

Functional Screening of Plant Genes Suppressed Salt Sensitive Phenotype of Calcineurin Deficient Mutant through Yeast Complementation Analysis

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Understanding salt tolerance mechanisms is important for the increase of crop yields, and so, several screening approaches were developed to identify plant genes which are involved in salt tolerance of plants. Here, we transformed the *Arabidopsis* cDNA library into a salt-sensitive calcineurin (CaN)-deficient (*cnb1*) yeast mutant and isolated the colonies which can suppress salt-sensitive phenotype of *cnb1* mutant. Through this functional complementation screen, a total of 34 colonies functionally suppressed the salt-sensitive phenotype of *cnb1* yeast cells, and sequencing analysis revealed that these are 9 genes, including *CaS*, *AtSUMO1* and *AtHB-12*. Among these genes, the ectopic expression of *CaS* gene increased salt tolerance in yeast, and *CaS* transcript was up-regulated under high salinity conditions. *CaS*-antisense transgenic plants showed reduced root elongation under 100 mM NaCl treatment compared to the wild type plant, which survived under 150 mM NaCl treatment, whereas *CaS*-antisense transgenic plant leaves turned yellow under 150 mM NaCl treatment. These results indicate that the expression of *CaS* gene is important for stress tolerance in yeast and plants.

Key words : NaCl, salt tolerance, yeast genetic system

Introduction

Plants frequently encounter various environmental stress conditions such as high salinity and drought. These stresses, especially high salinity, change from altered gene expression and cellular metabolism to plant growth, development, and productivity [15, 19]. Many researchers have been trying to identify plant genes which are involved in stress tolerance mechanisms through forward genetic approaches.

Late embryogenesis-abundant (*LEA*) genes are induced by salt stress and abscisic acid (ABA), and function in homeostasis as molecular shields under stress conditions [12, 18]. These *LEA* genes were initially screened from barley and tomato cDNA libraries through colony hybridization [3, 8]. *Responsive to desiccation* (*RD*) genes of *Arabidopsis*, including *RD29A*, identified through the colony hybridization ap-

proach, are induced by ABA and desiccation treatments [30]. Especially, *RD29A* promoter involves dehydration responsive element (DRE), which is important for the rapid response of *RD29A* after dehydration or high salinity treatment [30, 31]. Two proteins, DREB1A and DREB2A, were identified as transcription factors to bind the DRE sequence of the *RD29A* promoter by using the yeast one-hybrid screening technique [11]. *DREB1A* transcript is induced under low-temperature conditions and the expression of *DREB2A* is positively regulated by dehydration. Transgenic *Arabidopsis* over-expressing each genes show tolerance to freezing and dehydration stress conditions [11].

During the last 10 years, global genome expression profiles in plants have been monitored through microarray technology. In *Arabidopsis*, expression of more than 7000 genes was monitored using a full-length cDNA microarray and 277, 53, and 194 genes were identified as drought-, cold-, and high salinity-inducible genes, respectively [22]. An oligomer microarray, covering about 37,000 genes of rice, was used to monitor genome expression profiling, and revealed organ specific gene regulation under drought or high salinity conditions [33]. Promoters of down-regulated genes were analyzed and novel promoter motifs associated with

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gene repressed by drought were suggested in rice shoot and panicle [33].

The numerous cellular mechanisms of salt tolerance in yeast are conserved in plant cells, and yeast has been used for screening of salt tolerance genes in plants [15, 23]. *Arabidopsis salt tolerance protein (STO)* and *salt tolerance zinc finger (STZ)* were identified as proteins complementing the salt-sensitive phenotype of yeast calcineurin mutants [10]. Expression of STZ was rapidly increased under abiotic stress conditions such as high salinity and drought. STZ protein has a bind ability to cause A(G/C)T repeats within an EP2 sequence, and functions as a transcription repressor to increase stress tolerance following growth retardation [21]. Even if the mRNA level of the *STO* gene was not increased, STO protein interacted with Myb transcription factor homologue, and overexpression of *STO* showed tolerance to salt stress conditions [16]. Plasma membrane Na^+/H^+ antiporter, salt tolerance locus SOS1 complements salt tolerance of the *nhx1hhx1* double yeast mutant, which is deficient in endogenous Na^+ transporters, and controls long-distance Na^+ transport in plants [24]. *Arabidopsis* vacuolar Na^+/H^+ antiporters, AtNHK1, 2, and 5, suppressed the salt- and hygromycin-sensitive phenotype of a yeast *nhx1* mutant [32]. In this study, we screened plant salt-stress related genes using a unicellular yeast genetic system. Among salt-stress related genes, we chose *CaS* gene, and examined the biological role of *CaS* in high salinity stress responses in plants.

Materials and Methods

Plant material and yeast strain

Arabidopsis thaliana (ecotype Columbia) was grown in a growth chamber at 22°C under conditions of 70% relative humidity and a 16 h light/8 h dark cycle. *Saccharomyces cerevisiae* YP9 strain (*Mata ura3 leu2 his3 trp1 ade2 lys2 cnb1::HIS3*, isogenic to YPH499) was mutated in calcineurin B subunit and was used for screening salt stress-related genes [14].

Screening of cDNA clones complementing a NaCl stress-sensitive strain of yeast YP9

An *Arabidopsis* expression library constructed pGAD424 vector (Clontech, CA, USA) was introduced into the mutant yeast by the LiOAc method [25]. Approximately 2.5×10^5 transformants were selected for leucine prototrophy by plating on synthetic complete medium lacking leucine (SC-Leu), and were pooled to yield a transformed yeast library. The

transformed yeast cells were then plated at a density of 10^7 cells per YPD (1% yeast extract/2% peptone/2% dextrose) plate supplemented with 1.1 M NaCl. Putative positive colonies were obtained 4 days after plating. Plasmid DNAs were isolated from the yeast cells [25] and were reintroduced into YP9 yeast cells to confirm complementation. cDNAs constructed pGAD424 vector were sequenced with the dideoxy termination method using a dye-terminator cycle sequencing kit [25]. The sequencing reaction was analyzed using an automatic sequencer (ABI, MD, USA).

Spot assay

The full-length of *CaS* gene was subcloned into pYES2, an expression vector with the URA3 selection marker and the galactose-inducible GAL1 promoter [25]. The construct was introduced into the YP9 strain by LiOAc method, and the transformed cells were plated on a SC-Ura plate. The transformed cells were grown in SC-Ura medium overnight. Aliquots (2 μl) from an exponentially growing culture of cells at an O.D.₆₀₀ of 0.1 were serially diluted (1:10, 1:100, 1:1,000) and were spotted onto YPGal (1% yeast extract/2% peptone/2% galactose) plates containing NaCl (1.1M). The photograph was taken after 3 day at 30°C.

Semi-quantitative RT-PCR analysis

2-week-old *Arabidopsis* seedlings were treated with NaCl (100 mM) for various durations (0, 0.5, 1 and 6 hr) [25], and total RNA from the *Arabidopsis* seedlings was isolated with RNeasy Plant Mini kit, according to manufacturer's instruction (Qiagen, USA). Semi-quantitative RT-PCR was performed with specific primers. *CaS*; 5'-GGACTCGTACACGG ATTCGG-3' and 5'-GTTCCGGACCTAGTGCCAAA-3'. *KIN1*; 5'-AGCGGGAGGTGTTAACTTCG-3' and 5'-TGGGGAGTTT GATCTTTC GCA-3'. *Actin*; 5'-TCGCTGACCGTATGAGC AAA-3' and 5'-TGGAGATCCACATCTGTA ACCA-3'.

Root growth measurement

To compare root elongation, seeds of wild-type and transgenic plants were washed with 2% NaOCl solution and were plated on 1/2 MS medium. Plates containing seeds were stratified at 4°C for 4 days and were then transferred to a growth chamber (22°C under a 16-h-light/8-h-dark regime) in a vertical orientation [29]. Seedlings grown for 7 days were transferred to 1/2 MS medium with or without NaCl. Seedlings were further incubated in the growth chamber for another 5 days and root elongation was then measured.

Three replicate plates were used for each treatment to ensure the reproducibility of the data.

Results

Screening of plant genes showing increased salt tolerance in yeast

Under abiotic stress conditions, such as high salinity, plants accumulate the phytohormone ABA, which controls regulatory signal networks for survival. It has been also suggested that salt tolerance mechanisms are similar between plants and yeast. To identify plant genes that confer salt tolerance in plant and yeast, *Arabidopsis* cDNA library was introduced to CaN null mutant (*cnb1*) yeast cells showing salt-sensitive phenotype. Approximately, 2.5×10^5 independent transformants were screened on YPD medium supplemented with 1.1 M NaCl, and more than 100 colonies showing remarkable NaCl tolerance were obtained. To validate this screening result, we rescued the plasmid from 40 salt tolerant transformants, and re-introduced these plasmids to *cnb1* yeast cells. As shown Fig. 1, under normal medium conditions the growth of each transformant is almost similar to that of *cnb1* yeast cells harboring only the vector. However, a total of 34 colonies functionally suppressed the salt-sensitive phenotype of *cnb1* yeast cells (Fig. 1). Through sequencing analysis, these plasmids were determined to represent 9 genes and we named these suppressors as SRGs (Salt-stress Related Genes, Table 1).

Expression of CaS genes in yeast gives rise to the increase of salt tolerance

To validate the usefulness of the yeast genetic system for screening plant salt tolerance genes, among SRG genes, we selected and studied the *CaS* gene, encoding extracellular

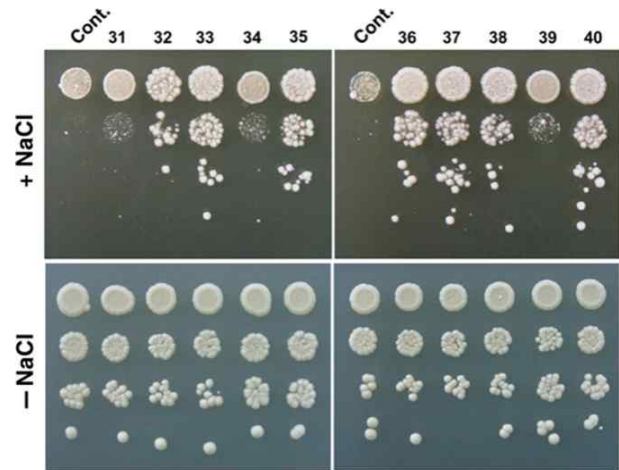


Fig. 1. Plant genes involved in salt stress tolerance were screened using a yeast genetic system. Plasmid DNAs which were isolated from the yeast cells suppressed salt sensitivity of the Δcnb mutant. 2 μ L of each cell was spotted on YPD medium with or without 1.1 M NaCl, and the photograph was taken after 3 days at 30°C.

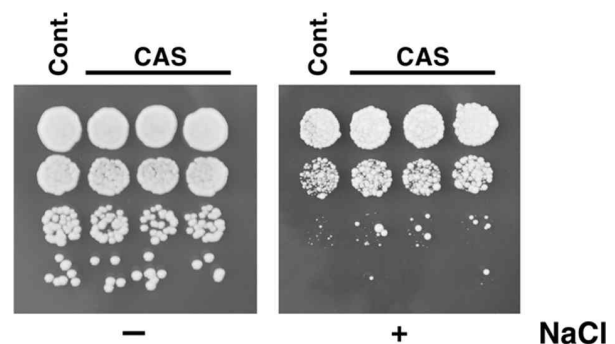


Fig. 2. Expression of *CaS* rescued the salt sensitive phenotype of Δcnb mutant. The YP9 yeast strain was transformed with the plasmid containing *CaS* gene. Transformed cells were tested for salt tolerance on YPGal medium as described in Materials and Methods. The photograph was taken after incubation for 3 days at 30°C.

Table 1. List of salt-stress related genes (SRGs)

Name	Protein information	Accession No.	Screened colony No.
SRG1	Elongation factor 1a	At1g07930	4
SRG2	Transcription factor AtHB-12	At3g61890	9
SRG3	Fructose 1,6-bisphosphate aldolase	At3g52930	3
SRG4	SUMO1	At4g26840	6
SRG5	APETELA3	At3g54340	4
SRG6	Reticulon	At1g64090	2
SRG7	Extracellular Ca^{2+} sensing receptor	At5g23060	3
SRG8	Ribosomal protein L19	At2g43010	1
SRG9	Unknown protein	At1g64090	2

cDNAs showing salt tolerance in yeast were rescued and sequenced with the dideoxy termination method, using a dye-terminator cycle sequencing kit. The sequencing reaction was analyzed using an automatic sequencer.

Ca^{2+} sensing receptor (At5g23060). *CaS* consists of a single transmembrane domain, two Ca^{2+} binding motifs, and a rhodanese homology domain [9]. It was reported that *CaS* exhibits a low-affinity/high-capacity Ca^{2+} binding ability, and repression of *CaS* gene disrupts Ca^{2+} signaling in guard cells [9, 27]. To further analyze whether the expression of *CaS* is able to confer tolerance to salt stress in yeast cells, we constructed full length *CaS* gene in the episomal plasmid pYES2, placing it under the control of the GAL1 promoter, and tested galactose-dependent phenotype to NaCl stress. The transformants (Ed note: Spell checking of scientific terms is also carried out for all papers) harboring full length of *CaS* gene suppressed the NaCl-sensitive phenotype of *cnb1* yeast cells (Fig. 2).

CaS is specifically induced by NaCl stress in plants.

To identify the physiological role of *CaS* in plants, RNA expression levels were analyzed under NaCl stress conditions. Total RNA was isolated from *Arabidopsis* seedlings treated with NaCl, and semi-quantitative RT-PCR was performed with *CaS* specific primers (Fig. 3). Transcript of *KIN1* gene, which was used as positive control for NaCl treatment, was increased by high salinity treatment. The expression level of *CaS* gene was induced under high salinity conditions and a maximum transcript level was reached at 1 h of NaCl treatment. This result indicated that the biological role of *CaS* may be to mediate the NaCl stress response in plants.

CaS antisense transgenic plants showed sensitive phenotype to salt stress.

A *CaS* antisense transgenic plant was kindly provided by

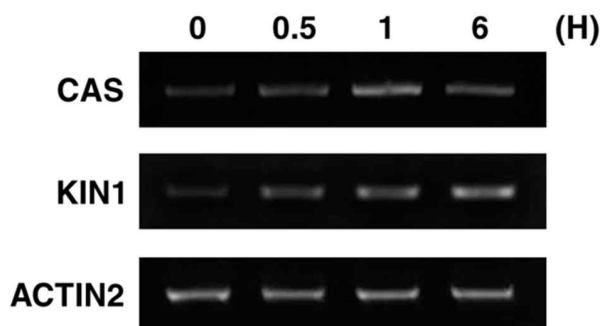


Fig. 3. Transcript level of *CaS* was increased by NaCl treatment. Total RNA was extracted from 2-week-old *Arabidopsis* seedlings treated with 100 mM NaCl for the indicated time, and semi-quantitative RT-PCR was performed using *CaS*-specific primers. *KIN1* was used as a positive control, and *ATCTIN2* was used as a quantitative control.

Prof. Zhen-Ming Pei of Duke University, USA. *CaS* transcript and protein was completely reduced in this *CaS* antisense transgenic plant [9]. To investigate the biological effect of *CaS* on salt stress tolerance, root elongation of *CaS* antisense transgenic plant was examined under salt stress conditions. No difference of root elongation was found between *CaS* antisense transgenic plants and wild-type plants under normal growth conditions. Root elongation of wild-type plants was not effected in MS medium containing 100 mM NaCl under our experiment conditions, however, root elongation of *CaS* antisense transgenic plants was reduced to 50% under the same conditions (100 mM NaCl treatment) (Fig. 4). Furthermore, root elongation of wild-type plants was severely impeded in MS medium containing 150 mM NaCl, but wild-type plants were still survived. In the case of *CaS*

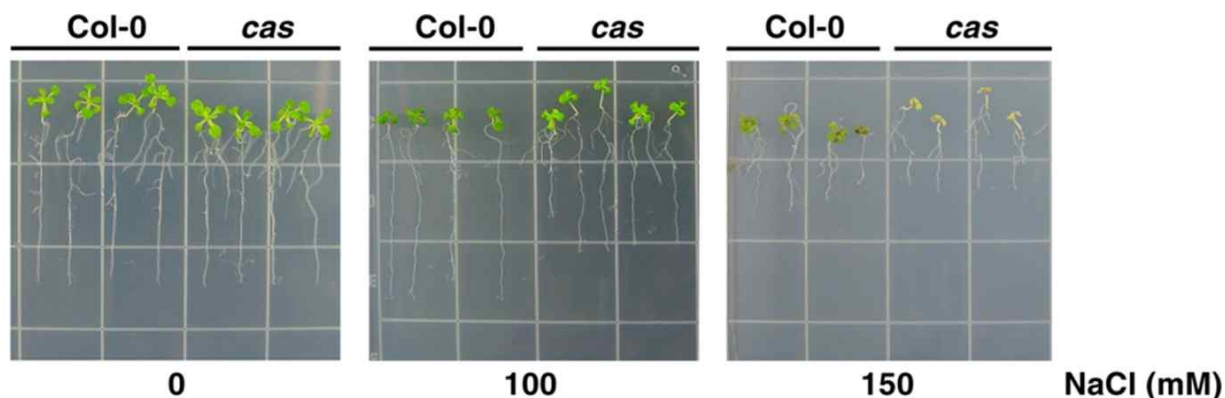


Fig. 4. *CaS*-antisense transgenic plants showed sensitive phenotype under high salinity conditions. 5-day-old seedlings of Col-0 and *CaS*-antisense transgenic plant were transferred to half-strength MS medium with or without NaCl. The photograph was taken after an additional 7 days.

antisense transgenic plants, root elongation was severely impeded and leaf color was altered to yellow under the experimental conditions (Fig. 4). This result suggests that the expression of *CaS* is important for salt tolerance and Ca^{2+} -mediated salt tolerance signaling is conserved between plant and yeast.

Discussion

Many stress-related genes in plants have been identified through diverse forward genetic approaches. However, the signal transductions and response mechanisms still remain to be understood. In this study, we used a yeast genetic system to identify plant genes showing salt tolerance, because genetic manipulation of yeast is a much simpler process than that of plants, and salt tolerance mechanisms, such as ion transport systems, are similar between plants and yeast [5]. Lippuner *et al.* used the salt-sensitive phenotype of yeast calcineurin mutants and identify two genes related to salt tolerance, *STO* and *STZ* [10]. Expression of *Arabidopsis* SR-like splicing proteins (AtRCY1) enhanced lithium and sodium tolerance in yeast and *Arabidopsis* [4]. Li-tolerant lipase 1 (AtLTL1) was screened for its functional expression in yeast [17]. *AtLTL1* expression showed a weak tolerant phenotype to salt stress in yeast, but overexpression of *AtLTL1* conferred salt tolerance in *Arabidopsis* [17]. We identified 9 kinds of salt tolerance genes, including *CaS*, using the yeast system (Table 1). Among these genes, *SRG4* encodes small ubiquitin-like modifier 1 (AtSUMO1) which is important in posttranslational modifications of proteins. ABA- and stress-responsive genes, such as *RD29* and *AtPLC1*, were up-regulated in *AtSUMO1* overexpression transgenic plant [13]. Recently, it has been determined that SUMOylated proteins are accumulated in response to drought stress, and double mutants of SUMO protease genes, *ots1* and *ots2*, showed salt sensitive phenotype in *Arabidopsis* [1, 2]. *SRG1* encodes a homeobox-leucine zipper domain transcription factor 12, AtHB-12, which regulates sodium exclusion in yeast [25].

Perception of stress cues and relay of the signals to switch on adaptive responses are the key steps leading to plant stress tolerance. In plant cells, calcium (Ca^{2+}) serves as a second messenger and is a major point of signaling cross-talk during abiotic stress signaling [20]. Phytohormone ABA induces cytosolic free Ca^{2+} concentration oscillation in guard-cells, and oscillations in cytosolic Ca^{2+} led to stomatal

closure [7, 26]. High salinity stress also increases cytosolic free Ca^{2+} concentration [6]. Recently, it was reported that NaCl-induced cytosolic free Ca^{2+} concentration was controlled through plasma membrane and intracellular Ca^{2+} permeable channels [28]. *CaS* gene was identified as an extracellular Ca^{2+} sensing receptor through a function screening assay in mammalian HEK293 cells [9]. *CaS* gene was mainly expressed in guard cells of leaf and *CaS* antisense transgenic plants had growth defects under low concentrations of Ca^{2+} [9]. *CaS* regulates concentrations of inositol 1,4,5-triphosphate (IP_3) and stomatal conductance in plant [27]. In here, we showed that the expression of *CaS* gene suppressed the salt-sensitive phenotype of *cnb1* yeast cells, and that *CaS* antisense transgenic plants were more sensitive than wild-type plants under high salinity conditions (Fig. 3 and 5). Even though yeast does not have guard cells, these results mean that cytosolic free Ca^{2+} concentration is important for salt tolerance in both yeast and plants.

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초록 : 애기장대의 염해 저항성 관련 유전자의 기능적 선별

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작물의 생산성 증가를 위해 염해 저항성 메커니즘을 이해하는 것이 중요하다. 식물의 염해 저항성에 관련된 유전자를 확보하기 위한 여러 가지의 선별방법이 개발되었다. 본 논문에서는 애기장대의 cDNA 라이브리를 염해 감수성 효모인 *cnb* 돌연변이체에 삽입하여 염해 감수성 표현형을 회복하는 콜로니를 선별하였다. 이 선별방법을 통하여 34종의 *cnb* 돌연변이체의 염해 감수성을 회복하는 콜로니를 선별하였으며, 염기서열분석을 통하여 *CaS*와 *AtSUMO1*, *AtHB-12* 등 9종의 유전자임을 확인하였다. 이들 유전자 중 *CaS*의 발현이 염해 저항성을 증가시키는 것과 염해 처리에 의해 *CaS*의 유전자의 발현이 증가되는 것을 확인하였다. *CaS* 발현억제 형질전환체는 100 mM 염처리에 의하여 뿌리생장이 저해되었다. 또한 150 mM 염처리에 의하여 *CaS* 발현억제 형질전환체의 잎에서 백화현상을 나타내었다. 이러한 결과를 통하여 *CaS* 유전자가 효모와 식물에서 염해 저항성에 중요한 유전자임을 증명하였다.