An Improved Grafting Technique for Mature Arabidopsis Plants Demonstrates Long-Distance Shoot-to-Root Transport of Phytochelatins in Arabidopsis[1][W]

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Phytochelatins (PCs) are peptides that function in heavy-metal chelation and detoxification in plants and fungi. A recent study showed that PCs have the ability to undergo long-distance transport in a root-to-shoot direction in transgenic Arabidopsis (*Arabidopsis thaliana*). To determine whether long-distance transport of PCs can occur in the opposite direction, from shoots to roots, the wheat (*Triticum aestivum*) PC synthase (*TaPCS1*) gene was expressed under the control of a shoot-specific promoter (*CAB2*) in an Arabidopsis PC-deficient mutant, *cad1-3* (CAB2::*TaPCS1*/*cad1-3*). Analyses demonstrated that *TaPCS1* is expressed only in shoots and that CAB2::*TaPCS1*/*cad1-3* lines complement the cadmium (Cd) and arsenic metal sensitivity of *cad1-3* shoots. CAB2::*TaPCS1*/*cad1-3* plants exhibited higher Cd accumulation in roots and lower Cd accumulation in shoots compared to wild type. Fluorescence HPLC coupled to mass spectrometry analyses directly detected PC2 in the roots of CAB2:*TaPCS1*/*cad1-3* but not in *cad1-3* controls, suggesting that PC2 is transported over long distances in the shoot-to-root direction. In addition, wild-type shoot tissues were grafted onto PC synthase *cad1-3 atpcs2-1* double loss-of-function mutant root tissues. An Arabidopsis grafting technique for mature plants was modified to obtain an 84% success rate, significantly greater than a previous rate of approximately 11%. Fluorescence HPLC-mass spectrometry showed the presence of PC2, PC3, and PC4 in the root tissue of grafts between wild-type shoots and *cad1-3 atpcs2-1* double-mutant roots, demonstrating that PCs are transported over long distances from shoots to roots in Arabidopsis.

Heavy metals are defined as metals that have a density of $\geq$5.0 g cm^{-3} and include elements such as cadmium (Cd), lead (Pb), mercury (Hg), and copper (Cu). Some of these metals are essential at low concentrations, such as iron (Fe) and Cu, but at higher concentrations these metals can become toxic. High concentrations of heavy metals are serious environmental concerns, as the U.S. Environmental Protection Agency lists Cd, Hg, Pb, and the metalloid arsenite among the 10 most hazardous contaminants at Superfund sites (http://www.atsdr.cdc.gov/clist.html).

A primary mechanism by which plants and fungi tolerate heavy-metal toxicity is through the production of small thiolate peptides called phytochelatins (PCs) that bind a variety of metals (Kondo et al., 1984; Grill et al., 1985). PCs, which have the chemical structure (γ-Glu-Cys)$_n$-Gly, where n = 2 to 11, are produced postranscriptionally through the enzyme PC synthase (PCS) using glutathione as a substrate (Grill et al., 1989; Ha et al., 1999; Clemens et al., 1999; Vatamaniuk et al., 1999, 2000, 2004).

The Arabidopsis (*Arabidopsis thaliana*) genome has two PCS genes: *AtPCS1* and *AtPCS2* (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999; Cazalé and Clemens, 2001; Lee and Kang, 2005). In the fungus *Schizosaccharomyces pombe*, complexes of heavy metals bound to PCs are transported across the tonoplast and sequestered in vacuoles by means of the ATP-binding cassette (ABC) transporter HMT1 (Ortiz et al., 1995). In plants, PCs are also sequestered into vacuoles (Salt and Rauser, 1995). Therefore, PCs were predicted not to undergo long-distance transport but to mainly aid in the sequestration of PC heavy-metal complexes into vacuoles. Recently, PCs were shown to undergo long-distance transport in a root-to-shoot direction when a wheat (*Triticum aestivum*) PCS, TaPCS1 (Clemens et al., 1999), was specifically targeted to Arabidopsis roots of the PC-deficient *cad1-3* mutant using an alcohol dehydrogenase 1 (ADH1) promoter (Gong et al., 2003). Furthermore, root-targeted, non-native (ADH1 promoter) expression of TaPCS1 unexpectedly enhanced the accumulation of Cd in the shoots and decreased Cd accumulation in roots compared to the *cad1-3* controls

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(Gong et al., 2003). These results imply that transported PCs play a role in the transport of Cd, but do not exclude the presence of additional long-distance Cd transport mechanisms.

To further analyze long-distance PC transport abilities and the role of PCs in Cd transport, here we address the question of whether PCs have the ability to undergo long-distance transport in the opposite direction—from shoots to roots. In this study, the *cad1-3* mutant, which is a recessive loss-of-function mutant in the Arabidopsis *PCS1* gene and exhibits no detectable PCs (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003), was transformed with a shoot-specific promoter, CAB2 (Millar and Kay, 1991), driving the expression of wheat TaPCS1 to determine whether shoot-to-root PC transfer is feasible. In addition, we pursued experiments to determine whether expression of Arabidopsis *PCS* genes under the spatial control of their native genomic promoters can mediate long-distance shoot-to-root transport. For these analyses, grafting techniques for mature Arabidopsis plants (Ayre and Turgeon, 2004) were modified for greatly improved success rates and wild-type shoots were grafted onto *cad1-3 atpcs2-1* double loss-of-function mutants. Fluorescence HPLC and coupled parallel mass spectrometry (MS) analyses of grafted plants demonstrate shoot-to-root transfer of PCs.

RESULTS

Shoot-Specific Expression of Wheat *TaPCS1* cDNA in *cad1-3*

The Arabidopsis *cad1-3* mutant containing a defective *AtPCS1* gene was selected as the background to target wheat *TaPCS1* expression because the mutant lacks detectable PCs (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003). The wheat gene *TaPCS1* was used to avoid co-silencing because it only has a 49.2% nucleotide identity to *AtPCS1*. A 199-bp region of the CAB2 promoter was linked to the wheat *TaPCS1* cDNA to drive expression of *TaPCS1*, and *cad1-3* plants were transformed with this construct. Sixteen independent homozygous lines of CAB2::*TaPCS1*/*cad1-3* were isolated, and three independent lines used for further analysis were selected based on showing the highest levels of the *TaPCS1* mRNA transcript as determined by quantitative reverse transcription (RT)-PCR of the 16 isolated homozygous lines isolated (data not shown).

Northern-blot analyses showed that expression of *TaPCS1* mRNA was specific to shoot tissue in all three independent lines (Fig. 1A). Shoot-specific expression of wheat *TaPCS1* was confirmed in stringent RT-PCR experiments (Fig. 1B). No *TaPCS1* mRNA was detected in root tissue even after 50 cycles of RT-PCR (Fig. 1B). In contrast, the positive control 35S:*TaPCS1*/*cad1-3* line showed strong expression of *TaPCS1* mRNA in both shoot and root tissue (Fig. 1A).

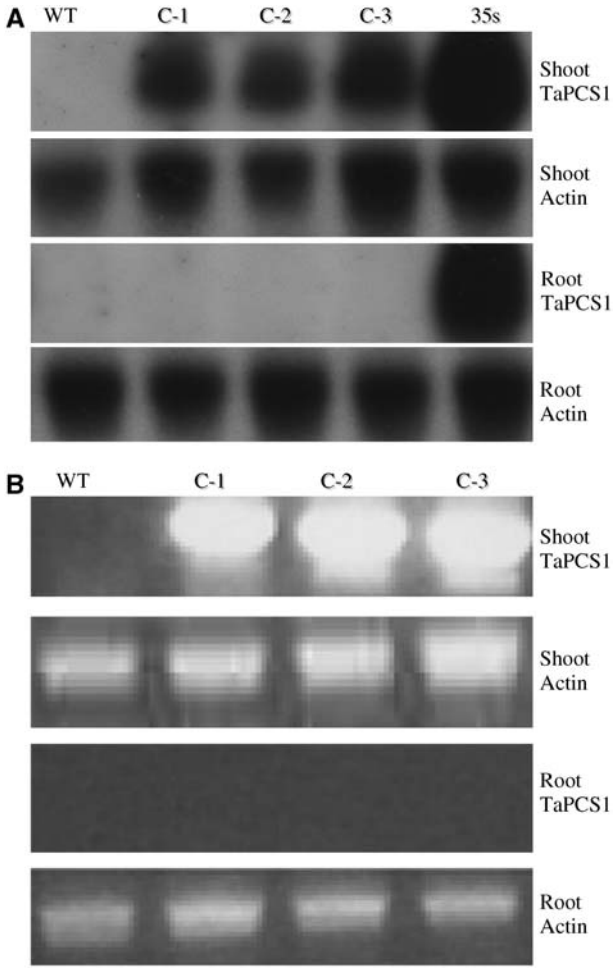

Figure 1. Expression of *TaPCS1* mRNA is targeted to the shoots of CAB2::*TaPCS1*/*cad1-3* plants. A, Northern blots probing *TaPCS1* expression in wild type (Col-0), three independent lines of CAB2::*TaPCS1*/*cad1-3* (C-1, C-2, C-3), and 35S::*TaPCS1*/*cad1-3* (35s). Actin was used as a loading control ($n = 2$). B, Fifty cycles of RT-PCR show lack of any *TaPCS1* expression in roots and strong *TaPCS1* expression in shoots. RT-PCR was performed with *TaPCS1*::c-myc fusion-specific primers. *Actin7* primers were used as a loading control ($n = 2$).

Transgenic Expression of cDNA Complements Heavy-Metal Sensitivity of *cad1-3* in Shoot Tissue But Not in Root Tissue

cad1-3 was unable to grow on plates containing 40 μM $CdCl_2$ or 80 μM KH_2AsO_4 (Fig. 2 ,C and D; $n = 60$ seedlings), which correlates with the lack of detectable PCs in *cad1-3*. Experiments showed that the shoot sensitivity of *cad1-3* can be complemented by both the CAB2::*TaPCS1* and 35S::*TaPCS1* constructs, as both of these lines produced green cotyledons, as did wild-type seedlings, when grown on Cd or arsenate (Fig. 2, C and D).

Root growth of the *cad1-3* mutant was also highly sensitive to Cd in comparison to wild-type seedlings (Fig. 3). The three CAB2::*TaPCS1*/*cad1-3* lines (C-1, C-2, and C-3) were not able to complement the root-sensitive phenotype of *cad1-3* and showed enhanced

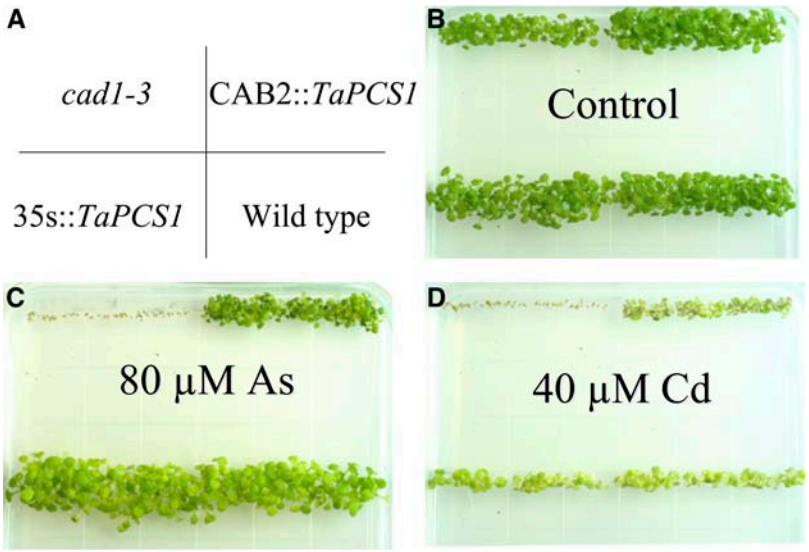

Figure 2. CAB2::*TaPCS1* expression complements the Cd and As sensitivity of *cad1-3* in shoots. For each image, seeds of four different lines were germinated as diagrammed in A: top left, *cad1-3* (*atpcs1*); top right, CAB2::*TaPCS1* (CAB2::*TaPCS1*/*cad1-3*); bottom left, 35S::*TaPCS1*(35S::*TaPCS1*/*cad1-3*); bottom right, wild type (Col-0 ecotype). All seeds were germinated and grown on 25% Murashige and Skoog medium for 14 d on medium containing no heavy metal as a control (B), 80 μM As (C), or 40 μM Cd (D).

growth inhibition by Cd^{2+} compared to wild type (Fig. 3). However, lines C-2 and C-3 showed significantly longer root growth in comparison to *cad1-3* (C-2, $P = 3.7 \times 10^{-4}$; C-3, $P = 1.1 \times 10^{-6}$).

PCs Are Transported in a Shoot-to-Root Direction

To determine whether PCs can undergo long-distance transport in the shoot-to-root direction, PC levels in shoot and root tissues were analyzed in the three independent CAB2::*TaPCS1*/*cad1-3* lines, as well as positive and negative controls. Fluorescence HPLC analyses of monobromobimane-labeled PCs from these tissues was performed to analyze PC levels (Fig. 4). PC2, PC3, and PC4 peaks were identified using synthesized PC standards and are shown in Figure 4, G and H. The HPLC flow was split to the fluorescence detector and to a mass spectrometer to simultaneously identify the M_rs of fluorescence HPLC peaks (Fig. 5). As previously reported (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003), *cad1-3* plants did not display any detectable PCs even when the fluorescence detector gain was amplified (Fig. 4, E and F; $n = 14$ of 14 plants). PCs were clearly detected in positive control wild-type shoot and root tissues of 4-week-old plants exposed to Cd (Fig. 4, A and B; $n = 15$ of 16 plants). In all three CAB2::*TaPCS1*/*cad1-3* lines, PC2, PC3, and PC4 were detected in shoot tissues (Fig. 4C; $n = 38$ of 40 plants). Interestingly, PC2 was clearly detected in the root tissue of all three CAB2::*TaPCS1*/*cad1-3* lines (Fig. 4D; $n = 38$ of 40 plants). Because *TaPCS1* mRNA was exclusively expressed in shoot tissues of the CAB2::*TaPCS1*/*cad1-3* lines (Fig. 1), the presence of PC2 in the roots indicated that PC2 underwent long-distance shoot-to-root transport in the transgenic CAB2::*TaPCS1*/*cad1-3*.

To identify and verify the presence of PCs in the root tissue of CAB2::*TaPCS1*/*cad1-3* plants, peaks detected by HPLC were analyzed by MS. The predicted and observed mass for PC2 standard labeled with two monobromobimane molecules at the +1 ion state was 920 *m/z* (Fig. 5A; $n = 30$). Peaks of identical mass were observed in both wild-type and CAB2::*TaPCS1*/*cad1-3* samples (Fig. 5, B and C; $n = 15$ of 16 plants for wild type; $n = 38$ of 40 plants for CAB2::*TaPCS1*/*cad1-3*). The larger background present in the CAB2::*TaPCS1*/*cad1-3* suggested a lower level of PC2 in the sample. In negative control experiments, no 920 *m/z* peak was found in the *cad1-3* extracts (Fig. 5D; $n = 14$ of 14 plants), supporting the hypothesis that PC2 is transported from shoots to roots in CAB2::*TaPCS1*/*cad1-3* plants.

Cd Accumulates in Roots of CAB2::*TaPCS1*/*cad1-3*

Cd levels in root and shoot tissues of 4-week-old plants exposed to 20 μM $CdCl_2$ for 4 d were analyzed

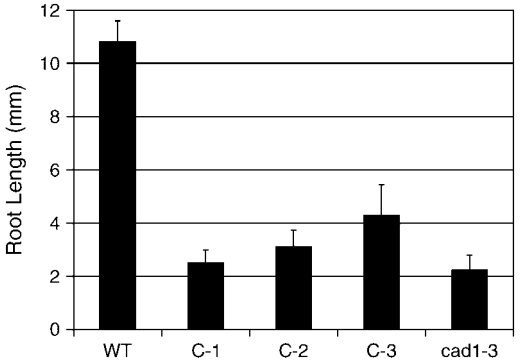

Figure 3. CAB2::*TaPCS1* expression in shoots of *cad1-3* does not recover the wild-type Cd-sensitive root growth. Wild type (WT), three CAB2::*TaPCS1*/*cad1-3* lines (C-1, C-2, C-3), and *cad1-3* seedlings were germinated on 25% Murashige and Skoog medium with no added Cd for 5 d and then transferred to plates containing 20 μM $CdCl_2$ for 3 d. Root length presented is a measure of new root growth after the transfer of seedlings to plates containing 20 μM $CdCl_2$. Data show mean values ± SEM; $n = 60$ plants per plant line.

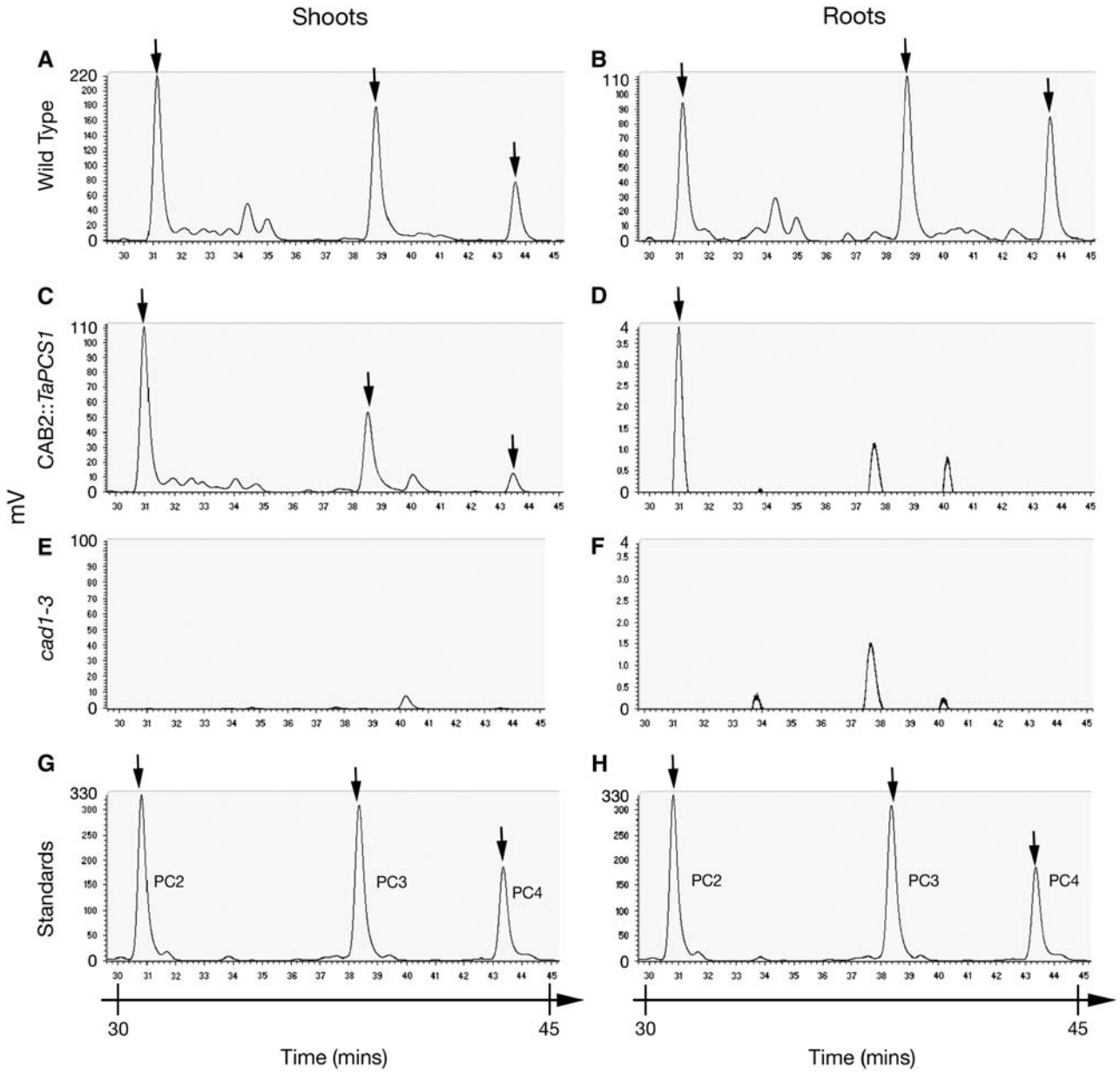

Figure 4. PCs detected in shoot tissue and PC2 in root tissues of CAB2::*TaPCS1*/*cad1-3* plants. Four-week-old plants grown in hydroponic conditions were exposed to 20 μM $CdCl_2$, and PCs in shoot and root tissue extracts were labeled with monobromobimane (shoot tissue extracts left column [A, C, and E]; root tissue extracts right column [B, D, and F]). PC2, PC3, and PC4, indicated by arrows, were detected by fluorescence HPLC and compared to synthesized standards (G and H). Note that the positions of arrows indicating PC peaks were calibrated using PC standard control experiments after every fifth sample. The slight shifts in PC peak retention times from experiment to experiment are due to the normal changes in the properties of the HPLC column over time. PC standards in G and H are identical and shown twice for visual analysis of all traces. E and F, *cad1-3* served as negative controls with a magnified *y* axis, and wild type (Col-0; A and B) served as positive control. $n = 13$ to 14 plants were analyzed for each of the three CAB2::*TaPCS1*/*cad1-3* lines.

by inductively coupled plasma-optical emission spectrometry (ICP-OES). *cad1-3* control plants showed Cd^{2+} overaccumulation in roots and reduced Cd^{2+} accumulation in shoots compared to wild-type plants (Fig. 6). These data are consistent with previous findings under different conditions that showed a contribution of *AtPCS1* (*CAD1*) to Cd^{2+} transfer from roots to shoots (Gong et al., 2003). CAB2::*TaPCS1*/*cad1-3* shoots showed slightly higher accumulation of Cd in shoot tissues in comparison to *cad1-3* (Fig. 6, black bars; C-1, $P = 2.5 \times 10^{-4}$; C-2, $P = 4.6 \times 10^{-5}$; C-3, $P = 3.9 \times 10^{-6}$; all compared to *cad1-3*). However, the overall effect of Cd distribution from shoot-specific expression of *TaPCS1* in *cad1-3* was relatively minor compared to *cad1-3*.

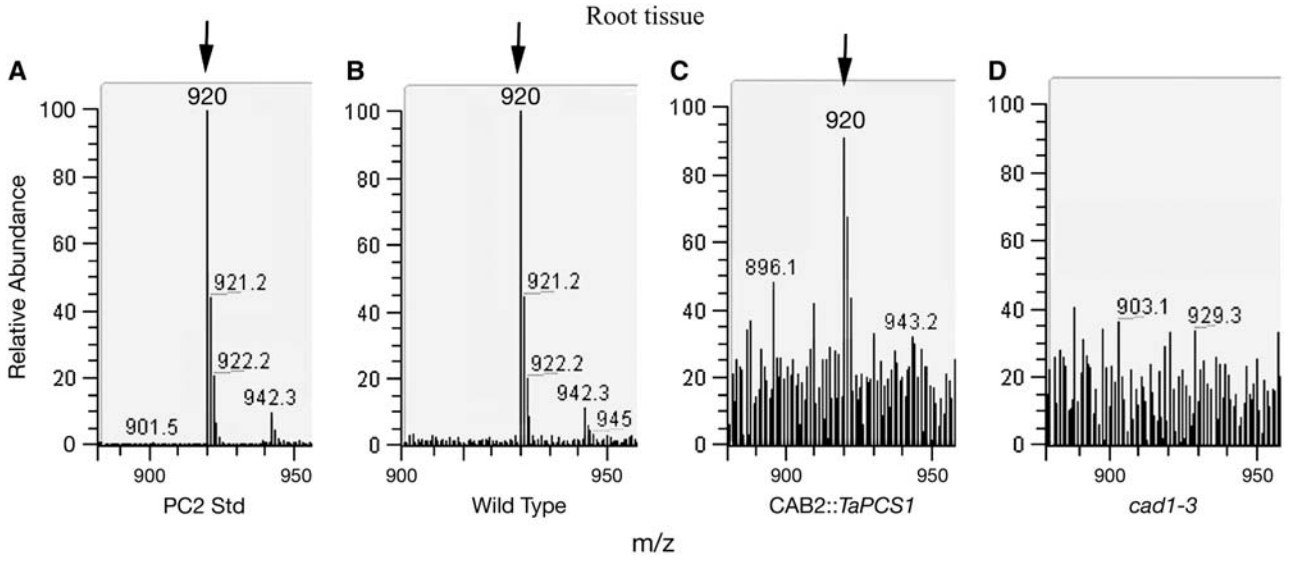

Figure 5. MS run concurrently with fluorescence HPLC confirms HPLC peaks as PCs in root tissues. A to D, Data from root samples. PC2 conjugated with two monobromobimane molecules showed a value of 920 *m/z* (*z* = ion charge) as indicated by arrows. A, Synthesized PC2 standard. B, Root sample from wild type (Col-0). C, Root sample from CAB2::*TaPCS1/cad1-3*. D, Root sample from *cad1-3*.

In CAB2::*TaPCS1/cad1-3* roots, higher concentrations of Cd^{2+} were observed in comparison to wild-type roots (Fig. 6; $n = 9$ plants per line; C-1, $P = 1.1 \times 10^{-4}$; C-2, $P = 2.1 \times 10^{-4}$; C = 3, $P = 4.1 \times 10^{-3}$). Lower concentrations of PCs were detected in root and shoot tissue of the CAB2::*TaPCS1/cad1-3* (Fig. 4, C and D) in comparison to wild type. The lower PC concentrations found in the roots of CAB2::*TaPCS1/cad1-3* compared to wild-type plants (Fig. 4, B and D) correlates with the dramatically lower concentration of Cd being translocated into shoot tissues of CAB2::*TaPCS1/cad1-3* in comparison to wild type (Fig. 6). Thus, CAB2::*TaPCS1/cad1-3* and *cad1-3* plants showed similar Cd accumulation patterns in both shoots and roots (Fig. 6), which may be attributable to the absence of PCs in roots during the initial Cd^{2+} exposure period of CAB2::*TaPCS1/cad1-3* roots (see "Discussion").

Improved Grafting of Mature Arabidopsis Plants

In this study, grafting experiments were pursued in mature plants to analyze long-distance transport of PCs in whole plants. Grafting techniques were originally developed for Arabidopsis seedlings (Turnbull et al., 2002). A previously developed successful grafting procedure for mature Arabidopsis plants showed a success rate of approximately 11% ($n = 13$ of 120 plants; Ayre and Turgeon, 2004). Modifications to the published grafting techniques in mature plants in this study led to an initial 84% ($n = 22$ of 25 plants) success rate after 10 d of growth. The high grafting success rate can be attributed to several modifications, including the growth of plants on hydroponic media in Magenta boxes rather than soil, which allowed a more sterile and humid environment (see "Materials and Methods"). In addition, this method does not require the bending of a steel pin for graft stabilization (Ayre and Turgeon, 2004), because the dense hydroponic sponge in which the plants are grown has the ability to securely hold the pin and grafted tissues together in comparison to soil-grown plants (Fig. 7, A and B; Supplemental Fig. 2). The use of a transverse cut with precision microscissors (Turnbull et al., 2002) also simplified the grafting technique and increased the success rate. The development of grafting in mature plants required that grafts show the ability to survive 10 d postgrafting and to initiate new organ development (Ayre and Turgeon, 2004). Supplemental Figure 2 depicts grafts between wild-type

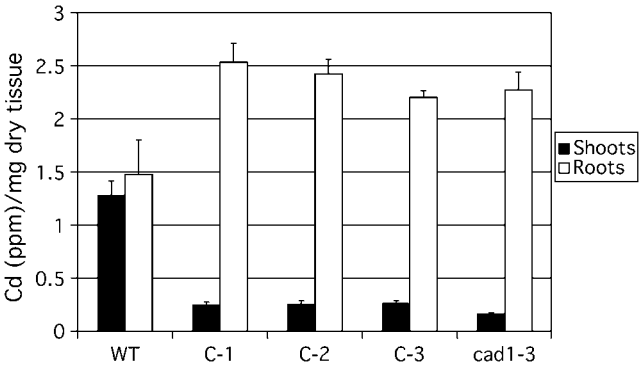

Figure 6. Cd overaccumulation in roots and reduced accumulation in shoots of CAB2::*TaPCS1/cad1-3* and *cad1-3* plants. Wild type (WT; Col-0), three independent lines of CAB2::*TaPCS1/cad1-3* (C-1, C-2, C-3), and *cad1-3* were grown under hydroponic conditions and exposed to 20 μM $CdCl_2$ for 4 d. Cd^{2+} accumulation in shoot and root tissues was determined by ICP-OES. Data show mean values ± SEM; $n = 9$ plants per line.

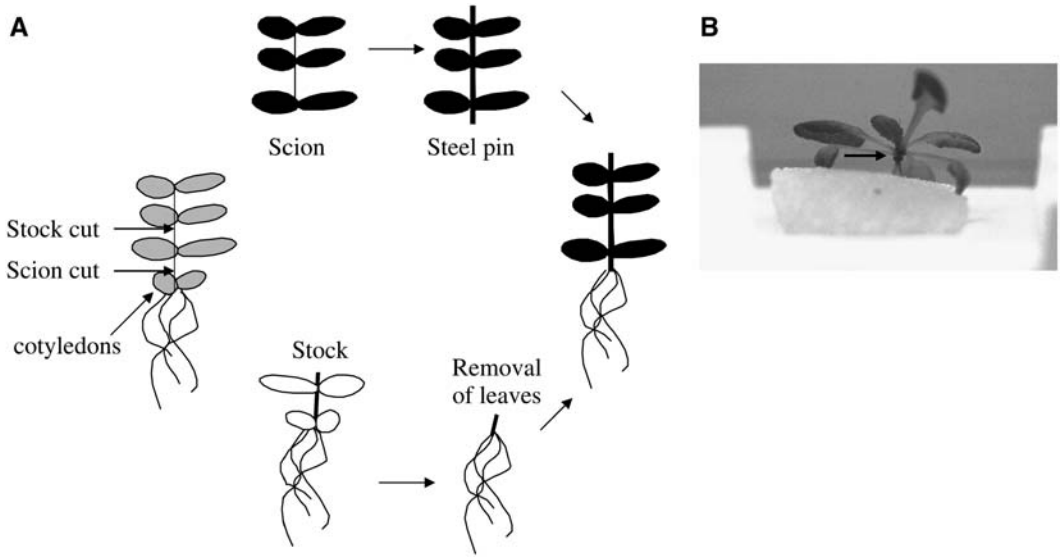

Figure 7. Grafting of mature Arabidopsis plants. A, Diagram of grafting technique. The top portion illustrates preparation of a portion of the graft, labeled as scion, not containing the root system in which a horizontal transverse cut was made in the stem directly above the cotyledons (see "Materials and Methods"). The bottom portion illustrates preparation of the stock, the portion of the graft containing the root system in which a transverse cut was made in the stem directly above the first set of rosette leaves and the subsequent removal of the cotyledons and lowest rosette leaves. Scion and stock were secured together with a steel pin. B, Grafted mature Arabidopsis plant at day 1. Arrow points to grafting junction between the shoot and root tissue. Graft junction did not come into direct contact with hydroponic sponge (bottom). See "Materials and Methods" for details.

shoots and *cad1-3 atpcs2-1* roots 10 d postgrafting, showing new organ development.

Grafts Demonstrate Shoot-to-Root PC Transfer

The CAB2::*TaPCS1*/*cad1-3* results presented above provide evidence that non-native *CAB2* promoter-driven expression of the wheat *TaPCS1* cDNA enables long-distance shoot-to-root transport of PCs. However, the question as to whether Arabidopsis PCSs expressed under their native promoters enable shoot-to-root PC transport remains unknown. To directly analyze this question and to further investigate the deduced shoot-to-root transport, grafting experiments were performed with a double mutant in both Arabidopsis *AtPCS* genes. A T-DNA insertion mutant in the *AtPCS2* gene was isolated from the Wisconsin T-DNA population (Krysan et al., 1999) and PCR screening of an F2 population led to the isolation of a homozygous T-DNA insertion in the *AtPCS2* gene (*atpcs2-1*). *atpcs2-1* contains a T-DNA insertion in the sixth intron of the *AtPCS2* gene. RT-PCR analysis was performed, which showed impaired mature mRNA transcript in *atpcs2-1* (Supplemental Fig. 1).

Experiments consisting of shoot growth and root growth response analyses to Cd, Cd accumulation, and PC quantification suggested that *atpcs2-1* had no easily discernible phenotype in comparison to its wild-type ecotype (Wassilewskija [Ws]) under the imposed conditions (Chen, 2005). These data are consistent with studies indicating a putative minor or unknown function of *AtPCS2* (Cazalé and Clemens, 2001; Lee and Kang, 2005). As expected, the *cad1-3 atpcs2-1* double-mutant plants showed similar phenotypes to *cad1-3* plants and produced no detectable PCs when analyzed with fluorescent HPLC-MS (Supplemental Fig. 3, C and D; $n = 13$ of 13 plants). To directly test the ability of natively expressed *AtPCS* genes to mediate long-distance PC transport in the shoot-to-root direction, 4-week-old shoot (scion) tissues from wild-type plants (Ws × Columbia [Col-0] F2 individuals) were grafted to 4-week-old *cad1-3 atpcs2-1* double-mutant root (stock) tissues. The grafts were then exposed to Cd for a period of 72 h at 4 d after grafting. Positive control grafts between wild-type shoots and wild-type roots and negative control grafts between *cad1-3 atpcs2-1* shoots and *cad1-3 atpcs2-1* roots were also performed.

Extracts from grafted plants containing wild-type shoot and *cad1-3 atpcs2-1* root tissue were labeled with monobromobimane and were analyzed by fluorescence HPLC-MS. Interestingly, PC2, PC3, and PC4 were detected in the roots of *cad1-3 atpcs2-1* grafted to wild-type shoots, indicating transport of PCs in a shoot-to-root direction (Fig. 8D; $n = 12$ of 20 root samples). Wild-type shoots (Ws × Col-0 F2 individuals) were grafted onto wild-type roots (Ws × Col-0 F2 individuals) to serve as a positive control and showed PCs in roots and shoots (Fig. 8, A and B; $n = 7$ of 12 for PCs in roots; $n = 10$ of 12 plants for PCs in shoots). *cad1-3 atpcs2-1* shoots were grafted onto *cad1-3 atpcs2-1* roots to serve as a negative control and showed no PCs (Fig. 8, E and F; $n = 9$ of nine plants).

We identified and confirmed the fluorescent HPLC peaks using directly coupled MS (liquid

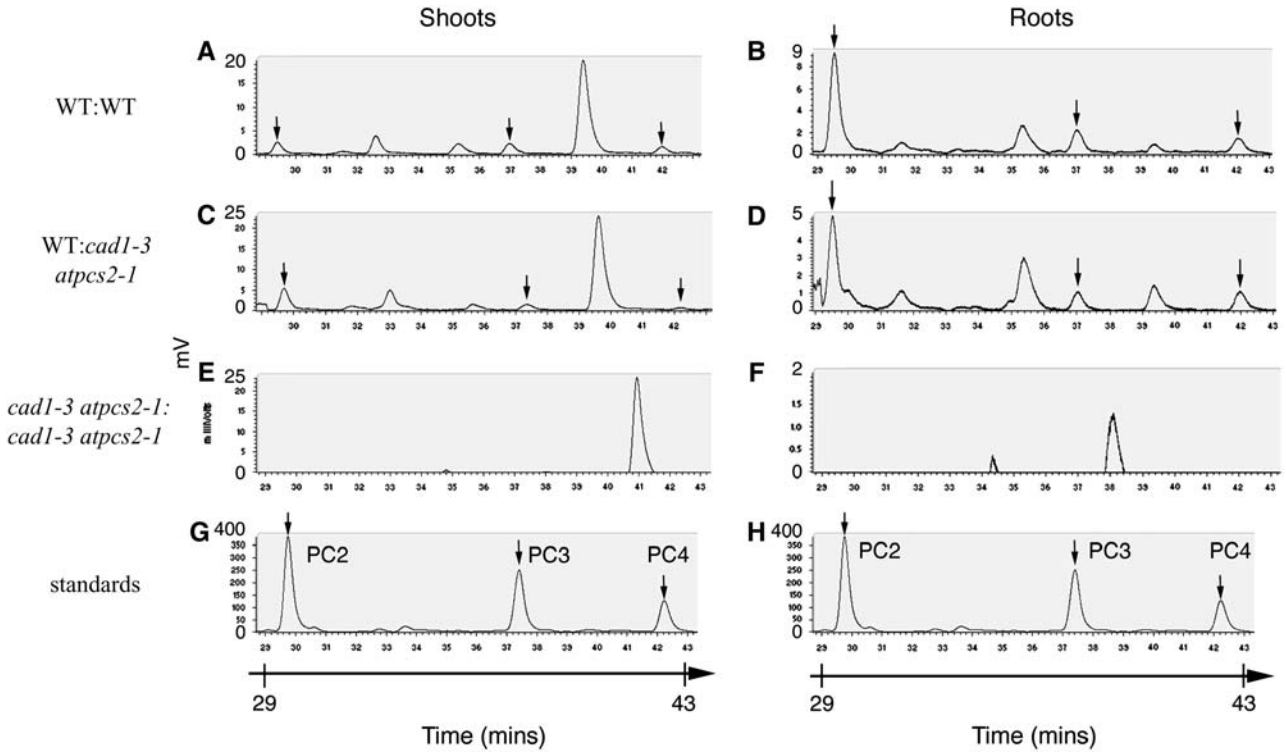

Figure 8. PCs are transported from shoots to roots in grafts between wild-type shoots and *cad1-3 atpcs2-1* double-mutant roots. Plant were grown under hydroponic conditions and tissues extracted for PC analyses prepared 7 d postgrafting, which included a 3-d exposure to 20 μM $CdCl_2$ (shoot tissue extracts left column [A, C, E, and G]; root tissue extracts right column [B, D, F, and H]). PCs were labeled with monobromobimane and detected by fluorescence HPLC. A and B, Grafts between wild-type (Ws × Col-0 F2) shoots and roots (n = 7). C and D, Grafts between wild-type (Ws × Col-0 F2 individuals) shoots and *cad1-3 atpcs2-1* roots (n = 12). Note that the y axes are not identical and amplified in D and F. E and F, Grafts between *cad1-3 atpcs2-1* shoots and roots (n = 9). G and H, Synthesized PC standards. Note that the positions of arrows indicating PC peaks were calibrated using PC standard control experiments after every fifth sample. The slight shifts in PC peak retention times from experiment to experiment are due to the normal changes in the properties of the HPLC column over time. G and H are identical traces and shown twice to facilitate visualization above fluorescence HPLC traces. y axes represent millivolts (mV), and x axes represent retention time (min).

chromatography-MS). The mass spectrometer analyses of the PC2 standard labeled with two monobromobimane molecules showed the predicted mass of the +1 ion at 920 *m/z* (Fig. 9G); PC3 standard labeled with three monobromobimane molecules showed the predicted mass of the +2 ion at 672 *m/z* (Fig. 9H); and the PC4 standard labeled with four monobromobimane molecules showed the predicted mass of the +2 ion at 883 *m/z* (Fig. 9I). In plants with wild-type shoots grafted to *cad1-3 atpcs2-1* roots, directly coupled MS showed peaks and masses corresponding to PC2, PC3, and PC4 in both shoots (data not shown) and roots (Fig. 9, A–C; n = 12 of 20 root samples). Positive control grafts between wild-type (Ws × Col-0) shoots and wild-type (Ws × Col-0) roots showed peaks with identical masses corresponding to these PCs in coupled MS (data not shown), whereas negative control grafts between *cad1-3 atpcs2-1* shoots and *cad1-3 atpcs2-1* roots showed no detectable PCs (Fig. 9, D–F; n = 9 of nine plants). The presence of PCs in *cad1-3 atpcs2-1* root tissues that had been grafted to wild-type shoots (Fig. 9, A–C), which were clearly absent in the *cad1-3 atpcs2-1* mutant (Fig. 9, D–E), demonstrates that PCs are natively transferred in a shoot-to-root direction in Arabidopsis.

DISCUSSION

The role of PCs in mediating heavy-metal detoxification in plants and fungi is well established (Kondo et al., 1984; Grill et al., 1985; Grill, 1987; Ortiz et al., 1992, 1995; Howden et al., 1995; Ha et al., 1999; Cobbett, 2000). A recent study has shown that PCs have the ability to travel in a root-to-shoot direction when the wheat TaPCS1 protein was expressed under the control of the ADH promoter in Arabidopsis roots of PC-deficient plants (Gong et al., 2003). Controls show enhanced Cd^{2+} accumulation in *cad1*-3 roots, which was reduced by TaPCS1 expression in *cad1-3* roots (Gong et al., 2003). This study focused on two main questions. (1) Can PCs be transferred in the opposite direction from shoots to roots in Arabidopsis? (2) Is native promoter expression of Arabidopsis *PCS* genes in shoots sufficient for shoot-to-root transport of PCs to occur, as analyzed in grafting experiments,

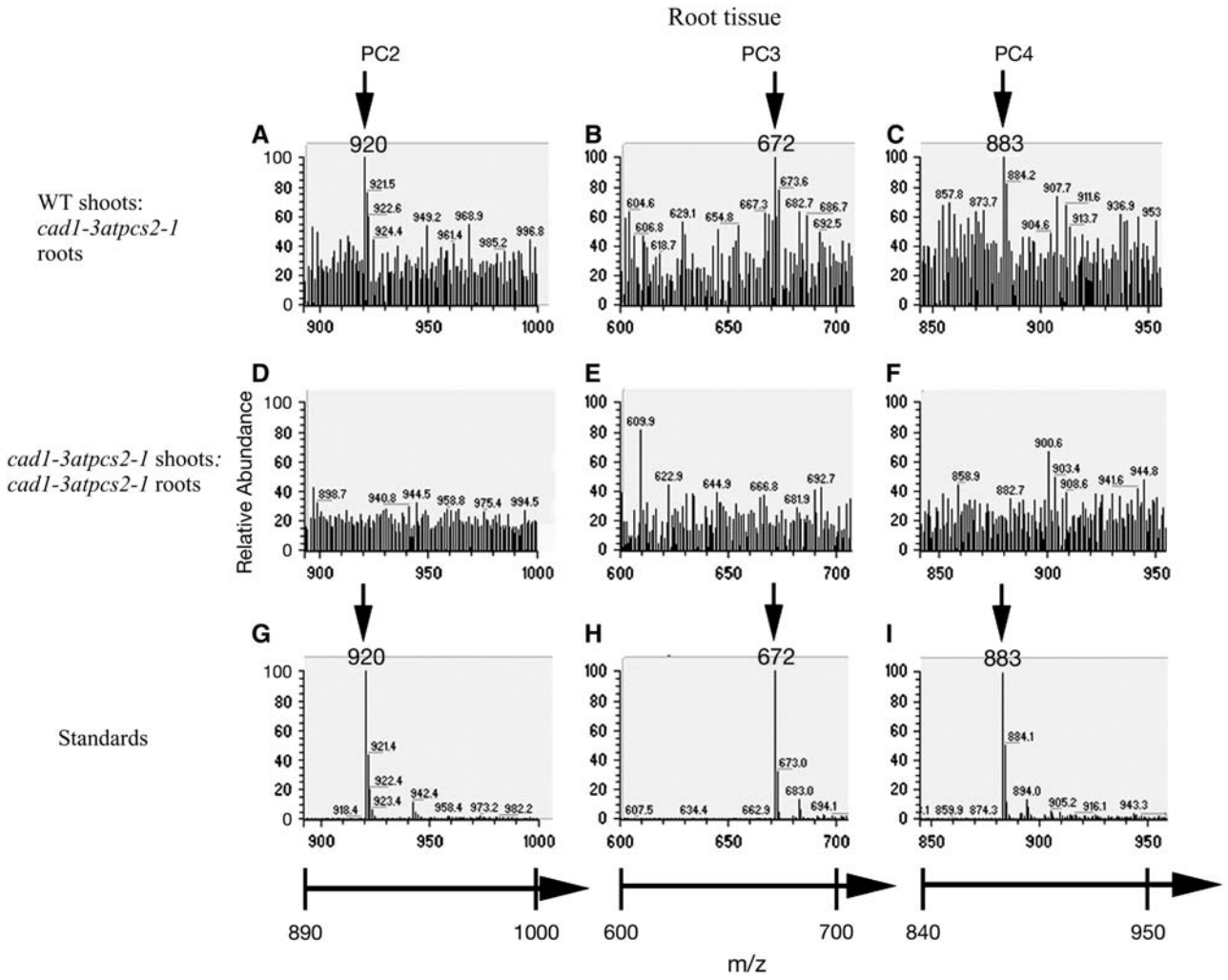

Figure 9. MS run concurrently with fluorescence HPLC confirms PCs in root samples. A to F, Data from root samples. A, B, and C, Raw MS data from root samples from plants with wild-type shoots grafted to *cad1-3 atpcs2-1* roots. D to F, Data from root samples from plants with *cad1-3 atpcs2-1* shoots grafted to *cad1-3 atpcs2-1* roots. G to I, PC standards. PC2 at the +1 ion state was 920 *m/z* (*z* = ion charge), PC3 at the +2 ion state was 672 *m/z*, and PC4 at the +2 ion state was 883 *m/z* as indicated by arrows. *y* axis represents relative abundance percentage and *x* axis represents mass per ion charge (*m/z*) as labeled.

or does the native shoot-to-root response differ from the non-native CAB2 promoter-directed *TaPCS1* expression? We have found that PC2 [$(\gamma\text{-EC})_2$G] can travel in a shoot-to-root direction using non-native CAB2::*TaPCS1* expression (Figs. 4 and 5). Although PC2, PC3, and PC4 were detected in the shoot tissues of CAB2::*TaPCS1*/*cad1-3* (Fig. 4C), the presence of only PC2 in the root tissue of CAB2::*TaPCS1*/*cad1-3* may be due to the expression of *TaPCS1* in CAB2-expressing photosynthetic cells and/or the use of a wheat *TaPCS1* gene instead of the native Arabidopsis gene *AtPCS1*. The smaller M_r of PC2, in comparison to PC3 and PC4, may have allowed PC2 to move from CAB2-expressing cells more easily to vascular tissues, which would be in line with the finding that PCs can undergo long-distance transport. In addition, root tissues of CAB2::*TaPCS1*/*cad1-3* plants overaccumulate Cd at levels roughly similar to *cad1-3* in comparison to wild-type plants (Fig. 6). To analyze whether native PC shoot-to-root transport occurs, we adapted and further developed a grafting method in mature plants resulting in a substantial increase in the percentage of viable grafted plants. To further unequivocally test whether PCs can be transported from shoots to roots, we pursued grafting experiments using *atpcs1* (*cad1-3*) *atpcs2-1* double-mutant roots. Grafting experiments demonstrated shoot-to-root transfer of the PCs $(\gamma\text{-EC})_2$G (PC_2), $(\gamma\text{-EC})_3$G (PC_3), and $(\gamma\text{-EC})_4$G (PC_4) with natively expressed *AtPCS* genes in shoots.

AtPCS1 Aids in Cd Translocation

Previous research and this study showed that the PC-deficient mutant *cad1-3* (*AtPCS1*; Howden et al., 1995; Ha et al., 1999) shows increased accumulation of Cd^{2+} in roots compared to wild type (Fig. 6; Gong et al.,

2003). In this study, CAB2::*TaPCS1/cad1-3* lines also showed Cd^{2+} overaccumulation in roots compared to wild-type controls, even though CAB2::*TaPCS1/cad1-3* plants contained PC2, PC3, and PC4 in shoots (Fig. 4C) and transferred PC2 to roots (Fig. 4D).

Heavy metals induce PC synthesis in plants (Grill, 1987; Maitani et al., 1996; Vatamaniuk et al., 2000). The overaccumulation of Cd^{2+} in roots of CAB2::*TaPCS1/cad1-3* plants (Fig. 6) may be due to lack of PC production directly in roots during initial Cd exposure before the transport of PC2 from the shoot tissues of CAB2::*TaPCS1/cad1-3* occurred.

Roots are the first tissues to experience Cd toxicity. In roots, Cd has been observed to damage nucleoli, alter the synthesis of RNA, inhibit ribonuclease activity (Shah and Dubey, 1995), and reduce absorption of nitrate by inhibiting nitrate reductase activity in shoots (Hernández et al., 1997). CAB2::*TaPCS1/cad1-3* seedlings grown in the presence of Cd display less root growth and are therefore more sensitive to Cd in comparison to wild-type seedlings (Fig. 3). However, the roots of two of the three independent lines of CAB2:*TaPCS1/cad1-3* (C-2 and C-3) were approximately 1.4 and 1.9 times longer in comparison to *cad1-3*, respectively (Fig. 3; C-2, $P = 3.7 \times 10^{-4}$; C-3, $P = 1.1 \times 10^{-6}$ compared to *cad1-3*). This may be due to the presence of low levels of transported PC2 to root tissues from shoot tissues (Figs. 4 and 5). Thus, *cad1-3* root tissues have a reduced ability to effectively transport Cd to shoots.

In contrast to CAB2::*TaPCS1/cad1-3* and *cad1-3* plants, root-specific *TaPCS1* expression, or 35S::*TaPCS1/cad1-3*, and wild-type plants express an active version of PCS in root tissues at the time of initial Cd exposure, and concomitantly show reduced Cd^{2+} accumulation in roots and enhanced Cd^{2+} accumulation in shoots (Fig. 6; Gong et al., 2003). In addition, the low levels of PC2 observed in the root tissues of CAB2::*TaPCS1/cad1-3* (Fig. 4) appear not to be sufficient to effectively control the root-to-shoot balance of Cd^{2+} transport to shoot tissues. The large differences in Cd^{2+} accumulation in roots and shoots between *cad1-3* (*atpcs1*) and wild-type plants provide direct genetic evidence for a role of *AtPCS1* in Cd^{2+} distribution in Arabidopsis (Fig. 6). As PCs have been biochemically demonstrated to bind to a variety of heavy metals (Kondo et al., 1984; Grill et al., 1985) and the long-distance transport mechanisms of nonessential heavy metals remain relatively unknown, it is possible that one of the physiological roles of transported PCs is to bind and aid in long-distance transport of heavy metals to different tissue types. Arabidopsis overexpressing *TaPCS1* in root tissues was shown to transport higher levels of Cd to shoot tissues, thus suggesting a role of PCs in Cd transport (Gong et al., 2003). Additional PC-independent long-distance heavy-metal transport from roots to shoots also clearly exists (Fig. 6). In monocots, phytosiderophores, which are low-molecular-mass secondary amino acids, have been shown to chelate Fe and aid in the uptake of Fe through transporters such as maize (*Zea mays*) yellow stripe 1 (Curie et al., 2000). Although in dicots, such as Arabidopsis, the strategy for Fe uptake does not involve phytosiderophores and chelation, it is conceivable that for nonessential heavy metals dicots employ a strategy that involves peptide chelators such as PCs.

PCS Overexpression in Wild-Type Backgrounds

Recent reports have analyzed an important question different from this study, namely, whether overexpression of *PCS* cDNAs in wild-type backgrounds (rather than in *cad1-3* mutants) can enhance metal resistance (Gisbert et al., 2003; Lee et al., 2003b; Sauge-Merle et al., 2003; Li et al., 2004; Pomponi et al., 2005). These studies address the questions of (1) whether wild-type PCS activities are already saturated in the wild-type background of the analyzed plant species and (2) whether single *PCS* gene overexpression can further enhance traits, including heavy-metal resistance. These studies have shown interesting and differential results, depending on the analyzed plant species and applied toxic metals. For example, overexpression of *AtPCS1* in Arabidopsis wild-type plants caused sensitivity to Cd^{2+} in two studies (Lee et al., 2003b; Li et al., 2004). But, interestingly, the same plants in one of these studies showed a dramatically enhanced resistance to arsenic (As; Li et al., 2004).

Different plant species show different responses to single-gene *PCS* overexpression (Gisbert et al., 2003; Pomponi et al., 2005). It appears that multigene approaches may be needed to dramatically enhance Cd^{2+} accumulation in transgenic wild-type plants because intermediates, such as Cys and glutathione, can limit the production of PCs (Clemens et al., 2002). In support of a multigenic systems approach to enhancing heavy-metal metabolism, in *Escherichia coli* cooverexpression of three proteins in the PC biosynthesis pathway—PCS, Ser acetyltransferase, and γ-glutamyl-Cys synthase—resulted in the overaccumulation of PCs and Cd in bacterial cells and showed that single-gene overexpression in the PC biosynthetic pathway had limited effects (Wawrzynska et al., 2005). This study and a previous study (Gong et al., 2003) did not analyze TaPCS1 overexpression in wild-type backgrounds, but focused on characterizing the genetic function of *AtPCS1* in root-to-shoot Cd transfer and on long-distance PC transport by characterizing effects of transgenic *TaPCS1* expression and grafting relative to PC-deficient mutant lines.

Shoot-to-Root Transport of PCs and Possible Transport Mechanisms

To directly analyze whether PCs have the ability to undergo long-distance shoot-to-root transport, we generated and analyzed double loss-of-function mutant plants in the two Arabidopsis PCS genes, *AtPCS1* (*CAD1*; Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al.,

1999) and *AtPCS2* (Cazalé and Clemens, 2001; Lee and Kang, 2005). As expected, the *cad1-3 atpcs2-1* double-mutant plants showed no detectable PCs in all plant samples analyzed, providing a genetically robust PCS-free mutant background for long-distance transport analyses (Supplemental Fig. 3, C and D). Grafting of *cad1-3 atpcs2-1* double-mutant roots to wild-type shoots clearly showed transfer of PC2, PC3, and PC4 from shoot to roots (Figs. 8, C and D, and 9, D and F). However, in general, lower levels of PCs were found in grafted plants (Fig. 8, A–D) compared to nongrafted plants (Fig. 4, A and B; Supplemental Fig. 3, E and F). These differences in PC levels may be in part due to different growth conditions that were required for grafts and, in particular, due to the grafting procedure and the relative intactness of the vascular phloem and xylem pathways at the graft junction because the positive control wild-type shoots grafted to wild-type roots (Fig. 8, C and D) also exhibited lower PC levels compared to nongrafted wild-type plants (Fig. 4, A and B; Supplemental Fig. 3, E and F).

The mechanisms by which PCs undergo long-distance transport in plants remain unknown. Previous research in *Brassica juncea* showed that Cd was mainly found in xylem sap extracts as complexes with nitrogen-containing compounds that are not reminiscent of PCs (Salt et al., 1995). We show that PCs undergo long-distance transport in a shoot-to-root direction, which cannot be mediated by the xylem and implicates phloem transport as a long-distance transport pathway. Phloem transport of PCs would likely require several types of PC transporters for phloem loading and unloading, which remain unidentified. PCs are small peptides, and completion of the Arabidopsis genome sequence has revealed a number of potential peptide transporter families for PCs. Peptide transporters have been placed into two groups based on their energy source: (1) the oligopeptide transporter (OPT) and peptide transporter (PTR) families, which use proton-motive force; and (2) ABC-type transporters, which use ATP hydrolysis as an energy source.

A member of an OPT proton-motive force transporter family was shown to translocate tetra- and pentapeptide substrates when expressed in yeast (Koh et al., 2002). Expression of AtOPT4 in *S. pombe* mediated the uptake of Lys-Leu-Gly-[^{3}H]Leu (Koh et al., 2002). In addition, the *Saccharomyces cerevisiae* oligopeptide transporter, ScOPT1, was recently shown to display a higher affinity for PC2 in comparison to reduced or oxidized glutathione and oligopeptides, including the tetrapeptide GGFL (Osawa et al., 2006).

Members of a different proton-motive force transporter family, PTR, which is also known as the proton oligopeptide transporter family, have shown the ability to transport small peptides. For example, AtPTR1 recognizes a broad spectrum of di- and tripeptides, is localized to the plasma membrane, and is expressed in vascular tissues throughout Arabidopsis plants, suggesting a role in long-distance peptide transport (Dietrich et al., 2004).

The ABC-type transporter superfamily is represented by a large gene family in Arabidopsis, with approximately 130 members (Sanchez-Fernandez et al., 2001). ABC-type transporters transport substrates ranging from small ions to large macromolecules (Sanchez-Fernandez et al., 2001). In *S. pombe*, the heavy-metal tolerance factor 1 (*SpHMT-1*) gene encodes an ABC-type transporter and has been shown to mediate uptake of PC-Cd^{2+} complexes into *S. pombe* vacuoles (Ortiz et al., 1995). Recently, a HMT1 homolog in *Caenorhabditis elegans* was identified (CeHMT-1) and shown to be required for Cd tolerance (Vatamaniuk et al., 2005). Arabidopsis lacks a direct HMT1 homolog. To date, the identification of an Arabidopsis gene encoding the vacuolar transporter for PCs has not been reported. A magnesium (Mg) ATP-energized transport pathway for PCs and PC-Cd^{2+} complexes, analogous to the ABC-type transporter, has been characterized in vacuolar membrane vesicles isolated from oat (*Avena sativa*) roots (Salt and Rauser, 1995). Recently, the Arabidopsis ABC-type transporter AtPDR12 was shown to contribute to Pb resistance by serving to exclude Pb and/or Pb-containing compounds (Lee et al., 2005).

PCs may also serve as signaling molecules to communicate heavy-metal content between different tissue types. However, because different heavy metals have been shown to differentially activate PCS, it is possible that PCs would serve as signaling molecules for the stronger inducers, such as Cd, silver (Ag), bismuth (Bi), and Pb, rather than for weaker PCS inducers, such as zinc (Zn), Cu, Hg, and gold (Au; Grill et al., 1989). PCs transported from shoots to roots (Figs. 4, 5, 8, and 9), for example, might serve as a signal for roots to down-regulate nutrient transporters in roots to prevent further uptake of heavy metals. Cd competes with the physiological transport of nutrients such as calcium (Ca), Fe, Mg, manganese (Mn), Cu, and Zn, as Cd^{2+} is transported by transmembrane nutrient transporters in plants (Clarkson and Lüttge, 1989; Rivetta et al., 1997; Clemens et al., 1998, 2002; Grotz et al., 1998; Curie et al., 2000; Pence et al., 2000; Picard et al., 2000; Thomine et al., 2000, 2003; Connolly et al., 2002; Papoyan and Kochian, 2004).

In conclusion, long-distance shoot-to-root transport of PC peptides is revealed in this study in non-native promoter-driven CAB2::*TaPCS1/cad1-3* and native promoter-driven grafts between wild-type shoots and *cad1-3 atpcs2-1* roots. Shoot-specific targeting of wheat *TaPCS1* in *cad1-3* restores Cd and As tolerance of leaves. In addition, improved grafting techniques were developed for mature Arabidopsis plants to analyze long-distance transport of PCs. Further characterization of shoot-to-root long-distance PC transport mechanisms and transport regulatory pathways for PCs will elucidate the functions and pathways of PCs in heavy-metal processing in plant biology.

MATERIALS AND METHODS

DNA Constructs and Plant Transformation

The CAB2::*TaPCS1* plasmid was constructed by modifying the ADH::*TaPCS1*::c-myc/pBI121 binary expression vector (Gong et al., 2003) by digestion with the two restriction enzymes *Bam*HI and *Hin*dIII and subcloning in the CAB2 promoter to replace the ADH promoter. The DNA sequence for the promoter sequence of CAB2 was constructed by PCR recovery from a plasmid containing the CAB2 promoter. All PCR products were confirmed by sequencing (Retrogen). The CAB2::*TaPCS1* construct was introduced into the PC-deficient Arabidopsis (*Arabidopsis thaliana*) mutant *cad1-3* by direct *Agrobacterium tumefaciens*-mediated transformation using the floral-dip technique (Clough and Bent, 1998).

Plant Material, Growth Conditions, and Metal Stress Treatments

For growth in petri dishes, Arabidopsis seedlings were grown on 25% Murashige and Skoog basal medium (Sigma), 1 mM MES, 1% agar, and the indicated concentrations of heavy metals (Lee et al., 2003a). Seedlings used for shoot growth analyses were grown horizontally under a 16-h-day/8-h-night period and those used for root analyses were grown vertically under 24-h light periods.

CAB2::*TaPCS1*/cad1-3 and grafted plants used for ICP-OES and fluorescence HPLC-MS analysis were grown in 80 mL of hydroponic medium as described (Arteca and Arteca, 2000), with minor modifications. Plants were germinated in petri dishes and grown vertically for the first 4 d and subsequently moved to a horizontal position for an additional 2 d to bend the hypocotyl to aid in successful transplantation onto the hydroponic sponge. Plants were grown on sponges (Jaece Industries; catalog no. L800-B; www.jaese.com) in Magenta boxes (Sigma; catalog no. V 8505) at 24°C under a 16-h-day/8-h-night period and hydroponic media were replaced every 3 to 4 d. After reaching the bolting stage (approximately 4-week-old plants), the hydroponic medium was replaced with 50 mL of the same medium, to which 20 μM $CdCl_2$ were added for a period of 4 d for Cd accumulation analyses, whereas the hydroponic medium containing 20 μM $CdCl_2$ was added for a period of 3 d for induction of PCs.

For analysis of Cd^{2+}-dependent root growth, seeds were sterilized and plated on plates containing 25% Murashige and Skoog medium, 1 mM MES, 1% agar type A (Sigma), cold treated at 4°C for 48 h, and grown vertically under 24-h-light growth room conditions for 5 d. Seedlings were then transferred to 25% Murashige and Skoog medium, 1 mM MES, 1% agar plates containing 20 μM $CdCl_2$ for an additional 72 h of vertical growth. *P* values were calculated using one-tailed, homoscedastic parameters.

Generation and Isolation of an *atpcs2* Insertion Mutant and *cad1-3 atpcs2-1*

An *AtPCS2* T-DNA insertion mutant was isolated from the Wisconsin T-DNA collection by PCR screening (Krysan et al., 1999). A *cad1-3 atpcs2-1* double mutant was generated by crossing *cad1-3* (Col-0 background) with *atpcs2-1* (Ws background), and homozygous *cad1-3 atpcs2-1* lines were identified through PCR genotyping of the F2 population. The isolation of the corresponding wild type for *cad1-3 atpcs2-1* was generated by crossing Ws to Col-0 and generating a Ws × Col-0 F2 population.

Grafting of Mature Arabidopsis Plants

Plants were grown under hydroponic conditions until the six- to eight-rosette leaf stage (approximately 4 weeks old) in 80-mL hydroponic medium (see above) in Magenta boxes under a 16-h-day/8-h-night period at 22°C temperature conditions with the lids ajar to allow for air exchange. The component of the graft containing the root structure (stocks) was prepared for grafting using a horizontal transverse cut through the rosette stem above the first true rosette leaves using precision microscissors (Fine Science Tools; catalog no. 15031-14; www.finescience.com; Fig. 7A). The cotyledon and rosette leaves remaining on the stock were subsequently removed at the base of the petiole using microscissors. Scions, the portion of the graft not containing the root system, were prepared using a horizontal transverse cut through the rosette stem above the cotyledons using precision microscissors (Fig. 7A). Scion and stock were attached using a steel pin (1-mm diameter; Fine Science Tools; catalog no. 26002-10) by impaling both the scion and the stock at the center of the stem to securely attach both tissues together. Then the pin, with the attached scion and stock, was inserted into the hydroponic sponge to secure the graft (Fig. 7A). The graft site, where the stock meets scion, was held approximately 0.25 cm above the hydroponic sponge by the steel pin to avoid adventitious root growth (Fig. 7B). Any grafts observed to have adventitious root growth were immediately discarded. The lids of the Magenta boxes were then closed to ensure high humidity for 7 d. Grafts that produced new leaves and floral organs postgrafting and continued to grow after 7 to 10 d were scored as successful grafts (Supplemental Fig. 2).

Several of the grafts that were scored as successful, by initiating new organ development postgrafting, displayed purple leaves, which were likely due to anthocyanin production and interpreted as indicators of stress (Supplemental Fig. 2). Wild-type shoots of such stressed individuals also showed transfer of PCs to the *cad1-3 atpcs2-1* double-mutant roots. Grafting experiments to analyze long-distance shoot-to-root PC transfer were initially developed using plants 10 d postgrafting, which included a 3-d 20 μM Cd treatment. However, 7 d postgrafting, including a 3-d 20 μM Cd treatment, proved to be a better time period for PC analyses and these conditions were used for the illustrated PC analyses.

Northern Blotting and RT-PCR

Total RNA was extracted from shoots and roots using the TRIzol reagent (Invitrogen). RNA gel blotting, probe labeling, and hybridizations were accomplished using standard protocols recommended by the manufacturers. Northern blots were probed with the *Actin7* (At5g09810) gene as a loading control and with *TaPCS1*. First-strand cDNA was synthesized from DNAseI-digested total RNA using Maloney murine leukemia virus reverse transcriptase (Promega) and PCR performed on a PE GeneAmp 9700 (Perkin-Elmer Applied Biosystems) with 50 PCR cycles using Biolase *Taq*DNA polymerase (DocFrugal).

ICP-OES Analyses

Cd-treated plants grown under hydroponic conditions were first washed and then separated into root and shoot tissues. Shoot tissues were separated immediately below the cotyledons and above the hydroponic sponge. Root tissues were separated below the hydroponic sponge, approximately 3 cm below cotyledons. Shoots were rinsed three times in deionized water. For ICP-OES analyses of root tissue, roots were rinsed in deionized water, washed in 100 mL of 100 mM $CaCl_2$ on an orbital shaker (Bellco Glass) for 5 min at approximately 135 rotations/min (speed setting no. 4), and then washed in 100 mL of deionized water on the orbital shaker (speed setting no. 4) for an additional 3 min. Both shoot and root tissues were dried at 60°C overnight. The dry weight was recorded and then the tissues were digested in 70% trace metal-grade nitric acid (Fisher Scientific) overnight. Samples were then boiled for 30 min to ensure complete digestion and diluted to a final concentration of 5% nitric acid with deionized water (Gong et al., 2004). *P* values were calculated using one-tailed, homoscedastic parameters.

PC Analyses

CAB2::*TaPCS1*/*cad1-3* plants were grown under hydroponic conditions until they bolted (approximately 4 weeks) and then incubated in hydroponic media containing 20 μM $CdCl_2$ for 72 h. Twenty micromolar $CdCl_2$ was added to successfully grafted plants. Plants were rinsed three times in deionized water and root and shoot tissue were separated and lyophilized for 24 h. Shoot and root tissues were separated as described in "Materials and Methods." PCs were derivatized with monobromobimane as described (Fahey and Newton, 1987; Clemens et al., 1999; Sneller et al., 2000). Synthesized standards were used for the identification of PCs $(\gamma\text{-EC})_2G$ (PC_2), $(\gamma\text{-EC})_3G$ (PC_3), $(\gamma\text{-EC})_4G$ (PC_4). The fluorescence HPLC column (Ultrasphere C18 ion-pair HPLC column; Beckmann catalog no. 235335) was directly coupled to the mass spectrometer (LC-MS) by splitting the 1-mL/min flow in which 900 μL/min of the flow went to the fluorescence detector (FL3000 fluorometer; Spectrasystem) and HPLC (Surveyor LCQ Advantage; ThermoFinnigan), and 100 μL/min were electrosprayed in parallel directly into the mass spectrometer (Surveyor LCQ Advantage; ThermoFinnigan). PC standards were run after every fifth sample to monitor the migration and changes in retention time

of PC peaks due to normal alterations in the properties of the HPLC column over time.

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