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Overexpression of a putative maize calcineurin B-like protein in *Arabidopsis* confers salt tolerance

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Abstract The calcineurin B-like proteins (CBLs) represent a unique family of calcium sensors in plants. Although extensive studies and remarkable progress have been made in *Arabidopsis* (*Arabidopsis thaliana*) *CBLs*, their functions in other plant species are still quite limited. Here, we report the cloning and functional characterization of *ZmCBL4*, a novel CBL gene from maize (*Zea mays*). *ZmCBL4* encodes a putative homolog of the *Arabidopsis* CBL4/SOS3 protein, with novel properties. *ZmCBL4* has one copy in maize genome and harbors seven introns in its coding region. *ZmCBL4* expressed differentially in various organs of the maize plants at a low level under normal condition, and its expression was regulated by NaCl, LiCl, ABA and PEG treatments. Expression of 35S::*ZmCBL4* not only complemented the salt hypersensitivity in *Arabidopsis sos3* mutant, but also enhanced the salt tolerance in *Arabidopsis* wild type at the germination and seedling stages. Moreover, the LiCl tolerance in all of the *ZmCBL4*-expressing lines increased more significantly as compared with the NaCl tolerance, and in consistent with this, it was found that the expression of *Arabidopsis AtNHX8*, a putative plasma membrane Li^+/H^+ antiporter gene identified recently, was induced in these transgenic lines under LiCl stress. The *ZmCBL4*-expressing *Arabidopsis* lines accumulated less Na^+ and Li^+ as compared with the control plants. This study has identified a putative maize CBL gene which functions in the salt stress-elicited calcium signaling and thus in the tolerance to salinity.

Keywords CBL gene · Maize · Expression · Salt tolerance

Abbreviations
ABA Abscisic acid
PEG Polyethylene glycol

Introduction

High soil salinity results in both ionic and osmotic stresses for plants. The ionic stress disrupts ion homeostasis in plant cells, resulting in excess toxic ions, for example, Na^+, in the cytosol and a deficiency of essential ions such as K^+ (Zhu 2001a, b, 2002). Therefore, plants have to develop mechanisms to maintain the ion homeostasis in the cytoplasm. Previous studies have indicated that various ion transporters, pumps and channels play crucial roles in these processes, and their expression and activities are regulated by some signaling pathways (Xiong et al. 2002; Zhu 2003; Reddy and Reddy 2004). Salt stress is known to trigger a rapid and transient increase of free calcium concentration in plant cells (Lynch et al. 1989; Knight et al. 1997; Knight 2000; Pauly et al. 2000). As such, Ca^{2+} signaling processes

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are one of the earliest events in salt signaling and may play an essential role in the ion homeostasis and salt tolerance in plants (Lynch et al. 1989; Knight et al. 1997; Zhu 2003; Reddy and Reddy 2004). Little was known about the salt stress-elicited calcium signaling processes in plant cells until the SOS pathway was revealed in *Arabidopsis* (Zhu 2000). This study began with genetic screening for SOS (salt overly sensitive) *Arabidopsis* mutants from an EMS or fast-neutron mutagenized M2 population or T-DNA insertion lines, which resulted in the discovery of five genes namely, *AtSOS1–5* (Wu et al. 1996; Liu and Zhu 1998; Liu et al. 2000; Shi et al. 2000, 2002b, 2003; Shi and Zhu 2002c). Subsequent studies indicated that *SOS1*, *2* and *3* function in the same signaling pathway and their functions are Ca^{2+} dependent (Halfter et al. 2000). Loss-of-function mutation in each of them causes the plants to be hypersensitive both to NaCl and LiCl stresses (Liu and Zhu 1997, 1998; Liu et al. 2000; Shi et al. 2000). The *Arabidopsis AtSOS3*, also known as *AtCBL4*, encodes a small N-myristoylated calcium-binding protein with four predicted EF-hands (Sánchez-Barrena et al. 2005). The SOS3 protein has sequence similarities with both the regulatory B-subunit of calcineurin (CNB) in yeast and the neuronal calcium sensor (NCS) in animals (Liu and Zhu 1998). Both N-myristoylation and Ca^{2+} binding are required for its function in perceiving and relaying the Ca^{2+} signal elicited by salt stress (Ishitani et al. 2000). The *Arabidopsis AtSOS2* (*AtCIPK24*) encodes a serine/threonine protein kinase consisting of a conserved N-terminal catalytic kinase region and a unique regulatory C-terminal region that is required for interacting with SOS3 and functioning in plant (Albrecht et al. 2001; Guo et al. 2001, 2004). The SOS2 protein kinase is the target of SOS3 calcium sensor in vivo (Shi et al. 1999; Halfter et al. 2000). The expression of *AtSOS2* was up-regulated by salt stress (Liu et al. 2000). The *Arabidopsis AtSOS1* encodes a plasma membrane Na^+/H^+ antiporter, which is directly involved in removing the excess Na^+ out of the cell. The expression of *AtSOS1* in the wild-type roots was up-regulated by NaCl stress and partially controlled by *AtSOS3* and *AtSOS2* (Shi et al. 2000). The activation of SOS1 Na^+/H^+ antiporter also required SOS3 and SOS2 proteins (Qiu et al. 2002). In the SOS pathway, a short chain of calcium signal transduction was proposed in that the SOS3 senses cytosolic calcium changes elicited by salt stress and physically interacts with and activates SOS2. The SOS3–SOS2 complex may be recruited to the plasma membrane by the myristoylated SOS3, where the SOS1 Na^+/H^+ antiporter activity is activated through phosphorylation by the activated SOS2 and thereby regulates ion homeostasis (Halfter et al. 2000; Zhu 2002; Qiu et al. 2002). Reconstruction of the *Arabidopsis* SOS system in yeast provided additional evidences for the interaction between SOS3 and SOS2 and their regulation of SOS1. The SOS3–SOS2 complex phosphorylated and activated SOS1 and further reduced Na^+ accumulation in yeast cells. Coexpression of *AtSOS1*, *2*, and *3* dramatically increased the Na^+ tolerance of a yeast mutant lacking all the endogenous Na^+ transporters (Quintero et al. 2002).

Very recently, it has been reported that the SOS salt tolerance pathway was identified from rice (*Oryza sativa*). OsSOS1, the rice functional homolog of the *Arabidopsis* SOS1 displayed the ability for Na^+ excluding from yeast cells and suppressed the salt sensitivity of a *sos1-1* mutant of *Arabidopsis*. OsCIPK24 and OsCBL4, the putative rice counterparts of the *Arabidopsis* SOS2 and SOS3 proteins, also exhibited the functional similarity to AtSOS2 and AtSOS3 (Martínez-Atienza et al. 2007).

The *Arabidopsis* CBL1 and CBL9 calcium sensors also play roles in response to salt stress (Cheong et al. 2003; Pandey et al. 2004). These two CBL proteins share ~90% sequence identity (Kolukisaoglu et al. 2004). Although having distinct response to exogenous ABA, the expressions of both *AtCBL1* and *AtCBL9* were highly inducible by salt, cold and drought stresses. Both *cbl1* and *cbl9* null mutants displayed reduced salt tolerance and *CBL1*-over-expressing transgenic plants showed enhanced salt tolerance (Cheong et al. 2003; Pandey et al. 2004). These findings suggest that either AtCBL1 or AtCBL9 functions as a positive regulator in salt stress response pathway. Xu et al. (2006) demonstrated that the *Arabidopsis* CBL1 and CBL9 proteins are also involved in the CIPK23 activated, AKT1-mediated K^+ uptake pathway. Both CBL1 and CBL9 localize to the plasma membrane. Low-K^+ stress signals may trigger the cytosolic Ca^{2+} signal and lead to the activation of CBL1 and CBL9, and then the CBL proteins interact with and activate CIPK23 and may recruit CIPK23 to the plasma membrane, where AKT1, a K^+ transporter, is phosphorylated by CIPK23. As the result, AKT1 is activated for K^+ uptake under low-K^+ conditions.

In spite of the extensive studies and remarkable progress in the *Arabidopsis* salt-elicited SOS/CBL-CIPK calcium signaling pathways, information about these signaling modules in other plant species is still quite limited. Here we present the cloning and characterization of *ZmCBL4*, a novel CBL gene from maize. *ZmCBL4* encodes a putative homolog of the *Arabidopsis* CBL4/SOS3 protein, with novel properties. *ZmCBL4* expressed differentially in various plant organs under normal condition. The expression of *ZmCBL4* in young seedling roots was up-regulated by salt stress and ABA, but down-regulated by PEG treatment. Expression of 35S::*ZmCBL4* not only complemented the salt hypersensitivity in *Arabidopsis sos3* mutant, but also enhanced the salt tolerance in *Arabidopsis* wild type. Moreover, the LiCl tolerance in all of the *ZmCBL4*-expressing lines increased more significantly as compared with the NaCl tolerance, and in consistent with this, it was

found that the expression of *Arabidopsis AtNHX8*, a putative plasma membrane Li^+/H^+ antiporter gene identified recently, was induced in these transgenic lines under LiCl stress. To the best of our knowledge, this is the first report of maize *CBL* genes.

Materials and methods

Maize material and stress treatments

Maize inbred line Han21 was used in this study. The grains were surface-sterilized in a solution of 0.5% Clorox plus 0.05% Triton X-100 for 20 min and rinsed five times with tap water and then sown in pots filled with sand. The pots were placed in a greenhouse and watered once every 3 days with tap water. The three-leaf-stage seedlings were carefully removed from the sand and washed clean with tap water. After the three-day adaptive culture in Hoagland solution as described by Zheng et al. (2004), the seedling roots were immersed in MS nutrient solution supplemented with 20% PEG(w/v), 100 μM(±)-*cis,trans*-ABA, 250 mM NaCl or 20 mM LiCl for 0, 2, 6, 12 and 24 h, respectively. The shoots and roots from all the treated seedlings were harvested separately at the indicated time points and stored at –76°C for RNA extraction.

Database searches and isolation of *ZmCBL4* gene

To identify maize CBL genes, we performed database searches for the predicted CBL mRNA sequences, expressed sequence tags (ESTs), or genomic sequences by sequence comparison with previously identified *Arabidopsis* CBLs in different maize databases using TBLASTN. The sequences or ESTs obtained were further assembled and compared pairwise to identify nonredundant sequences. Among the identified putative maize CBL unique genes, one mRNA sequence (BT018770), which encodes a predicted protein with the highest similarity to *Arabidopsis* SOS3/CBL4, was chosen for further study and designated as *ZmCBL4*.

The complete coding region of the cDNA and the genomic DNA of *ZmCBL4* were amplified by PCR approach using the specific primer pairs, 5′-GATCCATG GGCTGCGCGACGTCCAA-3′ (forward) and 5′-GTGG GTCACCATACGCAGATGTACGCAAAC-3′ (reverse). The condition for amplification was at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, at 62°C for 30 s and at 72°C for 1 min and 30 s, plus a final extension at 72°C for 10 min. Three independent PCR products were purified and cloned into pGEM T-easy vector (Promega) and sequenced.

Real-time quantitative PCR

Total RNA was extracted from the stored (–76°C) maize samples using the hot-phenol method and was treated with RQI DNase (Promega) to remove the genomic DNA contamination. The first strand cDNA was synthesized from the total RNA (5 μg) with M-MLV reverse transcriptase (Promega) and used as the template for subsequent PCR amplification.

The real-time quantitative PCR (RT-qPCR) for examination of *ZmCBL4* expression was carried out with an ABI Prism 7900 HT sequence detection system (Applied Biosystems). The maize α-tubulin gene was used as internal control for normalization of the template cDNA. The specific primers were designed according to the guidelines with help of the Primer Express 2.0 software (Applied Biosystems). The primers for *ZmCBL4* were 5′-TCAGTG TGTTCCACCCTAAAGCA-3′ (forward) and 5′-ATCAAG CAGCGCCAAGACCAT-3′ (reverse). The primers for maize *α-tubulin* were 5′-GAGCATGGCATTCAGGCTGA CG-3′ (forward) and 5′-TCAACAAAAACAGCACGGGG CA-3′ (reverse). The expected sizes of the amplified fragments were all 128 bp. Each PCR was repeated at least four times in a total volume of 25 μl containing 1 × SYBR Green I PCR Master Mix (ABI), 200 nM of each primer and 1 μl 1:10 diluted template cDNA, using 96-well optical-grade PCR plate and the matched optical-grade membrane. The PCR protocol was as follows: an initial denature step consisting of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C and 18 s at 72°C, and an additional cycle of 15 s at 95°C, 15 s at 60°C and 15 s at 95°C for melting curve analysis. The specificity of the PCR products was verified by their migrating rate on a 2.0% (w/v) agarose gel at the expected size and by sequencing. The data obtained were analyzed with SDS 2.2 software (Applied Biosystems). The relative expression of *ZmCBL4* under each stress condition or in different organs was calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and the error bars indicate SD (n = 4).

Arabidopsis transformation and stress tolerance characterization

The *ZmCBL4* cDNA in the sense orientation was cloned into the *Nco*I/*Bst*EII restriction enzyme site of vector p3301 (pCAMBIA3301) under the control of 35S promoter from the cauliflower mosaic virus. The p3301-*ZmCBL4* construct was introduced into *Agrobacterium tumefaciens* GV3101 strain cells. The *Arabidopsis sos3* mutant and wild type (ecotype Columbia) were used in this study. The plants were grown at 22°C under long-day conditions (16-h-light/8-h-dark cycle) to the flowering stage for plant

transformation. The transformation was carried out by the floral dip method (Clough and Bent 1998). The T_1 transgenic plants were screened by spraying with 0.5‰ (v/v) phosphinothricin (ppt) solution and confirmed by PCR approach. The T_2 seeds were plated on MS (Murashige and Skoog 1962) agar plates containing 7 mg/l ppt and the transgenic lines with a 3:1(resistant: sensitive) segregation ratio were selected to produce T_3 seeds. The T_3 lines displaying 100% ppt resistance were considered homozygous and used for further experiments. Northern blot analysis was performed to confirm the expression of *ZmCBL4* in the transgenic lines. The *sos3* mutant and the transformed wild type with empty vector p3301 were used as the controls.

The salt tolerance analysis of the transgenic lines at the seedling stage was carried out using the root-bending assay described as Liu and Zhu (1998). The MS agar plates containing 1% (w/v) agar and 2% (w/v) sucrose, pH 5.7 were supplemented with different concentrations of NaCl (100, 125, 150 or 175 mM) and LiCl (12, 15, 17 or 20 mM). For germination test, the surface-sterilized seeds were sown on the MS agar plates supplemented with different concentrations of NaCl (125, 150, or 175 mM), LiCl (12, 15, 17, or 20 mM), KCl (90 or 120 mM), ABA (0.75, 1.0, 1.25, 1.5, or 1.75 μM) and mannitol (300, 350, or 400 mM). The plates were maintained at 4°C for 4–5 d and then incubated in a growth room at 22°C under long-day conditions (16-h-light/8-h-dark cycle) at $\sim 100\mu E\ m^{-2}\ s^{-1}$. At least 60 seeds were sown for each line and the percentage of germinated seeds that developed green cotyledons were calculated in all assays 5 d after germination. For seedling fresh weight assay, 30 ten-day-old seedlings growing on vertical MS agar plates (1.2%) supplemented with different concentrations of NaCl (125, 150, or 175 mM) and LiCl (12, 15, or 20 mM) were weighed. All experiments were performed in triplicate and the error bars indicated the standard deviation (SD).

Southern blot and Northern blot analyses

For Southern blot analysis, genomic DNA (30 μg) from maize inbred line Han21 seedlings was digested with *Eco*RI, *Eco*RV, *Hin*dIII *Bam*HI, *Kpn*I, or *Sac*I, separated by electrophoresis on a 0.8% agarose gel, transferred to nylon membrane (Amersham), and hybridized for 16 h at 65°C with α-^{32}p-dCTP-labeled complete coding region of the *ZmCBL4* cDNA as a probe. The membrane was washed with 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS for 15 min at 65°C, respectively.

For Northern blot analyses, the transgenic and control *Arabidopsis* seedlings growing on vertical MS agar plates for 2 weeks were treated by immersing the roots in MS nutrient solution supplemented with 250 mM NaCl or 20 mM LiCl for 5 h. The seedlings were collected and washed two times with double distilled water (ddH_2O), and then stored at –76°C for RNA extraction. Total RNA was extracted using hot-phenol method, fractionated in denaturing formaldehyde agarose gel (1.2%), blotted onto nylon membranes (Amersham), and hybridized for 20 h at 65°C with α-32p-dCTP-labeled specific probes. For *ZmCBL4* expression, the complete coding region of *ZmCBL4* cDNA was used as a probe. For *AtSOS1* expression, a 500 bp cDNA fragment in the 5′ region of the open reading frame was used as the probe and amplified by PCR using the primers, 5′-TTGTGAAGGTCACGTTTCCGTAT-3′ (forward) and 5′-TGGTAACTTTCGCTTGGTAGGC-3′ (reverse); for *AtNHX8* expression, a 321 bp cDNA fragment was used as the probe and amplified by PCR using the primers, 5′-TCTGACTTGAAGAAGCTCCTGAG-3′ (forward) and 5′-TCAAAGCCAAAAAGGATTGATTGAA-3′ (reverse). The membranes were washed with 2 × SSC, 0.5% SDS; 1 × SSC, 0.5% SDS; 0.5 × SSC, 0.5% SDS and 0.1 × SSC, 0.1% SDS for 15 min at 65°C, respectively.

The hybridization signals for both Southern- and Northern-blotting were imaged using a phosphorimager. The ethidium bromide-stained rRNA bands in the agarose gel were shown as a RNA loading control.

Measurement of ion contents

Twelve-day-old *Arabidopsis* seedlings growing on vertical MS agar plates (1.0%) were transferred onto MS, MS supplemented with 125 mM NaCl or 12 mM LiCl agar plates and placed vertically. After growing for 6 d, the seedlings were collected and washed five times with ddH_2O, and then dried at 80°C for 3 days. Approximately 4 mg (dry weight) of the samples were dry-ashed for 6 h in quartz dishes at 570°C in a muffle furnace. The samples were digested in 2 ml 1:1 diluted concentrated HCl with ddH_2O and then diluted to a total volume of 20 ml with ddH_2O prior to ion measurement. The K^+, Na^+, and Li^+ contents in the solutions were determined with an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer, Boston, MA, USA). All the experiments were repeated three times and the SD was showed as the error bar.

Results

Isolation of *ZmCBL4* gene and comparison of its amino acid sequence

Database searches together with bioinformatics analysis led to the identification of nine putative maize CBL unique

mRNA sequences, and one of them (BT018770), which encodes a predicted protein with the highest sequence similarity (57% of identities) to *Arabidopsis* CBL4/SOS3, was designated as *ZmCBL4* and chosen for further study.

ZmCBL4 has an open reading frame (ORF) of 633 bp and encodes a predicted polypeptide of 211 amino acids. The cDNA and genomic DNA fragments with the complete coding region of *ZmCBL4* were cloned by PCR approach and sequenced. The cDNA ORF sequences from three independent positive clones were identical. This sequence was compared with the original sequence in the public databases (BT018770) and four nucleotide differences were found between them, which resulted in the changes of three amino acid residues (data not shown). Alignment with several representatives of *Arabidopsis* and rice CBLs exhibited that the predicted ZmCBL4 contains all of the four elongation factor (EF) hand motifs found in other CBL proteins (Fig. 1A). ZmCBL4 protein also carries the consensus myristoylation sequence MGXXXS/T at its amino terminus. The clustering pattern of ZmCBL4 with 10 AtCBLs showed that it is more similar to AtCBL4 than to others (Fig. 1B).

Genomic organization of ZmCBL4

Sequencing results demonstrated that the genomic DNA of *ZmCBL4* is 1994 bp (accession number: EF405963). Comparison of the genomic sequence with its corresponding cDNA sequence revealed that the genomic copy of *ZmCBL4* harbors seven introns in its coding region (Fig. 2A), which is similar to the case in most *AtCBLs* and *OsCBLs*. Southern blot analysis showed that *ZmCBL4* has only one copy in maize genome (Fig. 2B).

Expression of ZmCBL4 is regulated by abiotic stresses and ABA

To gain insight into the possible function of *ZmCBL4*, we initially examined the expression patterns of this gene. However, because the transcript level of *ZmCBL4* was too low to be detected by Northern blot analysis (data not shown), the real-time PCR approach was used to detect its expression. Under NaCl stress, the *ZmCBL4* expression was down-regulated in shoots (Fig. 3A). At 2 h after treatment, the transcript level of *ZmCBL4* was decreased to 0.33 ± 0.20 times that at the initial time point (time 0 h), and it reached the lowest level at 12 h after treatment. However, the *ZmCBL4* expression was up-regulated in roots under NaCl stress (Fig. 3B). At 12 h after treatment, the *ZmCBL4* mRNA level increased to 4.01 ± 0.56 times that at the initial time point, and it reached more than 11 times at 24 h after treatment. Under LiCl stress, the expression pattern of *ZmCBL4* in roots was similar to that of NaCl stress, whereas the expression in shoots increased during the first 2 h and then decreased to the basal level (Fig. 3A, B). ABA treatment (100 μM) caused a more rapid and more obvious up-regulation of *ZmCBL4* expression as compared with the induction by NaCl and LiCl. This change was stronger in roots than that in shoots and showed a wavelike pattern during the whole treatment period (Fig. 3A, B). In addition, a slight kinetic difference was observed between roots and shoots in response to ABA. By contrast, PEG treatment (20%) resulted in a rapid and strong down-regulation of *ZmCBL4* both in shoots and roots (Fig. 3A, B).

We also examined the expression of *ZmCBL4* in shoots and roots of young seedlings and in various organs of adult maize plants. As shown in Fig. 3C, the expression level was most abundant in ears but was very low in mature leaves, stalks and tassels. When the transcript level in mature leaves was used as the calibrator (its *ZmCBL4* mRNA level was given as 1), the relative fold changes in other organs were between 1.05 ± 0.13 and 10.98 ± 0.92. Interestingly, the transcript level in shoots was noticeably higher than that in roots at the seedling stage while the transcript level in adult roots was almost three times that in adult leaves. We performed three independent assays with RNA isolated from different samples of shoots/leaves and roots and obtained similar results each time.

Taken together, the expression patterns of *ZmCBL4* suggest its specific or differential regulatory roles in stress responses and developmental signaling processes in maize.

Constitutive expression of ZmCBL4 complements the salt hypersensitivity of *Arabidopsis sos3* mutant

As mentioned above, among the nine putative maize CBL proteins, CBL4 had the highest sequence similarity to *Arabidopsis* SOS3 protein. The salt-inducible expression also suggested a possible role of *ZmCBL4* in salt stress signaling pathway. These findings led us to hypothesize that *ZmCBL4* may be a homolog of *AtSOS3* and may function underlying the mechanism similar to SOS3 in the salt responsiveness. To test this hypothesis, we first constructed transgenic lines of 35S::*ZmCBL4* in the *Arabidopsis sos3* mutant background. Thirty independent transgenic homozygous T_3 lines were obtained and six of them were selected to examine the expression of *ZmCBL4* by Northern blot analysis. As shown in Fig. 4A, *ZmCBL4* was strongly expressed in all six transgenic lines and all of them were selected for phenotypic characterization.

A preliminary assay on the salt tolerance of the six transgenic lines showed that these transgenic lines had

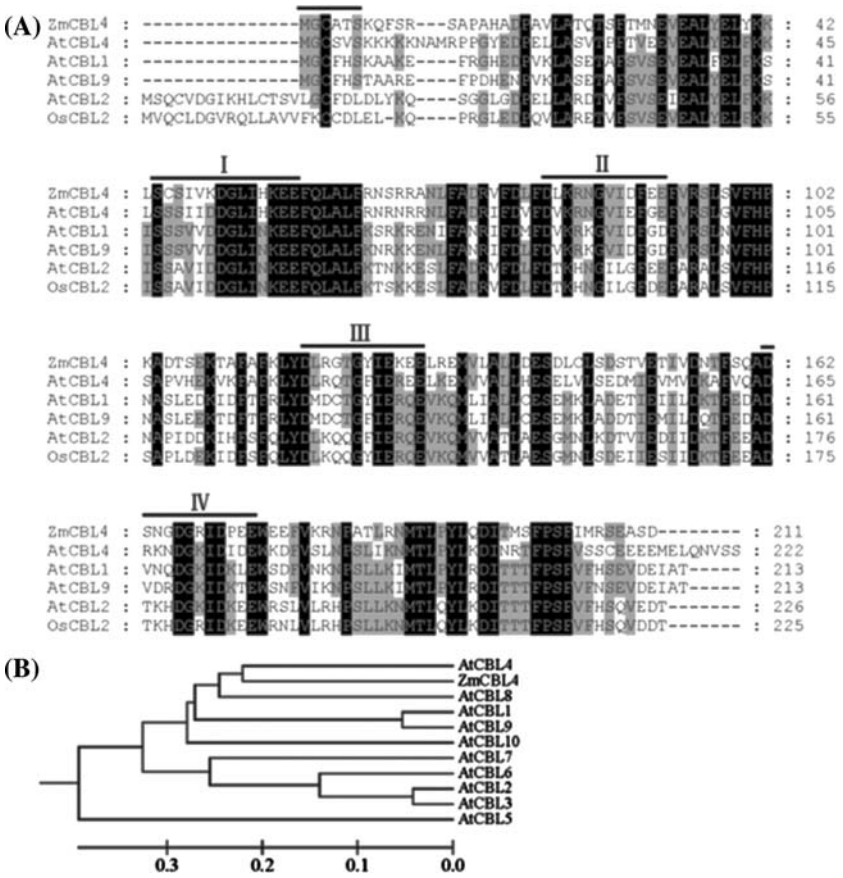

Fig. 1 Sequence analyses of ZmCBL4. (**A**) Alignment of the deduced amino acid sequences of maize CBL4, *Arabidopsis* CBL1, CBL2, CBL4/SOS3, CBL9, and rice OsCBL2. Hyphens indicate gaps introduced to maximize the sequence alignment. Identical residues are highlighted in black, and similar residues are highlighted in gray (60% similarity), respectively. The location of the myristoylation sequence at N- terminus was lined above. The four predicted EF hands are showed as I–IV. (**B**) Phylogenetic relationship of ZmCBL4 with 10 *Arabidopsis* CBLs. Multiple sequence alignment was performed using the program ClustalX1.83, and the phylogenetic tree was constructed using the Neighbor-Joining method. The accession numbers of *Arabidopsis* and rice CBLs are as follows: AtCBL1, AAC26008; AtCBL2, AAC26009; AtCBL3, AAC26010; AtCBL4/SOS3, AAG28402; AtCBL5, AAG28401; AtCBL6, AAG28400; AtCBL7, AAG10059; AtCBL8, AAL10300; AtCBL9, AAL10301; AtCBL10, AAO72364; the rice OsCBL2, AL713904

similar salt tolerance. Therefore, the detailed analyses were later performed using three independent transgenic lines, SS1, 13 and 23. On MS agar plates supplemented with various concentrations of NaCl (100–175 mM), the root-bending phenotypes of the transgenic lines were similar to those of wild type seedlings, whereas the *sos3* seedlings exhibited no significant root growth. Under different concentrations of LiCl (10–20 mM), the transgenic seedlings displayed a better growth both in roots and shoots than the wild type seedlings, whereas the root growth of the *sos3* seedlings was inhibited completely (Fig. 4C, D).

We also examined the salt tolerance of these materials at seed germination and post-germination stages. On the MS agar plates supplemented with different concentrations of NaCl (125–175 mM), the germination rates and subsequent growth of the transgenic lines were similar to the wild type (Fig. 4F, H, J). Under LiCl stress (12–20 mM), however, all of the transgenic lines showed much higher germination rates and significantly better subsequent growth than the wild type (Fig. 4 G, I, K). For example, at 15 mM LiCl, the average germination rate of the transgenic lines was 69.81 ± 4.36% after 5 d of incubation at 22°C, whereas the germination rate of the wild type was only 13.66 ± 2.51% on the same conditions. After 10 days under the same LiCl stress, the average fresh weight of the transgenic lines was 45.12 ± 2.58 mg per 30 seedlings, which was significantly higher than that of the wild type seedlings (14.63 ± 1.76 mg per 30 seedlings). The *sos3* mutant displayed hypersensitivity to both NaCl and LiCl stresses, just a few of the seeds developed green cotyledons when the concentration of NaCl or LiCl was increased to 150 mM or 15 mM, respectively (Fig. 4F–K).

The evidences presented thus far clearly indicate that the expression of *ZmCBL4* completely complemented the salt hypersensitive phenotype of the *sos3* mutant and that ZmCBL4 is a functional homolog of AtSOS3. No

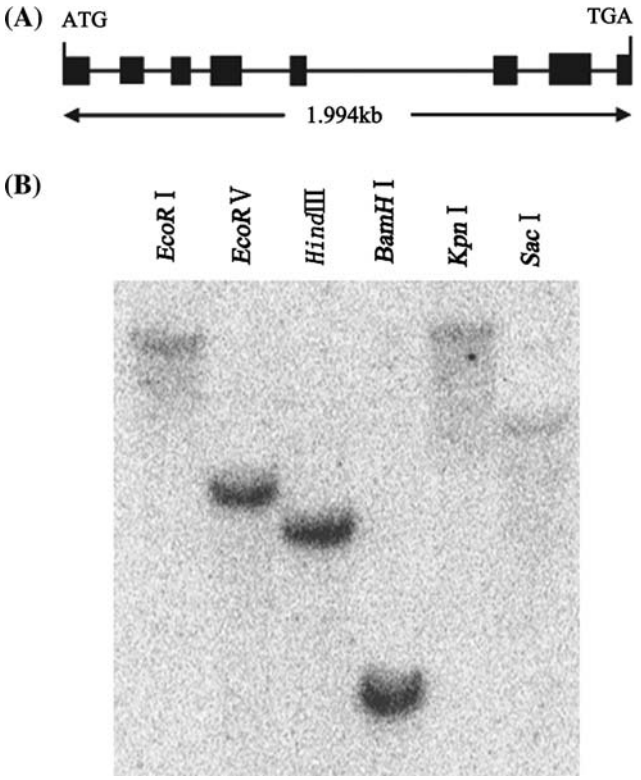

Fig. 2 Gene structure and genomic organization of *ZmCBL4*. (**A**) Gene structure of *ZmCBL4*. Filled boxes indicate exons, and lines between boxes indicate introns. (**B**) Southern blot analysis of *ZmCBL4* to determine the copy number in the maize genome. Genomic DNA (30 μg) was completely digested with the enzyme indicated

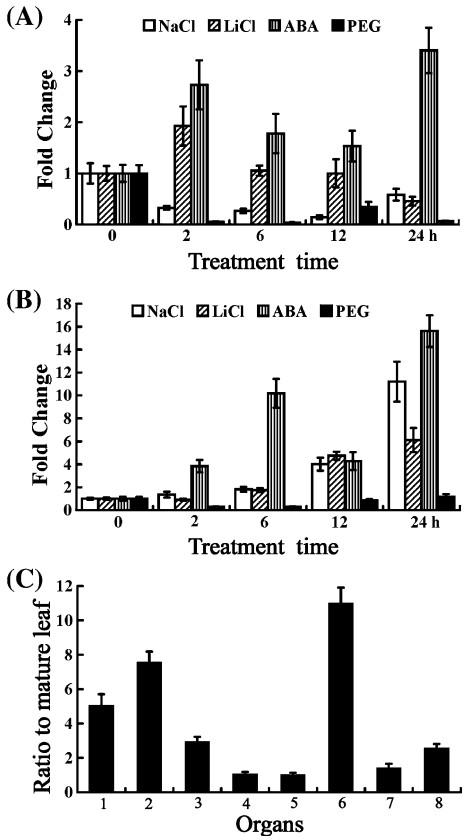

Fig. 3 Expression of *ZmCBL4* in maize. The maize α-tubulin gene was used as the internal control for normalization of the template cDNA. Each PCR was repeated at least four times and the error bars represent the SD. (**A**) and (**B**) Expression of *ZmCBL4* in shoots and roots, respectively, under different treatments. The transcript level at time 0 h (untreated control) was used as the calibrator whose *ZmCBL4* mRNA level was given as 1. (**C**) Expression of *ZmCBL4* in various organs. 1, young roots; 2, shoots; 3, mature roots; 4, stalks; 5, mature leaves; 6, ears; 7, tassels; 8, silks. The transcript level in mature leaves was used as the calibrator and its *ZmCBL4* mRNA level was given as 1

significant difference was observed between the transgenic lines and the *sos3* mutant or wild type on MS medium (Fig. 4B, E, H–K) or MS media containing different concentrations of KCl, mannitol or ABA (data not shown).

Constitutive expression of ZmCBL4 enhances the salt tolerance of wild type Arabidopsis

To further investigate the function of *ZmCBL4*, we also constructed transgenic lines of 35S::*ZmCBL4* in the *Arabidopsis* wild-type background and obtained 20 independent homozygous T_3 lines. To confirm the expression of *ZmCBL4*, Northern blotting was performed on six of them. As shown in Fig. 5A, all of the transgenic lines tested produced the *ZmCBL4* transcript and the four lines showing higher expression levels, namely SW16, 17, 18 and 19, were selected for further study.

The seed germination and subsequent growth of the transgenic lines and the control wild type were compared under different conditions. No significant differences were observed in all the materials examined on MS medium or MS media supplemented with different concentrations of KCl, mannitol, or glucose (data not shown). However, under various concentrations of NaCl or LiCl, the transgenic lines showed significantly higher germination rates and better seedling growth than the wild type (Fig. 5B–G). Figure 5B and C illustrated the germination and subsequent seedling growth of the transgenic lines and wild type on MS medium supplemented with 150 mM NaCl or 17 mM LiCl, respectively. Figure 5D and E presented the results of more detailed analyses of germination rates on MS media supplemented with different concentrations of NaCl and LiCl, separately, after 5 days of incubation at 22°C, and the significant differences between the transgenic lines and wild type existed. Figure 5F and G further showed the fresh weights of the transgenic and control wild-type seedlings on these stress media after incubating for 10 d at 22°C, and the differences between them were

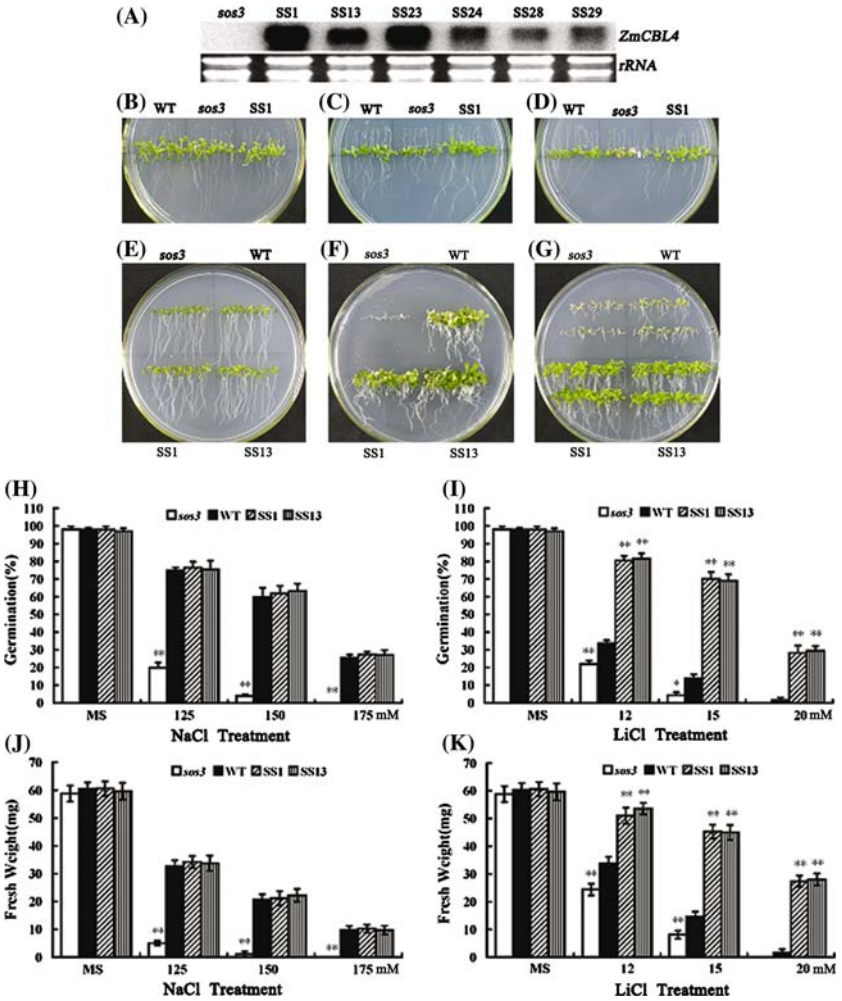

Fig. 4 Constitutive expression of *ZmCBL4* complemented the NaCl and LiCl hypersensitive phenotypes of the *Arabidopsis sos3* mutant. *sos3*, *Arabidopsis sos3* mutant; WT, wild-type; SS1, 13, 23, 24, 28 and 29, six independent transgenic lines, respectively. (**A**) Expression of *ZmCBL4* in *Arabidopsis sos3* mutant background. Twenty micrograms of total RNA from each material was loaded. The ethidium biomide-stained rRNA bands in the agarose gel were shown as a RNA loading control. (**B**), (**C**) and (**D**) The results of root-bending assays: 5-day-old seedlings were transferred from MS medium to the MS agar plates (**B**), MS agar plates supplemented with 100 mM NaCl (**C**) or 12 mM LiCl (**D**), and then the plates were placed vertically on a rack with roots upside down. The photographs were taken at 12 d after transferring to 22°C. (**E**), (**F**) and (**G**) Seed germination and subsequent growth on MS agar plates (**E**), MS agar plates supplemented with 125 mM NaCl (**F**) or 15 mM LiCl (**G**), and the photographs were taken at 8, 12 and 15 d, respectively, after transferring to 22°C. (**H**) and (**I**) Seed germination percentages on MS agar plates supplemented with different concentrations of NaCl and LiCl, respectively. The seeds (each with at least 60 seeds) that developed green cotyledons were scored at 5 d after transferring to 22°C. (**J**) and (**K**) Fresh weight (mg/30 seedlings) of seedlings on vertical MS agar plates supplemented with different concentrations of NaCl and LiCl, respectively. All the values are means±SD (n = 3), and SD was shown as error bar. * and ** represent significantly different from the control WT at $P < 0.05$ and 0.01 level, respectively, by student's *t*-test

also significant. All of the results indicate that constitutive expression of *ZmCBL4* in *Arabidopsis* enhanced their NaCl and LiCl tolerance during the early developmental stages. Moreover, it is necessary to mention that the salt-tolerant properties of the transgenic lines remained for at least 1 month, when the transgenic plants were still green and quite strong but the control wild-type plants became weak and yellow (under 150–175 mM NaCl stress) or died (under 15–20 mM LiCl stress) (data not shown). Later, the media became dried.

Constitutive expression of ZmCBL4 induces the transcription of Arabidopsis AtSOS1 and AtNHX8

Previous study has shown that expression of *AtSOS1* is inducible in the wild-type seedling roots by NaCl stress, but in *sos3* mutant, no up-regulation of *SOS1* expression was found in either shoots or roots under NaCl stress (Shi et al. 2000). To investigate whether the *ZmCBL4* expression really rescued the *AtSOS1* expression in the transgenic *sos3* lines, we examined the *AtSOS1* mRNA level in the

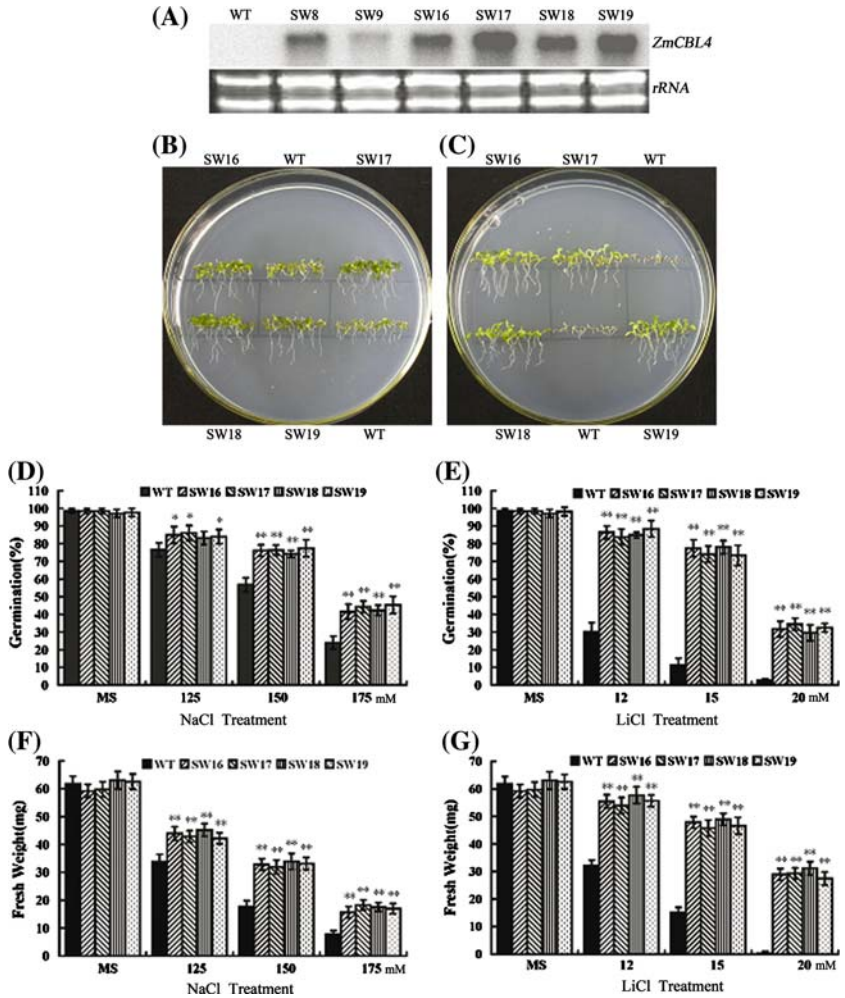

Fig. 5 Constitutive expression of *ZmCBL4* in *Arabidopsis* wild type enhanced the salt tolerance. WT, wild type; SW8, 9, 16, 17, 18, and 19, six independent transgenic lines, respectively. (**A**) The expression of *ZmCBL4* in *Arabidopsis* wild type background. Twenty micrograms of the total RNA from each material was loaded. The ethidium biomide-stained rRNA bands in agarose gel were shown as a RNA loading control. (**B**) and (**C**) Seed germination and subsequent growth of each material on MS agar plates supplemented with 150 mM NaCl or 17 mM LiCl, respectively. The photographs were taken at 10 d after transferring to 22°C. (**D**) and (**E**) Seed germination percentage of each material on MS agar plates supplemented with different concentrations of NaCl and LiCl, respectively. The seeds that developed green cotyledons were scored at 5 d after transferring to 22°C. (**F**) and (**G**) Seedling fresh weight (mg/30 seedlings) growing on vertical MS agar plates supplemented with different concentrations of NaCl and LiCl, respectively. All the values are means ± SD (n = 3), and SD was shown as error bar. * and ** are significantly different from the control WT at $P < 0.05$, 0.01, respectively, by student's *t*-test

ZmCBL4-expressing *sos3* seedlings by Northern blot analysis. As shown in Fig. 6A, the transcripts of *AtSOS1* were detected in the transgenic lines SS1 and SS23, but no signal was seen in the control *sos3* mutant, wild type and the transgenic line SS13 under NaCl stress. In normal growth condition, the *AtSOS1* mRNA was not detectable in all the materials tested (data not shown). This result indicates that *ZmCBL4* at least partially rescued the transcription of *AtSOS1* in the transgenic *sos3* lines.

During phenotype characterization of the transgenic lines, we noticed that the LiCl tolerance in all of the transgenic lines including SS1-23 and SW16-19 increased more significantly as compared with the NaCl tolerance. Moreover, Northern blot analysis showed that the expression level of *AtSOS1* in the transgenic seedlings was very low. This contradiction might imply that new monovalent cation:proton antiporter(s) other than SOS1 be activated by ZmCBL4 and responsible for LiCl tolerance. Recent study has shown that *Arabidopsis AtNHX8*, a highly homologous gene of *AtSOS1*, encodes a putative plasma membrane Li^+/H^+ antiporter and functions specifically in lithium extrusion and ion homeostasis in *Arabidopsis* (An et al. 2007). To the best of our knowledge, this is the only specific Li^+/H^+ antiporter identified so far. Therefore, the *AtNHX8* was selected as the candidate and Northern blotting was performed for its expression. Under normal

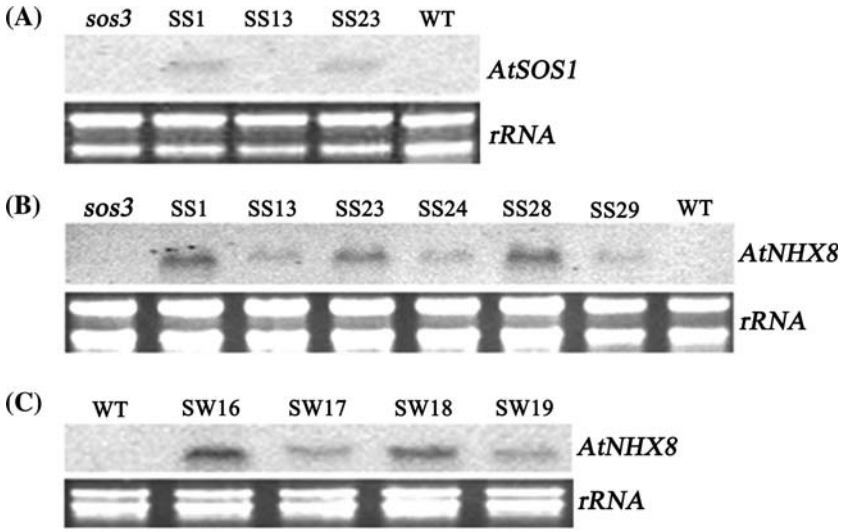

Fig. 6 Constitutive expression of *ZmCBL4* in *Arabidopsis* induced the transcription of *AtSOS1* and *AtNHX8*. (**A**) *AtSOS1* expression in the *ZmCBL4*-expressing transgenic *sos3* mutant seedlings under NaCl stress (250 mM NaCl for 5 h). (**B**) and (**C**) *AtNHX8* expression in the *ZmCBL4*-expressing *sos3* mutant and wild type seedlings, respectively, under LiCl stress (20 mM LiCl for 5 hours). Forty (for *AtSOS1*) or twenty (for *AtNHX8*) micrograms of total RNA from each material was loaded. The ethidium biomide-stained rRNA band in the agarose gel was shown as a RNA loading control

growth condition, the *AtNHX8* transcript was not detectable in any of the seedlings examined including wild type, the *sos3* mutant, and the transgenic lines (data not shown). Under LiCl stress, however, all the transgenic lines produced moderate or low levels of *AtNHX8* mRNA, whereas no signal was detected in the *sos3* and wild type seedlings (Fig. 6B, C). This finding indicates that constitutive expression of *ZmCBL4* induced the transcription of *AtNHX8*, which might account for the enhanced LiCl tolerance of *ZmCBL4*-transgenic lines.

The transgenic seedlings accumulate less Na^+ or Li^+ under NaCl or LiCl stress

To investigate whether constitutive expression of *ZmCBL4* altered the accumulation of Na^+, Li^+, and K^+, we measured the contents of these cations in control and transgenic seedlings under normal growth condition, NaCl and LiCl stresses. As shown in Fig. 7A, when the seedlings of the controls and *ZmCBL4*-expressing *sos3* lines were grown on MS medium supplemented with 125 mM NaCl or 12 mM LiCl, the K^+ contents in all of the seedlings were reduced. Further, the reductions were less strong in the transgenic and wild type seedlings as compared with the *sos3* mutant seedlings. The Na^+ contents were similar in all of the seedlings grown on MS medium or MS medium containing 12 mM LiCl. Under NaCl stress, however, the transgenic and wild type seedlings accumulated significantly less Na^+ than did the *sos3* mutant seedlings (Fig. 7B). No Li^+ was detected in the seedlings grown on MS medium or MS medium supplemented with 125 mM NaCl. After 6 days subjected to 12 mM LiCl stress, however, both the *sos3* mutant and wild type seedlings accumulated significantly more Li^+ than did the transgenic lines (Fig. 7C).

The changes of K^+, Na^+ and Li^+ contents in the transgenic wild type and control wild type seedlings were shown in Fig. 7D–F, respectively. The transgenic lines also accumulated significantly less Na^+ or Li^+ but relatively more K^+ as compared with the control wild type seedlings under NaCl or LiCl stress.

Together, these results indicate that *ZmCBL4* is involved in the Na^+, Li^+ and K^+ homeostasis in the transgenic *Arabidopsis* lines under the corresponding salt stresses.

Discussion

ZmCBL4 may be involved in different calcium signaling pathways

Calcium plays a crucial role as a second messenger in various environmental and developmental signaling pathways in plant cells. The calcium sensors are immediate downstream components after calcium changes and function as a threshold factor in linking the calcium signature to the downstream components in the pathways (Cheong et al. 2003; Reddy and Reddy 2004). CBLs are newly identified calcium sensors unique to plants. Recent studies have demonstrated that most CBL genes are differentially involved in various stress and/or developmental signaling processes (Kudla et al. 1999; Albrecht et al. 2003; Cheong et al. 2003; Pandey et al. 2004; Hwang et al. 2005; Mahajan et al. 2006). We show here that *ZmCBL4* expressed differentially in various maize organs tested under normal growth conditions, and that the expression level of *ZmCBL4* changed when the seedlings were subjected to different abiotic stresses and ABA treatment. Unlike many other salt tolerance genes that are preferentially expressed in roots but not in aerial tissues, the

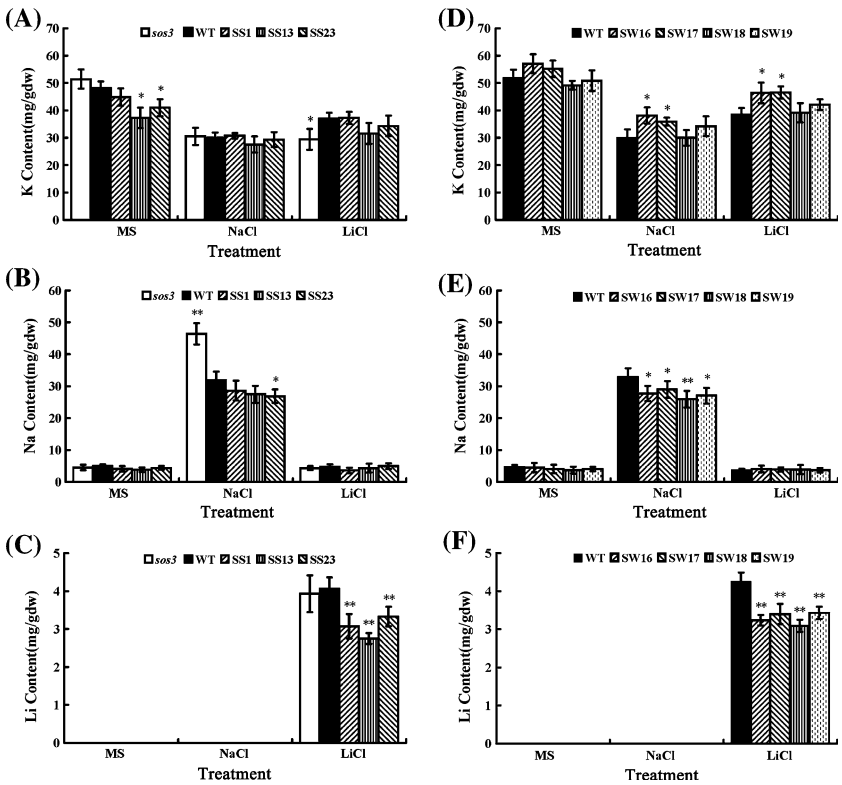

Fig. 7 *ZmCBL4*-expressing *Arabidopsis* seedlings accumulated less Na^+ or Li^+ than did the control *Arabidopsis* seedlings under salt stress. (**A**), (**B**) and (**C**) K^+, Na^+ and Li^+ contents of the *ZmCBL4*-expressing *sos3* mutant seedlings on MS agar plates, MS agar plates supplemented with 125 mM NaCl or 12 mM LiCl, respectively. (**D**), (**E**) and (**F**) K^+, Na^+ and Li^+ contents of the *ZmCBL4*-expressing wild type seedlings on MS agar plates, MS agar plates supplemented with 125 mM NaCl or 12 mM LiCl, respectively. Values are means ± SD ($n = 3$), and SD was shown as error bar. * and ** are significantly different from the control WT at $P < 0.05$, 0.01, respectively, by student's *t*-test

ZmCBL4 transcript levels in maize shoots and ears were obviously higher than those in roots under normal growth condition (Fig. 3C). This tissue-specific expression pattern suggests its other function in addition to that for response to salt stress. Salt stress is known to elicit a rapid increase in the free calcium concentration in the cytosol (Lynch et al. 1989; Knight et al. 1997; Knight 2000; Pauly et al. 2000). The maize CBL4, a putative homolog of *Arabidopsis* SOS3 calcium sensor, is proposed to sense this salt-triggered calcium signal and relay it downstream. The up-regulation of *ZmCBL4* expression in roots under NaCl or LiCl stress seems consistent with its function in salt response (Fig. 3B). However, the significant induction appeared 12 h after exposure to NaCl or LiCl stress if a 2-fold induction criterion was used. This is actually too slow to account for any significant contribution toward the rapid transduction of the stress signal. There exists a low level of *ZmCBL4* expression in maize roots under normal growth condition (Fig. 3C), and perhaps this basal mRNA and its corresponding protein function during the early exposure to salt stress, while for a sustained response, the induced transcript and its protein may be required and thus play a major role.

Unlike many other stress-regulated genes that are induced not only by various stresses but also by ABA, the expression of *AtSOS1*, an ultimate component in the SOS pathway, was not inducible by exogenous ABA (Shi et al. 2000). In addition, the *Arabidopsis sos* mutants, including the *sos1, 2* and *3* mutants, did not show altered response to ABA treatment (Liu and Zhu 1998; Shi et al. 2000; Liu et al. 2000). This phenotype again suggests that the *Arabidopsis* SOS pathway is not regulated by ABA, or not involved in ABA-mediated signaling processes. However, our data indicate that *ZmCBL4* expression is up-regulated rapidly and obviously by ABA treatment (Fig. 3A, B), which suggests its involvement in ABA-mediated signaling pathway. Some other CBL genes, for example, the *Arabidopsis AtCBL9*, are also implicated in the plant response to ABA (Pandey et al. 2004).

By contrast to ABA, PEG treatment negatively regulated the expression of *ZmCBL4*. Although the meaning of this down-regulation is unknown, it is clear that *ZmCBL4* is responsive to osmotic stress and may further influence the related signaling processes in maize.

Taken together, the expression patterns of *ZmCBL4* in various organs or under stresses suggest its involvement in the developmental and stress signaling pathways in maize. Like *AtSOS3* (Liu et al. 2000), the expression level of *ZmCBL4* is also low, which is consistent with its regulatory role.

A calcium sensor for ion homeostasis and salt tolerance

The *Arabidopsis* SOS pathway is the first established calcium-dependent salt stress signaling pathway in plant cells. In the SOS pathway, the SOS3/CBL4 protein is

known to perceive and relay the calcium signal elicited by salt stress to its target SOS2, which then activates SOS1 (Halfter et al. 2000; Quintero et al. 2002). SOS1 is a plasma membrane Na^+/H^+ antiporter and directly functions in removing excess Na^+ from the cell (Qiu et al. 2002). In line with this module, the *SOS1* gene expression and SOS1 transporting activity are partially controlled by SOS3 and SOS2, and in *sos3* mutant plants, both the *SOS1* transcription and the SOS1 activity cannot be induced or activated by salt stress (Shi et al. 2000; Qiu et al. 2002). Very recently, the SOS pathway was identified from rice and was demonstrated a high degree of functional similarity to its *Arabidopsis* counterpart (Martínez-Atienza et al. 2007). In the present study, the sequence similarity and the salt-induced expression pattern made *ZmCBL4* a logical homolog of *AtSOS3* and may function in salt stress. To test this hypothesis, we performed transgenic assays both in *Arabidopsis sos3* mutant and wild type backgrounds. The root-bending assay, the seed germination and seedling growth experiments showed that constitutive expression of *ZmCBL4* not only suppressed completely the NaCl hypersensitivity but also enhanced the LiCl tolerance in the transgenic *sos3* lines as compared with the wild type *Arabidopsis*. We further provide evidence for *ZmCBL4* rescuing the *AtSOS1* transcription in the *sos3* transgenic lines by Northern blot analysis, although the signals were weak (Fig. 6A). Shi et al (2000) reported that the *AtSOS1* gene expression was salt-induced in the roots of wild-type *Arabidopsis* seedlings but not in the shoots. Unexpectedly, no *AtSOS1* mRNA was detected in the NaCl treated-wild type seedlings in our Northern blotting assay. The failure, together with the weak Northern blotting signals of *AtSOS1* detected in the transgenic lines (Fig. 6A), may result from the NaCl treatment and subsequent RNA loading. Perhaps the treatment duration (5 h) with 250 mM NaCl is not long enough and the total RNA loading of 40 μg is still not sufficient for successful detection of *SOS1* transcripts by Northern blotting analysis. The whole-seedling RNA but not the root RNA used in our assay may be another possible reason. Constitutive expression of *ZmCBL4* in the wild type background also enhanced the NaCl and especially LiCl tolerance during the seed germination and seedling growth. These data clearly demonstrate that *ZmCBL4* is a functional homolog of *AtSOS3* and it can increase the salt tolerance of the transgenic wild-type lines.

Maintaining low level of Na^+ and a high K^+/Na^+ ratio in the cytosol is critical for cellular metabolism and salt tolerance in glycophytes (Zhu et al. 1998; Qiu et al. 2002; Zhu 2003). The AtSOS3 protein is required for maintaining sodium and potassium ion homeostasis and salt tolerance in *Arabidopsis* (Liu and Zhu 1998; Halfter et al. 2000; Qiu et al. 2002). Our data also indicate that constitutive expression of *ZmCBL4* enable the transgenic *Arabidopsis* seedlings to maintain low levels of Na^+ and Li^+ but relatively high levels of K^+ under salt stress, which provides additional evidence to certify that the *ZmCBL4* is a functional homolog of *AtSOS3*. A yeast two-hybrid assay indicated that some rice CBLs could interact with CIPKs from *Arabidopsis* (Hwang et al. 2005). Perhaps the maize CBL4 protein heterogenously expressed in *Arabidopsis* can sense the calcium signal triggered by salt stress and interact with *Arabidopsis* SOS2, and then the ZmCBL1-AtSOS2 complex activates or enhances the activity of SOS1 in the *sos3* mutant or wild type seedlings.

Overexpression of ZmCBL4 induces the transcription of Arabidopsis AtNHX8

In plants, the Na^+/H^+ antiporters play an important role in transporting monovalent cations including Na^+, K^+, and Li^+. There are more than 40 genes that encode putative Na^+/H^+ antiporters in *Arabidopsis* genome (Maser et al. 2001; Brett et al. 2005). These antiporters fall into three families, and one of them is the monovalent cation:proton antiporter-1 (CPA1) family, which includes eight members from AtNHX1 to AtNHX8 (Maser et al. 2001). AtNHX1 and AtNHX7 (identical to SOS1) have been well characterized. AtNHX1 is localized to and contributes a major portion of the Na^+/H^+- exchange activity in tonoplast, where it mainly sequesters Na^+ and K^+ and to a much lesser extent Li^+ (Apse et al. 2003; Qiu et al. 2004). Recent evidence indicates that the *Arabidopsis* tonoplast Na^+/H^+ exchanger is also a target for the SOS pathway. However, in this tonoplast SOS pathway, the Na^+/H^+ exchanger activity is regulated by SOS2 while not by SOS3. An unknown component regulates SOS2 (Qiu et al. 2003). AtNHX7/SOS1 is the first identified plasma membrane target for the *Arabidopsis* SOS pathway. SOS1 enables Na^+ to efflux across the plasma membrane and controls long-distance Na^+ transport from root to shoot (Shi et al. 2002a). Loss-of-function mutation in SOS1 renders the plants hypersensitive both to NaCl and LiCl stresses, although the transport experiment showed that SOS1 was specific for Na^+ and could not transport Li^+ or K^+ (Zhu 2003 and the references therein). AtNHX8 is a highly similar homolog of AtNHX7/SOS1 and it is predicted to be a plasma membrane Na^+/H^+ antiporter with 756 amino acid residues, which exhibits ~72% identity with the SOS1 sequence over a stretch of 760 amino acids (Maser et al. 2001; Ward 2001; Brett et al. 2005). Recent functional characterization demonstrated that AtNHX8 specifically functions in lithium but not sodium extrusion and in ion homeostasis in *Arabidopsis* (An et al. 2007). To the best of our knowledge, this is the only specific Li^+/H^+ antiporter identified so

far. In the present study, overexpression of *ZmCBL4* strongly increased the LiCl tolerance both in the *Arabidopsis sos3* mutant (Fig. 4G, I, K) and the wild type backgrounds (Fig. 5C, E, G). In line with this phenotype, Northern blot analysis demonstrated that the *AtNHX8* transcript obviously accumulated in all of the transgenic lines under LiCl stress, but was not detectable in the control *sos3* mutant and wild type seedlings in the same condition (Fig. 6B, C). Although the *AtNHX8* gene expression does not necessarily mean its corresponding product to be responsible for the enhanced LiCl tolerance, it is clear that the *AtNHX8* expression is induced by *ZmCBL4* at the transcription level in a LiCl stress-induced manner. Moreover, from the seed germination and seedling growth (Fig. 4F–K) and ion contents (Fig. 7B, C), it is not difficult to find that the difference in NaCl tolerance is more obvious than that in LiCl tolerance between the *Arabidopsis sos3* mutant and wild type under our experimental conditions. These phenomena probably indicate that the SOS pathway or the SOS1 Na^+/H^+ exchanger regulated by SOS3 may function mainly in extruding Na^+ but not Li^+ in *Arabidopsis*. Our findings, together with other evidence mentioned above, make it conceivable that the AtNHX8 Li^+/H^+ antiporter may play a major role in removing Li^+ out of the cell in the *ZmCBL4*-expressing *Arabidopsis* lines. Perhaps in these transgenic lines, the maize CBL4 protein cannot only induce the *AtNHX8* gene expression, but also may activate the corresponding AtNHX8 transporting activity through interacting with and then activating some *Arabidopsis* CIPKs including SOS2 in a LiCl stress-dependent manner. Of course, this suggestion will need to be proved with more experimental data.

Taken together, our results demonstrate that ZmCBL4 is a functional homolog of AtSOS3, and that its function for improving the tolerance of transgenic plants to NaCl and LiCl is related to the positive regulation of *AtSOS1* and *AtNHX8* expression. The facts that overexpression of *ZmCBL4* enhances NaCl and LiCl tolerance in wild type *Arabidopsis*, which is important for the improvement of crop salt tolerance by genetic manipulation, and that *AtNHX8* is involved in the response of the *ZmCBL4*-expressing lines to LiCl stress represent the novel properties of *ZmCBL4* because the similar functions have not been reported for either *AtSOS3* or *OsCBL4* (Guo et al. 2004; Martínez-Atienza et al. 2007).

Whether *ZmCBL4* functions on salt tolerance and/or other physiological aspects in maize itself awaits further experimentation. At present, the transformation of *ZmCBL4* into maize is on the way in our laboratory.

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