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Identification of novel genes responsible for salt tolerance by transposon mutagenesis in *Saccharomyces cerevisiae*

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Abstract *Saccharomyces cerevisiae* strains tolerant to salt stress are important for the production of single-cell protein using kimchi waste brine. In this study, two strains (TN-1 and TN-2) tolerant of up to 10 % (w/v) NaCl were isolated by screening a transposon-mediated mutant library. The determination of transposon insertion sites and Northern blot analysis identified two genes, MDJ1 and VPS74, and revealed disruptions of the open reading frame of both genes, indicating that salt tolerance can be conferred. Such tolerant phenotypes reverted to sensitive phenotypes on the autologous or overexpression of each gene. The two transposon mutants grew faster than the control strain when cultured at 30 °C in rich medium containing 5, 7.5 or 10 % NaCl. The genes identified in this study may provide a basis for application in developing industrial yeast strains.

Keywords Yeast · Transposon · Salt tolerance

Introduction

Kimchi is a traditional, fermented Korean food prepared with different vegetables, spices and ingredients and is an important dietary source of vitamins, minerals and other nutrients [1]. The waste brine from the kimchi manufacturing process is released and eventually results in serious environmental pollution. The waste brine, which contains 10–15 % NaCl and some sugars extracted from the vegetables, had been disposed of by physical and chemical methods such as separation, precipitation and chemical transformation of specific species [2]. The waste brine can also be reused as a substrate for the production of single-cell protein (SCP) and other useful materials [3]. Therefore, strains tolerant to salt stress are used for the production of SCP using kimchi waste brine, and for the removal of potential pollutants from wastewater [4]. The production of SCP normally uses waste materials as substrates, including agricultural wastes such as wood shavings, sawdust and corn cobs, food processing wastes, residues from alcohol production, hydrocarbons, or human and animal excreta [5]. However, there has not been much research on SCP production using waste brine compared with other waste material substrates. Furthermore, studies on waste brine have focused on the selection of strains tolerant of salt stress, rather than on the identification of genes affecting growth under salt stress. The aim of this study was to identify genes related to salt tolerance and to gain new insight into using waste brine from a kimchi factory for the production of SCP.

Saccharomyces cerevisiae has been widely used in ethanol-related industries because of its potent ethanol production and tolerance [6, 7]. During industrial ethanol fermentation, yeast cells are exposed to several environmental stresses including high ethanol concentration, temperature increments, high osmotic pressure and inhibitors, eventually resulting in decreased cell growth, viability and ethanol yield [8, 9]. Thus, yeast strains that can endure various stresses are highly desirable. To develop stress-tolerant strains, several strategies are used. Classic strategies include evolutionary adaptation [10], random chemical mutagenesis [11] and gene shuffling [12]. Alternate strategies include genome-wide DNA microarray analysis

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[13], screening of transposon-mediated deletion mutant libraries [14] or single gene knockout collections [15, 16], and global transcriptional machinery engineering (gTME) [17]. Although gTME is intriguing as a means to positively select stress-tolerant strains, its usefulness is debatable because the enhanced growth of the mutants was seen only on defined medium with low concentrations of leucine and did not improve in complex rich media supplemented with 8 % ethanol [18]. The other three alternate strategies are mostly indirect: a verification step is required following the identification of target genes, as identification of stress-sensitive genes helps to understand the molecular basis of stress tolerance, but does not ensure creation of stress-tolerant strains. Strategies for the isolation of tolerant yeast strains from natural resources have also been reported [19, 20].

A few studies demonstrated that the disruption of specific gene(s) led to creation of strains tolerant to several kinds of stressors [21, 22]. More recently, two clones with enhanced growth in 8 % ethanol and 10 osmotolerant clones were identified by screening of a single gene knockout library [23]. This study [23], in particular, paved the way for the use of transposon or single gene knockout deletion mutant libraries for positive (or direct) isolation of strains tolerant (not sensitive) to a certain stressor. A transposon mutant library can easily be 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 this study, we identified novel genes responsible for salt tolerance 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 single gene knockout mutants, respectively. The nonessential haploid *S. cerevisiae* deletion library was kindly obtained from 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 [SC; 0.67 % (w/v) Difco yeast nitrogen base without amino acid, and 2 % glucose, supplemented with appropriate nutrients] was used for the yeast transformation. *Escherichia coli* DH5α was used for plasmid construction and grown in Luria–Bertani medium (LB) supplemented with 100 mg/l of ampicillin at 37 °C.

Transposon mutagenesis

S. cerevisiae genomic library pools with transposon-mediated random insertions of mTn3-*lacZ/LEU2* were kindly provided by Yale Genome Analysis Center (New Haven, CT; http://ygac.med.yale.edu/mtn/reagent/avail_reagents/lacZ_LEU2_info_p.stm). The plasmid DNA from pools of the mTn3-mutagenized genomic library was digested with *Not*I and used for transformation of *S. cerevisiae* L3262 by the lithium acetate method as previously described [24, 25].

Isolation of salt-tolerant mutants

For the isolation of salt-tolerant mutants, the transformants were replica plated on YPD agar and YPD agar containing 10 % NaCl and incubated at 30 °C for 6 days. The mutants that grew well on both media were selected. To confirm the salt-tolerant mutations, the selected mutants were grown in YPD medium to an OD_{600} of 1.0 and serially diluted tenfold with sterile water. For salt tolerance, aliquots (5 μl) of each dilution were spotted onto NaCl-free YPD agar (control) and agar containing 5, 7.5 and 10 % NaCl, and then incubated at 30 °C for 1–6 days. Mutants that grew well on NaCl-containing YPD agar compared to the control strain were concluded to be salt-tolerant 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 transposon [25]. 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 [24]. DNA fragments for probe 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). *ACT1* gene was used as an internal control (Accession Number: NC_001138.5). The band intensities were quantified with NIH image J, version 1.61 (National Institutes of Health, Bethesda, MD, USA).

Table 1 Strains, plasmids and oligonucleotides used in this study

Strain or plasmid		Characteristics or sequence	Source or reference
Strains			
S. cerevisiae	L3262	*MAT-α; ura3-52 leu2-3,112 his4-34*	[43]
	BY4741	*MAT-a; hisΔ1 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*	Stratagene
Plasmid			
pRS316-GAPDH		pRS316 plasmid with P_{GAPDH}	This study
pRS-GAMDJ1		pRS316-GAPDH carrying 1536 bp of ORF (*MDJ1* gene)	This study
pRS-GAVPS74		pRS316-GAPDH carrying 1038 bp of ORF (*VPS74* gene)	This study
pRS316		*Saccharomyces/E. coli* plasmid vector for cloning, Ap^r *URA3*	ATCC
pRSMDJ1		pRS316 carrying ORF plus promoter and terminator sequences (*MDJ1* gene)	This study
pRSVPS74		pRS316 carrying ORF plus promoter and terminator sequences (*VPS74* gene)	This study
Oligonucleotides (sequence 5′ → 3′)			
ACT1-F		ATGGATTCTGAGGTTGCTGCTT	This study
ACT1-R		TAGAAACACTTGTGGTGAACGA	This study
GAPDH-F		ACCACAGTCCATGCCATCAC	This study
GAPDH-R		TCCACCACCCTGTTGCTGTA	This study
For overexpression experiments			
MDJ1-F		CGCGGATCCATGGCTTTCCAACAAGGT	This study
MDJ1-R		CGCCTTAAGTTAATTTTTTTTGTCACCTT	This study
VPS74-F		CGCGGATCCATGTCTACTTTACAACGT	This study
VPS74-R		CGGGTCGACTCATAATAGCATATCCATT	This study
For complementation experiments			
MDJ1-FU		CGCGGATCCAATTTCTACGTCAATGGGTTTT	This study
MDJ1-FL		CGCGGCCGCTTCCACGCCTGCGTTGCTAAA	This study
VPS74-FU		CGCGGCCGCGCCATTGCTGGATTTCTCTTCT	This study
VPS74-FL		CGGGTCGACTCTAGAAAATTAGTACTGGG	This study

The restriction enzyme sites are underlined

Ap^r ampicillin resistance, *ATCC* American type culture collection, VA USA

Gene cloning

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. 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) from genomic DNA prepared by standard protocols [24]. PCR fragments were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and verified by sequencing.

Quantitative reverse-transcription PCR (qRT-PCR)

All strains were cultured in YPD medium containing 5 % NaCl. After 6 h, total RNA was prepared using the UltraspectTM-II RNA system (Biotecx Laboratories, Inc., Houston, TX, USA) and single-stranded cDNA was then synthesized from isolated total RNA using the avian myeloblastosis virus (AMV) reverse transcriptase. A reaction mixture totaling 20 µL containing 1 µg total RNA, 1× reverse-transcription buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1 % Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs), 0.5 U RNase inhibitor, 0.5 µg oligo(dT), and 15 U AMV reverse transcriptase was incubated at 42 °C for 15 min, heated to 99 °C for 5 min, and then incubated at 0–5 °C for 5 min. Transcript levels were quantified by quantitative reverse-transcription PCR (qRT-PCR) using the iQ5 qRT-PCR detection system (Bio-Rad). Amplifications were performed using IQ SYBR green supermix (Bio-Rad) and gene-specific primers (Table 1). Two-step cycling was performed by amplification with an initial preheating step of 10 min at 95 °C, 30 cycles at 95 °C for 15 s and 60 °C for 30 s, and a final 10-min elongation step at 72 °C. The level of GAPDH gene was

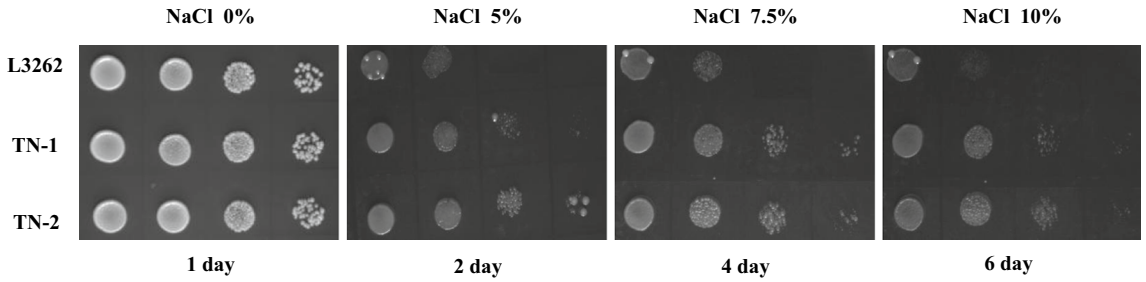

Fig. 1 Identification of salt-tolerant strains. Tenfold serial dilutions of TN 1 and 2 were spotted on YPD plates containing 0, 5, 7.5 or 10 % NaCl and incubated at 30 °C for 1 day (0 %), 2 days (5 %), 4 days (7.5 %) or 6 days (10 %)

used as a loading control. The relative mRNA level (normalized to the level of GAPDH) of each specific transcript was determined with the BioRad software according to the $2^{-\Delta\Delta CT}$ method [26]. The transcript level of wild-type cells at time point zero was set to 1. For the correct calculation of transcript abundance, the PCR efficiency was determined by dilution series with genomic DNA. Analyses were performed using triplicate technical replicates from duplicate biological cultures. To determine melting temperatures for the amplification products of the specific primers, the temperature was raised after qRT-PCR from 65 to 95 °C, and fluorescence was detected continuously.

Results

Isolation of salt-tolerant strains

Approximately 3,000 transformants were selected as leucine prototrophs and replica-plated on both YPD agar and YPD agar containing 10 % (w/v) NaCl. After incubation at 30 °C for 6 days, around 40 mutants were initially selected as salt-tolerant strains that grew in the presence of high NaCl. However, 38 of the mutants grew very slowly. Two strains, designated TN-1 and TN-2, were therefore selected for further analysis following a spot assay on YPD plates containing 0, 5, 7.5 or 10 % NaCl (Fig. 1).

Identification of disrupted genes

Sequencing of genes disrupted by transposon insertion revealed the integration sites depicted in Fig. 2. The sites were located in the ORF of *MDJ1* in TN-1 and in the ORF of *VPS74* in TN-2. The transposon insertion positions are summarized in Table 2. To confirm the disruptions in TN-1 and TN-2, the expression levels of *MDJ1* and *VPS74* were examined by Northern blot analysis (Fig. 3). The expression of *MDJ1* and *VPS74* was not detected as expected. Thus, the transposon insertions completely affected *MDJ1* and *VPS74*.

Contribution to salt tolerance

The deletions of *MDJ1* and *VPS74* to salt tolerance were individually investigated with knockout mutants for the two genes picked from the *S. cerevisiae* single gene knockout collection with a BY4741 background. When the cultures of Δ*mdj1* and Δ*vps74* were spot-assayed on YPD plates containing 5 % NaCl, all of them showed enhanced growth compared to control BY4741 on YPD agar containing 5 % NaCl (Fig. 4). Thus, single gene knockout mutants of *MDJ1* and *VPS74* were salt tolerant.

Confirmation of tolerance by complementation

The finding that TN-1 and TN-2 acquired salt tolerance through the deletions of specific genes implied that complementation of those genes should render cell stress-sensitive. To test this hypothesis, each gene was autologously expressed by cloning amplified DNA fragments presumed to contain the gene's own promoter, ORF and terminator (ORF ± 700 bp) into pRS316 and transforming the vector into TN-1 or TN-2, respectively. The control was conducted by transformation of pRS316. When the spot assay was performed on YPD agar containing 5 % NaCl, the tolerance of complemented cells was significantly reduced compared with the TN mutants (Fig. 5). The ORF of each gene was also PCR-amplified, cloned into pRS316-GAPDH for overexpression, and transformed into strain L3262. pRS316-GAPDH was used as the control plasmid. When the spot assay was performed with 5 % NaCl, overexpressed strains were sensitive to salt stress (Fig. 6). The dosage effect of MDJ1 and VPS74 was determined by quantitative RT-PCR. Figure 7 shows that the expression levels of both MDJ1 and VPS74 were significantly higher under the control of the GAPDH promoter than under their endogenous promoters. These results indicate that sensitivity to salt stress may be dependent on the expression levels of genes MDJ1 and VPS74.

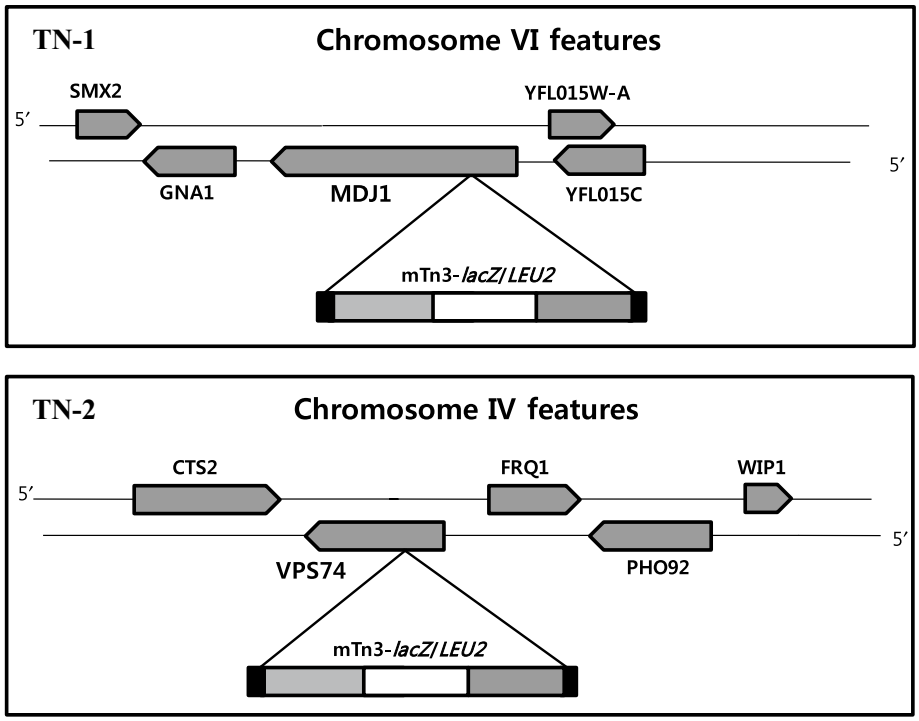

Fig. 2 Determination of transposon insertion sites. Transposon insertion sites were determined by sequencing of recovered transposons from two salt-tolerant strains. Surrounding genetic loci are shown

Table 2 Identified genes that can cause the salt-tolerant phenotype

Strain	Disrupted gene[a]	Function[a]	Insertion position[b]
TN-1	*YFL016C/MDJ1*	Co-chaperone that stimulates HSP70 protein Ssc1p ATPase activity; involved in protein folding/refolding in the mitochondrial matrix	1,306 bp
TN-2	*YDR372C/VPS74*	Golgi phosphatidylinositol-4-kinase effector and PtdIns4P sensor; interacts with the cytosolic domains of cis and medial glycosyltransferases	871 bp

[a] Annotation from the Yeast Genome Database (YGD)

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

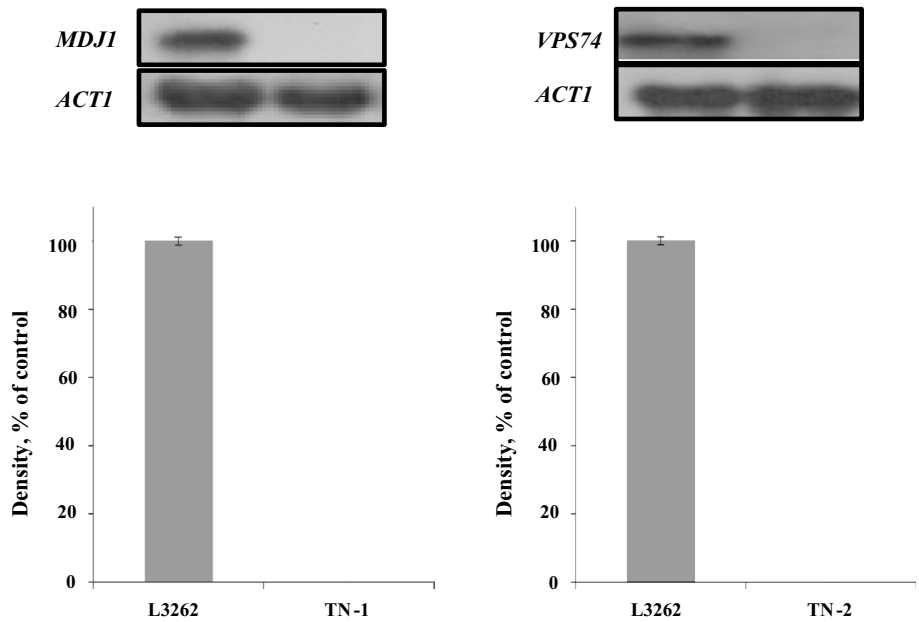

Fig. 3 Expression of genes affected by transposon insertion. Northern blot analysis was performed with total RNAs prepared from L3262, TN-1 and TN-2 mutants. Signals were relatively quantified using a scanning densitometer, normalized with *ACT1*, and represented as % control. *Vertical bars* represent the standard errors for three replicate analyses

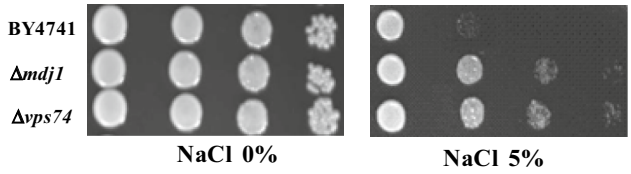

Fig. 4 Salt tolerance of single gene knockout mutants. Tenfold serial dilutions of single gene knockout mutants were spotted on YPD plates containing 0 and 5 % NaCl and incubated at 30 °C for 1 day (0 %) or 2 days (5 %). BY4741 were used as control

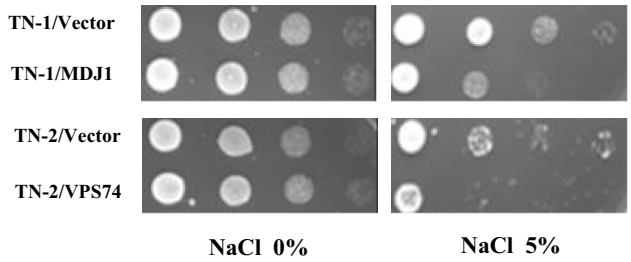

Fig. 5 Restoration of stress sensitivity by complementation. Tenfold serial dilutions of complemented strains were spotted on YPD plates containing 0 and 5 % NaCl and incubated at 30 °C for 1 day (0 %) or 2 days (5 %). Control strains were constructed by transforming pRS316 into TN-1 and TN-2

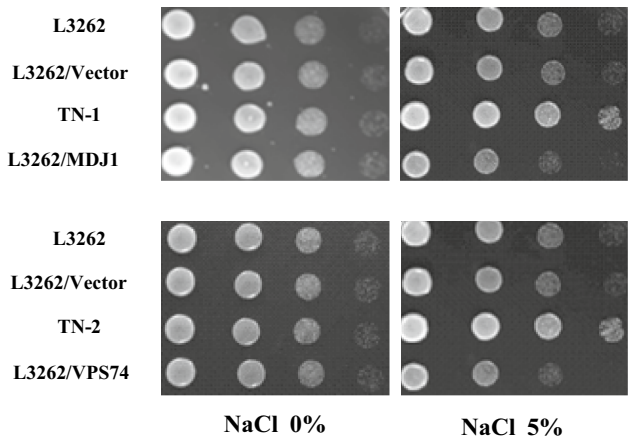

Fig. 6 Effect of overexpression on stress sensitivity. Tenfold serial dilutions of over-expressing strains were spotted on YPD plates containing 0 and 5 % NaCl and incubated at 30 °C for 1 day (0 %) or 2 days (5 %). Control strains were constructed by transforming pRS316-GAPDH into L3262

Growth of TN mutants at salt stress

As shown in Fig. 8, the growth of TN mutants was significantly better than that of the control in YPD medium containing NaCl. No significant differences between the control and TN mutants were observed in YPD medium without NaCl. Therefore, the salt-tolerant phenotype of TN mutants was the result of disrupted genes. In addition, two genes identified may be required for growth under salt stress. Interestingly, the cell growth of all strains was not altered in medium containing 10 % NaCl after 60 h. This result might be explained, due to impaired growth later caused by high salt stress.

Discussion

Resistance to stress is an important phenotype for industrial organisms. In yeast and other organisms, most cellular pathways to stresses are reprogrammed with numerous genes being regulated [27]. Although a large number of the involved genes have been identified, little is currently known about how tolerance is achieved against various stressors. In salt-tolerant organisms, many studies have focused on glycerol synthesis [28, 29]; to attenuate salt stress, salt-tolerant organisms accumulate large amounts of compatible solutes such as glycerol to avoid water loss [30]. It has recently been reported that the deletion of *IRC22* induced salt tolerance in yeast [31]. *IRC22* is a nonessential gene for yeast growth, and its function is unknown. It has been reported that IRC22 protein interacted with ubiquitin-associated proteins such as DSK2 [31]. In this study, we initially isolated two strains with enhanced tolerance to NaCl by screening 3,000 transposon-mediated disruption mutants. We identified two genes, *MDJ1* and *VPS74*, responsible for enhanced salt tolerance.

MDJ1 encodes a co-chaperone that stimulates HSP70 protein Ssc1p ATPase activity. Ssc1p is involved in protein folding/refolding in the mitochondrial matrix, is required for proteolysis of misfolded proteins, and is a member of the HSP40 (DnaJ) family of chaperones. MDJ1/HSP70 has been shown to participate in reactivation of mitochondrial proteins, including DNA polymerase [32, 33]. There has been no report of an association of *MDJ1* with salt tolerance in *S. cerevisiae*. However, in stress conditions such as a heat shock, the role of MDJ1 was protection of mitochondrial proteins, but the biochemical activity of mitochondrial DNA polymerase was not affected by deletion of MDJ1 in optimal growth condition [34], suggesting that MDJ1 plays an important role in stress adaptation. Thus, the role of *MDJ1* in salt tolerance needs to be verified in *S. cerevisiae*.

VPS74 encodes Golgi phosphatidylinositol-4-kinase effector and PtdIns4P sensor; interacts with the cytosolic domains of cis and medial glycosyltransferases; and in the PtdIns4P-bound state, mediates the targeting of these enzymes to the Golgi. VPS74 interacts with the catalytic domain of Sac1p, the major cellular PtdIns4P phosphatase, to direct dephosphorylation of the Golgi pool of PtdIns4P. Tetramerization of VPS74 is required for function. The

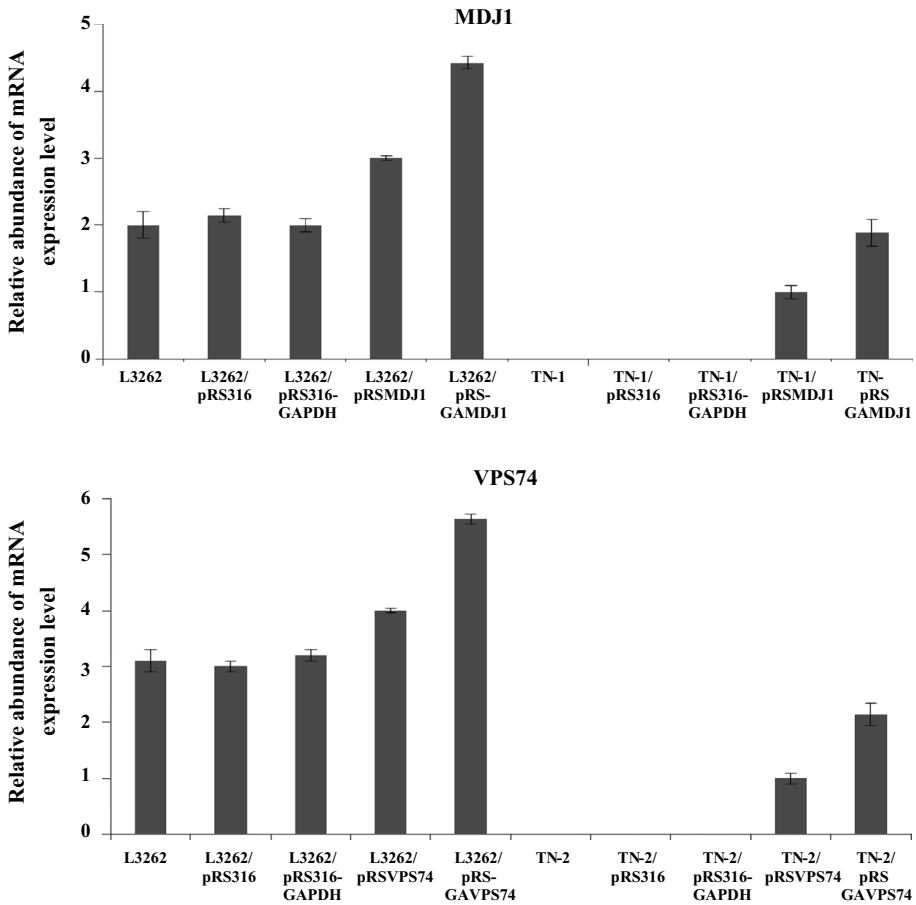

Fig. 7 Dosage effect of *MDJ1* and *VPS74*. The effect was determined by quantitative reverse-transcription PCR. Values were normalized to glyceraldehyde 3-phosphate dehydrogenase before calculating changes. Control strains were TN-1and TN-2, respectively

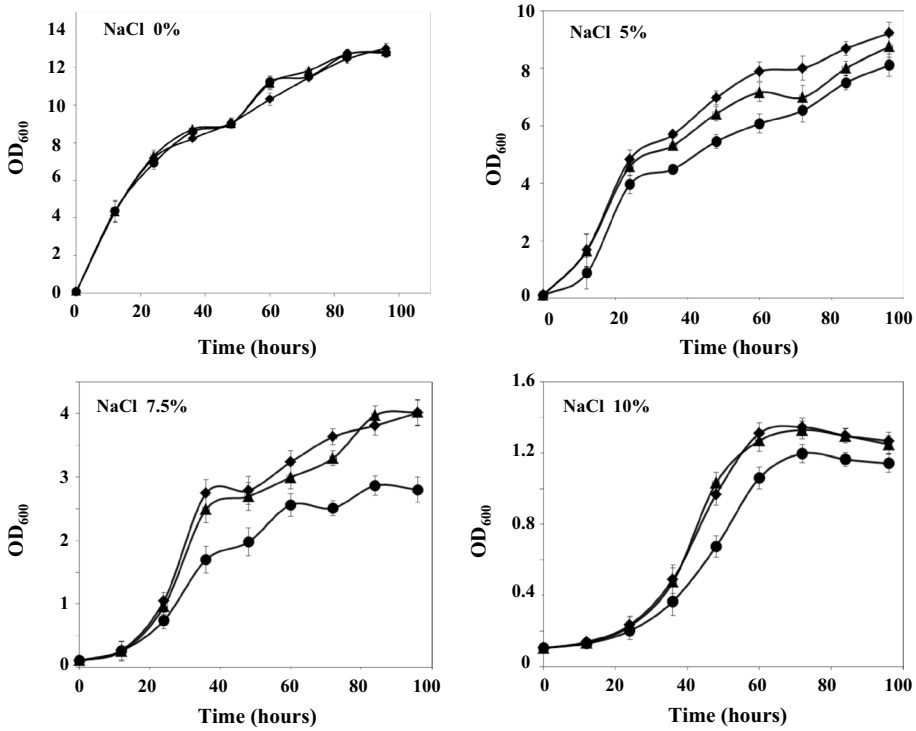

Fig. 8 Growth profiles of TN mutants. Exponentially growing cells (0.5 OD_{600}) were grown at 30 °C in YPD containing 0, 5, 7.5 or 10 % NaCl. Cell growth was monitored. L3262 (*closed circles*), TN-1 (*closed triangles*) and TN-2 (*closed squares*). *Vertical bars* the standard errors for three replicate analyses

protein is an ortholog of human GOLPH3/GPP34/GMx33 [35]. It was reported that VPS74 is required for the proper localization of several Golgi glycosyltransferases and modulation of cell wall integrity [35], suggesting that VPS74 controls a variety of cellular functions. Deletion of VPS35 in budding yeast results in rapamycin hypersensitivity [36], although the role of VPS74 remains to be elucidated. Thus, VPS74 may be responsible for salt tolerance. In relation to ethanol stress, the deletion mutant of VPS74 was sensitive to 8 % ethanol [23]. Therefore, we suggest that the mechanisms of ethanol and salt tolerance are different [37, 38], or VPS74 may generate multiple protein isoforms [39]. Further studies will be needed to elucidate the regulatory mechanisms affecting stress tolerance. By screening a single gene deletion library, 10 deletion strains, ALD6, HOC1, PRO1, SCP1260, SKY1, TIP1, UBP6, YKL161C, YNR004W, and YNR036C, were identified as tolerant to osmotic stress [23]. The absence of overlap in these tolerance genes with our data suggests different mutation methods and library; however, the identification of new genes will provide useful information for understanding the mechanisms of salt tolerance.

SCP has the potential to be developed into a very important source of supplemental protein that could be used in livestock feeding. Thus, the production of SCP from various wastes has been extensively studied on the basis of yeast biomass [5, 40, 41]. Wastewater treatment by yeast is known to be suitable for industrial disposal where the chemical oxygen demand (COD) is high. The treatment capacity of yeast is 3–5 times higher than for the general bacterial-activated sludge method, because the sludge floc formed by yeast is air permeable and sinks easily by sedimentation [42]. Therefore, it may be significant that the yeast strains tolerant to salt stress were used for the production of SCP from kimchi waste brine and for removal of pollutants from wastewater.

This study has revealed that two genes (MDJ1 and VPS74) are responsible for enhanced salt tolerance. These genes required for a salt-tolerant phenotype have not previously been reported. The interrelationship between these genes could not be deduced from their annotated functions. Studies on MDJ1 and VPS74 may help understand at the molecular level how salt tolerance is achieved. One aim of developing strains with enhanced tolerance to salt stress is to provide information for industrial applications. The novel genes identified in this study will be helpful to improve industrial yeast strains for potential application in SCP production using waste brine derived from kimchi production.

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