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Molecules and Cells

Constitutive Overexpression of the Calcium Sensor *CBL5* Confers Osmotic or Drought Stress Tolerance in *Arabidopsis*

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Calcium serves as a critical messenger in many adaptation and developmental processes. Cellular calcium signals are detected and transmitted by sensor molecules such as calcium-binding proteins. In plants, the calcineurin B-like protein (CBL) family represents a unique group of calcium sensors and plays a key role in decoding calcium transients by specifically interacting with and regulating a family of CBL-interacting protein kinases (CIPKs). In this study, we report the role of *Arabidopsis* CBL5 gene in high salt or drought tolerance. *CBL5* gene is expressed significantly in green tissues, but not in roots. *CBL5* was not induced by abiotic stress conditions such as high salt, drought or low temperature. To determine whether the *CBL5* gene plays a role in stress response pathways, we ectopically expressed the CBL5 protein in transgenic *Arabidopsis* plants (35S-CBL5) and examined plant responses to abiotic stresses. *CBL5*-overexpressing plants displayed enhanced tolerance to high salt or drought stress. *CBL5* overexpression also rendered plants more resistant to high salt or hyperosmotic stress during early development (i.e., seed germination) but did not alter their response to abiscisic acid (ABA). Furthermore, overexpression of *CBL5* alters the gene expression of stress gene markers, such as *RD29A*, *RD29B* and *Kin1* etc. These results suggest that *CBL5* may function as a positive regulator of salt or drought responses in plants.

INTRODUCTION

Plant have exhibited regulatory mechanisms against critical environmental abiotic stresses such as high salt, drought, cold and low K^+ by perceiving, transducing and responding to signals at the molecular, cellular and physiological levels. The Ca^{2+}-dependent pathway plays a pivotal role in the regulation of almost all biological processes including response to abiotic stresses in plants (Batistic and Kudla, 2009; Knight, 2000; Lee et al., 2008; Luan, 2008; Sanders et al., 2002; Xiong et al., 2002). It has been reported that calcium sensor proteins in plants, such as calmodulin (CaM), calcineurin B-like proteins (CBLs), and calcium-dependent protein kinase (CDPK) sense and transmit local changes in the concentration of Ca^{2+} to their target proteins (Luan et al., 2002; Sanders et al., 2002). Among calcium sensor proteins, CBLs that are most similar to calcineurin B and neuronal calcium sensor (NCS) proteins perform critical roles in diverse Ca^{2+}-dependent processes in plants by interacting with a specific group of protein kinases designated as CBL-interacting protein kinases (CIPKs) (Batistic and Kudla, 2004; 2009; Luan, 2008; Luan et al., 2002). In *Arabidopsis*, at least 10 CBLs and 25 CIPKs have been reported, and they form a network-like signaling system for specific and synergistic stimulus–response coupling (Batistic and Kudla, 2004; Luan et al., 2002). Several CBLs and their target CIPKs have been shown to function in the response to several abiotic stresses in plants (Batistic and Kudla, 2009; Luan, 2008; Luan et al., 2009; Xu et al., 2006; Zhu, 2003). The interaction between CIPK24 and CBL4 has been identified by a genetic screen and had found to play a role in salt tolerance in *Arabidopsis* by regulating the downstream component SOS1, a putative Na^+/H^+ antiporter (Chinnusamy et al., 2004; Shi et al., 2000). It has been reported that CBL10 seems to interact with and function through CIPK24 in the salt-tolerance pathway. However, unlike CBL4, which functions mainly in the roots, CBL10 expression and function is observed almost exclusively in the shoots and leaves (Kim et al., 2007; Quan et al., 2007). CBL1 also functions under drought, high salt, and hyperosmotic stresses in plants (Albrecht et al., 2003; Cheong et al., 2003). CBL9 can function differently from CBL1 in some aspects of plant physiology, including response to abscisic acid (ABA) and ABA biosynthesis during seed germination (Pandey et al., 2004). Among the CBL families in *Arabidopsis*, CBL1, CBL4, CBL5, and CBL9 possess a highly conserved N-myristoylation motif at their N-terminus, like CNB and many NCS proteins (Batistic et al., 2008; Ishitani et al., 2000). In the salt overly sensitive (SOS) signaling system, the N-

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myristoylation motif of CBL4 is necessary for salt tolerance (Ishitani et al., 2000). As described above, most N-myristoylation motif containing CBL proteins such as CBL1, CBL4 and CBL9 play unique and critical roles in plant abiotic stress signaling pathways (Batistic and Kudla, 2009; Batistic et al., 2008; Ishitani et al., 2000; Luan, 2008; Luan et al., 2009). However, there is no evidence concerning the function of CBL5 protein in *Arabidopsis*. Thus, we attempted to understand the role of CBL5 by analyzing the expression patterns of stress-responsive genes and the tolerance of transgenic plants to abiotic stresses by using *CBL5*-overexpressing transgenic *Arabidopsis* plants.

MATERIALS AND METHODS

Plant materials and stress treatments

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown in a greenhouse under long-day conditions (16-h-light/8-h-dark cycle) to the flowering stage for plant transformation and RNA analysis as described previously (Cheong et al., 2003; Pandey et al., 2004). For treatment under different stress conditions, 3-week-old seedlings grown on Murashige and Skoog (MS) basal medium were used (Murashige and Skoog, 1962). For NaCl treatment, 300 mM NaCl was added to the plants on MS plates and incubated at room temperature under white light. For drought treatment, 4-week-old plants grown in potting soil were carefully removed and dehydrated on filter paper as described by Yamaguchi-Shinozaki and Shinozaki (1994).

Generation of *CBL5*-overexpressing transgenic plants

To make the *CBL5* overexpression construct (35S-CBL5), the full-length *CBL5* cDNA (At4g01420) was cloned into pBI121 vector with *Xba*I and *Bam*HI sites using *CBL5-Xba*I (ATTCTAGAATGGGATGTGTTTGCAGCAAG) and *CBL5-Bam*HI (ACGGATCCTTACCGGAGAAAGGTTGGGAA) primers. The orientation and the junction of the cloned fragment were confirmed by DNA sequencing. This construct was transferred into *Agrobacterium tumefaciens* GV3101 and used for *Arabidopsis* transformation. Seeds from these plants were harvested and subsequently screened on selection medium (half-strength MS medium, 1× Gamborg's vitamins, and 50 μg/ml kanamycin) for transformants. The putative transformants (T_1) were rescued from plates and grown in a greenhouse under long-day conditions. T_2 plants were raised to produce seeds that were germinated to produce T_3 plants. Those T_2 plants that produced 100% kanamycin-resistant plants in the T_3 generation were considered as homozygous transformants and were used for further experiments.

For plant transformation, *Arabidopsis* plants were grown in a greenhouse under long-day conditions (16-h-light/8-h-dark cycle) for 4 weeks before a floral-dip procedure was performed (Clough and Bent, 1998). Briefly, *Agrobacterium* cells were grown in Luria-Bertani broth for 24 h at 30°C. The cells were collected by centrifugation and resuspended in infiltration medium (half-strength MS medium, 5% sucrose, 1× Gamborg's vitamins, 0.044 μM benzylamino purine, and 0.04% Silwet L77) to an OD_{600} of 1.5 to 2.0. Plants were dipped into this suspension for 30 s and then transferred to a greenhouse.

Germination assay

Approximately 100 seeds each from the wild-type (WT) and 35S-CBL5 lines were planted in triplicate on MS medium with different concentrations of NaCl, or mannitol and incubated at 4°C for 4 d before being placed at 23°C under long-day conditions. Germination (emergence of the radical) was scored daily for 5 days.

Drought and salt tolerance analyses

For the drought-tolerance assays, water was withheld from 4-week-old soil-grown plants for specified times. To minimize experimental variations, the same number of plants in each comparison group was grown on the same tray. Watering was withheld from the plants for 3 weeks before photographs were taken and survival rates were counted. For salt-tolerance assays, 4-week-old, long-day-grown plants that were starting to bolt were treated with 300 mM NaCl once every 3 days for 2 weeks. Photographs were taken 10 days after the first treatment. To analyze of root growth and fresh weight (FW) measurements, 4-day-old seedlings from vertical MS plates were transferred onto MS plates supplemented with salts. Plants of different genotypes were placed on the same plate, and three replicate plates were used for each treatment. Ten seedlings from each genotype were collected on each plate, and their total FW was measured. The number of salt-treated plants expressed as a percentage of the number of plants grown under normal conditions was used to present the data. All experiments were repeated at least three times, and results from one representative experiment are shown in figures. Survival rates and standard errors were calculated from the results of three independent experiments.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

To analyze the gene expression by RT-PCR, total RNA (0.2 μg) from plants was heated to 65°C for 7 min and then subjected to reverse transcription reaction using SuperScript II RNase H^- reverse transcriptase (200 units per reaction; Invitrogen, USA) with oligo(dT) primer for 50 min at 42°C. PCR amplification was performed as follows: initial denaturation at 94°C for 2 min followed by 25 cycles of incubations at 94°C for 20 s, 55°C for 40 s, and 72°C for 1.0 min, with a final extension at 72°C for 10 min using the specific forward and reverse primers (see below) with AmpliTaq polymerase (2 units per reaction; Perkin-Elmer). *Actin* expression level was used as a quantitative control. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide under UV light. Primers used for RT-PCR as follow; CBL5-F:ATGGGATGTGTTTGCAGCAAGCAATTAG, CBL5-R:TTACCGGAGAAAGGTTGGGAAAATCCTC, RD29A-F:GAATGGTGCGACTAAGATGTTTAGGAA, RD29A-R:GTACAGATTCAGTGGGTTTGGTGTAAT; RD29B-F:GTGAAGATGACTATCTCGGTGGTC, RD29B-R:GCCTAACTCTCCGGTGTAACCTAG; RAB18-F:ATGACGAGTACGGAAATCCGATGG, RAB18-R:TATGTATACACGATTGTTCGAAGC; COR47-F:ATGGCTGAGGAGTACAAGAACAACGTT, COR47-R:TCTTCTTCTTCTTCTCCTTCTTTTCCT; COR15A-F:ATGGCGATGTCTTTCTCAGGAGCTGTT, COR15A-R:TTTTATCCGTCACGAAATCTGAAGCTT; KIN1-F:CAGACCAACAAGAATGCCTTCCAAG, KIN1-R:CTCTTTCCCGCCTGTTGTGCTCC; DREB2A-F:GACCTAAATGGCGACGATGT, DREB2A-R:TCGAGCTGAAACGGAGGTAT; ACTIN-F:CATCAGGAAGGACTTGTACGG, ACTIN-R:GATGGACCTGACTCGTCATAC

RESULTS

CBL5 is predominantly expressed in green tissues

Members of the calcium sensors CBL family that regulate ion homeostasis are predominantly expressed in the roots and contribute to ion uptake or exclusion from the roots (Liu and Zhu, 1998; Xu et al., 2006). To identify CBLs that may contribute to salt tolerance by an alternative mechanism, we analyzed the expression patterns of all CBLs based on public gene chip

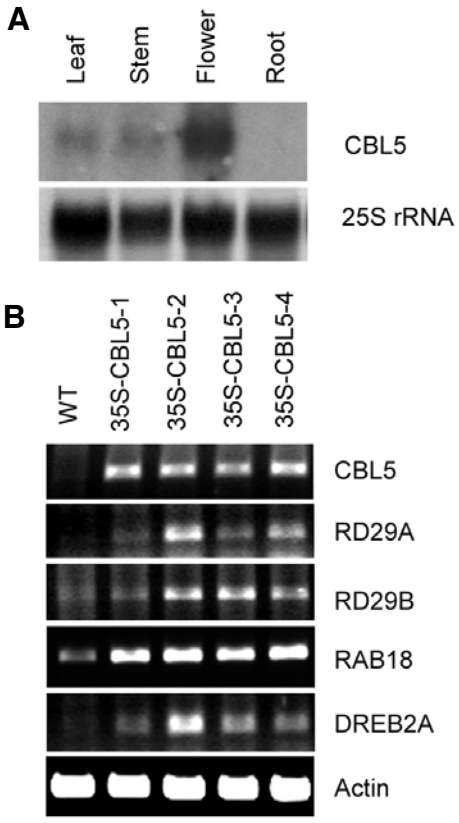

Fig. 1. Expression pattern of the *CBL5* gene and the constitutive overexpression of *CBL5* in *Arabidopsis* (35S-CBL5). (A) Expression pattern of the *CBL5* gene. Northern blot analysis of *CBL5* mRNA levels in rosette leaves, stems, flowers, and roots of 4-week-old *Arabidopsis* plants grown under long-day conditions. (B) Constitutive overexpression of *CBL5* in *Arabidopsis* (35S-CBL5) by the analysis of stress-responsive gene expression using RT-PCR.

databases. This preliminary search revealed that most *CBL* genes were significantly expressed in root tissues with the exception of *CBL5* and *CBL10,* which appeared to be predominantly expressed in the aerial tissues of the plant. To confirm and extend the analysis of the *CBL5* expression pattern, we examined *CBL5* mRNA levels in different plant organs by Northern blot analysis. The analysis indicated significant expression levels of the *CBL5* gene in green tissues, but not in the roots (Fig. 1A). Further gene expression analyses indicated that *CBL5* was not induced by abiotic stress conditions such as high salt, drought or low temperature (data not shown). The AtGenExpress stress expression data set (http://arabidopsis.org/info/expression/ATGenExpress.jsp) confirmed the observed expression patterns: in addition, it did not indicate a significant increase in expression levels of *CBL5* mRNA in response to abiotic stresses.

Constitutive expression of *CBL5* modifies stress-responsive gene expression under normal conditions

To determine whether the CBL5 expression level plays a role in stress response pathways, we ectopically expressed the CBL5 protein in transgenic *Arabidopsis* plants (35S-CBL5) and examined the stress-responsive gene expression in these transgenic plants. Homozygous T_3 lines were obtained by selfing the 30 original T_0 transformants, and four lines were selected for further analysis. We examined *CBL5* mRNA levels in these plants by RT-PCR analysis and found that all four independent lines showed a significant increase in mRNA levels compared with that of the wild-type control lines (Fig. 1B). To determine whether *CBL5* overexpression has any effect on stress response pathways in transgenic plants, we chose to monitor the expression patterns of several stress gene markers including *RD29A*, *RD29B*, *RAB18* and *DREB2A* in the control and in the *CBL5*-overexpressing transgenic lines. The *RD29A* (responsive to desiccation) gene has been shown to be responsive to ABA, drought, cold, and salinity, whereas *RD29B* and *RAB18* are induced more specifically by ABA and drought stress (Yamaguchi-Shinozaki and Shinozaki, 1994). *DREB2A* is more specifically responsive to ABA and osmotic stress (Liu et al., 1998). *DREB* genes are often induced earlier than *RD/KIN* genes: this is consistent with the fact that DREB proteins are transcriptional activators for *RD/KIN* genes. As shown in Fig. 1B, all 35S-CBL5 transgenic plants produced high levels of mRNA corresponding to *RD29A/B*, *RAB18* and *DREB2A.* The results indicated that *CBL5* overexpression in transgenic plants activated the expression of numbers of late stress-responsive genes even under "normal" conditions

35S-CBL5 transgenic plants display enhanced tolerance to high salt, mannitol or drought stresses

We examined 35S-CBL5 transgenic plants under normal growth conditions in the greenhouse and did not observe significant differences from the WT plants. In germination and post-germination assays under various abiotic stress conditions, we found that the 35S-CBL5 transgenic plants were specifically resistant to high salt or mannitol stress. We tested this possibility by a seed germination assay on medium containing ABA and found no difference between control and 35S-CBL5 transgenic plants (data not shown). Interestingly, the same assay on medium containing high concentrations of NaCl or mannitol indicated that the 35S-CBL5 transgenic seeds were more tolerant of these conditions than the control seeds (Fig. 2). For example, on medium containing 250 mM NaCl, 58-68% of the 35S-CBL5 transgenic seeds had germinated within 5 days, whereas only 19% of the control seeds had germinated during the same period (Fig. 2A). On medium containing 500 mM mannitol, 70% of the 35S-CBL5 transgenic seeds had germinated within 5 days, whereas only 17% of the control seeds had germinated during the same period (Fig. 2B). These results indicate that *CBL5* overexpression rendered transgenic plants more resistant to high salt or hyperosmotic stress during early development (i.e., seed germination) but did not alter their response to ABA. 35S-CBL5 transgenic seedlings were also more resistant to high-salt medium following their transfer from the normal medium (Fig. 3). The cotyledons of WT plants were bleached 6 days after transfer to medium containing 200 mM NaCl, whereas the cotyledons of 35S-CBL5 transgenic lines were not as severely affected (Fig. 3A). Under the same stress treatment of high salt, more than 70% of 35S-CBL5 transgenic plants had survived compared with a 10% survival rate in control plants (Fig. 3B). We also performed post-germination assays using several different NaCl concentrations and scored the fresh weight (FW) of seedlings as a growth indicator. As shown in Fig. 3C, the 35S-CBL5 transgenic plants were consistently more resistant to high salt than the WT plants when grown in medium containing 150 or 200 mM NaCl. To determine the root growth of *CBL5*-overexpressing transgenic plants, we tested the 35S-CBL5 transgenic plants on medium containing several different concentration of NaCl. The measured root growth indicated that the 35S-CBL5 transgenic plants displayed

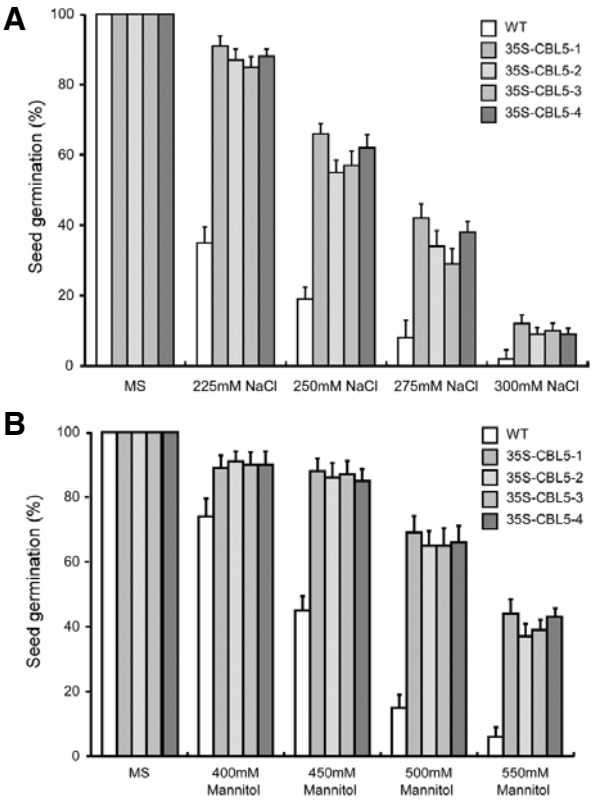

Fig. 2. Seed germination assays of 35S-CBL5 transgenic plants at 5 days under high-salt or mannitol conditions. Germination rates of WT and 35S-CBL5 seeds after 5 days of incubation at 23°C on MS medium containing different concentrations of NaCl or mannitol. The results shown are the mean +/- SE of three independent experiments.

more resistance to NaCl (Fig. 3D).

To extend the high salt-, mannitol- or drought-tolerance assays to adult plants, we treated 5-week-old plants that had just started to bolt with 300 mM NaCl or drought treatment (Cheong et al., 2003). Under control conditions (treated with water), the growth of 35S-CBL5 transgenic plants was similar to the WT plants. However, the elongation of the stems in 35S-CBL5 transgenic plants was highly resistant on NaCl or drought treatment (Fig. 4A). On 300 mM NaCl treatment, the shoot length of 35S-CBL5 transgenic plants was 3 times higher than that of WT plants (Fig. 4B).

To address the potential involvement of CBL5 in plant drought stress responses, both 35S-CBL5 transgenic and WT plants were grown in soil under continuous-light conditions, and subsequently, water was withheld for 10 days (Fig. 4A). As shown in Fig. 4C, the FW of the 35S-CBL5 transgenic plants indicated that they were consistently more resistant than the WT plants when grown under high-salt (300 mM NaCl) or drought conditions. Rosette leaves of 35S-CBL5 transgenic plants showed more resistance than the WT plants at 7 days after 300 mM NaCl, drought or 400 mM mannitol treatment (Fig. 4D). Therefore, these results support the idea that CBL5 may play an important role in plant osmotic stress responses.

Overexpression of *CBL5* alters the stress induction of stress gene markers

As shown in Fig. 1B, the altered expression of some stress gene markers in *CBL5*-overexpressing plants indicates that CBL5 might have altered stress signaling pathways. To examine this possibility further, we determined whether *CBL5* overexpression had altered the expression pattern of stress-responsive genes under conditions of high salt or drought stresses. Comparative expression analyses of the described stress gene markers were performed by quantitative RT-PCR on RNA isolated from plants grown under non-stress and under stress conditions (Fig. 5). Consistent with previous studies (Liu et al., 1998; Tahtiharju et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994), high-salt treatment induced the expression of stress gene markers such as *RD29A/B*, *KIN1/2*, and *COR genes* in the control plants. We

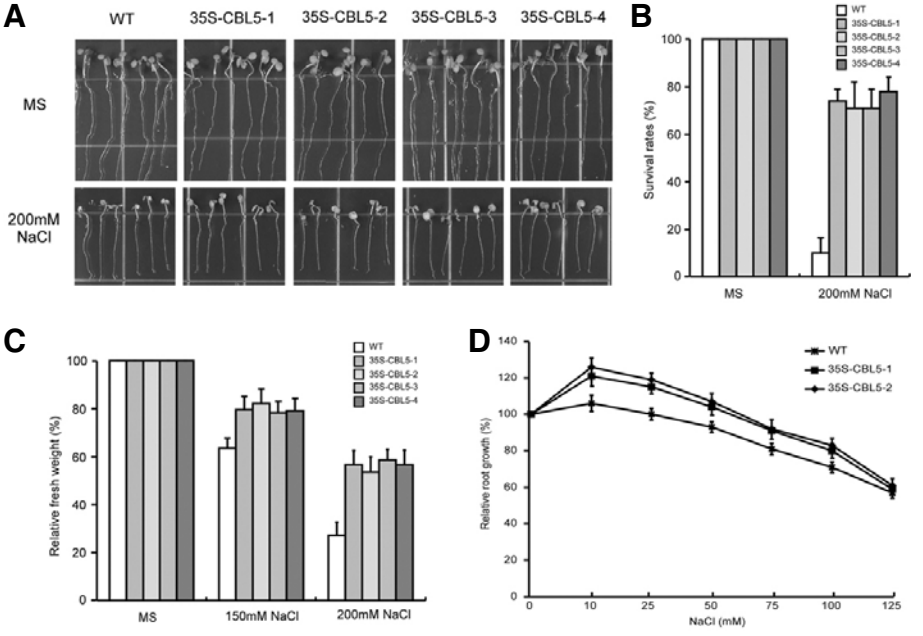

Fig. 3. Resistance of 35S-CBL5 transgenic plants against high-salt treatments. (A) Post-germination assay of 35S-CBL5 transgenic seedlings. Four-day-old seedlings were transferred to 0.5X MS medium without (upper panel) or with (lower panel) 200 mM NaCl. Photographs were taken at 6 days after the transfer. (B) Relative survival rates of 35S-CBL5 transgenic seedlings grown on 200 mM NaCl media. Four-day-old seedlings were transferred to 0.5X MS medium with or without 200 mM NaCl. Survival rate was measured at 6 days after the transfer. (C) Relative fresh weight (FW) of 35S-CBL5 transgenic seedlings grown on 200 mM NaCl media. Four-day-old seedlings were transferred to 0.5X MS medium with or without 200 mM NaCl. Rate was measured 6 days after transfer. (D) Relative root growth 35S-CBL5 transgenic seedlings grown on NaCl Four-day-old seedlings were transferred to 0.5X MS medium with or without various concentrations of NaCl. Rate was measured 6 days after transfer. The results shown are the mean +/- SE of three independent experiments.

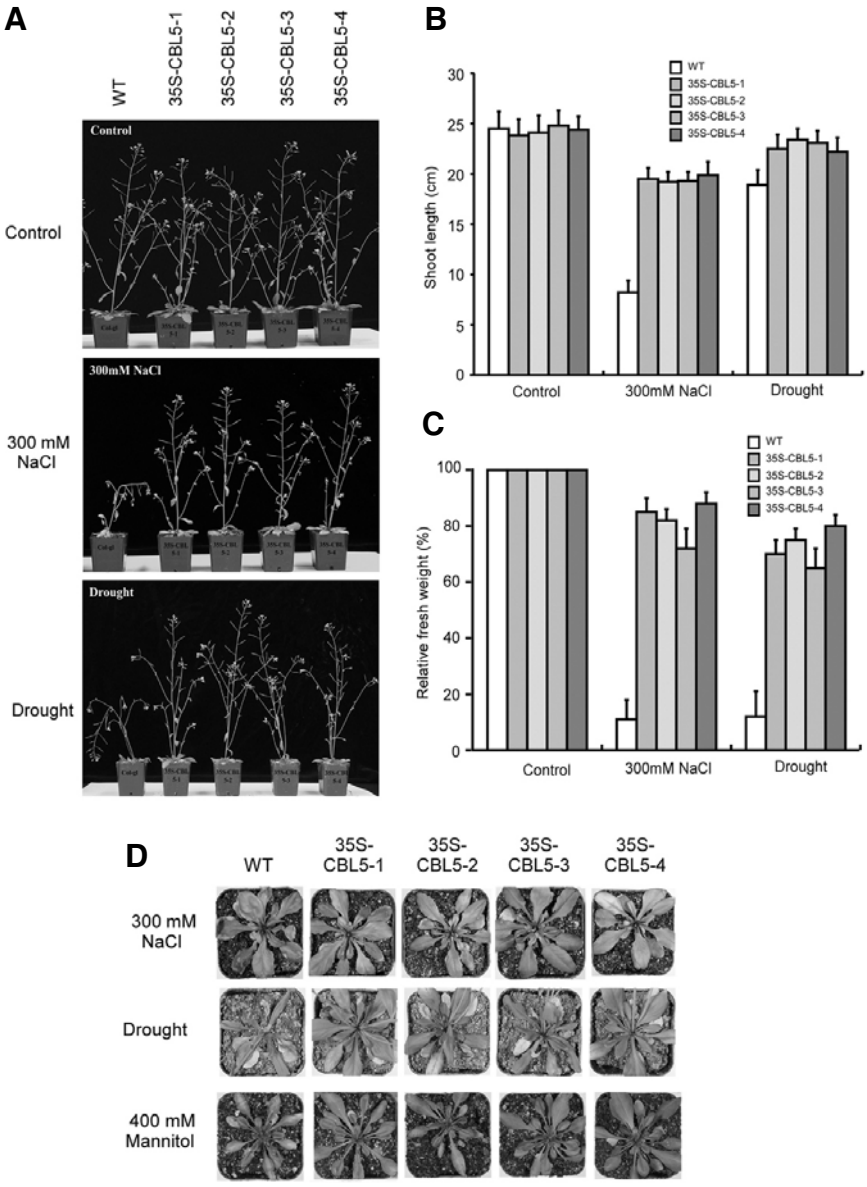

Fig. 4. 35S-CBL5 transgenic plants are resistant to high salt, drought, or mannitol stresses. (A) Plant growth of mature 35S-CBL5 transgenic plants on NaCl or drought treatment. Upper panel, plants treated with water. Middle panel, plants treated with 300 mM NaCl. Lower panel, plants treated with drought. (B) Shoot length of 35S-CBL5 transgenic plants after treatment with 300 mM NaCl or drought. (C) Survival rates of 35S-CBL5 transgenic plants after treatment with 300 mM NaCl or drought. (D) Rosette leaves of mature plants after treatment with 300 mM NaCl, drought, or 400 mM mannitol.

selected the key marker genes *RD29A/B*, *KIN1* and *COR15a/47*, whose expression has been responsive to multiple stress signals including osmotic stress (Gilmour et al., 1998; Pandey et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki and Shinozaki, 1994). In 35S-CBL5 transgenic plants, most of these stress-responsive genes including *RD29A/B*, *KIN1*, *COR15a* and *COR47* were highly induced on 300 mM NaCl stress treatment. Under drought stress conditions, only *RD29A* and *RD29B* genes were hyperinduced. The *RD29A/B*, *KIN1*, *COR15a* and *COR47* were also induced much more strongly in the 35S-CBL5 transgenic plants in compared to the WT plants.

DISCUSSION

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 they function as a threshold factor in linking the calcium signature to the downstream components in the signaling pathways (Luan et al., 2002; Sanders et al., 2002). CBLs are newly identified calcium sensors unique to plants. Several studies have demonstrated that most CBL genes are differentially involved in various stress and/or developmental signaling processes (Albrecht et al., 2003; Batistic and Kudla, 2009; Cheong et al., 2003; Luan, 2008; Pandey et al., 2004).

In *Arabidopsis*, at least 10 CBLs have been reported to be involved in a network-like signaling system for specific and synergistic stimulus-response coupling (Batistic and Kudla, 2004; Luan et al., 2002). Among them, CBL1, CBL4, CBL5 and CBL9 possess a highly conserved N-myristoylation motif like many calcineurin-B subunit (CNB) and NCS proteins (Batistic et al., 2008; Ishitani et al., 2000). Myristoylation is a post-translational modification and has a significant role in protein-protein interaction or in protein-membrane attachment (Ishitani et al., 2000). Most N-myristoylation motif containing CBL proteins such as CBL1, CBL4, and CBL9 play unique and critical roles in plant abiotic stress signaling pathways (Batistic and Kudla, 2009; Batistic et

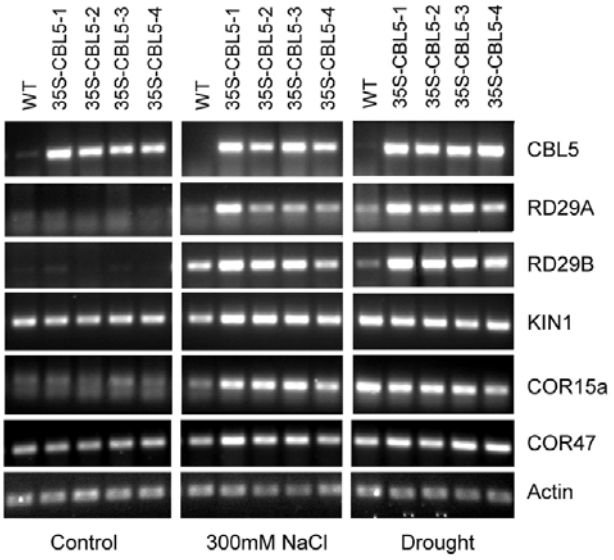

Fig. 5. Expression of stress-responsive genes in WT and 35S-CBL5 transgenic plants induced by 300 mM NaCl or drought treatments.

al., 2008; Ishitani et al., 2000; Luan, 2008). In addition, the calcium sensors CBL1 and CBL9 have been shown to localize to the plasma membrane, where they form alternative complexes with their target kinases (Cheong et al., 2007; D'Angelo et al., 2006). Recently, studies have also demonstrated that both CBL1 and CBL9 target CIPK23, which functions in the regulation of potassium uptake and stomatal movements (Cheong et al., 2007; Lee et al., 2007; Li et al., 2006; Luan et al., 2009; Xu et al., 2006).

Here, we have studied the function of CBL5 in abiotic stress responses using the *CBL5*-overexpressing transgenic (35S-CBL5) *Arabidopsis* plants. CBL1 and CBL4 calcium sensors that function in regulating ion homeostasis exhibit a predominant expression in roots and contribute to ion uptake or exclusion from roots (Cheong et al., 2003; 2007; Liu and Zhu, 1998; Xu et al., 2006). By analyzing the *CBL5* expression pattern, we found significant expression levels of the *CBL5* gene in green tissues, but not in roots (Fig. 1A). This expression pattern is very similar to that of CBL10 (Kim et al., 2007). As shown in Fig. 1B, all 35S-CBL5 transgenic plants produced high levels of mRNA for *RD29A/B*, *RAB18,* and *DREB2A* under normal conditions. These results indicated that overexpression of *CBL5* in transgenic plants activated the expression of a number of late stress-responsive genes. 35S-CBL5 transgenic plants display enhanced tolerance to high salt, mannitol, or drought stresses. In germination and post-germination assays under various abiotic stress conditions, the 35S-CBL5 transgenic plants were specifically resistant to high salt or mannitol stresses (Figs. 2 and 3) and we found no difference in ABA conditions between control and 35S-CBL5 transgenic plants (data not shown). As shown in Fig. 4, the elongation of the stems in 35S-CBL5 transgenic plants was highly resistant upon high NaCl or drought treatment. The shoot length of 35S-CBL5 transgenic plants was 3 times higher than that of WT plants on NaCl treatment. The FW of 35S-CBL5 transgenic plants was consistently more resistant than the WT plants when grown under high-salt (300 mM NaCl) or drought conditions. Thus, CBL5 may play an important role in plant osmotic stress responses.

Furthermore, the overexpression of *CBL5* alters the expression of stress-responsive gene markers (Fig. 5). In 35S-CBL5 transgenic plants, most of stress-responsive genes including *RD29A/B*, *KIN1*, *COR15a* and *COR47* were induced by 300 mM NaCl stress treatment. Under drought stress conditions, only *RD29A* and *RD29B* genes were hyper-induced. The same genes were induced much more strongly in the 35S-CBL5 transgenic plants. Previous studies demonstrated that osmotic stresses, such as high salt and drought induced the expression of stress gene markers, such as *RD29A/B*, *KIN1/2*, and *COR* genes in the control plants (Gilmour et al., 1998; Kim et al., 2009; Liu et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Tahtiharju et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). These results suggest that CBL5, like CBL1, may function as a positive regulator in high-salt, drought, or osmotic stress signaling pathways (Cheong et al., 2003).

Earlier studies demonstrated that CBL1 functions as a positive regulator of salt or drought responses and as a negative regulator of cold response in plants. Constitutively overexpressing *CBL1* revealed a proportional positive correlation of *CBL1* transcript levels with the amount of *RD29A* mRNA under non-stressed conditions. *CBL1*-overexpressing plants showed enhanced tolerance to salt or drought, but reduced tolerance to freezing: this suggests that the amount of CBL1 is a rate-limiting factor in calcium-mediated stress signaling, at least with respect to the level of expression of downstream target genes (Albrecht et al., 2003; Cheong et al., 2003). In contrast, *cbl1*-null mutant plants showed enhanced cold induction and reduced drought induction of stress-responsive gene expressions. The mutant plants also displayed less tolerance to salt or drought but enhanced tolerance to freezing.

It has also been known that the functional specificity defined by different CBL-CIPK complexes is a crucial feature for the functional versatility of the CBL-CIPK network. An example of CBL-dependent target specificity of CBL-CIPK complexes is the CBL1/9-CIPK1 complex that is differentially regulated by two highly related CBL proteins (D'Angelo et al., 2006). While interaction of the CBL1-CIPK1 complex is important for regulating downstream processes during salt stress, the CBL9-CIPK1 complex mediates the response to the stress-hormone ABA (D'Angelo et al., 2006; Pandey et al., 2004). These results suggest that the calcium sensor moiety of the CBL-CIPK complex is crucial for determining the activity toward defined phosphorylation targets. Recent findings also revealed that only CBL1 and CBL9 activate CIPK23 for the phosphorylation of AKT1 (Cheong et al., 2007; Xu et al., 2006). Another example is CBL4/CBL10-CIPK24 complexes in high-salt signaling pathways. High-salt conditions induce calcium signals that activate CBLs. In the case of Na^+ stress, elevated levels of Ca^{2+} activate CBL4/SOS3 and CBL10 although the gene expression of *CBL4* or *CBL10*, like *CBL5*, is not changed by stress treatment. Both these calcium sensors can interact with CIPK24/SOS2 to form alternative calcium sensor-kinase complexes. The CBL4/SOS3-CIPK24/SOS2 complex targets the plasma membrane-localized Na^+/H^+ exchanger SOS1 whereas the CBL10-CIPK24/SOS2 complex regulates ion transport processes at the vacuolar membrane. However, unlike CBL4/SOS3, which functions mainly in the roots, gene expression and function of CBL10 is observed almost exclusively in the shoots and leaves (Kim et al., 2007; Luan et al., 2009; Shi et al., 2000). Remarkably, CIPK23 can also interact with CBL5 and CBL8 to form calcium sensor-kinase complexes, but the sensor moieties in these complexes are unable to achieve the activity toward the target AKT1 (Xu et al., 2006). Thus, other specific CIPK proteins may mediate the function of CBL5 in osmotic or drought stress signaling in plants. It will be very interesting and important to elucidate the mecha-

nisms responsible for this remarkable specificity.

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