (XCG016), PTDH3–ELO2 (XCG017) and PTEF1–ELO2 (XCG010) were spotted on YNB plates at 0 M and 1.0 M NaCl. (B) Maximum specific exponential growth rates of strains XCG002, XCG016, XCG017, and XCG010 in YNB supplemented with increasing NaCl concentration. The half-maximal inhibitory concentration (IC50) was calculated by the fitting curve to the data. (C and D) Growth
curves of strains XCG002, XCG016, XCG017, and XCG010 at 0 M and 1.0 M NaCl. (E) The survival rates of strains XCG002, XCG016, XCG017, and XCG010 over a range of NaCl doses (0.00, 0.25, 0.50, 0.75, 1.00 M).

All data are presented as mean values of three independent experiments. Error bars indicate the standard deviations. **, P < 0.01.

**FIG. 3.** Overexpression of ELO2 enhanced very long fatty acids contents. (A) Fatty acids contents in strains XCG002, XCG016, XCG017, and XCG010 at 0 M NaCl. (B) Fatty acids contents in strains XCG002, XCG016, XCG017, and XCG010 at 1.0 M NaCl. (C) The fatty acid average length of strains XCG002, XCG016, XCG017, and XCG010 at 0 M and 1.0 M NaCl. (D) Unsaturation/saturation ratio of strains XCG002, XCG016, XCG017, and XCG010 at 0 M and 1.0 M NaCl. All data are presented as mean values of three independent experiments. Error bars indicate the standard deviations. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**FIG. 4.** Overexpression of ELO2 changed complex sphingolipids contents. (A-F) Phospholipid content (including phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI), phosphocholine (PC), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE)) changed in strain XCG002 and strain XCG010 at 0 M and 1.0 M NaCl. (G) The ratio of complex sphingolipid (including phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), mannosyldiinositol phosphorylceramide (M(IP)_2C)) content in strain XCG010 to that of control strain XCG002 changed at 0 M and 1.0 M NaCl. All data are presented as mean values of three independent experiments. Error bars indicate the standard deviations. *, P < 0.05; **, P < 0.01.
FIG. 5. Complex sphingolipid improved osmotic tolerance. (A) The mRNA level of the complex sphingolipid biosynthesis genes in strains XCG002 and XCG010 at 0 M NaCl. (B) The mRNA level of complex sphingolipid biosynthesis genes in strains XCG002 and XCG010 at 1.0 M NaCl. (C) The ratio of complex sphingolipid content in strain XCG018 (deletion of LAC1 in strain XCG010) to that of the control strain XCG002 changed at 0 M and 1.0 M NaCl. (D) Strains XCG002, XCG010, and XCG018 were spotted on plates containing with or without 1.0 M NaCl. (E) The survival rates of strains XCG002, XCG010, and XCG018 over a range of NaCl doses (0.00, 0.25, 0.50, 0.75, 1.00 M). All data are presented as mean values of three independent experiments. Error bars indicate the standard deviations. *, P < 0.05; **, P < 0.01.

FIG. 6. Increased complex sphingolipids changed membrane integrity. (A and B) Fluorescent microscope analysis of membrane integrity in the XCG002, XCG010, and XCG018 cells at 0 M (A) or 1.0 M NaCl (B). Under the view of a confocal fluorescence microscope, all cells showed red fluorescence with an integral membrane while only cells with a damaged membrane showed green fluorescence. Cell with a damaged membrane can be stained by SYTOX green and cell with integral or damaged membrane all can be stained by FM4-64. The scale is 25 μm. (C and D) Flow cytometry analysis of membrane integrity in strains XCG002, XCG010, and XCG018 cells at 0 M or 1.0 M NaCl. All data are presented as mean values of three independent experiments.
A. 

B. 

C. 

D. 

E. 

F. Differently regulated genes in response to NaCl

up-regulated genes
down-regulated genes

wild-type XCG001 wild-type XCG001

484 180 236 437 68 125

G. Differently regulated genes in the XCG001 strain

up-regulated genes
down-regulated genes

0 M NaCl 1.5 M NaCl 0 M NaCl 1.5 M NaCl

199 13 118 243 28 207
