Functional analysis of CAX2-like transporters isolated from two ecotypes of *Sedum alfredii*

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Abstract

Two genes encoding CAX2-like proteins were isolated from a Zn/Cd hyperaccumulating ecotype (HE) and non-hyperaccumulating ecotype (NHE) of *Sedum alfredii* Hance, and they were named *SaCAX2h* and *SaCAX2n*, respectively. Both SaCAX2h:eGFP and SaCAX2n:eGFP proteins were localized to the vacuolar membrane of tobacco epidermal cells and yeast mutants. Heterologous expression of SaCAX2h or SaCAX2n in the Δzrc1 yeast mutant increased Cd content in yeast cells. Yeast complementary assay also revealed that both the transporters could suppress Ca and Mn hypersensitivity and enhance Ca and Mn accumulation in a K667 yeast mutant. The expression patterns of the two genes were different under the Cd treatment. Transcription of *SaCAX2h* was down-regulated in roots and up-regulated in shoots whereas transcription of *SaCAX2n* was down-regulated in shoots after the exposition to Cd. Furthermore, over-expression of SaCAX2h enhanced metal accumulation in the tobacco plants. The Cd content increased by 17 - 19 % in shoots and 31 - 36 % in roots; the Ca content of the transgenic plants increased by 31 - 32 % in shoots, and the Mn content increased by 60 - 79 % in shoots and 22 - 29 % in roots. These results indicate that SaCAX2h was responsible for Ca and Mn sequestration into vacuoles, and over-expression of SaCAX2h enhanced Cd accumulation in the transgenic tobacco.

Additional key words: cadmium, calcium, manganese, tobacco, yeast mutant.

Introduction

During the past decade, physiological studies have revealed a basic understanding of metal tolerance and accumulation mechanisms, including enhanced metal uptake, increased xylem loading, and increased sequestration into leaf vacuoles (Clemens *et al*. 2002, Verbruggen *et al*. 2009, Krämer *et al*. 2010). Several groups of metal transporters have been found to play important roles in Zn/Cd tolerance and accumulation. The AhHMA4 of a Zn/Cd hyperaccumulator *Arabidopsis halleri* is important transporter for Zn/Cd xylem loading (Talke *et al*. 2006, Courbot *et al*. 2007, Hanikenne *et al*. 2008). NcHMA4 is suggested to be involved in Cd transport and possibly in Cd hyperaccumulation in another Zn/Cd hyperaccumulator *Noccaea caerulescens* (Bernard *et al*. 2004). AtHMA3, OsHMA3, and NcHMA3g are required for Cd detoxification as they are responsible for Cd sequestration into vacuoles (Gravot *et al*. 2004, Morel *et al*. 2009, Ueno *et al*. 2010, 2011). Another family of transporters named AtCAXs (CAXs of *A. thaliana*) have also been analyzed for Cd transport ability. A comparison of five different *AtCAX* genes indicated that CAX4 and CAX2 have a high Cd transport ability and selectivity into tonoplast vesicles (Korenkov *et al*. 2007 a,b).

The cation exchangers (CAXs) belong to a Ca^{2+}/cation antiporter (CaCA) superfamily, and they are group of proteins transporting cations of the cytosol to maintain their optimal ionic concentration in the cell (Shigaki *et al*. 2006). Six CAXs of *Arabidopsis* (*AtCAX1 - AtCAX6*) are divided into two subclasses. AtCAX1, AtCAX3, and AtCAX4 are clustered into type I-A group whereas AtCAX2, AtCAX5, and AtCAX6 belong to type

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Abbreviations: CAF - Cys-Ala-Phe domain; CAX - cation exchanger; GFP - green fluorescent protein; HE - hyperaccumulating ecotype; HMA - heavy metal ATPase; MTP - metal tolerance protein; NHE - non-hyperaccumulating ecotype; ORF - open reading frame; RACE - rapid-amplification of cDNA ends.

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I-B group, and they are predicted to possess 11 transmembrane domains (Shigaki *et al.* 2006). Another five AtCAXs, which were named CAX**7** to CAX11, show a limited primary amino acid sequence homology with any CAX; they have been renamed CCX1 to CCX5, respectively (Shigaki *et al.* 2006). In *planta*, seven AtCAXs have been found to transport Cd^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+} to varying degrees, CAX2 and CAX4 have a high Cd^{2+} transport and selectivity in tonoplast vesicles (Korenkov *et al.* 2007a). Over-expression of *AtCAX1cd* enhances Cd tolerance and accumulation in petunia plants (Wu *et al.* 2010). In Zn/Cd hyperaccumulators *A. halleri* and *N. caerulescens*, some of the *AtCAXs* homology genes are highly expressed or differentially expressed in response to Zn (Van de Mortel *et al.* 2006, 2008, Weber *et al.* 2006). However, *AhCAX2* shows no evidence in Cd or Zn tolerance activity when expressed in yeast cells, and the authors suggested that AhCAX2 does not appear to be directly involved in metal detoxification or accumulation in *A. halleri*.

The hyperaccumulating ecotype (HE) of *Sedum alfredii* is a Zn/Cd hyperaccumulator found in China. Compared with the non-hyperaccumulating ecotype (NHE) of *Sedum alfredii* which is sensitive to Zn and Cd, HE plants can accumulate Cd in leaves up to a maximum of 9 000 mg kg^{-1}(d.m.) and also can accumulate Zn in shoots up to 2 % (m/m) of d.m. (Yang *et al.* 2002, 2004, 2006, Xiong *et al.* 2004). Vacuoles have been suggested to be the primary site of accumulation of a number of heavy metals (Conn and Gilliham 2010). Protoplasts isolated from leaves of HE plants have been found to take up 1.5-fold more ^{65}Zn as compared to NHE plants (Yang *et al.* 2006); a higher expression of *SaMTP1* in shoots should be responsible for Zn accumulation in HE plants (Zhang *et al.* 2011). It has also been found that Cd in HE plants accumulate preferentially in parenchyma tissues (pith, cortex, and mesophyll) which then consist of cells with large vacuoles (Tian *et al.* 2011). The AtCAX transporters are localized in the tonoplast and some of them have been suggested to play a role in Cd transport. In this study, *CAX2* genes of HE and NHE plants were isolated. Then we analyzed *1*) the function of SaCAX2h and SaCAX2n using yeast mutant strains; *2*) the effects of Cd treatment on expression of *SaCAX2h* and *SaCAX2n*; and *3*) the role of the *SaCAX2h* gene in Ca, Mn, and Cd accumulation.

Materials and methods

Plants, growth conditions, and Cd treatment: Seeds of *Sedum alfredii* Hance HE plants were collected from an old Pb/Zn mining area in Quzhou city of the Zhejiang province, P.R. China, and seeds of NHE plants were from tea gardens of Hangzhou, the Zhejiang province, P.R. China. After the seeds were germinated, one-month-old seedlings with equal growth were selected and subjected to 4-d exposure to one-fourth-, one-half-, and full-strength nutrient solution containing 2 mM Ca^{2+}, 4 mM NO_3^-, 1.6 mM K^+, 0.1 mM $H_2PO_4^-$, 0.5 mM Mg^{2+}, 1.2 mM SO_4^{2-}, 0.1 mM Cl^-, 10 mM H_3BO_3, 0.5 mM $MnSO_4$, 5 mM $ZnSO_4$, 0.2 mM $CuSO_4$, 0.01 mM $(NH_4)_6{\cdot}Mo_7O_{24}$, and 100 mM Fe-EDTA. After one month, three plants of each ecotype were transferred to a hydroponic solution containing 2 µM $CdCl_2$ for 8 d the nutrient solution was renewed every 4 d. The untreated plants were used as control.

Microscopic imaging Cd in leaves of HE plants was conducted according to the method described by Tian *et al.* (2009). After the HE plants were treated with 100 µM $CdCl_2$ for 8 d, fresh leaves of the control plants and Cd treated plants were cross-sectioned into slices (thickness < 0.5 mm). The samples were then immersed in a diluted *Leadmium*™ *Green AM* dye (*Invitrogen*, Carlsbad, USA) in the dark for 30 min. After washing with double-distilled water three times, the samples were analyzed using a laser scanning confocal microscope (*LSM5Pascl*, *Carl Zeiss*, Göttingen, Germany).

Isolation of *CAX2* cDNAs from HE and NHE plants and sequences analysis: The full length cDNAs of *SaCAX2h* and *SaCAX2n* were isolated in two steps. Degenerate oligonucleotide primers were designed according to highly conserved regions of *AtCAX* cDNAs; *CAX2* cDNA fragments were isolated from the HE and NHE plants by nested PCR amplification. Primers for a rapid-amplification of cDNA ends (RACE) were designed based on the isolated *CAX2* fragment sequences. The 3' region was isolated using a *ReverTra Ace -α-*® kit (*Toyobo*, Osaka, Japan) and the 5' region was isolated using a RACE system (*Invitrogen*). The *CAX2* cDNAs from the HE and NHE plants were named SaCAX2h and SaCAX2n, respectively. Alignment of CAX2 sequences was performed using *ClustalW* (http://clustalw.ddbj.nig.ac.jp/top-e.html).

Plasmid DNA constructs: Gateway technology was used to construct yeast expression vectors and a 35S-SaCAX2h vector. The open reading frames (ORFs) of *SaCAX2h* and *SaCAX2n* were amplified using primers CAX2-F, 5'-CACCATGGTTGGAGTTGGTGTCC-3' and CAX2-R, 5'-TTATGGTGTTCCTACCACATC ATCATC-3'. The amplified fragments were subcloned into pENTR/D-TOPO (*Invitrogen*). The pENTR/D-TOPO entry vector containing the SaCAX2h or SaCAX2n coding sequence was designated pENTR-SaCAX2h and pENTR-SaCAX2n, respectively. Subsequent attL substrate and attR substrate (LR) recombination reactions between the destination (pDR195) and entry vector (pENTR-SaCAX2h or pENTR-SaCAX2n) generated yeast expression vectors; an LR recombination reaction between the pH7WG2.0 and entry vector pENTR-SaCAX2h generated the

35S-SaCAX2h vector.

To construct 35S:SaCAX2h-eGFP and 35S:SaCAX2n-eGFP vectors, the ORFs of *SaCAX2h* and *SaCAX2n* without the stop codon were amplified. After digestion with restriction enzymes (KpnI and XabI), SaCAX2h or SaCAX2n was ligated to pCAMBIA1300g-GFP vector. To construct pDR195/SaCAX2h-eGFP and pDR195/SaCAX2n-eGFP vectors, regions of SaCAX2h-eGFP and SaCAX2n-eGFP were amplified from the 35S:SaCAX2h-GFP and 35S:SaCAX2n-GFP vectors. After digestion with XhoI and BamHI restriction enzymes, they were ligated to pDR195. The sequence accuracy of the genes in the recombined vectors was confirmed by sequencing.

Subcellular localization of SaCAX2h and SaCAX2n in plant cells: The subcellular localization of SaCAX2h:eGFP and SaCAX2n:eGFP fusion proteins were performed by *Agrobacterium*-mediated transient expression of SaCAX2h:eGFP or SaCAX2n:eGFP in tobacco epidermal cells. After the 35S:eGFP vector and 35S:SaCAX2h:eGFP or 35S:SaCAX2n:eGFP vector were introduced into *Agrobacterium tumefaciens* strain LBA4404, leaves of one-month-old tobacco seedlings (*Nicotiana benthamiana* Domin) were transformed as described by Courbot *et al.* (2007). Fluorescence of eGFP was observed through a laser scanning confocal microscope (*LSM510*; *Carl Zeiss*, Jena, Germany); excitation of GFP fluorescence at 488 nm.

Yeast strains and assays for metal tolerance: *Saccharomyces cerevisiae* Δzrc1 and its K667 mutant (*cnb1::*LEU2 *pmc1::*TRP1 *vcx1Δ*) were used in this study. Yeast transformation was carried out using the Li-acetate transformation method (Gietz *et al.* 1995). For Cd tolerance test, *Δzrc1* transformants expressing pDR195, SaCAX2h, SaCAX2h:eGFP, SaCAX2n, or SaCAX2n:eGFP were spotted onto a synthetic defined (SD) selective medium with or without 40 µM Cd. In Ca and Mn tolerance assays, the K667 yeast transformants were grown in a liquid SD medium without leucine, tryptophan, and uracil. For Ca test, 0.01 cm^3 of each dilution was spotted onto YP medium + 2 % glucose (YPD) solid medium plates with or without 50 or 75 mM $CaCl_2$; for Mn tolerance test, 0.01 cm^3 of each dilution was spotted onto solid SD selective medium plates (without leucine, tryptophan, and uracil) containing 5 or 6 mM $MnCl_2$. The plates were incubated at 30 °C for 3 - 4 d. Fluorescence of eGFP and FM4-64 in yeast cells was observed using a laser scanning confocal microscope (*LSM510;* excitation at 488 and 543 nm, respectively).

To measure the metal tolerance of the yeast transformants in a liquid medium, the yeast strains were grown to saturation in selection media at 30 °C. Then the Δzrc1 transformants were inoculated into an SD selection medium supplied with 40, 50, 60, or 70 µM $CdCl_2$. The K667 transformants were inoculated into a YPD medium supplemented with 25, 50, or 75 mM $CaCl_2$, or an SD selection medium containing 4, 5, 6, or 7 mM $MnCl_2$. Absorbance at 600 nm (A_{600}) was measured after 24-h incubation at 30 °C.

Determination of intracellular Cd, Ca, and Mn content in yeast: The yeast transformants were grown in a liquid SD selective medium at 30 °C overnight. To determine the Cd content in the Δzrc1 transformants, cells were adjusted to $A_{600} = 0.2$ in the presence of 10 µM Cd; for Ca and Mn measurements, the K667 mutants were incubated with 1 mM Ca or 200 µM Mn. After 48 h incubation, the cells were collected and washed once with distilled water, once with 20 mM Na_2EDTA, and once more with distilled water. Dry mass was determined after drying at 85 °C for 48 h. The cells were resuspended in concentrated HNO_3, incubated at 95 °C for 2 h, and diluted with distilled water. The Cd, Ca, and Mn content were determined using inductively coupled plasma optical emission spectrometry (*ICP-MS*, *7500A*, *Agilent*, CA, USA).

Transcription analysis of *CAX2* genes in HE and NHE plants: Total RNA from shoots and roots of the HE and NHE plants with or without the heavy metal treatment were prepared using an *RNeasy Mini* kit (*Qiagen*, USA). First-strand cDNA was synthesized with a *ReverTra Ace-α-®* kit (*Toyobo*). The sizes of the amplified fragments were confirmed by gel electrophoresis and sequencing. A real-time RT-PCR was performed in *Eppendorf* (Hamburg, Germany) *Realplex²* with *SYBR green I* and *Ex Taq* reagents (*Takara*, Dalian, China). Common primers for the real-time RT-PCR were designed according to a sequence similarity between the two ecotypes of *Sedum* (caxRT F: 5'-GATTCCTTT CTGCGTGGTTG-3', caxRT R: 5'-CTTGCGGCGACT ATCAGG-3'). *Actin* primers of both the ecotypes were designed according to a published *actin* sequence (Chao *et al.* (2010); actin F: 5'-TGTGCTTTCCCTCTATGCC-3', actin R: 5'-CGCTCAGCAGTGGTTGTG-3'). For absolute quantification of *CAX2* transcription, a series of dilutions of plasmids (from 1×10^{-1} to 10^{-6} ng) were made to generate standard curves.

Analysis of SaCAX2h over-expressed transgenic plants: The constructed 35S-SaCAX2h vector was introduced into an *A. tumefaciens* streain LBA4404 and then transformed into tobacco (*Nicotiana tabacum* L. cv. Petit-Havana SR1) according to the method of Helmer *et al.* (1984). Surface-sterilized T_1 seeds of two transgenic tobacco lines were germinated on Gamborg (B5) plates containing hygromycin L (30 mg dm^{-3}) to select hygromycin-resistant seedlings. For Cd tolerance analysis, the seedlings were transferred to a B5 medium containing 150 µM Cd for 14 d. To determine Ca, Mn, and Cd content in plants, both the wild-type and transgenic plants were transferred to hydroponic culture. One-month-old plants were then treated with 100 µM $CdCl_2$ for one week. Ca, Mn, and Cd content in plant tissues was measured as described by Yang *et al.* (2004).

A portion (0.1 g) of plant samples was digested in a high-purity acid mixture (HNO_3:$HClO_4$, 4:1, v/v) at 200 to 220 °C. Concentrations of Ca, Mn, and Cd in the solutions were determined using ICP-MS.

Statistical analysis: Data of the quantitative real-time RT-PCR analysis and Cd, Ca, and Mn content were processed using the *SPSS* package (*v.16.0*), analysis of variance (*ANOVA*) was also performed on the data sets. Graphical work was carried out using *SigmaPlot* (*v. 10*).

Results

After the treatment with 100 µM Cd for 8 d, leaves of the HE plants were harvested to analyze the cellular distribution of Cd in leaf cross sections. As shown in Fig. 1, bright and green fluorescence which indicated the combination of Cd with the *Leadmium Green AM* dye was observed in vacuoles of mesophyll cells of the Cd-treated plants. In contrast, the untreated plants did not possess any green fluorescence which indicated no Cd accumulated in their vacuoles.

The full length cDNAs encoding CAX2-like proteins were isolated from the HE and NHE plants and they were named as *SaCAX2h* (GenBank acc. No. JQ012930.1) and *SaCAX2n* (acc. No. JQ012931.1). Both genes encoded proteins of 449 amino acids and the amino acids of SaCAX2h and SaCAX2n proteins were 96 % identical, and they also showed 68 and 70 % identities with AtCAX2, respectively. The two transporters contained a nine-amino acid region which was called "Ca^{2+} domain". They also possessed a CAF (Cys-Ala-Phe) domain which is responsible for Mn^{2+} specificity in AtCAX2 (Shigaki *et al*. 2003). Two α-repeats and signature motif regions for CAXs within these repeats were also observed in the two transporters (Fig. 2).

The subcellular localization of SaCAX2h and SaCAX2n were investigated by transiently expressing the C-terminal eGFP fusion protein of SaCAX2h or SaCAX2n in tobacco epidermal cells. Green fluorescence of eGFP alone was observed in the plasma membrane and nucleus (Fig. 3*A*-*C*). Red auto-fluorescence of chloroplasts separated green fluorescence of SaCAX2h-eGFP and the plasma membrane, which indicated SaCAX2h-eGFP was localized to the tonoplast (Fig. 3*D*-*G*). The SaCAX2n-eGFP was also found to be localized to the vacuolar membrane (Fig. 3*H*-*K*).

A drop test shows that both SaCAX2h and SaCAX2n increased Cd sensitivity in the Δzrc1 yeast cells, and the yeast cells expressing the SaCAXs:eGFP fusion proteins showed a similar growth under the Cd treatments (Fig. 4*A*). The localization of the eGFP fused protein reveals that SaCAXs:eGFP was localized to the vacuolar membrane of the Δzrc1 yeast cells (Fig. 4*B*). Cadmium tolerance analysis in the liquid medium also shows an increased Cd sensitivity in the *SaCAX2s* expressed cells (Fig. 5*A*), however, the yeast cells expressing *SaCAX2h* or *SaCAX2n* accumulated a significantly higher Cd content and Ca content after the incubation in 10 µM Cd for 48 h (Fig. 5*B*).

The Ca and Mn transport ability of the two transporters were also analyzed. The SaCAX2h, SaCAX2n, and SaCAX2s:eGFP fused proteins suppressed Ca^{2+} and Mn^{2+} hypersensitivity in the K667 yeast mutant, and SaCAXs:eGFP was also localized to the vacuolar membrane of the K667 yeast cells (Fig. 6*A*,*B*). In consistence with the drop test, SaCAX2s enhanced Ca and Mn tolerance in the yeast mutant (Fig. 7*A*,*B*). The yeast cells expressing SaCAX2s accumulated a 20 % higher content of both Ca and Mn than the yeast cells containing the empty vector (Fig. 7*C*).

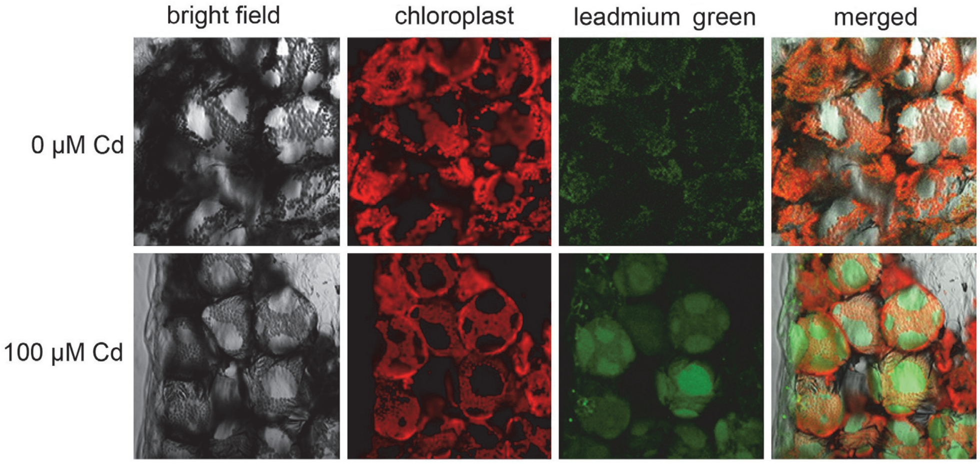

Fig. 1. Visualization of Cd in leaf cross-sections of the hyperaccumulating ecotype of *Sedum alfredii* using a *Leadmium^{TM} Green AM* dye. Cadmium localization in leaf cross-sections of control plants and plants treated with 100 µM $CdCl_2$ for 8 d. The green fluorescence indicates the binding of the dye with Cd; the red fluorescence indicates auto-fluorescence of chloroplasts.

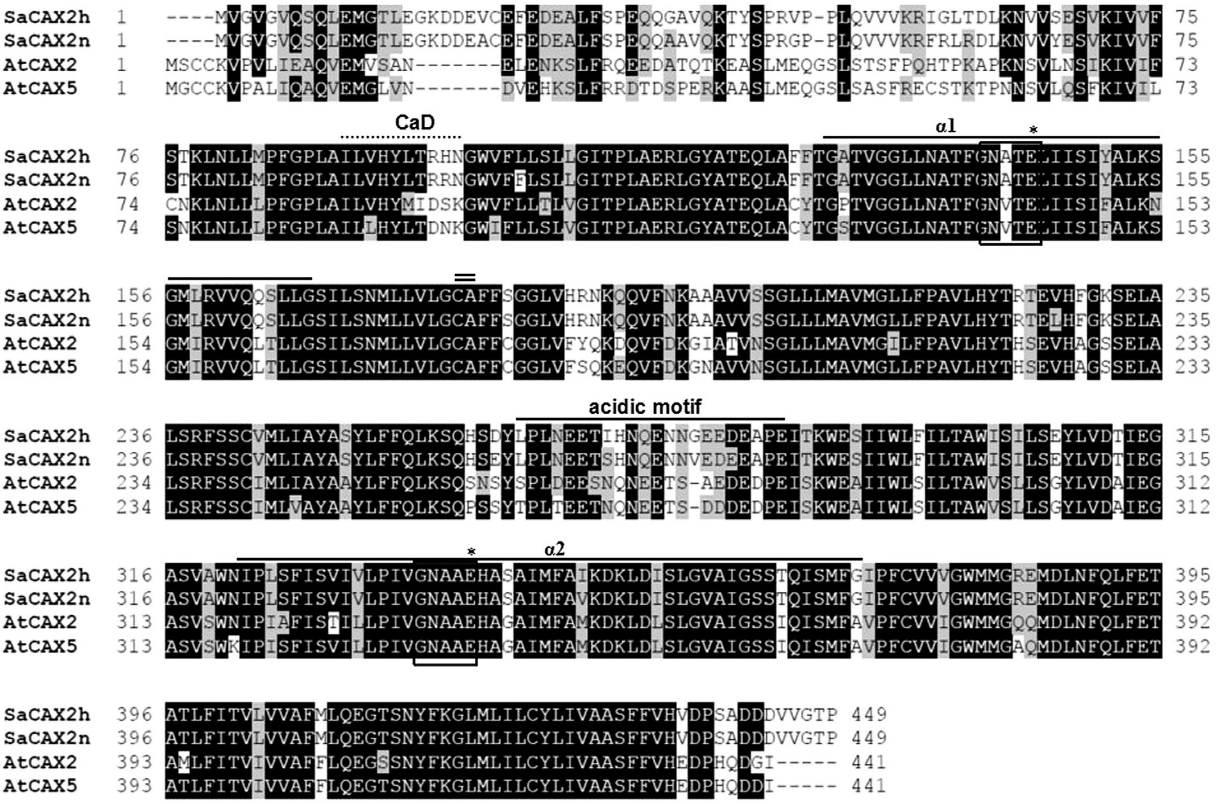

Fig. 2. The alignment of predicted amino acid sequences of polypeptides encoded by *SaCAX2h*, *SaCAX2n*, *AtCAX2,* and *AtCAX5*. The alignment of deduced amino acid sequences of SaCAX2h and SaCAX2n compared with AtCAX2 and AtCAX5. A nine-amino acid region (CaD) is included in this investigation (the *dotted line*). A hydrophilic motif rich in acidic residues is *overlined*. Amino acids specific for Mn^{2+} domain (Cys-Ala-Phe, CAF) of AtCAX2 are denoted with the *double lines*. Two α-repeats are indicated with the *lines*, and signature motif regions for CAXs within these repeats are indicated with the *box*. Two conserved glutamic acid residues are indicated with the *asterisks*.

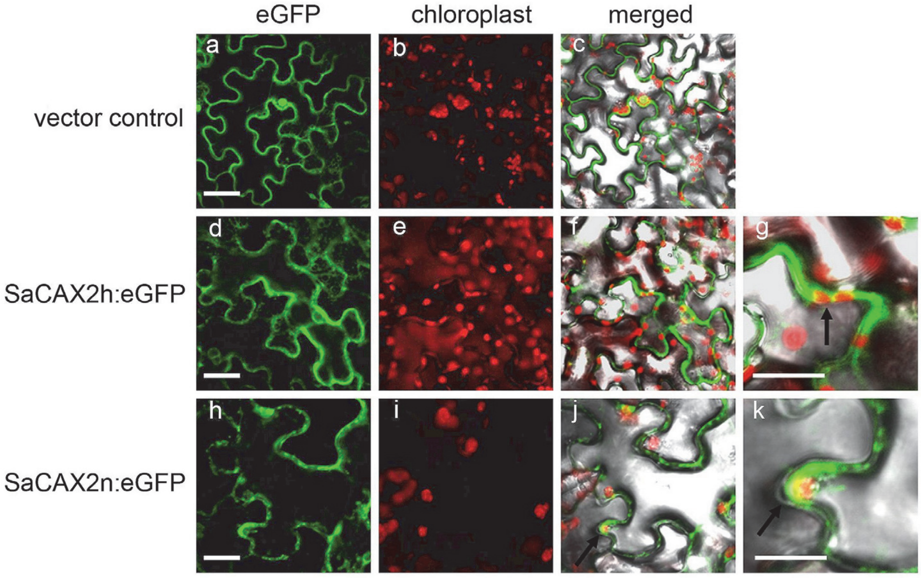

Fig. 3. Subcellular localization of SaCAX2s. Confocal images show eGFP fluorescence (*green*) and auto-fluorescence of chloroplasts (*red*) in tobacco epidermal cells (*a-c*: localization of eGFP alone in tobacco epidermal cells; *d-g*: localization of SaCAX2h:eGFP fusion protein; *h-k*: localization of SaCAX2n:eGFP fusion protein). The magnification views of *f* and *j* are shown in *g* and *k*. The *black arrows* indicate the tonoplast. *Bars* = 50 µm.

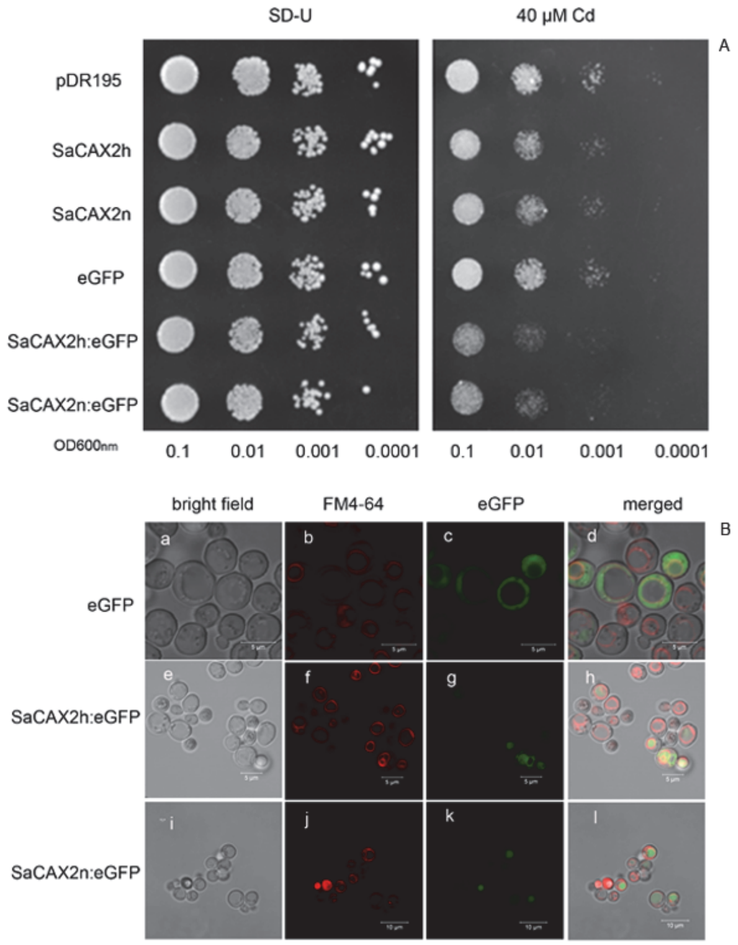

Fig. 4. The Cd transport activity analysis of SaCAX2h and SaCAX2n using the *Δzrc1* yeast. *A* - Transformants Δzrc1 expressing pDR195, SaCAX2h, SaCAX2n, eGFP, SaCAX2h:eGFP, or SaCAX2n:eGFP were grown on plates supplied with 0 or 40 µM $CdCl_2$. After incubation at 30 °C for 3 - 4 d, the plates were photographed. *B* - Localization of eGFP, SaCAX2h:eGFP, or SaCAX2n:eGFP in yeast cells. Bright field images of the Δzrc1 transformants (*a*, *e*, *i*), FM4-64 fluorescence images (*b*, *f*, *j*), eGFP fluorescence images (*c*, *g*, *k*), and artificially merged images (*d*, *h*, *l*) are shown. Fluorescence of GFP was excited at 488 nm, and red fluorescence was excited at 543 nm.

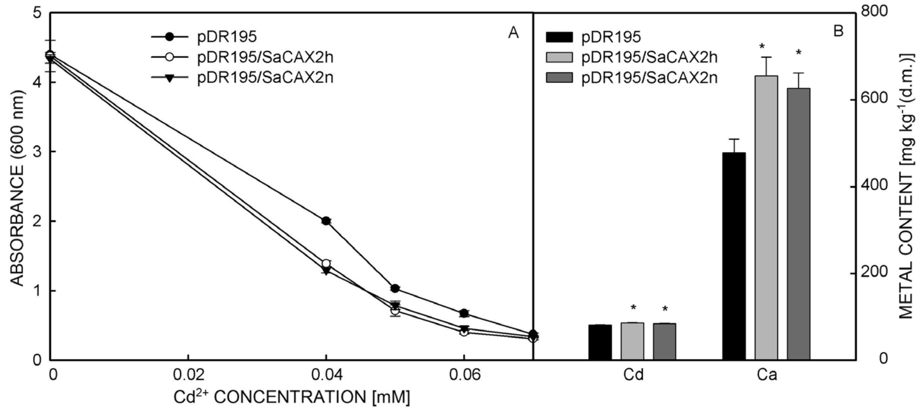

Fig. 5. Content of Cd and Ca in Δzrc1 yeast cells expressing SaCAX2s. *A* - The Δzrc1 transformants expressing pDR195, SaCAX2h, or SaCAX2n were grown in a liquid SD selective medium containing 0, 40, 50, 60, or 70 µM $CdCl_2$. Absorbance at 600 nm was measured after 24-h incubation. *B* - The Δzrc1 transformants expressing pDR195, SaCAX2h, or SaCAX2n were grown in a liquid SD selective medium in the presence of 10 µM $CdCl_2$. The cells were incubated at 30 °C for 48 h, Cd and Ca content was measured by an ICP-OES. Means ± SEs from six different colonies. *Asterisks* indicate means significantly different at $P < 0.05$.

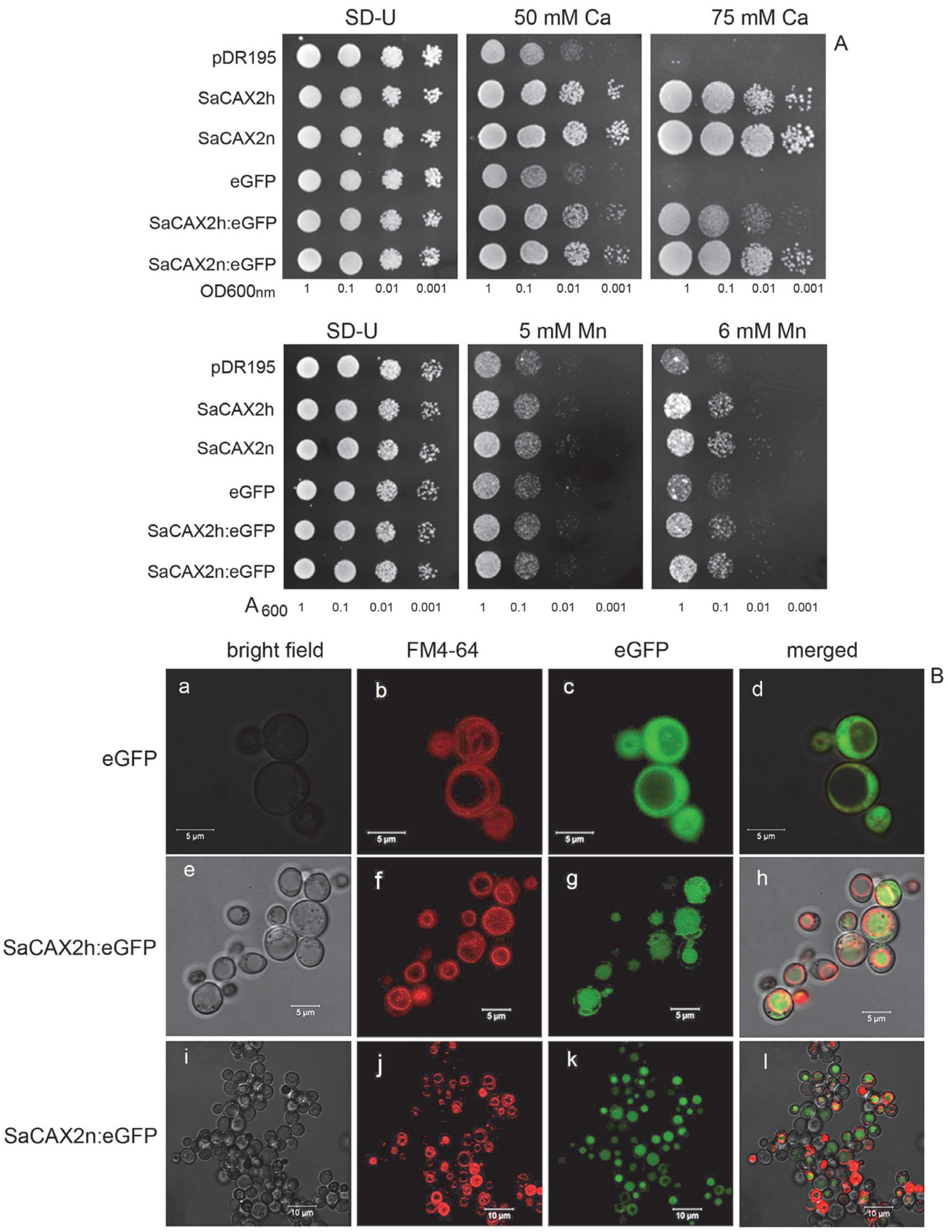

Fig. 6. Transport activity of SaCAX2h and SaCAX2n in the K667 yeast mutant. *A* - Yeast cells (K667) expressing an empty vector (pDR195), SaCAX2h, SaCAX2n, eGFP, SaCAX2h:eGFP, or SaCAX2n:eGFP were spotted onto a selection SD medium containing uracil, onto a YPD medium containing 50 or 75 mM Ca, and a selection SD medium containing uracil and 5 or 6 mM Mn. The pictures were taken after 4 d incubation at 30 °C. *B* - Localization of eGFP, SaCAX2h:eGFP, or SaCAX2n:eGFP in yeast cells. Bright field images of *Δzrc1* transformants (*a*, *e*, *i*), FM4-64 fluorescence images (*b*, *f*, *j*), eGFP fluorescence images (*c*, *g*, *k*), and artificially merged images (*d*, *h*, *l*) are shown. Fluorescence of GFP was excited at 488 nm and the red fluorescence was excited at 543 nm.

The transcriptions of *SaCAX2h* and *SaCAX2n* were measured by the real-time RT-PCR (Fig. 8). The HE and NHE plants possessed almost equal *CAX2* mRNA amounts under the control conditions. The expression patterns of *SaCAX2h* and *SaCAX2n* in plants subjected to Cd were quite different. In the HE plants, *CAX2* mRNA in shoots was significantly up-regulated, and it was significantly down-regulated in roots whereas it was significantly reduced in shoots of the NHE plants.

The transgenic tobacco plants over-expressing *SaCAX2h* were constructed to investigate the role of SaCAX2h in *planta*. The over-expression of *SaCAX2h* enhanced plant growth on the B5 medium containing 150 µM Cd (Fig. 9*A*). The Cd, Ca, and Mn content of the two transgenic lines was determined after the plants were treated with 100 µM Cd in the hydroponic solution for one week. The two transgenic lines (OX6 and OX9) accumulated 19 and 17 % higher Cd in shoots, and the Cd content in roots of OX6 and OX9 was 36 and 31 % higher than in the non-transgenic (NT) plants (Fig. 9*B*). These transgenic lines accumulated 32 and 31 % more Ca in shoots, and the Ca content in roots of the NT and transgenic plants was not significantly different (Fig. 9*C*). The OX6 and OX9 lines accumulated significantly more Mn in shoots (60 - 79 %) and roots (22 - 29 %) than the NT plants (Fig. 9*D*).

Discussion

In this study, Cd localization in the leaf cross sections of the HE plants was investigated using the *Leadmium^{TM} Green AM* dye. The results indicate that the vacuoles of mesophyll cells were one of the Cd storage sites in the HE plants. Parenchyma cells, especially in stems, have been found to accumulate the majority of Cd in the HE, and Cd accumulation is likely associated with calcium pathways (Tian *et al.* 2011). These results are indicative of a critical role of vacuolar sequestration in the HE plants. In another Zn/Cd hyperaccumulator *N. caerulescens*, the vacuoles of mesophyll cells were found to store most Zn and Cd (Ma *et al.* 2005). Among known membrane transporters, *AtHMA3*, *OsHMA3, NcHMA3*, and *AtCAXs* have been found to transport Cd into vacuoles (Korenkov *et al.* 2007a,b, Morel *et al.* 2009, Ueno *et al.* 2010, 2011). However, unlike HMA3 transporters, CAX transporters of heavy metal hyperaccumulators were less analyzed although some of *AtCAXs* homology genes are highly expressed or differentially expressed in response to Zn in *A. halleri* and *N. caerulescens* (Weber *et al.* 2006, Van de Mortel *et al.* 2006, 2008). In this study, two *CAX2*-like genes were isolated from the HE and NHE plants (*SaCAX2h* and *SaCAX2n*) using the RACE method, and the function of two transporters in Cd, Ca, and Mn accumulation was characterized.

Both SaCAX2h:eGFP and SaCAX2n:eGFP fusion proteins were localized to the vacuolar membrane of the tobacco epidermal cells and yeast cells, and the eGFP fused proteins were functional in yeast. The yeast complementation assays reveal that both SaCAX2h and SaCAX2n suppressed Ca hypersensitivity in the yeast K667 mutant. The Ca tolerance analysis in the liquid culture showed the transport ability of SaCAX2h was lower than of SaCAX2n although the deduced amino acids sequences of these two proteins shared a 96 % identity. A nine amino acid region (CaD) is involved in a CAX-mediated Ca^{2+} specificity and is highly variable among the plant CAX-like transporters (Shigaki *et al.* 2001); there was only one amino acid difference in this region between SaCAX2h and SaCAX2n. The CAF domain has been found to be responsible for Mn^{2+} specificity in AtCAX2 (Shigaki *et al.* 2003); SaCAX2h and SaCAX2n also possessed this domain and they could suppress Mn hypersensitivity in the yeast to the same level. For AtCAX1, AtCAX2, and AtCAX5, only expression of an N-terminal truncated form in yeast can mediate Ca and Mn transport, which suggests that the N-terminal regulates transport activity (Pittman *et al.*, 2001, 2002). However, this is not the case of LeCAX2, SaCAX2h, and SaCAX2n (Edmond *et al.* 2009).

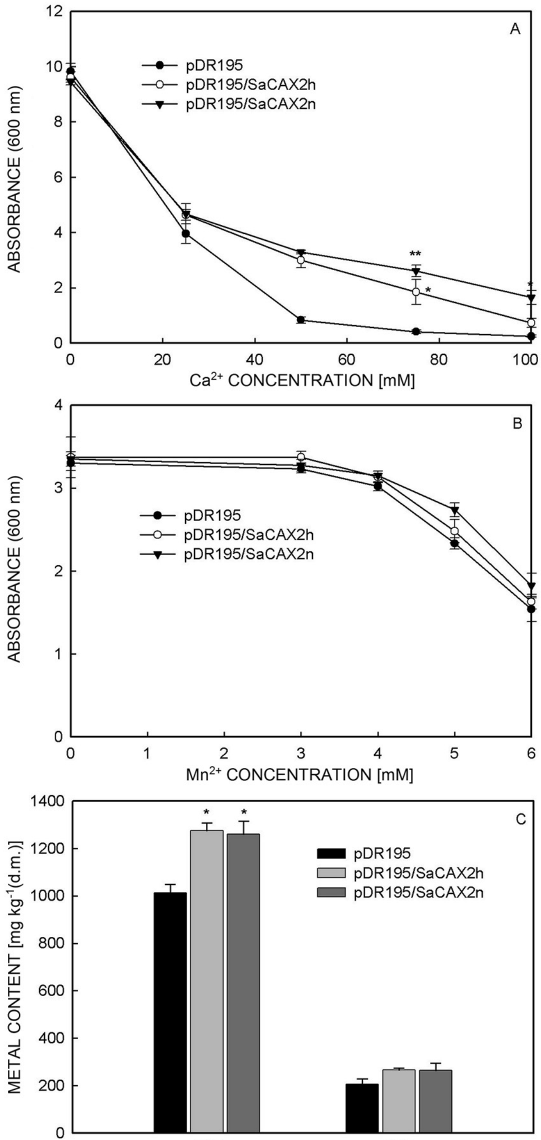

Fig. 7. The content of Ca and Mn in K667 yeast cells expressing SaCAX2s. *A* and *B* - The K667 transformants expressing pDR195, SaCAX2h, or SaCAX2n were grown in a liquid SD selective medium containing a range of Ca or Mn concentrations. Absorbance at 600 nm was measured after 24-h incubation. *C* - The K667 transformants expressing pDR195, SaCAX2h, or SaCAX2n were grown in a liquid SD selective medium in the presence of 1 mM $CaCl_2$ or 200 µM $MnSO_4$. The cells were incubated at 30 °C for 48 h, Ca and Mn content was measured by an ICP-OES. Means ± SEs from six different colonies. *Asterisks* indicate means significantly different at $P < 0.05$.

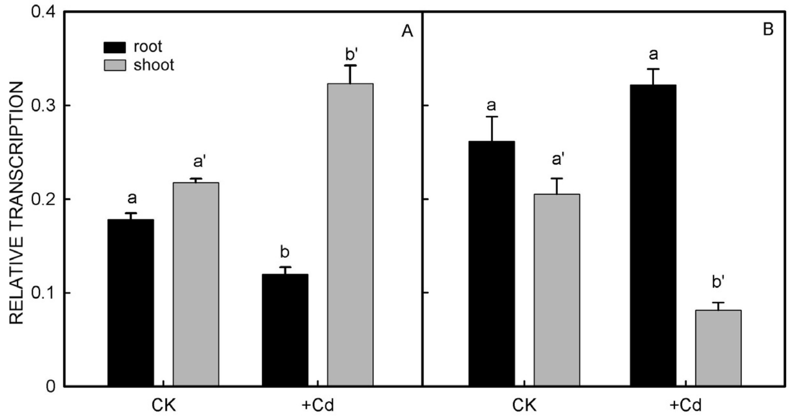

Fig. 8. Expression patterns of *SaCAX2h* and *SaCAX2n* in response Cd in HE and NHE plants. The expression of *CAX2* genes in roots and shoots of the HE plants (*A*) and NHE plants (*B*) after treatment with 2 µM Cd for 8 d. Data shown are the transcriptions of *SaCAX2h* and *SaCAX2n* relative to *actin*. Means ± SEs of three PCR repetitions for each of three biological replicates. Different letters indicate means significantly different at $P < 0.05$.

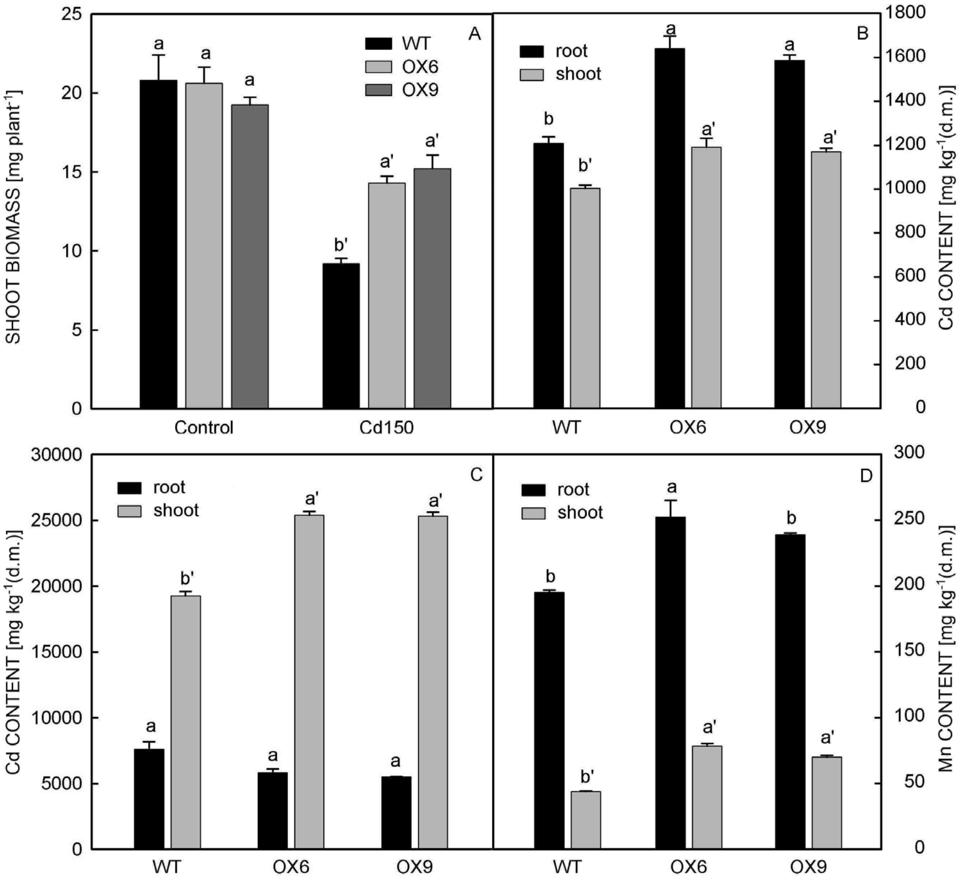

Fig. 9. Metal tolerance and accumulation analyses of transgenic tobacco plants over-expressed SaCAX2h. *A* - The shoot biomass of NT plants and two transgenic lines after treatment with 150 µM $CdCl_2$ for 14 d. *B to D* - Cd, Ca, and Mn content in NT and transgenic plants after treatment with 100 µM $CdCl_2$ for 7 d in a hydroponic culture. Means ± SEs ($n = 3$). Different letters indicate means are significantly different from NT tobacco at $P < 0.05$.

In planta, tobacco plants over-expressing AtCAX2 accumulate more Ca and Mn than only vector expressing plants (Hirschi *et al.* 2000). In this study, over-expression of SaCAX2h also enhanced Ca and Mn accumulation in the tobacco plants.

The role of SaCAX2s in Cd tolerance and accumulation was also determined. Although the expression of *SaCAX2s* enhanced Cd sensitivity in the Δzrc1 yeast mutant, the Cd and Ca content in the yeast cells significantly increased. Therefore, we speculate that the enhanced Ca sequestration caused that much more Ca and Cd were absorbed by the yeast cells. Similarly, the expression of *AhCAX2* in the YYA4 yeast mutant resulted in an exacerbated Cd-hypersensitivity (Becher *et al.* 2004), but the authors did not measure the Cd content in yeast. Among AtCAXs, only AtCAX4 has been found to slightly rescue the Cd sensitivity of the *AtIRT1*-expressing yeast strain (Cheng *et al.* 2002); AtsCAX1 enhances Cd and Zn tolerance in yeast only after the H^{338} was mutated to N (Shigaki *et al.* 2005). However, the maximal Cd transport ability in isolated root cell tonoplast of the AtCAXs over-expressing plants shows that AtCAXs can transport Cd^{2+} to varying degrees in tobacco tonoplast vesicles, thus CAX2 and CAX4 have higher Cd transport and selectivity (Korenkov *et al.* 2007a,b).

In the HE and NHE ecotypes of *Sedum* plants, *SaCAX2h* transcription in shoots was up-regulated when subjected to Cd whereas *SaCAX2n* was down-regulated. This indicates that interaction between Ca and Cd was quite different between the HE and NHE plants. It has been revealed that the distribution pattern of Cd in both stems and leaves of the HE is very similar to Ca; however, in the NHE plants, Cd is distributed in a very similar pattern to that of Zn but not Ca (Tian *et al.* 2011). It seems that the expression patterns of *SaCAX2s* were consistent with the Ca/Cd distribution patterns in the two ecotypes of *Sedum*. *In planta*, the over-expression of *SaCAX2h* significantly enhanced Cd accumulation in both roots and shoots of the transgenic tobacco plants. Over-expression of *AtCAX2* in KY160 or KY14 tobacco plants also enhances Cd accumulation in roots, but the Cd content in shoots is not significantly changed (Hirschi *et al.* 2000, Korenkov *et al.* 2007b). Korenkov *et al.* (2007b) speculated that perhaps 0.02 μM Cd used in their experiment or 0.1 μM Cd used by Hirschi *et al.* (2000) exceeds a level that allows root retention and reduction of Cd translocation to shoots. In our study, the Cd content in the hydroponic culture was 100 μM and the transgenic plants over-expressing *SaCAX2h* accumulated significantly higher amount of Cd than NT plants in roots and shoots. It seems that different concentrations of Cd could cause different Cd distribution patterns in transgenic tobacco plants.

Based on the results, we conclude that SaCAX2h was responsible for Ca and Mn sequestration into vacuoles and was also possibly involved in Cd accumulation in the HE plants. Further experiments will be designed to analyze Cd transport ability in tonoplast vesicles of *SaCAX2h* over-expressing plants.

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