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Genomic reconstruction to improve bioethanol and ergosterol production of industrial yeast *Saccharomyces cerevisiae*

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Abstract Baker's yeast (*Saccharomyces cerevisiae*) is the common yeast used in the fields of bread making, brewing, and bioethanol production. Growth rate, stress tolerance, ethanol titer, and byproducts yields are some of the most important agronomic traits of *S. cerevisiae* for industrial applications. Here, we developed a novel method of constructing *S. cerevisiae* strains for co-producing bioethanol and ergosterol. The genome of an industrial *S. cerevisiae* strain, ZTW1, was first reconstructed through treatment with an antimitotic drug followed by sporulation and hybridization. A total of 140 mutants were selected for ethanol fermentation testing, and a significant positive correlation between ergosterol content and ethanol production was observed. The highest performing mutant, ZG27, produced 7.9 % more ethanol and 43.2 % more ergosterol than ZTW1 at the end of fermentation. Chromosomal karyotyping and proteome analysis of ZG27 and ZTW1 suggested that this breeding strategy caused large-scale genome structural variations and global gene expression diversities in the mutants. Genetic manipulation further demonstrated that the altered expression activity of some genes (such as *ERG1*, *ERG9*, and *ERG11*) involved in ergosterol synthesis partly explained the trait improvement in ZG27.

Keywords *Saccharomyces cerevisiae* · Bioethanol · Ergosterol · Genome reconstruction · Tolerance

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Introduction

Bioethanol is considered as an alternative biofuel to petrol and is attracting increased attention because it has fewer negative environmental effects and is renewable. Yeast (*Saccharomyces cerevisiae*) is the most suitable microorganism used for bioethanol fermentation in industrial scale because yeast has higher ethanol yield and tolerance than other microbes [2, 4, 10, 19]. Using fermentable sugars from starch or molasses, bioethanol can be produced at concentrations of 8–15 % by natural or engineered *S. cerevisiae* strains within 24–70 h depending on the process [3, 11, 21, 32]. Although bioethanol can alleviate environmental pollution, the high cost of bioethanol production is a disadvantage compared with petroleum. The extraction of certain physiologically active substances from yeast biomass can decrease the cost of bioethanol production [24].

One of the most economically important components of yeast biomass is ergosterol which could be used as a precursor of vitamin D2 and other sterol drugs, such as cortisone, brassinolide, and progesterone [23, 26]. Many fungi can synthesize ergosterol as the predominant sterol component of plasma membrane, but *S. cerevisiae* strains usually produce higher content of ergosterol. This sterol has been confirmed to contribute to tolerance to ethanol [1, 7, 13], arsenite [17], vanillin [13], and D-limonene [18] in yeast strains. Currently, commercial ergosterol is mainly obtained by the fermentation of *S. cerevisiae*

biomass. The ergosterol biosynthesis pathway of *S. cerevisiae* involves multiple genes whose expression can be adjusted by transcriptional regulation [12, 28]. To improve the production of ergosterol in yeast, significant efforts including process innovation and strain breeding have been carried out. He et al. constructed high ergosterol-producing yeast strains by hybridizing haploid strains with different genetic backgrounds [14]. Overexpression of sterol C-24 (28) reductase and sterol acyltransferase can reportedly increase ergosterol formation in yeast strains [14, 22]. Shang et al. reported the optimization of the high-cell density cultivation of *S. cerevisiae* GE-2 for simultaneously producing ergosterol and glutathione [23].

In this study, a novel method of producing *S. cerevisiae* strains that can increase the productions of bioethanol and ergosterol was developed. The genome of the bioethanol-producing *S. cerevisiae* strain ZTW1 was first reconstructed, and the resulting mutants were selected by a special procedure. As a result, a total of 27 mutants with higher ethanol and ergosterol production were obtained through this strategy. A comparative study of the highest performing mutants ZG27 and ZTW1 was then performed to examine the possible mechanisms underlying the phenotype alteration of the mutants. Our results suggested that the large-scale genomic structure variations could modify the global gene expression pattern of yeast cells and thus yield mutants with altered phenotypes.

Materials and methods

Strains and culture conditions

S. cerevisiae strain ZTW1 (CCTCC M 2013061) was first isolated in our lab and has been used for bioethanol production at an industrial scale by the Henan Tianguan Fuel Ethanol Co. Ltd., China. *S. cerevisiae* strain ZG27 (CCTCC M 2014111) was a mutant from ZTW1 obtained in this study. BYZ1 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ*) was a laboratory *S. cerevisiae* strain that served as a control in pulsed-field gel electrophoresis (PFGE) and array-comparative genomic hybridization (aCGH) experiments. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used as the hosts for construction and amplification of all plasmids used in this study. Yeast extract–peptone–dextrose (YPD) medium (10 g/L yeast extracts, 20 g/L peptone, and 20 g/L glucose, pH 5.5) was used to culture yeast cells. *E. coli* cells were cultured in Luria broth (LB) medium (10 g/L peptone, 5 g/L yeast extracts, and 5 g/L NaCl, pH 7.0) at 37 °C.

Genome reconstruction and mutant selection

Genome reconstruction was performed by treatment with the antimitotic drug methyl benzimidazole-2-yl-carbamate (MBC; Adamas-beta, Shanghai, China) followed by sporulation and hybridization. *S. cerevisiae* ZTW1 was cultured with 25 mL YPD medium containing 35 μg/mL MBC at 30 °C for 18 h (initial concentration of ZTW1 was ~2×10^7 cells/mL), and then 3×10^8 cells were collected by centrifugation (6,000 rpm for 5 min at 4 °C) and cultured in 10 mL sporulation medium (10 g/L KAc, pH 6.0) at 28 °C for 5 days. All asci in the sporulation medium were collected by centrifugation (12,000 rpm for 5 min at 4 °C) and the ascospores were released as previously described [36]. Approximately, 2×10^8 free spores were then collected (12,000 rpm for 15 min at 4 °C) and randomly mated in 25 mL YPD medium for 2 days. Hybrids (3×10^8 cells) were collected by centrifugation (5,000 rpm for 5 min at 4 °C) and cultured in 10 mL YPD with 20 % (v/v) ethanol for 2 h to kill the cells with weak ethanol tolerance. Finally, 50 μL yeast culture was aspirated and spread onto each plate for mutant screening.

Stress tolerance test

For the tolerance tests, yeast cells were pre-cultured in 25 mL YPD with an initial concentration of 3×10^6 cells/mL for 18 h at 30 °C. The yeast culture was then diluted to a concentration of 5×10^7 cells/mL, and 4 μL of tenfold serial dilutions of each sample was spotted onto YPD plates with certain stressors. To determine the viability of the strains after the application of ethanol, yeast cells (6×10^7) were collected and resuspended by 3 mL ethanol (10, 15, and 20 %; v/v) and then cultured at 30 °C in a thermostatic water bath. 100 μL of the treated cells was then aspirated at certain time points (0 h was taken as the control) and spread onto YPD plates at 30 °C for 2 days to calculate the survival rates. Experiments of stress tolerance test were repeated three times.

Measurement of intracellular nucleotide leakage

Yeast cells were cultured under the same condition as described in stress tolerance test. Cells (6×10^8) were harvested and washed by sterile water until the absorbance of the supernatant at 260 nm was negligible. The cells were suspended in 3 mL ethanol with certain concentrations and incubated at 30 °C. After low-speed centrifugation (4,000 rpm for 5 min) to remove cells, the absorbances of supernatant at 260 and 280 nm were measured using a NanoDrop™ Spectrophotometer (Thermo, Wilmington, De, USA). The concentration of nucleotide leakage was calculated as described in our previous study [29].

Ethanol fermentation and ergosterol measurement

A fermentation medium was prepared using corn flour with a two-enzyme method. Briefly, 8.8 L water and 4 kg corn flour were mixed, and α-amylase [35 Novo alpha-amylase Unit (KNU)/g corn; Liquozyme® Supra, Novozymes, Beijing, China] was added to the mixture. The corn mash was kept in an autoclave at 105 °C for 1.5 h for liquefaction. Glucoamylase [1 Amyloglucosidase Unit (AGU)/g corn; Suhong GA II, Novozymes, Beijing, China] was used to saccharify the mash at 60 °C for 10 h. The solid substance in the corn mash was removed by centrifugation (6,000 rpm for 10 min). The glucose concentration of the fermentation medium is 265.2 g/L. Yeast cells were first grown in 50 mL YPD for 20 h (initial concentration is ~3×10^6/mL) and cells were then transferred into 300 mL of fermentation medium with an initial concentration 5×10^7/mL. Fermentations were then performed in 1 L flasks (covered with eight layers of gauze) for 65 h at 33 °C and 100 rpm. Samples of fermentation broth for metabolite measurement were immediately centrifuged (12,000 rpm for 10 min at 4 °C), filtered through 0.45 mm cellulose nitrate filter (Whatman, Maldstone, UK), and stored at −20 °C until analysis. Glucose and ethanol concentrations were measured by high-performance liquid chromatography (HPLC; Agilent, Palo Alto, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C, and were detected using a refractive index detector (Agilent, Palo Alto, CA, USA). The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.6 mL/min.

Ergosterol was analyzed using external standard method by HPLC [18]. Prior to HPLC determination, the alkaline hydrolysis of cells (1×10^9) in a 3 mL alcohol alkali solution (25 g NaOH was dissolved in 60 ml ddH_2O and absolute ethanol was added to a volume of 100 mL) at 95 °C was performed for 3 h followed by extraction in 5 mL *n*-heptane (Sigma-Aldrich, St Louis, MO, USA). HPLC was performed using an Agilent 1100 machine (Palo Alto, CA, USA) equipped with a Zorbax SB-C18 column (250 × 4.6 mm; Agilent, Palo Alto, CA, USA). Ergosterol was detected using a UV detector (Agilent, Palo Alto, CA, USA) set at 282 nm. The mobile phase (methanol: water = 95: 5) was used to dissolve ergosterol.

PFGE and aCGH experiment

Yeast cells were grown at 30 °C in 25 mL YPD media for 30 h and collected by centrifugation (6,000 rpm for 10 min at 4 °C). Yeast cells (3×10^8) were washed twice by sterile water and resuspended in 50 μL lyticase solution (3 U/μL). An equal volume of 1.6 % low melt agarose was added to the cell/lyticase suspension to give a final agarose concentration of 0.8 %. The samples were transferred to plug molds and kept at room temperature for about 30 min. The plugs were pushed out into a 50 ml Falcon tube containing ESP buffer [0.5 M EDTA (pH 9.0), 1 M Tris (pH 9.0), 10 % *N*-Laurylsarcosine, 80 mg/mL Proteinase K], and then incubated at 50 °C for 24 h. The plugs were washed with TE buffer for three times and PFGE was performed on a Bio-Rad DRIII apparatus.

aCGH experiment was performed to detect the copy number variations in the genome of mutant ZG27. Total genomic DNA from BYZ1 (a standard diploid *S. cerevisiae* strain used as a control) and ZG27 was isolated with the yeast DNA kit (OMEGA, GA, USA) and then sonicated. The shearing DNA (200–1,000 bp) of BYZ1 and ZG27 was labeled with Cy5/Cy3 and hybridized to *S. cerevisiae* CGH 385 K Whole-Genome Tiling Arrays (NimbleGen, Madison, WI, USA). Scanning and data analysis were performed as previously described [38]. The microarray data have been deposited in the NCBI Gene Expression Omnibus [GEO: GSE31872].

iTRAQ and pathway enrichment analysis

Yeast cells were first cultured in 25 mL YPD medium for 18 h with an initial cell concentration of 3×10^6 cells/mL. Protein of yeast cells (2×10^9) was then extracted (Yeast Buster™ Protein Extraction Reagent, Merck, Darmstadt, Germany) and quantified using the 2-D Quant kit (GE Healthcare, Piscataway, NJ, USA). The protein samples (100 μg each) of *S. cerevisiae* strains ZTW1 and ZG27 were then reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, digested with trypsin (Promega, Madison, WI, USA), and labeled with iTRAQ reagents according to the standard iTRAQ protocol (AB SCIEX, Foster City, CA, USA). The protein digests obtained from ZTW1 and ZG27 were labeled with iTRAQ reagent 117 and 121, respectively, and pooled together. The pooled peptides were dried in a Speedvac and dissolved in 1 mL buffer A (10 mM KH_2PO_4 in 25 % acetonitrile at pH 2.8) and loaded onto a 4.6 × 250 mm strong cation-exchange chromatography (SCX) column (Phenomenex, Torrance, CA, USA). The peptides were eluted at a flow rate of 1 ml/min with a gradient of buffer A for 10 min, 5–35 % buffer B (25 mM NaH_2PO_4, 1 M KCl in 25 % acetonitrile, pH 2.8) for 11 min, and 35–80 % buffer B for 1 min. Elution was monitored by measuring the absorbance at 214 nm, and the fractions were collected every 1 min. The collected peptides were desalted with a strata-X 33 μm PolyRevStage SPE (Phenomenex, Torrance, CA, USA) following the manufacturer's instructions. The MS analysis was performed on a TripleTOF 5600 system (AB SCIEX,

Foster City, CA, USA) in Information-dependent Mode. MS spectra were acquired across the mass range of 400–1,800 *m/z* in high-resolution mode (>30,000) using 250 ms accumulation time *per* spectrum. Generation of peak lists was performed with the Proteome Discoverer 1.3 Software. Software Mascot 2.3.02 (Matrix Science, London, United Kingdom) was used to identify and quantify proteins by searching the yeast protein database in the *Saccharomyces* Genome Database (SGD; www.yeastgenome.com). The search parameters were designed as follows: trypsin was chosen as the enzyme with one missed cleavage allowed; fixed modifications of carbamidomethylation at Cys; variable modifications of oxidation at Met; peptide tolerance was set at 0.05 Da; and MS/MS tolerance was set at 0.1 Da. KEGG pathway enrichment analysis of differently expressed proteins between the two strains was performed using KOBAS software based on KEGG pathway database [33].

Real-time quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR experiment was used to determine the expression activity of certain genes at the mRNA level. Yeast cells were cultured in 25 mL YPD medium for 18 h (initial cell concentration is 3×10^6 cells/mL). Total RNA from yeast cells (3×10^8) was extracted using Fungal RNAout Kit (TIANDZ, Bejing, China). qRT-PCR experiments were performed using an ABI Prism 7500 instrument (AB SCIEX, Foster City, CA, USA). Each sample was tested in triplicate in a 96-well plate with a final reaction volume of 20 µl using the SYBR® PrimeScript® RT-PCR kit (Takara, Dalian, Shandong, China). The expression activity was quantified and normalized using *ALG9*, *TAF10*, *UBC6*, and *TFC1* as the reference genes [39]. The primers used in qRT-PCR experiment are listed in Table S1 (Supplemental material).

Genetic manipulation

To overexpress genes *ERG1*, *ERG9*, and *ERG11* in ZTW1, these genes were cloned (ZTW1 genome DNA was used as the template of polymerase chain reaction) into the plasmid pYZ (under the control of *PGK1* promoter) and transformed into ZTW1 by the previously described LiAc/SS carrier DNA/PEG methods [37]. pYZ was derived from pYES2 (Invitrogen, Carlsbad, CA, USA), but the URA3 marker in this plasmid was replaced with a Zeocin-resistant marker (*Ble*^{r}). The primers used to clone these genes are listed in Table S2 (Supplemental material). The transformants were selected on the YPD plates (pH was adjusted to 7) containing 55 µg/mL Zeocin (Invitrogen, San Diego, CA, USA).

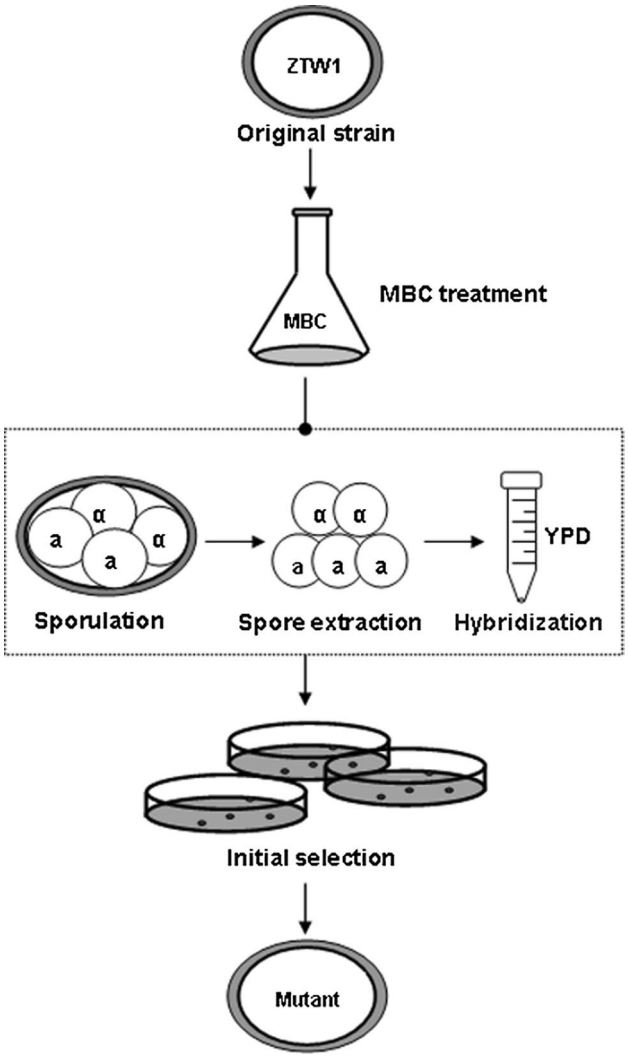

Fig. 1 Procedures of the method of improving ergosterol content and ethanol production in yeast based on genome reconstruction. The original strain ZTW1 was first treated with MBC and the resulting cells were sporulated and hybridized. The hybrids were diluted and plated onto the selection plates containing certain stressors. The details of this breeding strategy are described in the section of "Materials and methods"

Results

A novel method of improving ethanol and ergosterol production in yeast

Figure 1 describes the breeding strategy used to improve the production of ethanol and ergosterol of yeast in this study. The original *S. cerevisiae* strain ZTW1 was chosen for genome reconstruction, which involved two unit processes, i.e., treatment with MBC and a round of asexual propagation (sporulation and random hybridization). MBC is an antimitotic drug that can disrupt the function of the mitotic spindle and cause chromosome

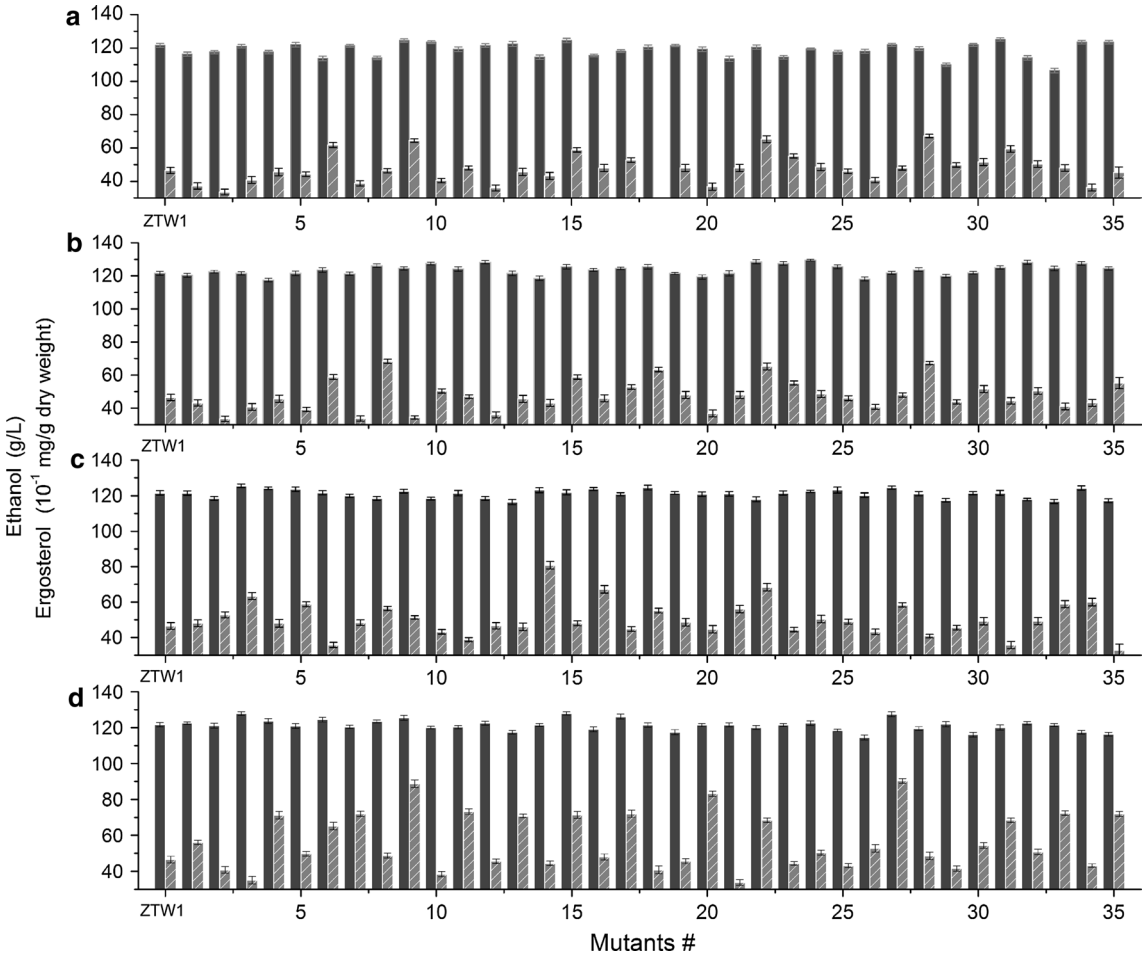

Fig. 2 Comparison of ergosterol contents and ethanol productions between ZTW1 and the mutants from **a** normal YPD, **b** ethanol, **c** vanillin, and **d** $CuSO_4$ selection plates. Pillars represent the ergosterol content (*gray*) and final ethanol concentration (*dark gray*) of mutants and ZTW1. Yeast was grown in 25 mL YPD at 30 °C for 18 h (initial cell concentration was 3×10^6/mL), and the cells (1×10^9) were collected for ergosterol determination. Ethanol fermentation test was performed as described in the "Materials and methods" section. Experiments were performed three times in triplicate, and *error bars* indicate ±SD

nondisjunction, resulting in chromosomal aberration in progenies [34]. Asexual propagation, in which chromosomal rearrangement and random distribution of chromosomes occur [8], was used to accelerate the genome structural variation in the progenies of the near-triploid strain, ZTW1. After genome reconstruction, a procedure was then designed for the initial screening of mutants with desired traits: the resulting mutants were treated with 19 % (v/v) ethanol for 2 h to kill the cells with less ethanol resistance, and the viable cells were spread onto normal YPD plates and three types of selection plates containing 13 % (v/v) ethanol, 1.8 g/L vanillin, and 7 mM $CuSO_4$, respectively. A total of 140 colonies that first appeared on the selective plates were randomly picked to determine their ergosterol content and ethanol production described in Materials and methods (Fig. 2). Without the addition of inhibitors, only three strains (numbers 9, 15, and 31) out of the 35 mutants (randomly picked from the normal YPD plates after hybridization) showed both higher ethanol and ergosterol than ZTW1 (Fig. 2a). Interestingly, the ratio of desired mutants was much higher among the colonies from the three selected plates but to different degrees (Fig. 2b–d). The three screening plates yielded eight (number 6, 8, 10, 15, 17, 22, 23, and 35 in Fig. 2b), seven (number 3, 5, 14, 16, 18, 27, and 34 in Fig. 2c), and nine (numbers 4, 6, 8, 9, 15, 17, 24, 27, and 33 in Fig. 2d) mutants with higher ethanol and ergosterol productions, respectively. Overall, however, a significant correlation was observed between ergosterol content and ethanol production among the all mutants (Pearson's correlation coefficient = 0.23, $P < 0.05$).

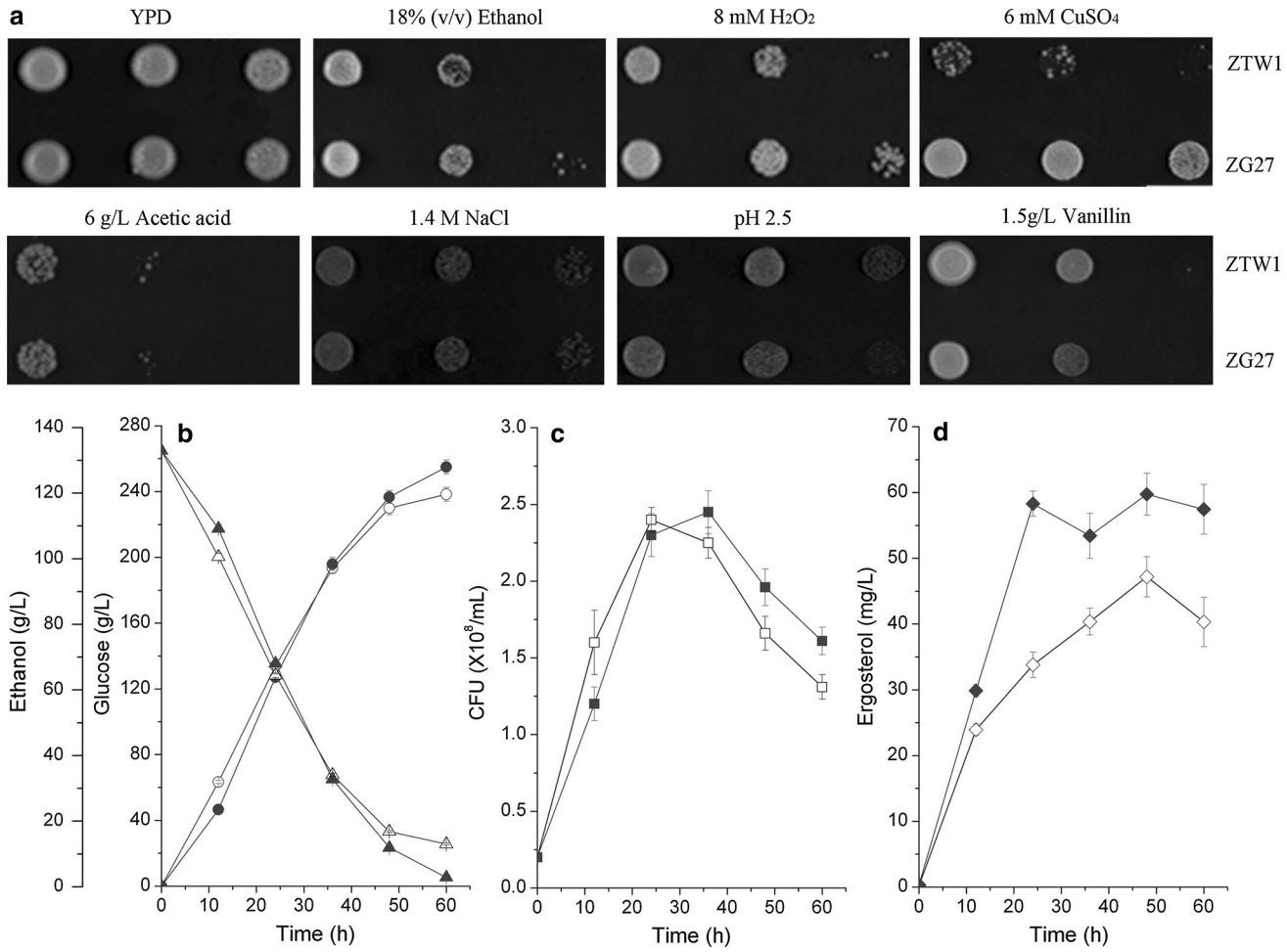

Fig. 3 Comparison of stresses tolerance and fermentation performances of ZTW1 and ZG27. **a** Stress tolerance test of ZTW1 and ZG27. 4 μL of tenfold serial dilutions of each sample was spotted onto plates with indicated stresses. Typical data from one of three independent experiments are shown. Comparison of **b** ethanol production, **c** colony-forming units (CFU), and **d** ergosterol production of ZTW1 (*empty*) and ZG27 (*solid*) during ethanol fermentation. Ethanol fermentation was performed three times in triplicate, and *error bars* indicate ±SD

Comparison of ethanol fermentation performances and ergosterol contents between ZTW1 and the mutant ZG27

The proposed strategy yielded a total of 27 mutants (three from the normal YPD plates and 24 from the YPD plates with stressors) that showed improved productions of ethanol and ergosterol (Fig. 2). Strain ZG27 (number 27 in Fig. 2d), which accumulated the most ergosterol among all the 140 mutants (Fig. 2), was chosen to compare stress tolerances and fermentation performances with the parental strain, ZTW1. ZG27 was more tolerant to the stressors 18 % (v/v) ethanol, 8 mM H_2O_2, and 6 mM $CuSO_4$ than ZTW1, but no distinctive difference was observed in the tolerances to osmotic pressure (1.4 M NaCl) and acetic acid (6 g/L) between these two strains (Fig. 3a). ZG27 had a slightly weaker growth under the stress of low pH and vanillin (Fig. 3a). Regarding fermentation performances, ZG27 showed a slightly lower fermentation rate at the early-to-mid stage (0–40 h) but produced 7.9 % more ethanol at the end of fermentation (at the time point of 60 h) than ZTW1 (Fig. 3b; $P < 0.05$, *t* test) with higher viability as ethanol accumulated in the fermentation medium (Fig. 3c). Moreover, this strain also synthesized more ergosterol during fermentation, achieving 43.2 % higher production than ZTW1 at the point of 60 h (Fig. 3d; *t* test, $P < 0.01$). High-concentration of ethanol was the main stressor during the late stage of bioethanol fermentation, especially under very high-gravity conditions (the concentration of fermentation substrate is usually more than 260 g/L) [1, 29]. During the ethanol fermentation process, an increased ethanol concentration could gradually reduce cell viability by causing destruction of cell membrane and thus leakage of cell contents [29]. As illustrated by Fig. 4a, the viability of ZTW1

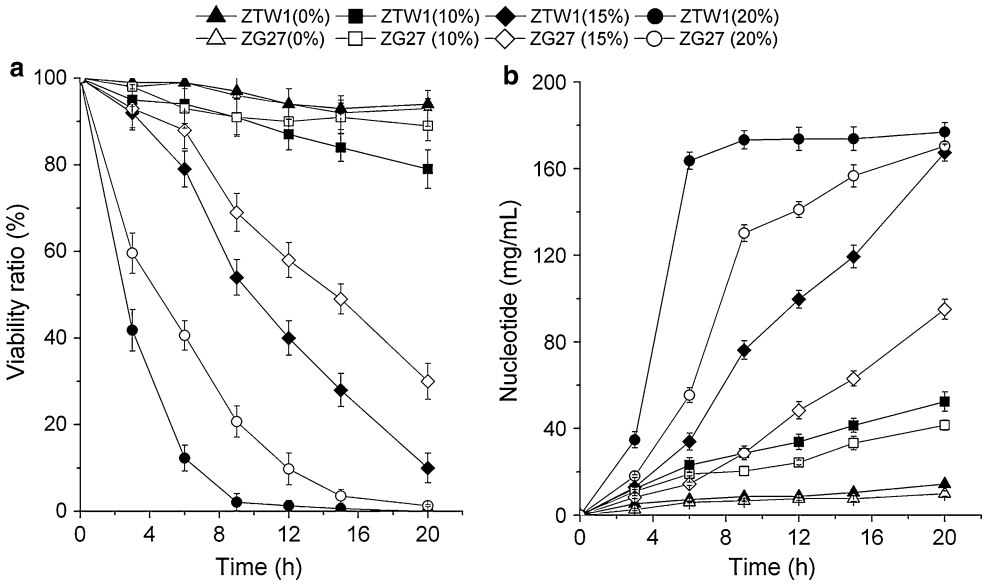

Fig. 4 Survival rate and membrane integrity of ZTW1 (*solid*) and ZG27 (*empty*). **a** The different survival rates of ZTW1 and ZG27 after the application of different concentrations [0 % (*triangles*), 10 % (*squares*), 15 % (*diamonds*), and 20 % (*circles*) (v/v)] of ethanol for 0–20 h. **b** Time course of extracellular nucleotide concentration in cell suspension of strains. Yeast cells were suspended in aqueous solution with 0 % (*triangles*), 10 % (*squares*), 15 % (*diamonds*), and 20 % (*circles*) (v/v) ethanol and incubated at 30 °C. Concentration of nucleotide that leaked into the supernatant was measured every few hours as described in the section of "Materials of methods". Experiments were performed three times in triplicate, and *error bars* indicate ±SD

and ZG27 could be greatly affected when exposed to a high concentration of ethanol [15 % (v/v) and 20 % (v/v)], but ZG27 kept a higher survival rate than ZTW1 at any time point (0–20 h). Consistent with this result, the nucleotide that leaked into the supernatant of strain ZG27 was always less than that of ZTW1 after the application of ethanol and the differences widened with the increase of ethanol concentration (Fig. 4b). Thus, the enhanced ethanol production in ZG27 at the late stage of ethanol fermentation may be partly due to its higher ability to keep membrane integrity and viability than ZTW when exposed to high concentration of ethanol. These results demonstrated that our breeding method could effectively change the complex traits (such as stress tolerance, fermentation performances, and physiological factors) of *S. cerevisiae*.

Comparison of genome structures of some mutants and ZTW1

Theoretically, the possible bases underlying the changed traits of mutants obtained in this study included DNA sequence variations resulting from homologous recombination during meiosis and genome structural variations (such as insertions, deletions, translocation, and inversions). To determine whether large-scale chromosomal rearrangements had occurred during the breeding process of ZTW1, PFGE experiment was used to reveal the karyotypes of ZG27 and other four mutants (numbers 6, 8, 17, and 33 in Fig. 2d; selected from $CuSO_4$ plates) with higher productions of ethanol and ergosterol. Distinct differences were observed in both the lengths of certain chromosomes and chromosome numbers between mutants and their original strain ZTW1 (Fig. 5a). This result indicates that our breeding strategy can produce yeast mutants with diverse genomic structures. Higher resolution results pertaining to the structural variations in the genome of ZG27 were obtained through aCGH analysis (Fig. 5b). Our previous work had revealed that ZTW1 is a near-triploid strain and some large chromosomal segments (whole chromosome 9, 31–378 kb on chromosome 11, and 1–97 kb on chromosome 14) have four copies and some segments (727–777 kb on chromosome 14) have only two copies [20]. The purple and olive green colored regions in Fig. 5b show the amplified and underrepresented chromosomal regions in ZG27 genome, respectively, compared with ZTW1 genome. Although the ploidy of ZG27 was similar with that of ZTW1 revealed by intracellular DNA content using flow cytometry (data not shown), aCGH experiment showed that ZG27 has one less copy of chromosome 3 and the 727–777 kb region on chromosome 14 than ZTW1 (Fig. 5b). This strain also has one more copy of two large DNA regions (31–378 kb on chromosome 11 and 1–97 kb on chromosome 14) than ZTW1. In other words, ZG27 has only one copy of 727–777 kb on chromosome 14, two copies of chromosome 3, and four copies of 31–378 kb on chromosome 11 in a single cell. These copy number

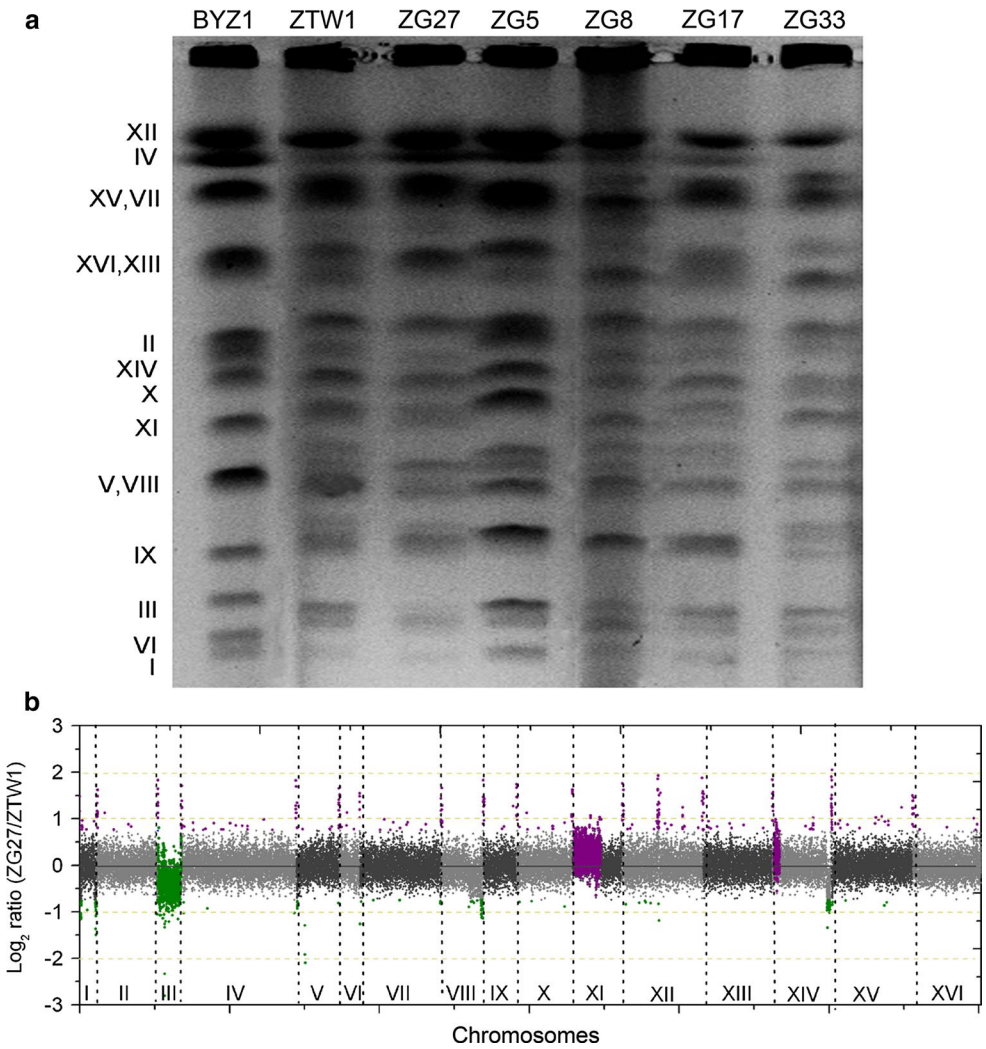

Fig. 5 Genome structural variations of the mutants compared with parent strain ZTW1. **a** PFGE of the chromosomes in BYZ1 and YJS329. The standard diploid strain BYZ1 was used as a control. **b** The amplified regions and underrepresented regions in the genome of ZG27 compared with ZTW1 are colored in *violet* and *green*, respectively, revealed by aCGH analysis (color figure online)

variations of chromosomal segments or a whole chromosome might be an important genetic mechanism underlying the changed phenotypes of ZG27.

Proteome analysis of different gene expression levels in ZG27 and ZTW1

To understand the molecular basis that confers the desired traits to mutants, ZG27 was chosen for iTRAQ-based proteome analysis compared with ZTW1. A total of 25.4 % yeast genes (1,675 out of 6,607) annotated by SGD database could be detected in the two samples. Using software Mascot, we identified 640 differently expressed genes (DEGs; $P < 0.05$) between these two strains (Table S3 in Supplemental material). Compared with ZTW1, 428 DEGs were up-regulated, and 212 DEGs were repressed in ZG27 ($P < 0.05$).

Pathway enrichment analysis of the 298 DEGs (fold change >0.5 and $P < 0.05$) showed that the up-regulated genes (234) in ZG27 mainly fell within the biosynthesis of secondary metabolites (including ergosterol metabolism), amino acids metabolism, proteasome, and glycolysis/gluconeogenesis (Hypergeometric test, $P < 0.05$; Table 1). The 64 down-regulated genes were mainly involved in glyoxylate and dicarboxylate metabolism, peroxisome, and oxidative phosphorylation (Hypergeometric test, $P < 0.05$; Table 1). To confirm the accuracy of iTRAQ experiment, qRT-PCR was used to reveal the expression levels of 20 DEGs detected in iTRAQ experiment. Most of these genes showed high coincidence in expression levels detected in iTRAQ and qRT-PCR (Table S1 in Supplemental material). These results demonstrated that our breeding strategy could modify the global gene expression patterns of yeast cells, offering the possibility of altering their phenotypes to a large extent.

Altered expression activity of the genes involved in ergosterol biosynthesis pathway

The ergosterol biosynthesis pathway in *S. cerevisiae* is required for generation of a major constituent of its

Table 1 Pathway enrichment analysis of the differently expressed genes between ZTW1 and ZG27

Term	*P* value	Genes
Up-regulated genes		
Biosynthesis of secondary metabolites	2.23E−04	*TRP2 CDC19 ARG1 LPD1 ARG4 ADH3 ERG1 HMG1 GAD1 ILV1 PDB1 SFA1 SOL4 ARO3 ADH1 ADE2 IDH2 HIS1 TKL2 LYS2 ADE16 HIS6 ERG11 AMD1 AGX1 ADH6 PRS3 HEM2 COQ5*
Alanine, aspartate and glutamate metabolism	9.44E−04	*CPA2 UGA1 ARG1 AGX1 ARG4 GFA1 GDH1 URA2 GAD1 UGA2*
Tyrosine metabolism	1.21E−03	*ARO8 SFA1 ADH1 ADH3 ALD2 ALD3 UGA2*
Lysine biosynthesis	8.99E−03	*ARO8 HOM6 LYS12 LYS2 LYS21*
Degradation of aromatic compounds	1.21E−02	*SFA1 ADH1 ADH6 ADH3*
Proteasome	1.93E−02	*RPT6 SCL1 PRE9 PRE3 PRE5 PUP3 PRE10 PRE6*
Glycolysis/gluconeogenesis	3.63E−02	*PDB1SFA1 ADH6 LPD1 ADH1 ADH3 ALD2 YMR099C ALD3 CDC19 TDH1*
Down-regulated genes		
Glyoxylate and dicarboxylate metabolism	4.75E−05	*MLS1 CTA1 CIT2 ICL1 FDH1*
Peroxisome	1.89E−02	*YAT1 CTA1 PEX14*
Oxidative phosphorylation	2.93E−02	*VMA10 ATP3 ATP15 ATP16*
Protein processing in endoplasmic reticulum	4.02E−02	*PDI1 SSB2 KRE5 SIL1*

plasma membrane. There are 21 genes (*HMG1-2*, *ERG1-13*, *ERG20*, *ERG25-28*, and *MVD1*) involved in the biochemical reactions of this pathway (Fig. 6). iTRAQ analysis of ZTW1 and ZG27 showed that 8 genes (*HMG1*, *ERG1*, *ERG5*, *ERG8*, *ERG9*, *ERG11*, *ERG13*, and *ERG20* that encodes HMG-CoA reductase, squalene epoxidase, C-22 sterol desaturase, squalene synthase, phosphomevalonate kinase, farnesyl-diphosphate farnesyl transferase, 3-hydroxy-3-methylglutaryl-CoA synthase, and lanosterol 14-alpha-demethylase, respectively; red colored in Fig. 6) out of these 21 genes had higher expression levels in ZG27 than in ZTW1; and that 5 genes (*ERG6*, *ERG10*, *ERG12*, *ERG26*, and *MVD1* that encodes Delta(24)-sterol C-methyltransferase, acetoacetyl-CoA thiolase, mevalonate kinase, C-3 sterol dehydrogenase, and mevalonate pyrophosphate decarboxylase; green colored in Fig. 6) were slightly down-regulated in ZG27 than in ZTW1, but with no significance ($P > 0.05$); and the other 8 genes (gray colored in Fig. 6) were not detected in the iTRAQ experiment. qRT-PCR also confirmed the enhanced expression activates of genes *HMG1*, *ERG1*, *ERG5*, *ERG9*, and *ERG11* in ZG27 than in ZTW1 (Fig. 6). The altered expression activity of ergosterol biosynthesis genes might account for the different ergosterol contents between the two strains. To determine the physiological effects of up-regulation of the genes involved in ergosterol biosynthesis, three differently expressed genes (*ERG1*, *ERG9*, and *ERG11*; Fig. 6) between the two strains were overexpressed in ZTW1 through genetic manipulation. The engineered strains accumulated 9.3, 8.7, and 9.1 % more ergosterol than ZTW1 when *ERG1*, *ERG9*, or *ERG11* was overexpressed, respectively ($P < 0.05$, *t* test; Fig. 7). Figure 7 also shows that the

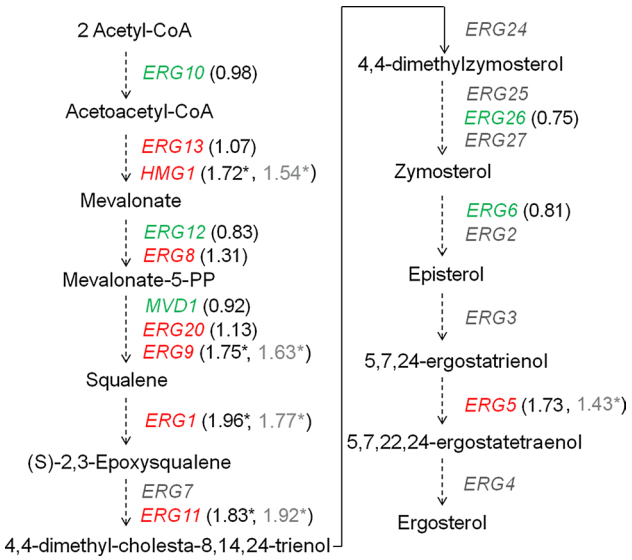

Fig. 6 The expression activities of the genes involved in ergosterol biosynthesis pathway of ZTW1 and ZG27 determined by iTRAQ. *Red* and *green* indicate the genes were expressed at a higher level and lower level in ZG27 than in ZTW1, respectively. *Gray* indicates the genes were not detected in iTRAQ experiment. The values from iTRAQ data and qRT-PCR experiments were shown in *black* and *gray* in brackets if available, respectively. *Asterisk* indicates a significant difference at the 0.05 level between these two strains (color figure online)

combined overexpression of any two of these three genes could increase the ergosterol content of ZTW1 to a large extent than overexpression of a single gene. The engineered strain (ZTERG1-11) with co-expression of *ERG1* and *ERG11* accumulated more ergosterol than five other engineered strains, which is 16.3 % higher than that of ZTW1.

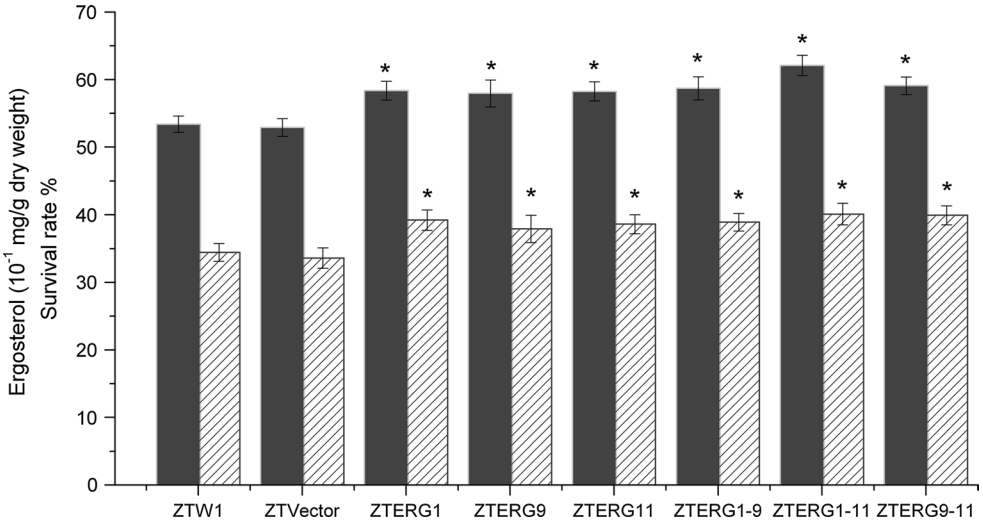

Fig. 7 Effects of overexpression of certain genes on ergosterol synthesis and ethanol tolerance of ZTW1. *Dark gray bars* indicate the ergosterol contents of ZTW1 and engineered strains (ZTERG1, ZTERG9, ZTERG11, ZTERG1-9, ZTERG1-11, and ZTERG9-11); and *white bars* indicate that overexpression of *ERG1*, *ERG9*, and *ERG11* in ZTW1 improved its survival rate after treatment with 20 % (v/v) ethanol for 4 h. ZTW1 harboring the empty vector (pYZ) was named as ZTvector. Experiments were performed three times in triplicate, and *error bars* indicate ±SD. *Asterisk* indicates a significant difference at $P < 0.05$ between ZTW1 and engineered strains by the *t* test

Besides, the engineered strains (ZTERG1, ZTERG9, ZTERG11, ZTERG1-9, ZTERG1-11, and ZTERG9-11) showed slightly but significantly increased viabilities (with a 14.0, 10.2, 12.4, 13.1, 16.6, and 16.0 % higher survival rate than ZTW1, respectively) after treatment with 20 % (v/v) ethanol than the wild strain ($P < 0.05$, *t* test; Fig. 7). However, even the engineered strain (ZTERG1-11) with the highest ergosterol content still produced ~35 % less ergosterol than that of ZG27. It seems that genetic manipulation of limited number of genes in ZTW1 could not enhance ergosterol content to a large extent.

Discussion

Generally, *S. cerevisiae* strains are naturally in diploid form. Recent studies have reported that genome structural variations, even the copy number variations of whole chromosomes, frequently appear in the genomes of laboratory evolved and industrial strains [6, 9, 35]. Some studies have demonstrated that this genomic mutation can contribute to specified traits in yeast. For example, Stambuk and colleagues [25] confirmed that amplification of the genes involved in the biosynthesis of vitamins B6 and B1 conferred an ability to grow more efficiently in certain bioethanol yeast strains in industrial environments; and Chang et al. [9] reported that several copper-tolerant *S. cerevisiae* strains isolated from the natural environment show segmental duplications in chromosomes 7 and 8 of their genomes. These findings prompted us to develop the present breeding strategy based on genome reconstruction (combination of MBC treatment c and meiosis) to effectively change the traits of yeast (Fig. 1). After genome reconstruction, an initial screening process was designed to aid the selection of mutants with desired traits. The mutants were first treated with high concentration of ethanol because this stressor can greatly affect the viability of yeast cells and thereby cause sluggish fermentation. Then, the viable mutants were spread onto YPD plates with certain stressors, including ethanol, vanillin, and $CuSO_4$. Although the content of cellular ergosterol has been proven to affect tolerance to ethanol [4] and vanillin of yeast [13], $CuSO_4$ was suggested to be more suitable as the selective pressure to obtain mutants with higher ergosterol content [57 % mutants (20 out of 35) picked from $CuSO_4$ plates had a higher ergosterol content than ZTW1; Fig. 2]. We chose $CuSO_4$ in this work because we found that a correlation existed between copper tolerance and ergosterol content in yeast cells (unpublished data). Finally, a total of 24 mutants (8 from the ethanol plates, 7 from vanillin plates, and 9 from $CuSO_4$) with improved ethanol and ergosterol productions were obtained. Overexpression of certain enzymes in the ergosterol biosynthesis pathway has been reported in previous studies to improve ergosterol production by 20–50 % in yeast strains [14, 21, 30]. Compared with genetic engineering approaches, our strategy was more effective in changing the ergosterol content between the mutants and original strain because certain strains (such as ZG27) obtained in study produced 90 % more ergosterol than ZTW1 under the growth condition (Fig. 2d). The mutant strains obtained

from this nongenetic engineering breeding approach can also be applied in countries that do not permit the use of strains with recombinant DNA molecules for industrial production. Using PFGE and aCGH analysis, we confirmed that our breeding method could cause large-scale chromosomal rearrangements in yeast cells, yielding mutants with diverse genomic structures compared with their parent strain (Fig. 4). Compared with ZTW1 genome, ZG27 genome showed a different copy number of ~811 kb regions located on chromosomes 3, 11, and 14. Some modifications of genome ZG27 are specific ally located on those regions in ZTW1 that already showed differences with a euploid strain, but certain novel genomic structural variations (e.g., decreased copy of chromosome 3) were new events that appeared on ZG27 genome (Fig. 4b).

Using iTRAQ technology, we detected a total of 298 differently expressed genes at the protein level in ZG27 compared with its parent strain ZTW1 (fold change >0.5; $P < 0.05$). Pathway enrichment analysis (based on KEGG database; http://www.genome.jp/kegg/) revealed that these DEGs were mapped onto many metabolism pathways, and Table 1 shows the 11 enriched pathways. These results indicate that the gene expression pattern and physiological metabolism of ZTW1 had been modified to a large extent after genomic reconstruction. Specifically, some genes (*HMG1*, *ERG1*, *ERG9*, and *ERG11*) involved in ergosterol biosynthesis pathway showed significantly higher expression in ZG27 than in ZTW1 at both mRNA and protein levels (Fig. 6). Besides, genetic manipulations confirmed that the overexpression of *ERG1*, *ERG9*, and *ERG11* not only enhanced the ergosterol content in ZTW1, but also the ethanol resistance of this strain (Fig. 7). These results suggested that the up-regulation of these genes in ZG27 contributed to its improved traits (higher ergosterol content and ethanol resistance), consistent with a previous finding that ergosterol exerts a protective effect when yeast encounters ethanol stress [1, 27, 37]. Although large-scale genomic structural variations were observed between ZTW1 and ZG27, the regions with genes *HMG1*, *ERG1*, *ERG9*, and *ERG11* had not been covered. This result suggests the changed gene expression of these genes in the two strains that were caused by indirect regulation effect rather than gene dosage changes. Recently, Caspeta et al. [16] reported that altered sterol composition, from ergosterol to fecosterol (caused by mutations in gene *ERG3*), renders *S. cerevisiae* thermotolerant. This work and our findings suggest alteration of plasma membrane component might be a common evolutionary strategy for yeast to response to a changed environment. In addition to ergosterol, other altered physiological factors may also affect the ethanol resistance of ZG27 because this trait involves multiple metabolic processes or pathways, including trehalose metabolism [20, 31], antioxidative system [5], and membrane lipid composition [15, 30].

To the best of our knowledge, this work was the first one on the development of an approach to obtain yeast mutants that can enhance the productions of both ethanol and ergosterol. In addition to obtaining certain strains with potential application in the co-production of bioethanol and ergosterol, we also suggested that genomic reconstruction serves as an important molecular basis of altering the global transcription pattern and physiological status of yeast cells, thereby altering their traits for industrial application.

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