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Enhanced Accumulation of Metals in Bottle Gourd Plants Expressing an *Arabidopsis* Cation/H^+ Exchanger

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ABSTRACT

In plants, cation sequestration from the cytosol to the vacuole is thought to be an important component for ion tolerance and its potential for use in phytoremediation. The *Arabidopsis* vacuolar exchanger *CAX2* may be a key mediator in this machinery. Here we demonstrate that transgenic bottle gourd plants expressing CAX2 have more calcium (Ca^{2+}) than wild-type. In addition, expression of CAX2 in bottle gourd showed a significant increase of the two metals tested (Mn^{2+} and Cd^{2+}). Furthermore, transgenic bottle gourd seedlings showed no phenotypic alterations during the whole growth period. These results indicate that intact CAX2 has broad substrate specificity, and suggest that the modulation of this exchanger might be meaningful for the future strategies to improve plant ion tolerance.

Keywords: CAX2, ion tolerance, phytoremediation
Abbreviations: CAX2, cation exchanger 2; **MES**, 2-morpholinoethanesulfonic acid monohydrate; ***npt*II**, neomycin phosphotransferase; **PCR**, polymerase chain reaction

INTRODUCTION

Manipulation of vacuolar exchange activity may be an important component of genetic modifications to improve plant productivity and ion tolerance. Overexpression of an *Arabidopsis* vacuolar Na^+/H^+ antiporter in plants enhanced salinity tolerance (Apse *et al*. 1999). Expression of sCAX1, a deregulated vacuolar Ca^{2+}/H^+ exchanger from *Arabidopsis*, in tobacco (*Nicotiana tabacum*) increased Ca^{2+} accumulation and Ca^{2+}-related stress sensitivity (Hirschi 1999). Ectopic expression in tobacco of AtMHX, an *Arabidopsis* Mg^{2+} and Zn^{2+} vacuolar antiporter, also increased sensitivity to Mg^{2+} and Zn^{2+} (Shaul *et al*. 1999). Thus, dysregulated expression of vacuolar antiporters can impart positive (salinity tolerance) or negative (ion sensitivity) effects on plant growth.

Previously, two *Arabidopsis* genes, *CAX1* (for calcium exchanger 1) and *CAX2* were identified by their ability to suppress mutants of yeast defective in vacuolar Ca^{2+} transport (Hirschi *et al*. 1996; Shigaki *et al*. 2003). CAX1 biochemical activities in yeast vacuoles correlate well with those described for plant vacuolar Ca^{2+}/H^+ antiport activities, and recent evidence suggests that CAX1 plays a role in plant Ca^{2+} homeostasis (Hirschi 1999). Biochemical activities of CAX2 in yeast suggest that this gene product has a low affinity for Ca^{2+} (Hirschi *et al*. 1996). In yeast, either *CAX1* or *CAX2* can compensate for the absence of the endogenous vacuolar Ca^{2+}/H^+ exchanger (Hirschi *et al*. 1996). The functional redundancy of *CAX1* and *CAX2* suggests that loss-of-function cation exchanger mutations may not reveal a perceived phenotype. Ectopic expression of *CAX1* in tobacco caused Ca^{2+} deficiency-like symptoms (Hirschi 1999; Shigaki and Hirschi 2006), suggesting that heterologous *CAX2* expression might provide useful insights into *CAX2* function (Diener and Hirschi 2000). Actually, the tobacco plants expressing CAX2 contained more Ca^{2+} than wild-type plants although these plants were as vigorous as controls, and showed a significant increase of other metal ions, Cd^{2+} and Mn^{2+} (Hirschi *et al*. 2000).

Recently, bottle gourd (*Lagenaria siceraria* Standl.), which is an archaeological plant species (Erickson *et al*. 2005), has attracted public attention because it is used as a rootstock for watermelon in Far Eastern countries (Davis *et al*. 2008). Grafting onto bottle gourd seedling generally provides resistance to soil-borne diseases such as watermelon fusarium wilt (*Fusarium oxysprum* f. sp. *niveum*), and confers tolerance to low-soil temperature. However, a recent epidemic of sudden wilt in cucurbit crops was caused by some abiotic/biotic stresses such as high nutrient soil following a progressive salinization of irrigated land, and other soil-borne diseases (Edelstein *et al*. 1999; Gal-On *et al*. 2005; Park *et al*. 2005). Consequently, to improve the performance of bottle gourd rootstock for cucurbit scions through genetic engineering it might be meaningful to control the stresses.

Our approach here was to evaluate the potential for increasing the cation levels of bottle gourds through ectopic expression of an *Arabidopsis* cation/H^+ exchanger. Our results suggest that modulation of cation exchangers could make an important contribution toward developing a plant material for phytoremediation.

MATERIALS AND METHODS

Agrobacterium-mediated transformation of bottle gourd

Agrobacterium tumefaciens LBA 4404 octopine (Hoekema *et al*. 1983) carrying the binary vector pBI121 was used for the transformations. The plasmid was reconstructed with the T-DNA consisted of *nos-pro*/*nptII*/*nos-ter*/*35S*/*CAX2*/*nos-ter* (**Fig. 1A**), and then introduced into *A. tumefaciens* using the freeze-thaw method (Holsters *et al*. 1978). The bacteria were grown for 2 days in YEP medium [10 $g \cdot L^{-1}$ yeast extract (Becton, Dickinson and Co., Sparks, USA), 10 $g \cdot L^{-1}$ peptone (Becton, Dickinson and Co.), 5 $g \cdot L^{-1}$ NaCl (Sigma-Aldrich, St. Louis, USA)] containing 50 $mg \cdot L^{-1}$ kanamycin monosulfate (Sigma-Aldrich) and 50 $mg \cdot L^{-1}$ rifampicin (Sigma-Aldrich) at 28°C on a rotary shaker (220 rpm) until an

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OD_{600} = 1.0 was obtained.

Bottle gourd (*Lagenaria siceraria* Standl.) transformation was performed via *Agrobacterium*-mediated transformation method using the cotyledon explants of G5 inbred line as previously reported (Han *et al*. 2005) except that 100 mg·L^{-1} kanamycin monosulfate was used as a selective agent for selecting transgenic shoots.

DNA isolation and Southern blot analysis

Bottle gourd genomic DNA was extracted from leaf tissue as previously described (Paterson *et al*. 1983). For Southern blot analysis, DNA (5-10 μg) was digested with *Xba*I (which made only one cut in the T-DNA region and another cut elsewhere in the plant DNA), and then separated by electrophoresis in a 0.9% agarose gel, and then blotted onto a nylon membrane (Zeta-probe GT membrane; Bio-Rad Laboratories, Hercules, USA) according to the manufacturer's instructions. The probe for the *CAX2* gene was isolated from an *Xba*I-*Sac*I (1.4-kb) restriction fragment of the plasmid (Hirschi *et al*. 2000). The membrane was pre-hybridized overnight at 65°C in 7% sodium dodecyl sulfate (SDS; Sigma-Aldrich) SDS and 0.25 M Na_2HPO_4, and then hybridized overnight at 65°C in the same solution containing the probe labeled with ^{32}P-dCTP using NEBlot kit (New England BioLabs, Ipswich, USA). The membrane was washed twice for 30 min each with 20 mM Na_2HPO_4 and 5% SDS at 65°C, and then washed twice again for 30 min each with 20 mM Na_2HPO_4 and 1% SDS 65°C. The membrane was exposed to X-ray film at -80°C.

RNA isolation and Northern blot analysis

Total RNA was also extracted from the newly developing young leaves of the randomly selected PCR-positive plants using the RNeasy Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For Northern blot analysis, total RNA (7 μg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde, blotted onto a Zeta-Probe GT membrane (Bio-Rad Laboratories) according to the manufacturer's instructions. Hybridization and washing were as previously described in Southern blot analysis.

Detection of Ca^{2+} using the acetoxymethyl (AM) ester of Fluo-4

The pollen grains from mature *CAX2*-expressing and wild-type plants were incubated in a modified incubation solution that containing 11.4 μM Fluo-4/AM ester (Molecular Probes, Eugene, USA), 0.02% Pluronic F-127 (Molecular Probes, Eugene, USA), 0.1% digitonin (Fluka BioChemie, GmbH, Buchs, Switzerland), 22.45 mM (pH 6.0) 2-morpholinoethanesulfonic acid monohydrate (MES) (Fluka BioChemie). Materials preparation and Fluo-4/AM ester loading were performed as previously described (Gee *et al*. 2000).

Fluorescence from the pollens was detected using an inverted microscope (Axiovert 100M; Carl Zeiss, Germany) coupled with a scanning laser confocal system (LSM 510; Carl Zeiss, Germany). Argon laser light (488 nm wavelength, 70% power) was used to excite the Ca^{2+}-binding Fluo-4 and the emission light was measured at 520 nm.

Progeny segregation analysis

Segregation of the *npt*II gene in the three independent T_1 progenies (TCB-1, TCB-2, and TCB-5 line) was confirmed by PCR analyses. For PCR amplification of *npt*II gene (AY781296 in Nucleotide D/B; National Center for Biotechnology Information, USA), one set of primers were designed and used: 5′-GAGGCTATTCGG CTATGACTG-3′ for forward primer; and 5′-ATCGGGAGCGGC GATACCGTA-3′ for reverse primer. The amplification of *npt*II involved 35 cycles of 95°C for 30 s, 70.5°C for 45 s, and 72°C for 1 min using a thermal cycler (iCycler; Bio-Rad Laboratories). The de-coated seeds of TCB-1 T_1 line progenies were also sown in the MS medium (Murashige and Skoog 1962) with 100 mg·L^{-1} kanamycin monosulfate for screening of resistant ones. After four weeks of sowing, the number of seedlings that normally rooted was recorded.

Metal analysis

After kanamycin-mediated selection of T_2 seedlings derived from self-pollinated T_1 plants (a population showing the segregation pattern of 3:1), eight homozygous T_2 populations were selected. Six *CAX2*-expressing bottle gourd seedlings were established on three independent sets of the household hydroponic culture baths (Hi-Green; Gwang Myeong Control Electric, Korea), which were equipped with waterproof electric motors that continuously circulated the medium at 5-L per min. Each bath contained 12 L of medium that was changed with the progressively fortified MS medium (Murashige and Skoog 1962) with 20 μM $CdCl_2$ every six days. De-ionized water was used for preparing all medium, and the pH of the medium was adjusted to 5.8 using 1 N HCl and 1 N NaOH before applying. Each root and shoot from bottle gourd plants grown on the hydroponic for 28 days was dried at 70°C for 4 days. Dried samples were ground, and then a total of 0.25 g (dry weight) was digested using a ternary solution (HNO_3: H_2SO_4: $HClO_4$ = 10: 1: 4 ratio by volume). Total Ca^{2+}, Mn^{2+}, and Cd^{2+} contents per gram of dry weight were determined by inductively coupled plasma emission spectrophotometry (Integra XM2; GBC, Australia).

RESULTS

Generation of *CAX2*-expressing bottle gourd

To examine the role of CAX2 in ion homeostasis, we expressed *CAX2* driven by the *Cauliflower mosaic virus* 35S promoter (35S *pro*) in bottle gourd plants (**Fig. 1A**). We generated 15 *CAX2*-expressing independent lines. First, we examined the presence of the selectable marker genes by PCR analysis: Excepted 650-bp fragments of *npt*II were amplified in 15 putative transgenic plants (data not shown). We randomly selected and confirmed 10 transgenic bottle gourd events by Southern blot analysis. As demonstrated in **Fig. 1B**, the transgenic bottle gourd plants contained one to five copies of copy numbers of the *CAX2* expression vector. The plants termed TCB-1, TCB-4, TCB-5, TCB-6, TCB-8, and TCB-9 appeared to contain single insertions. The RNA gel blot showed that the *CAX2* transcript accumulated in all of the transgenic lines (**Fig. 1C**, part of the data are shown). The inability to detect *CAX2* homology in the control lines by southern or northern analysis may be due to the stringency of hybridization used in this study.

Detection of Ca^{2+} using the acetoxymethyl (AM) ester of Fluo-4

The Fluo-4/AM ester was loaded as a probe to detect free Ca^{2+} in the cells of *CAX2*-expressing bottle gourd that may sequestrate Ca^{2+} into the compartments, especially the vacuoles. The Fluo-4·AM-loaded transgenic pollen clearly showed much higher intensity of fluorescence than non-transgenic pollens (**Fig. 2**). However, no detectable difference in fluorescence intensity was observed in the internode sections (data not shown). The present suggests that the higher intensity of fluorescence in transgenic pollens could indicate more Ca^{2+} and/or faster efflux into cytoplasm for pollen germination than the non-transgenic pollens for the same duration of incubation for dye-loading.

Segregation analysis and selection of non-segregating population

Each T_0 plant (TCB-1, TCB-2, and TCB-5 line) were self-pollinated to generate T_1 progenies. The T_1 populations were analyzed to determine the presence of the *npt*II genes by PCR analysis. The segregation ratios of the selectable marker gene in each progeny were 15: 1 (positive: negative) in TCB-2 T_1 population, and 3: 1 in TCB-1 and TCB-2 T_1 populations (**Table 1**). We also confirmed the expression of *npt*II by kanamycin resistance test in T_1 seedlings. While wild-type seedlings formed no adventitious roots on the MS medium containing 100 mg·L^{-1} kanamycin monosulfate, the

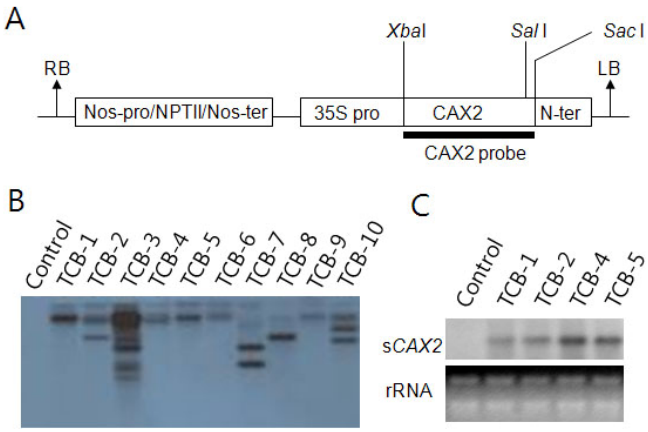

Fig. 1 Molecular analyses of *CAX2*-expressing bottle gourds. (**A**) T-DNA regions of pCaMV35S::CAX2. RB, Right border; LB, left border; Nos-pro, nopaline synthase promoter; NPTII, neomycin phosphotransferase gene; Nos-ter, nopaline synthase terminator; 35Spro, CaMV 35S promoter; CAX2, cation exchanger 2; N-ter, nopaline synthase terminator. (**B**) Southern blot analysis of the transgenic bottle gourd plants. Five to 10 μg of genomic DNA of randomly selected PCR-positive bottle gourd plants were digested with *Xba*I, and hybridized with the *CAX2* probe. Lanes: Control, negative control (wild-type bottle gourd); TCB-1 to TCB-10, transgenic bottle gourds lines with CAX2 gene. (**C**) Northern blot analysis of transgenic bottle gourd plants. Seven μg of total RNA from bottle gourd leaves were hybridized with the *CAX2* probe. Ethidium bromide-stained rRNA (*bottom*) is shown as a loading control. Lanes: Control, negative control (wild-type bottle gourd); TCB-1, TCB-2, TCB-3, and TCB-5, transgenic bottle gourd lines with CAX2 transcripts.

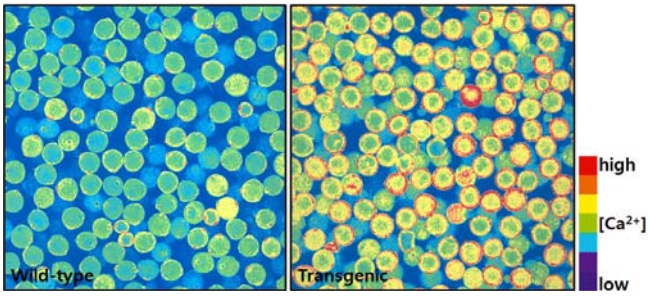

Fig. 2 Confocal scanning laser microscope (CSLM) images. The pollen of *CAX2*-expressing and wild-type were incubated in 11.4 μM Fluo-4/AM ester solutions. Argon laser light (488 nm wavelength, 70% power) was used to excite the Ca^{2+}-binding Fluo-4 and the emission light was measured at 520 nm. Rainbow color enhanced visualization of Ca^{2+}-binding Fluo-4 distribution and ratio-metrically indicated the intensity of fluorescence, where red is the maximum and violet is minimum intensity.

Table 1 Segregation ratios of *nptII* in T_1 progenies of transformed bottle gourd inbred line G5.

T_0 parent	Detection of transgene in T_1 plants		Expected segregation ratio	χ^2-value	*P*-value
	Positive	Negative			
TCB-1	73	28	3:1	0.399	0.527
TCB-2	66	8	15:1	2.627	0.105
TCB-5	21	8	3:1	0.103	0.748

All χ^2 values indicate the best fit among expected ratios.

resistant seedlings among the TCB-1 T_1 progenies vigorously rooted, exhibiting 3:1 ratio [resistant (R):susceptible (S)] (**Fig. 3**).

We selected one line, TCB-5, to generate the T_2 progenies along with T_1, which contained one copy of the construct with a strong expression of the transcript (**Fig. 1B, 1C**), and showed a 3: 1 segregation ratio (**Table 1**). The non-segregating T_2 population of TCB-5 line was subjected to further analysis of calcium and metal accumulation in hydroponic culture.

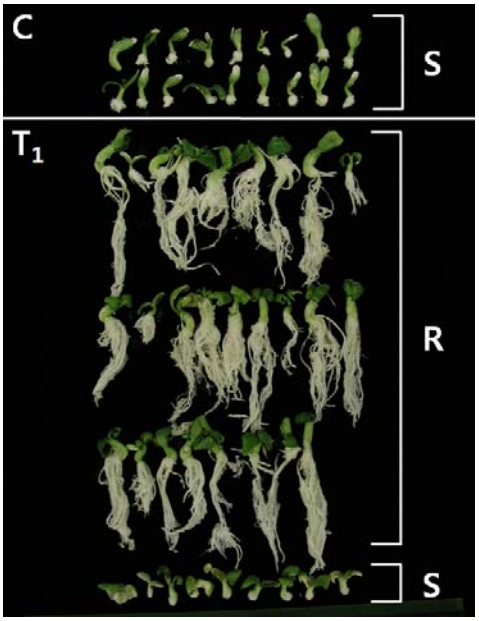

Fig. 3 Bioassay for *nptII* in the T_1 bottle gourd progeny. To evaluate kanamycin resistance, ten decoated seeds were cultured on MS medium supplemented with 100 mg·L^{-1} kanamycin monosulfate. After 4 weeks of culture, the wild-type seedlings did not root (C), while the *nptII*-expressing seedlings segregated 3:1 ratios as vigorously rooted or not (T1). S, susceptible seedlings to kanamycin; R, resistant seedlings to kanamycin.

CAX2-expressing plants accumulate increased cation contents

In this study, we used a hydroponic culture system to precisely assess the ionic alterations and sensitivity of the CAX2-expressing transgenic bottle gourd. As shown in *CAX2*-expressing bottle gourds grown on soil (data not shown), all of the *CAX2*-expressing T_2 lines did not alter the morphology or growth characteristics in hydroponic culture as well (**Fig. 4A, 4B**).

To ascertain whether CAX2 expression altered ion levels, metallic cations including Ca^{2+} were measured in the shoots and roots of transgenic plants. As shown in **Fig. 4C**, *CAX2*-expressing plants contained almost two fold of total Ca^{2+} and Mn^{2+} in root parts when compared to wild-type plants. In addition, the aerial parts of *CAX2*-expressing plants contained approximately 12 to 15% more the cations than those of wild-type plants. Moreover, Cd^{2+} levels were also double in CAX2-expressing root tissues with a 15% increase in the content of Cd^{2+} in the aerial parts. Meanwhile, there was no significant increase in any other metal ion (Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}) levels in both parts of *CAX2*-expressing bottle gourd plants (data not shown).

DISCUSSION

While the ectopic expression of N-terminus-truncated *Arabidopsis* CAX1 (sCAX1) showed Ca^{2+} deficiency-like symptoms in tobacco and tomato (Hirschi 1999; Park *et al*. 2005), the intact *CAX2* expression in bottle gourd plants did not alter the phenotype under a soil cultivation (data not shown). These results infer that the ectopic expression of CAX exchangers accompany a distinguished phenotypes depending to the existence of auto-inhibitory domain of CAX. In the present study, we showed that an intact *CAX2*-expressing bottle gourd plants also had no adverse phenotypic alterations in a hydroponic culture (**Fig. 3A, 3B**). Our finding indicates that the use of *CAX2* eliminates the severe Ca^{2+} deficiency-like symptom associated with the expression of the high capacity Ca^{2+} transporter, sCAX1 (Hirschi 1999; Park *et al*. 2004, 2005).

A number of ion-specific fluorescent indicators are commercially available now (e.g. http://www.probes.com).

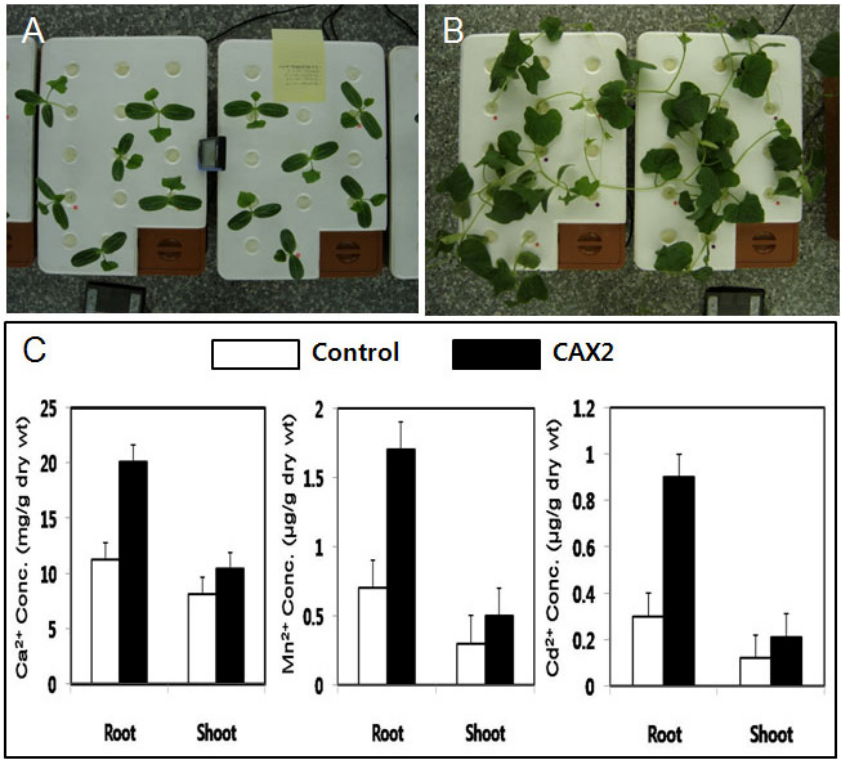

Fig. 4 Phenotype of *CAX2*-expressing bottle gourd plants under hydroponic culture, and their cation contents in shoots and roots. (**A**) Phenotypes of both *CAX2*-expressing (left) and wild-type (right) bottle gourd plants, which were grown a hydroponic culture system after 3 days (**A**) and 10 days (**B**) of transplanting. The media were progressively fortified every six days. After ten days of supplying the medium, the contents of cations were measured by inductively coupled plasma emission spectrophotometer (**C**). Data represent the mean values (±S.E.) obtained from three independent hydroponic culture systems in which eight bottle gourd seedlings were established. Control, negative control (wild-type bottle gourd); CAX2, *CAX2*-expressing bottle gourd.

It then becomes possible to measure and compare calcium fluctuations occurring simultaneously in different organelles. The technology to visualize fluctuations in cytosolic Ca^{2+} levels and its incorporation into the molecular genetics is an exciting new vista in plant biology (Allen *et al*. 2000; Kiegle *et al*. 2000). In this study, detectable difference of fluorescence intensity was observed only in their pollen grains (**Fig. 2**). This may be a result of loading feasibility into pollen through the germ pore or of a high level of activity of *CAX2* driven by the CaMV35S promoter (Mesa *et al*. 2004). In fact, in a previous study, transgenic bottle gourd plants expressing the GUS gene driven by the CaMV35S promoter showed a high level of GUS activity in their pollen grains as compared to other tissues (Han *et al*. unpublished data).

In plant cells, one component for engineering ion tolerance in plants appears to be the manipulation of plant vacuolar transporters. Increased expression of Na^+/H^+ antiporter confers increased sodium accumulation and thus increased salt tolerance in *Arabidopsis* (Apse *et al*. 1999). Expression of a vacuolar Zn^{2+} and Mg^{2+} transporter in tobacco confers heightened sensitivity to these specific ions (Shaul *et al*. 1999). We demonstrated here that expression of intact CAX2 in bottle gourd plants altered the Ca^{2+}, Cd^{2+}, and Mn^{2+} contents of aerial part (**Fig. 4C**). Mn^{2+} is a plant micronutrient that is required for many enzyme-catalyzed reactions (Marschner 1995). Mn^{2+} toxicity can also be an important factor limiting plant growth, particularly in acidic and poorly drained soils (Horst 1988). Cd^{2+} can also be toxic to plants, but levels encountered in natural and agricultural environments are generally below toxic levels (Wagner 1992). Mechanisms of Cd^{2+} accumulation in plants have been characterized (Wagner 1992; Rea *et al*. 1998). Several hypotheses concerning the physiological mechanisms of Mn^{2+} tolerance have also been proposed (Gonzales and Lynch 1999). CAX2 expression in transgenic crops could potentially alleviate Mn^{2+} toxicity problems and aid in phytoremediation of Cd^{2+} through sequestration of these ions into the vacuole. Furthermore, the *CAX2*-expressing plants demonstrated only modest increases in Cd^{2+} and Mn^{2+} accumulation in the aerial part (**Fig. 4C**). This suggests that future approaches to increase Mn^{2+} tolerance and Cd^{2+} phytoremediation potential will have to also include control of root uptake, long distance metal transport, and additional tolerance factors to accommodate high concentrations of these ions (Raskin *et al*. 1994).

In conclusion, the ectopic expression of the intact cation/H^+ exchanger (CAX2) in bottle gourd plants produces altered phenotypes in cation contents without any adverse effects, unlike deregulated expression of the high affinity Ca^{2+}/H^+ exchanger (sCAX1). The s*CAX1*-expressing plants accumulate high levels of Ca^{2+} but have symptoms of Ca^{2+} deficiency (Hirschi 1999). In contrast, even though intact *CAX2*-expressing bottle gourd plants accumulated Ca^{2+} levels comparable with s*CAX1*-expressing ones, these plants were, for the most part, as vigorous as wild-type plants. These findings suggest that regulating the expression of V-type metal transporters with broad substrate spectrum may have an important impact on the production of a plant material for phytoremediation.

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