Journal of Plant Biology, January 2008, 51(1) : 25-34

Overexpression of a *Thellungiella halophila* CBL9 Homolog, *ThCBL9*, Confers Salt and Osmotic Tolerances in Transgenic *Arabidopsis thaliana*

Zhibin Sun†, Xingyun Qi†, Pinghua Li, Chunxia Wu, Yanxiu Zhao, Hui Zhang, and Zenglan Wang*
Key Lab of Plant Stress Research, College of Life Science, Shandong Normal University,
88 Wenhua East Road, Jinan 250014, China

The calcineurin B-like (CBL) proteins, comprising a large subfamily of calcium sensors in plant cells, play an important role in many stress responses. We cloned a gene from the halophyte *Thellungiella halophila* that is homologous to *AtCBL9* in *Arabidopsis thaliana*. The 1008-bp *ThCBL9* contains an ORF of 639 bp and encodes 213 amino acids, with a 5′-untranslated region of 193 bp and a 3′-untranslated region of 176 bp. Its amino acid sequence shares high homology with AtCBLs. *ThCBL9* is up-regulated by ABA, NaCl, and PEG in *Thellungiella* leaves. Using molecular biological methods, we over-expressed *ThCBL9* in *A. thaliana* and found that this enhanced tolerances to both high salt and osmotic stress in transgenic *Arabidopsis*.

Keywords overexpression, stress tolerance, *ThCBL9*, *Thellungiella halophila*

Soil salinity is a major abiotic stress that reduces plant growth. It is one of the leading factors limiting agricultural productivity. Nevertheless, sessile plants have several mechanisms for coping with varying salt concentrations in their environment. Such stress triggers a rapid and transient increase of free calcium concentration in the cells (Lynch et al., 1989; Knight et al., 1997; Knight, 2000; Pauly et al., 2000). Therefore, this Ca^{2+} signaling process is one of the earliest events in salt-signaling, and may play an essential role in ion homeostasis and plant salt tolerance (Lynch et al., 1989; Knight et al., 1997; Zhu, 2003).

The divalent cation Ca^{2+} is an essential plant nutrient. As a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol, it is required for structural functions in the cell wall and membranes (Marschner, 1995). Several families of Ca^{2+} sensors have been identified in higher plants, including CaMs, CaM-like proteins, calcineurin B-like (CBL) proteins, and Ca^{2+}-dependent protein kinases (CDPKs) (Kim et al., 2000). CBL proteins possess three typical EF-hand calcium binding motifs that act *in vitro* (Kudla et al., 1999; Luan et al., 2002; Nagae et al., 2003). Many contain an N-terminal conserved domain for myristoylation that often targets proteins to cell membranes (Luan et al., 2002; Kolukisaoglu et al., 2004).

AtSOS3 (*AtCBL4*) plays an important role in salt tolerance (Liu and Zhu, 1998). It is strongly expressed in roots but at only background levels in shoots, and is not induced by NaCl treatment (Quan et al., 2007). Based on genetic analysis, SOS3, SOS2, and SOS1 work in the same pathway (Zhu et al., 1998). AtSOS3 senses cytosolic calcium changes elicited by salt stress (Ishitani et al., 2000), and then physically interacts with and activates SOS2 (Halfter et al., 2000; Liu et al., 2000). This SOS3/SOS2 kinase complex then phosphorylates and activates SOS1 to rebuild ion homeostasis in the root cells (Zhou and Wang, 2002; Quan et al., 2007). As a multi-functional protein kinase, AtCIPK24 (AtSOS2) regulates different aspects of salt tolerance by interacting with AtSOS3 (AtCBL4) in the roots, and with AtSCABP8 (AtCBL10) in the leaves and stems (Kim et al., 2007; Quan et al., 2007).

Expression of *AtCBL1* shows tissue/organ specificity under normal growing conditions; the accumulation of AtCBL1 protein, as with its mRNA, is induced by drought, salt, cold, wounding, and ABA (Kudla et al., 1999; Cheong et al., 2003) AtCBL9 acts as a common element in the ABA-signaling and stress-induced ABA biosynthesis pathways (Pandey et al., 2004). *AtCBL9* is expressed ubiquitously at all developmental stages, but its expression in seedlings is also induced to a high degree by ABA and to a lesser extent by salt, drought, and cold (Pandey et al., 2004). *Atcbl9* mutant plants can become hypersensitive to ABA or to high concentrations of salt and mannitol, and the expression of genes involved in the ABA-signaling pathway is enhanced in those plants. AtCBL1 and AtCBL9 and their common target kinase-CIPK23 form alternative complexes at the plasma membrane, where they jointly activate the AKT1 channel, thereby increasing K^+ uptake capacity (Li et al., 2006; Xu et al., 2006). Furthermore, the plasma membrane-localized CBL1- and CBL9-CIPK23 complexes simultaneously regulate K^+ transport processes, not only enhancing its uptake in the roots, but also operating in the stomata guard cells to affect transpiration water loss from the leaves (Cheong et al., 2007). Lee et al. (2007) have now found a more complex CBL-CIPK network via heterologous model systems; the importance of this network in *Arabidopsis thaliana* awaits confirmation by genetic analysis.

Despite the extensive studies and remarkable progress made toward revealing the CBLs-CIPKs signaling pathways in *Arabidopsis*, information is still quite limited about these signaling modules in other plant species. Although salt cress (*Thellungiella halophila*) is a close relative of *A. thaliana*

*Corresponding author; fax +86-531-86180764
e-mail wangzlsd@yahoo.com.cn

†These authors contributed equally to this work.

(Bressan et al., 2001; Zhu, 2001), sharing similarities in both their physiological and genetics aspects, the former exhibits much higher stress tolerance than the latter (Inan et al., 2004). This enhanced tolerance may result from differences in basic biochemical and physiological activities, as well as the regulation of an exquisite signaling pathway.

Here, we obtained the cDNA of an *AtCBL9*-homologous gene -- *ThCBL9* -- from the NaCl-treated cDNA library of *T. halophila* (Wang et al., 2004). ThCBL9 was the first calcium sensor protein studied in that species, and its expression can be induced by treatment with ABA, NaCl, or PEG. Using molecular biological methods, we over-expressed *ThCBL9* in *A. thaliana* and measured certain physiological characteristics to examine how salt and osmotic stress tolerances might be affected in transgenic *Arabidopsis*.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Plants of *Thellungiella halophila* (collected from Shandong Province, China), wild-type *Arabidopsis thaliana*, and *ThCBL9*-overexpressing *A. thaliana* (Columbia ecotype) were used. Unless stated otherwise, all were grown in 9-cm-diam. pots containing soil, perlite, and vermiculite (2:1:1), with irrigation from a Hoagland solution. Growing conditions in the greenhouse included a 16-h photoperiod, a day/night thermoperiod of 23°C/18°C, and a day/night relative humidity of 60%/80%.

Isolation of *ThCBL9* cDNA

Through large-scale partial sequencing of cDNA clones randomly selected from an NaCl-treated cDNA library of *T. halophila* leaves, we obtained the cDNA that putatively encodes ThCBL9 (Wang et al., 2004). The full-length nucleotide sequence of *ThCBL9* (GenBank Accession No. EU169239) was achieved by sequencing on an Applied Biosystem 373 Automated DNA sequencer (ABI/Perkin-Elmer, Foster City, CA, USA).

Stress Treatments

To examine *ThCBL9* expression, 30-day-old *T. halophila* seedlings were exposed to various stress conditions. For ABA treatment, 50 μM (±)-*cis*, *trans*-ABA solution in water was sprayed to cover the entire foliar area. The salt or dehydration treatments were applied through irrigation, using either 200 mM NaCl or 25 mM PEG 6000. Plain Hoagland solution served as the control.

After 24 h of treatment, total RNA was extracted from the rosette leaves according to the method of Chomczynski and Sacchi (1987). DNase I-treated, total RNA (5 μg) was denatured and subjected to reverse transcription reactions with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania).

Real-Time PCR Analysis

Real-time PCR was performed with the DNA Engine Opticon Real-Time PCR System (Bio-Rad, USA), using SYBR Green I to monitor the double-stranded DNA products. Expression levels of *ThActin-2* were monitored with forward (5′-CAGTTTTCCCGAGTGTTGTTGGTAGG-3′) and reverse (5′-ACCTCTTTTGGATTGTGCTTCGTCA-3′) primers as a quantifying control. The *ThCBL9* gene-specific forward (5′-TACAATATCCGAAATCGTTGAATTAGCG-3′) and reverse (5′-AAATTCTCTCGAAGCCGTGGAATG-3′) primers were used to detect *ThCBL9* transcript levels. Relative gene expression following the stress treatments was calculated by comparing transcript levels with those from untreated seedlings, the latter being assigned a value of '1'.

Generation of *ThCBL9*-Overexpressing Transgenic Plants

To make our *ThCBL9* overexpression construct, *ThCBL9* cDNA was cut from the pBK-CMV plasmid with *Bam*HI and *Kpn*I. This fragment was sub-cloned into the *Bam*H and *Kpn* sites of the binary vector pROKII under the cauliflower mosaic virus 35S promoter. The resulting recombinant plasmid, pROKII-*ThCBL9*, was introduced into *Agrobacterium tumefaciens* strain GV3101 by a freeze-thaw procedure (Hooykaas, 1988). This resulting strain was used for transformation. *Arabidopsis* plants (4 weeks old) were infected with *A. tumefaciens* by the floral dipping method (Clough and Bent, 1998) and grown in the greenhouse. These were self-fertilized and their seeds plated on an MS medium containing 30 mg L^{-1} kanamycin to screen for transformants.

PCR amplifications were performed in the presence of the forward primer 5′-CTTCTCGGGTTTCTTCGT-3′ and reverse primer 5′-TCACGTGGCAATCTCATCC-3′ for *ThCBL9*, using genomic DNA extracted from transgenic and wild-type *A. thaliana* leaves by the CTAB method.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from the rosette leaves of transgenic and wild-type *Arabidopsis* plants according to the method of Chomczynski and Sacchi (1987). For Northern blot analysis, 20 μg of total RNA was electrophoresed on a 1.2% (w/v) agarose gel containing formaldehyde, then transferred onto a positively charged nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden) with 20×SSC buffer (1×SSC = 150 mM NaCl and 15 mM sodium citrate; pH 7.0). DNA probes were labeled by a random primer DNA labeling kit (TaKaRa) with a portion of the cloned cDNA fragment and [^{32}P] dCTP. To assess relative quantities, the loaded RNA was stained with ethidium bromide after electrophoresis. The membrane was pre-hybridized for 4 to 5 h at 65°C in Church buffer (0.25 M Na_2HPO_4, 7% [w/v] SDS, 2 mM EDTA, and a 1% BSA fraction). Hybridization was carried out overnight at 65°C before the blot was first washed twice (15 min each) at low stringency (2×SSC containing 0.1% SDS at 65°C), then re-washed for 30 min at high stringency (0.2×SSC containing 0.1% SDS at 65°C) (Sambrook and Russell, 2001).

Germination and Seedling Growth Assays

Approximately 100 seeds each from the wild-type and T3 transgenic *Arabidopsis* lines were surface-sterilized and sown in triplicate on MS media containing NaCl (0, 50, 100, 150, or 200 mM), sorbitol (0, 200, 250, 300, 350, or

400 mM), or ABA (0,1, 2, 3, 4, or 5 µM). At 4 d after stratification at 4°C, these MS plates were transferred to a 22 to 23°C chamber for continuous incubation, and the germination rate (i.e., emergence of radicle) was scored daily for 7 d.

For early growth assessments, 5-day-old seedlings from vertical plates containing MS media were placed with their roots pointing downward onto vertically oriented plates with MS media that was supplemented with different concentrations of NaCl (0, 100, 150, or 200 mM). Each plate contained five seedlings per wild type or transgenic line, and five replicate plates were used for each treatment. Root lengths were marked at the onset of treatment, and their increases were monitored for 7 d. Plants were photographed after 3 weeks.

Determinations of Fresh and Dry Weights

Four-week-old wild-type and transgenic *Arabidopsis* plants were treated with NaCl concentrations that were stepped up at 50-mM increments every other day until the final desired concentration (0, 50, 100, 150, or 200 mM NaCl) was achieved. At 10 d after treatment, shoot fresh weights from each line were recorded immediately after harvesting; shoot dry weights were measured after oven-drying the samples (20 per line per treatment, analyzed in parallel) at 70°C for 48 h.

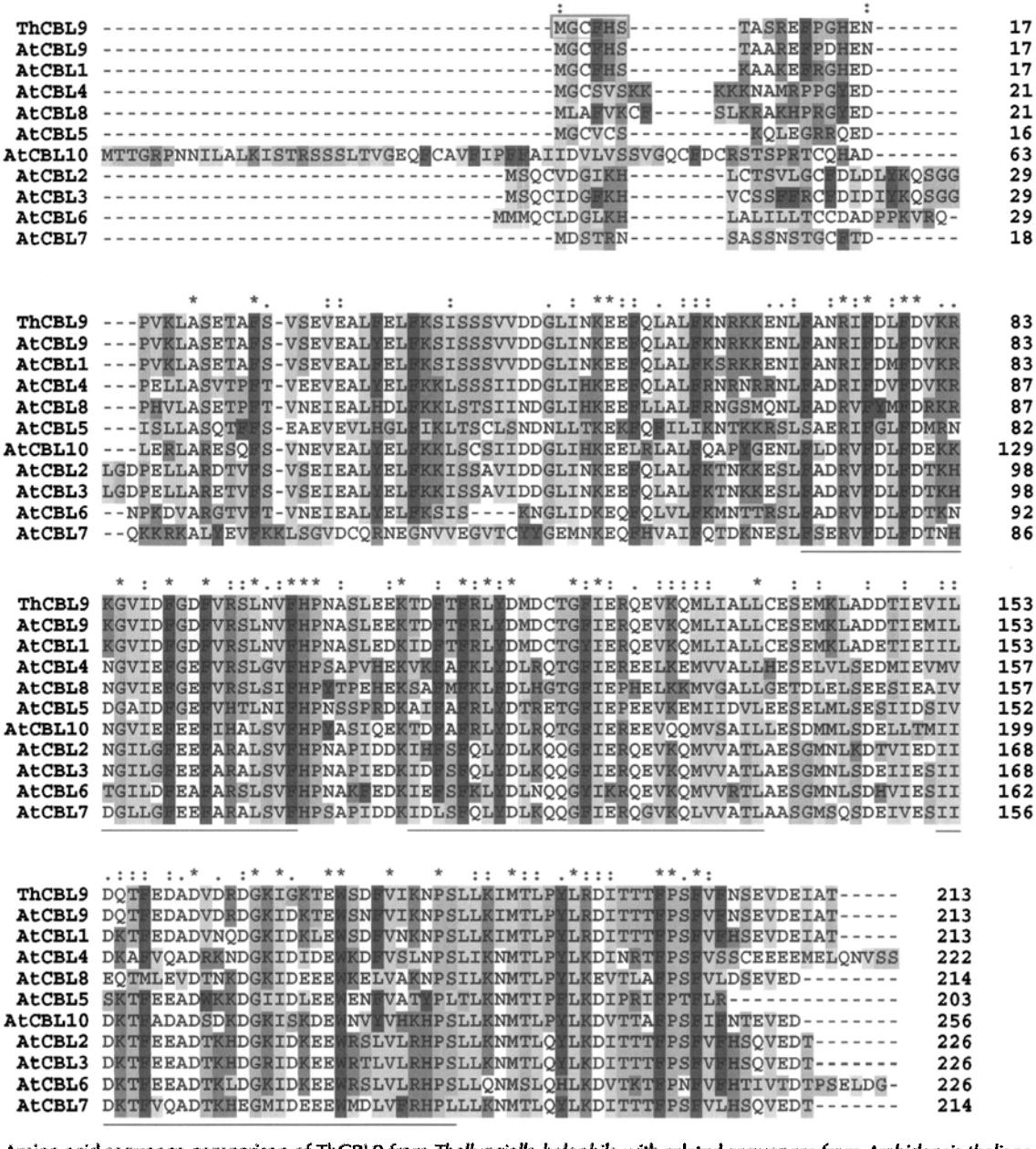

Figure 1. Amino acid sequence comparison of ThCBL9 from *Thellungiella halophila* with related sequences from *Arabidopsis thaliana* AtCBL1 (GenBank Accession No. AF076251), AtCBL2(AF076252), AtCBL3(AF076253), AtCBL4(Y18870), AtCBL5(AF290435), AtCBL6 (AF192884), AtCBL7(AL078465.1), AtCBL8(AF069300.1), AtCBL9 (AF411958), and AtCBL10(NP849485). Solid lines below sequence indicate EF-hand motifs. Asterisks indicate amino acid conserved in all sequences. Dashes represent gaps to maximize alignment. Sequences in box indicate N-myristoylation motif (MGxxxS/T[K]).

Measurement of Photosynthesis and Transpiration Rates in Leaves

Following these NaCl treatments, the *in vivo* photosynthetic rate, concentration of intercellular CO_2 (Ci), photosynthetic efficiency (Fv/Fm), and transpiration rate of the leaves were determined with an automatic photosynthesis-measuring apparatus (Ciras-2; PPSystems, Hitchin, Hertfordshire, UK), as described by Qiu et al. (2003). Three independent experiments with 20 leaves were conducted for each NaCl treatment.

Measurement of Ion Contents

Na^+ and K^+ contents of the shoots and roots were assessed by atomic absorption spectrometry (Hitachi Z-8000) (Wang and Zhao, 1995). Experiments were performed in triplicate, with 15 plants per replicate pooled for each determination.

Accession Numbers

Sequence data associated with our study can be found in the GenBank data libraries under the following accession numbers: *ThCBL9*, EU169239; and *ThActin-2*, DN774023.

RESULTS

Isolation and Sequence Analysis of *ThCBL9*

The full-length *ThCBL9* cDNA (1008 bp) contains an ORF of 639 bp encoding 213 amino acids, with a 193-bp 5′-untranslated region and a 176-bp 3′-untranslated region. ThCBL9 shares high similarity with other genes, including AtCBL9 at 97% and AtCBL1 at 90% (Fig. 1). The main differences are in their N-terminals. However, all three contain the N-myristoylation motif (MGxxxS/T[K]).

ThCBL9 Expression in *Thellungiella halophila* Leaves under Stress

Because *AtCBL*s possess high nucleotide sequence similarities (data not shown) and *Thellungiella halophila* shares higher cDNA identity with *Arabidopsis thaliana* (Bressan et al., 2001), we performed real-time PCR reactions to check transcript levels of *ThCBL9* under three stress conditions. Expression was up-regulated 12-, 6-, and 13-fold by ABA, NaCl, and PEG treatment, respectively (Fig. 2), indicating that transcription of *ThCBL9* is stress-induced.

Exogenous *ThCBL9* Expression in Transgenic *Arabidopsis* Lines

The *ThCBL9* coding sequence, preceded by the CaMV 35S promoter, was sub-cloned into binary vector pROKII. After the recombinant was introduced by floral-dipping, 35 kanamycin-resistant plants were generated. These were then selected to identify the transgenic homozygote and 6 T3 transgenic homozygote lines for use in PCR and expression analysis of exogenous *ThCBL9*.

PCR-amplification produced specific bands of approx. 0.67 kb from the transgenic lines but not from wild-type plants (data not shown). Southern blots also showed that signaling bands existed only in the transgenic lanes (data not shown), thereby demonstrating that *ThCBL9* did integrate into the genome of *A. thaliana*.

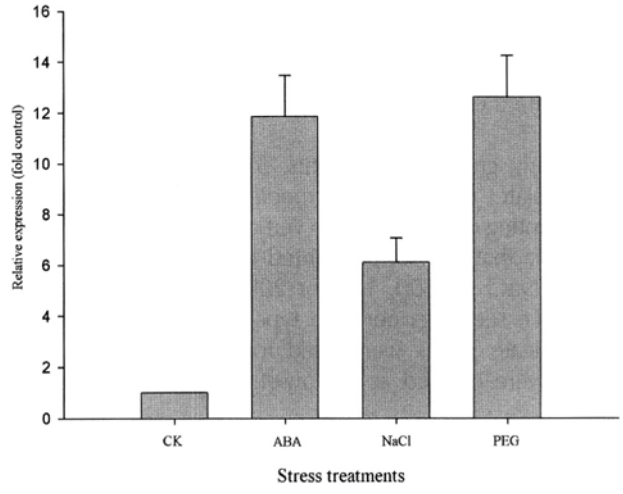

Figure 2. Real-time PCR analysis of *ThCBL9* expression in *Thellungiella halophila* leaves in response to stresses. Plants were grown in soil for 30 d, then treated with ABA (50 μM), NaCl (200 mM), PEG 6000 (25 mM), or plain Hoagland solution (as control). Total RNA was extracted from rosette leaves after 24 h of treatment. After DNase I was applied, 5 μg total RNA was denatured and subjected to reverse transcription reaction, the product of which was used for real-time analysis. Error bars represent SD (n = 3).

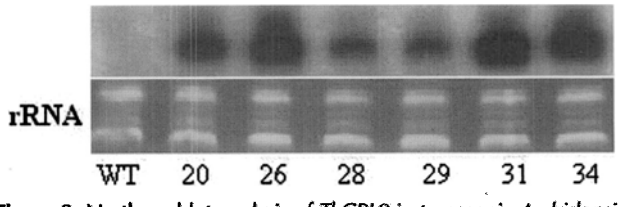

Figure 3. Northern blot analysis of *ThCBL9* in transgenic *Arabidopsis thaliana*. Total RNA (20 μg) was analyzed by RNA gel blotting. Ethidium bromide-stained rRNA band in agarose gel is shown as loading control.

Our northern blot analysis revealed that *ThCBL9* transcripts were more abundant in transgenic plants than in the wild type, with especially large amounts being detected in Lines 20, 26, 31, and 34 (Fig. 3). This result is powerful evidence for the integration of *ThCBL9* into *Arabidopsis* genome and, furthermore, for the overexpression of that exogenous gene.

Seed Germination and Enhanced Early Growth of Transgenic *Arabidopsis*

NaCl treatment

All transgenic lines had better germination rates than the wild type after 3 d on an MS medium containing NaCl (Fig. 4A). As the salt concentration was increased, rates declined for both genotypes, although germination was inhibited to a greater extent in the wild type. For example, at 200 mM NaCl, the wild-type rate decreased to 60.89% compared with 68.27% or 69.08% for the transgenics. Our germination time course on an MS medium containing 100 mM NaCl (Fig. 4B) also showed that rates were greater for transgenic plants in the first 2 d. At 7 d later, wild-type root

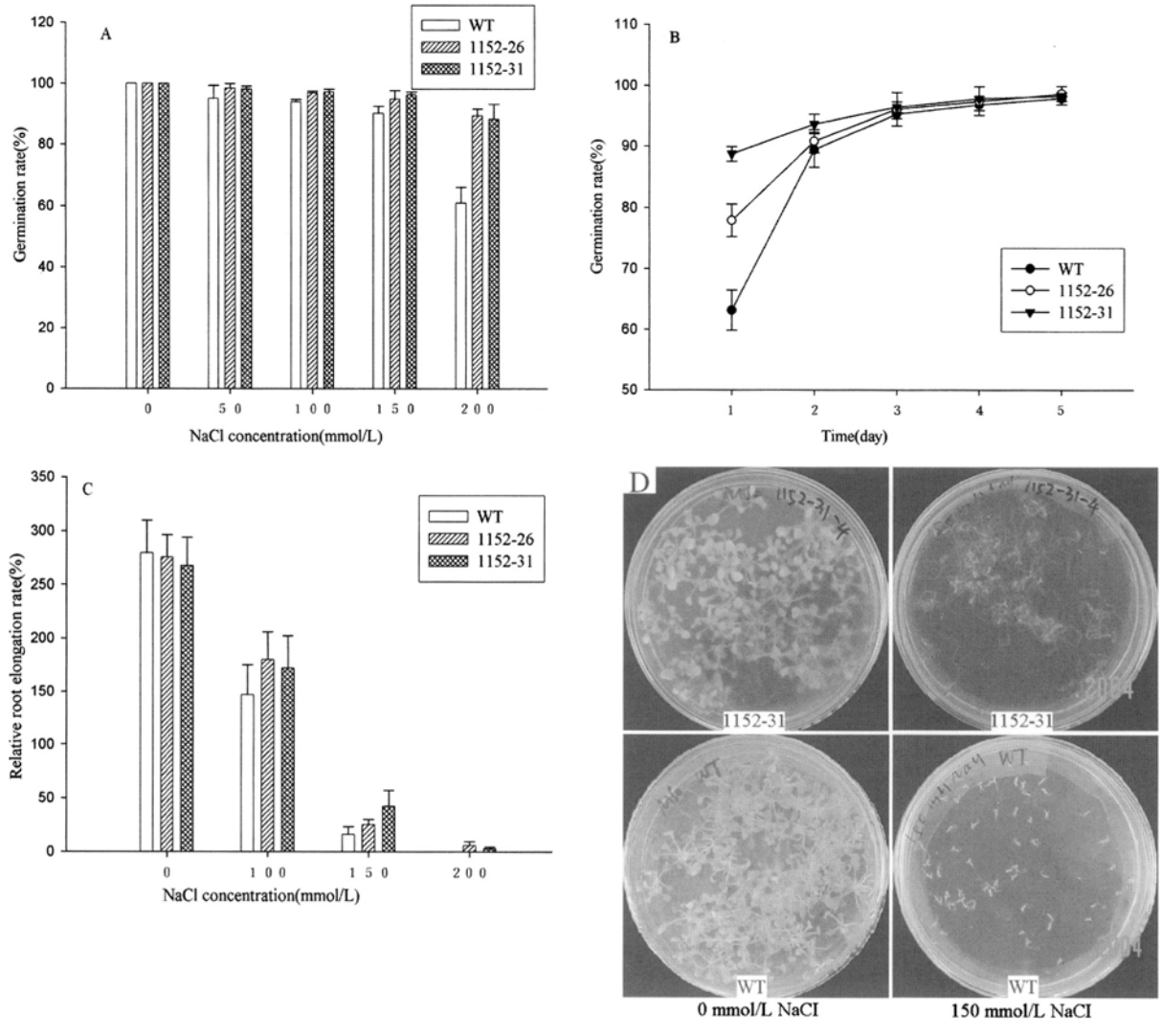

Figure 4. Germination and seedling growth under NaCl treatment. **A,** Effect of salt on seed germination at 3 d after incubation at 23°C. **B,** Germination time course (days after incubation at 23°C) on MS containing 100 mmol L^{-1} NaCl. Data in **A** and **B** are average values and standard errors from 3 experiments (n=100). **C,** Effect of salt on relative root growth of 5-day-old wild-type and transgenic *Arabidopsis* seedlings that were transferred from non-NaCl medium to MS medium supplemented with NaCl. Inhibition of root growth in presence of NaCl is relative to mean growth on MS medium without NaCl, and was measured 7 d after transfer. Data are average values and standard errors of 5 experiments (n=5). **D,** Influence of salt on growth of wild-type seedlings and transgenic *Arabidopsis thaliana* plants over-expressing *ThCBL9*.

growth was much more severely inhibited than that of the transgenic lines on MS media containing 150 or 200 mM NaCl (Fig. 4C). After 3 weeks, the transgenic plants grew as well as the wild type on MS media, indicating that the overexpression of exogenous *ThCBL9* did not influence development of the transformant seedlings. However, as the NaCl concentration increased to 150 mM, growth of both wild types and transgenic lines was diminished, although the latter continued to have better performance (Fig. 4D).

Sorbitol treatment

The effect of sorbitol on germination followed a trend similar to that observed with NaCl (Fig. 5A). Rates for transgenic lines were 96.19% versus 89.01% for wild types when plants were treated with 250 to 300 mM sorbitol. At a concentration of 300 mM, all transgenic lines had greater germination success. For example, at Day 3, rates for the germination of transgenic lines were all >89% whereas, even at Day 6, the rate for wild-type seed was still <83% (Fig. 5B).

ABA treatment

When the ABA concentration was increased, the wild-type germination rate decreased while that of the transgenic plants remained almost the same (Fig. 6A). For example, on the MS medium containing 2 μM ABA, the transgenic lines had higher germination rates than the wild type on Day 1, with the rate of the former increasing again at Day 2, but then remaining at that same pace afterward. In contrast, the wild-type rate did not peak until Day 4 but, even then, germination values were still lower than for the transgenics (Fig. 6B). This demonstrates that the overexpression of *ThCBL9* in *Arabidopsis* causes the germination process by transgenic seeds to become insensitive to ABA. However, after germination was complete, the growth of both transgenic and wild-type seedlings was equally and severely inhibited by ABA (data not shown).

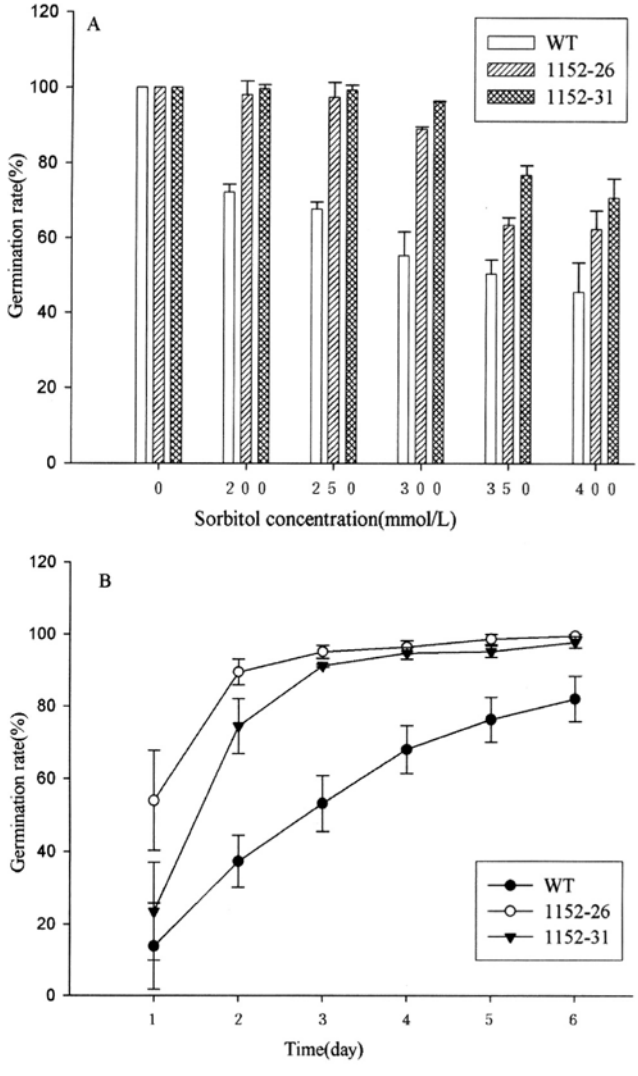

Figure 5. A, Effect of sorbitol on seed germination at 3 d after incubation at 23°C. **B,** Germination time course (days after incubation at 23 °C) on MS containing 300 mmol L^{-1} sorbitol. Data are average values and standard errors from 3 experiments (n=100).

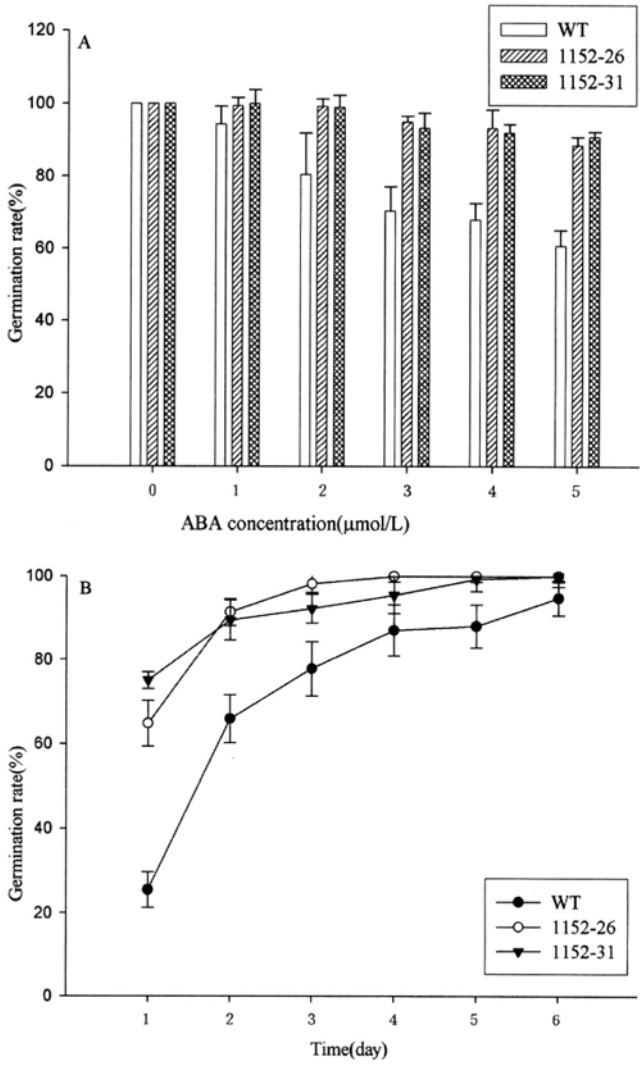

Figure 6. A, Effect of ABA on seed germination at 3 d after incubation at 23°C. **B,** Germination time course (days after incubation at 23°C) on MS containing 2 μmol L^{-1} ABA. Data are average values and standard errors from 3 experiments (n=100).

Evaluation of Salt Tolerance in Transgenic Plants

Whole-plant growth, and fresh and dry weights

Fresh and dry weights are commonly used as indices of plant growth. Here, our transgenic lines had obviously better performance than the wild type under NaCl stress (Fig. 7A), with values for both parameters being higher in the former type when all were treated at various NaCl concentrations (Fig. 7B, C). This indicated that the transgenic plants possess stronger NaCl tolerance.

Net photosynthesis rate, Ci values, transpiration rate, and maximum quantum efficiency of PSII (Fv/Fm)

Compared with the trend in net photosynthesis rates for the wild type, that of the transgenics declined much more smoothly (Fig. 8A). Although the concentrations of intercellular CO_2 in both wild-type and transgenic leaves decreased as NaCl was increased, Ci values in the latter were always higher (Fig. 8B). At 100 mM NaCl, the wild-type transpiration rate showed a sharp drop while that of the transgenic lines always remained at the same level (Fig. 8C). However, both genotypes had decreased photosynthetic efficiencies following the application of salt stress (Fig. 8D). For example, at 10 d after treatment with 200 mM NaCl, values for Fv/Fm decreased about 21.90% in the wild type but only 19.14% in the transgenics. This suggests that the PSII for transgenic lines undergoes less severe inhibition.

Na^+ and K^+ contents

A critical aspect of salt tolerance in plant cells is the maintenance of a low concentration of toxic Na^+ and a high concentration of K^+ in the cytoplasm. Here, the Na^+ concentration in shoots and roots was lower in the transgenic lines than in the wild type (Fig. 9A, B). Concurrently, the K^+ concentration in the transgenics was higher than in the wild type.

DISCUSSION

A close relative of *Arabidopsis thaliana*, *Thellungiella halo-*

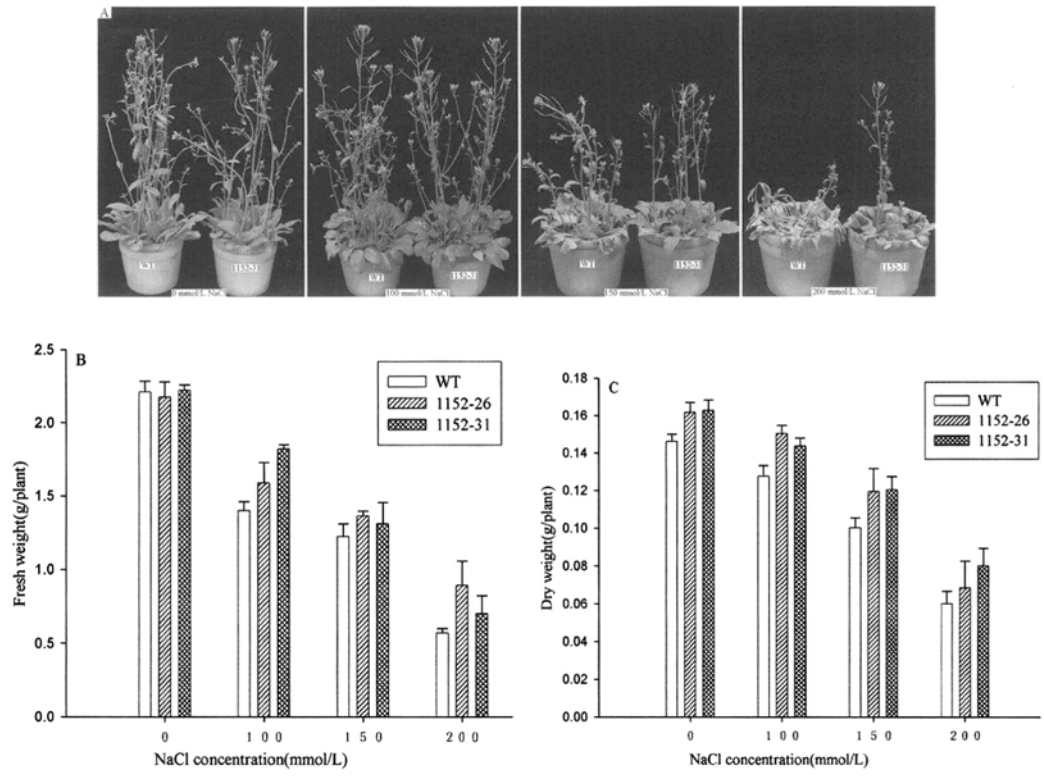

Figure 7. **A**, Whole-plant growth, **B**, fresh weights, and **C**, dry weights of seedlings under NaCl stress. Photograph was taken 10 d after 4-week-old *Arabidopsis thaliana* plants were treated with different concentrations of NaCl. Data are average values and standard errors from 3 experiments (n=20).

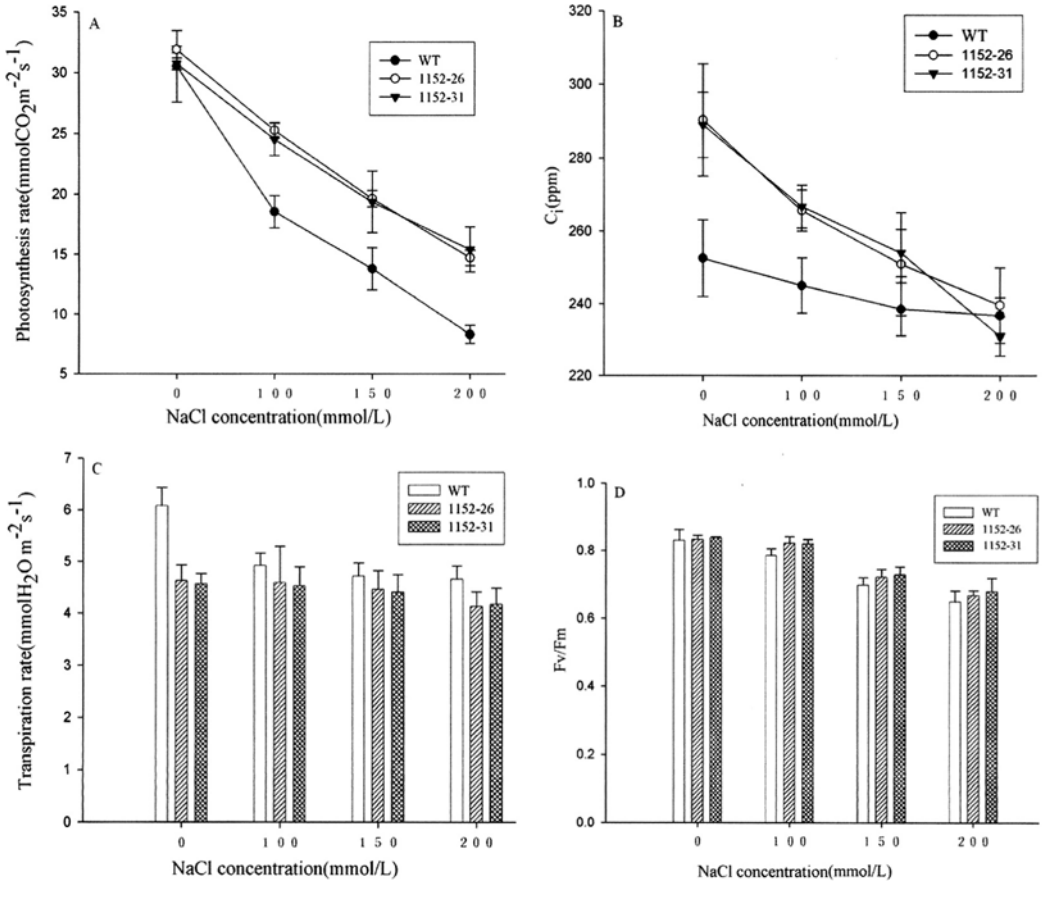

Figure 8. Effect of NaCl on net photosynthesis rate (**A**), intercellular CO_2 concentration (Ci) (**B**), transpiration rate (**C**), and maximum quantum efficiency of PSII (Fv/Fm) (**D**) for wild-type and *ThCBL9*-overexpressing *Arabidopsis thaliana*. Data are average values and standard errors from 3 experiments (n=10).

phila has a genome that is approximately twice as large and shares 90 to 95% identity at the cDNA level (Bressan et al., 2001; Zhu, 2001). However, although they are similar, the latter has a much greater tolerance to stresses from salt, drought, and low temperatures (Bressan et al., 2001; Inan et al., 2004). *T. halophila* has become an important model for abiotic stress research (Amtmann et al., 2005). Some microarray results have shown that the expression patterns for many of those homologous genes differ between *T. halophila* and *A. thaliana* under salt stress, with several being constitutively expressed in *Thellungiella* but induced only by NaCl in *Arabidopsis* (Taji et al., 2004; Gong et al., 2005). Therefore, this specialization in gene expression may contribute to higher tolerance.

Calcineurin B-like (CBL) proteins are a unique family of calcium sensors in plants (Kudla et al., 1999; Luan et al., 2002), and the CBLs-CIPKs network is involved in several signaling processes (Cheong et al., 2003; Kim et al., 2003; Li et al., 2006). Most of the progress in CBL research has been achieved with *Arabidopsis*, and information about these signaling modules in other plant species is still quite limited.

Our comparison of amino acid sequences showed that ThCBL9 shared the highest identity with AtCBL9 (Fig. 1). ThCBL9 also possesses an N-terminus myristoylation and palmitoylation motif (MGCxxS/T[K]) (Fig. 1), which is the signal and enhancer for membrane localization (Luan et al., 2002; Navarro-Lérida et al., 2002; Kolukisaoglu et al., 2004). The AtCBL9 protein appears to act as a negative regulator of ABA-signaling, which leads to the inhibition of seed germination. When CBL9 functioning is disrupted, the *cbl9* mutant displays ABA hypersensitivity during germination (Pandey et al., 2004). Our data demonstrated that lines of *ThCBL9*-overexpression transgenic *A. thaliana* were insensitive to ABA during that stage (Fig. 6), thereby verifying that CBL9 is a negative regulator of ABA-signaling via heterologous expression.

CBLs, CIPKs, and the CBL-CIPK network may also be involved in such signaling processes (Cheong et al., 2003; Kim et al., 2003; Pandey et al., 2004). For example, AtCBL1 and AtCBL9 interact with CIPK23 through the C-terminal nonkinase domain and active CIPK23 (Shi et al., 1999). Furthermore, the myristoylation on the N terminus of CBL1/9 recruits the CBL1-CBL9-CIPK23 complexes to the cell membranes (Xu et al., 2006; Cheong et al., 2007) to phosphorylate AKT1, a voltage-gated shaker-type K channel (Hirsch et al., 1998). Those CBL1/9-CIPK23 complexes activate AKT1 and enhance K^+ uptake under conditions of low potassium (Li et al., 2006; Xu et al., 2006). The plasma membrane-localized CBL1-CBL9-CIPK23 complexes simultaneously regulate K^+ transport processes in both roots and stomata guard cells (Cheong et al., 2007).

The ability of plants to exclude toxic ions (e.g., Na^+), while taking up or retaining others (e.g., K^+) is a key determinant of salt tolerance (Maathuis and Amtmann, 1999). Our northern blots showed that *ThCBL9* was highly expressed in *Arabidopsis* (Fig. 3). ThCBL9 may have been assembled into those CBL1-CBL9-CIPK23 complexes, further enhancing AKT1 activity, and causing *ThCBL9* transgenic plants to accumulate higher K^+ contents than by the wild type (Fig. 9B). The SOS pathway in *A. thaliana* helps maintain the ion

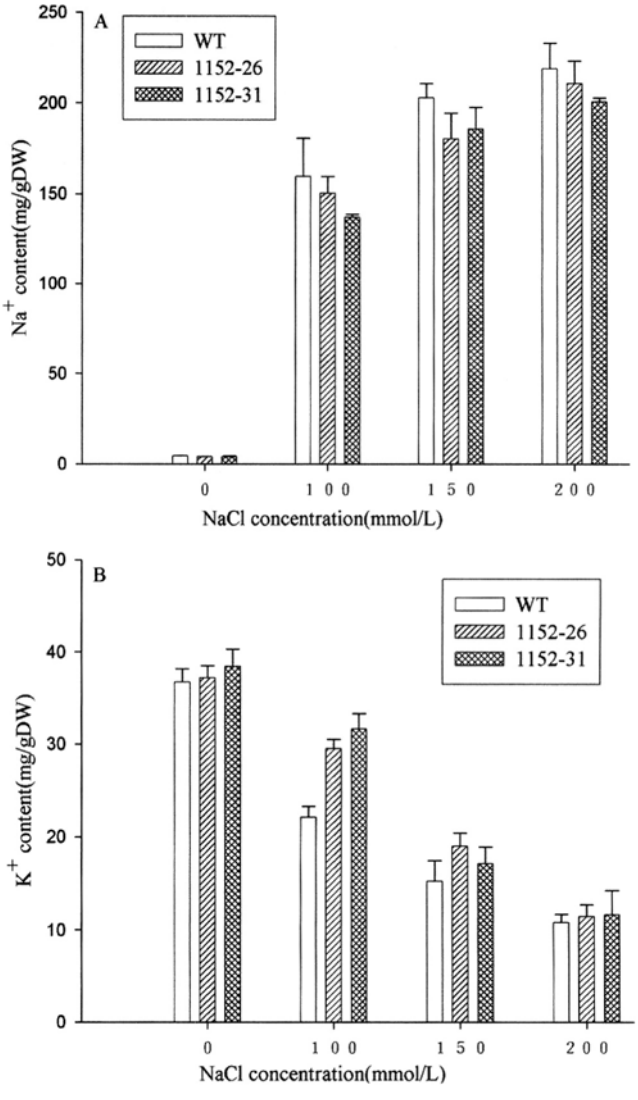

Figure 9. Effect of NaCl on contents of Na^+ (A) and K^+ (B) in wild-type and transgenic *Arabidopsis thaliana* over-expressing *ThCBL9*. Data are average values and standard errors from 3 experiments.

equilibrium of cells under salt stress (Zhu, 2003). Multiple CBLs can associate with multiple CIPKs (Lee et al., 2007). Here, the ThCBL9 protein may have joined in that SOS pathway to keep the transgenic plants at a lower intracellular Na^+ concentration than was measured in the wild type, especially under salt stress (Fig. 9A).

Maintaining an intercellular environment of higher K^+ and lower Na^+ can help transgenics alleviate ion toxicity (Maathuis and Amtmann, 1999), such that those plants achieve higher tolerance to toxins due to *ThCBL9* overexpression. When treated under NaCl on the MS medium, those transgenic plants also germinated earlier (Fig. 4A, B) and had better root elongation (Fig. 4C) and overall growth (Fig. 4D).

Our transgenic plants that over-expressed *ThCBL9* had higher salt tolerance and improved growth (Fig. 7A) than the wild type when NaCl was applied to the soil. In addition, either fresh or dry weight values were greater in the transgenic lines, all further evidence of the high salt tolerance found in transgenic lines (Fig. 7B, C). These observations may directly demonstrate that the photosystem in transgenic

plants is better protected than in the wild type under conditions of elevated salt content (Fig. 8). Overexpression of ThCBL9 may also confer improved tolerance to ion toxicity by intracellular enzymes.

Taken together, our results show that ThCBL9 is a functional homolog of AtCBL9, and that its ability to improve the tolerance of transgenic plants to ABA, NaCl, and osmotic stress is related to the putative multiple functions of ThCBL9. These include acting as a negative regular of ABA-signaling, as a calcium sensor member assembled in the CBL1-CBL9-CIPK23 complexes to activate AKT1, and/or as a participant in the SOS pathway to enhance the activation of SOS1

ACKNOWLEDGMENTS

This work was supported in part by the National Basic Research Program of China (Grant No. 2006CB100100), and by the Natural Science Foundation of Shandong Province, China (Grant No. Y2006D27).

Received August 16, 2007; accepted November 17, 2007.

LITERATURE CITED

Amtmann A, Bohnert HJ, Bressan RA (2005) Abiotic stress and plant genome evolution. Search for new models. Plant Physiol 138: 127-130

Bressan RA, Zhang C, Zhang H, Hasegawa PM, Bohnert HJ, Zhu JK (2001) Learning from the *Arabidopsis* experience. The next gene search paradigm. Plant Physiol 127: 1354-1360

Cheong YH, Kim KN, Pandey GK, Gupta R, Grant JJ, Luan S (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. Plant Cell 15: 1833-1845

Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim BG, Lee SC, Kudla J, Luan S (2007) Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in *Arabidopsis*. Plant J 52: 223-239

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159

Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735-743

Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. Plant J 44: 826-839

Halfter U, Ishitani M, Zhu JK (2000) The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc Natl Acad Sci USA 97: 3735-3740

Hirsch RE, Lewis BD, Spalding EP, Sussman MR (1998) A role for the AKT1 potassium channel in plant nutrition. Science 280: 918-921

Hooykaas, PJJ (1988) *Agrobacterium* molecular genetics. In SB Gelvin, RA Schilperoort, DPS Verma, eds, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp A4: 1-13

Inan G, Zhang Q, Li PH, Wang ZL, Cao ZY, Zhang H, Zhang C, Quist TM, Goodwin SM, Zhu J, Shi H, Damsz B, Charbaji T, Gong Q, Ma S, Fredricksen M, Galbraith DW, Jenks MA, Rhodes D, Hasegawa PM, Bohnert HJ, Joly RJ, Bressan RA, Zhu JK (2004) Salt cress. A halophyte and cryophyte *Arabidopsis* relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. Plant Physiol 135: 1718-1737

Ishitani M, Liu J, Halfter U, Kim CS, Shi W, Zhu JK (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. Plant Cell 12: 1667-1678

Kim BG, Waadt R, Cheong YH, Pandey GK, Dominguez-Solis JR, Schultke S, Lee SC, Kudla J, Luan S (2007) The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in *Arabidopsis*. Plant J 52: 473-484

Kim KN, Cheong YH, Gupta R, Luan S (2000) Interaction specificity of *Arabidopsis* calcineurin B-like calcium sensors and their target kinases. Plant Physiol 124: 1844-1853

Kim KN, Cheong YH, Grant JJ, Pandey GK, Luan S (2003) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. Plant Cell 15: 411-423

Knight H (2000) Calcium signaling during abiotic stress in plants. Intl Rev Cytol 195: 269-324

Knight H, Trewavas AJ, Knight MR (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J 12: 1067-1078

Kolukisaoglu U, Weinl S, Blazevic D, Batistic O, Kudla J (2004) Calcium sensors and their interacting protein kinases: Genomics of the *Arabidopsis* and rice CBL-CIPK signaling networks. Plant Physiol 134: 43-58

Kudla J, Xu Q, Harter K, Gruissem W, Luan S (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci USA 96: 4718-4723

Lee SC, Lan WZ, Kim BG, Li L, Cheong YH, Pandey GK, Lu G, Buchanan BB, Luan S (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc Natl Acad Sci USA 104: 15959-15964

Li L, Kim BG, Cheong YH, Pandey GK, Luan S (2006) A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in *Arabidopsis*. Proc Natl Acad Sci USA 103: 12625-12630

Liu JP, Zhu JK (1998) A calcium sensor homolog required for plant salt tolerance. Science 280: 1943-1945

Liu J, Ishitani M, Halfter U, Kim CS, Zhu JK (2000) The *Arabidopsis thaliana* SOS2 gene encodes a protein kinase that is required for salt tolerance. Proc Natl Acad Sci USA 97: 3730-3734

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W (2002) Calmodulins and calcineurin B-like proteins: Calcium sensors for specific signal response coupling in plants. Plant Cell 14: S389-400

Lynch J, Polito VS, Lauchli A (1989) Salinity stress increases cytoplasmic Ca^{2+} activity in maize root protoplasts. Plant Physiol 90: 1271-1274

Maathuis FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: The basis of cellular K^+/Na^+ ratios. Ann Bot 84: 123-133

Marschner H (1995) Mineral Nutrition of Higher Plants, 2nd ed. Academic Press, London

Nagae M, Nozawa A, Koizumi N, Sano H, Hashimoto H, Sato M, Shimizu T (2003) The crystal structure of the novel calcium-binding protein AtCBL2 from *Arabidopsis thaliana*. J Biol Chem 278: 42240-42246

Navarro-Lérida I, Álvarez-Barrientos A, Gavilanes F, Rodriguez-Crespo I (2002) Distance-dependent cellular palmitoylation of de-novo-designed sequences and their translocation to plasma membrane subdomains. J Cell Sci 115: 3119-3130

Pandey GK, Cheong YH, Kim KN, Grant JJ, Li L, Hung W, D'Angelo C, Weinl S, Kudla J, Luan S (2004) The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and bio-

synthesis in *Arabidopsis*. Plant Cell 16: 1912-1924

Pauly N, Knight MR, Thuleau P, van der Luit AH, Moreau M, Trewavas AJ, Ranjeva R, Mazars C (2000) Control of free calcium in plant cell nuclei. Nature 405: 754-755

Quan RD, Lin HX, Mendoza I, Zhang YG, Cao WH, Yang YQ, Shang M, Chen SY, Pardo JM, Guo Y (2007) SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect *Arabidopsis* shoots from salt stress. Plant Cell 19: 1415-1431

Qiu N, Lu Q, Lu C (2003) Photosynthesis, photosystem eifficiency and the xanthophyll cycle in the salt-adapted halophyte *Atriplex centralasiatica*. New Phytol 129: 479-486

Sambrook J, Russell WD (2001) Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Press, New York

Shi J, Kim KN, Ritz O, Albrecht V, Gupta R, Harter K, Luan S, Kudla J (1999) Novel protein kinases associated with calcineurin B-like calcium sensors in *Arabidopsis*. Plant Cell 11: 2393-2405

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* microarray. Plant Physiol 135: 1697-1709

Wang B, Zhao K (1995) Comparison of extractive methods of Na and K in wheat leaves (in Chinese). Plant Physiol Commun 31: 50-52

Wang ZL, Li PH, Fredricksen M, Gong ZZ, Kim CS, Zhang C, Bohrtert HJ, Zhu JK, Bressan RA, Hasegawa PM, Zhao YX, Zhang H (2004) Expressed sequence tags from *Thellungiella halophila*, a new model to study plant salt-tolerance. Plant Sci 166: 609-617

Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. Cell 125: 1347-1360

Zhou XF, Wang XZ (2002) Research progress in salt tolerance genes of SOS in *Arabidopsis thaliana*. Yi Chuan 24: 190-192

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6: 66-71

Zhu JK (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6: 441-445

Zhu JK, Liu J, Xiong L (1998) Genetic analysis of salt tolerance in *Arabidopsis*. Evidence for a critical role of potassium nutrition. Plant Cell 10: 1181-1191