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Two poplar calcineurin B-like proteins confer enhanced tolerance to abiotic stresses in transgenic *Arabidopsis thaliana*

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Abstract

Calcium is a critical component in a number of plant signal transduction pathways and the calcineurin B-like protein (CBL) family is a unique group of calcium sensors regulating a family of CBL-interacting protein kinases (CIPKs). In this study, two poplar *CBL* genes, *PeCBL6* (GenBank acc. No. DQ907710) and *PeCBL10* (GenBank acc. No. DQ899956), were characterized in transgenic *Arabidopsis thaliana* particularly with regard to its role in abiotic stress resistance. Expression of the two *CBL* genes in poplar was induced by cold, drought, or high salinity, but not by abscisic acid (ABA) treatment. In *Arabidopsis thaliana, PeCBL6* was found in the nucleus and *PeCBL10* in the tonoplast. Transgenic *Arabidopsis* plants overexpressing *PeCBL6* or *PeCBL10* showed enhanced tolerance to high salinity, drought, and low temperature. These results suggested that *PeCBL6* and *PeCBL10* may function as positive regulators of salt, drought, and temperature responses.

Additional key words: over-expression of *CBL* genes, *Populus euphratica*, transgenic plants.

Introduction

Drought, high salinity, and freezing are prevalent environmental stresses that strongly influence the survival of plants (Mohavedi *et al*. 2012). Plants use calcium ion as a second messenger in mediating a number of various signal transduction pathways including responses to abiotic stresses (Knight 2000, Sanders *et al*. 2002, Xiong *et al*. 2002, Lee *et al*. 2008, Luan 2008, Batistič and Kudla 2009). Ca^{2+} spatial and temporal dynamics is critical for proper coupling of defined stimuli with the corresponding specific responses (Allen *et al*. 2000, 2001, Sanders *et al*. 2002). Such stimulus-specific elevations in cytosolic Ca^{2+} concentration are referred to as 'calcium signatures'. An additional regulation and specificity is achieved by Ca^{2+}-binding proteins that function as signal sensor proteins (Luan *et al*. 2002, Batistič and Kudla 2004). Such proteins sense changes in the local calcium concentration and relay the information to downstream responses, such as phosphorylation cascades, protein translocation processes, and finally regulation of gene expression (Luan *et al*. 2002, Sanders *et al*. 2002). These calcium sensors, such as calmodulin (CaM), calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs), bind Ca^{2+} and change their conformation (Zielinski 1998, Harmon *et al*. 2000, Luan *et al*. 2002, Sanders *et al*. 2002).

CBLs 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; Luan *et al*. 2002, Batistič and Kudla 2004, 2009, Luan 2008). CBLs were initially identified from *Arabidopsis thaliana*. Subsequent genome-wide analysis has revealed ten CBL members in *Arabidopsis*,

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Abbreviations: ABA - abscisic acid; CaM - calmodulin; CaMV - *Cauliflower mosaic virus*; CBF - C-repeat binding factor; CBL - calcineurin B-like protein; CDPK - calcium-dependent protein kinase; CIPK - CBL-interacting protein kinase; COR - cold-regulated; CTAB - cetyltrimethylammonium bromide; DRE - dehydration responsive element; DREB - DRE-binding protein; F_v/F_m - variable to maximum chlorophyll fluorescence ratio (photochemical efficiency of photosystem 2); WT - wild-type.
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rice, and poplar (Zhang *et al*. 2008). In *Arabidopsis*, at least 10 CBLs and 26 CIPKs have been reported, and they form a network-like signaling system for specific and synergistic stimulus-response coupling (Weinl and Kudla 2009). For example, it has been reported that CBL10 seems to interact with CIPK24 in the salt tolerance pathway. In addition, CBL10 expression and function is observed almost exclusively in the shoots and leaves (Kim *et al*. 2007, Quan *et al*. 2007). However, the function of CBLs and the interactions between CBL and CIPK in poplar are less known.

Populus euphratica, the most important species for large-scale forestation projects on saline desert sites in China, can tolerate salt concentrations of up to 450 mM NaCl (Ottow *et al*. 2005). Characterization of *CBL* genes from *P. euphratica* would help to improve understanding the surprising resistance of this tree to environmental stresses. Here, we report two *CBL* genes, *PeCBL6* and *PeCBL10*, from salt-treated *P. euphratica*. Expression patterns and sub-cellular localization were investigated. In addition, transgenic *Arabidopsis* plants were evaluated for tolerance to multiple stresses.

Materials and methods

Plants and growth conditions: Two-year-old *Populus euphratica* Oliv. trees, collected from Mongolia, were planted in individual pots (10 dm^3) containing loam soil and placed in a greenhouse at Beijing Forestry University. For analyzing gene expression, similarly developed trees (50 - 60 cm in height, 40 - 50 leaves) were subjected to various stress treatments. Salt stress was induced by watering young plants with 300 mM NaCl. The ABA treatment was performed by spraying the leaves with 200 μM ABA solution. Dehydration was induced by removing plants from the plots and exposing them (on filter paper) to air at relative humidity of 70 %, temperature of 25 °C, and irradiance of 120 μmol m^{-2} s^{-1} for 24 h. The cold treatment was applied by transferring the young trees to a growth chamber set at temperature of 4 °C and a 16-h photoperiod (irradiance of 40 μmol m^{-2} s^{-1}) for 24 h. For all treatments, leaves, stems, and roots from different plants were collected at different time points and immediately frozen in liquid nitrogen for later use.

Seeds of *Arabidopsis thaliana* L. (ecotype Columbia-0 and *cbl10* mutant) were sterilized, germinated, and grown on solid Murashige and Skoog (MS) medium supplemented with 3 % sucrose and 0.7 % agar at 22 °C under a 16-h photoperiod (irradiance of 120 μmol m^{-2} s^{-1}) in a tissue culture room. Plantlets were transplanted into mixture of soil and *Vermiculite* (1:2) and grown in a greenhouse under a 9-h photoperiod, irradiance of 150 μmol m^{-2} s^{-1}, temperature of 22 °C, and relative humidity of 70 % until the flowering stage suitable for plant transformation and RNA analysis, as described previously (Cheong *et al*. 2003, Pandey *et al*. 2004). The *cbl10* mutant allele (SALK-056042) was isolated from the T-DNA-transformed *Arabidopsis* collection from the *Arabidopsis* Biological Resource Center (http://www.biosci.ohiostate.edu/pcmb).

qRT-PCR analysis: To analyze gene expression by quantitative real-time PCR (qRT-PCR), total RNA from each sample was extracted by the cetyltrimethyl-ammonium bromide method (CTAB) (Chang *et al*. 1993) and 1 μg of RNA was used for the reverse transcription reaction. Each reaction contained 0.005 cm^3 of *SYBR Green*, 2 ng of cDNA sample, and 200 nM gene-specific primers in a final volume of 0.01 cm^3. Forty PCR cycles were performed according to the following temperature scheme: 94 °C for 30 s, 62 °C for 30 s, and 68 °C for 1 min (Chen *et al*. 2009). The relative value for the expression level of *PeCBL6* and *PeCBL10* was calculated by the $2^{-\Delta\Delta CT}$ method using the poplar *PeActin* gene as an internal control (Livak and Schmittgen 2001, Chen *et al*. 2009, Ye *et al*. 2009). Each PCR assay was carried out for three biological replicates. The results from the repeated experiments were consistent and so only those for one set of experiments are shown. The primer sequences are shown in Table 1.

Plasmid constructions: To analyze the sub-cellular localization of *PeCBL6* and *PeCBL10*, both were fused to GFP in pBI121 under the cauliflower mosaic virus (CAMV35S) promoter yielding the *pBI121-PeCBL6-GFP* and *pBI121-PeCBL10-GFP* constructs. The primer sequences used are shown in Table 1. For gene overexpression and complementation, the coding region of *PeCBL6* and *PeCBL10* were cloned into pCAMBIA1301-PMI (Yang *et al*. 2009) under the CAMV35S promoter yielding the *pCAMBIA1301-PMI-PeCBL6-GFP* and *pCAMBIA1301-PMI-PeCBL10-GFP* constructs. The primer sequences are shown in Table 1.

Plant transformation and sub-cellular localization analysis: For plant transformation, *Agrobacterium tumefaciens* strain GV3101 cells carrying the constructs (*pCAMBIA1301-PMI-PeCBL6-GFP* and *pCAMBIA1301-PMI-PeCBL10-GFP*) were used to transform these constructs into wild-type (WT) *Arabidopsis* and *cbl10* mutants by the floral dip method (Clough and Bent 1998). Seeds were harvested from transgenic plants and subsequently screened on selection medium (half-strength MS medium, 1× Gamborg's vitamins, and 4 mM mannose). The surviving plants were further confirmed by PCR.

For sub-cellular localization analysis, *Agrobacterium tumefaciens* GV3101 cells carrying the constructs (*pBI121-PeCBL6-GFP* and *pBI121-PeCBL10-GFP*) were used to transform WT *Arabidopsis* by the floral dip method (Clough and Bent 1998). Seeds were harvested from these plants and subsequently screened on selection

medium (half-strength MS medium, 1× Gamborg's vitamins, and 50 μg cm^{-3} kanamycin). A *35S-GFP* vector was transformed as a control. The untransformed *Arabidopsis* also served as a control. We then visualized green fluorescence signals using a laser-scanning confocal microscope (*Leica*, Heerbrugg, Switzerland).

Germination assay: For salt and drought treatments, approximately 100 seeds, each from the wild-type (WT), the *cbl10* mutant, the complemented line, and the overexpressing lines, were planted in triplicate on MS medium with different concentrations of NaCl (0, 50, 100, 150 mM) and mannitol (0, 100, 200, 300 mM) and incubated at 4 °C for 2 d before being placed to 23 °C under 16-photoperiod. For cold treatment, these four types of plants were planted in triplicate on MS medium and then transferred to -2 °C for 12 h. After that step, plants were placed back to 23 °C. Germination (emergence of the radicle) was scored on the 7th day.

Drought, salt, and freezing tolerance analyses: To analyze fresh mass, 20-d-old seedlings from vertical MS plates were transferred onto MS plates supplemented with different concentrations of NaCl (0, 50, 100, 150 mM) and mannitol (0, 100, 200, 300 mM) or transferred to -2 °C for 12 h before placing to 23 °C under 16-h photoperiod. There were different genotypes of plants on the same plate and three replicate plates were used for each treatment. Ten seedlings from each genotype were collected on each plate. After 8 d, their total fresh mass was examined.

For salt tolerance assays, 4-week-old plants were treated with 300 mM NaCl every 3 d for 2 weeks. Survival rates were counted 10 d after the first treatment. For the drought-tolerance assays, water was withheld from 4-week-old, soil-grown plants for 3 weeks. Then the survival rates were counted. For the cold treatment, 4-week-old plants grown in soil in a growth chamber (22 ± 2 °C, 16-h photoperiod) were treated at 4 °C for 12 h for acclimation. After that, the plants were subjected to freezing at -7 °C for 6 h. After this treatment, plants were transferred back to 4 °C and incubated for 12 h. Then the plants were transferred to a growth chamber and surviving plants were scored after 8 d (Cheong *et al*. 2003).

To further determine, whether the *PeCBL6* and *PeCBL10* expression level plays a role in stress response pathways, we examined the stress-responsive gene expression in these transgenic plants. Furthermore, chlorophyll content and photochemical efficiency were measured as previously described by Chen *et al*. (2009). Briefly, the chlorophyll was extracted with 80 % acetone. The absorbance at 652 nm was determined by a *Ultrospec 3100* UV/visible spectrophotometer (*Amersham Biosciences*). Photochemical efficiency was monitored using a *Dual-PAM-100* (*Walz*, Effeltrich, Germany) fluorimeterat room temperature.

Data analysis: Three technical replicates were performed. Differences among treatments were analyzed by one-way *ANOVA* using the LSD test at $P < 0.05$.

Table 1. Primer sequences used for qRT-PCR, RT-PCR, and plasmid constructions.

Type	Gene	Forward primers	Reverse primers
RT-PCR	*GFP*	ctggtcgagctggacggcgacg	cacgaactccagcaggaccatg
qRT-PCR	*PeCBL6*	gtttttcatcctcgcgctccca	agcatttgacatctgccggacttct
	PeCBL10	tgttccatccctacgcacc	attttaccatccttgtcggcatc
	KIN1	cagaccaacaagaatgccttccaag	ctctttcccgcctgttgtgctcc
	DREB1A	gatcaatgaactcattttctgcttt	cgtcgcatcacacatctcat
	actin	catcaggaaggacttgtacgg	gatggacctgactcgtcatac
	PeActin	gtcctcttccagccatctc	ttcggtcagcaataccagg
Constructions	*pBI121-PeCBL6*	ggatccatggacagaagctcaagctc	ggatccaagtcctcaacttctgtgttgaaa
	pBI121-PeCBL10	ggatccatggatttcaacaacaacaacag	ggatccaaatcctcaacctcagtgttgaa
	pCAMBIA1301-PeCBL6	ggatccatggacagaagctcaagctc	ggatccaagtcctcaacttctgtgttgaaa
	pCAMBIA1301-PeCBL10	ggatccatggatttcaacaacaacaacag	ggatccaaatcctcaacctcagtgttgaa

Results

The *PeCBL6* (GenBank acc. No. DQ907710) and *PeCBL10* (acc. No. DQ899956), homologous with *AtCBL10*, possess four EF-hand motifs (Fig. 1), and their proteins were 62 and 68.6 % identical to the CBL10 protein in *Arabidopsis thaliana*, respectively (Fig. 2). Their amino acid variation is mainly accounted for in the divergence of their N- and C-terminal domains (Batistič *et al*. 2010). qRT-PCR showed that *PeCBL6* and *PeCBL10* were expressed in all organs, with the highest levels in roots under common conditions (Table 2). Their expressions in leaves and roots were induced by salt, cold, and dehydration but not ABA application (Table 2).

In *Arabidopsis* plants carrying the constructs *pBI121-PeCBL6-GFP* and *pBI121-PeCBL10-GFP*, green fluorescence signals were not observed throughout the cells, but exclusively in the vacuolar membrane of cells trans-

Table 2. Quantitative real-time PCR of *PeCBL6* and *PeCBL10* in leaves under various treatments in *Populus euphratica*. The expression levels were normalized to that of *PeActin* and the levels of the *PeCBL6* and *PeCBL10* transcripts in the controls were set at 1.0. Means ± SD (n = 3). Under control conditions, the expression in stem was 0.96 ± 0.22 and 1.02 ± 0.08, respectively.

Type		*PeCBL6*				*PeCBL10*			
		ABA	NaCl	drought	cold	ABA	NaCl	drought	cold
Leaf	1 h	1.41±0.09	1.18±0.05	1.10±0.04	1.59±0.19	1.11±0.36	1.32±0.36	1.14±0.13	1.38±0.13
	3 h	1.03±0.09	3.20±0.15	4.84±0.58	2.00±0.41	0.81±0.01	3.22±0.94	1.36±0.43	1.73±0.48
	6 h	1.22±0.10	2.57±0.57	2.27±0.18	2.18±0.48	0.63±0.17	2.22±0.51	1.96±0.37	2.04±0.07
	12 h	1.43±0.28	0.76±0.11	1.91±0.24	4.10±0.59	1.19±0.47	1.70±0.74	2.41±0.46	3.59±0.60
	24 h	0.97±0.41	0.99±0.10	1.77±0.21	0.54±0.33	0.61±0.39	1.25±0.45	3.64±0.26	2.27±0.82
Root	3 h	1.08±0.57	13.67±1.87	20.82±0.40	15.57±1.24	0.61±0.33	16.73±3.07	18.02±6.08	10.34±3.67

Table 3. Effect of NaCl, mannitol, or cold on germination rate of different *Arabidopsis* lines. Data are means ± SD (n = 3). Different letters indicate statistically significant differences at $P < 0.05$ according to the LSD test. Germination of control was 100 %.

Line	NaCl [mM]			Mannitol [mM]			Cold
	50	100	150	100	200	300	
cbl10	94.67±3.52abc	66.67±10.97gh	26.67±7.09m	96.67±2.08abc	64.67±8.50hi	31.00±7.81m	34.00±6.56lm
WT	99.00±1.00ab	90.00±3.61bcd	41.67±3.21kl	98.67±0.58ab	88.00±4.36cd	44.00±3.00k	64.33±10.1hi
cbl10/PeCBL6	100.00±0.00a	94.67±1.53abc	57.00±6.25ij	—	—	—	74.33±9.29fg
35S-PeCBL6	100.00±0.00a	98.00±2.00ab	75.67±6.66ef	—	—	—	83.33±5.77de
cbl10/PeCBL10	98.67±1.53ab	92.33±3.06abc	52.67±4.51j	100.00±0.00a	92.00±3.61abc	64.00±7.81hi	—
35S-PeCBL10	100.00±0.00a	97.00±1.73abc	70.33±4.62fgh	100.00±0.00a	96.33±2.89abc	74.00±8.00fg	—

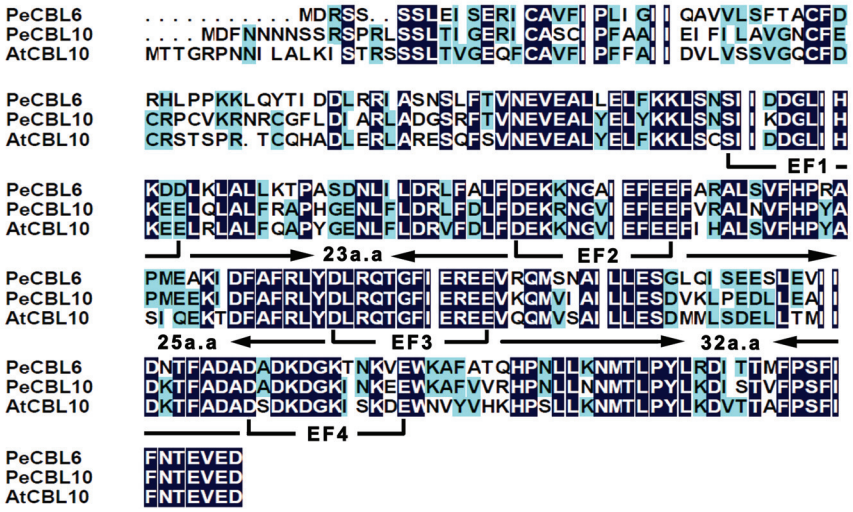

Fig. 1. Deduced amino acid sequence of *PeCBL6*, *PeCBL10*, and *AtCBL10*. Four predicted EF-hand motifs (EF1-4) are indicated. There are 23 amino acids between EF1 and EF2; 25 amino acids between EF2 and EF3; and 32 amino acids separate EF3 from EF4. *Black* and *colored shading* indicate identical and conserved amino acid residues, respectively.

formed with *35S-PeCBL10-GFP* (Fig. 3*G-I*) demonstrating that *PeCBL10* encoded a vacuolar membrane-localized protein. On the other hand, fluorescence in the cell transformed with *35S-PeCBL6-GFP* was detected exclusively in the nucleus (Fig. 3*D-F*), indicating that *PeCBL6* encoded a nuclear-localized protein.

The stress-inducible expression suggested a possible role of *PeCBL6* and *PeCBL10* in abiotic stress signaling. To study the functions of *PeCBL6* and *PeCBL10*, the constructs *pCAMBIA1301-35S-PeCBL6-GFP* and *pCAMBIA1301-35S-PeCBL10-GFP* were transformed into WT *Arabidopsis* and *cbl10* mutants and 12 independent transgenic lines were generated. Three independent transgenic lines (L1–L3) were selected for transient analysis. Under normal conditions, the expression of GFP was detected in the transgenic lines

but not in the WT plants. The expression levels of *PeCBL6* and *PeCBL10* among the transgenic lines were slightly different (Fig. 4). Compared to the mutant, the germination of lines overexpressing *35S-PeCBL6* or *35S-PeCBL10* was less reduced under 150 mM NaCl. Under the 300 mM mannitol treatment, the germination rate of *35S-PeCBL10* transgenic plants was more than twice as great as of the *cbl10* mutants. Under cold treatment, the lines overexpressing *35S-PeCBL6* showed higher germination rates in comparison with the WT and *cbl10* mutant ($P < 0.05$) (Table 3). Furthermore, we scored the fresh mass (FM) and survival rate of seedlings as a growth indicators. The both transgenic plants were more resistant to 150 mM NaCl than the WT plants and the mutant. The *35S-PeCBL10* transgenic plants accumulated more biomass and had higher survival rate than WT plants when treated by 300 mM mannitol. In addition, the *35S-PeCBL6* transgenic plants exhibit enhanced cold resistance (Table 4). Consistently, the *35S-PeCBL6* transgenic plants had higher chlorophyll content and F_v/F_m than the mutant plants under high salinity and cold treatments. When treated by 150 mM

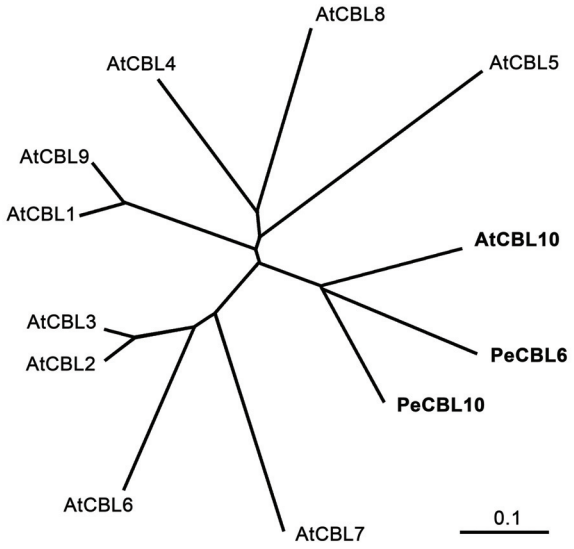

Fig. 2. Phylogenetic relationship of *PeCBL6*, *PeCBL10*, and *AtCBL10*. The phylogenetic tree was constructed using neighbor-joining method.

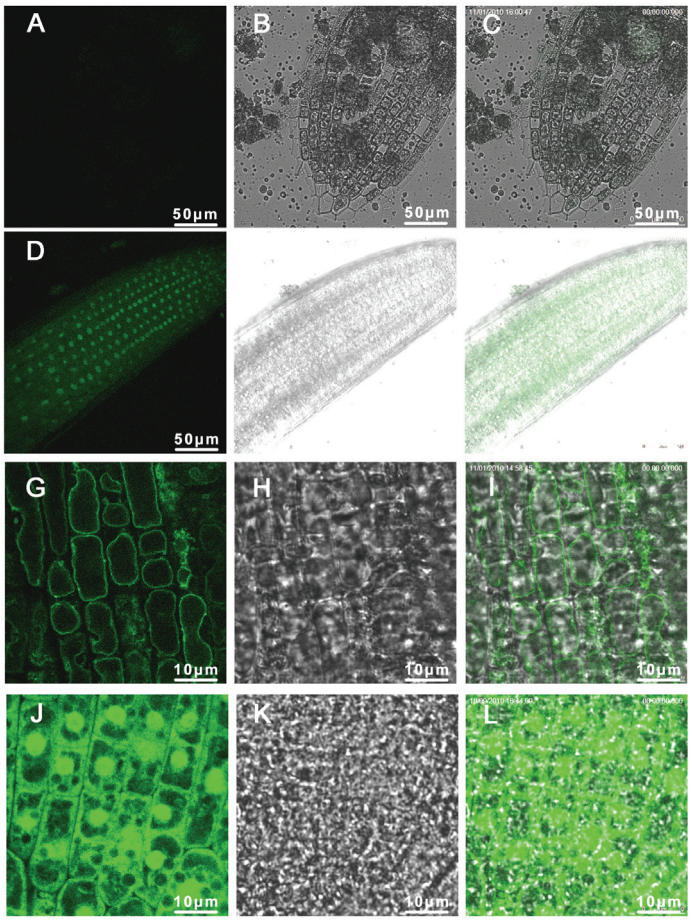

Fig. 3. Sub-cellular localizations of *PeCBL6* and *PeCBL10* by GFP fusion expression in transgenic *Arabidopsis* carrying *p35S:GFP* (as a control), *p35S:PeCBL6-GFP,* and *p35S:PeCBL10-GFP*. The apical cells were photographed under a dark or light field by fluorescence microscopy. *A*,*B* - untransformed control; *J*,*K* - *p35S:GFP*; *D*,*E* - *p35S:PeCBL6-GFP*; and *G*,*H* - *p35S:PeCBL10-GFP*. *C*, *F*, *I*, *L* - merged images.

NaCl or 300 mM mannitol, the chlorophyll content and F_v/F_m ratio of the *35S-PeCBL10* transgenic lines were higher than that of the control plants showing the improved ability of transgenic lines to utilize radiation under stress. To further determine whether *PeCBL6* and *PeCBL10* play a role in stress response pathways, we chose to monitor the expression patterns of cold-inducible (*KIN1*) and dehydration responsive element binding protein (*DREB1A*) by the qRT-PCR method in these four types of plants (*cbl10* mutant, wild-type, complementation, and overexpression seedlings). Under normal conditions, the gene expression of *KIN1* in overexpressing plants slightly increased compared with that of the WT and the mutant (Table 5). However, under 150 mM NaCl, the gene expression of *KIN1* significantly more increased in both *PeCBL6*- and *PeCBL10*-overexpressing plants than in WT and *cbl10* mutant. In addition, the *DREB1A* gene exhibited a higher expression pattern in *PeCBL6*-overexpressing plants than in the mutant under cold stress and was not induced in *PeCBL10*-overexpressing plants and *cbl10/PeCBL10*

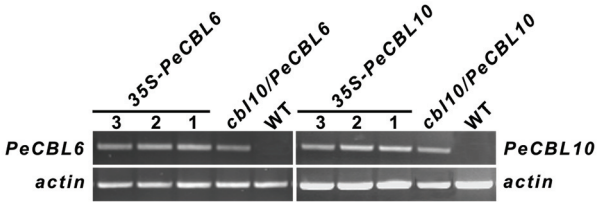

Fig. 4. Expression analysis of the *PeCBL6* and *PeCBL10* examined by RT-PCR in the WT, three independent T3 overexpression transgenic lines (*35S-PeCBL6*, *35S-PeCBL10*), and the complemented line (*cbl10/PeCBL6*, *cbl10/PeCBL10*). The *Arabidopsis actin* gene was used as a loading control.

Table 4. Effect of NaCl, cold, and droughts on fresh mass [mg seedling^{-1}], survival rate [%], chlorophyll content [mg g^{-1}(f.m.)], and F_v/F_m in different lines. Data are means ± SD (n = 3). The different letters in the same column indicate statistically significant differences at $P < 0.05$ according to the LSD test.

Treatments	Line	Fresh mass	Survival rate	Chlorophyll content	$[F_v/F_m]$
Control	cbl10	46.33±3.05a	100.00±0.00a	1.16±0.06a	0.83±0.01a
	WT	47.00±3.00a	100.00±0.00a	1.15±0.11a	0.83±0.01a
	cbl10/PeCBL6	46.67±1.86a	100.00±0.00a	1.17±0.03a	0.83±0.01a
	35S-PeCBL6	47.33±2.88a	100.00±0.00a	1.17±0.12a	0.82±0.01a
	cbl10/PeCBL10	46.67±2.08a	100.00±0.00a	1.14±0.06a	0.84±0.01a
	35S-PeCBL10	47.33±3.22a	100.00±0.00a	1.18±0.04a	0.84±0.01a
NaCl	cbl10	15.33±2.08i	20.33±3.22h	0.40±0.04i	0.39±0.04h
	WT	21.00±2.65gh	37.00±3.61g	0.53±0.03gh	0.52±0.02f
	cbl10/PeCBL6	30.00±3.46bcde	49.67±6.11f	0.61±0.03efg	0.63±0.02de
	35S-PeCBL6	33.33±3.06b	69.33±8.39cd	0.78±0.18cd	0.72±0.03c
	cbl10/PeCBL10	26.33±3.06def	59.33±5.13e	0.62±0.05efg	0.62±0.02de
	35S-PeCBL10	31.67±1.53bcd	82.33±6.03b	0.81±0.02bcd	0.76±0.06b
Cold	cbl10	13.00±2.65i	26.33±7.37h	0.58±0.03fgh	0.45±0.03g
	WT	21.67±2.08fgh	47.00±4.36f	0.71±0.01de	0.52±0.02f
	cbl10/PeCBL6	26.67±5.13cdef	64.67±6.03de	0.74±0.06d	0.66±0.02d
	35S-PeCBL6	32.00±3.46bc	76.33±11.02bc	0.90±0.08b	0.74±0.02bc
Drought	cbl10	12.33±2.08i	26.33±7.02h	0.48±0.02hi	0.40±0.03h
	WT	17.33±1.16hi	48.00±3.00f	0.58±0.01fgh	0.45±0.03g
	cbl10/PeCBL10	25.00±3.61efg	65.67±7.10de	0.69±0.03def	0.61±0.03e
	35S-PeCBL10	29.33±4.04bcde	78.33±2.52bc	0.89±0.02bc	0.73±0.01bc

Table 5. Expression of *KIN1* and *DREB1A* in different lines under control conditions, NaCl, or cold treatments. Means ± SD of three independent experiments.

Line	*KIN1* control	NaCl	*DREB1A* control	cold
cbl10	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
WT	1.46±0.04	2.29±1.13	0.23±0.11	5.77±0.85
cbl10/PeCBL6	3.10±0.57	7.25±1.71	0.39±0.31	7.15±0.27
35S-PeCBL6	3.11±0.53	10.25±2.53	0.48±0.44	8.69±0.58
cbl10/PeCBL10	3.10±0.53	5.46±1.57	0.30±0.07	0.26±0.09
35S-PeCBL10	3.61±1.54	6.99±0.76	0.36±0.01	0.30±0.10

plants (Table 5). These results indicated that over-expression of *PeCBL6* and *PeCBL10* in transgenic plants activated the expression of a number of stress-responsive genes and further indicated that *CBL* genes may play an important role in adaptation to various environmental conditions.

Discussion

P. euphratica is known for its high salt tolerance (Chen *et al*. 2003, Ottow *et al*. 2005). It is the only arboreal species that has established in the world's largest shifting sand desert, the Taklimakan Desert (Bruelheide *et al*. 2003), which is characterized by wide temperature fluctuations as well as salinity and aridity (Sharma *et al*. 1999, Gries *et al*. 2003). Thus, studies on this tree species will improve our understanding of the abiotic stress resistance mechanisms of woody plants (Watanabe *et al*. 2000, Gu *et al*. 2004, Chen *et al*. 2009).

In this study, *PeCBL6* and *PeCBL10*, two members of the *PeCBL* family, homologous with *AtCBL10*, were characterized (Figs. 1, 2). In *Arabidopsis thaliana, PeCBL6* was localized in the nucleus and *PeCBL10* was targeted to the tonoplast membrane (Fig. 3). Transgenic *Arabidopsis* overexpressing *PeCBL6* displayed signifi-cantly increased tolerance to high salinity and cold stress and transgenic plants overexpressing *PeCBL10* improved salinity and drought tolerance at the both seedling or adult stages. The chlorophyll content and photochemical efficiency (F_v/Fm) of *35S-PeCBL6* and *35S-PeCBL10* transgenic plants under stress conditions confirmed that they are more resistant than the WT plants and the mutants (Table 4). Previous studies demonstrated that salinity and drought induced the expression of stress genes such as *RD29A/B*, *KIN1/2*, and *COR* genes in WT plants (Yamaguchi-Shinozaki and Shinozaki 1994, Tahtiharju *et al*. 1997, Gilmour *et al*. 1998, Liu *et al*. 1998, Shinozaki and Yamaguchi-Shinozaki 2000, Kim *et al*. 2009). *DREB1A* (or *CBF3*) is induced specifically by cold (Liu *et al*. 1998). In our experiments, the *KIN1* gene was induced remarkably in *PeCBL6*- and *PeCBL10*-overexpressing plants under salinity. In addition, the expression of the *DREB1A* gene was largely induced under cold stress in *PeCBL6*-overexpressing plants. These results demonstrated that *PeCBL6* and *PeCBL10* might play an important role in coping with various environmental stresses including salinity, drought, and freezing. Further investigation should be made to assess whether overexpressing *CBL* genes in transgenic poplar can enhance its stress tolerance without causing growth retardation.

Here, the expressions of *PeCBL6* and *PeCBL10* in roots are observed stronger than that in leaves and stems (Table 2) no matter whether under control condition or stress treatments which is different from the expression of *CBL10* in *Arabidopsis*. Obviously, while plants are exposed to abiotic stresses, the roots are usually the first to encounter the new environment. Under high salinity, Na^+ sequestration into the vacuoles is one of the most useful methods for managing the stress. After transporting excess Na^+ into the vacuole, the cells reduce water potential and can continue to absorb water. The maintenance of low salt concentration in the cytosol is of great importance for salt adaptation of plants (Greenway and Munns 1980, Munns and Tester 2008, Thapa *et al*. 2011). In this report, *PeCBL10* was targeted to the tonoplast membrane (Fig. 3*G-I*) and might play an important role in ion or water transport. On the other hand, *PeCBL6* was localized in the nucleus, suggesting that it might regulate the expression of some other genes. The fact that *KIN1* and *DREB1A* were induced more in *PeCBL6*-overexpressing plants under stress treatments suggested that *PeCBL6* might enhance the resistance of *P. euphratica* by regulating the expression of transcription factors. These results suggest that *P. euphratica* has faster, more efficient and more sophisticated ways to fight stresses and indicate that the *PeCBL-PeCIPK* complex plays an important role in plant abiotic stress responses. Therefore, further research is still needed to focus on this complicated regulation network.

In conclusion, two CBL-genes, *PeCBL6* and *PeCBL10*, were isolated. Transgenic *Arabidopsis* over-expressing *PeCBL6* displayed significantly increased tolerance to high salinity and cold stress. *PeCBL10* transgenic plants exhibited a stronger tolerance to salt and osmotic stresses in comparison with the control plants. Further experiments are now required to examine their protein-protein interactions using a yeast two-hybrid assays between PeCBLs and PeCIPKs and to define in more detail the biochemical and physiological functions of *PeCBL6* and *PeCBL10* in response to various stresses in poplar.

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