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

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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.

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

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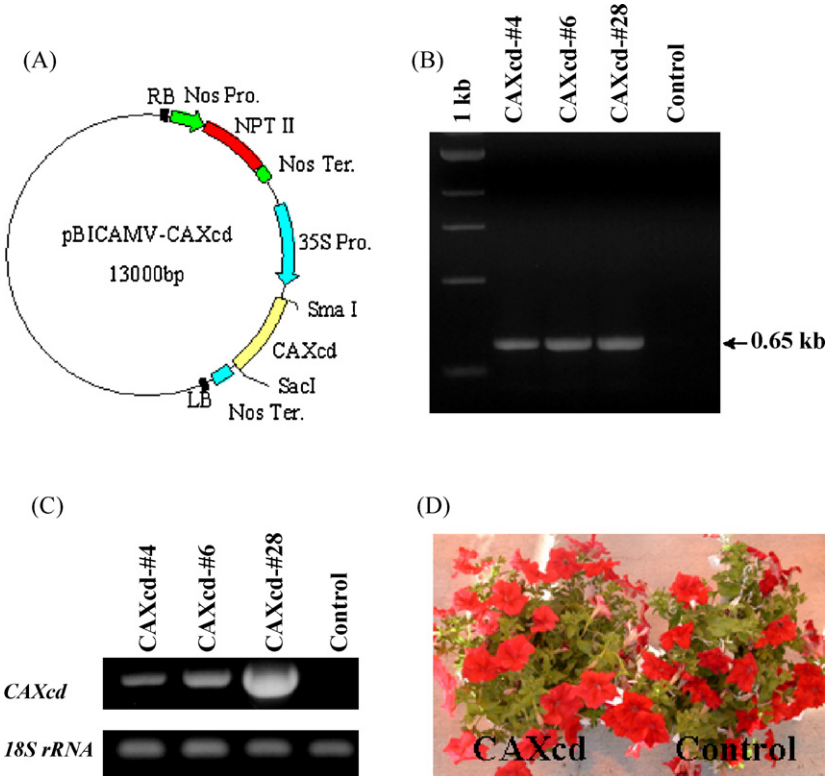

Fig. 1. Molecular analyses of T1 transgenic petunia plants. (A) Vector used for petunia transformation. Abbreviations: RB, right border; LV, left border; Nos Pro., nopaline synthase promoter; NPT II, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator; 35S Pro., cauliflower mosaic virus (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