Author's personal copy

Journal of Plant Physiology 168 (2011) 167–173

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Expression of an *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1 variant in petunia enhances cadmium tolerance and accumulation

Qingyu Wu[a], Toshiro Shigaki[b], Kimberly A. Williams[a], Jeung-Sul Han[c], Chang Kil Kim[d], Kendal D. Hirschi[b,e], Sunghun Park[a,*]

[a] *Department of Horticulture, Forestry and Recreation Resources, Kansas State University, Manhattan, KS 66506, USA*
[b] *United States Department of Agriculture/Agricultural Research Service, Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX 77030, USA*
[c] *Department of Ecological Environment Conservation, Kyungpook National University, Sangju 742-711, Republic of Korea*
[d] *Department of Environmental Horticulture, Kyungpook National University, Sangju 742-711, Republic of Korea*
[e] *Vegetable and Fruit Improvement Center, Texas A&M University, College Station, TX 77845, USA*

ARTICLE INFO

Article history:
Received 24 November 2009
Received in revised form 17 June 2010
Accepted 18 June 2010

Keywords:
Cadmium
CAX
Petunia
Phytoremediation
Transporter

ABSTRACT

Phytoremediation is a cost-effective and minimally invasive technology to cleanse soils contaminated with heavy metals. However, few plant species are suitable for phytoremediation of metals such as cadmium (Cd). Genetic engineering offers a powerful tool to generate plants that can hyperaccumulate Cd. An *Arabidopsis* CAX1 mutant (CAXcd), which confers enhanced Cd transport in yeast, was ectopically expressed in petunia to evaluate whether the *CAXcd* expression would enhance Cd tolerance and accumulation *in planta*. The *CAXcd*-expressing petunia plants showed significantly greater Cd tolerance and accumulation than the controls. After being treated with either 50 or 100 μM $CdCl_2$ for 6 weeks, the *CAXcd*-expressing plants showed more vigorous growth compared with controls, and the transgenic plants accumulated significantly more Cd (up to 2.5-fold) than controls. Moreover, the accumulation of Cd did not affect the development and morphology of the *CAXcd*-expressing petunia plants until the flowering and ultimately the maturing of seeds. Therefore, petunia has the potential to serve as a model species for developing herbaceous, ornamental plants for phytoremediation.

© 2010 Elsevier GmbH. All rights reserved.

Introduction

Cadmium (Cd) is highly toxic to humans, even at low concentrations. Exposure levels of 30–50 μg per day have been linked to increased risk of bone fracture, cancer, kidney dysfunction and hypertension (Satarug et al., 2003). Among the various sources of Cd intake, it is estimated that approximately 70% is from vegetable components of the human diet (Wagner, 1993). Increasingly, human food is under threat from the Cd pollutions of agricultural lands due to industrial activities. In order to reduce human consumption of Cd through food products, there is an urgent need to remove Cd from contaminated soils. Unfortunately, remediation of soils by physical or chemical means requires expensive operations that often result in secondary pollution (Lasat, 2002).

Phytoremediation, the use of plants and their associated microbes for environmental cleanup (Doty, 2008), has been gaining popularity because it is economically feasible and involves minimum disturbance of the surrounding environment (Raskin et al., 1997). Despite the significant advancement in understanding of metal tolerance and hyperaccumulation, phytoremediation of Cd still requires a breakthrough technology, mainly because Cd tolerance and hyperaccumulation have been identified in only a limited number of species compared with other heavy metals, such as zinc (Zn) and arsenic (As) (Ma et al., 2001; McGrath et al., 2001). Therefore, genetic engineering provides a means to heighten Cd tolerance and accumulation in plants.

Various mechanisms confer Cd tolerance and accumulation in plants (Verbruggen et al., 2009). Among them, vacuolar sequestration of Cd through either phytochelatin dependent or independent pathways is relatively well studied (Clemens et al., 1999; Hirschi et al., 2000; Song et al., 2003; Korenkov et al., 2007a; Wojas et al., 2009). Vacuolar transporters provide an important mechanism for metal sequestration into vacuoles; therefore, manipulation of vacuolar transport activity may be an essential component of genetic modifications to improve Cd tolerance and accumulation.

Among the transporters thought to be capable of moving Cd into the vacuole, CAXs (cation/H^+ exchangers) have been well characterized (Hirschi et al., 2000; Korenkov et al., 2007a). CAX antiporters are a group of proteins that export cations out of the cytosol to maintain ion homeostasis across biological membranes (Pittman and Hirschi, 2003). They are energized by the pH gradient established by proton pumps such as the H^+-ATPase and

* Corresponding author. Tel.: +1 785 532 4412; fax: +1 785 532 6949.
E-mail address: shpark@ksu.edu (S. Park).

0176-1617/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2010.06.005

Author's personal copy

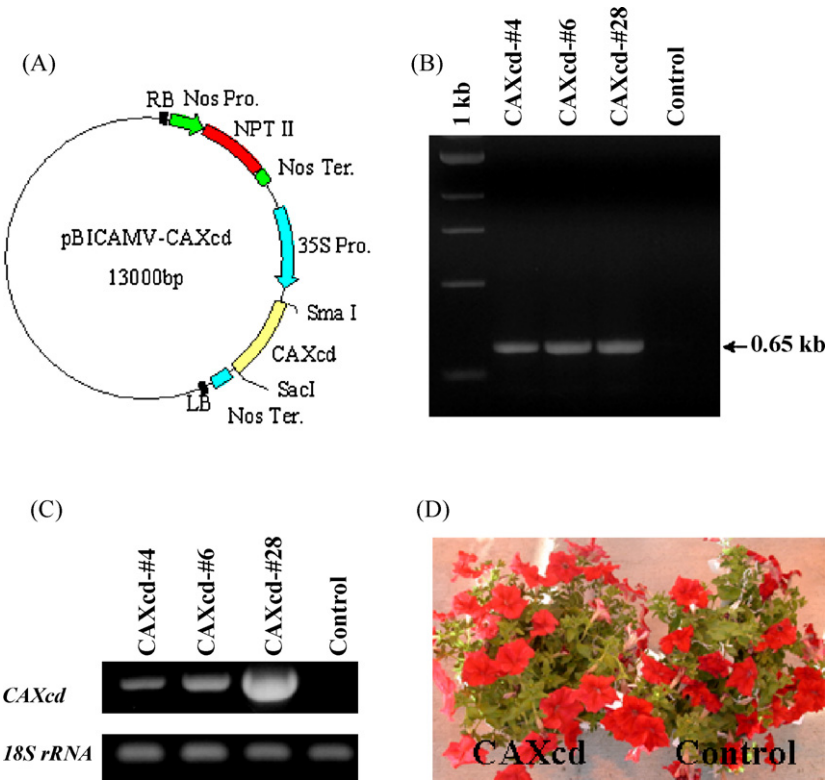

Fig. 1. Molecular analyses of T1 transgenic petunia plants. (A) Vector used for petunia transformation. Abbreviations: RB, right border; LV, left border; Nos Pro., nopaline synthase promoter; NPT II, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator; 35S Pro., cauliflower mosaic virus (CaMV) 35S promoter. (B) PCR analysis of transgenic petunia plants. Lane1 kb, 1 kb ladder; lanes 4, 6, and 28, the numbers of the transgenic lines; lane control, wild type petunia plant. (C) RT-PCR analysis of transgenic petunia plants. Lanes 4, 6, and 28, the numbers of the transgenic lines; lane control, wild type petunia plant. (D) Phenotype of wild type and *CAXcd*-expressing petunia plants. Control, wild type petunia plant; CAXcd: *CAXcd*-expressing #28 petunia plant.

H^+-pyrophosphates (Gaxiola et al., 2002). In *Arabidopsis*, there are six CAXs, namely, CAX1–CAX6 (Shigaki and Hirschi, 2006). All the *Arabidopsis* CAXs so far tested have the capability to transport Cd, but CAX2 and CAX4 have the strongest Cd transport capabilities (Korenkov et al., 2007a,b). In earlier work using tobacco, it was shown that expression of the *Arabidopsis CAX2* results in enhanced Cd transport in root tonoplast vesicles (Hirschi et al., 2000). In addition, expression of *CAX2* and *CAX4* in tobacco results in higher tonoplast Cd^{2+}/H^+ antiporter activity, Cd accumulation and Cd tolerance (Korenkov et al., 2007a,b). The transgenic *Arabidopsis* plants overexpressing *CAX4* also display increased accumulation and tolerance of Cd which is presumably resulting from increased Cd sequestration into the vacuole (Mei et al., 2009). A site-directed mutagenesis approach was used to alter His^{338} of an activated N-terminal truncated form of *Arabidopsis* CAX1 (sCAX1) to all possible amino acids, and it was found that the H338N variant has high apparent Cd transport (Shigaki et al., 2005). We term this mutant CAXcd throughout this paper and this transporter variant provides a potential tool to generate novel Cd accumulators.

Particular model plants have been widely used to characterize phytoremediation-related genes (Hirschi et al., 2000; Song et al., 2003; Gorinova et al., 2007; Korenkov et al., 2007b; Wojas et al., 2009). The most widely used plant is *Arabidopsis*; however, it is often not a convenient plant for physiological and biochemical analyses (Cobbett, 2003), nor an appropriate species for practical implementation of phytoremediation. While tobacco is commonly employed to investigate the metal homeostasis capability of foreign genes through engineering (Hirschi et al., 2000; Gorinova et al., 2007; Korenkov et al., 2007a,b; Wojas et al., 2009), petunia may be also a good candidate to use as a model species to study Cd phytoremediation. Besides possessing the common advantages of model plants, including a short life cycle, ease of transformation, well characterized molecular genetics, and availability of large sets of mutants, petunia provides advantages over *Arabidopsis* for phytoremediation research, such as its amenability to biochemical analysis because of its large leaves and flowers (Gerats and Vandenbussche, 2005). Moreover, petunia has significant commercial potential for phytoremediation, as petunias ranked as the number two bedding plant in wholesale value produced in the U.S. in 2008 (Agricultural Statistics Board, 2009). Petunias are widely used in commercial landscapes because of their drought tolerance; wide range of flower colors, forms, and growth habits; long season of bloom; and universal dependability for excellent garden performance (Still, 1988). Despite developing a fine-textured root system, petunias grow prolifically and produce thousands of seeds per plant. In addition, petunia has been widely used as an indicator crop to evaluate heavy metal uptake from waste-based substrates (Burger et al., 1997; Klock, 1997; Bucher and Schenk, 2000).

CAXcd expression facilitates Cd sequestration into yeast vacuoles (Shigaki et al., 2005). It was therefore our goal to investigate whether expression of *CAXcd* can confer Cd tolerance in plants while simultaneously developing petunia as a model plant for the phytoremediation research of Cd.

Materials and methods

Bacterial strains and plasmids

The *CAXcd* open reading frame (Shigaki et al., 2005) was cloned into pBICaMV binary vector by using 5′-*Sma*I and 3′-*Sac*I restriction sites (Fig. 1A). The *sCAX1* was also cloned into pBICaMV binary vector with the same procedure as *CAXcd* (Park et al., 2005). The plasmids were introduced into *Agrobacterium tumefaciens* strain

Author's personal copy

LBA4404 (Hoekema et al., 1983) using the freeze–thaw method (Holsters et al., 1978).

Plant material, transformation, and growth conditions

Petunia x hybrida 'Dreams™ Red', a grandiflora type, was used as the research material. The petunia transformation was performed via *Agrobacterium*-mediated transformation using leaf disc explants. Seeds of petunia were sterilized and cultured on Murashige–Skoog (MS) inorganic salt medium (Murashige and Skoog, 1962) with 30 g/L sucrose, pH 5.7, and solidified using 8 g/L TC agar (Sigma, St. Louis, MO). The petunia leaves after 4 weeks growth *in vitro* were excised and cultured on MS inorganic salts with 100 mg/L inositol, MS vitamins, 3% (w/v) sucrose, 1 mg/L BA (6-benzyl-aminopurine), 0.1 mg/L NAA (naphthaleneacetic acid) and 0.8% (w/v) TC agar. At the end of the 1-day preculture, the leaves were dipped in an *Agrobacterium* culture, blotted and recultured on the same media for 72 h. Leaf sections were then cultured on a selection medium containing MS inorganic salts, 3% (w/v) sucrose, 100 mg/L inositol, MS vitamins, 1 mg/L BA, 0.05 mg/L NAA, 100 mg/L kanamycin, 250 mg/L Clavamox® (Pfizer Animal Health, Exton, PA), and 0.8% (w/v) TC agar. Cultures were maintained at 25 °C under a 14-h photoperiod. After 4–6 weeks (subcultured once at 2–3 weeks), regenerated shoots were transferred to rooting medium for 2 more weeks, then established in soil. All plants were watered as needed. Once a week they were watered with Miracle-Gro (Scotts Miracle-Gro Products, Port Washington, NY). The temperature of the growth chamber was maintained within a range of 23–25 °C under a 14-h photoperiod.

Molecular analysis of CAXcd-expressing petunia transformants

PCR analysis

Petunia genomic DNA of the transformants and wild type plants was isolated from 100 mg of fresh leaves using the DNeasy Plant Mini-Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The *CAXcd*-specific primers CAXcdF 5′-ATG TCT TCT TCT TCT TTG AG-3′ and CAXcdR 5′-CAA TGT AGC TGA TCA ACA TAA C-3′ were used to amplify a 650-bp fragment, which demonstrates the presence of transformed foreign DNA.

RT-PCR analysis

Total RNA was isolated using the Qiagen Plant RNeasy kit according to the manufacturer's instructions. RNA for RT-PCR was treated with RNase-free DNase prior to the synthesis of first-strand cDNA by oligo (dT) priming using moloney murine leukaemia virus-reverse transciptase (BD Biosciences Clontech, Palo Alto, CA, USA). One microliter of the reverse transcription reaction solution was used as a template in a 25 μl PCR solution. The PCR primers for amplifying the *CAXcd* and *sCAX1*were same as the PCR primers above. The 18s rRNA gene (primers 5′-GGC GCG CAA ATT ACC CAA TC-3′ and 5′-CTA TAA TGT TAT CCC ATG CT-3′) was used as an internal control of RNA quantity.

Cadmium and mineral analysis

Seeds (T1) from the *CAXcd*-expressing petunia lines and the wild type were initially germinated on a MS selection medium containing MS inorganic salts, 3% (w/v) sucrose, MS vitamins, and 100 mg/L kanamycin (only for the *CAXcd*-expressing petunia lines), and 5 plants of similar size from each of the 2- and 3-week-old seedling lines were transferred to agar-solidified MS media containing four different concentrations of Cd^{2+} (0 and 50 μM $CdCl_2$ for 2-week-old seedlings and 100 and 200 μM $CdCl_2$ for 3-week-old seedlings) for 6 weeks. The development and growth status of both wild type and *CAXcd*-expressing petunia was observed during the Cd treatment. At the end of the growth period of 6 weeks, the petunia leaves were harvested and washed 4–5 times with deionized water to thoroughly remove any shoot surface contamination by the medium. The plant leaves were dried at 70 °C for 4 days, and a total of 0.25 g (dry weight) from each of the petunia lines were digested in a mixture of nitric and perchloric acids [7:1 (v/v)] following standard methods (Jones and Case, 1990). Cadmium, macro- and micro- nutrient element accumulations per gram of dry weight were determined by inductively coupled plasma spectrometry. A standard reference material for ion concentration was NIST Apple [the National Institute of Standards and Technology (www.nist.gov)].

Development and growth of CAXcd-expressing petunia

For tracking development and growth of the *CAXcd*-expressing petunia until flowering under Cd stress, 3-week-old *CAXcd*-expressing petunia plants after germination in initial selection media containing 100 mg/L kanamycin were transferred and grown in the magenta boxes containing agar-solidified MS medium with 100 μM $CdCl_2$ for 8 more weeks.

Results

CAXcd expression in petunia

We generated more than 20 independent *CAXcd*-expressing petunia lines, and three of them (CAXcd-4, -6, and -28) were randomly selected for analyses of Cd tolerance, accumulation and adaptation in subsequent *CAXcd*-expressing generations. When screening on 100 mg/L kanamycin selection medium, the T1 seeds of these three lines showed a segregation pattern of 3:1 for the kanamycin resistance marker gene (data not shown). We confirmed the stable integration of the *CAXcd* gene in T1 generation transformants by PCR (Fig. 1B), and the expression level of the introduced *CAXcd* was determined by RT-PCR (Fig. 1C). The morphology and growth characteristics of the *CAXcd*-expressing petunia were indistinguishable from that of wild type (Fig. 1D).

CAXcd-expressing petunia plants have enhanced tolerance to Cd

In order to examine whether ectopic expression of the *CAXcd* in petunia confers increased tolerance to Cd, 2-week-old *CAXcd*-expressing plants (CAXcd- 4, -6, and -28), after germinating on a MS selection medium, were evaluated for Cd tolerance by growing on MS solid media containing 50, 100, or 200 μM $CdCl_2$. The wild type leaves exhibited severe yellowing symptoms, and the growth was significantly inhibited at 50 μM $CdCl_2$, whereas the *CAXcd*-expressing lines were less affected at the same $CdCl_2$ concentration (Fig. 2A). At 100 μM $CdCl_2$ concentration, even though the *CAXcd*-expressing plants were also stressed and delayed in growth, the wild type plants showed minimal growth and eventually died at 14 weeks (Fig. 2B). However, when challenged with 200 μM $CdCl_2$, neither the *CAXcd*-expressing nor wild type plants grew and eventually died (Supplementary data 2A–B).

We tracked the growth status of wild type and *CAXcd*-expressing petunia plants in detail at 50 μM $CdCl_2$ concentration over different treating periods. In the first 4-week treatment, both wild type plants and *CAXcd*-expressing plants showed chlorosis (Fig. 2C); however, the *CAXcd*-expressing plants showed much more vigorous growth in shoots and roots than the wild type plants. The obvious toxicity of Cd for the first several weeks indicates that both wild type plants and *CAXcd*-expressing plants require an adjustment period to the stress. After the 6-week Cd treatment, the *CAXcd*-expressing plant leaves had partially recovered from

Author's personal copy

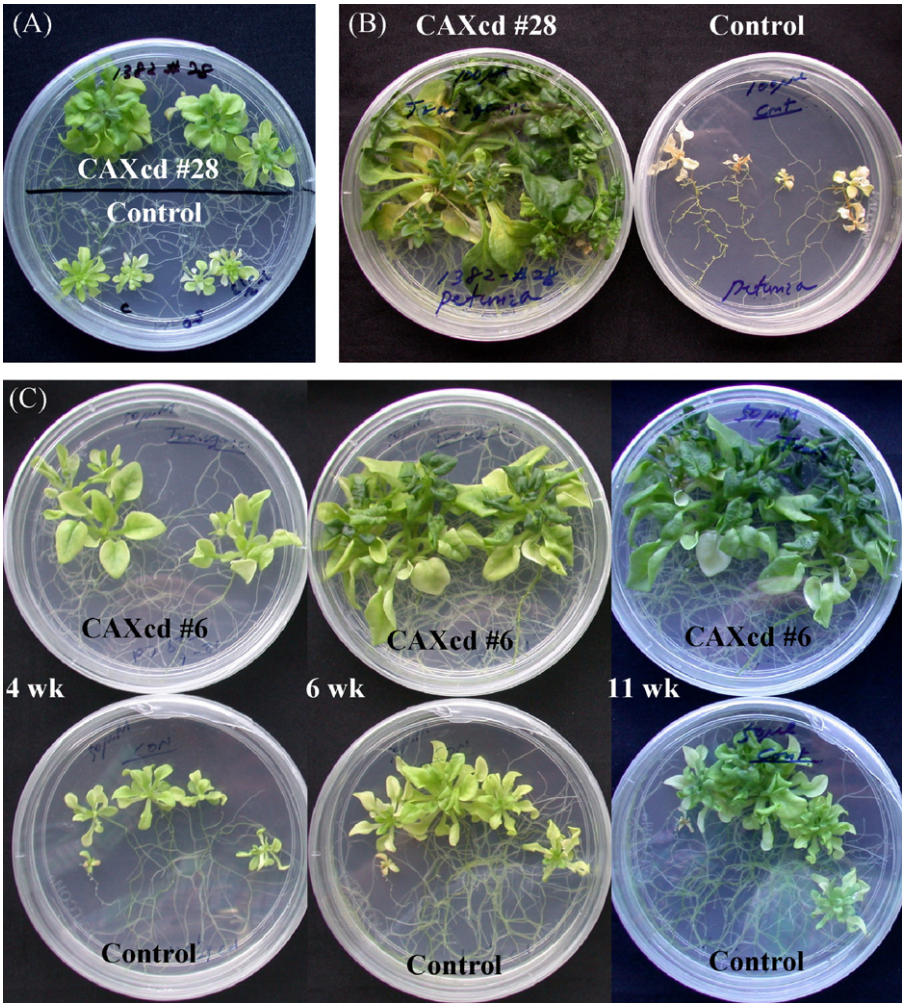

Fig. 2. Cadmium tolerance tests of transgenic petunia plants. (A) Phenotype of *CAXcd*-expressing #28 and wild type petunia treated with 50 μM $CdCl_2$ for 5-week. (B) Phenotype of *CAXcd*-expressing #28 and wild type petunia plants treated with 100 μM $CdCl_2$ for 11-week. (C) Phenotypes of *CAXcd*-expressing #6 and wild type petunia plants treated with 50 μM $CdCl_2$ for different weeks. Control, wild type petunia. CAXcd, *CAXcd*-expressing petunia.

chlorosis, while the wild type plants still showed symptoms of severe chlorosis (Fig. 2C). After 5 more weeks, the *CAXcd*-expressing plant's leaves became a darker green, which indicated that the transgenic plants adapted more to Cd stress than the wild type petunia plants (Fig. 2C).

CAXcd-expressing petunia plants accumulate more Cd

In order to evaluate whether the *CAXcd*-expressing plants accumulate more Cd in parallel with the tolerance, 2-week-old (for 50 μM $CdCl_2$) or 3-week-old (for 100 μM $CdCl_2$) wild type and *CAXcd*-expressing petunia lines, after germination on a MS selection medium, were transferred and grown for 6 weeks on MS solid media containing 50 and 100 μM $CdCl_2$, respectively. After 6-week treatment in 100 μM $CdCl_2$, the *CAXcd*-expressing lines grew vigorously while the wild type plants showed minimal growth (Fig. 3A). *CAXcd*-expressing petunia plants accumulated significantly more Cd than wild type plants. When the Cd concentration in the medium was 50 μM, the *CAXcd*-expressing plants accumulated 2.5-fold Cd compared to the wild type plants (Fig. 3B). At 100 μM $CdCl_2$, the Cd concentration in plant leaves reached approximately 500 mg/kg dry weight. (Fig. 3B). Among these *CAXcd*-expressing lines, line #28 appeared to have higher transgene expression than two other lines possibly due to genomic position effects (Fig. 1C). However, there was no significant correlation between the *CAXcd* expression level and Cd accumulation. We also generated more than 10 independent *sCAX1*-expressing petunia lines, and three of them (sCAX1-1, -2, and -4) were randomly selected for analyses of Cd tolerance and accumulation (Supplementary data 1 and 3); however, the Cd concentration of *sCAX1*-expressing petunia leaves did not show any significant differences as compared with that of wild type plants (Fig. 3B, part of the data is shown).

Effects of Cd accumulation on the absorption of mineral elements in CAXcd-expressing petunia plants

CAXcd-expressing petunia plants accumulated significantly more Cd than wild type plants. To ascertain whether *CAXcd* expression altered mineral contents, 3-week-old wild type and *CAXcd*-expressing petunia lines were transferred and grown for 6 weeks on media containing 0 and 100 μM $CdCl_2$, respectively, and total accumulation of other ions (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) was measured in plant leaves. When grown in MS solid media without Cd, no significant differences were observed for any ion levels including divalent cations in both *CAXcd*-expressing lines and wild-type lines (data not shown). However, when the Cd concentration in the medium was 100 μM, total accumulation of other ions (Ca, Fe, K, Mg, Mn, P, S, and Zn) was significantly increased in both *CAXcd*-expressing lines and wild-type lines, and the *CAXcd*-expressing petunia plants accu-

Author's personal copy

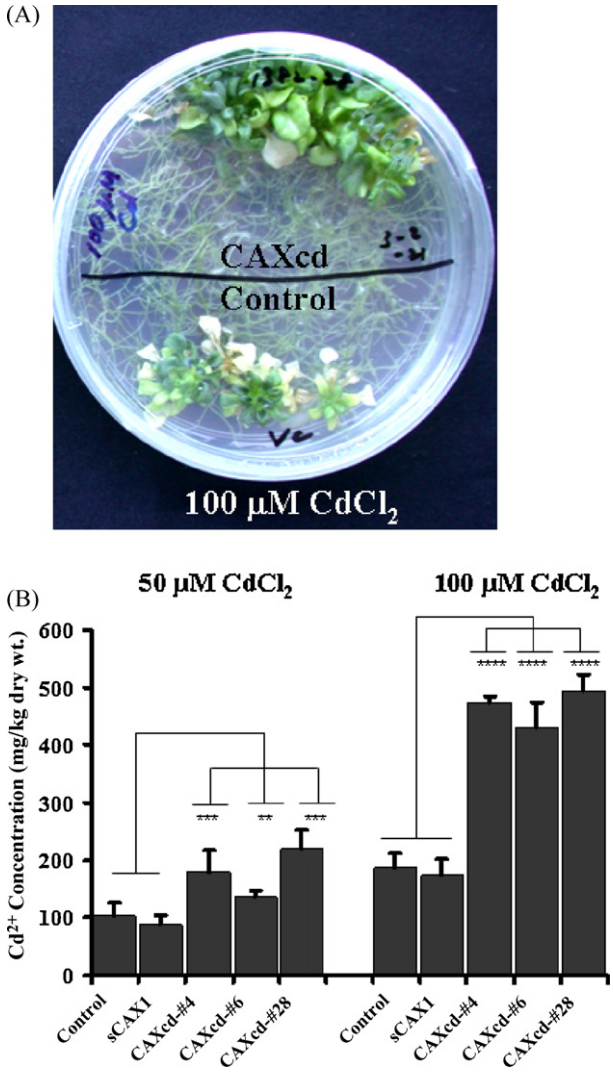

Fig. 3. Concentrations of cadmium in the *CAXcd*-expressing petunia leaves after 6-week treatment of 50 and 100 μM $CdCl_2$, respectively. (A) Phenotype of *CAXcd*-expressing #28 and wild type petunia after 6-week treatment of 100 μM $CdCl_2$. Control, wild type petunia; CAXcd, *CAXcd*-expressing #28 petunia. (B) Concentrations of cadmium in leaves of *CAXcd*-expressing and wild type petunia. Data represent the values obtained from the means (±SD) of three independent analyses [one replicate: analysis of petunia plants harvested from at least 5 plates (5 plants per plate)]. Control, wild type petunia. sCAX1, *sCAX1*-expressing #2 petunia. CAXcd, *CAXcd*-expressing petunia. #4, 6, and 28, the numbers of the transgenic lines. Student *t* test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

mulated significantly more Cd and less Cu than wild type plants (Fig. 3B and Fig. 4)

Development and growth characteristics of the CAXcd-expressing petunia until flowering under Cd stress

We next sought to determine whether the development and growth of the *CAXcd*-expressing plants was affected by elevated levels of Cd in the growth media (100 μM $CdCl_2$). After germination in 100 mg/L kanamycin selection medium, 3-week-old *CAXcd*-expressing petunia plants were transferred and grown in media containing 100 μM $CdCl_2$ for 8 more weeks. Even though the *CAXcd*-expressing plants were slightly stressed and delayed in growth, they did not show any significant phenotypic differences compared to wild-type lines (Fig. 5). This observation suggested that the *CAXcd*-expressing petunia plants have the potential to remain fertile when grown in high Cd containing environments.

Discussion

In this study, we observed that the ectopic expression of *CAXcd* in petunia plants conferred tolerance and rapid adaptation to Cd, supporting the idea that the CAXcd may contribute to Cd sequestration into vacuoles of plant cells. Cd toxicity affects organisms mainly by competing with other essential cations and induction of oxidative stress in the cytoplasm (Cherian and Oliveira, 2005). Sequestration, which separates heavy metals in cellular compartments where the metal can do the least harm to vital cellular processes, is one of the most effective ways to detoxify Cd (Pilon-Smits, 2005).

We postulate that CAXs help to determine the duration and amplitude of cytosolic Ca^{2+} oscillations (Hirschi, 1999; Pittman et al., 2002), and various CAX variants have been investigated to identify differences in transport properties (Pittman et al., 2002; Park et al., 2005; Mei et al., 2007). The transport of Cd by CAXcd may be similar to calcium (Ca) transport by CAXs (Salt and Wagner, 1993; Pittman and Hirschi, 2003) due to their near-identical ionic radii. However, CAXcd has a higher Cd affinity than other CAXs (Shigaki et al., 2005). Thus, expression of the *CAXcd* in petunia plants could minimize the amount of time that Cd is retained in the cytosol and reduce toxicity. Here we show that CAXcd has specific effects on Cd tolerance and accumulation that are not apparent when *sCAX1* is expressed in petunia plants (Supplementary data 3). These results strongly suggest that variants of CAXs can be engineered to preferentially transport specific substrates *in planta*.

Our results suggested that ectopic expression of *CAXcd* appeared to increase Cd accumulation in the shoot; however, previous studies indicated that expression of *CAX2* and *CAX4* in tobacco did not increase the Cd accumulation in the shoot (Korenkov et al., 2007b). The difference may result from the different Cd concentrations in the two experiments. In this paper, we treated the petunia seedlings by 50 or 100 μM Cd, while the tobacco seedlings were treated by 0.02 μM Cd in the Korenkov et al. study. For this low Cd level, the difference of Cd accumulation in tobacco shoots may be below the limit of detection. In addition, Korenkov's further study suggested that when treated with 3 μM Cd, both the *CAX2* and *CAX4* expressing tobacco plants accumulated significantly more Cd than control in both shoots and roots (Korenkov et al., 2007a). However, the plant materials used (tobacco and petunia), the genes transformed (*CAXcd*, *CAX2*, and *CAX4*) and the growing conditions (hydroponic solution and agar) are totally different in those experiments. Thus, it may not be appropriated that the difference will result from the different Cd concentrations. It should also be noted that there may be a difference between tobacco and petunia in the ability of Cd translocation to the shoots or accumulation in the shoots. Further studies will be required to directly compare the Cd transport ability of CAXcd with that of other CAXs in tobacco or petunia.

Ectopic expression of *CAXs* may cause adverse effects in plants. We speculate this is due to altered Ca homeostasis within the cells (Hirschi, 1999). *CAX1*-expressing plants are often sensitive to ion stresses, and in tobacco and tomato, tip burning of young leaves and severe stunting caused by over-sequestration of Ca (Hirschi, 1999; Park et al., 2005). However, *CAXcd* greatly reduces Ca transport due to the putative filter domain of H338N which tends to be more selective to Cd than Ca (Shigaki et al., 2005); therefore, expression of the *CAXcd* in petunia plants may not cause altered growth in our assay conditions. Indeed, the *CAXcd*-expressing petunia plants accumulated significantly more Cd, but not Ca, than wild type plants when grown in 100 μM $CdCl_2$ (Fig. 4); therefore, the lack of deleterious morphological phenotypes in the *CAXcd*-expressing petunia plants may correlate with the levels of Ca accumulation and homeostasis. However, it is also possible that no adverse effect in the *CAXcd*-expressing petunia may result not from the different levels of Ca transport between CAXcd and CAX1, but from

Author's personal copy

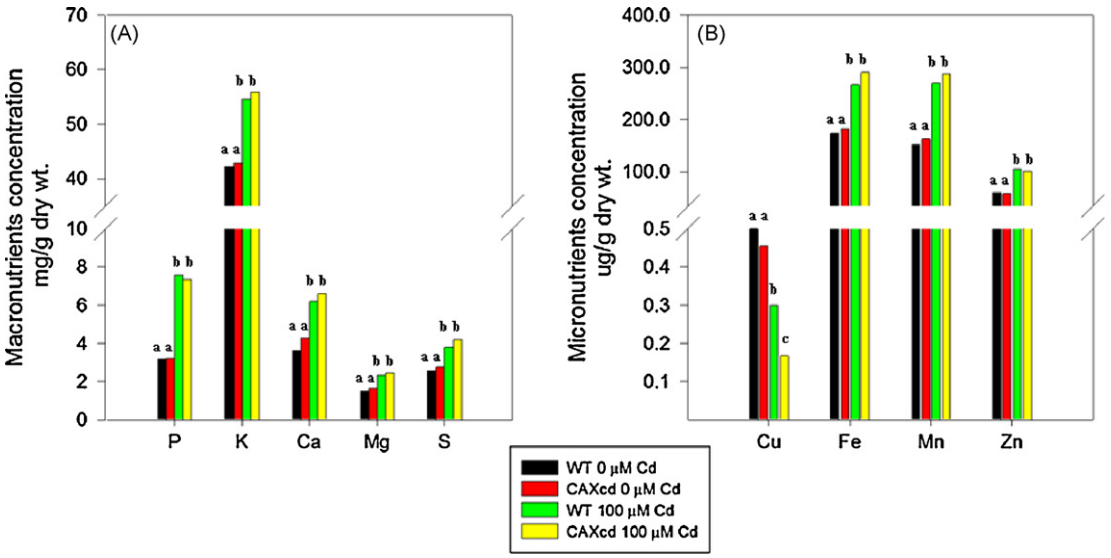

Fig. 4. Concentrations of other ions in wild type and *CAXcd*-expressing petunia leaves after 6-week treatment of 0 and 100 μM $CdCl_2$, respectively. WT 0 μM, wild type plants grown in solid MS medium without Cd; CAXcd 0 μM, *CAXcd*-expressing plants grown in solid MS medium without Cd; WT 100 μM, wild type plants grown in solid MS medium with 100 μM $CdCl_2$; CAXcd 100 μM, *CAXcd*-expressing plants grown in solid MS medium with 100 μM $CdCl_2$. (A) Macronutrient concentration. (B) Micronutrient concentration. Data represent the values obtained from the means of three independent analyses. Means accompanied by the same letter are not significantly different using student *t* tests ($p < 0.05$) within each element (P, K, Ca, Mg, S, Cu Fe, Mn, Zn).

intrinsic differences between petunia and other plant species in which these CAXs were expressed. While the *CAXcd*-expressing petunia plants have increased Cd when compared to wild type plants at 100 μM $CdCl_2$ concentration, they have reduced level of Cu (Fig. 4). In previous reports, high external metal ions can compete for other metal ion binding sites, thus reducing uptake of a particular metal (Marschner, 1995). Theoretically, this competition could also occur at sites of intracellular transport (Wu et al., 2002). Thus, high Cd potentially interferes with proper uptake of Cu into intracellular locations. However, more direct transport studies will need to be done to further determine the substrate specificity of CAXcd.

One of the most efficient phytoremediation strategies for Cd would be phytoextraction where Cd is removed from contaminated soils by accumulating within the tolerant plants. Here we have examined the correlation between Cd tolerance and accumulation in the *CAXcd*-expressing petunia plants. The results indicated that the *CAXcd*-expressing petunia plants showed not only high Cd tolerance, but also up to 2.5-fold higher Cd accumulation when compared with wild type plants (Fig. 3B). The increased Cd accumulation in the plant tissues did not adversely affect growth and development (Fig. 5). It should be noted that soil is a complex media, which may not be properly mimicked by agar. Moreover, plants under agar-solidified medium conditions could suffer from diminishing amount of nutrients, lack of space, and high humidity. Therefore, further tests must be performed under various soil conditions (or hydroponic systems) to establish the impact of *CAXcd*-expressing petunia plants in physiologically relevant conditions.

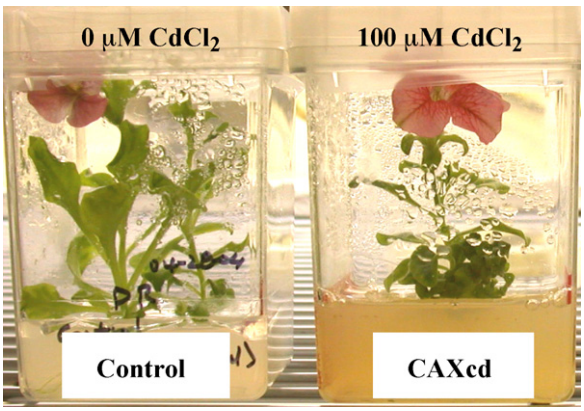

Fig. 5. Development and growth characteristics of the *CAXcd*-expressing petunia under Cd stress. Control, wild type petunia plant grown on MS solid media without Cd for 11 weeks; CAXcd, 3-week-old *CAXcd*-expressing #28 petunia plant grown on MS solid media containing 100 μM $CdCl_2$ for 8 more weeks.

This work also explores the utility of genetically engineered petunia plants for Cd phytoremediation. Very few species have been identified as hyperaccumulators of Cd to date (McGrath et al., 2001). Furthermore, there are a number of limitations for the identified hyperaccumulator species to clean up the contaminated soils. For example, *Thlaspi caerulescens* is a model plant for the study of heavy metal hyperaccumulation and tolerance, but it is extremely difficult to propagate large numbers of plants and requires a relatively long vernalization period for inducing flowering. In addition, some hyperaccumulator plants grown in normal potting soil in greenhouse appear to be highly sensitive to various pests, such as fungi and arthropods (Assuncao et al., 2003). However, petunia is relatively easy to grow, even under conditions of drought, and can also be planted at a high density of 75–100 plants per m^2. After treatment with 100 μM $CdCl_2$ for 6 week, the Cd accumulation reached approximately 500 mg/kg dry weight which significantly exceeds the definition of Cd hyperaccumulating plants (Peer et al., 2006). More importantly, the *CAXcd* expression petunia plants can complete their life cycle when exposed to this Cd stress (Fig. 5). Finally, another benefit of the *CAXcd*-expressing petunia as a pytoremediator is that it could have a high ornamental value in polluted soils.

In this report, we have demonstrated that ectopic expression of the *CAXcd* in petunia can significantly increase Cd tolerance and accumulation. This study suggests an important role of *CAXcd* in Cd sequestration and detoxification *in planta*. It also suggests that petunia could be a model plant for phytoremediation research.

Author's personal copy

Future studies will focus on the characterization of other Cd detoxification genes by employing petunia as a model plant.

Acknowledgements

This research was supported by the NIHHS RDA-KSU Cooperative Research Project (SHP, KAW and JSH), the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (CKK) and the U.S. Department of Agriculture Grant CSRESS#2005-34402-16401 Designing Foods for Health (SHP and KDH).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.06.005.

References

Agricultural Statistics Board. Floriculture crops 2008 Summary. NASS, USDA; 2009.
Assuncao AGL, Schat H, Aarts MGM. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytol 2003;159:351–60.
Bucher AS, Schenk MK. Characterization of phytoavailable copper in compost-peat substrates and determination of a toxicity level. J Am Soc Hortic Sci 2000;125:765–70.
Burger DW, Hartz TK, Forister GW. Composted green waste as a container medium amendment for the production of ornamental plants. HortScience 1997;32:57–60.
Cherian S, Oliveira MM. Transgenic plants in phytoremediation: recent advances and new possibilities. Environ Sci Technol 2005;39:9377–90.
Clemens S, Kim EJ, Neumann D, Schroeder JI. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 1999;18:3325–33.
Cobbett C. Heavy metals and plants model systems and hyperaccumulators. New Phytol 2003;159:289–93.
Doty SL. Enhancing phytoremediation through the use of transgenics and endophytes. New Phytol 2008;179:318–33.
Gaxiola RA, Fink GR, Hirschi KD. Genetic manipulation of vacuolar proton pumps and transporters. Plant Physiol 2002;129:967–73.
Gerats T, Vandenbussche M. A model system for comparative research: petunia. Trends Plant Sci 2005;10:251–6.
Gorinova N, Nedkovska M, Todorovska E, Simova-Stoilova L, Stoyanova Z, Georgieva K, et al. Improved phytoaccumulation of cadmium by genetically modified tobacco plants (*Nicotiana tabacum* L.). Physiological and biochemical response of the transformants to cadmium toxicity. Environ Pollut 2007;145:161–70.
Hirschi KD. Expression of *Arabidopsis* CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell 1999;11:2113–22.
Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ. Expression of *Arabidopsis* CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol 2000;124:125–33.
Hoekema A, Hirsch PR, Hooykaas PJJ, Schillperoort RA. A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature 1983;303:179–80.
Holsters M, De Waele D, Depicker A, Messens E, Van Montagu M, Schell J. Transfection and transformation of A. tumefaciens. Mol Gen Genet 1978;163:181–7.
Jones J, Case V. Sampling, handling, and analyzing plant tissue samples. In: Westerman R, editor. Soil testing and plant analysis. Madison: Soil Science Society of America; 1990. p. 389–427.
Klock KA. Growth of dianthus and petunia in media amended with composted urban waste. J Environ Hortic 1997;15:135–7.
Korenkov V, Hirschi K, Crutchfield JD, Wagner GJ. Enhancing tonoplast Cd/H antiport activity increases Cd, Zn, and Mn tolerance, and impacts root/shot Cd partitioning in *Nicotiana tabacum* L. Planta 2007a;226:1379–87.
Korenkov V, Park S, Cheng NH, Sreevidya C, Lachmansingh J, Morris J, et al. Enhanced Cd^{2+}-selective root tonoplast-transport in tobaccos expressing *Arabidopsis* cation exchangers. Planta 2007b;225:403–11.
Lasat MM. Phytoextraction of toxic metals: a review of biological mechanisms. J Environ Qual 2002;31:109–20.
Ma LQ, Komar KM, Tu C, Zhang WH, Cai Y, Kennelley ED. A fern that hyperaccumulates arsenic. Nature 2001;409:579–80.
Marschner H. Mineral nutrition of higher plants. New York: Academic Press; 1995.
McGrath SP, Lombi E, Zhao FJ. What's new about cadmium hyperaccumulation? New Phytol 2001;149:2–3.
Mei H, Cheng NH, Zhao J, Park S, Escareno RA, Pitman JK, et al. Root development under metal stress in *Arabidopsis thaliana* requires the H^+/cation antiporter CAX4. New Phytol 2009;183:95–105.
Mei H, Zhao J, Pittman JK, Lachmansingh J, Park S, Hirschi KD. *In planta* regulation of the *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1. J Exp Bot 2007;58:3419–27.
Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plantarum 1962;15:473–97.
Park S, Cheng NH, Pittman JK, Yoo KS, Park J, Smith RH, et al. Increased calcium levels and prolonged shelf life in tomatoes expressing *Arabidopsis* H^+/Ca^{2+} transpoter. Plant Physiol 2005;139:1194–206.
Peer WA, Mahmoudian M, Freeman JL, Lahner B, Richards EL, Reeves RD, et al. Assessment of plants from the Brassicaceae family as genetic models for the study of nickel and zinc hyperaccumulation. New Phytol 2006;172:248–60.
Pilon-Smits E. Phtoremediation. Annu Rev Plant Biol 2005;56:15–39.
Pittman JK, Hirschi KD. Don't shoot the (second) messenger: endomembrane transporters and binding proteins modulate cytosolic Ca^{2+} levels. Curr Opin Plant Biol 2003;6:257–62.
Pittman JK, Shigaki T, Cheng NH, Hirschi KD. Mechanism of N-terminal autoinhibition in the *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1. J Biol Chem 2002;277:26452–9.
Raskin I, Smith RD, Salt DE. Phytoremediation of metals: using plants to remove pollutants from the environment. Curr Opin Biotechnol 1997;8:221–6.
Salt DE, Wagner GJ. Cadmium transport across tonoplast of vesicles from oat roots. J Bio Chem 1993;268:12297–302.
Satarug S, Baker JR, Urbenjapol S, Haswell-Elkins M, Reilly PEB, Williams DJ, et al. A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. Toxicol Lett 2003;137:65–83.
Shigaki T, Barkla BJ, Miranda-Vergara MC, Zhao J, Pantoja O, Hirschi KD. Identification of a crucial histidine involved in metal transport activity in the *Arabidopsis* cation/H^+ exchanger CAX1. J Biol Chem 2005;280:30136–42.
Shigaki T, Hirschi KD. Diverse functions and molecular properties emerging for CAX cation/H^+ exchangers in plants. Plant Biol 2006;8:419–29.
Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, et al. Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat Biotechnol 2003;21:914–9.
Still SM. Manual of herbaceous ornamental plants. Champaign: Stipes Publishing; 1988.
Verbruggen N, Hermans C, Schat H. Molecular mechanisms of metal hyperaccumulation in plants. New Phytol 2009;181:759–76.
Wagner GJ. Accumulation of cadmium in crop plants and its consequences to human health. Adv Agron 1993;51:173–212.
Wojas S, Hennig J, Plaza S, Geisler M, Siemianowski O, Sklodowska A, et al. Ectopic expression of Arabidopsis ABC transporter MRP7 modifies cadmium root-to-shoot transport and accumulation. Environ Pollut 2009;157:2781–9.
Wu Z, Liang F, Hong B, Young JC, Sussman MR, Harper JF, et al. An endoplasmic reticulum-bound Ca^{2+}/Mn^{2+} pump, ECA1, supports plant growth and confers tolerance to Mn^{2+} stress. Plant Physiol 2002;130:128–37.