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Isolation and Characterization of Salt Stress Signaling Components from Yeast *Saccharomyces cerevisiae.*

Jiyoung Lee, Dongjin Shin, Boyoung Lee and Dae-Jin Yun†

Plant molecular Biology and Biotechnology Research Center, and Department of Molecular Biology, Gyeongsang National University, Chinju 660-701, Korea.

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Abstract To identify novel components involved in the salt stress signaling pathway of yeast cells, we used mTn3-mediated transposon tagging library and screened mutants displaying enhanced tolerance to NaCl. Southern blot analysis indicated that more than 80% of the *sre* (salt resistant) mutants possessed only one insertion of the tagged transposon, suggesting that the NaCl resistant phenotype was mediated by a single gene in the majority of the mutants. To define the role of *SRE* genes in the salt stress signaling pathway, we introduced NaCl stress-inducible *ENA1*::*LacZ* construct into the *sre* mutants and examined the expression of β-galactosidase activity. Interestingly, we could detect high level of β-galactosidase activity without any NaCl treatment in the *sre*-3, 4, 6, and 7 mutants. These results indicate that *SRE*-3, 4, and 7 gene are components of salt stress signaling pathway of yeast cells.

Key words: salt stress, signaling components, *saccharomyces cerevisiae*

Introduction

In the yeast *Sacchromyces cerevisiae*, two signaling cascades mediate osmotic adjustment and appropriate ion homeostasis under NaCl stress; a mitogen activated protein (MAP) kinase cascade and a calcineurin, Ca^{2+}/calmodulin dependent protein phosphatase, regulated cascade (Fig. 1). At high osmolarity, two distinct cell-surface osmosensors activate a MAP kinase cascade that eventually up-regulates glycerol production and accumulation [1,2]. Tyrosine-and PP2C threonine/serine-phosphatase may also regulate this pathway by dephosphorylating these MAP kinases [2]. Na^+ stress also activates a calcineurin-dependent pathway that regulates the activity of Na^+ influx and efflux transporters [3,4]. Calcineurin deficient mutants fail to convert the K^+ transport system to the high affinity state that facilitates better discrimination for K^+ over Na^+ [4,5] and exhibit reduced expression of the *ENA1* gene that encodes a plasma membrane sited P-type ATPase essential for Na^+ efflux [4,6]. The net result is substantially greater Na^+ accumulation and consequently an extremely salt sensitive phonotype. Redundant protein phosphatases PPZ1 and PPZ2 regulate negatively the calcineurin-based pathway (Fig. 1) [7].

In this work, we describe identification of components involved in the salt stress signaling pathway of yeast. We constructed yeast mutant library pools using mTn3 transposon and screened *sre* mutants displaying increased salt resistance. Among the *sre* mutants we screened, the *sre*3, 4, 6, and 7 mutants were defective in repression of *ENA1*::*lacZ* reporter plasmid. These results indicate that the mTn3-mediated transposon tagging system is an effective method for cloning of components involved in the negative regulation of salt stress signaling pathway of yeast cell.

MATERIALS AND METHODS

Culture media and growth conditions.

The *S.cerevisiae* strain BWG7a (*MATa ade1-100 leu2-3,112 ura3-52 his4-519*) was used in this study. Standard procedures for yeast culture and growth media preparation were followed [8]. Yeast transformation was performed by the lithium acetate method [9].

Construction of yeast mutant library and screening of *sre* mutant

The mTn3 insertion library DNA was digested with *Not*I to make homologous recombination with host DNA [10]. Linearized DNA was transformed into an yeast strain BWG7a. Yeast mutant library pool was obtained by selecting Leu^+ transformants.

Measurement of sensitivity to NaCl

For spot assays, aliquots (2 $\mu\ell$) from an exponentially growing culture to be tested at an OD_{600} of 0.1 and serial dilution

†Corresponding author
Phone: 82-55-751-6256, Fax: 82-55-759-9363
E-mail: djyun@nongae.gsun.ac.kr

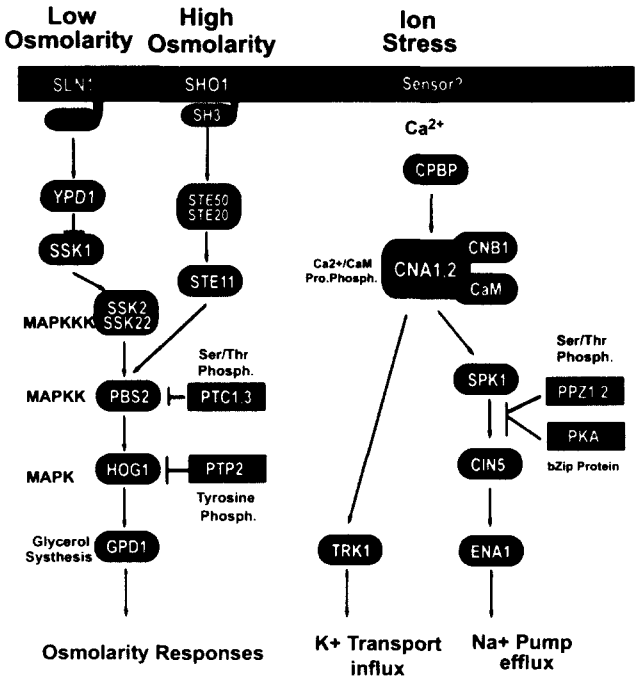

Fig. 1. Osmotic and Na+ ion stress mediated signaling pathways in yeast *Saccharomyces cerevisiae*.

(1:10, 1:100) were spotted onto YPD plates containing 1.5 M NaCl. The plates were examined for the presence of surviving cells after 2 days at 30℃. For measurement of NaCl tolerance in liquid culture, cultures were started at an OD_{600} of 0.01 from diluted overnight cultures, and incubated at 30℃ with shaking for 16-20 h, and the OD_{600} reached were determined with appropriate dilutions.

Southern blot analysis

For Southern blot analysis, genomic DNA was prepared [11] and 5 μg of the genomic DNA was digested with restriction endonuclease *Eco*RI. Hybridization and washing were carried out as described [12]. The probe was prepared by digesting *lacZ* gene with *Bam*HI and *Hpa*I.

β-galactosidase assay

A reporter gene construct *ENA1::lacZ* was introduced into the *sre* mutants [13]. Transformed yeast strains were grown on SD liquid media until saturation and diluted into YPD. Logarithmically growing cells (OD_{600} of 0.5 to 0.8) were then transferred to fresh YPD containing either without or with 1 M NaCl. After 1 hour incubation, cells were harvested and β-galactosidase activity was determined as described.

RESULTS AND DISCUSSION

Construction of mTn3-mediated Transposon tagging library and screening of *sre* mutants.

Yeast *S. cerevisiae* is a useful model fungus to study salt stress response of plants [14,15]. Although many yeast genes involved in salt stress resistance were cloned and the signaling pathways were characterized (See Fig. 1), the genes and mechanisms involved in the salt sensitivity are still elusive. Thus, cloning and functional characterization of novel genes involved in the salt sensitivity will greatly help to understand the salt stress response of yeast cells.

For cloning genes involved in salt sensitivity of yeast cells, we used yeast mTn3 insertion library and screened for mutants displaying increased salt tolerance. The mTn3 insertion library is yeast genomic library constructed in an *E.coli* plasmid and mutagenized with a mTn3 derivative that contains *lacZ*, yeast *LEU2* and *E.coli* β-lactamase [16]. The mTn3 insertion library DNA was digested with *Not* I to release vector sequences. The linearized DNA was transformed into yeast strain BWG7a and Leu+ transformants were selected on YPD plates supplemented with 1.5M NaCl to identify salt resistant colonies (Fig. 2). By using this method, we could screen total 10 *sre* mutants.

To confirm the phenotype of the screened mutants, we performed both spot assay and growth inhibition assay. As shown in figure 2A, the wild-type cells were sensitive to

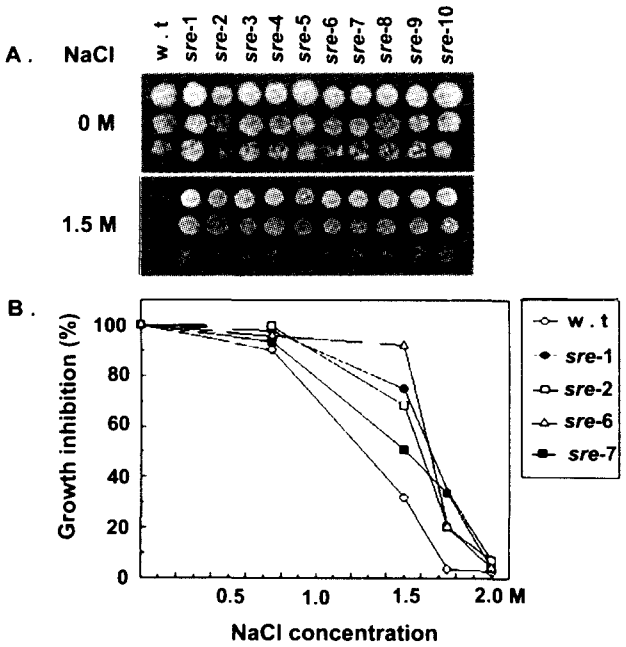

Fig. 2. Mutants displaying increased salt tolerance. (A) Growth of a wild-type and *sre* mutant strains on YPD solid medium. Cells were grown to logarithmic phase and diluted to an OD_{600} of 1.0. A 10-fold serial dilution series was plated on rich glucose media containing either without or with 1.5M NaCl. (B) Measurement of NaCl tolerance of strains shown in (A) in liquid culture. Exponentially growing cultures were diluted to OD_{600} of 0.01 and grown for 16-20 hrs with shanking at 30℃ in the presence of the indicated amounts of NaCl. The final OD_{600} was determined by appropriate dilutions. The values are normalized to the OD_{600} of control cultures grown without NaCl and given as percentage.

1.5 M NaCl. However, the screened *sre* mutants were resistant to 1.5 M NaCl. Similar results were obtained when we performed growth inhibition assay. Tolerance to NaCl was determined in 0.5-ml liquid cultures containing various concentrations of NaCl. As shown in figure 2B, most of *sre* mutant strains that we tested had varying degrees of growth on NaCl containing media. However, comparing wild-type, the *sre* mutants were clearly resistant to NaCl. Taken together, these results demonstrate that the products of *sre* genes were required for sensitivity to NaCl.

Analysis of *sre* mutants by Southern blot

It has been reported that some of yeast transformants obtained from the mutagenized library contain more than one mTn3 insertion [14]. Therefore, we decided to determine whether the phenotype of *sre* mutants were mediated by a single insertion of mTn3 transposon. For this, we carried out Southern blot analysis using integrated *lacZ* as a probe (Fig. 3B). As shown in figure 3, we could detect only a single band from more than 80% of *sre* mutants. These results indicate that most of NaCl tolerant *sre* mutants contain mutation in single locus of the chromosome and the salt tolerance phenotype is caused by a single gene mutation.

Genetic analysis of *sre* genes using *ENA1::LacZ* constructs

Since *sre* mutation caused salt resistant phenotype, it could be possible that some of the *SRE* genes could suppress the NaCl stress signal transduction pathway in yeast that turns on the transcription of genes encoding proteins involved in the regulation of the internal concentration of Na^+. To investigate this, we examined whether mutation of *sre* gene

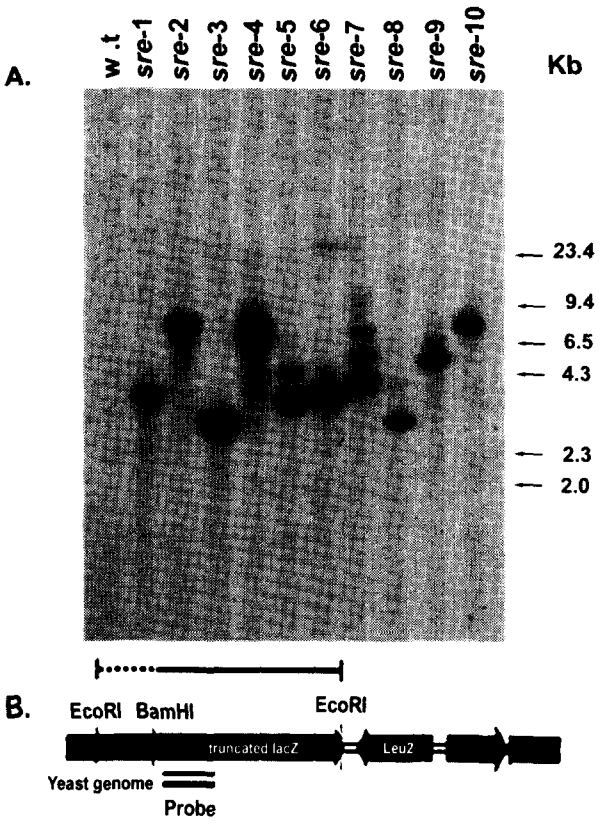

Fig. 3. Estimation of foreign gene integration in the *sre* mutants. (A) The yeast genomic DNA was prepared from a wild-type and *sre* mutant strains. The DNA was digestion with *EcoRI* and Southern blot analysis was performed as described in the Materials and Methods. Lower panel (B) shows schematic description of mTn3 transposon inserted in the *sre* mutants and *lacZ* gene used as probe for Southern blot analysis.

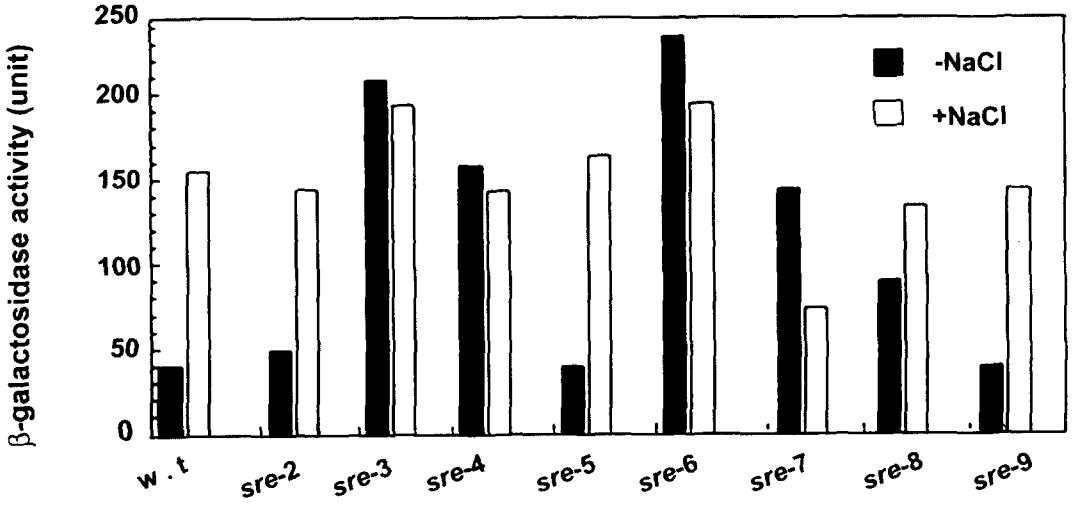

Fig. 4. Analysis of the *ENA1* gene activation in the *sre* mutants. Reporter gene construct *ENA1-LacZ* (Cunningham *et al.*, 1994) was introduced into the *sre* mutants. Yeast cells harboring the plamisd were cultured at 30℃ overnight and inoculated to an OD_{600} of 0.1 into YPD medium supplemented for 1 hr without (solid bars) or with (open bars) 1.0 M NaCl. Cells were harvested from cell extracts prepared at various time, and β-galactosidase activity was measured as described in the Materials and Methods.

could influence the activation of NaCl inducible genes in yeast. It has been shown that the *ENA1* gene, which encodes a plasma membrane P-type ion pump thought to be responsible for Na^+ and Li^+ ion efflux [17], is induced by high concentration of Na^+ ion [18]. Therefore, we introduced a reporter gene, *ENA1::lacZ*, to the *sre* mutants and β-galactosidase activity was measured in the cell extracts prepared from the transformed cells. As show in the figure 4, the level of *lacZ* expression is low in the wild type without salt treatment but increased after treated with 1M NaCl. However, the level of β-galatosidase activity was markedly enhanced in the *sre3, 4, 6,* and *7* without any salt stress treatments. These results indicate that the responsible *SRE* genes are negative regulators of salt stress signaling pathway of yeast cell.

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