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Improvement of oxidative stress tolerance in *Saccharomyces cerevisiae* through global transcription machinery engineering

Hongwei Zhao · Jingyuan Li · Beizhong Han · Xuan Li · Jingyu Chen

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Abstract Excessive oxidative stress poses significant damage to yeast cells during fermentation process, and finally affects fermentation efficiency and the quality of products. In this paper, global transcription machinery engineering was employed to elicit *Saccharomyces cerevisiae* phenotypes of higher tolerance against oxidative stress caused by H_2O_2. Two strains from two plasmid-based mutagenesis libraries (Spt15 and Taf25), which exhibited significant increases in oxidative stress tolerance, were successfully isolated. At moderate H_2O_2 shock ($\leq$3.5 mM), a positive correlation was found between the outperformance in cell growth of the oxidation-tolerate strains and H_2O_2 concentration. Several mutations were observed in the native transcription factors, which resulted in a different transcriptional profile compared with the control. Catalase and superoxide dismutase activities of the two mutants increased under H_2O_2 stress conditions. Fermentation experiments revealed that the mutant strain taf25-3 has a shorter lag phase compared to the control one, indicating that taf25-3 had improved adaptation ability to H_2O_2-induced oxidative stress and higher fermentation efficiency. Our study demonstrated that several amino acid substitutions in general transcription factors (Spt15 and Taf25) could modify the cellular oxidation defense systems and improve the anti-oxidation ability of *S. cerevisiae*. It could make the industrial ethanol fermentation more efficient and cost-effective by using the strain of higher stress tolerance.

Keywords Oxidative stress · *Saccharomyces cerevisiae* · Transcription factors · Biofuel · Inhibitors

Introduction

Saccharomyces cerevisiae has been used as a primary ethanol-producing microorganism in the food and pharmaceutical industries due to its genetic tractability, utilization of a broad variety of feedstock, and production of numerous valuable products [7]. Meanwhile, ethanol produced from renewable resources such as lignocelluloses is considered to be a cost-effective and reproducible alternative to fossil fuels [22]. In this sense, *S. cerevisiae*, the preferred microorganism for ethanol production from lignocellulosic biomass, has become increasingly important. However, fermentation imposes a number of stresses on yeast cells, including substrate and/or product inhibition, heat shock, oxidative stress, osmotic stress, and exposure to toxic molecules and byproducts [8, 23]. Among these stress factors, oxidative stress was suggested to be the most severe one that affects fermentation performance. Oxidative stress arises from an imbalance between

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H. Zhao · B. Han · J. Chen (✉)
College of Food Science and Nutritional Engineering, China Agricultural University, No. 17 Tsinghua East Road, Haidian District, PO Box 398, Beijing 100083, China
e-mail: chenjy@cau.edu.cn

H. Zhao · X. Li
Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN, USA

H. Zhao · X. Li
Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN, USA

J. Li
College of Food Science and Engineering, Qingdao Agricultural University, Qingdao, China

generation and elimination of reactive oxygen species (ROS), such as hydroxyl radical ($^{\cdot}OH$), super-oxide anions ($O_2^{\cdot-}$), and non-radical reactive species, which includes hydrogen peroxide (H_2O_2) and singlet oxygen [26]. ROS pose significant damage to cellular constituents including DNA, lipids, and proteins, and to cellular redox balance [20]. It has been testified that active dried yeast (ADY) may encounter oxidative stress due to the intracellular accumulation of ROS during the ADY-making procedure and that the sensitivity of yeast to air-drying stress is correlated with its oxidative stress tolerance [25]. It has also been reported that during must fermentation wine yeast are subject to oxidative stress as a result of the synergistic activities of nutritional and environmental stress factors [6]. To solve these problems, a strain that displays a phenotype of higher tolerance to ROS would be highly desirable for the economic feasibility of industrial-scale ethanol production.

Cellular responses to oxidative stress are controlled by multiple genes that are widely distributed throughout the genome. However, deletion or over-expression of a single gene can hardly reach a global phenotype optimum due to the complexity of metabolic landscapes [3]. Alper and coworkers [4] firstly reported an approach termed "global transcription machinery engineering (gTME)", which allows for global perturbations of the transcriptome to quickly and more effectively optimize phenotypes, and have shown the application of gTME to *S. cerevisiae* for improved glucose/ethanol tolerance by mutating the components of the RNA polymerase II (RNA Pol II) transcription factor D (TFIID), namely TATA-binding protein (*SPT*15) and one of the TATA-binding protein-associated factors (*TAF*25). The underlying principle of gTME involves establishing mutant libraries of transcription factors, screening for phenotypes with desired properties, and iterating the process in a directed evolution manner [15].

gTME has successfully been applied to elicit new cellular phenotypes, resulting in improved stress tolerances, metabolite production, and substrate utilization. For example, Liu et al. [17] recently increased the xylose metabolism, tolerance, and adaptation of *S. cerevisiae* to corn cob acid hydrolysate, and Yang et al. [28] mutated the factor *SPT*15 and obtained five strains with enhanced ethanol tolerance (ETS1–5) of *S. cerevisiae* on rich media. To the best of our knowledge, however, the application of gTME in developing oxidation-tolerant strains has not been reported. In this work, transcription factors *SPT*15 and *TAF*25, which encode TBP and Taf(II)25, respectively, were subject to random mutagenesis by using error-prone PCR and transformed into *S. cerevisiae* BY4741 to obtain strains tolerant to ROS.

Materials and methods

Strains and culture methods

S. cerevisiae strain BY4741 (*MATa*; *his3*Δ*1*; *leu2*Δ*0*; *met15*Δ*0*; *ura3*Δ*0*) used as the host strain and template for PCR amplification was obtained from Invitrogen Co., Carlsbad, CA, USA. *E. coli* DH5α competent cells (Takara Bio. Inc., Japan) were used as a host for preparation of the recombinant plasmid. YPD medium (10 g/l yeast extract, 20 g/l Bacto Peptone, and 20 g/l glucose) was used for the cultivation of the BY4741 wild-type strain and SC-URA medium (6.7 g/l yeast nitrogen base, 20 g/l glucose, and a mixture of appropriate nucleotides and amino acids without URA) was used for selection and growth of transformants. LB medium containing 100 μg/ml of ampicillin and SOC medium (2 % Bacto-tryptone, 0.5 % yeast extract, 0.05 % NaCl, 0.0186 % KCl, 0.0952 % $MgCl_2$, 0.3603 % glucose) was prepared for the routine cultivation and transformation of *E. coli*. Other chemical reagents used in this experiment are domestic productions.

The BIO-RAD Gene Pulser Xcell Total System #165-2660 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for electroporation of *E. coli* DH5α and *S. cerevisiae* BY4741 in accordance with the operation manual.

Library construction

The wild type of *SPT15* and *TAF25* fragments were PCR-amplified from genomic DNA properly and cloned into pZHW4, which was obtained from our previous studies. It contains a strong constitutive promoter *ADH1* and *CYC1* terminator and the selection marker genes of *URA3* and ampicillin resistance gene.

Error-prone PCR was performed for the fragment mutagenesis via an optimized reaction and mutated fragments were purified and were digested for 2 h at 37 °C using *EcoR* I and *Xho* I for *SPT15* mutants and *Hind* III and *EcoR* I for *TAF25* mutants (NEB, New England Biolabs Beijing LTD, Beijing, China) and ligated for 30 min at 23 °C to pZHW4 plasmid digested properly. The two mutant libraries were transformed into *E. coli* DH5α and plated onto LB-agar plates containing 100 μg/ml of ampicillin, cultivated for 12–16 h at 37 °C. The whole forming colonies were scraped off to create a liquid library and the total size for each of the two libraries was approximately 10^5 cfu/ml.

Plasmids were isolated using a E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek, Norcross, GA, USA) and transformed into *S. cerevisiae* strain BY4741 using electrotransformation method. Yeast transformation mixtures

were plated on a total of 10–φ150 mm dishes containing SC-URA agar medium for each of the two libraries (one for *SPT15* and one for *TAF25*). These transformants were scraped off the plates and placed into a liquid SC-URA suspension for phenotype selection. In order to eliminate the effects of the blank plasmid (those not expressing either the *SPT15* or the *TAF25*) and overexpression of the wild-type protein (either *SPT15* or *TAF25*), the control strains that harbored the unmutated version of either the Spt15 or Taf25 protein and the blank plasmid were constructed and named as C1, C2, C3, respectively. The plasmids of the mutant strains were sequenced and aligned using NCBI online BLAST service.

Phenotype selection

The screening procedure of mutant strains was performed as previously described [4]. Samples from the liquid libraries were placed into SC-URA medium containing elevated H_2O_2 concentration from 2.0 to 2.5 mM for the accumulation of the tolerant strains. Following this selection phase, these mixtures were plated onto SC-URA plate to ensure single-colony isolation with proper diluting. Six mutants of each of the two libraries were selected and re-numbered and growth rate were measured.

Growth yield assays

Mutant and control strains were grown overnight in 10 ml of culture volume in an 18 × 180-mm culture tube with cap. Medium containing six of the following conditions: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 mM H_2O_2 were dispensed in 10-ml aliquots into 18 × 180-mm culture tubes with caps. The initial inoculation was $OD_{600} = 0.05$. Strains were cultivated by placing the tubes vertically at 28 °C with 200 RPM orbital shaking for 18 h, and then the tubes were vortexed and cell densities were measured by taking optical density at 600 nm.

Spot assay

To test the tolerance of mutants and wild-type strain toward extremely high H_2O_2 stress, shock experiments were performed with much higher concentrations of H_2O_2. Overnight cultures were prepared and adjusted to an OD_{600} value of 1.0. The H_2O_2 was added to the cultures to a final concentration of 2.0 and 6.0 mM, which represented moderate and extreme stress. The cultures were treated in PBS buffer (pH = 7.4) with the above H_2O_2 stress at 28 °C for 2 h and then serially diluted and 5 μl of each dilution was dropped on SC-URA plates. After 2 days of incubation at 28 °C, plates were photographed.

Evaluation of intracellular ROS level using confocal microscopy

Intracellular ROS level was determined as described by Allen et al. [2] with slight modification. Cells were cultured overnight and adjusted to the same cell concentration of ~10^6 cells/ml (OD_{600} ~ 0.08–0.20). The cells were challenged by 10.0 mM H_2O_2 stress in PBS buffers (pH = 7.4) for 2 h and then stained using ROS monitor probe kit (DCFH-DA, Beyotime Institute of Biotechnology, Shanghai, China) as per the operation instruction. DCFH-DA is a nonpolar dye, converted into the polar derivative DCFH by cellular esterase that are no-fluorescent but switched to highly fluorescent DCF when oxidized by intracellular ROS and other peroxides. The positive control was treated with ROSup reagent (affiliated to the ROS monitor probe kit) for 30 min at 28 °C, then washed twice with PBS buffer and stained as the above operations. The negative control was stained directly without any stress factors. All of the operations were done fast and the treated cells were kept in the dark before immediate detection in order to prevent the fluorescence quenching.

While the cells were prepared, the laser scanning confocal microscope (Leica TCS SP5, Mannheim, Germany) was used to measure intracellular ROS level of yeast cells (excitation, 488 nm; emission, 525 nm). After sufficient vertex, 10 μl of each dilution were added to the glass slide and covered by the coverslip immediately before performing the confocal microscope analysis. For the quantification of the fluorescence, the confocal images were analyzed using Leica Application Suite Software V 1.3.1. Briefly, the relative fluorescence intensity from every treated group was semi-quantified by calculating average pixel intensity in green channel. The final value of each measurement presented its average (± standard deviation, SD) from three separate replications.

Measurement of enzyme activities

Catalase (CAT) and superoxide dismutase (SOD) activities were determined by using the catalase assay kit and the total superoxide dismutase assay kit with NBT, respectively (Beyotime Institute of Biotechnology, Shanghai, China). Yeast cells were harvested at the log phase and treated in PBS buffer (pH = 7.4) with 0 and 2.0 mM H_2O_2 for 5 h at 28 °C. After centrifugation at 4 °C, the cell pellets were washed twice with PBS buffer and then re-suspended in PBS buffer. The MiniBeadbeater-16 (BioSpec Products Inc., Bartlesville, OK, USA) was used for the cell disruption. To avoid possible thermal effects on the enzymes, the cell suspensions were placed in 2-ml screw-cap microvials containing 1 ml of small cold glass beads (0.5 mm in diameter, Sigma) and violently agitated for 45 s and then cooled down for 1 min in an ice bath (in total five cycles).

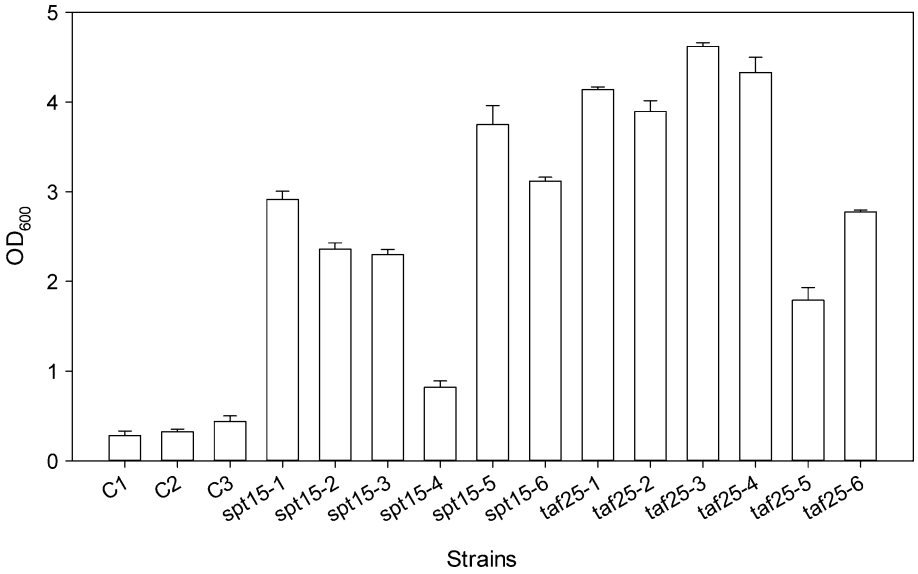

Fig. 1 Isolation of oxidation-tolerant mutants. C1: strain harboring un-mutated chromosomal copy of Spt15; C2: strain harboring un-mutated chromosomal copy of Taf25; C3: strain harboring the blank pZHW4 plasmid. Other strains are the mutants. All of the control and mutants were cultured in 2.5 mM H_2O_2 containing SC-URA medium at 28 °C for 17 h. Mean ± SD values calculated from three independent experiments are presented

Fermentation

To test the fermentation characteristics of the mutant strain taf25-3 and control strain C3, a 500-ml scale shake flask test was performed to simulate the fermentation environment. The inoculum was pre-cultured twice before the fermentation. The initial inoculation was $OD_{600} = 0.1$ in the 250-ml medium containing 4.0 mM H_2O_2. Fermentations were run in biological replicates for 96 h and samples were taken every 12 h for measuring OD_{600}, ethanol, and residual glucose concentrations. The ethanol and residual glucose contents were measured using a Waters 2996 HPLC system (Waters Corp., Milford, MA, USA) consisting of an Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) resin-based column (300 × 7.8 mm), a guard column containing the same material, and a 2414 RI detector. The HPLC was run under isocratic conditions with a flow rate of 0.6 ml/min and a mobile phase of 0.005 M H_2SO_4 at 65 °C.

Statistical analysis

The experiments were conducted in three biological replicates and one-way ANOVA followed by Tukey's HSD test was used to perform statistical analysis. Data were presented as the mean ± SD. A p value <0.05 was considered significant.

Results

Library construction and selection

To select oxidation-tolerant mutants, strains were inoculated with elevated levels of H_2O_2 from 2.0 to 2.5 mM.

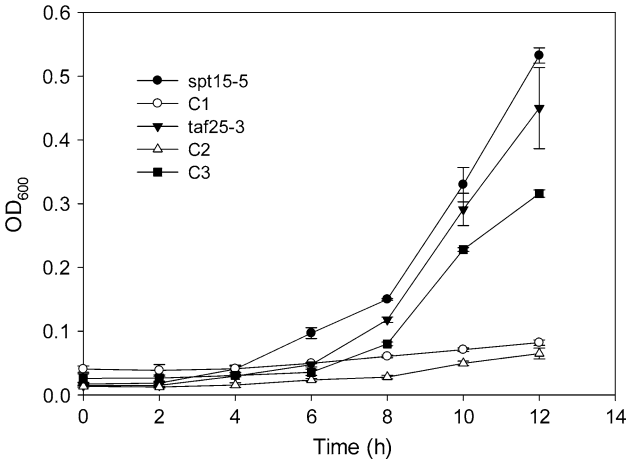

Fig. 2 Growth curves of control groups and oxidation-tolerant mutants. All the strains were cultivated in SC-URA medium with 1.5 mM H_2O_2 stress at 28 °C for 12 h. The OD_{600} values (mean ± SD, $n = 3$) were measured every 2 h

After this challenging cultivation, six mutants were selected for each library. Figure 1 presents the cell concentrations of the control and mutant strains when incubated with 2.5 mM H_2O_2, where C1, C2, and C3 represent the strain harboring un-mutated chromosomal copy of Spt15, un-mutated chromosomal copy of Taf25, and blank pZHW4 plasmid, respectively. As shown in Fig. 1, the growth of both spt15 and taf25 mutants were improved significantly compared to the control strains under oxidative stress, where spt15-5 and taf25-3 showed ten times-higher growth than C1 and C2. These results also verified that the above improvement was attributed to the mutated transcription factors. It was also found that the growth rates of C1 and C2 strains were lower than that of C3. This was again observed in Fig. 2,

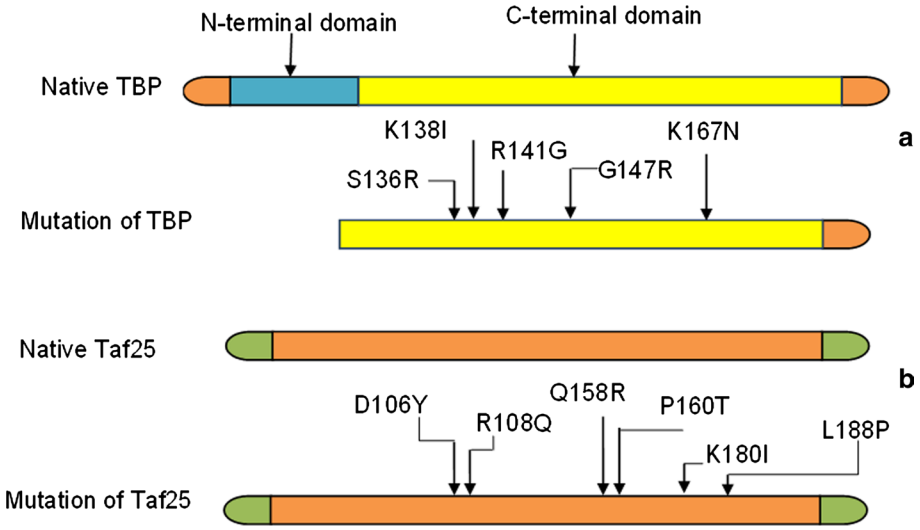

Fig. 3 The location of mutations in TBP and Taf25 proteins in spt15-5 strain (**a**) and taf25-3 strain (**b**). The mutated TBP in spt15-5 strain showed a truncated factor containing five mutations in the C-terminal conserved region

which describes the time courses of growth of the control and mutated strains in SC-URA medium with 1.5 mM H_2O_2 for 12 h. During the cultivation, the cell concentrations of the two mutants increased significantly, while the growth of C1 and C2 strains were shown to be repressed. This could be attributed to the oxidation and overexpression of the endogenous, unmutated chromosomal copy of Spt15 and Taf25, which might increase the burden for yeast cells. Therefore, in the following section, the C3 strain harboring the blank plasmid was chosen as the control group.

The sequence characteristics of these altered genes conferring the improved phenotype are shown in Fig. 3 and online resources. For spt15-5, there occurred 24 site mutations and three gaps throughout the whole sequence (shown in online resource 1). Interestingly, these mutated sits and gaps caused the formation of a truncated factor (lost N-terminal domain), which could increase the overall oxidation tolerance significantly (Fig. 3a). The truncated factor remained the DNA interaction surface on C-terminal conserved domain of TBP_eukaryotes. Five mutation sites, in which serine was substituted for arginine (S136R), and similarly, K138I, R141G, G147R, K167N, respectively, were labeled in the schematic in Fig. 3a. For taf25-3, seven site mutations without gaps resulted in six amino acid replacements, including D106Y, R108Q, Q158R, P160T, K180I, and L188P (shown in Fig. 3b and online resource 2).

Figure 4 illustrates the performance of spt15-5 and taf25-3 over a wide range of H_2O_2 concentrations. The results suggest that both spt15-5 and taf25-3 mutants outperformed the control at all H_2O_2 concentration tested. At low (<2.0 mM) and high (>3.5 mM) H_2O_2 concentrations, the growth of the mutants was comparable to that of the control. In contrast, the mutants showed considerably enhanced growth over the control strain when the H_2O_2 concentration was between 2.0 and 3.5 mM, where the

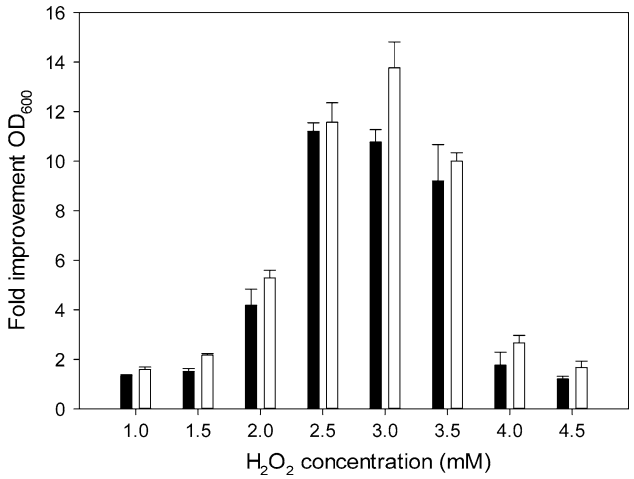

Fig. 4 Fold improvement in OD_{600} values for spt15-5 (*black bar*) and taf25-3 (*white bar*) compared with control at different H_2O_2 concentrations. Mutants and control strains were grown overnight in 10 ml of SC-URA medium in an 18 × 180-mm culture tube with a cap and then subcultured in elevated levels of H_2O_2 concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mM) with an initial inoculation of OD_{600} = 0.05 for 18 h at 28 °C. All data represent the mean values ± SD from three independent experiments

maximum fold improvement OD_{600} values were 11.2 for spt15-5 at 2.5 mM and 13.76 for taf25-3 at 3.0 mM.

Spot assay

To determine the cell vitality at very high H_2O_2 concentrations, H_2O_2 shock experiments were performed with 2.0 and 6.0 mM H_2O_2, which respectively represented moderate and extreme stress to cells in this study. The results are illustrated in Fig. 5. After shocking with 2.0 mM H_2O_2 for 2 h, around 100-fold mutant cells survived compared with the control; under the extreme stress treatment, about

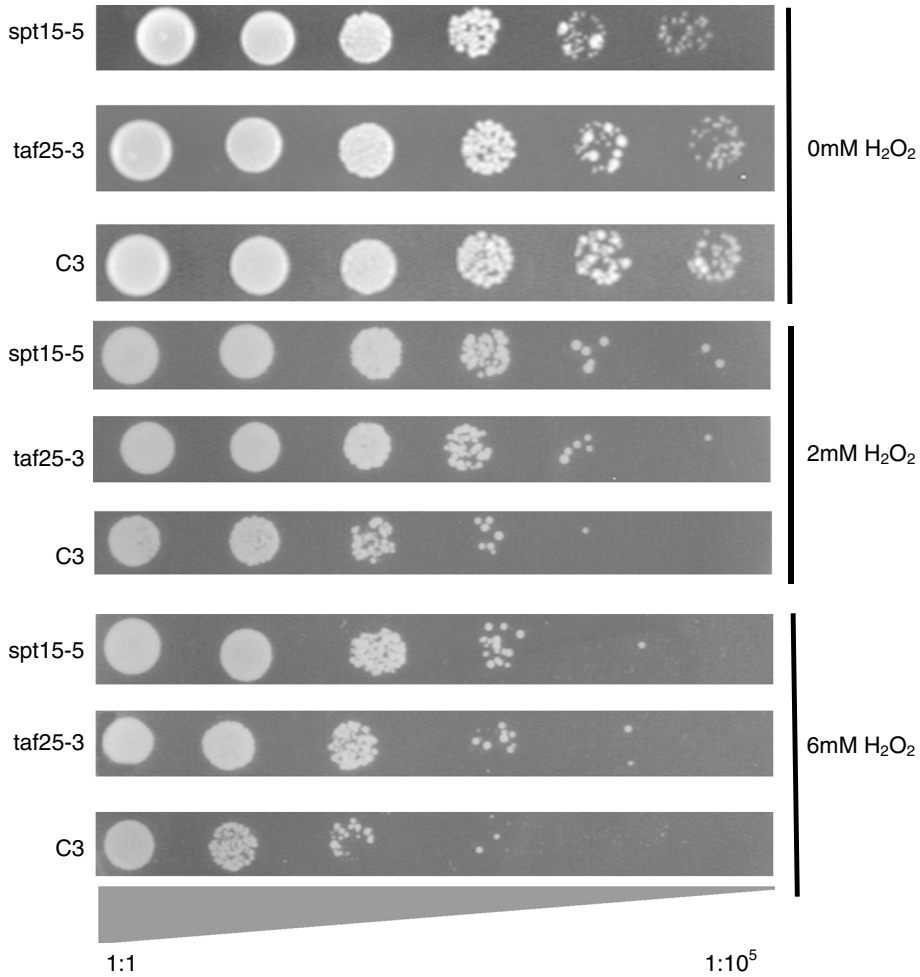

Fig. 5 H_2O_2 sensitivity assay. The viabilities of the strains were tested after shocking with 0, 2.0, and 6.0 mM H_2O_2 for 2 h at 28 °C. The *triangle* below the panel indicates a tenfold serial dilution of plated cells ($1:1–1:10^5$, *from left to right*)

tenfold mutant cells survived comparatively. These results indicated that the oxidation-tolerant mutants were far more robust than the control strain in both moderate and extreme oxidative stress caused by H_2O_2.

Intracellular ROS assay of mutants

In various microorganisms, the intracellular ROS level increases significantly when the cells are challenged by oxidative stress or toxic substances [11], indicating that the intracellular ROS level can be used as an indicator of cellular-tolerant capacity toward oxidation particles. To test if the mutants that performed higher oxidation tolerances could decrease intracellular ROS level under oxidation conditions, intracellular ROS level assay was performed. Figure 6a shows the images of dyeing cells of the mutants, wild-type, positive, and negative controls. The positive control was used to optimize the parameters of the confocal microscope. After obtaining a clear fluorescent image of the dyeing cells, the whole parameters were fixed and the ROS level for each sample was then measured. The negative control emitting weak fluorescence indicated a very low ROS level in yeast cells without H_2O_2 stress treatment. To semi-quantify the intracellular ROS level, the fluorescent cells were circled on the images by using the Leica Application Suite Software V 1.3.1. For each sample, more than 100 cells were examined. Figure 6b shows the relative fluorescence intensity of these samples. The cells harboring blank plasmid accumulated 52.86 and 89.36 % more intracellular ROS compared to spt15-5 and taf25-3, where the relative fluorescence intensity was 8.56. The decreased relative fluorescence intensity in spt15-5 and taf25-3 mutants indicated that ROS was removed significantly in spt15-5 and taf25-3 cells ($p < 0.05$).

ROS buffering abilities of mutant strains

To determine whether the differences seen in the intracellular contents of ROS were due to differences between the ROS buffering abilities of mutants and control strains, the activities of the scavenging enzymes including catalase (CAT) and superoxide dismutase (SOD), which are regarded as the first-line defense in response to oxidative stress, were monitored. Table 1 shows the CAT and SOD activities of all the samples treated with or without H_2O_2 stress. No significant

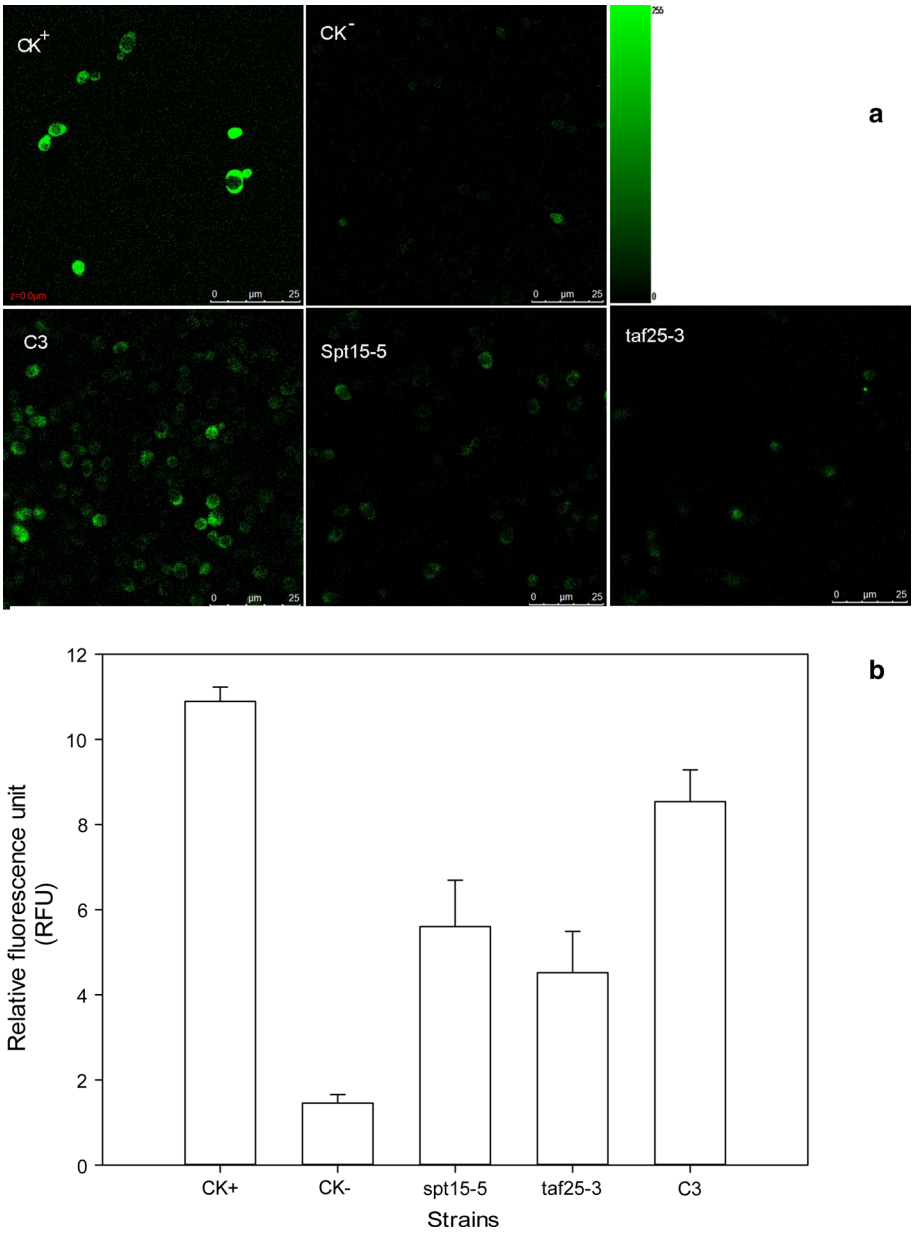

Fig. 6 Intracellular ROS assay of mutants and control. Cells were cultured overnight and adjusted to the same cell concentration of ~10^6 cells/ml (OD_{600} ~ 0.08–0.20). CK^+ represents the positive control that was treated with ROSup reagent and then stained. CK^- represents the negative control that was stained directly without any stress factors. **a** The fluorescence images were obtained by using Leica TCS SP5 (excitation, 488 nm; emission, 525 nm). **b** Suite Software V 1.3.1 was applied to semi-quantify the intracellular ROS level, and values are presented as the mean ± SD of three independent experiments

differences were found in CAT and SOD activities between the mutant strains and control strain treated without H_2O_2 stress. However, a significant increase of CAT and SOD activities was observed in spt15-5 and taf25-3 strains compared with the control strain ($p < 0.05$). Besides, the SOD activity of taf25-3 strain was higher than that of the spt15-5 strain, suggesting that the taf25-3 strain possessed more robust ROS buffering capacity than the spt15-5 strain.

Comparison of the fermentation capacity between taf25-3 and C3

Since the strain taf25-3 showed the highest resistance to high-level H_2O_2-induced oxidative stress, laboratory-scale fermentation experiments using taf25-3 and C3 strains were carried out under micro-aerobic conditions. Although the final yields of ethanol were approximately equal, as shown in Fig. 7b, different fermentation behaviors were observed for taf25-3 and C3 over time. At the beginning, both strains grew very slowly due to the H_2O_2-induced oxidative stress. However, a rapid increase in cell mass was found at 24 h for taf25-3, which was 12 h earlier than that of the control strain. After that, the two strains achieved the maximum biomass at 84 h without significant differences ($p > 0.05$) (Fig. 7a).

For ethanol production and glucose consumption profiles, the taf25-3 strain also showed great improvement compared to the control. To evaluate the effect of the H_2O_2

Table 1 CAT and SOD activities of mutants and control

Strains	CAT activity (U/mg prot.)	SOD activity (U/mg prot.)
0 mM H_2O_2 treatment		
spt15-5	$17.52^{a} \pm 2.84$	$140.53^{a} \pm 7.90$
taf25-3	$15.80^{a} \pm 1.54$	$142.80^{a} \pm 3.95$
C3	$15.55^{a} \pm 2.52$	$140.43^{a} \pm 3.72$
2.0 mM H_2O_2 treatment		
spt15-5	$7.67^{a} \pm 1.25$	$128.24^{a} \pm 4.40$
taf25-3	$6.84^{a} \pm 0.05$	$155.36^{b} \pm 4.55$
C3	$4.97^{b} \pm 0.43$	$92.65^{c} \pm 3.31$

Yeast cells were harvested at the log phase and treated in PBS buffer (pH = 7.4) with 0 and 2.0 mM H_2O_2 for 5 h at 28 °C; and then cells were disrupted using MiniBeadbeater-16 (Biospec, USA) under low-temperature conditions. Results are the mean ± SD of three independent experiments

Superscript lowercase letters represent the significant difference at $p < 0.05$ level

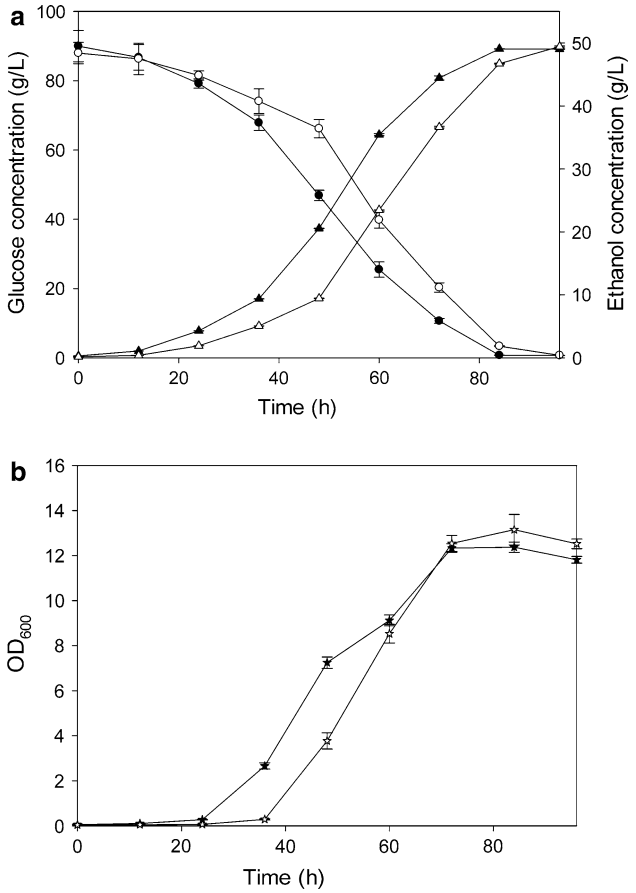

Fig. 7 Time course profiles for the ethanol fermentation by taf25-3 and C3 in the presence of 4.0 mM H_2O_2 under micro-aerobic conditions: **a** growth curve (*pentagram*); **b** glucose consumption curve (*circle*) and ethanol production curve (*triangle*). Mutant strain taf25-3 (*black symbols*), control strain C3 (*white symbols*). Results are the mean ± SD of three independent experiments

Table 2 Comparison of fermentation abilities in experimental strains

	taf25-3	C3
Glucose consumption rate (g glucose l^{-1} h^{-1})	$1.76^{a} \pm 0.03$	$1.35^{b} \pm 0.04$
Ethanol metabolic yields	$0.96^{a} \pm 0.01$	$0.97^{a} \pm 0.02$
Ethanol volumetric production rate (g ethanol l^{-1} h^{-1})	$0.64^{a} \pm 0.03$	$0.51^{b} \pm 0.02$

Glucose consumption rate was calculated by determining the slope of the steepest portion of the glucose concentration curve; ethanol metabolic yields was calculated by dividing the observed ethanol concentrations by the theoretical ethanol concentrations (0.51 times the total concentration of consumed glucose); ethanol volumetric production rates were calculated by dividing the maximum ethanol concentration by the fermentation time required to reach that concentration. Values listed are the mean ± SD of three independent experiments

Superscript lowercase letters represent the significant difference at $p < 0.05$ level

on ethanol production, ethanol metabolic yields and ethanol volumetric production rates were calculated (Table 2). No significant differences were found in the yields of ethanol between the two strains under H_2O_2-induced oxidative stress; they all achieved approximately 96 % of the theoretical yield. However, from 24 h, the taf25-3 took a 12-h lead in ethanol production compared with the control strain until 72 h fermentation, when the ethanol concentration reached 44.45 g/l for taf25-3 and 36.65 g/l for the control, corresponding to a 25.5 % increase in ethanol volumetric productivity. This trend was also confirmed by glucose consumption, which was shown to be significantly faster for taf25-3 (1.76 ± 0.03 g glucose l^{-1} h^{-1}) compared to the control strain (1.35 ± 0.04 g glucose l^{-1} h^{-1}), as calculated in Table 2.

Discussion

In recent years, gTME has mainly been applied in the research of improving ethanol tolerance of both *E. coli* and *S. cerevisiae* [5, 28]. However, oxidation stress is also considered a serious problem associated with ethanol fermentation in that the presence of ethanol and other inhibitors could trigger oxidation stress, especially in hypoxic fermentation of a high-sugar-containing medium [14]. Therefore, in this study we employed gTME method to improve the oxidation tolerance of *S. cerevisiae* BY4741 strain. We used a different plasmid, pZHW4, which was constructed based on the main frame of pYES2 plasmid in which the *GAL1* promoter was replaced by *ADH1* promoter to carry on the mutated transcription factors. Two mutants, spt15-5 and taf25-3, were isolated and examined for oxidation tolerance (Fig. 2). As expected, both mutants exhibited improved performance under oxidative conditions. This

indicated the flexibility and applicability of gTME in altering complicated genotype–phenotype interactions. In other words, the mutation libraries constructed in this study could be further used for screening strains with enhanced tolerance to other stresses such as biomass hydrolyses inhibitors, heat shock, osmotic pressure, and so on.

The sequence characteristics of the altered *SPT15* and *TAF25* genes conferring improved properties were shown in online resource 1 and 2. Several mutations occurred in both mutated genes, with those of spt15-5 localized to the C-terminal core of TBP (~180 residues), in which the region is highly conserved and contains two 77-amino-acid repeats that produce a saddle-shaped structure that straddles the DNA; this region is responsible for the initiation by binding to the TATA box and interacting with transcription factors and regulatory proteins [13, 24]. Due to the random mutations in SPT15 gene, the TBP protein was truncated. The N-terminal region was lost but 11 residues that compose the C-terminal core region remained. From the improved phenotype of spt15-5 mutant, it is not difficult to deduce that the truncated TBP protein not only maintained the capacity binding to the RNA polymerase I, II, and III core enzyme and TATA box, but increased the binding affinity and efficiency during the initiation period of transcription by RNA polymerases I, II, and III. Taf25, also known as Taf10 or Taf23, is the TATA binding protein (TBP) associated factor. It is one of several Tafs that bind TBP and is involved in forming the transcription factor IID (TFIID) complex, which plays an important role in the recognition of promoter DNA and the assembly of the preinitiation complex [27]. Mutations located in Taf25 protein can also perform a novel interaction that prevails over the wild-type, endogenous ones. Although it was difficult to analyze the mechanism by which spt15-5 and taf25-3 altered the transcription profiles from the sequence data, the sequence-specific observations did not affect the main results presented here: the successful genetic engineering method for improving the oxidation tolerance in yeast from the transcription factors mutant libraries.

ROS buffering ability plays an important role in yeast cells' oxidation-stress tolerance. CAT and SOD are involved in the first defense systems that scavenge excessive ROS [9]. The ROS scavenging ability could be reflected by the intracellular ROS content. Laser confocal microscopy was used for intracellular ROS content detection at the single-cell level (Fig. 6). The green fluorescence intensity varies according to the mutants and control. In both the spt15-5 and taf25-3 strains, the intracellular ROS level is significantly lower than the C3 strain. This indicates that the ROS buffering system was more efficient in spt15-5 and taf25-3 than that in C3. CAT and SOD are the two important enzymes that protect cells from oxidation stress. It has been reported that overexpression of the genes *SOD1* or *SOD2* could improve the activity of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase and then result in an increase of cell viability in yeast [12]. In our research, the mutants displayed higher CAT and SOD activities, which could partially explain the reduced ROS level and improved viability of these strains.

Our results also suggest a stimulation mechanism associated with the oxidation defense system. At a very low H_2O_2 concentration, the growth rates of spt15-5 and taf25-3 were almost equal to that of the control strain. Similarly, as reported by Alper et al. [4], the basal growth rate of *spt15-300* and *taf25-300* in the absence of ethanol and glucose stress was not different from that of the control. It was also noticed that the activities of CAT and SOD were not increased when no stress was applied (Table 1). Therefore, we can conclude that the stress defense system needs the stimulation of some stress factors to work as a stress scavenger.

The fermentation abilities of the taf25-3 strain and C3 strain were also evaluated in laboratory-scale fermentation. During fermentation, the mutant strain taf25-3 showed a more powerful adaptive ability to the oxidation stress conditions (Fig. 7a), which could be confirmed from the significant increases in ethanol productivity and glucose consumption rate (Table 2) compared to the control strain C3. Yeast adaptation to inhibitor-induced stresses is manifested at the genome level and likely during the lag phase. The duration of the lag phase may be interpreted as a measurement of varied levels of tolerance to inhibitors during ethanol fermentation [18, 19]. This process could include the repair of macromolecule damage that accumulated during stationary phase and the synthesis of cellular components necessary for growth and great changes could be observed in the gene transcription expresses in the cell [10]. Ma and Liu [21] reported that more than 300 genes, including *YAP1*, *YAP5*, *YAP6*, and *HSF1*, showing statistically significant differential expression responses that affect yeast adaptation ability to the inhibitor stresses potentially during the lag phase. The Yap proteins family has been proven to play a major role in the regulation of the transcriptional responses to oxidative stress in yeast [16]. Yap 1p and Yap 2p could active the target genes *CTT1*, *SOD1*, *SOD2*, encoding catalases and superoxide dismutase regarded as the important enzyme defense systems when the cells were exposed to oxidative stress [1]. The mutant strain taf25-3, in our study, was testified to maintain higher CAT and SOD activities than the control one (Table 1); therefore, it could withstand the H_2O_2-induced oxidative stress more effectively and enter the log phase earlier (Fig. 7). That is, the higher oxidative stress-tolerant strain could shorten the fermentation time and therefore make the ethanol production more cost-effective. Our research demonstrated the applicability of gTME to improve the oxidation tolerance

capacity in yeast cells. An attempt of further modification of other transcription factors through gTME could have the potential to obtain other phenotype improvement.

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