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Athb-12, a homeobox-leucine zipper domain protein from *Arabidopsis thaliana*, increases salt tolerance in yeast by regulating sodium exclusion

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Abstract

An *Arabidopsis* cDNA clone that encodes Athb-12, a homeobox-leucine zipper domain protein (HD-Zip), was isolated by functional complementation of the NaCl-sensitive phenotype of a calcineurin (CaN)-deficient yeast mutant (*cnbΔ*, regulatory subunit null). CaN, a Ca^{2+}/calmodulin-dependent protein phosphatase, regulates Na^+ ion homeostasis in yeast. Expression of Athb-12 increased NaCl tolerance but not osmotic stress tolerance of these *cnbΔ* cells. Furthermore, expression of two other HD-Zip from *Arabidopsis*, Athb-1 and -7, did not suppress NaCl sensitivity of *cnbΔ* cells. These results suggest that Athb-12 specifically functions in Na^+ ion homeostasis in yeast. Consistent with these observations, expression of Athb-12 in yeast turned on transcription of the NaCl stress-inducible *PMR2A*, which encodes a Na^+/Li^+ translocating P-type ATPase, and decreased Na^+ levels in yeast cells. To investigate the biological function of Athb-12 in *Arabidopsis*, we performed Northern blot analysis. Expression of *Athb-12* was dramatically induced by NaCl and ABA treatments, but not by KCl. In vivo targeting experiments using a green fluorescent protein reporter indicated that Athb-12 was localized to the nucleus. These results suggest that Athb-12 is a putative transcription factor that may be involved in NaCl stress responses in plants.

Keywords: A homeobox-leucine zipper domain protein; Salt tolerance; Na^+ ion homeostasis; *Arabidopsis*; Yeast

When plants are exposed to excessive salinity, apoplastic levels of Na^+ and Cl^- alter aqueous and ionic thermodynamic equilibria, resulting in hyperosmotic stress and ionic imbalance and toxicity [1]. Therefore, survival and growth is dependent on the capacity of the plant to re-establish, expeditiously, cellular osmotic and ionic homeostasis, i.e., to adapt to the stress environment [2]. Tolerance to salt stress is mediated by processes that restrict Na^+ uptake across the plasma membrane (presumably due to a combination of reduced influx and increased efflux), facilitate Na^+ and Cl^- sequestration into the vacuole, and regulate compatible osmolyte production and accumulation [3]. The coordinate control of these processes is essential for the osmotic adjustment and ion homeostasis that is required for salt adaptation.

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In response to NaCl, plants express numerous genes to adapt to high salinity stress. Identification and characterization of these genes has made valuable contributions to the understanding of the processes that occur during NaCl stress [4,5]. In addition, molecular genetic approaches using the model plant *Arabidopsis* have led to the identification of a regulatory pathway and determinants for Na^+ ionic homeostasis. Included amongst these are SOS (for salt overlay sensitive) proteins, sodium transporters, protein kinases, and others [6–9]. However, despite such advances, the understanding of the plant adaptation to salt stress is far from complete and much remains to be discovered about regulatory signals and pathways that control these processes.

A unicellular yeast system has proven to be a very useful model to study the mechanisms underlying tolerance to saline stress in higher plants [10,11], since numerous cellular mechanisms of NaCl tolerance in yeast are conserved in plant cells. The gain of function by overexpression in episomal plasmids has recently proven to be a very successful approach leading to the isolation of several halotolerance genes [12]. In yeast, Na^+ stress activates a calcineurin (CaN)-dependent pathway that regulates the activity of Na^+ influx and efflux transporters. CaN is a Ca^{2+} and calmodulin-dependent protein phosphatase consisting of a catalytic subunit (CNA) and a regulatory subunit (CNB). CaN-deficient mutants fail to convert the K^+ transport system to the high affinity state that facilitates better discrimination of K^+ over Na^+ [13,14] and exhibit reduced expression of *PMR2*, which encodes a plasma membrane P-type ATPase essential for Na^+ efflux [13,16]. Since yeast *can* mutants accumulate abnormally high levels of Na^+ ions due to reduced Na^+ efflux and a failure of K^+ uptake, the mutants cannot grow efficiently on medium containing sodium ions [13–16].

In plants, SOS3, which shares sequence identity with CaN, has a role in the adaptation to salt stress [6]. Under salt stress conditions, *Arabidopsis* sos3 mutants accumulate more Na^+ and retain less K^+ than wild-type plants [17]. The sos3-deficient mutants show hypersensitivity to high Na^+ or low K^+ and addition of external Ca^{2+} suppresses both phenotypes [18]. AtCBL, an *Arabidopsis* SOS homolog, functionally suppressed the salt-sensitive phenotype of CaN null mutant (*cnbΔ*) yeast cells when co-expressed with a rat CaN A subunit [19]. The expression of activated yeast CaN, a truncated form of CaN A subunit and CNB, enhanced salt tolerance in transgenic tobacco plants [20]. These results indicate the conservation of a Ca^{2+}-dependent salt-stress signaling pathway which regulates ion homeostasis and salt tolerance in both yeast and plants.

In this study, we report that an Athb-12, a homeo domain-leucine zipper protein (HD-Zip) from *Arabidopsis*, can suppress the Na^+-sensitive phenotype of *cnbΔ*. We present evidence that Athb-12 regulates *PMR2A*, a gene encoding a Na^+/Li^+ translocating P-type ATPase, whose expression is essential for Na^+ efflux in yeast. The net result is substantially less Na^+ accumulation and, consequently, an extremely NaCl tolerant phenotype. This is the first evidence that a plant homeodomain protein is functionally involved in a cellular ion homeostasis response in eukaryotes.

Materials and methods

Screening of cDNA clones. To screen for cDNAs complementing a NaCl stress-sensitive strain of yeast (*Saccharomyces cerevisiae*) YP9 (*Mata ura3 leu2 his3 trp1 ade2 lys2 cnb1::HIS3*), an *Arabidopsis* expression library constructed pGAD424 vector (Clontech, Palo Alto, CA, USA) [21] was introduced into the mutant yeast by the LiOAc method [22]. Approximately 2.5×10^5 transformants were selected for leucine prototrophy by plating on synthetic complete medium lacking leucine (SC-Leu) and 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 and reintroduced into YP9 yeast cells to confirm the complementation. Plasmid inserts were sequenced with the dideoxy termination method using a dye-terminator cycle sequencing kit. The sequencing reaction was analyzed using an automatic sequencer (ABI, Columbia, MD, USA).

Spot sssay. The full-length and truncated Athb-12 were subcloned into pYES2, an expression vector with the GAL1 promoter, which is a galactose-inducible promoter, and the URA3 selection marker. The constructs were introduced into the YP9 strain and the transformed cells were plated on SC-Ura plates. The transformed cells were grown in YPD medium overnight, and 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, and 1:1000) and spotted onto YPGal (1% yeast extract/2% peptone/2% galactose) plates containing NaCl (1.1 M), LiCl (100 mM), KCl (1.4 M), or sorbitol (1.5 M). The growth was examined after 4 days at 30 °C.

Assay of PMR2A gene activation. The reporter gene construct *PMR2A::LacZ* [23] was introduced into the YP9 mutant strain harboring Athb-12, and the cells were plated on SC-Leu, Trp plates. Yeast cells harboring both genes were cultured at 30 °C overnight, diluted to an $O.D._{600}$ of 0.1, and then incubated in YPD medium with or without 1 M NaCl for 1 h. Cells were harvested by centrifugation at 3000*g* for 5 min and resuspended in 1 mL Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0). Cell extracts were prepared, and β-galactosidase activity was measured according to the manufacturer's protocol (Clontech).

Determination of intracellular ion content. To measure intracellular Na^+ and K^+ content, yeast cells were grown in YPGal medium. The YP9 strain transformed with the pYES2 vector, or expressing Athb-12 were grown in liquid YPGal medium with 1 mM KCl and 70 mM NaCl. When cultures reached an $O.D._{600}$ of 0.2, cells were collected by filtration, and their Na^+ and K^+ contents were determined using an Inductively Coupled Plasma Spectrophotometer, OPTIMA4300DV (ICP, Perkin–Elmer, Fremont, CA, USA). Ion content is presented as nmol of ion per mg dry weight of cell samples.

Northern blot analysis. Total RNA was purified from 2-week-old *Arabidopsis* seedlings treated with NaCl (100 mM), ABA (10 μM), and KCl (100 mM) for various times (0, 0.5, 1, 3, 6, and 12 h). Total RNA was isolated from the *Arabidopsis* seedlings according to a published protocol [21]. Samples (20 μg/lane) were electrophoresed on a 1.2% formaldehyde agarose gel and transferred to an Immobilon transfer membrane (Millipore, Billerica, MA, USA).

Membranes were UV cross-linked and hybridized to a full-length *Athb-12* probe in Church buffer (1% BSA, 1 mM EDTA, 0.25 M $NaHPO_4$ (pH 7.2), and 7% SDS at 65 °C) [21]. Following incubation, membranes were washed with 2× SSC for 10 min, 1× SSC for 10 min, and 0.5× SSC for 10 min, and rewashed with 0.1 SSC/0.1% SDS at 65 °C for 10 min.

Protoplast preparation. Leaf tissues (5 g) of 3- to 4-week-old *Arabidopsis* plants were cut into small squares (5–10 mm^2) with a new razor blade and incubated with 30 mL of enzyme solution containing 0.25% Macerozyme R-10 (Yakult Honsha, Osaka, Japan), 1.0% cellulase R-10 (Yakult Honsha), 500 mM mannitol, 1 mM $CaCl_2$, and 5 mM Mes–KOH (pH 5.6) at 22 °C for 9 h with gentle agitation (50–75 rpm). After incubation, the protoplast suspension was filtered through a 100 μm mesh and protoplasts were overlaid on 20 mL of 21% sucrose, and centrifuged at 720 rpm for 10 min. The intact protoplasts at the interface were transferred to a new falcon tube containing 30 mL W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes–KOH (pH 5.6)). The protoplasts were pelleted again by centrifugation at 500 rpm for 5 min and resuspended in 30 mL W5 solution. The protoplasts were then incubated on ice for 5 h for stabilization.

In vivo targeting of Athb-12. To construct *Athb-12:GFP*, *Athb-12* was amplified from an *Arabidopsis* cDNA library by PCR using primers designed from the nucleotide sequence information deposited in the EST database. PCR products were confirmed by nucleotide sequencing and were inserted into *Xba*I and *Bam*HI sites of plasmid p326-sGFP (kindly provided by Inhwan Hwang, POSTEC, Korea) to create chimeric GFP-fusion constructs under the control of the 35S promoter. Plasmid p326-sGFP is a pUC-based vector containing CaMV35S-sGFP-NOS3' for protoplast expression (S65T) [24]. The plasmid was introduced into *Arabidopsis* protoplasts that had been prepared from leaf tissues by polyethylene glycol-mediated transformation [25,26]. Expression of the fusion constructs was monitored at various time points after transformation and images were captured with a cooled CCD camera and a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany). The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; and emitter, 605DF50) and XF137 (exciter, 540AF30; dichroic, 500DRLP; and emitter, 585ALP; Omega Optical, Brattleboro, VT) for GFP and RFP, respectively. Data were then processed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA) and presented in pseudocolor format.

Results

Isolation of Arabidopsis cDNA clones that confer increased NaCl tolerance to a yeast CaN null mutant (cnbΔ)

To identify plant genes that can suppress the NaCl-sensitive phenotype of yeast *cnbΔ*, we transformed the cells with an *Arabidopsis thaliana* cDNA library containing inserts under the ADH1 promoter. After plating 2.5×10^5 transformants on YPD medium supplemented with 1.1 M NaCl, a total of 34 colonies that showed remarkable NaCl tolerance were obtained. Among these suppressors, two independent cDNA inserts, containing full-length ORFs but with different sizes of 5′ UTR, were identified as *Athb-12* (Accession No. At3g61890; Fig. 1A). Comparative amino acid sequence analysis of Athb-12 with other known proteins indicates that a homeobox domain (HD) and a leucine-zipper motif (Zip) exist in the central region, and an activation domain (AD) lies in the C-terminal region of Athb-12 (Fig. 1B). To analyze whether these domains are required for NaCl tolerance of yeast cells, we constructed a series of *Athb-12* deletion clones in the episomal plasmid pYES2, thus placing them under the control of a GAL1 promoter. We tested for a galactose-dependent NaCl tolerant phenotype and found that transformants harboring full-length *Athb-12* cDNA that contain HD, Zip, and AD suppressed the NaCl-sensitive phenotype of *cnbΔ* cells, but the constructs missing any one of these sequences did not (Fig. 1B). These results suggest that all of these domains are required for salt tolerance in yeast.

Athb-12 is specifically required for the Na^+ toxicity response in yeast

To further characterize the function of Athb-12, the growth of Athb-12 transformed yeast cells was compared in YPG supplemented with an increasing amount of NaCl, LiCl, KCl, or sorbitol. Athb-12 transformed cells had identical growth rates to the control strain grown in KCl or sorbitol. On the contrary, the transformants were more tolerant to NaCl or LiCl than the control strain. Li^+ is a toxic analog of Na^+ and 10 times more effective than Na^+ ion (Fig. 1C). Taken together, these results indicate that Athb-12 is involved in Na^+ and Li^+ tolerance but not osmo-tolerance.

More than 20 genes have been identified in *Arabidopsis* that encode proteins with an HD-Zip domain. To investigate whether other members of the family are also involved in the mechanism of NaCl tolerance in yeast, two Athb-12 homologs, Athb-1 [26] and -7 [27], were transformed into *cnbΔ* cells. The amino acid identities of the HD-Zip domains of Athb-12 and Athb-1 or Athb-7 were 34.2%, and 69.9%, respectively. Expression of Athb-12 dramatically enhanced NaCl tolerance of both wild-type and *cnbΔ* cells, however, transformants expressing Athb-1 or Athb-7 did not (Fig. 1D and data not shown). These data indicate that Athb-12 is a specific NaCl tolerant determinant among the HD-Zip gene family of *Arabidopsis*.

Athb-12 activates the NaCl stress response gene PMR2A in yeast

Since Athb-12 belongs to an HD-Zip transcription factor family, Athb-12 might activate transcription of the genes involved in sodium ion homeostasis in yeast. To investigate this possibility, we examined whether Athb-12 can regulate the expression of NaCl-inducible *PMR2A*, which encodes a plasma membrane Na^+-ATPase involved in Na^+ transport (Fig. 2A). After transforming both *PMR2A::lacZ*, a reporter gene, and

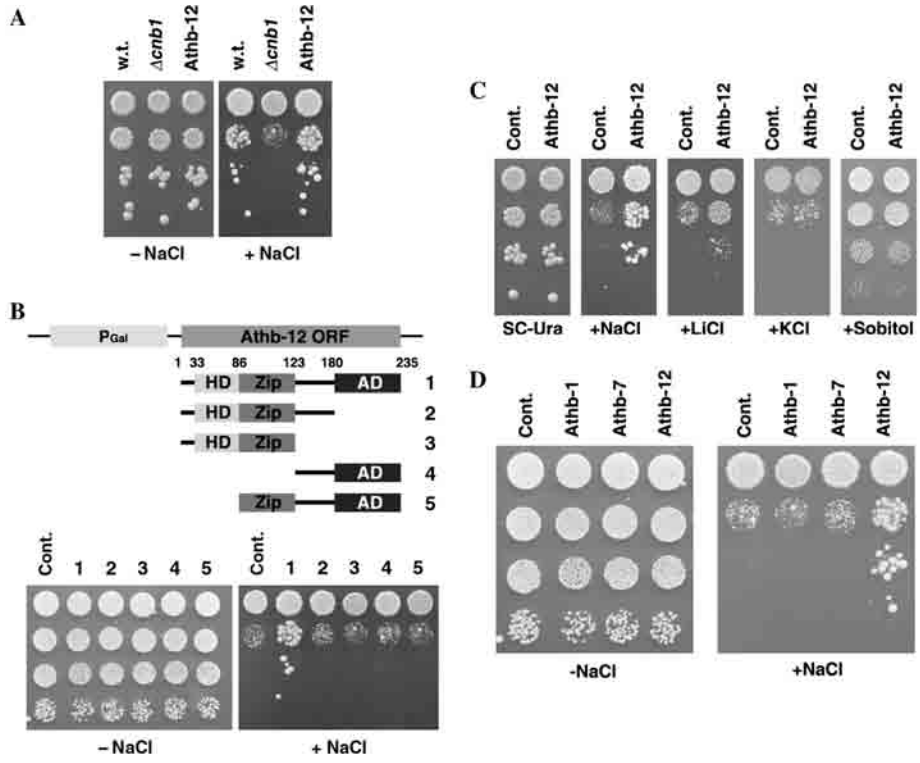

Fig. 1. Athb-12 is required for the Na^+ toxicity response. (A) Identification of Athb-12 as a salt tolerant determinant in yeast. The wild-type or *cnbΔ* cells were transformed with either empty vector (pGAD424) or the plasmid containing Athb-12. Transformed cells were tested for tolerance to 1.1 M NaCl as described in Materials and methods. Plates were photographed after incubation at 30 °C for 3 days. (B) Deletion analysis of Athb-12. Physical map of the Athb-12 segment cloned into plasmid pYES2 (top). Solid bars represent deletion clones originating from Athb-12. The capacity or inability of these constructs to confer resistance to NaCl (below) was tested as described in Materials and methods. HD, homeobox domain; Zip, leucine-zipper motif; and AD, activation domain. (C) The tolerance phenotype conferred by Athb-12 is specific to sodium and lithium. The YP9 yeast strain was transformed with either empty vector (pYES2) or the plasmid containing Athb-12. Plate assays were performed as described in Materials and methods. (D) Functional analysis of *Arabidopsis* HD-Zip isoforms in yeast cells. The YP9 strain was transformed with either empty vector (pYES2) or the plasmid containing Athb-1, -7, or -12. Complementation assays were performed as described in Materials and methods.

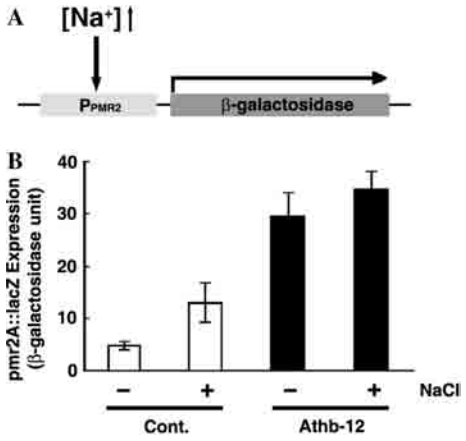

Fig. 2. Activation of *PMR2A* gene in CaN mutant cells by Athb-12. The reporter gene construct *PMR2A::LacZ* (A) was introduced into the YP9 strain with or without Athb-12. The double transformants were grown in YPD medium overnight at 30 °C, the overnight culture was diluted to an OD_{600} of 0.1 and inoculated onto YPD medium with or without 1 M NaCl for 1 h, and the cells were harvested. Cell extracts were prepared and assayed for β-galactosidase activity (B). Three independent experiments were carried out and similar results were obtained.

Athb-12 cDNA into *cnbΔ* cells, the galactosidase activities of the doubly transformed yeast cells were measured. The cells harboring *Athb-12* constructs exhibited significantly greater β-galactosidase activity than control cells (Fig. 2B), as well as significantly increased transcription of *PMR2A* (data not shown). These results indicate that Athb-12 positively regulates the expression of *PMR2A* and may potentiate Na^+ ion efflux in yeast.

Since expression of Athb-12 showed both a sodium ion tolerant phenotype (Fig. 1) and enhanced expression of *PMR2A* (Fig. 2), we next investigated whether Athb-12 can regulate Na^+-ion efflux. The initial rate of Na^+ uptake in both controls and transformants expressing Athb-12 was similar, but the *Athb-12* transformed cells clearly accumulate less Na^+ than control (Fig. 3), indicating that Athb-12 controls Na^+ ion homeostasis in yeast.

Athb-12 is specifically induced by NaCl stress in plants and localizes to the nucleus

To identify the physiological role of Athb-12 in plants, RNA expression levels were analyzed under

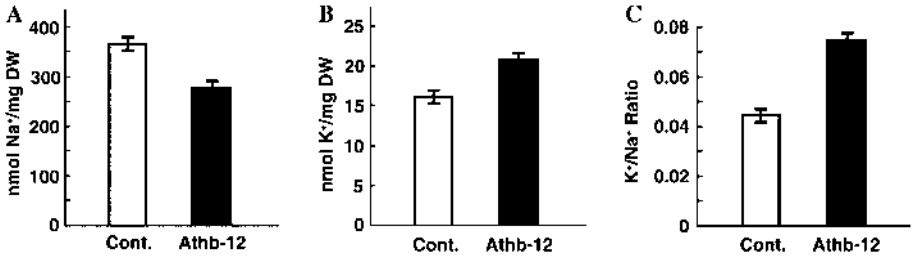

Fig. 3. Expression of the Athb-12 gene reduces Na^+ accumulation and improves K^+ status. The YP9 strain transformed with an empty vector (control), or expressing Athb-12 were grown in liquid YPGal medium with 1 mM KCl and 70 mM NaCl. When cultures reached OD_{600} of 0.2, cells were collected by filtration, and their Na^+ and K^+ contents (A, B, and C) were determined using ICP. Units are nmol of ion per mg dry weight of cell samples.

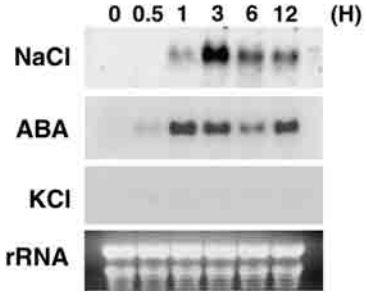

Fig. 4. *Athb-12* transcript accumulates during NaCl or ABA stress. Total RNA was prepared from 2-week-old *Arabidopsis* seedlings treated for various times with NaCl (100 mM), ABA (10 μM), or KCl (100 mM). Total RNA (20 μg) was loaded onto each lane. Equal loading for each lane was confirmed by prestaining gels with ethidium bromide (lower). RNA blots were probed with ^{32}P-labeled *Athb12*.

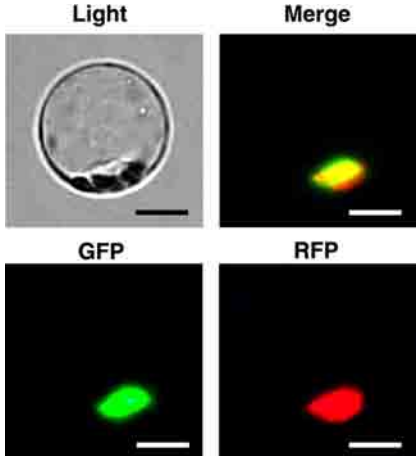

Fig. 5. Subcellular localization of Athb-12. Protoplasts prepared from *Arabidopsis* seedlings were co-transformed with two constructs *Athb-12:GFP* plus *NLS:RFP*. The transformed protoplasts were examined by fluorescence microscopy at 12 and 24 h after transformation. Green and red images are GFP and RFP signals, respectively. Bar indicates 20 μm.

various stress conditions. Total RNA was isolated from *Arabidopsis* seedlings treated with NaCl, KCl, or ABA, and Northern blot analysis was performed using Athb-12 cDNA as a probe (Fig. 4). Transcription of Athb-12 was induced either by NaCl or by exogenously applied ABA. These results indicate that one function of Athb-12 in plants may be to mediate the NaCl stress response.

The localization of Athb-12 in *Arabidopsis* protoplasts was investigated in vivo using green fluorescent protein (GFP) or red fluorescent protein (RFP) reporter gene fusion constructs [28,29]. Chimeric *Athb-12:GFP* was introduced into protoplasts isolated from wild-type *Arabidopsis* seedlings along with a *NLS:RFP* construct encoding a nuclear marker protein [30]. The subcellular distribution of the Athb-12:GFP green fluorescent signals clearly overlapped with those of the NLS:RFP red fluorescent signals indicating co-localization in the nucleus (Fig. 5).

Discussion

In this paper, we present evidence that Athb-12 participates in cellular sodium ion homeostasis regulation in yeast. We showed that (i) Athb-12 specifically suppresses the NaCl-sensitive phenotype of a CaN-deficient yeast mutant, (ii) Athb-12 turns on transcription of *PMR2A*, which encodes Na^+ translocating P-type ATPase, and (iii) Athb-12 decreases Na^+ levels in yeast cells. Although how Athb-12 functions in the NaCl stress response remains to be determined in planta, our results suggest that Athb-12 functions as a component of a pathway specific to the ion homeostasis pathway in yeast. Because the expression of Athb-12 was specifically induced by NaCl stress in plants and Athb-12 is localized to the nucleus, Athb-12 may be a putative transcription factor that is involved in the salt stress response in plants, similar to its activity in yeast

HD-Zip has a specific domain structure in which a homeodomain is accompanied by a leucine-zipper motif at the C-terminus [31–33]. Since these proteins interact with DNA and act as positive and negative regulators of gene expression, it has been suggested that these proteins may function as transcription factors [34]. In *Arabidopsis*, more than 20 HD-Zip isoforms have been

isolated; however, their functions are mostly unknown. Interestingly, we found that NaCl tolerance in yeast cells is enhanced by Athb-12 but not by other HD-Zip homologs, Athb-1 or Athb-7 (Fig. 1 and data not shown). In addition, the expression level of Athb-12 increased in response to exogenous NaCl or ABA. In contrast, KCl or sorbitol treatment did not result in a significant change in the expression level of Athb-12 under our experimental conditions. These results imply that Athb-12 is specifically involved in the NaCl stress response in plants.

Our results provide experimental evidence that modulation of a signaling pathway in yeast can impart a NaCl tolerant phenotype. NaCl tolerance of the Athb-12 expressing yeast may be the result of activating genes involved in the salt stress (or ion stress) adaptation mechanism. Our results indicate that the Athb-12 promotes the expression *PMR2A*, but did not increase the osmotic stress-inducible gene, *CTT1* [35] (Fig. 2 and data not shown). Consistent with these observations, we found that overexpression of Athb-12 did not modulate the osmotic balance of yeast cells (Fig. 1). These results collectively suggest that Athb-12 may specifically regulate NaCl tolerance rather than a generalized yeast stress response.

Although some differences do exist between yeast and plant, the yeast, *S. cerevisiae*, is a useful model system to study ion homeostasis and cellular responses to NaCl stress in higher plants [10,11,36,37]. For example, Pardo et al. [20] showed that activated yeast CaN functions to mediate salt tolerance in a salt-stress signaling pathway in plants. In addition, molecular genetic studies using *Arabidopsis* have indicated that SOS3, a CaN homolog, functions in a NaCl stress adaptation mechanism in plants. Since Athb-12 functions in a CaN-mediated NaCl stress signal transduction pathway in yeast, and the expression of Athb-12 in plants is specifically induced by NaCl and ABA, it is likely that Athb-12 is involved in ion homeostasis in plants. Additional studies in plants will be needed to fully elucidate the function of Athb-12 in the NaCl stress response of plants.

Acknowledgments

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