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Identification of novel genes responsible for ethanol and/or thermotolerance by transposon mutagenesis in *Saccharomyces cerevisiae*

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Abstract *Saccharomyces cerevisiae* strains tolerant to ethanol and heat stresses are important for industrial ethanol production. In this study, five strains (Tn *1–5*) tolerant to up to 15% ethanol were isolated by screening a transposon-mediated mutant library. Two of them displayed tolerance to heat (42 °C). The determination of transposon insertion sites and Northern blot analysis identified seven putative genes (*CMP2*, *IMD4*, *SSK2*, *PPG1*, *DLD3*, *PAM1*, and *MSN2*) and revealed simultaneous down-regulations of *CMP2* and *IMD4*, and *SSK2* and *PPG1*, down-regulation of *DLD3*, and disruptions of the open reading frame of *PAM1* and *MSN2*, indicating that ethanol and/or heat tolerance can be conferred. Knockout mutants of these seven individual genes were ethanol tolerant and three of them (*SSK2*, *PPG1*, and *PAM1*) were tolerant to heat. Such tolerant phenotypes reverted to sensitive phenotypes by the autologous or overexpression of each gene. Five transposon mutants showed higher ethanol production and grew faster than the control strain when cultured in rich media containing 30% glucose and initial 6% ethanol at 30 °C. Of those, two thermotolerant transposon mutants (Tn *2* and Tn *3*) exhibited significantly enhanced growth and ethanol production compared to the control at 42 °C. The genes identified in this study may provide a basis for the application in developing industrial yeast strains.

Keywords Bioethanol · Transposon · Ethanol tolerance · Thermotolerance

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Introduction

The demand for bioethanol as a sustainable alternative fuel is increasing. The yeast *Saccharomyces cerevisiae* has been widely used in bioethanol industries because of its potent ethanol production and tolerance (Schubert 2006). Yeast cells are usually exposed to several environmental stresses including high ethanol concentration, temperature increments, high osmotic pressure, and inhibitors during industrial ethanol fermentation, eventually resulting in decreased cell growth, viability, and ethanol yield (Casey and Ingledew 1986; Jones 1989; Suutari and Laakso 1994). Thus, yeast strains that can endure various stresses are highly desirable.

One of the most powerful emerging technologies for fuel ethanol production is the consolidated bio-processing (CBP) featuring simultaneous saccharification and fermentation of lignocellulosic biomass in one reactor (Lynd et al. 2005). In CBP, thermotolerant strains are compulsory, since the optimum temperatures for saccharification (45–50 °C) are different from that for fermentation (25–35 °C) (Araque et al. 2008; Weber et al. 2010). The use of thermotolerant yeast strains is also useful in reducing cooling costs, distillation costs, and faster fermentation rates and helps

to decrease the chance of contamination during fermentation. Several reports have addressed the development of thermotolerant strains by using ultraviolet mutagenesis (Unaldi et al. 2002), error-prone polymerase chain reaction (PCR) (Shimoda et al. 2006), and long-term adaptation to high temperatures (Araque et al. 2008).

The use of ethanol-tolerant yeast strains is important in itself for high yield ethanol production, since ethanol concentration continues to increase during fermentation to levels that are detrimental, even lethal, to cells. Several physiological changes occur in ethanol-tolerant yeast strains, including intracellular accumulation of trehalose (Kim et al. 1996), proline (Takagi et al. 2005), and ergosterol (Inoue et al. 2000). To obtain ethanol-tolerant strains, in addition to classic strategies such as isolating new yeasts from natural resources (Araque et al. 2008; Unaldi et al. 2002), evolutionary adaptation (Stanley et al. 2010), random chemical mutagenesis (Mobini-Dehkordi et al. 2008), and genome shuffling (Hou 2010), several strategies that help understand the molecular mechanisms of ethanol tolerance have recently been used. These alternate strategies include genome-wide DNA microarray analysis (Hirasawa et al. 2007), screening of a transposon-mediated deletion mutant library (Takahashi et al. 2001) or single gene knockout (SGKO) collections (Auesukaree et al. 2009; Fujita et al. 2006; Kubota et al. 2004; Teixeira et al. 2009; van Voorst et al. 2006; Yoshikawa et al. 2009), and global transcriptional machinery engineering (gTME) (Alper et al. 2006). Although gTME is intriguing as a means to positively select ethanol-tolerant strains, its usefulness is debatable (Baerends et al. 2009). In contrast, the other three alternate strategies are mostly indirect: a verification step is further required following the identification of target genes, as identification of ethanol-sensitive genes helps to understand the molecular basis of ethanol tolerance, but does not ensure creation of ethanol-tolerant strains. More seriously, a complicating issue concerns the huge number of target genes identified, representing as much as 5–10% of genes encoded in the yeast genome, which is the case for other stressors as well.

A few studies demonstrated that the disruption of a specific gene(s) leads to creation of strains tolerant to several kinds of stressors including ethanol (Kim et al. 1996; Yazawa et al. 2007). More recently, two clones with enhanced growth at 8% ethanol and ten osmotolerant clones were identified by screening of a SGKO library (Yoshikawa et al. 2009). This study, in particular, paved the way for the use of deletion mutant libraries constructed by transposon mutagenesis or SGKO for positive (or direct) isolation of strains tolerant (not sensitive) to a certain stressor. Transposon mutagenesis is a biological process that allows interruption of a host organism's chromosome, causing mutations that may modify the function of an extant gene on the chromosome. Collective mutations constitute a transposon mutant library, which can be easily constructed in strains with various genetic backgrounds. Mutants harboring up- or down-regulated genes can be generated by transposon insertion into regulatory regions, which is particularly important when expression levels matter, as for lethal genes. In the current study, we identified seven novel genes responsible for ethanol and/or thermotolerance by screening a transposon-mediated deletion mutant library based on *S. cerevisiae* L3262.

Materials and methods

Strains, media, and culture conditions

The strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *S. cerevisiae* L3262 and BY4741 were used as the transformation recipient and control for SGKO mutants, respectively. The nonessential haploid *S. cerevisiae* deletion library was kindly obtained from Dr. Wonkee Hur (Seoul National University, Seoul, South Korea) for the verification of identified genes. Unless otherwise mentioned, strains were grown in YPD medium (1% (*w*/*v*) Bacto yeast extract, 2% Bacto peptone, and 2% glucose) at 30 °C. Synthetic complete medium (0.67% (*w*/*v*) Difco yeast nitrogen base without amino acid, and 2% glucose, supplemented with appropriate nutrients) was used for the yeast transformation. For fermentation, the strains were precultured in YPD medium at 30 °C for five generations and then inoculated into 100 ml of YPD 30 medium (1% (*w*/*v*) Bacto yeast extract, 2% *w*/*v* Bacto peptone, 30% *w*/*v* glucose) in shake flasks (500 ml) to an OD_{600} of approximately 0.1. The cells were grown in a reciprocal shaker (120 rpm) at 30 °C or 42 °C. *Escherichia coli* DH5α was used for plasmid construction and grown in Luria–Bertani medium supplemented with 100 mg/l of ampicillin at 37 °C. For ethanol stress treatment, exponentially growing yeast cells were treated with or without 10% ethanol. Cells were collected 30 min after the addition of ethanol.

Transposon mutagenesis

S. cerevisiae genomic library pools with transposon-mediated random insertions of mTn*3*-*lacZ*/*LEU2* were kindly provided by the Yale Genome Analysis Center (New Haven, CT, USA; http://ygac.med.yale.edu/mtn/reagent/avail_reagents/lacZ_LEU2_info_p.stm). The plasmid DNA from pools of the mTn*3*-mutagenized genomic library was digested with *Not*I and used for transformation of *S. cerevisiae* L3262 by the lithium acetate method as previously described (Adams et al. 1997; Burns et al. 1994).

Table 1 Strains, plasmids, and oligonucleotides used in this study

Strain or plasmid		Characteristics or sequence source	Source or reference
Strains			
S. cerevisiae	L3262	*MAT-α; ura3-52 leu2-3,112 his4-34*	Kang et al. (2000), Teixeira et al. (2009); Stratagene
	BY4741	*MAT*-a; *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ura3-52::URA3*	
E. coli	DH5α	F^- *endA1 hisR17 supE44 thi-1* λ^- *recA1 gyrA96 relA1 Δ(lacZYA-argF)U169 F80d lacZ ΔM15*	
Plasmid			
pRS316-GAPDH		pRS316 plasmid with P_{GAPDH}	This study
pRS-GACMP2		pRS316-GAPDH carrying 1.8 kb of ORF (*CMP2* gene)	This study
pRS-GAIMD4		pRS316-GAPDH carrying 1.6 kb of ORF (*IMD4* gene)	This study
pRS-GASSK2		pRS316-GAPDH carrying 4.7 kb of ORF (*SSK2* gene)	This study
pRS-GAPPG1		pRS316-GAPDH carrying 1.1 kb of ORF (*PPG1* gene)	This study
pRS-GAPAM1		pRS316-GAPDH carrying 1.5 kb of ORF (*PAM1* gene)	This study
pRS-GADLD3		pRS316-GAPDH carrying 1.8 kb of ORF (*DLD3* gene)	This study
pRS-GAMSN2		pRS316-GAPDH carrying 2.1 kb of ORF (*MSN2* gene)	This study
pRS316		*Saccharomyces*/*E. coli* plasmid vector for cloning, Ap^r *URA3*	ATCC
pRSCMP2		pRS316 carrying ORF plus promoter and terminator sequences (*CMP2* gene)	This study
pRSSSK2		pRS316 carrying ORF plus promoter and terminator sequences (*SSK2* gene)	This study
pRSPPG1		pRS316 carrying ORF plus promoter and terminator sequences (*PPG1* gene)	This study
pRSPAM1		pRS316 carrying ORF plus promoter and terminator sequences (*PAM1* gene)	This study
pRSDLD3		pRS316 carrying ORF plus promoter and terminator sequences (*DLD3* gene)	This study
pRSMSN2		pRS316 carrying ORF plus promoter and terminator sequences (*MSN2* gene)	This study
Oligonucleotides (sequence 5′ → 3′)			
For overexpression experiments			
CMP2-F		CGCGGATCCATGTCTTCAGACGCTATA	This study
CMP2-R		CGCCTTAAGCTATTTGCTATCATTCTT	This study
IMD4-F		CGCGGATCCATGAGTGCTGCTCCATTG	This study
IMD4-R		CGGGTCGACTCAATTGTATAGACGTTT	This study
SSK2-F		CGCGGATCCATGTCGCATTCAGACTACTTC	This study
SSK2-R		CGGGTCGACCTACTCTCTTTCCTCAGTATC	This study
PPG1-F		CGCGGATCCATGGAATTGGACGAATGT	This study
PPG1-R		CGGGTCGACCTACAAGAAGTAATCAAC	This study
PAM1-F		CGCGGATCCATGACATCTGCATTAAGG	This study
PAM1-R		CGGGTCGACAAAGACAAGGGCTCGCGT	This study
DLD3-F		CGCGGATCCATGACGGCCGCACAT	This study
DLD3-R		CGGGTCGACTCAAATGTACTTGTA	This study
MSN2-F		CGCGGATCCATGACGGTCGACCATGAT	This study
MSN2-R		CGCCTTAAGTTAAAAAAATGGGGTCTA	This study
MSN4-F		GTCGCGACGCAAGAAGATACAGC	This study
MSN4-R		TGAGCCGGAGATGACTTGAGAATG	This study
CTT1-F		CAATACTCCCGTCTTCTTCCTCAG	This study
CTT1-R		GCTTGTTCGGGTGTCATTGTTT	This study
HSP12-F		TGACGCAGGTAGAAAAGGATT	This study
HSP12-R		GTGGACACGACCGGAAACATA	This study
For complementation experiments			
CMP2-FU		CGCGGATCCATGGCTGAGGTTCCTATGTTGTCT	This study
CMP2-FL		CGCGGCCGCCTGCCGGTGCCGATGGTTTA	This study
SSK2-FU		CGCGGCCGCCATGCATATCCCCCACGACTG	This study
SSK2-FL		CGGGTCGACAGCCAACCGCCTCTCAAT	This study
PPG1-FU		CGCGGATCCCGCCCTAAACTTTCCGACCTT	This study
PPG1-FL		CGGGTCGACTCCTGGGCCAACAACGATAGC	This study
PAM1-FU		CGCGGATCCCAAGATGAAACGCGCTGTATG	This study
PAM1-FL		CGGGTCGACCTTCGGCAACGTTTTCAATGG	This study
DLD3-FU		CGCGGATCCCTTTAAAGTGCTATG	This study

Table 1 (continued)

Strain or plasmid	Characteristics or sequence source	Source or reference
DLD3-FL	CGGGTCGACTAGTCGCTTTAAAGT	This study
MSN2-FU	CGCGGATCCCGGCCCGGAACGTACCTAAAG	This study
MSN2-FL	CGCGGCCGCAAGCGCCATATAAAACATTGA	This study

The restriction enzyme sites are underlined.

Ap^r ampicillin resistance, *ATCC* American Type Culture Collection, VA, USA

Isolation of ethanol and/or thermotolerant mutants

For the isolation of ethanol-tolerant mutants, the transformants were replica-plated on YPD agar and YPD agar containing 10% ethanol and incubated at 30 °C for 2 days. The mutants that grew well on both media were selected. They were cultured at 42 °C for 4 days to select for thermotolerant mutants. To confirm the ethanol- and thermotolerant mutations, the selected mutants were grown in YPD medium to an OD_{600} of 1.0 and serially diluted tenfold with sterile water. For ethanol tolerance, aliquots (5 μl) of each dilution were spotted onto ethanol-free YPD agar (control) and agar containing 10%, 12.5%, and 15% ethanol, and then incubated at 30 °C for 1–6 days. For thermotolerance, YPD agar spotted in replica were separately incubated at 30 °C (control) for 1 day and 42 °C for 3–6 days. Mutants that grew well on the ethanol containing YPD agar and/or 42 °C compared to the control strain were concluded to be ethanol and/or thermotolerant mutants.

Identification of disrupted genes

To reveal the disruption sites, genomic DNA fragments containing transposon were rescued as plasmids that were amplified in *E. coli* following the procedures described at http://ygac.med.yale.edu/mtn/reagent/avail_reagents/lacZ_LEU2_info_p.stm. Rescued plasmids were sequenced using a primer derived from the *lacZ* sequence of the inserted transposon (Burns et al. 1994). The genes of transposon insertion were analyzed using the BLAST server of the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

Northern blot analysis

Total RNAs were prepared as described (Adams et al. 1997). DNA fragments for probes were prepared by PCR from L3262 genomic DNA with the appropriate gene-specific primers (Table 1). Purified PCR fragments were radioactively labeled with ^{32}P-dCTP (GE Healthcare, Buckinghamshire, UK) with random primers. *ACT1* gene was used as an internal control. The band intensities were quantified with NIH image J, version 1.61 (National Institutes of Health, Bethesda, MD, USA).

Gene cloning and analytical methods

The open reading frames (ORFs) of identified genes were cloned into pRS316-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for overexpression experiments. The ORFs with their own promoter and terminator sequences were sub-cloned into pRS316 for complementation experiments. Amplified products and vectors were digested with an appropriate restriction enzyme and transformed into *E. coli* DH5α. The clones were confirmed by sequencing and were transformed into *S. cerevisiae* L3262 or corresponding transposon mutants. Genes were PCR-amplified with *pfu* DNA polymerase (Stratagene, Santa Clara, CA, USA) with genomic DNAs prepared by standard protocols (Adams et al. 1997). PCR fragments were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and verified by sequencing. Because of an intron in the middle of an ORF, the ORF of *IMD4* was constructed through reverse transcription (RT)-PCR. Single-stranded complementary DNA was then synthesized from 1 μg of total RNA with the *IMD4* downstream primer (IMD4-R). The PCR conditions were 95 °C for 3 min, 35 cycles of 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min with a final extension for 10 min at 72 °C. Ethanol concentrations were analyzed by high-pressure liquid chromatography (HPLC) using a Breeze2 apparatus (Waters, Milford, MA, USA). An Aminex HPX-87H ion exclusion (7.8 mm×300 mm) column (Bio-Rad, Hercules, CA, USA) and Waters 2414 refractive index detector were used with 0.5 mM H_2SO_4 as the mobile phase. Twenty microliters of sample was injected after equilibration, while temperature and flow rate were kept at 60 °C and 0.6 ml/min, respectively.

Results

Isolation of ethanol and/or thermotolerant strains

Approximately 3,000 transformants of the mTn*3*-mutagenized genome library were selected as leucine prototrophs and replica-plated to both YPD agar and YPD agar containing 10% ethanol. After incubation at 30 °C for 3 days, about 40 mutants were initially selected as ethanol-

tolerant strains that grew in the presence of ethanol. Five strains, designated Tn *1–5*, were selected following a spot assay on YPD plates containing 15% ethanol (Fig. 1a). Only Tn *2* and Tn *3* grew at 42 °C (Fig. 1b). Above 42 °C, no growth was observed. Tn *2* and Tn *3* were concluded to be both ethanol and thermotolerant.

Identification of disrupted genes

Sequencing of genes disrupted by transposon insertion revealed the integration sites depicted in Fig. 2. The sites were located between the terminator regions of *CMP2* and *IMD4* in Tn *1*, between the promoter regions of *SSK2* and *PPG1* in Tn *2*, in the ORF of *PAM1* in Tn *3*, between the terminator region of *RMD6* and promoter region of *DLD3* in Tn *4*, and in the ORF of *MSN2* in Tn *5*. The transposon insertion positions are summarized in Table 2. To determine which gene was affected in Tn *1*, Tn *2*, and Tn *4* and to confirm the disruptions in Tn *3* and Tn *5*, the expression levels of *CMP2*, *IMD4*, *SSK2*, *PPG1*, *PAM1*, *RMD6*, *DLD3*, and *MSN2* were examined by Northern blot analysis (Fig. 3). Compared to the control, the levels of *CMP2* and *IMD4* were about 10% and 20% lower, respectively. The levels of *SSK2* and *PPG1* were approximately 20% and 50% lower, respectively, indicating *PPG1* was affected to a greater degree than *SSK2*. The level of *DLD3* expression was about 25% lower but *RMD6* remained unchanged, indicating that *RMD6* was not affected. *RMD6* was ruled out for further experiments. The expression of *PAM1* and *MSN2* was not detected as expected. Thus, the transposon insertions partially affected *CMP2*, *IMD4*, *SSK2*, *PPG1*, and *DLD3* and completely affected *PAM1*and *MSN2*.

Contribution to ethanol and/or thermotolerance

The above data revealed the down-regulation of *CMP2* and *IMD4* in Tn *1* and *SSK2* and *PPG1* in Tn *2*, down-regulation of *DLD3* in Tn *4*, and the disruptions of *PAM1* and *MSN2* in Tn *3* and Tn *5*. The contribution of down-regulations or deletions of *CMP2*, *IMD4*, *SSK2*, *PPG1*, *PAM1*, *DLD3*, and *MSN2* to ethanol and/or thermotolerance was individually investigated with knockout mutants for the seven genes picked from the *S. cerevisiae* SGKO collection with a BY4741 background. When the cultures of Δ*cmp2*, Δ*imd4*, Δ*ssk2*, Δ*ppg1*, Δ*pam1*, Δ*dld3*, and Δ*msn2* were spot-assayed on YPD plates containing 10%, 12.5%, and 15% ethanol, all of them showed enhanced growth at all concentrations of ethanol compared to control BY4741 (Fig. 4a). When the spotted YPD plates were incubated at 43 °C, the temperature at which Tn *1–5* could not grow, Δ*ssk2*, Δ*ppg1*, and Δ*pam1* showed enhanced growth (Fig. 4b). However, these mutants did not grow at 44 °C (data not shown). This 1 °C difference in the maximum temperature for growth may be because BY4741 are more thermotolerant than L3262 (data not shown). Thus, SGKOs for all seven genes were ethanol tolerant and three of those for *SSK2*, *PPG1*, and *PAM1* were also thermotolerant.

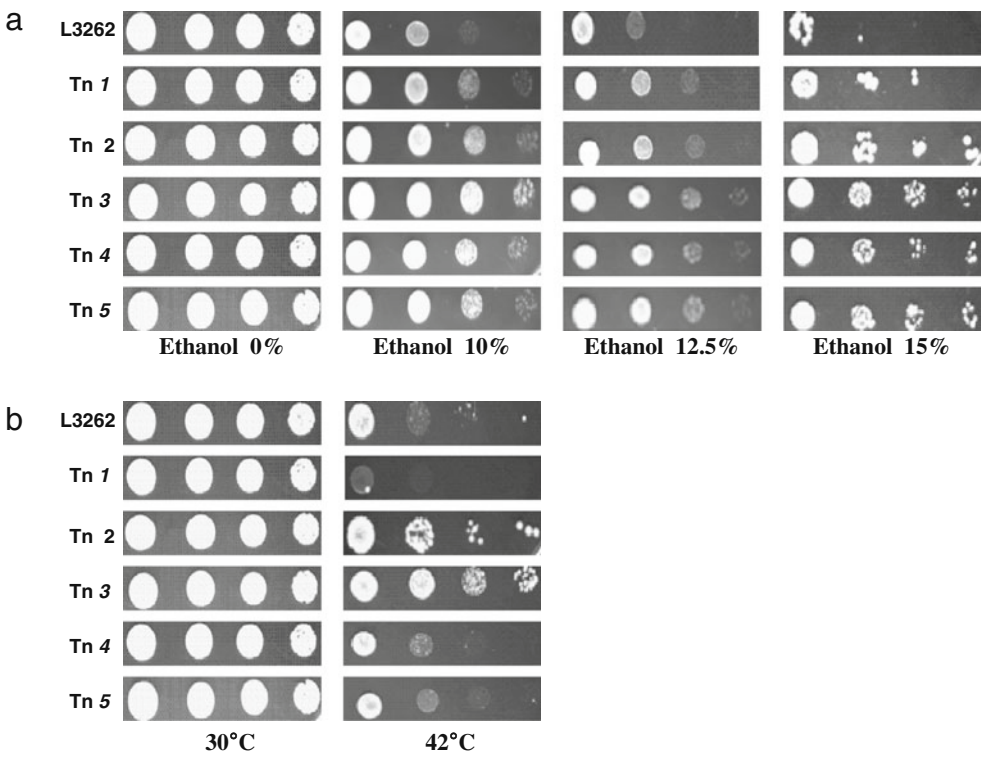

Fig. 1 Identification of ethanol and/or thermotolerant strains. **a** Tenfold serial dilutions of Tn *1–5* were spotted on YPD plates containing 0%, 10%, 12.5%, or 15% ethanol and incubated at 30 °C for 1 day (0%), 4 days (10%), 5 days (12.5%), or 7 days (15%). **b** Spotted YPD plates were incubated for 1 day (30 °C) or 4 days (42 °C)

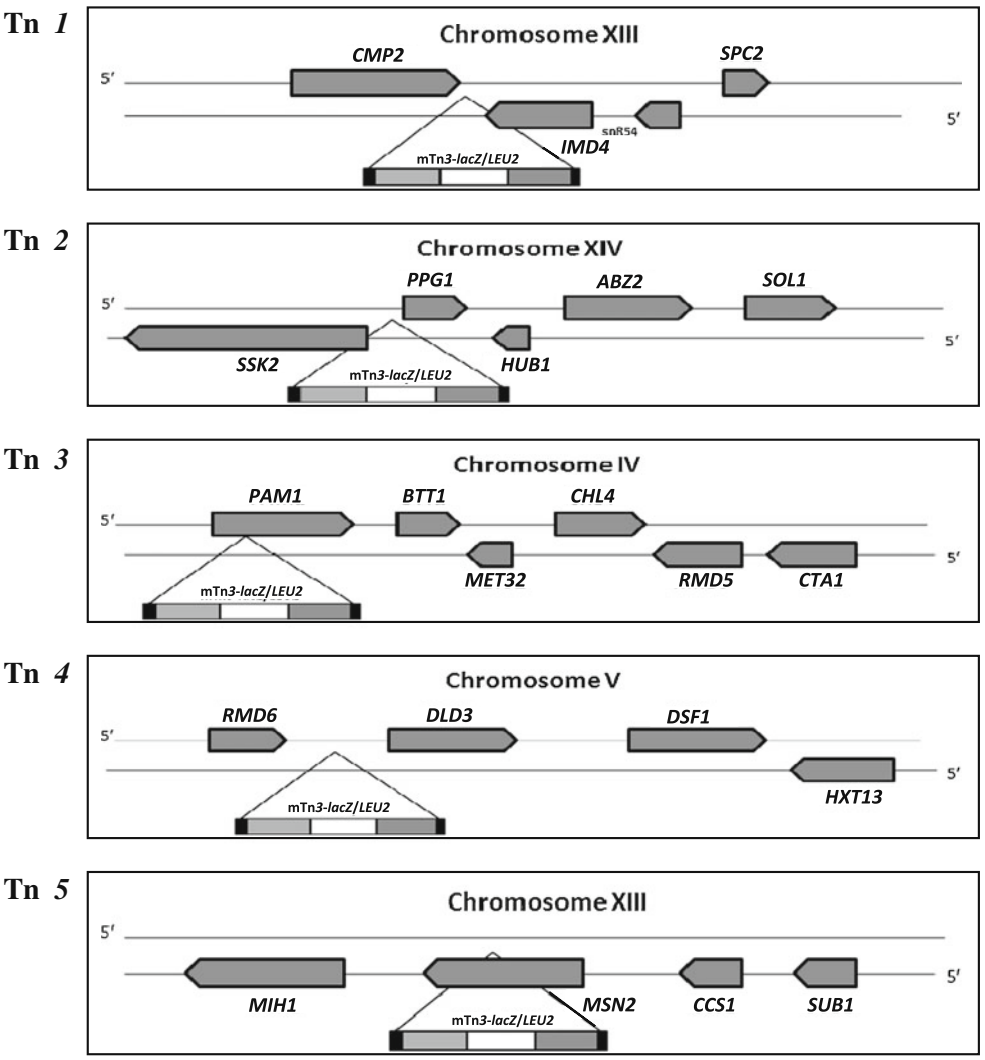

Fig. 2 Determination of transposon insertion sites. Transposon insertion sites were determined by sequencing of recovered transposons from five ethanol and/or thermotolerant strains. Surrounding genetic loci are shown

Confirmation of tolerance by complementation

The finding that Tn *1–5* acquired ethanol and/or thermotolerance through the deletions or down-regulation of specific genes implied that the complementation of those genes should render cells stress sensitive. To assess this speculation, each gene except for *IMD4* was autologously expressed by cloning amplified DNA fragments presumed to contain their own promoter, ORF, and terminator (ORF +/−700 bp) into pRS316 and transforming into the corresponding Tn *1–5*. In the case of *IMD4*, which has an intron in the middle of the ORF, its entire ORF was constructed by performing RT-PCR and cloned into pRS316-GAPDH, which overexpressed cloned DNA under the control of the GAPDH promoter. The control was constructed by transformation of pRS316. When the spot assay was performed on YPD agar containing 10% ethanol, the tolerance of complemented cells was significantly reduced compared to Tn mutants (Fig. 5a). Reduced thermotolerance was also observed when Tn *2* was complemented individually with *SSK2* and *PPG1* and when Tn *3* complemented with *PAM1* was incubated at 42 °C (Fig. 5b). Also, the ORF of each gene except for the formerly constructed *IMD4* was PCR-amplified, cloned into pRS316-GAPDH for overexpression, and transformed into L3262. pRS316-GAPDH was transformed as the control. When the spot assay was performed for ethanol (Fig. 6a) and heat (Fig. 6b), overexpressed strains were sensitive to both ethanol and heat stress.

Fermentation capacity of Tn mutants

It is generally accepted that ethanol-tolerant cells produce more ethanol than non-tolerant strains because of their higher cell viability. When the fermentation capacity was investigated in batch cultures with 30% glucose at 30 °C, no significant differences between the parent and Tn *1–5* were observed in spite of enhanced growth of mutants (data not shown). It was speculated that the parental strain is basically capable of producing such maximum level (14%)

Table 2 Identified strains with enhanced tolerance and genes affected

Strain	Disrupted gene[a]	Function[a]	Insertion position[b] (bp)	Gene expression	Enhanced tolerance
Tn *1*	*CMP2*	Calcineurin A; 1 isoform (the other is CNA1) of the catalytic subunit of calcineurin, a Ca^{2+}/calmodulin-phosphatase which regulates Crz1p (a stress response transcription factor)	110[c]	Down-regulation	Ethanol
	IMD4	Inosine monophosphate dehydrogenase, catalyzes the first step of GMP biosynthesis	90[c]	Down-regulation	
Tn *2*	*SSK2*	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway	−552	Down-regulation	Ethanol and heat
	PPG1	Putative serine/threonine protein phosphatase, required for glycogen accumulation	−25	Down-regulation	
Tn *3*	*PAM1*	Essential protein of unknown function; exhibits variable expression during colony morphogenesis	778	Disruption	Ethanol and heat
Tn *4*	*RMD6*	Protein required for sporulation	946[c]	No effect	Ethanol
	DLD3	D-Lactate dehydrogenase, part of the retrograde regulon which consists of genes whose expression is stimulated by damage to mitochondria	−994	Down-regulation	
Tn *5*	*MSN2*	Transcriptional activator related to Msn4p; activated in stress conditions	1,152	Disruption	Ethanol

[a] Annotation from the Yeast Genome Database.

[b] Insertion position is given with respect to the initiator ATG of each coding sequence

[c] Insertion position is given with respect to stop codon of each coding sequence

of ethanol from 30% glucose and that it was difficult to observe the effect of enhanced ethanol tolerance. Accordingly, it was necessary to establish a condition under which the final ethanol titer exceeds 14%. For this, we began fermentations in cultures of initial 0.5 OD_{600} cells and 6% ethanol. As shown in Fig. 7a and b, Tn mutants grew faster and produced more ethanol compared to the control.

We further examined the ethanol production capacity at 42 °C. Since ethanol evaporation at this temperature may affect the final titer, the amount of evaporated ethanol during the batch fermentation was calculated according to the calibration curve constructed with 4–10% ethanol standards (Abdel-Banat et al. 2010). An ethanol concentration of 4% evaporated slowly, while ethanol concentrations exceeding 4% evaporated rapidly. For instance, 8% ethanol reduced to 3.8% in 2 days and 10% ethanol reduced more drastically (data not shown). When fermentation profiles were created at 42 °C, the growths of Tn *2*, Tn *3*, and Tn *5* mutants were more rapid than that of the other strain (Fig. 7c) and the maximal ethanol concentrations of Tn *2* and Tn *3* strains were 1.5 and 2.4 times greater than that of the control strain, respectively, even without considering ethanol evaporation at 42 °C (Fig. 7d). Interestingly, Tn *5* did not show enhanced fermentation ability at 42 °C, although the growth of this strain was higher than that of the control strain unlike the solid medium. This result might be partly explained by the finding that the growth and the protein levels of yeast cells varied depending on the composition and physical state of the medium used (Cheung and Fischetti 1988; Mackey and Derrick 1982). The concentration of ethanol continued to decrease after 3 days, probably due to evaporation caused by heat and impaired growth later.

Expression of *MSN4* and stress response element-mediated genes

In this study, *MSN2* knockout clearly showed increased ethanol tolerance. Therefore, we investigated the expression level of *MSN4* in ethanol stress condition because Msn2p and Msn4p have partly redundant functions in the stress response (Estruch and Carlson 1993). Compared to control strains (L3262 and BY4741), the level of *MSN4* was increased in both Tn *5* and Δ*msn2* (Fig. 8). Msn2p and Msn4p bind to stress response elements (STRE) (CCCCT or AGGGG) within the promoters of stress-mediated genes and lead to transcriptional activation of these genes by various stresses (Causton et al. 2001). Thus, we compared the transcript levels of *CTT1* and *HSP12* which were known to be stress-mediated genes (Martínez-Pastor et al. 1996). As shown in Fig. 8, the levels of *CTT1* and *HSP12* in both Tn *5* and Δ*msn2* were higher than those of the control strains. Under no ethanol stress, the levels of *MSN4*, *CTT1*, and *HSP12* genes in strains examined were not changed (data not shown).

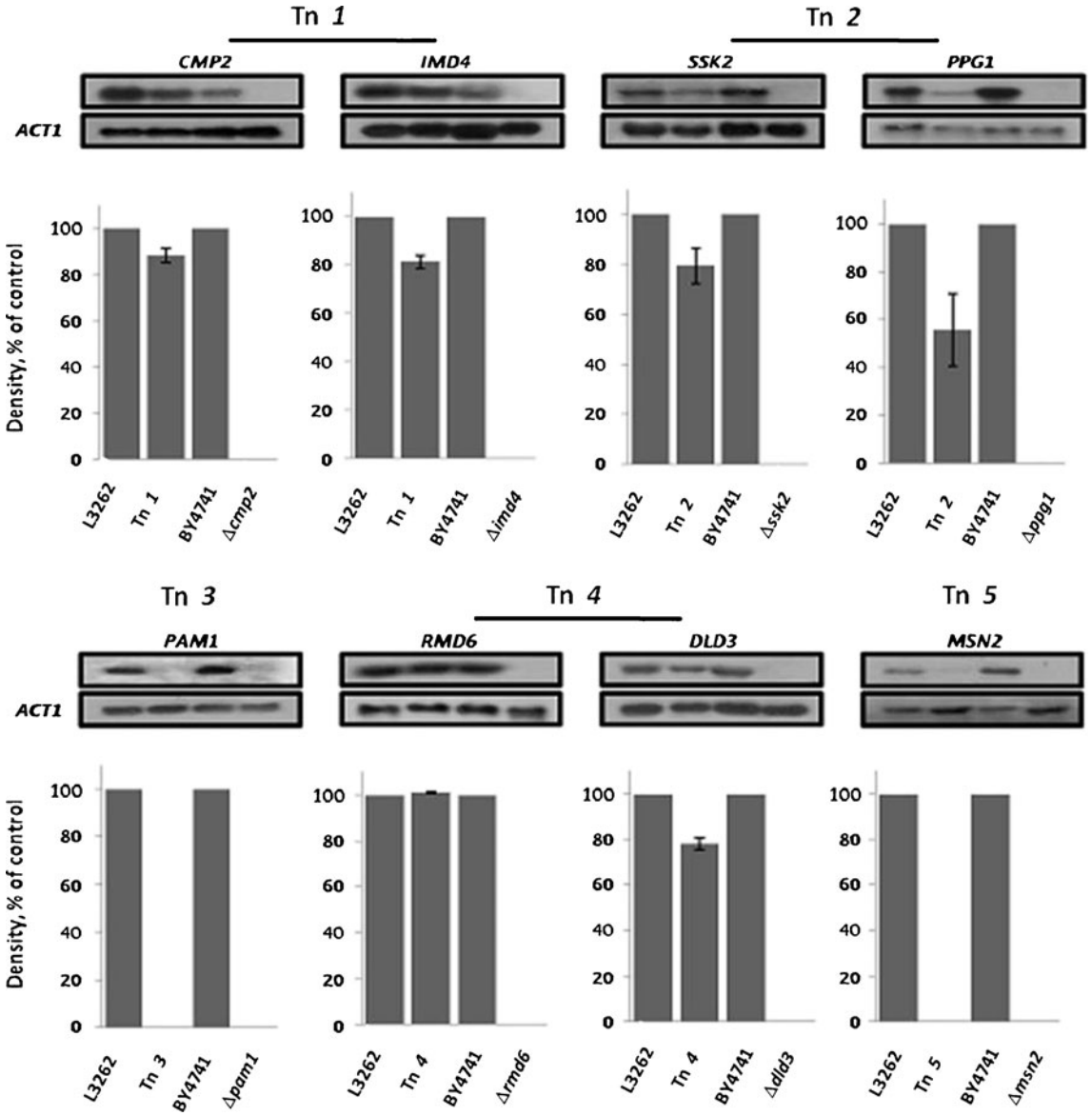

Fig. 3 Expression of genes affected by transposon insertion. Northern blot analysis was performed with total RNAs prepared from L3262, BY4741 (control), Tn *1–5*, and single gene knockout (SGKO) mutants. Signals were relatively quantified by using a scanning densitometer, normalized with *ACT1*, and represented as percent control. *Vertical bars* represent the standard errors for three replicate analyses

Discussion

In yeast and other organisms, most cellular pathways to stresses are reprogrammed with numerous genes being regulated (Kültz 2005). Although a large number of the involved genes have been identified, little is currently known about how tolerance is achieved against various stressors including ethanol and heat in terms of signaling pathways. It is generally accepted that the mechanisms of ethanol or thermotolerance are very complex and globally controlled and need to be understood through a comprehensive view of associated pathways (Ma and Liu 2010; Yoshikawa et al. 2009). Both ethanol and heat stresses cause similar changes in plasma membrane lipid composition (Okuyama and Saito 1979; Suutari et al. 1990) and a decrease in plasma membrane H^+-ATPase activity (Coote et al. 1994) and up-regulation of genes encoding for heat shock proteins (Ma and Liu 2010). These data suggest that some pathways required for ethanol tolerance overlap with those for thermotolerance. However, the strains constructed based on genome-wide investigations displayed only either ethanol or thermotolerance (Teixeira et al. 2009; Yoshikawa et al. 2009). In this study, we initially isolated 5 strains with enhanced tolerance to ethanol through the screening of 3,000 transposon-mediated disruption mutants; subsequently, 2 of the mutants were

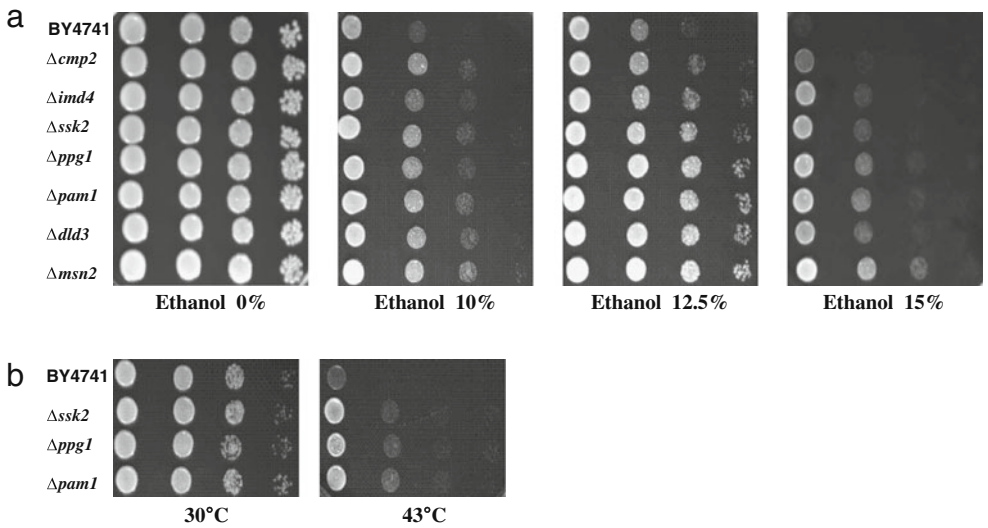

Fig. 4 Ethanol and/or thermotolerance of SGKO mutants. **a** Tenfold serial dilutions of SGKO mutants were spotted on YPD plates containing 0%, 10%, 12.5%, or 15% ethanol and incubated at 30 °C for 1 day (0%), 3 days (10%), 4 days (12.5%), or 5 days (15%). **b** Spotted YPD plates were incubated for 1 day (30 °C) or 3 days (43 °C)

found to have enhanced tolerance to heat. Those two strains may be useful for elucidating a possible interconnection between ethanol and thermotolerances.

We identified seven genes (*CMP2*, *IMD4*, *SSK2*, *PPG1*, *PAM1*, *DLD3*, and *MSN2*) associated with ethanol tolerance, three (*SSK2*, *PPG1*, and *PAM1*) of which were also related to thermotolerance by combination of transposon mutagenesis and SGKO mutants. In addition, down-regulation of *SSK2* and/or *PPG1* conferred tolerance to both ethanol and heat. Down-regulation of *CMP2* and/or *IMD4*, and of *DLD3*, was responsible for ethanol tolerance. As seen in Fig. 2, a transposon was inserted into the presumptive regulatory

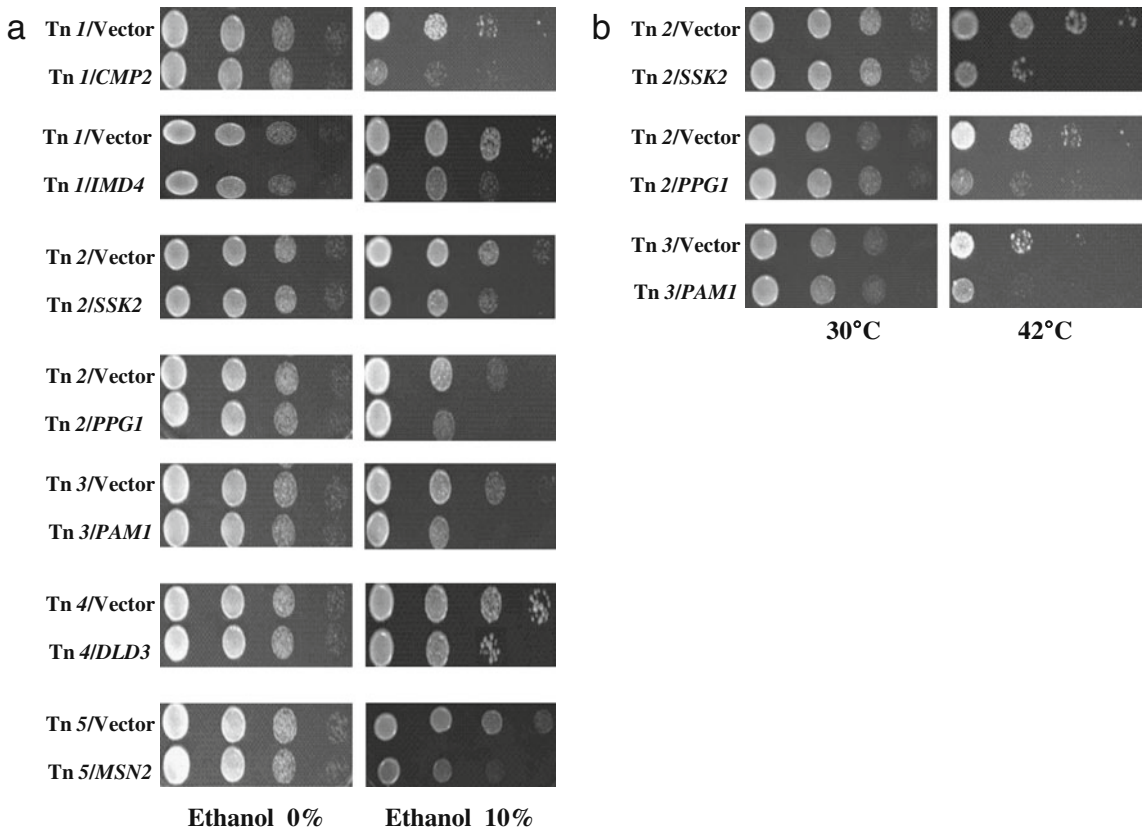

Fig. 5 Restoration of stress sensitivity by complementation. **a** Tenfold serial dilutions of complemented strains were spotted on YPD plates containing 0% and 10% ethanol and incubated at 30 °C for 1 day (0%) or 4 days (10%). **b** Spotted YPD plates were incubated for 1 day (30 °C) or 4 days (42 °C). Control strains were constructed by transforming pRS316 into Tn *1–5*. pRS316-GAPDH was transformed into Tn *1* for *IMD4* control, since the *IMD4* gene was placed under the control of the GAPDH promoter

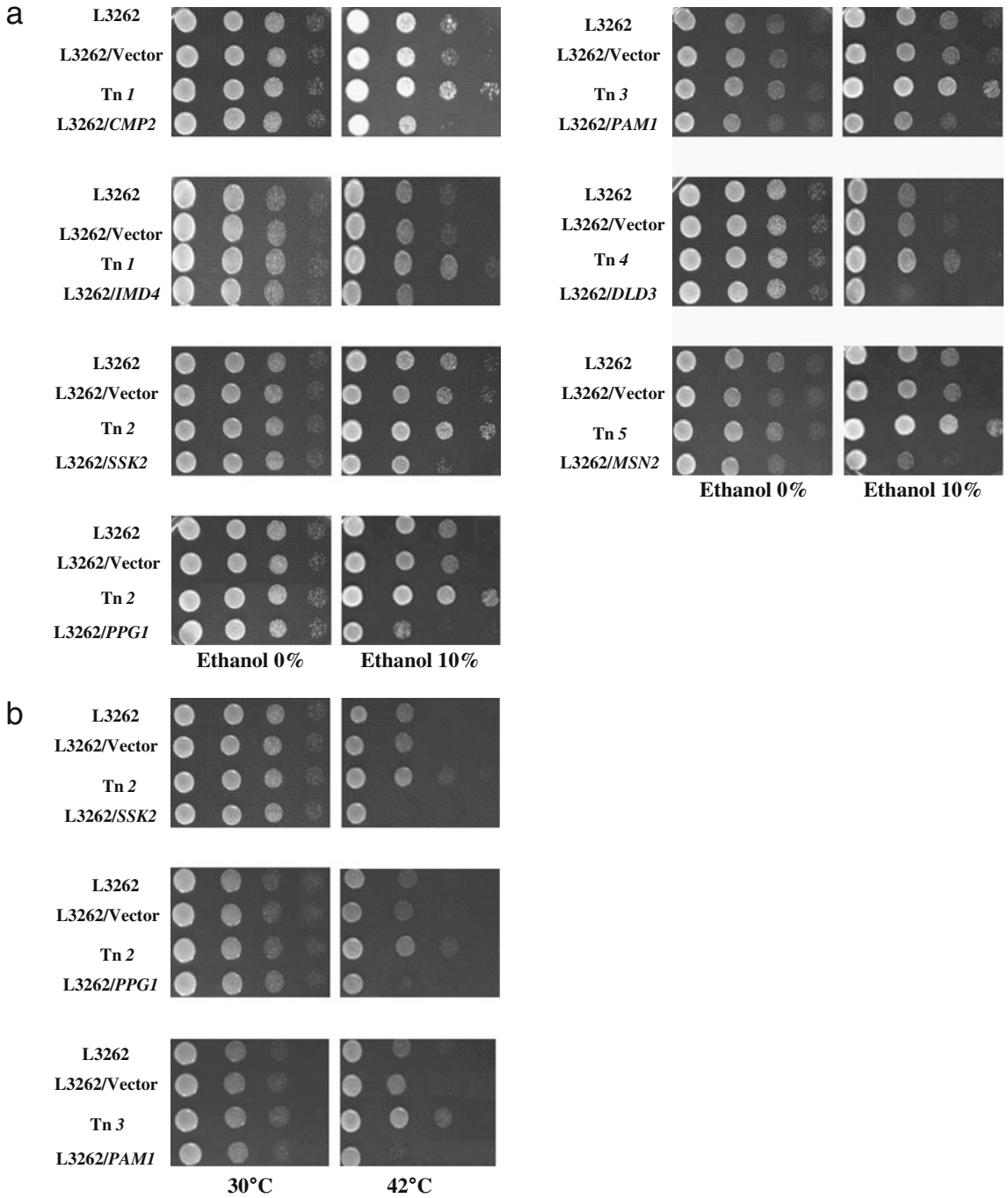

Fig. 6 Effect of overexpression on stress sensitivity. **a** Tenfold serial dilutions of overexpressing strains were spotted on YPD plates containing 0% and 10% ethanol and incubated at 30 °C for 1 day (0%) or 4 days (10%). **b** Spotted YPD plates were incubated for 1 day (30 °C) or 4 days (42 °C). Control strains were constructed by transforming pRS316-GAPDH into L3262

regions of the affected genes. This means that a library generated by transposon mutagenesis should theoretically consist of much more than 4,500 clones, an approximate number of clones in SGKO collections in which only ORFs are affected. In this study, we isolated five strains (Tn *1*–*5*) with enhanced ethanol tolerance with a library consisting of 3,000 clones, far less than the theoretical number. Accordingly, it is possible to isolate more strains with enhanced tolerance, if a properly represented library is screened. It should be noted that isolation of Tn *1*, Tn *2*, and Tn *4* showing down-regulation of affected genes would be impossible if SGKO collections were used.

In the case of Tn *1* and Tn *2*, in which two genes were down-regulated, we could not determine which gene was

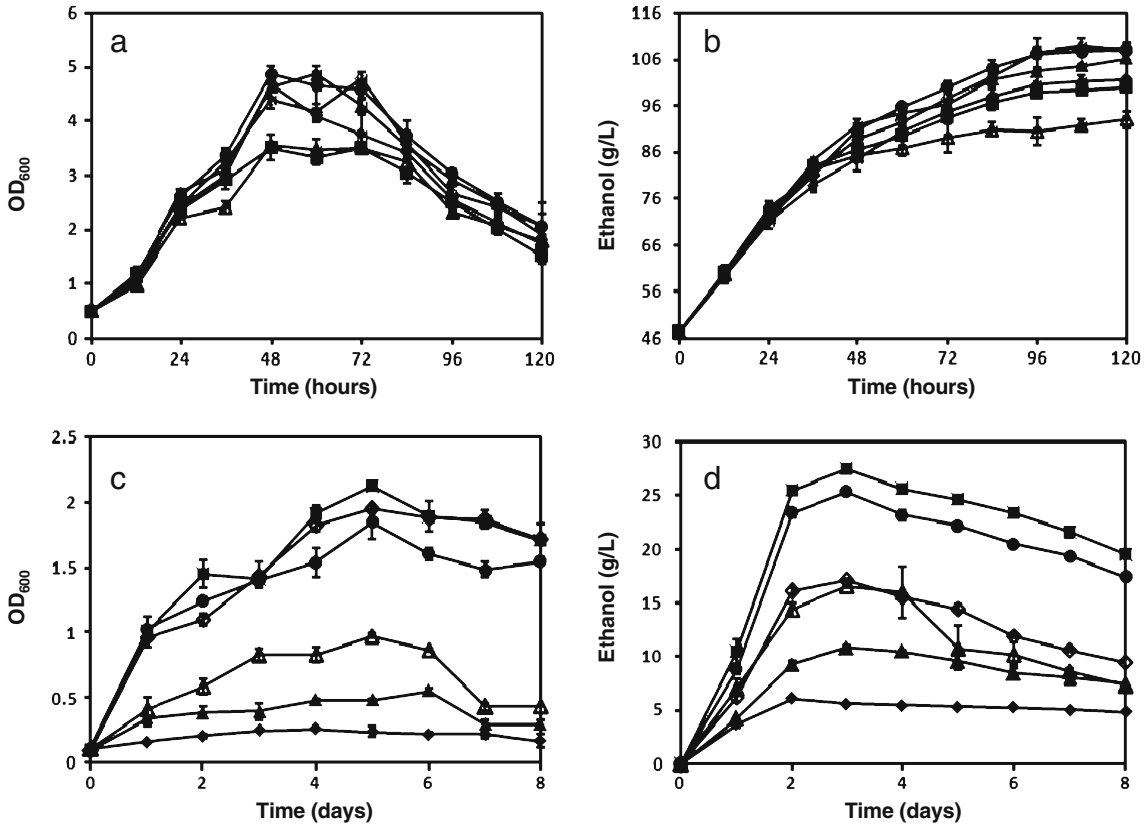

Fig. 7 Fermentation profiles of Tn mutants. Exponentially growing cells (0.5 OD_{600}) were grown at 30 °C in YPD supplemented 30% glucose and 6% ethanol. Cell growth (**a**) and ethanol production (**b**) were monitored during fermentation. Similar experiments without initial ethanol were performed at 42 °C to monitor cell growth (**c**) and ethanol production (**d**) during fermentation. L3262 (*open triangles*), Tn *1* (*closed triangles*), Tn *2* (*closed circles*), Tn *3* (*closed squares*), Tn *4* (*closed diamonds*), and Tn *5* (*open diamonds*). *Vertical bars* represent the standard errors for three replicate analyses. A calibration curve of ethanol was constructed, using a series of ethanol standards. The peak of ethanol appeared at 19 min, and the height of the peak was automatically calculated in computer-based Breeze 2 program (Waters) connected with HPLC

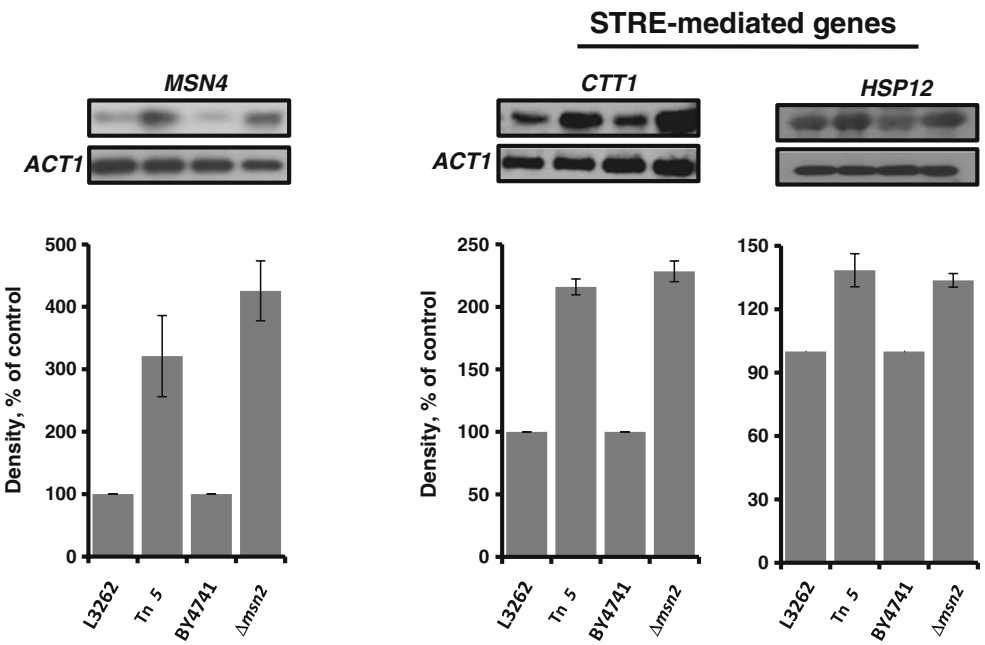

Fig. 8 Expression of *MSN4* and STRE-mediated genes (*CTT1* and *HSP12*) by ethanol stress. Cultures of L3262, BY4741 (control), Tn *5*, and Δ*msn2* strains growing exponentially on YPD were treated with 10% ethanol and further incubated for 30 min. Northern blot analysis was performed with total RNAs prepared from respective strains. Signals were relatively quantified by using a scanning densitometer, normalized with *ACT1*, and represented as percent control. *Vertical bars* represent the standard errors for three replicate analyses

really responsible for stress tolerance due to the lack of strains with down-regulation of only one gene. *CMP2* encodes calcineurin A, an isoform of the catalytic subunit of Ca^{2+}/calmodulin-dependent phosphatase, which regulates Crz1p (a stress response transcription factor). Calcineurin dephosphorylates Crz1p and causes its rapid translocation from the cytosol to the nucleus. Subsequently, Crz1p activates the transcription of genes related to cell survival (Cyert 2003; Panadero et al. 2007). *CRZ1* is crucial for cell survival under ethanol stress through its role in the ethanol-induced calcineurin/Crz1 pathway (Araki et al. 2009). On the other hand, calcineurin enhances the degradation of Msn2p (see below), leading to the insufficient induction of genes possessing the STRE (Takatsume et al. 2010). Thus, it remains contentious whether the calcineurin–Crz1p pathway plays a role in ethanol-protective mechanisms in yeast. The present observation that the *CMP2* knockout resulted in enhanced ethanol tolerance supports the latter. Meanwhile, *IMD4* encodes for an isozyme of inosine monophosphate dehydrogenase (IMPDH) that catalyzes the first step of GMP biosynthesis. There has been no report of an association of *IMD4* with ethanol tolerance in *S. cerevisiae*. However, in the lactic acid bacterium *Oenococcus oeni*, ethanol stress decreases the level of IMPDH, which in turn reduces the production of nicotinamide nucleotides, resulting in induction of glutathione reductase and, therefore, reduction of the NADPH level (Silveira et al. 2004), suggesting that the maintenance of redox balance plays an important role in ethanol adaptation (Bro et al. 2006; Silveira et al. 2004). Thus, both *CMP2* and *IMD4* may be responsible for ethanol tolerance, although the role of the latter need to be verified in *S. cerevisiae*.

SSK2 encodes for a mitogen-activated protein kinase kinase kinase (MAPKKK) of the HOG1 pathway (Posas and Saito 1998), which is essential for survival of yeast against various environmental stresses including high osmolarity (Albertyn et al. 1994; Brewster et al. 1993). The HOG1 pathway is regulated independently by two membrane-bound osmosensor proteins, Sln1p and Sho1p. Signals generated by Sln1p are delivered to Ssk2p, whereas those generated by Sho1p are delivered to MAPKKK Ste11p, with both pathways converging at the downstream Pbs2p (Winkler et al. 2002). The involvement of *SSK2* in ethanol stress has not been known. Meanwhile, heat stress involves the SHO1 pathway, irrelevant of *SSK2* (Winkler et al. 2002). Given these facts, the issue becomes how the disruption of *SSK2* induces both ethanol and thermotolerance. Our hypothesis is that the blocking of *SLN1* pathway caused by *SSK2* disruption enhances the signaling capability of the alternate *SHO1* pathway for heat. Whether ethanol tolerance is also mediated by the *SHO1* pathway will be an interesting issue to pursue. *PPG1* encodes for a putative serine/threonine protein phosphatase that is required for glycogen accumulation (Posas et al. 1993). Glycogen biosynthesis genes are highly expressed in response to ethanol shock (Hirasawa et al. 2007). Since the related data on *PPG1* were not presented in this study, it is premature to speculate on the inconsistency with our data that the disruption of *PPG1* conferred tolerance to both ethanol and heat.

Knockout of *PAM1* conferred tolerance to both ethanol and heat, and *DLD3*, and either knockout or down-regulation of *DLD3* was responsible for ethanol tolerance. *PAM1* encodes for the protein of unknown function. It was reported that overexpression of this gene inhibits growth and induces a filamentous phenotype by interfering with regulatory pathway that controls the cell shape (Hu and Ronne 1994). Since the inhibition of growth has been observed by overexpression of *PAM1* in yeast, on the contrary, the repression of *PAM1* might be the mechanism for ethanol and thermotolerance. *DLD3* encodes D-lactate dehydrogenase, part of the retrograde regulon consisting of genes whose expression is stimulated by damage to mitochondria (Chelstowska et al. 1999). Presently, there was no evidence connecting these genes to stress tolerance.

MSN2 encodes for a transcription factor that regulates the general stress response (Martínez-Pastor et al. 1996; Schmitt and McEntee 1996). Msn2p, in association with Msn4p, which has 41% identity with Msn2p, regulates the expression of about 200 genes in response to several stresses including heat and high ethanol concentration, by binding to the STRE element located in the promoters of these genes (Causton et al. 2001; Gasch et al. 2000; Martínez-Pastor et al. 1996). *MSN4* gene expression is induced by stress, while *MSN2* expression is constitutive (Gasch et al. 2000). While single deletion mutants of *msn2* and *msn4* have no obvious phenotype, *msn2* and *msn4* double null mutants are hypersensitive to carbon source starvation, heat shock, and osmotic and oxidative stresses. Overexpression of *MSN2* and *MSN4* genes decreases sensitivity to starvation and thermal stresses (Estruch and Carlson 1993). Thus, *MSN2* and *MSN4* work as a team in many biological reactions, although their individual contributions differ for specific genes and under particular stress conditions (Fujita et al. 2006). In relation with ethanol tolerance, overexpression of *MSN2* promotes ethanol tolerance and enhances the fermentation ability in sake yeast strains (Watanabe et al. 2009); in contrast, it has been shown that Msn2/4p-mediated stress responses decrease the initial rate of ethanol fermentation (Watanabe et al. 2011). In this study, *MSN2* knockout clearly showed increased ethanol tolerance with activation of *MSN4* and STRE-mediated genes under ethanol stress. Therefore, we suggest that *MSN4* may modulate ethanol stress response

on behalf of *MSN2* in Δ*msn2* strain and further studies will be needed to elucidate the regulatory mechanisms affecting ethanol tolerance and productivity.

This study has revealed that inactivation or down-regulation of seven genes (*CMP2*, *IMD4*, *SSK2*, *PPG1*, *PAM1*, *DLD3*, and *MSN2*) is responsible for enhanced ethanol tolerance, and inactivation or down-regulation three genes (*SSK2*, *PPG1*, and *PAM1*) is also responsible for enhanced thermotolerance. None has been reported to have such phenotype. The interrelationship between these genes could not be deduced from annotated functions. However, *CMP2*, *SSK2*, and *MSN2* are known to be involved in cell pathways (Cyert 2003; Martínez-Pastor et al. 1996; Posas and Saito 1998), which may help understand the molecular mechanism by which ethanol tolerance is achieved. Msn2p, the only transcription factor identified, is of particular concern, since it may negatively regulate some genes responsible for ethanol tolerance (Watanabe et al. 2011). Studies on *SSK2*, *PPG1*, and *PAM1* also may help understand at the molecular level how ethanol and thermotolerance are simultaneously achieved.

In conclusion, one of the aims of developing strains with enhanced tolerance to ethanol and heat stresses is to provide information for industrial applications besides understanding the mechanisms that underlie tolerance to stresses. The novel genes identified in this study would be helpful to improve industrial yeast strains in the capacity of ethanol production. Furthermore, through integrated application of genes identified, it will be explored to improve desired traits in ethanol-producing strains currently.

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