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***Full Length Article***

# Characterization of *CbCAX51*, a Cold Responsive $Ca^{2+}/H^{+}$ Exchanger from *Capsella bursa-pastoris* Modulating Cold Tolerance in Plants

**Highlighted References are Missing**

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## Abstract

The *CAX* (Calcium Exchanger) genes constitute a set of signal transductors that are important for plant growth and adaptation to environmental stresses, especially to low temperature. *CbCAX51*, a $Ca^{2+}/H^{+}$ exchanger was cloned form *Capsella bursa-pastoris*, has been reported as a cold inducible gene. Here we showed that the transcriptional level of *CbCAX51* in leaf and stem was higher than that in roots. Transcript of *CbCAX51* was continuously up-regulated by $Ca^{2+}$ and $Cu^{2+}$, and transiently up-regulated by $Zn^{2+}$ and $Li^{+}$, cold stress, GA and ABA. The CbCAX51 protein was predicted to be localized in vacuolar membrane by PSORT prediction, which was further confirmed by the subcellular detection of CbCAX51-GFP fusion protein. When *CbCAX51* was transformed into the cold-sensitive plant tobacco, it conferred cold tolerance in tobacco seedlings based on physiological studies. Likewise, overexpression of *CbCAX51* can activated some cold responsive genes in transgenic tobacco. Taken together, *CbCAX51* is involved in the cold signal transduction and modulation of downstream components to enhance cold tolerance in tobacco. © 2017 Friends Science Publishers

**Keywords:** *Capsella bursa-pastoris*; CbCAX51; $Ca^{2+}/H^{+}$ exchanger; Cold inducible gene; Cold tolerance

## Introduction

Calcium ion ($Ca^{2+}$), as second messenger, has a critical role in a variety of organisms, including plants. Like animals, plant cells induce a transient increase of $Ca^{2+}$ concentration in the cytoplasm in responses for a stimulus (Catala *et al*., 2003). After the transient elevation, cytosolic $Ca^{2+}$ activates transport systems, such as $Ca^{2+}$ pumps ($Ca^{2+}$-ATPases) and $Ca^{2+}/H^{+}$ exchangers (CAXs) at both the tonoplast and plasma membrane. Most of the cytosol $Ca^{2+}$ will be transported to intracellular organelles, the tonoplast the apoplast, resulted in plant cells with low cytosolic calcium (Sze *et al*., 2000; Hirschi, 2001). CAXs were firstly identified from yeast and then their diverse functions and molecular properties were identified in plants (Cunningham and Fink, 1996). At present, many CAX genes have been cloned from multiple plant species (Hirschi *et al*., 1996; Ueoka-Nakanishi *et al*., 1999; Maser *et al*., 2001; Kamiya and Maeshima, 2004; Pittman *et al*., 2004; Kamiya *et al*., 2005; 2006; Shigaki and Hirschi, 2006; Shigaki *et al*., 2006; Mei *et al*., 2009; Edmond *et al*., 2009; Manohar *et al*., 2011). In *Arabidopsis thaliana*, there are six genes encoding putative CAX proteins, among which *AtCAX1*, *AtCAX3* and *AtCAX4* specifically have high affinity to $Ca^{2+}$, whereas *AtCAX2* and *AtCAX5* have high affinity to other metal cations $Cd^{2+}$ and $Mn^{2+}$, including $Ca^{2+}$ (Hirschi *et al*., 2000; Cheng *et al*., 2003; Shigaki *et al*., 2006; Pittman and Hirschi, 2016). Correspondingly, these CAXs improve $Ca^{2+}$ accumulation (Hirschi, 1999), enhance tolerance to $Cd^{2+}$ (Korenkov *et al*., 2007a, b; Wu *et al*., 2011), and $Li^{+}$ and $Na^{+}$ (Luo *et al*., 2005) when expressed constitutively in plant tissues. In addition, some of CAXs are also involved in plant various stresses responses, including low temperatures, dehydration and high salt (Knight *et al*., 1996; Pittman *et al*., 2009; Han *et al*., 2011). Nevertheless, the fact that *Atcax*1 mutant showed stronger cold acclimation response and *CAX1* transgenic tobacco had higher cold sensitivity, but *cax3* mutant exhibited reduced salt tolerance, these demonstrated that different CAXs have distinct characteristics and play diverse roles in plant adaptation to various environmental conditions (Hirschi, 1999; Catala *et al*., 2003; Zhao *et al*., 2008).

Cold affects the fluidity of cellular membrane and increases their rigidity to induce the cell skeleton rearrangement in plants. The physical change of membrane is the basement biosensor which improves the $Ca^{2+}$ level (Yamamoto *et al*., 1989) and affects downstream cold responsive genes (Örvar *et al*., 2000). Cold damage in plant may be due to lack of calcium homeostasis and subsequent calcium toxicity, so the change of $Ca^{2+}$ levels is a key step in a cold-sensing mechanism that enables plants to adapt to it (Minorsky and Spanswick, 1989). *CAXs* participate in these



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$Ca^{2+}$ signaling and the changes of intracellular $Ca^{2+}$ levels were response to temperature, high salt and drought in transgenic plants/crops (Zhao *et al*., 2008; Xu *et al*., 2013; Yamada *et al*., 2014). The *CbCAX51* is a cold induced gene cloned from *Capsella bursa-pastoris* (Lin *et al*., 2008), which has a stronger capacity to prevent the cold damage than many other plants grown up in temperate district (Wu *et al*., 2012). But, the function of *CbCAX51* in dealing with damages caused by cold stress and how *CbCAX51* affects chilling responsive gene expression remain largely unknown.

## Materials and Methods

### Plant Materials

The seeds of *A. thaliana* Col-0 (Columbia) were obtained from ABRI: Columbus, OH, USA (the Arabidopsis Biological Resource Center). The seeds of *C. bursa-pastoris* were purchased from Shanghai Baiyulan Vegetable Seed Co. Ltd. The seeds of *N. benthamiana* or *N. tabacum* were stored in our Lab as previous described (Zhou *et al.*, 2012), the onion was purchased from Shanghai local market.

### Stress Treatment of *C. bursa-pastoris* and Transgenic Tobacco

To analyze the gene expression for cold induction, we treated the 28-day-old *C. bursa-pastoris* at 4°C for 4 h, 8 h, 24 h or 48 h. Meanwhile the seedlings were shifted from 22°C to 12°C for 4 days, 4°C for 4 days and 0°C for 2 h in turn for cold acclimation. For treatments of phytohormones, 16 day-old seedlings were respectively soaked with 5 μM GA (gibberllic acid), 300 μM MeJA (methyl jasmonate), 20 μM IAA (indole-3-acetic acid), 100 μM ABA (abscisic acid) solution for 1 h and 6 h. For treatments of ionic liquid, seedlings were respectively soaked with 80 mM KCl, 50 mM $MgCl_2$, 5 mM $ZnCl_2$, 30 mM LiCl, 0.1 mM $CuCl_2$, 80 mM $CaCl_2$ solution for 1 h, 4 h, 8 h and 24 h. For chilling and freezing treatments of tobacco, seedlings grown in the greenhouse at 22°C under a 16 h/8 h (light/dark cycle) for 21 days were shifted to 4°C for 24 h, then −4°C for 1h, 22°C for 2d.

### RNA and DNA Extraction and *CbCAX51* Sequence Amplification

Total RNA of the roots, stems and leaves from *C. bursa-pastoris* and the seeding from *N. tabacum* were extracted and determined according to the description in the previous reference (Zhou *et al.*, 2012). The cDNA was obtained using PrimeScript® RT Master Mix (TaKaRa Biotechnology (Dalian) Co., Ltd., China). The DNA extraction was conducted using CTAB method. The amplification of *CbCAX51* encoding sequence and promoter sequence, as well as the PCR procedure was described in reference (Zhou *et al*., 2010).

### Vector Construction

To construct overexpression vector p35S::CbCAX51, the *CAX51* cDNA sequence was inserted into the *Nco*I/*Nhe*I site of the pCAMBIA1304 vector (CAMBIA, Australia) replacing *GUS* gene with primers CbCAX-nco-F and CbCAX-nhe-R. To analyze the protein localization, *CbCAX51* cDNA sequence was cloned into *Nco*I/*Bgl*II site of the pCAMBIA1302 vector (CAMBIA, Australia) and fused with *GFP* gene with primers CbCAX-nco-F and CbCAX-bgl-R. To analyze the promoter activity, *CbCAX51* promoter sequences were cloned into the *Kpn*I/*Nco*I site of the pCAMBIA1301 vector (CAMBIA, Australia) before *GUS* gene with primers CbCAX-pr-F and CbCAX-pr-R. All primers sequences used in vector construction were listed in Table S1.

### Plant Transformation and Confirmation

The p35S::CbCAX51 vector was transferred into tobacco cells by leaf discs transformation method using Agrobacterium EHA105 based on the previous descriptions (Zhou *et al.*, 2014). The p35S::CbCAX51-GFP or pCbCAX51::GUS vectors was transferred into *Arabidopsis* (Col-0) by floral dip method using Agrobacterium GV3101 according to Lin *et al.*, 2016. The T1 positive transformers of tobacco were screened with hygromycin (25 mg/L) and confirmed by PCR. T3 and T4 homozygous lines were used for further analyses. The p35S::CbCAX51-GFP construct was transferred into *N. benthamiana* leaves and onion epidermis for transient gene expression assay based on the previous descriptions (Wu *et al.*, 2012).

### Fluorescence and Luminescence Imaging

The confocal laser scanning microscope (Zeiss 710, Germany) were used to observe the fluorescence signals. The images were analyzed with Zen software.

### Histochemical GUS Staining

Leaf, stem and root samples were collected from pCbCAX51::GUS transformants lines of *Arabidopsis* (Col-0) at 26°C and 4°C treatment for 1 d. GUS staining assay can refer the method described in reference (Lin *et al.*, 2016). The samples were then implanted with Technovit 7100 plastic embedding kit (Heraeus Kulzer, Germany) and semi-sectioned using Leica 2265 Rotary Microtome (Leica, Germany). The GUS activity was observed with Zeiss Scope A1 Microscope (Zeiss, Germany).

### Fluorescence Quantitative RT-PCR

The cDNA was synthesized by PrimeScript® RT Master Mix (TaKaRa). The FQ-PCR was carried out using real-time primers (Table 1) and by SYBR® Premix Ex Taq™ II (Perfect Real-Time; TaKaRa) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

**Table S1:** Primers for gene amplification and FQ-PCR in this paper

| Name | Sequence (5'--3') |
|---|---|
| CbCAX-F | ATGGCTGGAATCGTAACAGAAC |
| CbCAX-R | AGTTGAAGAAGCTCCTCCTGTT |
| CbCAX-nco-F | GCccatggTAATGGCTGGAATCGTAACAGAAC |
| CbCAX-bgl-R | GAagatctTCAGTTGAAGAAGCTCCTCCTGTT |
| CbCAX-nhe-R | CTgctagcTA AGTTGAAGAAGCTCCTCCTGTT |
| CbCAX-pr-F | GggtaccGTTATGAAGTTATTACCTCCCCGTTA |
| CbCAX-pr-R | TccatggCCTGAGGCACGCACTGACTCAAA |
| Hyg-F | GTCGAGAAGTTTCTGATCG |
| Hyg-R | GTTTCCACTATCGGCGAGTACT |
| GUS-F | GCTCTACACCACGCCGAACACCTG |
| GUS-R | TCTTCAGCGTAAGGGTAATGCGAGGTA |
| R-CbCAX51-F | CATTAGCCAACATTCCTCCTCCGACCA |
| R-CbCAX51-R | CTGACTGAAACAGACCTCTACGCTTG |
| R-NtDREB1-F | CAGGTAAGTGGGTGTGTGAAGTG |
| R-NtDREB1-R | TGCGATCTCGGCTGTTAGG |
| R-NtDREB3-F | TACAGGGGAGTGAGGAAGAGGA |
| R-NtDREB3-R | GCAGAAGGGAAAGTGCCAAG |
| R-NtTSI1-F | CCGTGGAGAGAAAAGCGAAA |
| R-NtTSI1-R | TCAAACCCAAAAGCGTCCA |
| R-NtERD10a-F | TGAGAAGAAGGGAATTATGGACAAG |
| R-NtERD10a-R | CGCAGCAGATTTTCTAGTGGTG |
| R-NtERD10b-F | ATCACACTGGAGGTACCATGGG |
| R-NtERD10b-R | CTTCTTCCTTCTTCCGCCTTG |
| R-NtActin-F | GGAAAGTCCTACCAGCATTG |
| R-NtActin-R | ATCTATTGTCTCCCACGAAG |
| R-GUS-F | GCTCTACACCACGCCGAACACCTG |
| R-GUS-R | TCTTCAGCGTAAGGGTAATGCGAGGTA |
| R-actin2F | TGAGAGATTCAGATGCCCAGAA |
| R-actin2R | TGGATTCCAGCAGCTTCCAT |

The reactions were performed in three technical replicates per sample for each run and each sample was tested on three biological replicates. The FQ-PCR procedure was described in reference (Zhou *et al.*, 2014). The *ACTIN* gene (HQ880662) from *C. bursa-pastoris*, the *ACTIN2* (AK230311) gene from *A. thaliana* and the *GAPDH* B-subunit gene (M14418) from *N. tabacum* were used as the control.

**Physiological Indexes Measurement**

Collected the leaves of each tobacco line after each treatment, incubated in 4 mL deionized water for 12 h. Ion leakage was measured with a DDS-11A conductivity meter (Shanghai SUOSHEN Electrical Equipment Co. Ltd., China) by the method from previous article (Wu *et al.*, 2012). Collected the leaves of each tobacco line after each treatment. The turgid weights (TW) of each sample and glucose content were measured by the method from previous article (Wu *et al.*, 2012). Three repeated experiments were performed and the data were analyzed by Student's *t*-test.

## Results

**Cold, Various Cations and Phytohormones can Induce *CbCAX51* Expression in *C. bursa pastoris***

Previous reports have established that several *CAX* genes from plants can be induced by the environmental stimuli and metal ion stresses, such as *AtCAX1* that was moderately induced by $Ni^{+}$, $Na^{+}$, nitrate and osmotic stress but highly induced by $Ca^{2+}$ in the media (Wang *et al.*, 2000). In addition, the expression of *AtCAX1* is induced specifically in leaves to cold, but repressed by dehydration, and not influence by ABA or by high salt (Catala *et al.*, 2003). To test the tissue specific expression pattern and the effects of various cation and abiotic stresses on *CbCAX51*, we analyzed its transcript levels by FQ-PCR. As shown in Fig. 1, *CbCAX51* was normally expressed in all three tissues with high level in leaves, middle in stems and low in roots (Fig. 1A). Under cold treatments, *CbCAX51* transcript level was transiently elevated to a peak in leaves, stems and roots at 4°C in 24 h, and then decreased at 48 h (Fig. 1A). A significant enrichment was also observed in leaves, roots and stems with the decrease of temperature during cold acclimation (Fig. 1B). Again the transcript level of *CbCAX51* in leaves was relatively higher than in stems and roots. These indicate that Cb*CAX51* can be transiently induced especially in leaves is in a temperature dependent manner.

The *CbCAX51* promoter contains several cis-acting elements involved in ABA, MeJA, IAA and GA (Zhou *et al.*, 2010). In the study, we analysis the expression of *CbCAX51* were response to these hormones. The results showed that *CbCAX51* was transiently activated at 1 h of ABA and GA application and gradually down-regulated by MeJA from 1 h to 6 h but not changed by IAA from 1 h to 6h (Fig. 1C). In the seedlings of *C. bursa-pastoris*, *CbCAX51* was transiently induced at 4 h of the LiCl and $ZnCl_2$ treatments and gradually up-regulated by $CaCl_2$ and $CuCl_2$ from 4 h to 24 h. In KCl and $MgCl_2$ treatments, *CbCAX51* was significantly repressed (Fig. 1D).

**CbCAX51 is Localized to Vacuolar Membranes of Cell**

Majority of the plant *CAXs*, such as *CAX1*, *CAX2*, *CAX3*, *CAX4* from *A. thaliana*, *OsCAX1a* from *O. sativa*, *Put-CAX1* from *P. tenuiflora* and *SsCAX1* from *S. salsa*, have been shown to be localized in vacuolar membranes (Hirschi *et al.*, 2000; Cheng *et al.*, 2002; 2005; Kamiya *et al.*, 2006; Liu *et al.*, 2009). In addition, some CAXs such as OsCAX3 from *O. sativa* and GmCAX1 from *G. max* are subcellular localized in plasma and plastid membranes (Qi, 2005; Luo *et al.*, 2005). For CbCAX51, no N-terminal signal peptide was found in PSORT analysis (http://psort.nibb.ac.jp) and Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) showed a prediction of vacuolar localization. For determine the localization of the CbCAX51 protein, GFP was the genetically fused to the C-terminus of CbCAX51 driven by a CaMV35S promoter (Fig. 2A) and then transiently expressed in *N. benthamiana* and onion epidermal cells. The results showed that the CbCAX51-GFP fusion protein was localized only around the periphery of tobacco leaves, quite different from the GFP control and DAPI-labeled nucleus

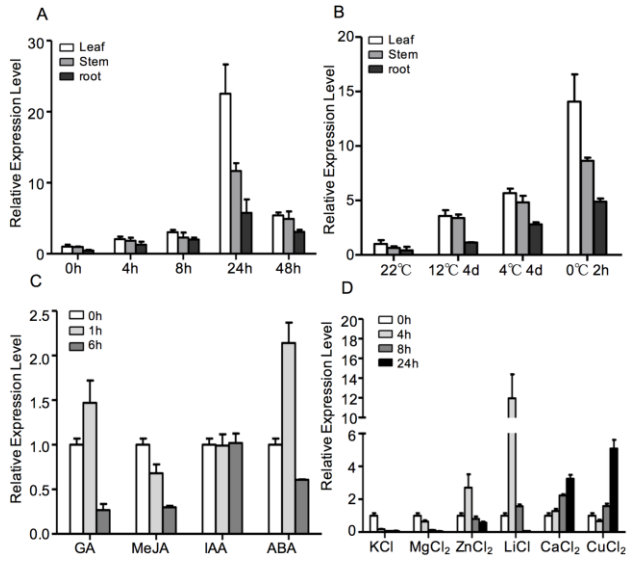


**Fig. 1:** Changes in *CbCAX51* mRNA in response to cold, metal ions and hormones using FQ-PCR analysis. *C. bursa-pastoris* was grown in the nutrient solution at 22°C under a 16 h light/8 h dark cycle. (A) The 28-day-old seedlings were treated at 4°C for 0, 4, 8, 24, and 48 h. (B) The 28-day-old seedlings at 22°C were shifted to 12°C for 4 days, 4°C for 4 days, 0°C for 2h in turn. (C) The 16-day-old seeding were treated with 5 μM GA, 300 μM MeJA, 20 μM IAA, 100 μM ABA for 1 and 6 h at 22°C under light. (D) The 16-day-old seeding were treated with 80mM KCl, 50 mM $MgCl_2$, 5 mM $ZnCl_2$, 30 mM LiCl, 0.1 mM $CuCl_2$, 80 mM $CaCl_2$ for 4, 8 and 24 h at 22°C under light. The *Actin* gene from *C. bursa-pastoris* (HQ880662) was used for normalization and the results were plotted as a ratio relative to the value in leaf of control seedlings

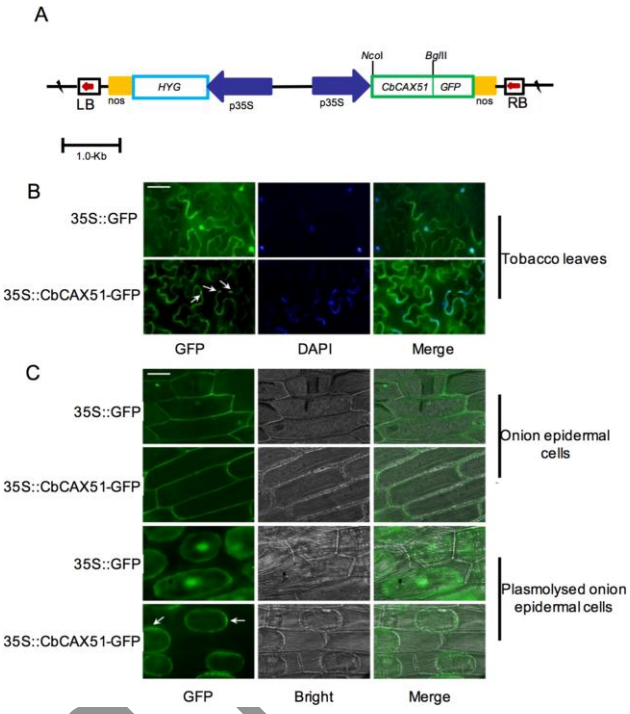


**Fig. 2:** Subcellular localization of the CbCAX51-GFP fusion protein in tobacco leaves and onion epidermal cells. (A) The CbCAX51-GFP fusion gene was under the control of the 35S promoter. (B) The CbCAX51-GFP was transiently expressed in tobacco leaves and DAPI staining was used to visualize the nucleus. (C) GFP signal of CbCAX51-GFP fusion protein was observed before and after plasmolysis in onion epidermal cells. The GFP (GFP), bright-field (bright) and merged (merge) images are shown. White arrows indicate the vesicle membrane space. The bars represent 10 μm

(Fig. 2B). Meanwhile, CbCAX51-GFP could be visualized in small vesicle like structures (as indicated by white arrows in Fig 2B) in the vacuolar membranes of tobacco leaves. In order to determine the localization of CbCAX51-GFP fusion protein more precisely, we expressed CbCAX51-GFP in onion epidermis with plasmolysis in 2 g/mL sucrose solution. The green fluorescence was clearly detected in vacuolar membranes of cell (Fig. 2C). So, it is through that CbCAX51 is a vacuolar membranes protein.

## CbCAX51 Promoter Exhibited Cold Inductive Activity

For better understanding of inductive and tissue specific patterns of *CbCAX51* expression in *A. thaliana*, three independent lines of 14 day-old transformed with pCbCAX51::GUS vector was tested using the histochemical GUS staining assay in 22°C and 4°C, respectively. The vector containing the promoter region (-1,152 to +10 bases) was fused with the GUS gene (Fig. 3A). FQ-PCR revealed the enhancement of GUS mRNA level in after 24 h of 4°C treatment (Fig. 3B). GUS activity obviously increased after 24 h of 4°C treatment in leaves and roots, and very notably in the leaves (Fig. 3C). These results shown that the promoter of *CbCAX51* were cold-induced. The tissue-specific expression of the CbCAX51 were analysis by GUS activity in leaves, stem and roots of 30 day-old *A. thaliana* plants. The results showed that the GUS was higher in protons translocation tissues like phloem of the root, vascular bundle of the stem and leaf vein, which also might be relevant to the gene function specificity (Fig. 3C).

## *CbCAX51*-Overexpression Tobacco Plants have Enhanced Chilling and Freezing Tolerance

Two lines of single copy 35S::CbCAX51 transgenic tobacco (Fig. 4A) were selected from more than 25 independent transgenic lines (L1:CbCAX51-3; L2:CbCAX51-10), that were used to further investigated of its function. Since the expression of *CbCAX51* is regulated by cold and cation. Two of twenty homozygous *CbCAX51* overexpression lines in

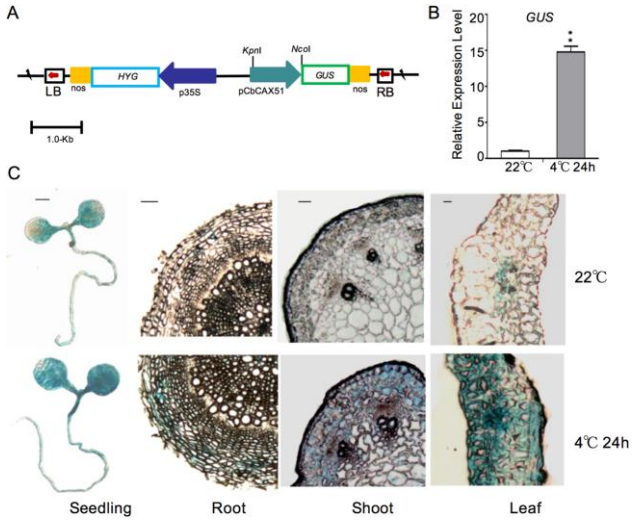


**Fig. 3:** Histochemical localization of GUS activity in transgenic *Arabidopsis* plants containing pCbCAX51::GUS fusion. (A) The GUS reporter gene was under the control of the CbCAX51 promoter. (B) Transcript levels of GUS reporter gene driven by pCbCAX51 in transgenic plants growing under 22°C or exposed to 4°C for 24 h. Actin gene was performed as an internal control. (C) GUS staining of transgenic seedlings containing pCbCAX51::GUS fusion growing under 22°C or exposed to 4°C for 24 h. The bars of 2.5-week-old seedlings represent 0.2 cm. Semi-thin sections were carried out in roots, shoots and leaves of 4.5-week-old transgenic seedlings after GUS staining. The bars represent 0.001 cm

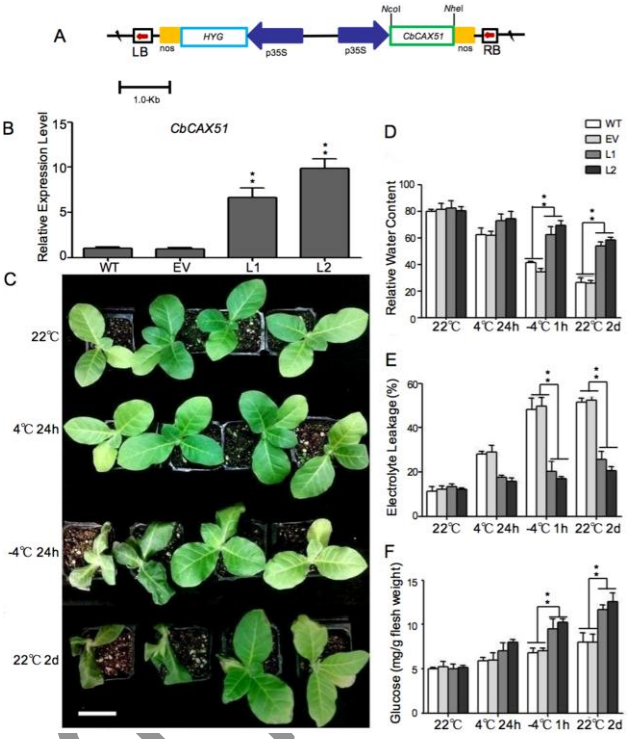


**Fig. 4:** The phenotypes of tobacco plants in bioassay for cold tolerance. (A) The *CbCAX51* gene was under the control of the 35S promoter. (B) The relative expression of *CbCAX51* in transgenic tobacco plants. The *Ntactin* gene was performed as an internal control. (C) Transgenic tobacco line L1, line L2, empty vector control (EV) and wild type (WT) growing at 22°C were treated in 4°C for 24 h, -4°C for 1 h and 22°C for 2 d in turn. In each time point the representatives of respective genotypes of plants were viewed from the top. The bar represents 10 cm. The relative water content (D), electrolyte leakage (E) and glucose content (F) of leaves from transgenic tobacco plants before and after cold treatment are shown. (SD, n = 3, **P* < 0.05, ***P* < 0.01)

T2 generation were subjected to chilling and freezing treatments. The expression of *CbCAX51* in L1 and L2 lines were much higher than that of the control (Fig. 4B).

But the growth and development of L1 and L2 were visually indistinguishable from wild-type plants (WT) or plants transformed with empty control vector (EV) before treatment, and after chilling treatment for 24 h at 4°C, WT, EV, L1 and L2 lines were still appear no obvious damage. While four plants wilted after 1 h of -4°C treatment, the WT and EV displayed more severe damages than L1 and L2. Subsequently *CbCAX51*-expressing tobacco L1 and L2 appeared to be more vigorous after 2-day recovery period under normal growth conditions (22°C) compared with WT and EV plants (Fig. 4C). The cold tolerance of the transgenic lines (L1 and L2) was further measured on the basis of electrolyte leakage, relative water content and glucose content. When compared with control plants, both two lines exhibited lower electrolyte leakage and higher relative water content that were indicated of reduced disruption of cell membranes after both chilling (4°C) and freezing (−4°C) application. Moreover, the transgenic plants accumulated more glucose that also contributed to the stability of the internal milieu and protection of bioactive components in plant cells (Fig. 4D).

The results of physiological index measurements shown that the *CbCAX51* can improve the cold tolerance in tobacco.

**Ectopic Expression of *CbCAX51* in Tobacco May Alter the Expression Patterns of CBF/DREB and Downstream Targets Genes under Chilling Stress**

The effects of *CbCAX51* ectopic expression on the chilling response of two CBF/DREB elements genes (*NtDREB1*-EU727155, *NtDREB3*-EU727157), two CBF downstream cold responsive genes (*NtERD10a*-AB049335, *NtERD10b*-AB049336) and a gene specialized for salt and disease tolerance (*NtTSI1*-AF058827) in tobacco plants were measured using FQ-PCR (Fig. 5). *NtDREB3* expression levels in both L1 and L2 lines were much higher

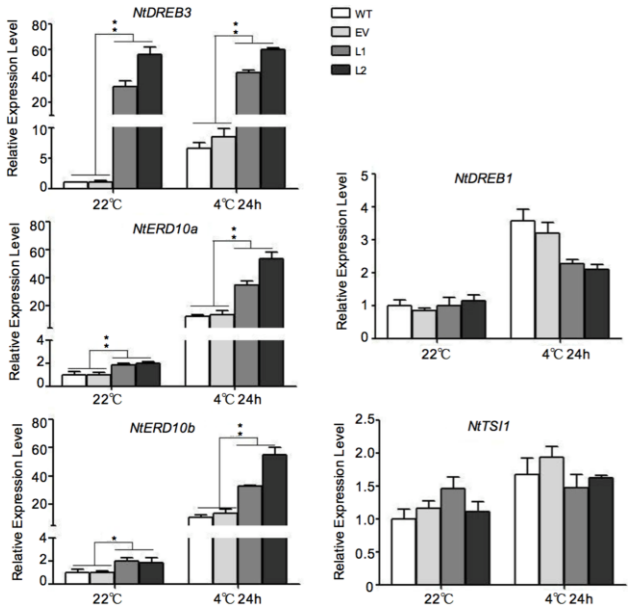


**Fig. 5:** The relative expression of cold responsive genes in transgenic tobacco plants. The relative transcripts of *NtDREB3* (A), *NtERD10a* (B), *NtERD10b* (C), *NtDREB1* (D), and *NtTSI1* (E) in transgenic tobacco plants before and after cold treatment were tested. (SD, n = 3, **P* < 0.05, ***P* < 0.01)

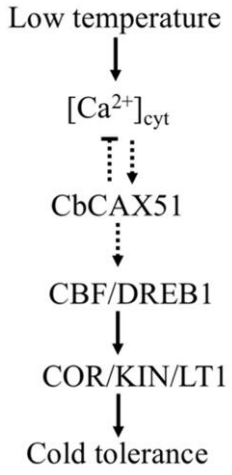


**Fig. 6:** Model for CbCAX51 regulation CBF/DREB in response to low temperature

than WT and EV at 22°C, and were increased in WT and EV but stayed the same way in L1 and L2 after 24 h of 4°C treatment (Fig. 5A). *NtERD10a* and *NtERD10b* expression levels in transgenic plants L1 and L2 were also higher than control at 22°C and elevated by 20 folds after 4°C treatment (Fig. 5B-C). *NtDREB1* expression was not changed at 22°C and only slightly increased to a lower level under chilling condition in L1 and L2 compared with WT/EV plants, indicating the negative role of *CbCAX51* in its cold induction (Fig 5D). *NtTSI1* expression level was not significantly altered between L1/L2 and WT/EV at both normal and chilling temperature (Fig 5E). These indicated that *CbCAX51* may diversely regulate CBF/DREB family factors and corresponding downstream targets in cold response.

## Discussion

The $Ca^{2+}$ as a second messenger playing an essential role during low temperature signal transduction (Knight *et al.*, 1991) and cold acclimation (Monroy *et al.*, 1993). In the plant cell, cold shock can cause a transient rise in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) (Knight *et al.*, 1991). The increased level of $Ca^{2+}$ is recognized by $Ca^{2+}$ receptors and transporters such as Calcium/calmodulin Regulated receptor-Like Kinase (CRLK1), CAXs, CaM independent Protein Kinases (CDPKs) and Calmodulin (CaMs), which can activate downstream proteins leading to phenotypic response of stress tolerance (Xiong *et al.*, 2002; Tuteja and Mahajan, 2007). During cold acclimation, the calcium signaling network related factors can act as either activators or repressors through regulating the balance of plant growth and cold response (Braam and Davis, 1990; Tähtiharju *et al.*, 1997). In *Arabidopsis*, knock-out mutation of *CRLK1* repressed cold induction of CBF/DREB signaling components including *CBF1*, *RD29A*, *COR15a* and *KIN1*, suggesting that CRLK1 acts as a positive regulator (Yang *et al.*, 2010). Meanwhile, T-DNA insertion mutants of *cax1* showed enhanced induction of CBF/DREB target genes (*KIN1*, *LTI78*, *COR47* and *AtP5CS2*) (Catala *et al.*, 2003). These evidences indicate that no matter positive or negative regulators controlled by $Ca^{2+}$ signaling, many of them typically modulate cold induction of genes involved in CBF/DREB dependent pathway, a key responsive signaling under cold stress.

*CbCAX51* from *C. bursa-pastoris* is a new cation exchangers (CAXs) gene (Lin *et al.*, 2008). *CbCAX51* has a high rate of identity in structure and sequence (87.8%) compared with previously characterized *AtCAX1* from *A. thaliana*. *CbCAX51* could be activated by cold treatment, but be inhibited by drought and not effect on ABA or salt (Lin *et al.*, 2008). In this study, the expression level of *CbCAX51* was first characterized in leaves, stem and roots of *C. bursa-pastoris* in response to low temperature. *CbCAX51* expression level reached a maximum peak in leaves, stem and roots after 24 h of 4°C treatment and leaves showed highest expression level. However, expression level of *AtCAX1*, the homolog with highest similarity of *CbCAX51*, reached a peak in leaves after 24 h of 4°C treatment, but did not apparently change in stem and decreased in roots (Catala *et al.*, 2003). These data indicate that *CbCAX51* is widely induced in whole seedlings in response to low temperature, while *AtCAX1* is enhanced specifically in leaves. In addition, expression analysis in

seedlings of *C. bursa-pastoris* showed that *CbCAX51* was transiently induced at 4 h of the $Li^+$ and $Zn^{2+}$ treatments, gradually up-regulated by $Ca^{2+}$ and $Cu^{2+}$ and down-regulated in $K^+$ and $Mg^{2+}$ treatments from 4 h to 24 h. The expression characteristics of *CbCAX51* in seedlings of *C. bursa-pastoris* in response to exogenous $Ca^{2+}$, $K^+$ and $Mg^{2+}$ is similar to *AtCAX1*, except $Cu^{2+}$ (Hirschi, 1999). The exogenous $Cu^{2+}$ does not appear to induce the expression of *AtCAX1* after 16 h incubation in seedlings of *A. thaliana.*

Different expression characteristics can lead to diverse molecular functions. Indeed, overexpression of *CbCAX51* caused stronger cold resistance together with upregulation of *NtDREB3*, *ERD10a* and *ERD10b*, which is more similar to *GhCAX3* and *SeCAX3* (Xu *et al.*, 2013; Zhang *et al.*, 2015), but opposite to *AtCAX1* (Hirschi, 1999). Results shown by Catala demonstrate that constitutive expression of At*CAX1* weakens the cold induced expression of DREB genes, alters the plant response to chilling, increases the the plant sensitivity to different ions, and affects growth. Interestingly, *CbCAX51* also reduced the cold induction of NtDREB1, suggesting the complexity of regulatory network between CAXs and DREBs. Further, heterogeneous expression of *CbCAX51* did not cause growth retardation in transgenic tobacco plants (Fig. 4B). As described in the work of Catala *et al.* (2003), *AtCAX1* was proposed to play a critical role in the restoration of $[Ca^{2+}]_{cyt}$ levels after cold induction of signaling components and modulating the accurate expression of CBF/DREB genes. The data we present here can also be applied to this model, whereas *CbCAX51* may adjust the $[Ca^{2+}]_{cyt}$ levels in a different way and diversely control the CBF/DREB genes and downstream targets leading to enhanced cold acclimation, rather than reduce their expression (Fig. 6). Together, given the high similarity in sequence CAXs from different species can still possess various expressional and functional characteristics. Since CbCAX51 has strong ability to regulate cold tolerance in plants without causing growth reduction, it can be concluded that *CbCAX51* has broad prospects in application of molecular breeding for cold tolerant crops.

## Conclusion

*CbCAX51* gene is a $Ca^{2+}/H^+$ exchanger from *Capsella bursa-pastoris*. We concluded that the expression level of *CbCAX51* in leaves and stems of plant was higher than that in roots and was continuously up-regulated by $Ca^{2+}$ and $Cu^{2+}$, and transiently up-regulated by $Zn^{2+}$ and $Li^+$, cold stress, GA and ABA. The CbCAX51 protein was a vacuolar membrane protein. The transgenic tobacco seedlings with *CbCAX51* overexpression acquired enhanced cold tolerance by activated downstream cold responsive genes.

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