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Functional analysis of a calcium-binding transcription factor involved in plant salt stress signaling

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Abstract **Calcium is known to serve as a secondary messenger to mediate salt stress signaling pathway. We found a calcium-binding basic/helix-loop-helix-type transcription factor (*AtNIG1*) as a salt stress-responsive gene by using the suppression subtractive hybridization. The AtNIG1 was targeted into nucleus and bound to $^{45}Ca^{2+}$, suggesting that AtNIG1 is a nuclear calcium-binding protein. In addition, AtNIG1 bound specifically to the E-box-DNA sequence (CANNTG), which is found in the promoter regions of many salt stress-related genes. Functional analyses with an *atnig1-1* knockout mutant revealed that the mutant plants show enhanced sensitivity to salt stress. Further analyses indicated that the *atnig1-1* plants have reduced survival rate, fresh weight, chlorophyll content, and protein content upon salt stress, suggesting that the AtNIG1 plays a critical role in plant salt stress signaling. Therefore, this study represents that AtNIG1 is the first known calcium-binding transcription factor involved in plant salt stress signaling.**

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Keywords: bHLH; Calcium; Salt stress; Transcription factor; *Arabidopsis*

1. Introduction

In plant cells, a variety of physiological and environmental stimuli induce the elevation of cytosolic Ca^{2+} concentration [1,2]. Ca^{2+} functions as an ubiquitous second messenger in a wide variety of cellular processes, especially as a secondary messenger in salt stress signaling pathway [1,2]. To decode an ubiquitous Ca^{2+} signal into specific biological information, various Ca^{2+}-binding proteins are considered to function as specific Ca^{2+} sensors. Genetic analyses have elucidated the salt overly sensitive (SOS) signaling pathway that controls salt stress response [3,4]. SOS3, a Ca^{2+}-binding protein, senses the Ca^{2+} change elicited by salt stress [5]. In addition, salt stress response was shown to be mediated by signaling pathways distinct from the SOS pathway, with the identification of several protein kinases activated by salt stress [4]. Mitogen-activated protein kinases (MAPKs) are activated by salt stress [6]. Calcium-dependent protein kinases have also been implicated in salt stress response in association with calcium signaling [7]. In addition, phospholipid signaling is closely related to salt stress [8]. Therefore, different signaling mechanisms are involved in plant salt stress signaling.

Salt stress triggers elevations in the cytosolic Ca^{2+} levels in plant cells. As a secondary messenger, Ca^{2+} activates signaling pathways and therefore influences multiple aspects of cellular functions [1,2]. Calcium sensors have been identified in plants, such as calmodulins (CaMs), Ca^{2+}-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) [9–11]. Despite considerable progress in understanding stress signal transduction, the mechanism of stress response remains largely unknown. Thus, the identification of novel signaling components will contribute to the clarification of stress signaling. Here, we report that the AtNIG1 is a novel calcium-binding bHLH transcription factor involved in plant salt stress signaling.

2. Materials and methods

2.1. Suppression subtractive hybridization (SSH)

For the PCR-based cDNA subtraction, the experiment was carried out according to the manufacturer's instructions (Clontech). *Arabidopsis thaliana* (Columbia) seedlings (2-week-old) were treated with 200 mM NaCl for 12 h and used as samples for the SSH.

2.2. Expression of recombinant AtNIG1 in Escherichia coli

The cDNAs coding for AtNIG1 and AtCaM4 were amplified by PCR with *AtNIG1*-specific primers and *AtCaM4*-specific primers. The nucleotide sequences of PCR products were confirmed by DNA sequencing. The amplified products were gel-purified and inserted into a pET32b expression vector (Novagen). The recombinant AtNIG1 and AtCaM4 were produced in *E. coli* strain BL21(DE3)pLysS, and then these proteins were purified using Ni-NTA chromatography (Qiagen) as described by the manufacturer. The amount of protein was estimated by the Bradford's method using a protein assay kit (BioRad).

2.3. Ca^{2+}-binding assay

The purified proteins were electrophoresed on SDS-denaturing gels. The AtNIG1 protein was then electrotransferred onto a polyvinylidene difluoride (PVDF). The blot was incubated in overlay buffer (50 mM KCl, 5 mM $MgSO_4$, and 25 mM Tris (pH 7.0) containing $^{45}Ca^{2+}$ (5 μCi/ml). After washing, the blot was exposed to a BAS image plate (Fujifilm).

2.4. Electrophoretic mobility shift assay (EMSA)

Oligonucleotides of the E-box (gat ccg ggt CANNTG tac cta cca acc tta aac ac) and the mE-box (gat ccg ggt CANNCC tac cta cca acc tta aac ac) were used in the EMSA. Double stranded probes were synthesized by the Klenow fragment (Promega) with [^{32}P]dCTP. The assay mixtures contained recombinant protein (0.5 μg), 1 ng of binding

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Abbreviations: bHLH, basic helix-loop-helix; RT, reverse transcription; ABA, abscisic acid; SSH, suppression subtractive hybridization; EMSA, electrophoretic mobility shift assay

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probe (1.0×10^6 cpm), 1 μg of poly(dI–dC), 20 mM Tris (pH 7.5), 50 mM KCl, 15% glycerol, and 0.5 mM DTT in a 20 μl reaction volume. The E-box probes were incubated at room temperature for 20 min and electrophoresed on an 8% polyacrylamide gel on a $0.5 \times$ TBE buffer. The gel was then dried and exposed to the BAS image plate (Fujifilm).

2.5. Subcellular localization of the AtNIG1

To produce the AtNIG1-smGFP fusion proteins, the full-length *AtNIG1* cDNA was inserted into the binary pCAMBIA1300 vector containing the *smGFP*. The construct was then introduced into anion epidermal cells using the biolistic PDS-1000 gene delivery system (Bio-Rad). The bombarded cells were examined under a fluorescence microscope using UV-blue light excitation (Zeiss).

Table 1
Potential salt stress-regulated transcription factors identified by the SSH method

Clone	Putative gene function	AGI no.
AtNIG1	bHLH transcription factor	At5g46830
AtNIG8	Trihelix DNA-binding protein	At5g28300
AtNIG18	bZIP-like protein	At4g34000
AtNIG28	Homeobox-Leu zipper	At3g61890
AtNIG32	bZIP-like protein	At2g48270
AtNIG46	Zinc finger-like protein	At4g23450
AtNIG54	Putative transcription factor	At5g47640
AtNIG61	AtMYB4	At5g26660
AtNIG75	MYB28-like protein	At5g61420
AtNIG81	Leu zipper-like protein	At1g45249

2.6. Characterization of a T-DNA insertional allele of the AtNIG1 gene

A T-DNA insertional mutant (*atnig1-1*; Salk_119765) were obtained from the Nottingham *Arabidopsis* Stock Centre [12]. The mutant seeds were grown in soil under long-day conditions (16 h light/8 h dark). For complementation analysis, the *AtNIG1* cDNA was inserted into the binary pCAMBIA1300 vector. Complementation construct carrying the *AtNIG1* transgene under the cauliflower mosaic virus 35S promoter (CaMV35S) was transformed into the *atnig1-1* plants using *Agrobacterium*-mediated floral dip method [13]. For seed germination assays, seeds were germinated on MS agar plates supplemented with different concentrations of NaCl, mannitol, or abscisic acid (ABA). Seeds were imbibed for 3 days at 4 °C to encourage synchronous germination and then moved under the constant white light (150 μmol m^{-2} s^{-1}). Seeds with emerging cotyledons were scored as germinated. For survival test, the *atnig1-1* and wild-type plants were transferred to MS plates containing 100 mM NaCl and cultured for 7 days. The treated plants were then transferred to soil under normal growth conditions for three weeks. Chlorophyll content was measured spectrophotometrically after extraction in 90% acetone [14]. Protein content was determined using a Bio-Rad protein assay kit (Bio-Rad).

2.7. Reverse transcription-PCR (RT-PCR)

Arabidopsis (Col-0) plants were grown in soil and subjected to salt stress conditions. *Arabidopsis* seedlings (2-week-old) were treated with 200 mM NaCl for varying lengths of time to ascertain their effects. Total RNA was extracted, and 1 μg of total RNA was used as a template for each RT reaction. The PCR conditions used were: 26–30 cycles of 94 °C for 40 s, 54 °C for 40 s, and 72 °C for 1–2 min, followed by 7 min of a final extension at 72 °C. The gene-specific primers used were: 5′-GTC CTG TCG CTT TAT GCT AGT-3′ (forward) and 5′-GGT GGT TTC ATG GAT ACC AGC-3′ (reverse) for *AtACT3*, 5′-GAC CCA AAG ACG GAG ACT CTT-3′ (Forward) and 5′-GCC AAG TGA TTG TGG AGA CTC T-3′ (reverse) for *RD29A*.

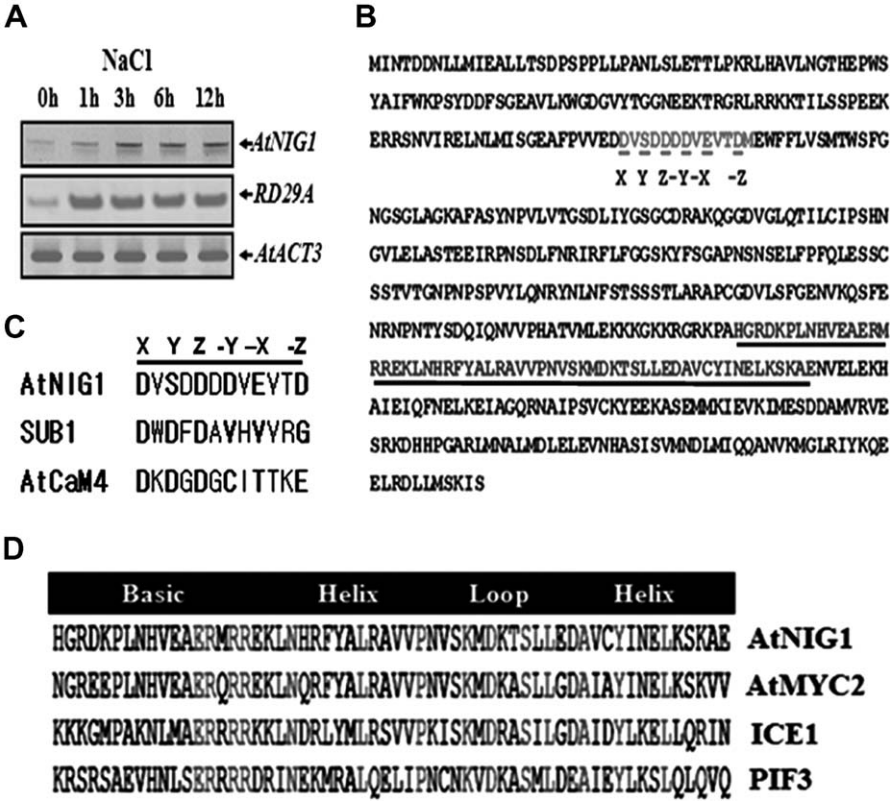

Fig. 1. The *AtNIG1* gene is a salt stress-inducible bHLH transcription factor. (A) Expression analysis of *AtNIG1* in salt stress condition (200 mM NaCl) from *Arabidopsis* seedlings (2-week-old) by RT-PCR. The actin gene (*AtACT3*) was used as a control. The *RD29A* was used as a control for salt stress. Three independent replicates were analyzed. (B) The deduced full-length AtNIG1 amino acid sequences. The calcium chelation loop with calcium ligating residues were denoted x, y, z, $-x$, $-y$, and $-z$. The bHLH domain of AtNIG1 was underlined. (C) Alignment of Ca^{2+} chelation loop of EF-hand domains. SUB1 (At4g08810). AtCaM4 (At1g66410). (D) The bHLH domain of the AtNIG1 was aligned with those of other bHLH proteins.

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