Long-distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*

Ji-Ming Gong, David A. Lee, and Julian I. Schroeder*

Division of Biological Sciences and Center for Molecular Genetics, Cell and Developmental Biology Section, University of California at San Diego, La Jolla, CA 92093-0116

Phytochelatin synthases (PCS) mediate cellular heavy-metal resistance in plants, fungi, and worms. However, phytochelatins (PCs) are generally considered to function as intracellular heavy-metal detoxification mechanisms, and whether long-distance transport of PCs occurs during heavy-metal detoxification remains unknown. Here, wheat *TaPCS1* cDNA expression was either targeted to *Arabidopsis* roots with the *Arabidopsis* alcohol dehydrogenase (*Adh*) promoter (Adh::TaPCS1/*cad1-3*) or ectopically expressed with the cauliflower mosaic virus 35S promoter (35S::TaPCS1/*cad1-3*) in the PC-deficient mutant *cad1-3*. Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* complemented the cadmium, mercury, and arsenic sensitivities of the *cad1-3* mutant. Northern blot, RT-PCR, and Western blot analyses showed *Adh* promoter-driven TaPCS1 expression only in roots and thus demonstrated lack of long-distance *TaPCS1* mRNA and protein transport in plants. Fluorescence HPLC analyses showed that under Cd^{2+} stress, no PCs were detectable in *cad1-3*. However, in Adh::TaPCS1/*cad1-3* plants, PCs were detected in roots and in rosette leaves and stems. Inductively coupled plasma atomic emission spectrometer analyses showed that either root-specific or ectopic expression of *TaPCS1* significantly enhanced long-distance Cd^{2+} transport into stems and rosette leaves. Unexpectedly, transgenic expression of TaPCS1 reduced Cd^{2+} accumulation in roots compared with *cad1-3*. The reduced Cd^{2+} accumulation in roots and enhanced root-to-shoot Cd^{2+} transport in transgenic plants were abrogated by L-buthionine sulfoximine. The presented findings show that (*i*) transgenic expression of TaPCS1 suppresses the heavy-metal sensitivity of *cad1-3*, (*ii*) PCs can be transported from roots to shoots, and (*iii*) transgenic expression of the *TaPCS1* gene increases long-distance root-to-shoot Cd^{2+} transport and reduces Cd^{2+} accumulation in roots.

Cadmium is a widespread nonessential toxic heavy metal, released into the biosphere mostly by modern industry (1). Phytochelatins (PCs) play an essential role in heavy-metal detoxification in plants and fungi (2, 3). PCs chelate heavy metals and then PC-metal complexes are translocated across the tonoplast and sequestered in vacuoles (4, 5), thus decreasing the heavy-metal content in the cytosol of yeast and plant cells. PCs are synthesized from glutathione by the enzyme PC synthase (PCS) (6–9). However, PC research has focused mainly on PC transport into vacuoles (4, 5), and it remains unknown whether long-distance transport of PCs or PCS occurs in plants.

Cd^{2+} is taken up by plants, and plants have been proposed to provide an efficient system for heavy-metal removal from soils (10, 11). Soil composition affects Cd^{2+} sensitivity. For example, silicon in soil reduces heavy-metal sensitivity (12, 13). Normally, Cd^{2+} concentrations in roots are at least 10 times greater than those in shoots (14). However, for efficient phytoextraction from soils, heavy metals must be translocated into aerial tissues for later harvest. Translocation of Cd^{2+} from roots to shoots has been studied in diverse plant species (15–18) and has been proposed to occur via the xylem of Indian mustard in a PC-independent manner (16). The molecular mechanisms for root-to-shoot Cd^{2+} transport remain largely unknown, and PCs are predicted not to undergo long-distance transport but to enhance vacuolar heavy-metal sequestration in the cells in which they are produced.

The *cad1-3* mutant is a recessive, loss-of-function mutation in the *Arabidopsis AtPCS1* gene (7) and shows no detectable PC levels under Cd^{2+} stress (19). In the present study, we addressed the following questions by targeted expression of the wheat *TaPCS1* cDNA in the *cad1-3* mutant. (*i*) Can PCSs or PCs be translocated from roots to shoots? (*ii*) Does targeted PCS expression in roots lead to Cd^{2+} trapping in roots, or does it enhance Cd^{2+} translocation and accumulation in shoots? and (*iii*) To what extent do PCs contribute to long-distance Cd^{2+} transport?

Materials and Methods

DNA Constructs and Plant Transformation. Two restriction enzyme sites, *Bam*HI and *Spe*I, were introduced into the wheat *TaPCS1* cDNA (9) by PCR. The *TaPCS1* PCR fragment was subcloned into pBluescript II SK(+) vector. A 3× *myc* tag DNA sequence was constructed by PCR recovery from a plasmid containing c-*myc* and then subcloned into the pGEM-T Easy vector (Promega). The subcloned *myc* fragment was recovered with *Spe*I and *Sac*I enzymes and then fused to the 3′ end of the *TaPCS1* ORF in the pBluescript II SK(+) vector at the *Spe*I site. All PCR products were confirmed by sequencing (Retrogen, La Jolla, CA). The fusion sequence was then digested with *Bam*HI and *Sac*I and subcloned into the binary expression vector pBI121, either with the cauliflower mosaic virus 35S or the *Arabidopsis* alcohol dehydrogenase (*Adh*) promoter (20). Both constructs were transformed into the PC-deficient *Arabidopsis* mutant *cad1-3* by direct *Agrobacterium tumefaciens*-mediated transformation using the floral dip technique (21). T2 seeds with 3:1 segregation on kanamycin plates were used for homozygote screening.

Plant Material, Growth Conditions, and Metal Stress Treatments. Seedling growth analyses were performed on one-quarter-strength Murashige and Skoog (MS) basal medium (Sigma) with 1 g/liter Mes, 0.8% agar, and the indicated concentrations of heavy metals. Homozygous transgenic plants and WT and mutant controls were grown in one-quarter-strength sterile hydroponic solution as described (22) at 24°C for a 16/8-h day/night period, with minor modifications. Plants were germinated on 0.5× MS plates with 0.6% sucrose for 2–3 days and then transferred to hydroponic solutions. The hydroponic medium was changed to refresh medium 2 days after transfer of plants and subsequently every 3–4 days. At 4 weeks of age, 20 μM $CdCl_2$ was applied to the hydroponic solution. Root, rosette leaf, and stem samples were then harvested at timed intervals. The glutathione synthesis inhibitor L-buthionine sulfoximine (BSO) was added as indicated. Before harvesting, root samples were

---

rinsed in deionized and filtered water for 5 min and then washed at room temperature in 25 mM CaCl$_2$ (pH ≈5) twice, 4 min each time.

Northern Blotting and RT-PCR. Total RNA was extracted from roots, rosette leaves, and stems by using TRIzol reagent (Invitrogen). RNA gel blotting, probe labeling, and hybridizations were performed by using standard protocols suggested by the manufacturers. First-strand cDNA was synthesized from DNaseI-digested total RNA by using Maloney murine leukemia virus reverse transcriptase (Promega), and PCR was performed on PE GeneAmp 9700 with 25 cycles with Biolase *Taq*DNA polymerase (DocFrugal, San Diego).

Protein Extraction and Western Blotting. Plant root, rosette leaf, and stem tissues (0.25–0.5 g) were homogenized in a glass homogenizer separately, with 1 ml of extraction buffer containing 25 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1 mM 2-mercaptoethanol, 1% (wt/vol) polyvinylpyrrolidone, and Complete EDTA-free (Roche Biochemicals) protease inhibitor mixture tablets (1 tablet per 50 ml of extraction buffer). Homogenates were centrifuged at $15,000 \times g$ for 15 min to remove the debris. The protein concentration of the crude extract was determined by the Bradford method (23) with BSA as standard.

Fifteen micrograms of protein from root, stem, and rosette tissues was separated on 10% SDS/PAGE gels and electroblotted onto nitrocellulose membrane (Bio-Rad). Membranes were blocked at room temperature for 1 h in TBST (10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween 20) containing 5% dry milk and then probed with the primary mAb against the c-*myc* tag at a dilution of 1:400 (Sigma) at 4°C overnight. After washing, the blot was incubated with horseradish peroxidase-conjugated anti-mouse secondary Ab (Sigma) at 1:5,000 dilution in blocking buffer for 1 h. After washing again in TBST, the ECL system (Amersham Pharmacia) was used to detect the antigen–Ab complexes.

HPLC Analyses of PCs in Plant Tissues. Four-week-old plants were exposed to 20 μM Cd^{2+} in hydroponic medium for 72 h, and then tissue extracts were prepared by using glass homogenizers and thiols labeled by monobromobimane as described (9, 24). Synthesized standards were used for the identification of PCs (γ-EC)$_2$G (PC$_2$), (γ-EC)$_3$G (PC$_3$), and (γ-EC)$_4$G (PC$_4$).

Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) Analysis. Heavy-metal-treated and control plant tissues were digested in concentrated nitric acid for 5–7 days at room temperature, and then samples were boiled until completely digested. Samples were diluted with Millipore filtered deionized water and briefly centrifuged. The diluted samples were then analyzed by ICP-AES.

Results

Root-Specific and Ectopic Expression of the Wheat *TaPCS1* Gene in *cad1-3*. The *cad1-3* mutant is a recessive, loss-of-function mutation in the *Arabidopsis AtPCS1* gene (7) and shows no detectable PC levels (ref. 19; see also below). Therefore, *cad1-3* was selected for targeted PCS expression. To target PCS expression in roots, the *Adh* promoter was introduced before the wheat *TaPCS1* cDNA. The wheat *TaPCS1* cDNA has 49.2% nucleotide identity to the *Arabidopsis* gene and was used here to avoid cosilencing. The *Adh* promoter is highly induced in mature plant roots by hypoxia (20, 25). Therefore, a hydroponic growth system was developed in which plants can grow under hypoxic conditions. For comparison with root-targeted lines, the *TaPCS1* cDNA was ectopically expressed by using the cauliflower mosaic virus 35S promoter. Independent transgenic lines for Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* with single-copy
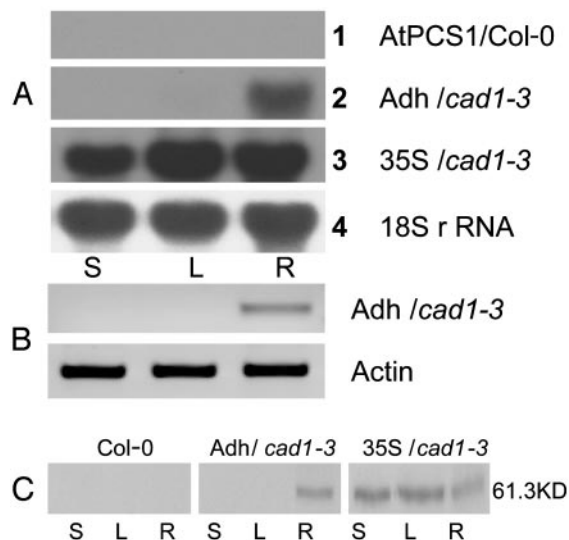

Fig. 1. *TaPCS1* mRNA and protein are not translocated from roots to shoots. (*A*) Northern blots probing *AtPCS1* expression in WT (Col-0) (row 1) and *TaPCS1* expression using the root promoter in Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*) (row 2) and the ectopic promoter in 35S/*cad1-3* (35S::TaPCS1/*cad1-3*) (row 3). 18S rRNA was used as loading control (row 4). (*B*) RT-PCR was performed with TaPCS1::myc fusion-specific primers. Actin was used as control. (*C*) Protein was extracted from stems (S), rosette leaves (L), and roots (R) of WT (Col-0), Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*), and 35S/*cad1-3* (35S::TaPCS1/*cad1-3*) plants. Western blot analyses were performed as described in *Materials and Methods*. WT protein extracts were used as negative controls.

insertions were generated. Two homozygous lines expressing each construct with high levels of TaPCS1 expression were chosen for detailed analyses.

As shown in Fig. 1*A*, no detectable *Arabidopsis AtPCS1* mRNA was observed in the WT(Col-0 ecotype) when *AtPCS1* probe was hybridized to native blots. 35S::TaPCS1/*cad1-3* plants grown under hydroponic conditions for 4 weeks showed transgenic expression of *TaPCS1* mRNA in stems, rosette leaves, and roots (Fig. 1*A*). However, in Adh::TaPCS1-transformed *cad1-3* mutant plants, *TaPCS1* mRNA was expressed only in roots (Fig. 1 *A* and *B*). No *TaPCS1* mRNA was detectable in stems and rosette leaves of Adh::TaPCS1/*cad1-3* plants even when using RT-PCR (Fig. 1*B*). The c-*myc* tag was added to the *TaPCS1* cDNA in all constructs to allow analyses of TaPCS1 protein expression and determine whether the PCS protein can be translocated. Western blot analyses with anti c-*myc* antibodies showed root-specific expression of Adh::TaPCS1/*cad1-3* and ectopic expression of 35S::TaPCS1/*cad1-3* in stems, rosette leaves, and roots (Fig. 1*C*).

Transgenic Expression of the *TaPCS1* Gene Suppresses the Heavy-Metal Sensitivity of the *cad1-3* Mutant. In the absence of metal stress, seedling growth analyses showed that all plants, including the *cad1-3* mutant, WT (Col-0), and transgenic plants expressing Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3*, germinated and grew similar to WT plants on 0.25× Murashige and Skoog plates (Fig. 2*A*). When heavy metals were added to the growth medium, the *cad1-3* mutant did not germinate under arsenate, mercury, and cadmium stress conditions (Fig. 2 *B–D*). However, WT, Adh::TaPCS1/*cad1-3*, and 35S::TaPCS1/*cad1-3* germinated and grew in 80 μM arsenate (Fig. 2*B*) and 10 μM mercury (Fig. 2*C*). In the presence of 40 μM cadmium, Adh::TaPCS1/*cad1-3* plants were slightly more sensitive to Cd^{2+} than WT and 35S::TaPCS1/*cad1-3* seedlings (Fig. 2*D*). The results showed that either targeted or ectopic expression of the wheat *TaPCS1* gene in the *cad1-3* mutant confers tolerance to
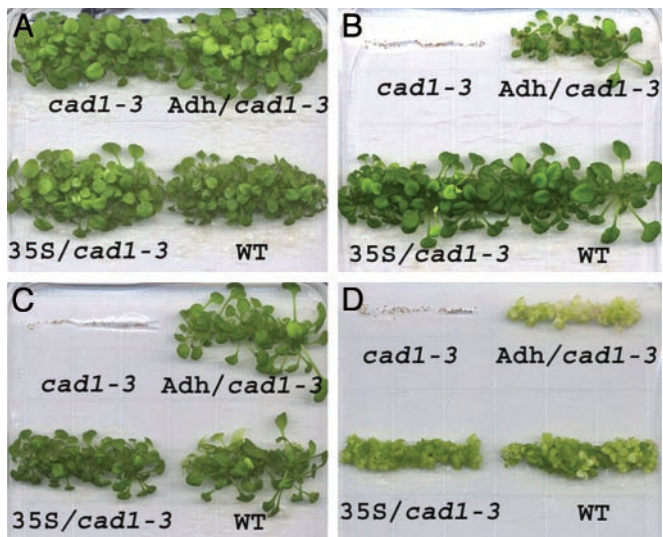
PLANT BIOLOGY

Fig. 2. TaPCS1 complements Cd^{2+}, Hg^{2+}, and As sensitivity in *cad1-3* at seedling stage. For all panels: *Upper Left*, *cad1-3*; *Upper Right*, Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*); *Lower Left*, 35S/*cad1-3* (35S::TaPCS1/*cad1-3*); and *Lower Right*, WT (Col-0). Seeds were plated and horizontally germinated on one-quarter-strength Murashige and Skoog medium with no added heavy metals (*A*), 80 μM KH_2AsO_4 (*B*), 10 μM $HgCl_2$ (*C*), and 40 μM $CdCl_2$ (*D*). Photographs were taken after 21 days.

cadmium, arsenate, and mercury, but no apparent increase of tolerance was observed in Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* compared with WT seedlings under the imposed conditions.

We next analyzed the effect of TaPCS1 expression in mature plants. When plants were 4 weeks old, 20 μM $CdCl_2$ was applied to the hydroponic solution. As shown in Fig. 3*A* after 3 days, roots and leaves of *cad1-3* plants showed brown coloration, which also has been observed for this mutant (19). In contrast, transgenic Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* plants showed root and rosette leaf growth similar to WT plants (Fig. 3*A*). However, further examination showed that the tissue just below the secondary shoot meristem wilted in stems of Adh::TaPCS1/*cad1-3* 3 days after Cd^{2+} addition, as indicated by arrows in Fig. 3*B*. In contrast, 35S::TaPCS1/*cad1-3* plants exposed to 20 μM Cd^{2+} showed normal growth in shoot meristems (Fig. 3*C*). These results show that transgenic expression of TaPCS1 suppresses heavy-metal sensitivity in mature *cad1-3* plants, but no significant increase of tolerance was observed compared with the WT.

PCs Can Be Transported from Roots to Shoots. PCs have been shown to detoxify heavy metals by chelation and subsequent sequestration into vacuoles in *Saccharomyces pombe* and oat roots (4, 5, 26). However, it remains unknown whether PCs are transported from roots to shoots in plants and whether PCs affect long-distance metal transport. To determine whether PCs can be transported from roots to shoots, we measured PC levels in roots, rosette leaves, and stems by using fluorescence HPLC with monobromobimane as a PC-binding label. As shown in Fig. 4*A*, no measurable PCs were detected in roots, rosette leaves, and stems of the *Arabidopsis* mutant *cad1-3*. Note that additional peaks of unknown origin ran at 35 min (Fig. 4*A*), which did not correspond to PC standards (Fig. 4*E*). The peaks were very small in *cad1-3* (note the 100-fold-magnified *y*-axis scale for *cad1-3*) (Fig. 4*A*). In contrast, in WT and 35S::TaPCS1/*cad1-3* plants, high levels of PC_2, PC_3, and PC_4 were detected in root, rosette leaf, and stem tissues (Fig. 4 *B* and *C*). Synthesized PC standards are shown in Fig. 4*E*. In Adh::TaPCS1/*cad1-3*, PC_2, PC_3, and PC_4
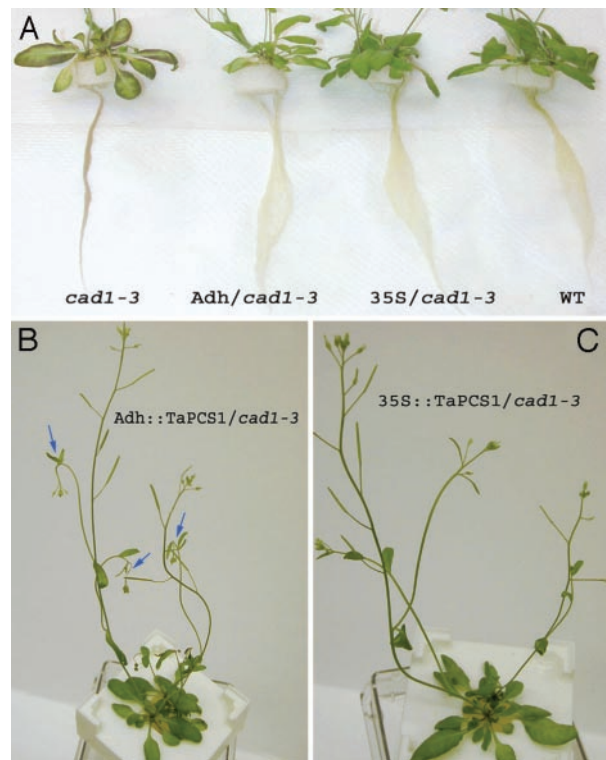

Fig. 3. Heavy-metal tolerance in *cad1-3* was restored completely or partially by ectopic or root-specific expression of TaPCS1. Four-week-old hydroponically grown *cad1-3* mutant, Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*), 35S/*cad1-3* (35S::TaPCS1/*cad1-3*), and WT plants were exposed to 20 μM $CdCl_2$ for 3 days. Phenotypes were assessed for roots and rosette leaves (*A*) and stems (*B* and *C*). (*B*) Blue arrows show sites sensitive to Cd^{2+} stress in Adh::TaPCS1/*cad1-3*.

were detected in roots at a level similar to those in WT and 35S::TaPCS1/*cad1-3* roots. Interestingly, PCs were also detected in stems and rosette leaves of Adh::TaPCS1/*cad1-3* (Fig. 4*D*), despite the finding that in Adh::TaPCS1/*cad1-3*, TaPCS1 protein and mRNA are root specific (Fig. 1). Both root-specific and ectopic expression of TaPCS1 did not increase PC levels significantly compared with those in the WT (Fig. 4). PC levels in rosette leaves and stems of Adh::TaPCS1/*cad1-3* plants were comparable in amplitude with the PC standards (Fig. 4*E*). These data suggest that PCs detected in Adh::TaPCS1/*cad1-3* shoots originate via translocation from roots.

Enhancement of Long-Distance Cd^{2+} Transport by Transgenic Expression of *TaPCS1*. One of the proposed prerequisites for bioremediation is that heavy metals are transported to and sequestered in aerial parts of plants. Experiments were pursued to analyze whether targeted expression of TaPCS1 affects Cd^{2+} distribution in *Arabidopsis*. Time-course analyses of Cd^{2+} accumulation in stems of 4-week-old plants showed that 3 days after 20 μM Cd^{2+} application, very little Cd^{2+} was transported to stems of the *cad1-3* mutant (Fig. 5*A*). In WT, no increase in Cd^{2+} accumulation was observed compared with *cad1-3* at 30 h, but at 54 h and 72 h, more Cd^{2+} was transported to WT stems than to *cad1-3* stems (Fig. 5*A*). Interestingly, dramatic increases in Cd^{2+} accumulation were observed in stems of Adh::TaPCS1/*cad1-3*, and 35S::TaPCS1/*cad1-3* plants compared with WT and *cad1-3* stems at 72 h (Fig. 5*A*; e.g., Adh::TaPCS1/*cad1-3* vs. *cad1-3*, $P < 0.002$ and 35S::TaPCS1/*cad1-3* vs. *cad1-3*; $P < 0.021$, $n = 6$). Furthermore, compared with *cad1-3*, no increase in Cd^{2+} accumulation was observed at 30 h in WT stems; however, in Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* stems,
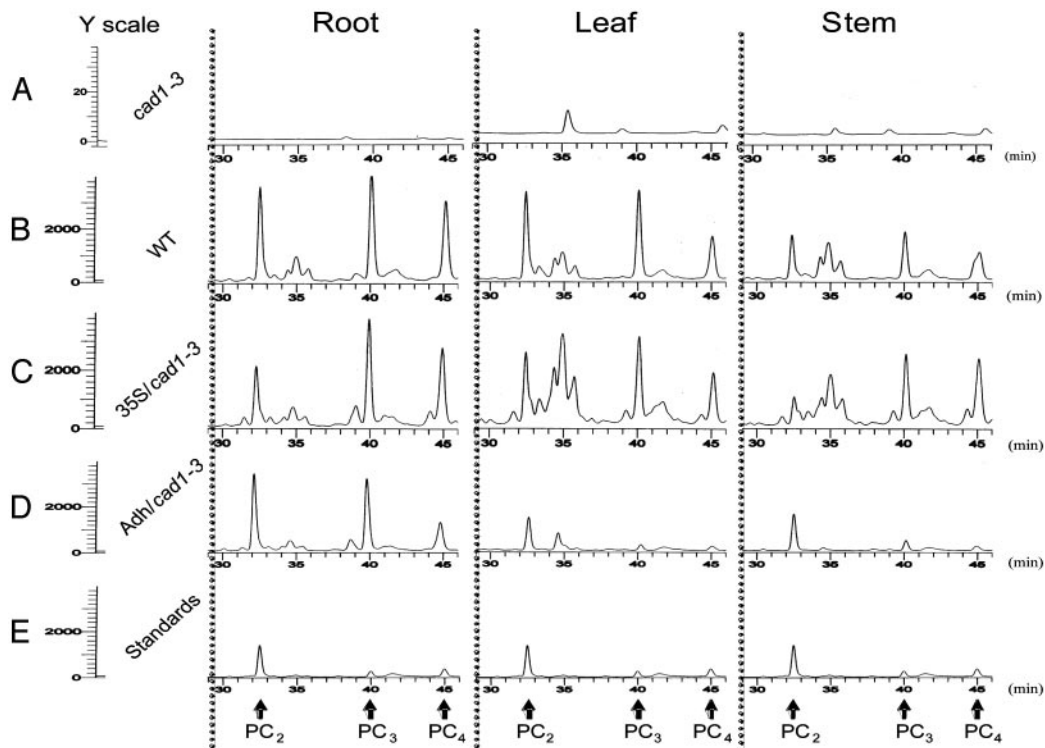
Fig. 4. PCs can be transported from roots to shoots. Four-week-old plants were exposed to 20 μM CdCl$_2$ for 3 days in hydroponic media, and PCs were extracted from root, leaf (rosette), and stem tissues of *cad1-3* (*A*), WT Col-0 (*B*), 35S/*cad1-3* (35S::TaPCS1/*cad1-3*) (*C*), and Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*) (*D*). PC levels were measured by fluorescence HPLC. (*E*) Synthesized PC$_2$, PC$_3$, and PC$_4$ (2 μM each) PC standards were measured, and three copies of standard measurements are illustrated for easy comparison to the measured samples. PC$_2$, PC$_3$, and PC$_4$ peaks are indicated by arrows.

more Cd^{2+} accumulated at 30 h compared with *cad1-3* (Fig. 5*A*; $P < 0.025$, $n = 6$). These data show that transgenic expression of TaPCS1 accelerated Cd^{2+} accumulation in stems. In rosette leaves (Fig. 5*B*), a similar Cd^{2+} accumulation pattern was observed in the WT and in the *cad1-3* mutant. In Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3*, dramatic increases in Cd^{2+} accumulation in rosette leaves were observed after 72 h when compared with WT and *cad1-3* (Fig. 5*B*; e.g., Adh::TaPCS1/*cad1-3* vs. *cad1-3*, $P < 0.00017$; 35S::TaPCS1/*cad1-3* vs. *cad1-3*, $P < 0.0066$, $n = 6$). These results showed that either root-specific or ectopic expression of *TaPCS1* substantially enhanced and accelerated long-distance Cd^{2+} transport.

Unexpectedly, targeted expression of *TaPCS1* did not enhance Cd^{2+} accumulation in roots (Fig. 5*C*). Adh::TaPCS1/*cad1-3*, 35S::TaPCS1/*cad1-3*, and WT plants showed a similar Cd^{2+} accumulation pattern in roots. Cd^{2+} accumulation was saturated 30 h after Cd^{2+} application. In contrast, a significant increase in Cd^{2+} accumulation was observed in *cad1-3* roots compared with roots of Adh::TaPCS1/*cad1-3*, 35S::TaPCS1/*cad1-3*, and WT (Fig. 5*C*). Furthermore, Cd^{2+} accumulation in *cad1-3* roots did not saturate within the analyzed 72-h period. Interestingly, these findings demonstrate that the presence of active PCS plays an essential role in maintaining reduced Cd^{2+} concentrations in *Arabidopsis* roots (Fig. 5*C*).

BSO Disrupts Cd^{2+} Homeostasis in Roots and Aborts Enhanced Long-Distance Cd^{2+} Transport in Transgenic TaPCS1 Plants. BSO inhibits glutathione biosynthesis and thus inhibits PC synthesis (19). Because transgenic expression of TaPCS1 enhanced long-distance Cd^{2+} transport (Fig. 5) but did not increase PC levels compared with WT (Fig. 4 *C* and *D*), we further analyzed whether PCs contribute to long-distance Cd^{2+} transport in the presence of BSO. Without BSO, WT, Adh::TaPCS1/*cad1-3*, and

35S::TaPCS1/*cad1-3* plants accumulated more Cd^{2+} in stems and rosette leaves but less in roots than *cad1-3* (Fig. 6*A*). In the presence of BSO, Cd^{2+} accumulation in Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* stems and rosette leaves was decreased significantly (Fig. 6*B*) ($P < 0.03$, $n = 3$), whereas in WT, no dramatic decrease was observed (Fig. 6*B*) ($P > 0.22$ in stems and $P > 0.068$ in rosette leaves, $n = 3$). Interestingly, addition of BSO mimicked the disruption of the *AtPCS1* gene in *cad1-3*, resulting in increased Cd^{2+} accumulation in roots of Adh::TaPCS1/*cad1-3*, 35S::TaPCS1/*cad1-3*, and WT plants (Fig. 6*B*). BSO had no significant effect on Cd^{2+} accumulation in *cad1-3* (Fig. 6*B*; $P > 0.47$, 0.25, and 0.67 in stems, rosette leaves, and roots, respectively; $n = 3$). These results demonstrate that PCs play a central role in maintaining low Cd^{2+} concentrations in roots and that PCs provide an essential mechanism for long-distance root-to-shoot Cd^{2+} transport.

Discussion

The functions of PCs in heavy-metal detoxification have been established at the intracellular and biochemical levels (2, 4, 5, 26, 27). In the present study, an important function of PCs in heavy-metal detoxification was revealed by targeting *TaPCS1* to root tissues of a PC-deficient mutant, *cad1-3*. The presented data unexpectedly demonstrate that root-to-shoot transport of PCs occurs and that PCs provide a major mechanism for regulating long-distance Cd^{2+} transport in *Arabidopsis*.

PCs Protect Against Cd^{2+} Overaccumulation in Roots. PCs are known to play an essential role in heavy-metal detoxification by chelating heavy metals in the cytosol and sequestering PC-Cd^{2+} complexes in vacuoles via transport across the tonoplast (4, 5). Because of the important functions of PCs in cellular metal sequestration, root-specific expression of TaPCS1 has been
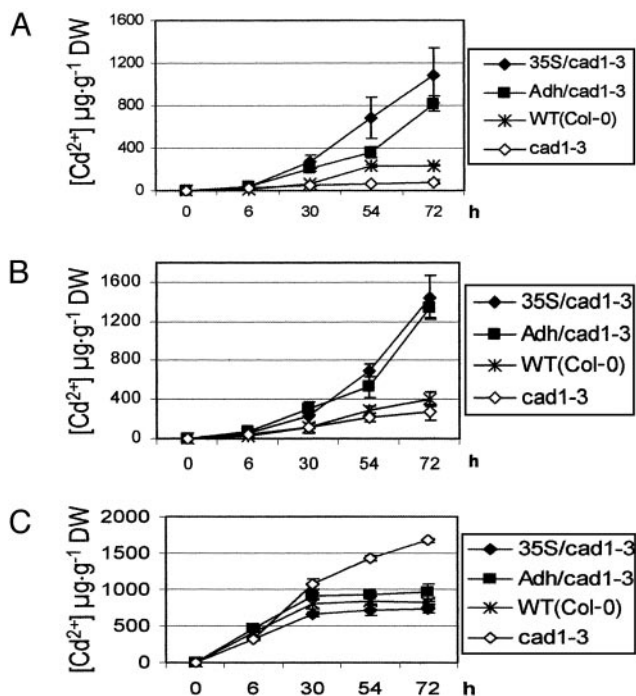
Fig. 5. Enhanced long-distance Cd^{2+} transport and reduced Cd^{2+} accumulation in roots by transgenic expression of TaPCS1. Four-week-old 35S/*cad1-3* (35S::TaPCS1/*cad1-3*), Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*), WT (WT Col-0), and *cad1-3* plants grown in hydroponic solution were exposed to 20 μM $CdCl_2$. Samples were harvested at timed intervals after Cd^{2+} exposure as indicated. Cd^{2+} accumulation in stems (*A*), rosette leaves (*B*), and roots (*C*) was determined by ICP-AES. Data show mean values $\pm$ SE, $n = 6$ plants.

Fig. 6. Short-term BSO exposure mimics the effect of *AtPCS1* gene disruption in *cad1-3* with respect to Cd^{2+} accumulation in roots and shoots. Four-week-old *cad1-3*, Adh/*cad1-3* (Adh::TaPCS1/*cad1-3*), 35S/*cad1-3* (35S::TaPCS1/*cad1-3*), and WT plants grown in hydroponic solution were pretreated with 0 mM/1 mM BSO for 12 h; 20 μM $CdCl_2$ was then applied to either the untreated plants (*A*) or the 1 mM BSO pretreated plants with 0.5 mM BSO for 54 h (*B*). Cd^{2+} accumulation in stems (S), rosette leaves (L), and roots (R) was determined by ICP-AES. Data show mean values $\pm$ SE, $n = 6$ and 3 plants in *A* and *B*, respectively.

expected to cause trapping of Cd^{2+} in plant roots by chelating and sequestering more Cd^{2+} into vacuoles.

Unexpectedly, both ectopic and root-specific expression of *TaPCS1* did not increase Cd^{2+} content significantly in root cells (Fig. 5*C*). On the contrary, transgenic expression of *TaPCS1* in *cad1-3* caused a reduction in Cd^{2+} accumulation in roots compared with *cad1-3*. The *cad1-3* mutant showed higher levels of Cd^{2+} accumulation in roots compared with WT and transgenic lines (Fig. 5*C*). Cd^{2+} accumulation in roots saturated in both transgenic and WT plants at 30 h after Cd^{2+} stress (Fig. 5*C*). These data show that Cd^{2+} accumulation in *Arabidopsis* roots is limited when PCs are present. Thus the PC-Cd^{2+} detoxification model derived from unicellular organisms (3, 4) is not completely applicable to multicellular organisms.

Consistent with the reduced Cd^{2+} accumulation in transgenic and WT plant roots (Fig. 5*C*), enhanced long-distance root-to-shoot Cd^{2+} transport occurred compared with *cad1-3* (Fig. 5 *A* and *B*). These findings suggest that, in addition to the known cellular protection function of PCs (28), PC-dependent long-distance Cd^{2+} transport contributes substantially to maintenance of low Cd^{2+} concentrations in roots. These data further implicate a model in which Cd^{2+} saturation in roots causes extra Cd^{2+} to be transported to shoots, thus contributing to maintenance of low Cd^{2+} levels in roots. This proposed "overflow protection mechanism" would contribute to maintaining a low Cd^{2+} content in roots by transporting extra Cd^{2+} to shoot tissues, and the process depends on PCs (Fig. 5*C*).

Enhanced Long-Distance Cd^{2+} Transport is PC-Dependent. In *cad1-3*, PCs were not observed in roots, rosette leaves, and stems (Fig. 4*A*). However, in Adh::TaPCS1/*cad1-3*, PCs were found in rosette leaves and stems (Fig. 4*D*), although PCS protein and
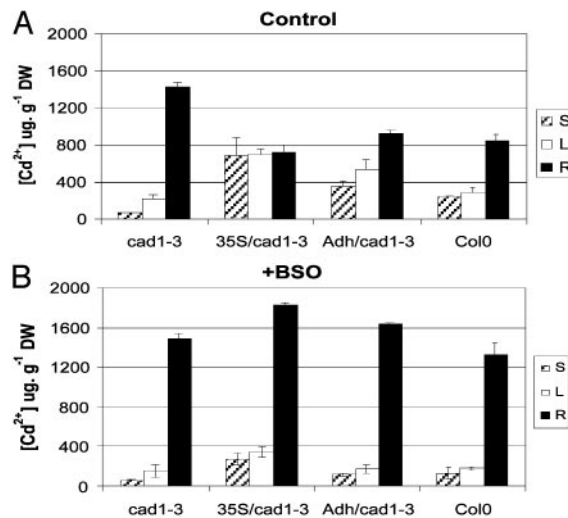
mRNA were detected only in roots (Fig. 1). These data show that long-distance PC transport occurs.

Interestingly, however, although much more Cd^{2+} was transported from roots to shoots in transgenic plants than in WT plants (Fig. 5 *A* and *B*), an increase in PC levels was not observed in transgenic plant shoots compared with WT plants (Fig. 4 *B–D*). Recent observations were reported showing that PC levels are generally lower in roots and slightly higher in shoots of the Zn/Cd hyperaccumulator *Thlaspi caerulescens* than in the non-accumulator *Thlaspi arvense*, despite the higher cadmium concentration in the hyperaccumulator (29). Interestingly, the present study shows that whole-tissue PC levels may not always be a good indicator of the roles of PCs in metal accumulation. Our data show that targeted PC synthesis rather than total tissue PC levels is important for Cd accumulation in *Arabidopsis* shoots. Surprisingly, in stems and rosette leaves of Adh::TaPCS1/*cad1-3* plants, lower PC levels were detected than in the WT. This finding may be partially attributed to the fact that PCs in Adh::TaPCS1/*cad1-3* originated from roots, whereas in 35S::TaPCS1/*cad1-3* and WT, PCs synthesis also occurred in shoots. Short-term pharmacological experiments were pursued by using BSO, a specific inhibitor of glutathione biosynthesis, which is required for PC biosynthesis. These experiments demonstrated that BSO significantly decreased Cd^{2+} accumulation in shoots and enhanced Cd^{2+} levels in roots of Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* plants, which further supports that the enhanced root-to-shoot Cd^{2+} transport in transgenic plants is PC-dependent.

Possible Mechanisms Regulating Long-Distance Cd^{2+} Transport. Our findings point to the question of why only the exogenous expression of TaPCS1 significantly enhanced root-to-shoot Cd^{2+} transport despite the fact that WT plants also synthesize PCs in roots. One possible answer is that transgenic expression of TaPCS1 may cause increased PCS accumulation in specific cell types such as vascular parenchyma cells, thus allowing more PC accumulation in these cells, which could further improve Cd^{2+} or PC-Cd^{2+} loading into vascular transport pathways. In addi-

tion, recombinant PCS protein alone is sufficient to encode for the enzymatic activity required for PC synthesis. As the transgenic wheat TaPCS1 protein differs in amino acid sequence (55% amino acid identity) from the native *Arabidopsis* AtPCS1 protein, it is conceivable that the transgenic protein acts constitutively and more independently from a possible regulatory network in *Arabidopsis*. The accelerated Cd^{2+} accumulation in Adh::TaPCS1/*cad1-3* and 35S::TaPCS1/*cad1-3* stems (Fig. 5*A*) supports this hypothesis. Similarly, a recent study has shown that transgenic altered subcellular targeting of methylmercury lyase enhances mercury detoxification in *Arabidopsis* (30). Further research using cell-specific promoters will be needed to determine important sites of PC synthesis for long-distance transport.

An alternative (nonexclusive to the above) model is also possible, in which the excessive PCS mRNA in transgenic plant roots before Cd^{2+} stress (Fig. 1*A*) may cause signal transduction events, reporting that plants have been challenged by severe Cd^{2+} concentrations, thus leading to up-regulation of long-distance Cd^{2+} transport mechanisms. These long-distance transport mechanisms could contribute to the overflow protection machinery.

In *Brassica juncea* WT plants, Cd^{2+} transport in the xylem sap was reported to be coordinated with oxygen or nitrogen ligands (16). In transgenic *Arabidopsis* plants, the presented data suggest that PC-assisted Cd^{2+} transport also occurs. The finding that the enhanced root-to-shoot Cd^{2+} transport was aborted by BSO addition in transgenic plants (Fig. 6) supports this model. Based on our PC standards (Fig. 4*E*) and the amplitude of PC_2, PC_3, and PC_4 peaks in rosette leaves and stems of Adh::TaPCS1/*cad1-3* plants, we roughly estimate that the stoi-

chiometry of Cd^{2+}/total PCs ranged approximately from 2.4:1 to 4.4:1. Recent research has suggested that Cd^{2+} binds to PC_3 with a stoichiometry of 3 Cd^{2+}/1 PC_3 in oat roots (5). This simple estimate suggests that PCs function as important components of root-to-shoot cadmium transport in Adh::TaPCS1/*cad1-3* plants. Note, however, that the present study does not exclude that PCs may function parallel to or contribute to other long-distance Cd^{2+} transport mechanisms. As suggested by the cadmium-induced secondary meristem wilting phenotype in mature Adh::TaPCS1/*cad1-3* plants (Fig. 3*B*), Cd^{2+} is slightly toxic in the stems of Adh::TaPCS1/*cad1-3*. These observations suggest the existence of less-detoxifying long-distance Cd^{2+} transport mechanisms. Furthermore, these observations suggest a function for local PC synthesis in stems for Cd^{2+} detoxification.

In conclusion, by developing a targeted expression system, we demonstrate that PCs can be transported from roots to shoots and that PCs play an essential role in maintaining reduced Cd^{2+} accumulation levels in roots and enhancing long-distance root-to-shoot Cd^{2+} transport.

1. Ayres, R. U. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 815–820.
2. Grill, E., Winnacker, E.-L. & Zenk, M. H. (1985) *Science* **230,** 674–676.
3. Mutoh, N. & Hayashi, Y. (1988) *Biochem. Biophys. Res. Commun.* **151,** 32–39.
4. Ortiz, D. F., Ruscitti, T., McCue, K. F. & Ow, D. W. (1995) *J. Biol. Chem.* **270,** 4721–4728.
5. Salt, D. E. & Rauser, W. E. (1995) *Plant Physiol.* **107,** 1293–1301.
6. Grill, E., Löffler, S., Winnacker, E.-L. & Zenk, M. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6838–6842.
7. Ha, S.-B., Smith, A. P., Howden, R., Dietrich, W. M., Bugg, S., O'Connell, M. J., Goldsbrough, P. B. & Cobbett, C. S. (1999) *Plant Cell* **11,** 1153–1164.
8. Vatamaniuk, O. K., Mari, S., Lu, Y.-P. & Rea, P. A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 7110–7115.
9. Clemens, S., Kim, E. J., Neumann, D. & Schroeder, J. I. (1999) *EMBO J.* **18,** 3325–3333.
10. Raskin, I., Kumar, N., Dushenkov, S. & Salt, D. E. (1994) *Curr. Opin. Biotechnol.* **5,** 285–290.
11. Raskin, I. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3164–3166.
12. Epstein, E. (1999) *Annu. Rev Plant Physiol. Plant Mol. Biol.* **50,** 641–664.
13. Epstein, E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11–17.
14. Chaney, R. L., Malik, M., Li, Y. M., Brown, S. L., Brewer, E. P., Angle, J. S. & Baker, A. J. (1997) *Curr. Opin. Biotechnol.* **8,** 279–284.
15. Yang, X., Baligar, V. C., Martens, D. C. & Clark, R. B. (1995) *J. Environ. Sci. Health B* **30,** 569–583.
16. Salt, D. E., Prince, R. C., Pickering, I. J. & Raskin, I. (1995) *Plant Physiol.* **109,** 1427–1433.
17. Jalil, A., Selles, F. & Clarke, J. M. (1994) *J. Plant Nutr.* **17,** 1839–1858.
18. Florijn, P. J., Knecht, J. A. & Van Beusichem, M. L. (1993) *J. Plant Physiol.* **142,** 537–542.
19. Howden, R., Goldsbrough, P. B., Andersen, C. R. & Cobbett, C. S. (1995) *Plant Physiol.* **107,** 1059–1066.
20. McKendree, W. L., Jr., & Ferl, R. J. (1992) *Plant Mol. Biol.* **19,** 859–862.
21. Clough, S. J. & Bent, A. F. (1998) *Plant J.* **16,** 735–743.
22. Arteca, R. N. & Arteca, J. M. (2000) *Physiol. Plant.* **108,** 188–193.
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
24. Sneller, F. E., van Heerwaarden, L. M., Koevoets, P. L., Vooijs, R., Schat, H. & Verkleij, J. A. (2000) *J. Agric. Food Chem.* **48,** 4014–4019.
25. Chung, H.-J. & Ferl, R. J. (1999) *Plant Physiol.* **121,** 429–436.
26. Ortiz, D. F., Kreppel, L., Speiser, D. M., Schreel, G., McDonald, G. & Ow, D. W. (1992) *EMBO J.* **11,** 3491–3499.
27. Grill, E. (1987) *Experientia* **52,** Suppl., 317–322.
28. Zenk, M. H. (1996) *Gene* **179,** 21–30.
29. Ebbs, S., Lau, I., Ahner, B. & Kochian, L. (2002) *Planta* **214,** 635–640.
30. Bizily, S. P., Kim, T., Kandasamy, M. K. & Meager, R. B. (2003) *Plant Physiol.* **131,** 463–471.