Molecular Soil Biology 2016, Vol.7, No.5, 1-5
http://msb.biopublisher.ca

Research Report Open Access

Identification and Characterization of a Cation^{2+}/H^{+} Antiporter AtCAX4 Gene from *Arabidopsis thaliana*

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Molecular Soil Biology 2016, Vol.7, No.5 doi: 10.5376/msb.2016.07.0005

Received: 05 May, 2016

Accepted: 01 Jun., 2016

Published: 02 Jun., 2016

Preferred citation for this article:

Jiangpo Chen, Testuo Takano, Shenkui Liu, and Yuanyuan Bu, 2016, Identification and Characterization of a Cation2+/H+ Antiporter AtCAX4 Gene from *Arabidopsis thaliana*, Molecular Soil Biology, 7(5): 1-5 (doi: 10.5376/msb.2016.07.0005)

Abstract AtCAX4 is a member of the Ca^{2+}/H^{+} exchangers (CAX) family was cloned from cDNA library of *Arabidopsis thaliana*. *AtCAX4* was expressed in all *Arabidopsis* plant organs, with the highest level in root under normal conditions by quantitative real-time PCR analysis. The expression of *AtCAX4* gene is response to Na^{+}, Ca^{2+} and Ba^{2+} in both the leaves and roots of *Arabidopsis*. In addition, the pYES2-*AtCAX4* transformants confers tolerance to Na^{+}, Ca^{2+} and Ba^{2+} ions in yeast. These results suggest that *AtCAX4* play important role in increasing the Na^{+}, Ca^{2+} or Ba^{2+} tolerance of yeast.

Keywords *AtCAX4*; Gene expression; Tolerance; Yeast

1 Introduction

The distribution of calcium (Ca^{2+}) and other ions among organelles and the regulation of cytoplasmic ion concentrations are important factors in plant development, growth and adaptation to stress (Berridge MJ., 2000; Cheng et al., 2005; Zhao et al., 2008). Ca^{2+}/H^{+} exchangers (CAXs) are a group of transporters that export $Ca^{2+}$and other cations from the cytosol to maintain optimal ionic concentrations in the cell (Cheng et al., 2003; Shigaki et al., 2006). CAX proteins are involved in various (Ueoka-Nakanishi et al., 1999) abiotic stress response pathways, in some cases as a modulator of cytosolic Ca^{2+} signaling, but in some situations, there is evidence of CAXs acting as a pH regulator (Shigaki and Hirschi, 2006; Toshiro et al., 2002).

Arabidopsis has six genes of the CAX family and other species also have *CAX* genes (Takehiro et al., 2004). *AtCAX1* and *AtCAX2* gene were mainly expressed in the leaves of *Arabidopsis thaliana*; while *AtCAX3* mainly expressed in root, especially in root tip (Manohar et al., 2011). *AtCAX4* mainly expressed in the apical and lateral root primordial (Mei et al., 2009; Hui et al., 2009). CAXs have been reported that not only transport Ca^{2+} into the vacuole, but also have a role in the transport of heavy metals (Korenkov et al., 2007). Such as, AtCAX2 was not only able to transport Ca^{2+} but also transport Mn^{2+}, Cd^{2+} and Zn^{2+}(Cheng et al., 2002). The specific expression of AtCAX4 in tobacco could enhance the Cd^{2+} accumulation, and reduce the accumulation of Cd^{2+} on the ground (Korenkov et al., 2007). Although CAXs has been reported to be involved in a number of important aspects of plant growth and development (Conn et al., 2011), the function of *AtCAX4* gene has not been yet well investigated. Here, the gene expression pattern of *AtCAX4* gene and yeast transformants response to Na^{+}, Ca^{2+} and Ba^{2+} ions were studied to explore the function of *Arabidopsis thaliana AtCAX4* gene.

2 Materials and Methods

2.1 Plasmid constructs and plant materials

The open reading frame (ORF) of *AtCAX4* was amplified form *Arabidopsis thaliana* cDNA Library using the primers FW: 5'-ATGTCTTCAATCAGTACGGAATCGTCTT-3' and RV: 5'-TTACCTTTTCGTTATTGTATGATTAGTT-3'. The amplified product was digested with *BamH*I and *Eco*RI,

and cloned into the yeast expression vector pYES2 to form *pYES2-AtCAX4* plasmid, which was confirmed by sequencing. This construct was used for yeast tolerance analysis.

2.2 Phylogenetic analysis, yeast transformation, and growth conditions

Full-length amino acids sequences were aligned using CLUSTALX, and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version MEGA6.0 (Lewis et al., 2013). Phylogenetic analyses were conducted using the neighbor joining (NJ) method implemented in MEGA6.0. The following accession numbers were used: OsCAX1 (GeneBank No: Q769E5), OsCAX2(Q5KQN0), OsCAX3 (Q6K1C4), OsCAX4 (Q6YXZ1), AtCAX1 (NP_973630.1), AtCAX2 (NP_566452.1), AtCAX3 (NP_190754.2), AtCAX4 (NP_568091.2), AtCAX5 (NP_175969.2), AtCAX6 (NP_175968.4), PucCAX1 (BAH01721), PucCAX2 (AFF18617), BnCAX1 (CDX91434.1), BnCAX2 (CDX82545.1), BnCAX3 (CDX90630.1), BnCAX4 (CDY40303.1) and ZmCAX1 (NP_001104999.2).

Yeast transformants were performed using a lithium acetate-based method (Gietz RD., 2006). The plasmids *pYES2-AtCAX4* and *pYES2* were introduced into the yeast strain K667. For the response assays, yeast transformants were cultured in solid yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) supplemented with different concentrations of NaCl (100, 300, 500, and 800 mM), $CaCl_2$ (10, 30, 40, and 50 mM), $BaCl_2$ (0.5, 1, 2, and 3 mM), KCl (1, 10, 50, and 100 mM), $MgCl_2$ (0.1, 10, 15, and 30 mM), $MnCl_2$ (0.1, 0.3, 0.5, and 1 mM). A yeast transformant of the *pYES2* empty vector was used as a control, and growth was monitored for 3-7 days at 30 °C.

2.3 Analysis of gene expression using real-time PCR

The *Arabidopsis* plants were cultured under an 8-h-light/16-h-dark cycle in a growth chamber. Roots, stems, leaves, panicle, and siliques of 2-months-old plants were sampled for qRT-PCR. A second batch of seedlings was pre-cultured for 2 weeks on 1/2 solid medium, and then treated with different concentrations of various ions: NaCl 150 mM, $CaCl_2$ 100 mM, $BaCl_2$ 1 mM. The shoots and roots were sampled after 0 h, 6 h, 12 h, and 24 h treatment and used for qRT-PCR analyses.

Total RNA was isolated using the Trizol method, and treated with RNase-free DNaseI. Gene-specific primers pairs FW: 5'-GGCTGAAGAATACGATGGGT-3' and RV: 5'-GGCTCCTTCTTCTTCCTTGT-3' were used for AtCAX4, while Actin-FW: 5'-GGCTGAAGAATACGATGGGT-3' and Actin-RV: 5'-GGCTCCTTCTTCTTCCTTGT-3' were used for Actin. Relative quantification using qRT-PCR reactions were performed with SYBR green I using the LightCycler®480 system II (Agilent, USA).

3 Results and Discussion

3.1 Cloning and characterization of AtCAX4

The full length of *AtCAX4* gene (GeneBank accession No: NM_120227.3) was 1472bp, and was identified from *A.thaliana* cDNA library. The open reading frame (ORF) was 1365 bp, and encoded a protein of 454 amino acids, with a predicted molecular mass of 49 kDa and a predicted *pI* of 6.5103. Phylogenetic analysis was performed based on the amino acid sequence of AtCAX4 and other published species CAXs amino acid sequence. Fig 1A shows that AtCAX4 is most close related to BnCAX4 with high affinity.

The polypeptide was 53%, 54%, and 42% identical to AtCAX1 (GeneBank accession No. NP_973630.1), AtCAX3 (NP_190754.2) and AtCAX2 (NP_566452.1) (Figure 1-A). The predicted AtCAX4 transmembrane domains showed that AtCAX4 contains 11 transmembrane domains, each transmembrane region contains about 40 amino acids. The N and C terminus of AtCAX4 were both in the inner of the membrane (Figure 1-B).

3.2 Gene expression pattern of *AtCAX4* under Na^+, Ca^{2+} and Ba^{2+} conditions

To investigate the pattern of *AtCAX4* expression under normal conditions, RNA was extracted from the Roots, stems, leaves, panicle, and siliques of plants grown for two month. Quantitative real-time PCR analyses showed that *AtCAX4* is expressed in all *A.thaliana* plant organs, with the highest expression in root and siliques (Figure 2). These results were consistence with the previous studies (Manohar et al., 2011).

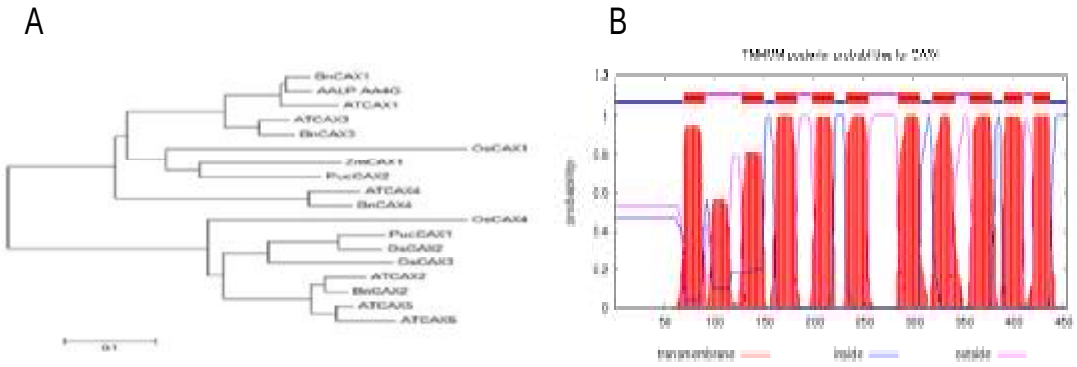

Figure 1 Sequence and phylogenetic analysis of AtCAX4

Note: (A) Amino acid sequence of AtCAX4 and phylogenetic trees analysis of CAX families; Sequence were obtained from the GeneBank database. The accession numbers are listed in the Material and methods section. (B) The transmembrane domains in AtCAX4 were predicted by the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/)

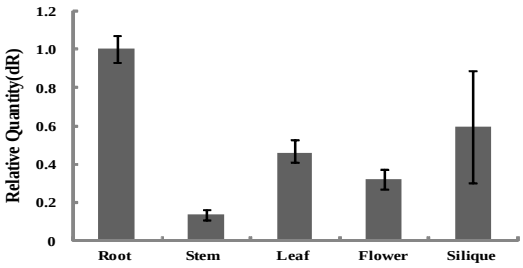

Figure 2 Quantative RT-PCR analysis of *AtCAX4* in different organs of *Arabidopsis thaliana*

Note: *AtCAX4* expression was normalized against *Actin* mRNA levels; the reported data are the means of three replicate experiments ±S.E

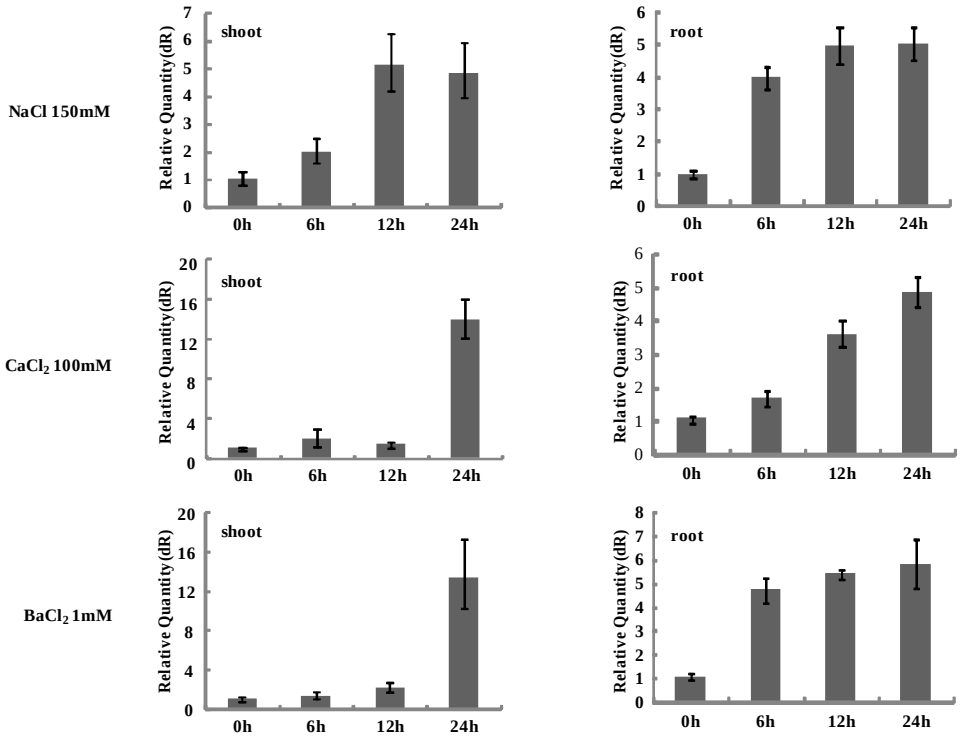

Figure 3 Quantative RT-PCR analysis of the expression of *AtCAX4* in response to NaCl, $CaCl_2$, and $BaCl_2$

Note: *AtCAX4* expression was normalized against *Actin* mRNA levels; the reported data are the means of three replicate experiments ±S.E

When plants were grown in the presence of 100mM NaCl, *AtCAX4* mRNA expression was induced within 6 h of treatment in both the leaves and roots (Figure 3). After stressing with 100 mM CaCl2, *AtCAX4* mRNA expression was increased gradually with increasing the treatment time, and peaked at 24 h in both the shoot and roots (Figure 3). These results suggested that the expression of *AtCAX4* gene is response to Na^+, Ca^{2+} and Ba^{2+} ions.

3.3 AtCAX4-overexpressing cells response to various cations in yeast

CAXs has been confirmed for some metal ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Na^+, Cd^{2+}) with transport function (Sunghun et al., 2005; Hirschi et al., 2000; Koren'Kov et al., 2007). As shown in Figure 4, growth of transgenic yeast cells carrying AtCAX4 was better on solid yeast YPG medium containing NaCl, $CaCl_2$, $BaCl_2$ than that of control cells, while the transformants did not significant difference with the empty vector transformants when grown on the medium containing KCl, $MgCl_2$ and $MnCl_2$, suggesting that AtCAX4 plays a role in response to $NaCl_2$, $CaCl_2$ and $BaCl_2$ in yeast.

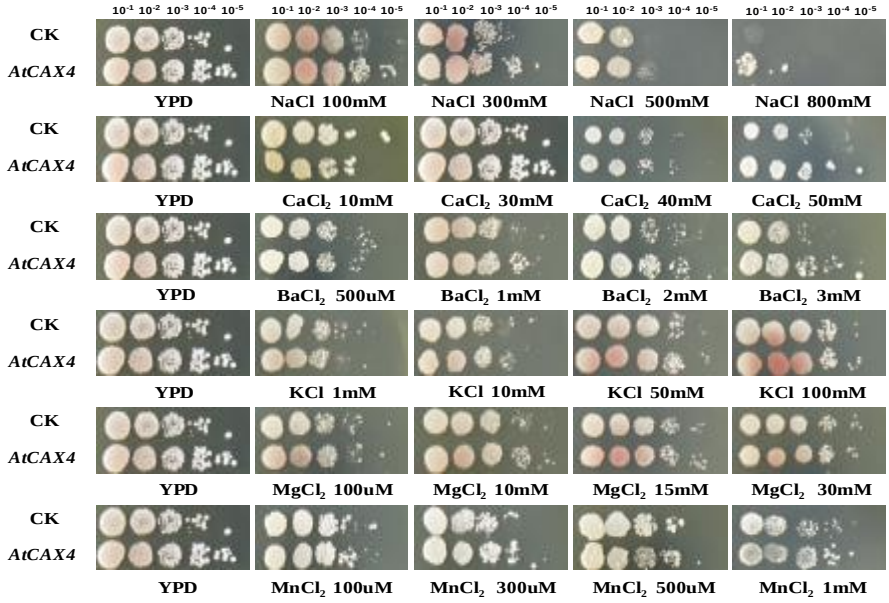

Figure 4 Growth of AtCAX4 transformants in response to various ions

Note: Yeast transformants were grown on YPD medium containing different concentrations of the following metal ions: NaCl, $CaCl_2$, $BaCl_2$, KCl, $MgCl_2$, and $MnCl_2$ in the presence of 2% (w/v) galactose. Growth was monitored for 3-7 days at 30°C

Acknowledgments

This work was supported by Heilongjiang Province foundation for Returnees (LC201405) awarded to Yuanyuan Bu. Further supported by the Program for Changjiang Scholars and Innovative Research Team in University (IRT13053) awarded to Shenkui Liu.

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