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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.**

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*.

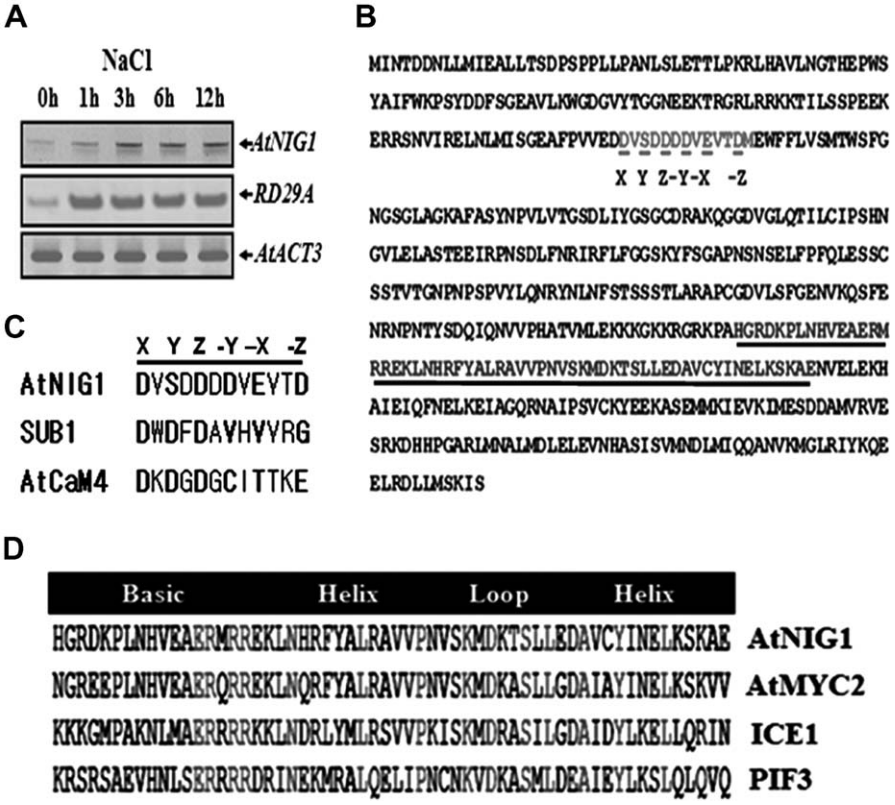

Fig. 1. The *AtNIG1* gene is a salt stress-inducible bHLH transcription factor. (A) Expression analysis of *AtNIG1* in salt stress condition (200 mM NaCl) from *Arabidopsis* seedlings (2-week-old) by RT-PCR. The actin gene (*AtACT3*) was used as a control. The *RD29A* was used as a control for salt stress. 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.

3. Results

3.1. AtNIG1 is a salt stress-responsive gene

The SSH was used to isolate salt stress (NaCl)-inducible genes in *Arabidopsis*. The SSH is known as a rapid method to screen differentially expressed genes in a short time [15]. *Arabidopsis thaliana* seedlings (Columbia) were treated with 200 mM NaCl for 12 h and used as samples for the SSH. Using this technique, we identified 95 clones in *Arabidopsis*. In addition, we found 10 transcription factors as potential salt stress-regulated genes, suggesting that various transcriptional mechanisms function in salt stress signal transduction pathways (Table 1). One of the clones (AtNIG1; NaCl-inducible gene; At5g46830) showed a significant sequence identity to other bHLH proteins. The expression pattern of the *AtNIG1* gene was investigated under high salt condition. The *AtNIG1* gene was up-regulated after exposure to high salt stress, suggesting that the *AtNIG1* may be involved in salt stress signaling (Fig. 1A).

3.2. AtNIG1 encodes a bHLH-type protein containing an EF-hand motif

To dissect the functional domains of AtNIG1 protein, AtNIG1 amino acid sequences were analyzed using InterProScan (http://www.ebi.ac.uk/interproscan/). The *AtNIG1* encoded a 511-residue polypeptide containing an EF-hand-like Ca^{2+}-binding motif at the N-terminal region (Fig. 1B). The EF-hand motif is known to consist of a Ca^{2+} chelation loop that bridges two α-helices producing a helix-loop-helix structure [16]. In the Ca^{2+}-binding loop, the six residues X, Y, Z, $-Y$, $-X$, and $-Z$ participate in binding Ca^{2+} (Fig. 1B and C) [16]. Fig. 1C shows the alignment of the amino acid sequences of the Ca^{2+}-binding loop. Database analyses revealed that the AtNIG1 contains a bHLH domain at its C-terminal region (Fig. 1B and D). *AtNIG1* gene is previously known as *AtbHLH028* [17]. In addition, the *Arabidopsis* genome contains a closely related gene (At5g46760), also referred to as ATR2/AtMYC3 [17].

3.3. AtNIG1 is a novel calcium-binding protein

To investigate whether AtNIG1 is a novel calcium-binding protein, we expressed and purified a recombinant AtNIG1 protein using a pET expression system. The purified recombinant AtNIG1 proteins were subjected to SDS–PAGE, then electroblotted onto a PVDF membrane. The membrane was then incubated with $^{45}Ca^{2+}$. The air-dried membrane was exposed and analyzed in a BAS image plate (Fujifilm). As shown in Fig. 2, AtNIG1 and *Arabidopsis* calmodulin (AtCaM4; a positive control) bound $^{45}Ca^{2+}$ whereas BSA (a negative control) did not, indicating that AtNIG1 is a calcium-binding protein.

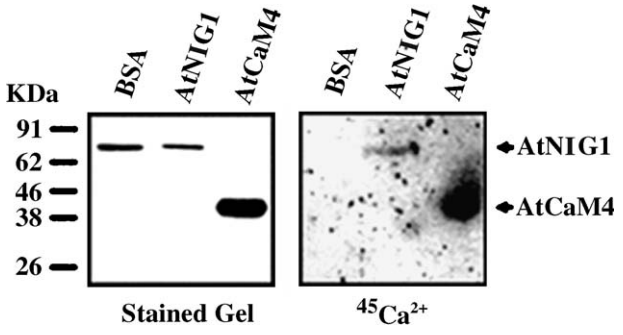

Fig. 2. The AtNIG1 is a novel calcium-binding protein. The purified AtNIG1 (1 μg), BSA (1 μg), and AtCaM4 (5 μg) were electrophoresed on SDS–PAGE and electrotransferred onto the PVDF membrane. The blot was then incubated in overlay buffer containing $^{45}Ca^{2+}$. After washing, the blot was exposed to BAS image plate (Fujifilm).

3.4. AtNIG1 is an E-box-binding protein

The bHLH proteins are known to bind to the E-box (CANNTG) [18]. To study the binding activity of AtNIG1 to this element, the DNA-binding analysis of AtNIG1 was performed using the E-box probe. The migration of this probe was shifted upon the addition of recombinant AtNIG1 protein, suggesting that AtNIG1 has a binding affinity to this element (Fig. 3A). In addition, the binding specificity of AtNIG1 to the E-box was

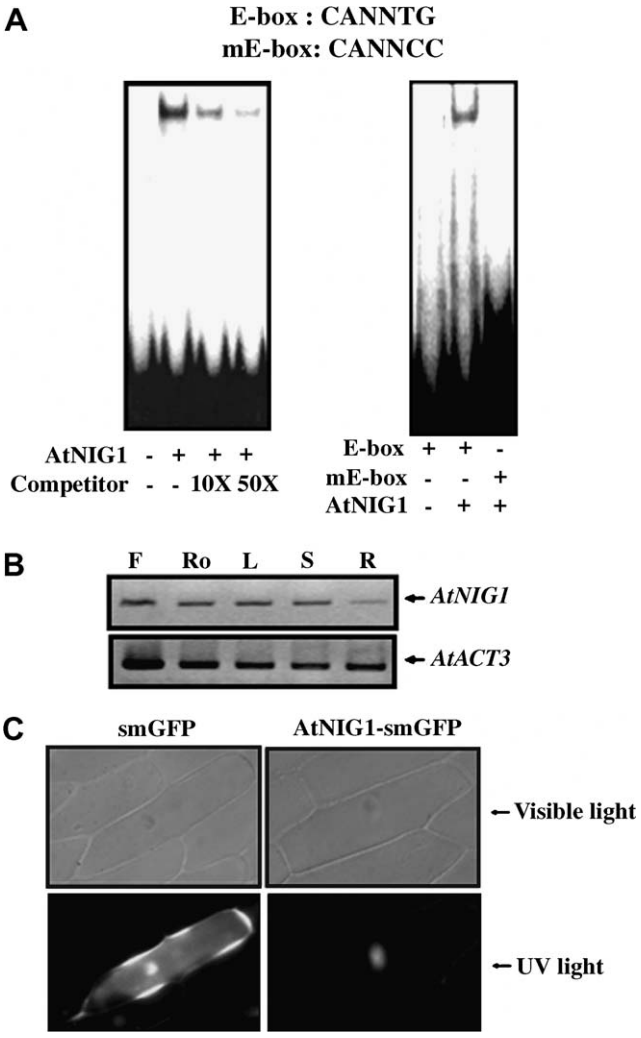

Fig. 3. The AtNIG1 is an E-box-binding nuclear protein. (A) The radiolabeled E-box probe was incubated with recombinant AtNIG1 (0.5 μg). The amount of competitor DNA was indicated at the bottom of each lane. In addition, the mutated E-box probe was incubated with the recombinant AtNIG1 protein. (B) RT-PCR analysis of *AtNIG1* gene using various tissues. S, stem; L, leaf; F, flower; R, root; Ro, rosette leaf. Three independent replicates were analyzed. (C) Onion epidermal cells were bombarded with gold particles coated with pCAMBIA1300-AtNIG1. Expression of the introduced genes was examined by a fluorescence microscopy.

examined using unlabeled E-box probe. The excess unlabeled E-box probes effectively competed with the labeled E-box probes (Fig. 3A). In addition, AtNIG1 did not bind to the mutated E-box, indicating that AtNIG1 protein binds to specifically to the E-box sequence (Fig. 3A).

3.5. *AtNIG1 is a nuclear protein*

To analyze the expression pattern of *AtNIG1* in different tissues, the tissue specificity of the *AtNIG1* gene was examined using RT-PCR. The *AtNIG1* gene was almost ubiquitously expressed in tissues (Fig. 3B). To examine the subcellular localization of AtNIG1 protein, green fluorescent protein (smGFP) was fused to the C-terminus of the AtNIG1, and the fusion protein was expressed in onion epidermal cells under the CaMV 35S promoter. The AtNIG1-smGFP expression was clearly detected in the nucleus, while the control smGFP was located throughout the cell, indicating that AtNIG1 is targeted into the nucleus (Fig. 3C).

3.6. *AtNIG1 is involved in salt stress signaling pathway*

To understand a biological role of AtNIG1 in salt stress signaling, we identified and obtained a T-DNA insertion mutant (*atnig1-1*; Fig. 4A) from the Salk T-DNA mutant populations. The *AtNIG1* gene contains no intron. Homozygous *atnig1-1* mutant was selected as recommended by Salk Institute Genomic Analysis Laboratory (http://www.signal.salk.edu/cgi-bin/tdnaexpress). RT-PCR analysis using the *AtNIG1*-specific primers gave no detectable amplification products, suggesting that the *atnig1-1* is a null mutant (Fig. 4B). We examined whether AtNIG1 is involved in salt stress response. First, we investigated whether the *atnig1-1* mutation affects seed germination in response to salt stress and ABA. The germination of the *atnig1-1* seeds showed a grater inhibition by NaCl, mannitol, or ABA compared with that of the wild-type seeds, indicating that the mutant seeds have increased sensitivity to salt stress and ABA (Fig. 4C). We analyzed the effect of salt stress and ABA on the growth of the mutant plants compared with that of the wild-type plants. Fig. 4D shows that the mutant plants growing MS plates containing 100 mM NaCl, 200 mM mannitol, or 0.5 μM ABA showed enhanced sensitivity to salt stress and ABA during growth and development. Genetic complementation of the *atnig1-1* mutation was performed with a CaMV35S-AtNIG1 cDNA transgene. The salt-sensitive phenotypes of *atnig1-1* plants were reverted to nearly the wild-type in the complemented mutant plants, suggesting that the salt-sensitive phenotype of the mutant is caused by direct disruption of *AtNIG1* gene (Fig. 4E).

3.7. *atnig1-1 plants show reduced survival rate, fresh weight, chlorophyll content, and protein content under salt stress*

Several growth parameters, such as survival rate, fresh weight, chlorophyll content, and protein content, were investi-

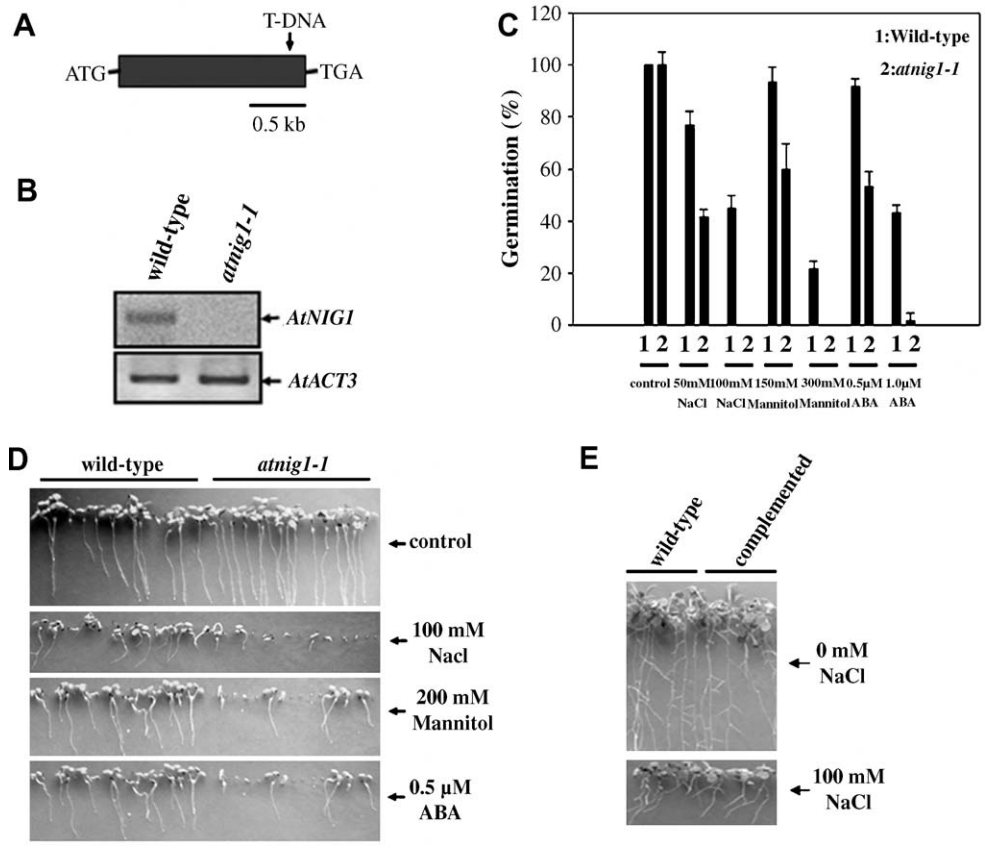

Fig. 4. Characterization of a T-DNA insertion mutant of the *AtNIG1* gene. (A) Position of a T-DNA insertion in the *atnig1-1* allele. (B) RT-PCR analyses of *AtNIG1* expression in wild-type and *atnig1-1* mutants. (C) Germination of the *atnig1-1* seeds was hypersensitive to NaCl, mannitol, and ABA. Germination was determined at 4 days. Error bars represent SE for average of three independent experiments ($n = 50$). (D) Comparison of growth inhibition by NaCl, mannitol, and ABA in wild-type and *atnig1-1* mutants (10-day-old). (E) Comparison of seedling growth by salt stress (NaCl) in wild-type and complemented (*atnig1-1*/*AtNIG1*) plants (12-day-old).

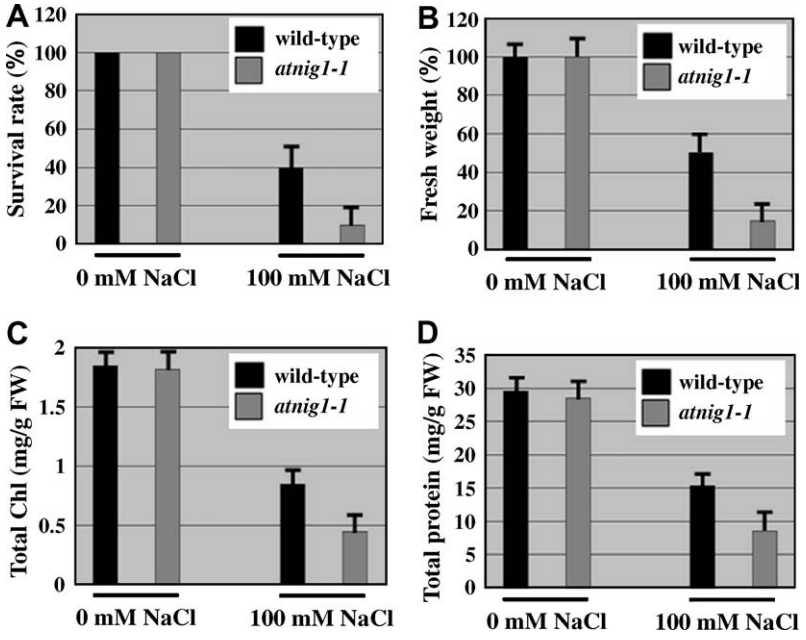

Fig. 5. The *atnig1-1* plants show reduced survival rate, fresh weight, chlorophyll content, and total protein content under salt stress. (A) The relative survival rate and SE were calculated based on three independent experiments. (B) The relative fresh weight of wild-type and *atnig1-1* plants under salt stress. Values are means ± S.D. ($n = 6$). The experiments were done in triplicates. (C) The relative chlorophyll content was measured spectrophotometrically after extraction in 90% acetone. Values are means ± S.D. ($n = 6$). Chl, total chlorophylls; FW, fresh weight. The experiments were done in triplicates. (D) The relative protein content was determined using a Bio-Rad protein assay kit (Bio-Rad). Values are means ± S.D. ($n = 6$). The experiments were done in triplicates.

gated as indicators to measure sensitivity of the *atnig1-1* plants to salt stress. The *atnig1-1* and wild-type were grown in MS plates containing 100 mM NaCl for 7 days and transferred to soil under normal growth conditions for three weeks. Upon salt stress, the survival rate of *atnig1-1* plants was largely decreased compared with that of the wild-type (Fig. 5A). The fresh weight of the *atnig1-1* plants in response to salt stress was also reduced compared with that of the wild-type (Fig. 5B). In addition, total chlorophyll content was decreased upon salt stress in the *atnig1-1* mutant plants, suggesting that the *atnig1-1* plants may have less photosynthetic capacities than the wild-type (Fig. 5C). We also used total protein content as an indicator of plant growth and measured it in the *atnig1-1* plants under salt stress. Upon salt stress, the total protein content was largely reduced in the *atnig1-1* plants (Fig. 5D). Thus, these results suggest that AtNIG1 protein plays an important role in salt stress signaling.

4. Discussion

Transcriptional regulation of gene expression is one of the fundamental processes in response to a variety of signals [19]. The *AtNIG1* encodes a bHLH-type transcription factor. Plant bHLH proteins function in transcriptional regulation involved in anthocyanin biosynthesis, phytochrome signaling, carpel and epidermal development, as well as stress response [18,20,21]. The *ICE1* encodes a bHLH protein that binds specifically to the MYC recognition site (CANNTG) in the *CBF3* promoter [22]. In addition, the bHLH protein (AtMYC2) has been shown to bind to the *RD22* promoter and activate the *RD22* gene expression [23]. Promoter regions of many salt stress-responsive genes contain the E-box sequence (data not shown). It is possible that the AtNIG1 binds to the promoter regions of these salt stress-regulated genes. Therefore, the AtNIG1 may be involved in salt stress signaling pathway by regulating these salt stress-responsive genes.

Many studies have elucidated Ca^{2+} as a secondary messenger in salt stress response [1,2]. The elevation of cellular Ca^{2+} is known to be a rapid response to salt stress. Ca^{2+} transduces the salt signal to downstream pathway by interacting with protein sensors. The identification of the SOS pathway revealed components and mechanisms to mediate plant response to salt stress [4]. The SOS3 is a Ca^{2+} sensor essential for transducing the salt stress-induced Ca^{2+} signal [5]. The SOS3 activates the SOS2 [24]. The SOS3–SOS2 complex controls both the expression and activity of the SOS1, a plasma membrane Na^+/H^+ exchanger [25]. The SOS2 is a Ser/Thr-type kinase, which is known to preferentially phosphorylate Ser or Thr residue in the motifs, such as RXX(S/T) or KXX(S/T) [26]. The AtNIG1 protein contains these motifs. Thus, it will be interesting to determine whether the SOS2 can interact with and phosphorylate the AtNIG1 in future investigation.

We do not know the exact physiological role of calcium-binding by AtNIG1 at present. We are trying to understand direct roles of calcium-binding in the AtNIG1 function for mediating plant salt stress signaling. Recent study showed that the role of Ca^{2+} depends on both its amplitude and its oscillations [27]. Ca^{2+}-binding proteins can be positive regulators or negative regulators in Ca^{2+} signaling. Compared with CaM, the AtNIG1 has a lower affinity to Ca^{2+} (Fig. 2). This may not be surprising because the AtNIG1 has only one Ca^{2+}-binding EF hand motif compared with four EF-hand motifs found in CaM. In addition, structural studies have demonstrated that EF-hands with D in position $-Z$ may be more likely to bind Mg^{2+}, because Mg^{2+} is smaller and requires only six

ligand-forming groups rather than seven as required by Ca^{2+} [28]. Therefore, we do not rule out the possibility that the AtNIG1 can bind Mg^{2+} as well.

Many Ca^{2+}-binding proteins have been identified in *Arabidopsis*, including several CaMs [11], touch-induced proteins TCH2 and TCH3 [29], centrin [30], CDPKs [9], CBLs [10], a NaCl-inducible protein [31], a protein phosphatase [32], channel proteins [33], and respiratory burst oxidase homologs [34]. Even though animal calcium-binding protein (DREAM) is known to be a transcriptional repressor involved in modulating pain, there is no report describing a calcium-binding transcription factor in plants [35]. Our data indicate that the AtNIG1 is the first known transcription factor involved in plant Ca^{2+}-binding.

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