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

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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.

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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 C\(_{\text{D}}\) emissions 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 transporters 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
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 Cd2+/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 His338 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., 2007b; 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 pBlCaMV binary vector by using 5′-SmaI and 3′-SacI restriction sites (Fig. 1A). The sCAX1 was also cloned into pBlCaMV binary vector with the same procedure as CAXcd (Park et al., 2005). The plasmids were introduced into Agrobacterium tumefaciens strain...
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 TCT TTG AG-3′ and CAXcdR 5′-CAA TGT TGC 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 leukemia virus-reverse transcriptase (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 sCAXI were same as the PCR primers above. The 18s RNA 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 Cd2+ (0 and 50 μM CdCl2 for 2-week-old seedlings and 100 and 200 μM CdCl2 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 CdCl2 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 CdCl2. The wild type lines exhibited severe yellowing symptoms, and the growth was significantly inhibited at 50 μM CdCl2, whereas the CAXcd-expressing lines were less affected at the same CdCl2 concentration (Fig. 2A). At 100 μM CdCl2 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 CdCl2, 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 CdCl2 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...
Cadmium tolerance tests of transgenic petunia plants. (A) Phenotype of CAXcd-expressing #28 and wild type petunia treated with 50 \( \mu \)M CdCl\(_2\) for 5-week. (B) Phenotype of CAXcd-expressing #28 and wild type petunia plants treated with 100 \( \mu \)M CdCl\(_2\) for 11-week. (C) Phenotypes of CAXcd-expressing #6 and wild type petunia plants treated with 50 \( \mu \)M 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 \( \mu \)M CdCl\(_2\)) or 3-week-old (for 100 \( \mu \)M 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 \( \mu \)M CdCl\(_2\), respectively. After 6-week treatment in 100 \( \mu \)M 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 \( \mu \)M, the CAXcd-expressing plants accumulated 2.5-fold Cd compared to the wild type plants (Fig. 3B). At 100 \( \mu \)M 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 \( \mu \)M CdCl\(_2\), respectively, and total accumulation of other ions (Ca, 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 \( \mu \)M, 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-
Evolutionary and Functional Analysis of CAXcd in Petunia Plants

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 Ca²⁺ 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 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.
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 CdCl₂ 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.

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 m². After treatment with 100 μM CdCl₂ 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.
Future studies will focus on the characterization of other Cd detoxification genes by employing petunia as a model plant.

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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.

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