BIOCELL
2008, 32(3): 229-235

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Isolation and molecular characterization of a *cax* gene from *Capsella bursa-pastoris*

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Keywords: *Capsella bursa-pastoris*, cation exchangers (CAXs), RACE, freezing tolerance

ABSTRACT: A new cation exchangers (CAXs) gene was cloned and characterized from *Capsella bursa-pastoris* by rapid amplification of cDNA ends (RACE). The full-length cDNA sequence of *cax* from *C. bursa-pastoris* (designated as *Cbcax51*) was 1754 bp containing a 1398 bp open reading frame encoding a polypeptide of 466 amino-acid residues with a calculated molecular mass of 50.5 kDa and an isoelectric point of 5.69. The predicted CbCAX51 contained an IMP dehydrogenase/GMP reductase domain, two Na^+/Ca^{2+} exchanger protein domains. Comparative and bioinformatics analyses revealed that CbCAX51 showed extensive homology with CAX from other plant species. The expression analysis by different treatments indicated that *Cbcax51* could be activated by cold triggering and was related to the cold acclimation process, but its expression is regulated negatively by drought and not affected by ABA or salt.

Introduction

Plants have multiple mechanisms to maintain appropriate intracellular levels of various ions (Fox and Guerinot, 1998), while plant vacuole plays a major role in the intracellular sequestration of various compounds (Marty, 1999). There are several kinds of transporters on the vacuolar membrane: Na^+/H^+, Ca^{2+}/H^+, Mn^{2+}/H^+, etc., helping to establish a concentration gradient of Na^+, Ca^{2+} and Mn^{2+} across the vacuolar membrane. Calcium (Ca^{2+}) is used by most cells to convert external signals into cytosolic information, which can drive processes that are required for full responses to a particular stimulus (Catala *et al*., 2003). Change in the cytosolic concentration of free Ca^{2+} is the basis for Ca^{2+} serving as a second messenger (Sze *et al*., 2000). Ca^{2+} levels in the cytosol fluctuate in response to growth, development, and environmental perturbations (Sanders *et al*., 1999; Curran *et al*., 2000). The alteration of Ca^{2+} partition is associated with the plants being more sensitive to environmental perturbations, such as cold. Since Ca^{2+} is an extremely common second messenger, localized Ca^{2+} spikes around the plant vacuole are also believed to play a pivotal role in determining signal specificity (Cheng and Hirschi, 2003). Therefore, the study of cation exchangers is important.

In plants, it has been established that Ca^{2+} plays an important role as a second messenger in many important physiological processes. Precise control of the amount of Ca^{2+} in the cytosol is essential for plants'

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Received on November 26, 2007. Accepted on July 3, 2008.

tolerance to some biotic or abiotic stresses. Cation exchangers are part of the ensemble of transporters that help modulate the duration of these Ca^{2+} signaling events (Ueoka-Nakanishi *et al*., 2000). CAX can function as Ca^{2+}/H^{+} and/or heavy metal/H^{+} exchangers (Hirschi *et al*., 2000). The CAX1 from *Arabidopsis* was the first plant gene encoding cation exchangers to be cloned. It was identified by screening a cDNA library from *Arabidopsis* for clones able to complement a yeast mutant defective in vacuolar Ca^{2+} transport. CAX1 may play a role in reducing cytosolic Ca^{2+} concentration to resting levels after Ca^{2+} increase in response to external stimuli. Transgenic tobacco plants overexpressing *cax1* show increased sensitivity to chilling temperatures (Hirschi, 1999), suggesting that it can play a role in plant adaptation to this environmental condition. Presently, more than ten *cax* from *Arabidopsis* have been cloned (Maser *et al*., 2001). From other plant species, *Vrcax1* (*Vigna radiata*) (Ueoka-Nakanishi *et al*., 1999) and *Oscaxs* (*Oryza sativa*) (Kamiya *et al*., 2006) were also cloned. All of the cloned plant *cax*, with the exception of *Oscaxs*, were determined to be localized to vacuolar membranes by expressing the *cax* fused to green fluorescent protein (GFP) gene and/or by immunochemical analysis (Pittman and Hirschi, 2001; Cheng *et al*., 2002; Cheng *et al*., 2003). Plasma and plastid membranes have also been reported to possess Ca^{2+}/H^{+} exchange activity (Kasai and Muto, 1990; Ettinger *et al*., 1999); however, an exchanger has not yet been identified.

However, there has been no report on the cloning of *cax* from *Capsella bursa-pastoris*, which is a wild grass belonging to the *Cruciferae* like *Arabidopsis*. It is considered to be a potential vegetable crop and can grow and set seeds normally at low temperatures. In this paper, we report the cloning of *cax* from *C. bursa-pastoris* by rapid amplification of cDNA ends (RACE)-PCR technology. Bioinformatics analysis and expression analysis revealed that the *C. bursa-pastoris cax* (*Cbcax51*) strongly resembles *cax* from other species, and is relevant to cold acclimation.

Materials and Methods

Plant materials

Seed *of C. bursa-pastoris* were germinated in sterilized soil. The plants were grown greenhouse at Fudan University, Shanghai, China, under continuous light at 28ºC.

Isolation of total RNA

About 1.0g materials of *C. bursa-pastoris* were powdered using liquid nitrogen with mortar and pestle and the total RNA was extracted using TRIZOL Reagent (GIBCO BRL) according to the manufacturer's instructions.

5' RACE of Cbcax51

According to the protocol of the SMART™ RACE cDNA Amplification Kit (Clontech), about 100ng of total RNA was reversely transcribed with primer 5'CDS primer coupled with (dC) tailing and SMART II A oligo, which annealed to the tail of the RNA and served as an extended template for PowerScript RT. The first round of PCR was performed with 5'CBCAX1 and universal primer A Mix (UPM) (provided in the kit). Subsequently, nested PCR was performed under normal PCR reaction conditions using 5'CBCAX2 and the nested universal primer A (NUP) (provided in the kit).

3' RACE of Cbcax51

According to the protocol of Rapid Amplification of cDNA Ends Kit (GIBCO BRL), an aliquot of isolated 100ng total RNA was reversely transcribed using the cDNA synthesis primer AP (provided in the kit). The gene-specific primer 3'caxGSP1 and universal amplification primer (UAP) was used to amplify the 3' end cDNA. Then, the gene-specific primer 3'caxGSP2 and abridged universal amplification primer (AUAP) was used to the nested PCR.

Generation of the full-length cDNA of Cbcax51

By comparing and aligning the sequences of the 5'-RACE and 3'-RACE products, the full-length cDNA sequence of *Cbcax51* was deduced and subsequently amplified with the primers FCBCAX and RCBCAX, using One Step PCR Kit (TaKaRa). All RT-PCR products were purified and cloned into pMD 18-T vector (TaKaRa), and sequenced. All the primers used in RACE were listed in Table 1.

Comparative and bioinformatics analysis

Comparative and bioinformatics analyses of *Cbcax51* were carried using computer programs available online. The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) encoded

by *Cbcax51* were analyzed and sequence comparison was conducted through database search using BLAST program (http://www.ncbi.nlm.nih.gov). Transmembrane analysis was carried out by TMHMM-Prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Expression analysis

To study the expression of the *Cbcax51* under different abiotic stress conditions such as low temperature, ABA, salt stress and drought, total RNA was extracted from seedlings of *C. bursa-pastoris* under various treatments, and then subjected to semi-quantitative reverse transcription (RT)-PCR for *Cbcax51* expression. For low temperature treatments, the 4-week-old seedlings grown under normal conditions were transferred to a growth chamber set to 4ºC for 4h, 8h, and 24h, respectively. For ABA treatment, after the 4-week-old seedlings were sprayed with 100 μM ABA, the seedlings grew for 4h, 8h, and 24h respectively. Salt stress was accomplished by watering plants with 250mM NaCl, after which the seedlings grew for 4h, 8h and 24h respectively. Drought was induced by removing 4-week-old seedling from soil and set them under room temperature for 0.5h, 1h and 4h respectively. After the treatments, leaves were frozen immediately in liquid nitrogen and stored at -70ºC until use. We use three plants per treatment. RT-PCRs using a One Step PCR Kit (TaKaRa) were performed at 50ºC for 30min followed by 20 cycles of amplification (94ºC for 45sec, 60ºC for 45sec, and 72ºC for 1min), using gene-specific primers Fcbcax1 and Rcbcax1 (Table 1). Two primers, 18SF and 18SR (Table 1), were also used to amplify 18S rRNA gene in the semi-quantitative RT-PCR as controls.

Results and Discussion

Cloning of the full-length cDNA of Cbcax51

By comparing and aligning the sequences of the 5'-RACE and 3'-RACE products, the full-length cDNA sequence of *Cbcax51* was deduced and subsequently amplified using One Step PCR Kit with the primers FCBCAX and RCBCAX (Table 1). The full-length cDNA of *Cbcax51* was 1754 bp and contained a 1398 bp complete ORF encoding a polypeptide of 466 of amino acids, a 16 bp of 5'- and 337 bp of 3' - untranslated sequences (Database Accession No.: AY660870). The translation stop codon and one putative polyadenylation signal site were recognized in the 3'-untranslated region, which were followed by a short poly (A) tail (Fig. 1).

TABLE 1.

Primers used for the cloning and analysis of *Cbcax51* gene

Primer	Primer sequence (5'→3')
5'CBCAX1	5'-TCATGTCAGAAACAAGAGCAGCCGG-3'
5'CBCAX2	5'-AGTCTCTGCGGTTGCCACGTATACG-3'
3'caxGSP1	5'- TGAACGC(A/G/C)AC(G/T/A)TGTGGAAACGCGAC-3'
3'caxGSP2	5'- ACCGGAAACAAGCCGATGTGAAC-3'
FCBCAX	5'-TGGTGAAATGGCTGGAATCGTAACAG-3'
RCBCAX	5'-TGGCCAAAACCCACAATGAGGAGTTG-3'
Fcbcax1	5'-CTCCTAATGGGTTTGTTGTGTCACTTGC-3'
Rcbcax1	5'-TCAAGGGTACGACGAATAAGCCAATCTG-3'
UPM	Long:5'-CTAATACGACTCACTATAGGGCAAGGAGTGGTATCAACGCAGAGT-3'
	Short: 5'-CTAATACGACTCACTATAGGGC-3'
NUP	5'-AAGCAGTGGTATCAACGCAGAGT-3'
18SF	5'-ATGATAACTCGACGGATCGC-3'
18SR	5'-CTTGGATGTGGTAGCCGTTT-3'

Characterization of CbCAX51

CAXs have in general 10-14 transmembrane (TM)-spanning domains along 400 amino acid residues. CAXs contain a central hydrophilic motif rich in acidic amino acid residues that bisect the polypeptide into two approximately equal segments (Waditee *et al*., 2004). The *Cbcax51* cDNA contains an open reading frame encoding a deduced 466 amino acids polypeptide with a molecular weight of 50.5 kDa and pI of 5.69 (Fig. 1). According to the result obtained from the PROSITE database (http://www.expasy.org/prosite), the CbCAX51 possess five putative glycosylation sites (Asn-Xxx-Thr/Ser), eight putative phosphor sites and nine myristyl sites. According to the result obtained from InterProScan (http://www.ebi.ac.uk /interpro/scan.html), the CbCAX51 contains an IMP dehydrogenase/GMP reductase domain and two Na^+/Ca^{2+} exchanger protein domains (Fig. 2A). The transmembrane analysis of CbCAX51, which was carried out online by SPLIT 4.0 SERVER, showed that like

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ACGCGGGGGTGGTGAA***ATG***GCTGGAATCGTAACAGAACCGTGGGCATCGGCGGAGAACGGAAACGCAAGCATGAC
            M  A  G  I  V  T  E  P  W  A  S  A  F  N  G  N  A  S  M  T
AGCGAAAGGATCGAGCAGAGAACTGAGACATGGAAGAACAGCTCACAACATGTCATCTTCTTC TCTAAGGAAGAA
 A  K  G  S  S  R  E  L  R  H  G  R  T  A  H  N  M  S  S  S  S  S  L  R  K  K
GTCAGACCTCCGAGTGATTCAGAAGGTTCCATACAAAGGTCTCAAAGATTTTCTCACCAATCT CCAAGAAGTCAT
 S  D  L  R  V  I  Q  K  V  P  Y  K  G  L  K  D  F  L  T  N  L  Q  E  V  I
CCTCGGCACTAAGCTCGCCATTCTTTTCCCGGCCATTCCTGCCGCCATTATCTGCACCTATTG TGGCGTCAGTCA
 L  G  T  K  L  A  I  L  F  P  A  I  P  A  A  I  I  C  T  Y  C  G  V  S  Q
GCCGTGGATATTTGGACTGAGCATGCTAGGACTGACACCTTTGGCTGAACGAGTCAGCTTTCT GACAGAGCAACT
 P  W  I  F  G  L  S  M  L  G  L  T  P  L  A  E  R  V  S  F  L  T  E  Q  L
AGCTTTCTACACCGGTCCAACATTGGGTGGTCTACTGAACGCAACGTGTGGAAACGCGACTGA ATTGATAATTGC
 A  F  Y  T  G  P  T  L  G  G  L  L  N  A  T  C  G  N  A  T  E  L  I  I  A
GATTCTGGCATTGACCAATAATAAGGTCGCAGTGGTGAAATATTCGCTGTTAGGTTCGATTTT GTCAAATCTTCT
 I  L  A  L  T  N  N  K  V  A  V  V  K  Y  S  L  L  G  S  I  L  S  N  L  L
TTTAGTTCTAGGGACTTCACTTCTCTGCGGCGGAATCGCTAATATCAGGAGGGAGCGGCGGTT CGACCGGAAACA
 L  V  L  G  T  S  L  L  C  G  G  I  A  N  I  R  R  E  R  R  F  D  R  K  Q
AGCGGATGTGAACTTCTTCTTACTCCTAATGGGTTTGTTGTGTCACTTGCTCCCATTGATGTT CGTATACGTGGC
 A  D  V  N  F  F  L  L  L  M  G  L  L  C  H  L  L  P  L  M  F  V  Y  V  A
AACCGCAGAGACTCCGGCTGCTCTTGTTTCTGACATGACACTGACTCTGTCGCGGGGCAGCAG TATTTTTATGTT
 T  A  E  T  P  A  A  L  V  S  D  M  T  L  T  L  S  R  G  S  S  I  F  M  L
GATCGGTTACATTGCATATCTCGTTTTCCAGCTTTGGTCTCACCGCCAATTGTTCGACGCACA AGATCAGGAAGA
 I  G  Y  I  A  Y  L  V  F  Q  L  W  S  H  R  Q  L  F  D  A  Q  D  Q  E  D
TGAGTATGATGACGATGTAGAGGAAGAAACCGCGGTGATTAGTTTTTGGAGCGGTTTTGCTTG GTTGGTTGGGAT
 E  Y  D  D  D  V  E  E  E  T  A  V  I  S  F  W  S  G  F  A  W  L  V  G  M
GACCCTTGTCATCGCATTGCTATCCGAGTATGTTGTTGCCACCATTGAGACCGCATCGGAATC ATGGAACCTATC
 T  L  V  I  A  L  L  S  E  Y  V  V  A  T  I  E  T  A  S  E  S  W  N  L  S
AGTAAGTTTCATAAGCATCATATTGCTTCCCATTGTTGGAAATGCGGCTGAACACGCTGGAGC CATCATTTTTGC
 V  S  F  I  S  I  I  L  L  P  I  V  G  N  A  A  E  H  A  G  A  I  I  F  A
CTTTAAGAACAAGCTCGACATATCATTGGGAGTTGCATTAGGCTCTGCGACTCAGATTGGCTT ATTCGTCGTACC
 F  K  N  K  L  D  I  S  L  G  V  A  L  G  S  A  T  Q  I  G  L  F  V  V  P
CTTGACCATAATCGTGGCGTGGATTCTAGGAATTAATATGGATCTTAACTTCAATCTCCTCGA AACCGGTTCTCT
 L  T  I  I  V  A  W  I  L  G  I  N  M  D  L  N  F  N  L  L  E  T  G  S  L
TGCTCTTTCCATTATCATCACTGCCTTCACATTACAGGATGGGACTTCACACTACATGAAGGG ACTGGTCCTCTT
 A  L  S  I  I  I  T  A  F  T  L  Q  D  G  T  S  H  Y  M  K  G  L  V  L  L
GCTTTGCTATTTCATTATTGCCTTCTGTTTCTTCGTCGACAAACTTCCTCAGAAACAACCAAA TGGTTTTCACAT
 L  C  Y  F  I  I  A  F  C  F  F  V  D  K  L  P  Q  K  Q  P  N  G  F  H  M
GGGACTTCAACAGATAAACAATGTTGTCACTGGAATCACTGGAACAGGAGGAGCTTCTTCAACT***TAA***ATGAAGTA
 G  L  Q  Q  I  N  N  V  V  T  G  I  T  G  T  G  G  A  S  S  T
CCAATGTTTTTTGGAAACAACATTTCCAAACCGAGACAATGGAGCATCGAAAAGAAAAAACAA AAAAACTTAAAC
CCCAAATATTCTTGATTCATTTTATGAAAGATTTGTCAGGGAGATTCCAGTGTTATTTCATAT TTTTTGGATGTT
TGGTGTCTTGATGAAGATGAGTCTTGATAAGTTTCTCAACTCCTCATTGTGGGTTTTGGCCAA TTGATGTTTTGA
TGTTGTCTCTCTATCTCTTTATGTTAATGTTTTTCCTCCTGAGCAGTTAAATAAAAATTATTT GATTTCTGTTCA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIGURE 1. The full-length cDNA sequence and the deduced amino acid sequence of *Cbcax51*. The start codon (ATG) and the stop codon (TAA) are shown in bold italically. The N-terminal regulatory region (NRR) is shown in wave underline, Ca^{2+} domain is shown in double underline, and a central hydrophilic motif is underlined.

AtCAX1 from *A. thaliana*, there were eleven predicted transmembrane helix positions (Fig. 2B). The CbCAX51 contain a central hydrophilic motif, rich in acidic amino acid residues (Fig. 1).

Comparative and bioinformatics analysis

Sequence alignment using Vector NTI Suite 8.0 and BLAST research showed that the predicted amino acid sequence of CbCAX51 had high identity with CAXs from other plant species, such as AtCAX1 (NP_181352), AtCAX2 (NP_566452), AtCAX3 (AAV85729), AtCAX4 (NP_568091) from *A. thaliana* and OsCAX1c (BAD83661) from *O. sativa*, with the identity of 87.8%, 41.9%, 76.8%, 53.7% and 47.5% respectively (Fig. 3). It demonstrated that CbCAX51 has high identity in structure and sequence to the previously characterized AtCAX1 from *A. thaliana* in its primary sequence. AtCAX1 functions as a cation exchanger and has been analyzed using yeast heterologous expression technology and T-DNA insertion mutant experimental systems (Cheng *et al*., 2003). Therefore, CbCAX51 might be the counterpart of AtCAX1 that modulated plant cation exchangers.

Expression analysis

Semi-quantitative RT-PCR was carried out to investigate the expression of *Cbcax51* under different abiotic stress. The *Cbcax51* expression was induced transiently in leaves, reaching a maximum level after 24h of exposing plants to low temperature (Fig. 4A). This result indicated that *Cbcax51* could be activated by cold triggering and was related to the cold acclimation process. It has been reported that the expression of *Atcax1* from *A. thaliana* was induced in response to stimuli such as low temperature in order to regulate the calcium content in cytosol. The changed cytosol calcium content activated the *cbf* gene expression-regulated pathway and transcriptional cascade leading to the expression of cold-responsive genes under cold stress (Wang *et al*., 2005), by activating the promoter of *cbf* gene to induce its expression. The CBF subsequently binds the DRE/CRT element in the promoter of the cold-regulated genes and activated transcription of them, thereby increasing plants' freezing tolerance. Therefore, it is possible that *Cbcax51*, which strongly resembled *Atcax1*, acts as a similar transcriptional regulator in *cbf* gene expression-

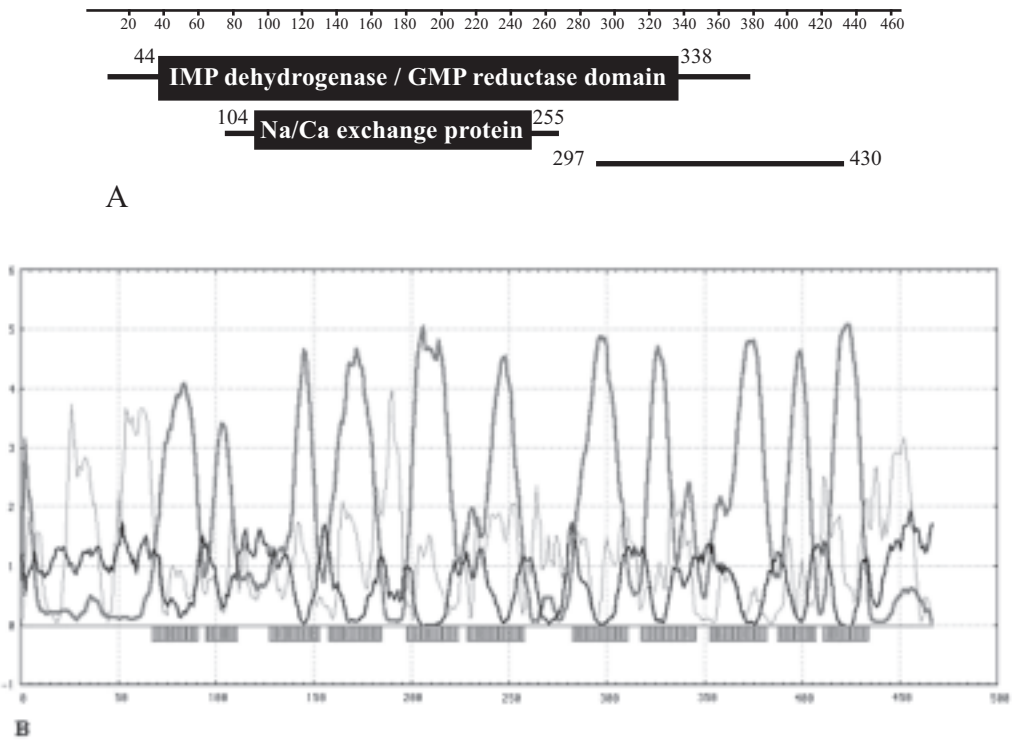

FIGURE 2. A. Predicted motifs of CbCAX51; **B.** Transmembrane analysis by SPLIT 4.0 SERVER. Gray line: predicted transmembrane helix preference (THM index); black line: Beta preference. (BET index); French Gray line: Modified hydrophobic moment index (INDA index); Gray boxes (below abscisa): Predicted transmembrane helix position. (DIG index).

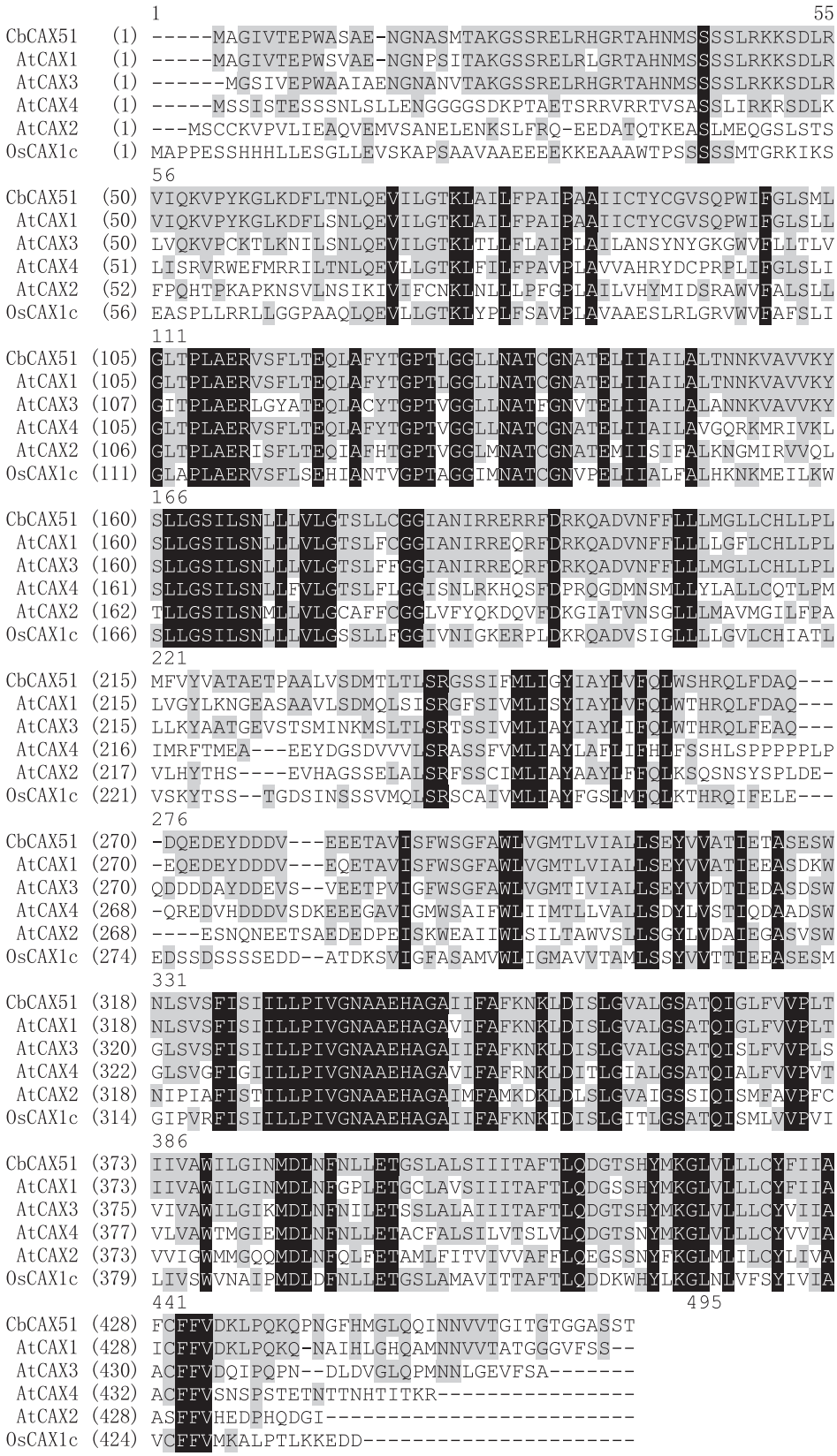

FIGURE 3. Multi-alignment of amino acid sequences of CbCAX51 and other CAX proteins. The identical amino acids are shown in black background and the conserved amino acids are shown in gray background. The aligned CAXs include sequences from *C. bursa-pastoris* (AY660870), *A. thaliana* (NP_181352, NP_566452, AAV85729 and NP_568091), and *O. sativa* (BAD83661).

regulated pathway in response to cold stress. Because many cold-inducible genes also are responsive to exogenous ABA and osmotic stresses (Thomashow, 1999), the effect of ABA, high salt and drought on *Cbcax51* mRNA accumulation was also analyzed. The expression of *Cbcax51* is not affected by ABA or high salt treatment; the gene transcripts were almost at the same level during the different time periods whether under ABA stress or high salt (not shown). Figure 4B shows that transcript level was lower than the control after the 4h dehydration treatment. Therefore, we infer that it is regulated negatively by drought. Overall, *Cbcax51* expression was inhibited by drought but was not affected by ABA and high-salt treatments, which is consilient with the report about the *Atcax1* in *A. thaliana* (Catala *et al*., 2003). Furthermore, although the significance of this expression pattern is not clear at present, it indicates that during *C. bursa-pastoris* development and in response to different adverse environmental situations, the regulation of *Cbcax51* is accomplished through several ABA-independent signal transduction pathways.

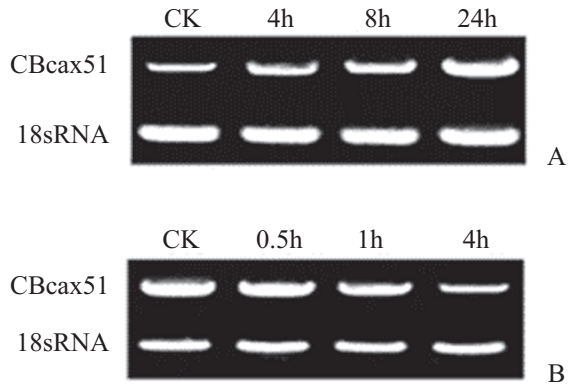

FIGURE 4. Expression pattern of *Cbcax51* by semi-quantitative RT-PCR analysis in response to different treatments. **A:** RT-PCR analysis with total RNA obtained from leaves of the seedling exposed at 4°C for 4h, 8h and 24h respectively; **B:** RT-PCR analysis with total RNA obtained from leaves of seedling that were removed from the soil and kept under room temperature for 0.5, 1 and 4h respectively. Equal RNA loading was used as the control, by amplifying *18S rRNA* for each treatment. CK indicates the control.

Acknowledgements

This research is financially supported by China National "863" High-Tech Program (2008AA10Z105), National Science Fund for Fostering Talents in Basic Science, Nation Science Education Base at Fudan University (J0630643).

References

Catala R, Santos E, Alonso JM, Ecker JR, Martinez-Zapater JM, Salinas J (2003). *Mutations in the Ca^{2+}/H^+ transporter CAX1 increase CBF/DREB1 expression and the cold-acclimation response in Arabidopsis*. Plant Cell 15: 2940-2951.

Cheng NH, Hirschi KD (2003). *Cloning and characterization of CXIP1, a novel PICOT domain containing Arabidopsis protein that associates with CAX1*. J Biol Chem. 278: 6503-6509.

Cheng NH, Pittman JK, Shigaki T, Hirschi KD (2002). *Characterization of CAX4, an Arabidopsis H^+/cation antiporter*. Plant Physiol. 128: 1245-1254.

Curran AC, Hwang I, Corbin J, Martinez S, Rayle D, Sze H, Harper JF (2000). *Autoinhibition of a calmodulin-dependent calcium pump involves a structure in the stalk that connects the transmembrane domain to the ATPase catalytic domain*. J Biol Chem. 275: 30301-30308.

Ettinger WF, Clear AM, Fanning KJ, Peck ML (1999). *Identification of a Ca^{2+}/H^+ antiport in the plant chloroplast thylakoid membrane*. Plant Physiol. 119: 1379-1386.

Fox TC, Guerinot ML (1998). *Molecular biology of cation transport in plants*. Annu Rev Plant Physiol Plant Mol Biol. 49: 669-696.

Hirschi KD (1999). *Expression of Arabidopsis CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity*. Plant Cell 11: 2113-2122.

Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ (2000). *Expression of Arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance*. Plant Physiol. 124: 125-133.

Kamiya T, Akahori T, Ashikari M, Maeshima M (2006). *Expression of the vacuolar Ca^{2+}/H^+ exchanger, OsCAX1a, in rice: cell and age specificity of expression, and enhancement by Ca^{2+}*. Plant Cell Physiol. 47: 96-106.

Kasai M, Muto S (1990). *Ca^{2+} pump and Ca^{2+}/H^+ antiporter in plasma membrane vesicles isolated by aqueous two-phase partitioning from corn leaves*. J Membr Biol. 114: 133-142.

Marty F (1999). *Plant vacuoles*. Plant Cell 11: 587-600.

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001). *Phylogenetic relationships within cation transporter families of Arabidopsis*. Plant Physiol. 126: 1646-1667.

Pittman JK, Hirschi KD (2001). *Regulation of CAX1, an Arabidopsis Ca^{2+}/H^+ antiporter. Identification of an N-terminal autoinhibitory domain*. Plant Physiol. 127: 1020-1029.

Sanders D, Brownlee C, Harper JF (1999). *Communicating with calcium*. Plant Cell 11: 691-706.

Sze H, Liang F, Hwang I, Curran AC, Harper JF (2000). *Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast*. Annu Rev Plant Physiol Plant Mol Biol. 51: 433-462.

Thomashow MF (1999). *Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms*. Annu Rev Plant Physiol Plant Mol Biol. 50: 571-599.

Ueoka-Nakanishi H, Nakanishi Y, Tanaka Y, Maeshima M (1999). *Properties and molecular cloning of Ca^{2+}/H^+ antiporter in the vacuolar membrane of mung bean*. Eur J Biochem. 262: 417-425.

Ueoka-Nakanishi H, Tsuchiya T, Sasaki M, Nakanishi Y, Cunningham KW, Maeshima M (2000). *Functional expression of mung bean Ca^{2+}/H^+ antiporter in yeast and its intracellular localization in the hypocotyl and tobacco cells*. Eur J Biochem. 267: 3090-3098.

Waditee R, Hossain GS, Tanaka Y, Nakamura T, Shikata M, Takano J, Takabe T, Takabe T (2004). *Isolation and functional characterization of Ca^{2+}/H^+ antiporters from cyanobacteria*. J Biol Chem. 279: 4330-4338.

Wang X, Sun X, Liu S, Liu L, Liu X, Sun X, Tang K (2005). *Molecular cloning and characterization of a novel ice gene from Capsella bursa-pastoris*. Mol Biol. 39: 21-29.