Fission Yeast HMT1 Lowers Seed Cadmium through Phytochelatin-Dependent Vacuolar Sequestration in Arabidopsis[1][C][W]

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Much of our dietary uptake of heavy metals is through the consumption of plants. A long-sought strategy to reduce chronic exposure to heavy metals is to develop plant varieties with reduced accumulation in edible tissues. Here, we describe that the fission yeast (*Schizosaccharomyces pombe*) phytochelatin (PC)-cadmium (Cd) transporter SpHMT1 produced in Arabidopsis (*Arabidopsis thaliana*) was localized to tonoplast, and enhanced tolerance to and accumulation of Cd^{2+}, copper, arsenic, and zinc. The action of SpHMT1 requires PC substrates, and failed to confer Cd^{2+} tolerance and accumulation when glutathione and PC synthesis was blocked by L-buthionine sulfoximine, or only PC synthesis is blocked in the *cad1-3* mutant, which is deficient in PC synthase. *SpHMT1* expression enhanced vacuolar Cd^{2+} accumulation in wild-type Columbia-0, but not in *cad1-3*, where only approximately 35% of the Cd^{2+} in protoplasts was localized in vacuoles, in contrast to the near 100% found in wild-type vacuoles and approximately 25% in those of *cad2-1* that synthesizes very low amounts of glutathione and PCs. Interestingly, constitutive *SpHMT1* expression delayed root-to-shoot metal transport, and root-targeted expression confirmed that roots can serve as a sink to reduce metal contents in shoots and seeds. These findings suggest that SpHMT1 function requires PCs in Arabidopsis, and it is feasible to promote food safety by engineering plants using *SpHMT1* to decrease metal accumulation in edible tissues.

Nonessential heavy metal(loid)s including cadmium (Cd^{2+}), arsenic (As), and lead (Pb^{2+}) are widespread in the biosphere due largely to industrial pollution. The adverse effects on agriculture and human health are well noted (Mohammed et al., 2011). In China alone, nearly one-fifth of the cultivated land, approximately 20 million hectares, are contaminated with Cd^{2+}, As, and/or Pb^{2+}, leading to approximately 12 million tons of contaminated grains that translate to approximately 20 billion Yuan of economic loss per year (Chen, 1996). In some areas, Cd^{2+} concentration in rice (*Oryza sativa*) was found as high as 1 to 2 mg/kg (Cheng, 2003), far beyond the highest level allowed by China National Standard (GB2762–2005). Heavy metal contamination is also widespread in Eastern Europe and is increasingly recognized in many parts of the developing world (Krämer, 2005). The past two decades have seen much discussion on the prospects of phytoremediation—the use of plants to remediate polluted soils (Cunningham and Ow, 1996; Salt et al., 1998; Singh et al., 2003). Likewise, much interest has been raised on the genetic improvement of crop varieties that could reduce dietary intake of these pollutants, as might be possible if toxic metals accumulate less in edible tissues (Ueno et al., 2010). Toward these aims, a better understanding of heavy metal allocation and detoxification in plants is of great importance.

To date, chelation and subcellular sequestration appear to represent essential metal detoxification mechanisms in yeasts (*Saccharomyces cerevisiae*) and plants (Hall, 2002; Verbruggen et al., 2009). The best-characterized heavy-metal-binding chemicals are small Cys-rich molecules, including metallothioneins, glutathione (GSH), and phytochelatins (PCs; Cobbett and Goldsbrough, 2002; Verbruggen et al., 2009). Metallothioneins are diverse proteins encoded by a superfamily in both animals and plants (Grennan, 2011). PCs are found in plants, algae, some fungi, and surprisingly in *Canenorhabditis elegans* (Grill et al., 1985; Vatamaniuk et al., 2001; Pal and Rai, 2010). PCs are serial oligopeptides with a general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$–11), and are synthesized from GSH by PC synthase (PCS; Grill et al., 1987, 1989; Clemens et al., 1999; Ha et al.,

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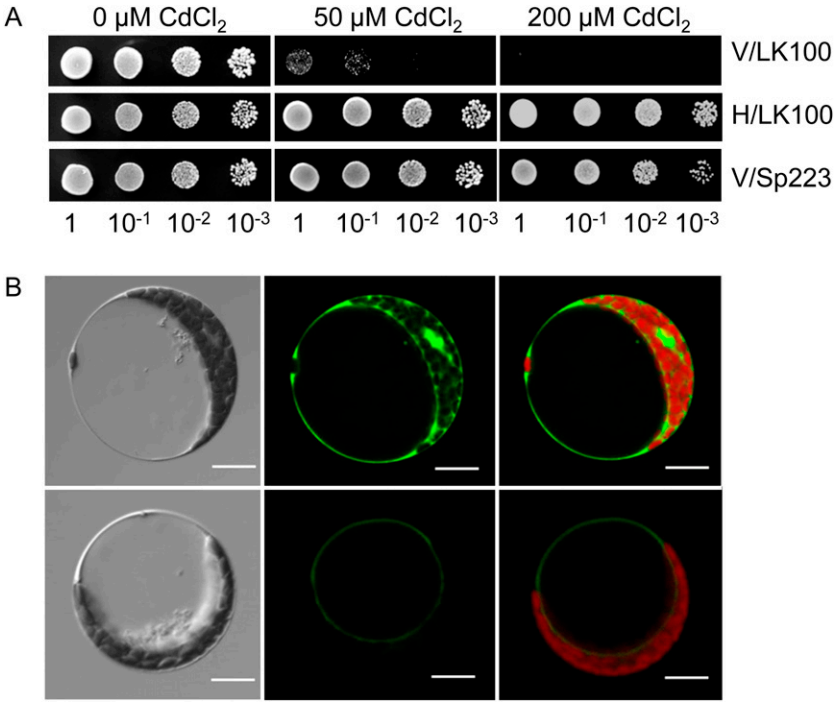

Figure 1. Rescue of Cd^{2+} sensitivity in *hmt1*⁻ mutant LK100 and subcellular localization analysis. A, LK100 transformed with empty vector pART1 (V/LK100) or c-myc-tagged *SpHMT1* (H/LK100). Wild-type strain Sp223 transformed with pART1 (V/Sp223) was used as positive control. B, Subcellular localization of *SpHMT1*. Left, bright field; middle, fluorescence; and right, merged images of Enhanced Yellow Fluorescent Protein (EYFP; top) and EYFP:SpHMT1 (bottom). Red color represents autofluorescence of chloroplasts. Bars = 20 μM.

1999; Vatamaniuk et al., 1999). PCs chelate Cd^{2+} with higher affinity than do GSH molecules (Kneer and Zenk, 1992; Howden et al., 1995a), and both types of complex can be sequestered into vacuoles (Ortiz et al., 1992; Li et al., 1996). In fission yeast (*Schziosaccharomyces pombe*), the inability of a Cd^{2+}-sensitive mutant to accumulate vacuolar PC-Cd led to the discovery of HMT1, a half-molecule ATP-binding cassette (ABC) transporter that recognizes PCs and PC-Cd (Ortiz et al., 1992, 1995). Likewise, a Cd^{2+}-sensitive mutant of yeast led to YCF1, a full-molecule ABC transporter that mediates vacuolar sequestration of a GS2-Cd complex [bis(glutathionato) Cd] (Li et al., 1996, 1997).

Subsequent studies have suggested that SpHMT1 might also transport GS2-Cd conjugates when overexpressed in fission yeast mutants that lack PCs for substrate (Prévéral et al., 2009). Whether GS2-Cd or glutathione S-conjugate (GS-X) are physiologically relevant substrates remains to be determined, as during Cd^{2+} stress, much more of the Cd ions are bound to PCs rather than to GSH, and GS2-Cd as well as GS-X transport activities are detected in vacuoles even in the absence of HMT1 (Ortiz et al., 1995). Nevertheless, this fact does raise the possibility that in fission yeast and *C. elegans*, HMT1 might have evolved from an ancient but more universal role in GS-X transport.

Despite the subsequent identification of CeHMT1, a *C. elegans* PC transporter, and more recently a *Drosophila melanogaster* HMT1 homolog that likely transports GSH conjugates, a HMT1 ortholog has not been identified in higher plants (Vatamaniuk et al., 2005; Sooksa-Nguan et al., 2009). The likelihood that higher plants have evolved a different PC transport system has received recent support from the discovery of two ABCC subfamily members of ABC transporters in Arabidopsis (*Arabidopsis thaliana*), for which vacuolar PC-As(III) transport was shown (Song et al., 2010). A most recent study further reported substantial decrease of vacuolar Cd^{2+} in the *atabcc1 atabcc2* mutant, indicating that AtABCC1 and AtABCC2 play a significant role in vacuolar Cd^{2+} sequestration. However, the relative contribution by PCs and GSH to this process remains to be determined, as GSH might possibly be a substrate of AtABCC1 and AtABCC2 under certain conditions (Park et al., 2012).

In this study, we found that PCs play a primary role in vacuolar Cd^{2+} sequestration in Arabidopsis, and ectopic expression of *SpHMT1* enhanced this process. SpHMT1 function requires PCs, as its effects are not seen in the PC-deficient mutant *cad1-3*. We further demonstrate the possibility of engineering plants using *SpHMT1* to improve food safety by reducing heavy metal translocation into plant edible parts.

RESULTS

Expression of *SpHMT1* in Arabidopsis

A *SpHMT1* expression construct pART1-SpHMT1 was generated containing the native 5′-untranslated region and a c-myc tag fused to the 3′ terminus of the *SpHMT1* gene. The construct complements the Cd^{2+}-sensitive phenotype in the *hmt1*⁻ strain LK100, showing that the c-myc tag did not substantially affect SpHMT1 activity (Fig. 1A; Supplemental Fig. S1). The c-myc-tagged *SpHMT1* fragment was placed under the control of the cauliflower mosaic virus 35S promoter and transferred to an *Agrobacterium tume-*

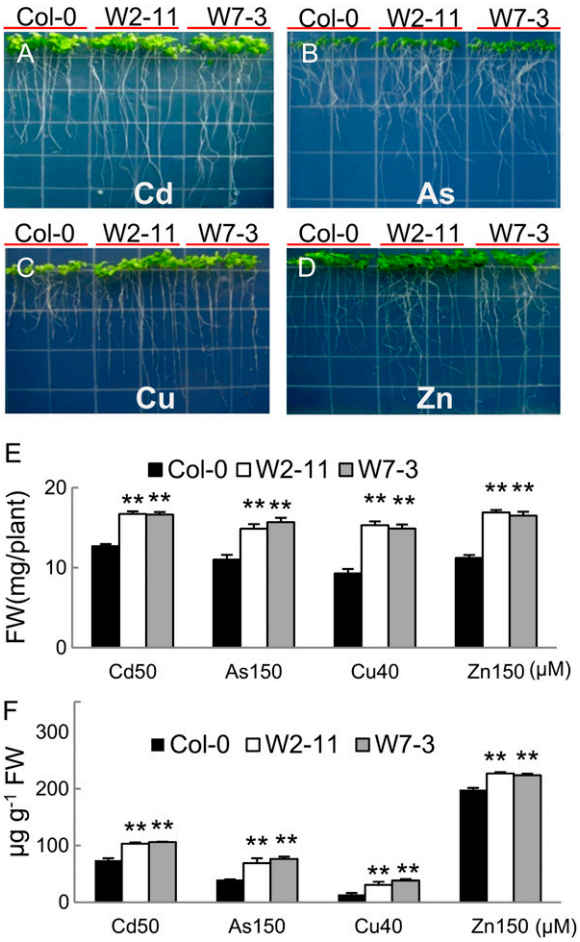

Figure 2. Enhanced metal tolerance and accumulation in *SpHMT1* plants. Surface sterile seeds plated onto one-fourth Murashige and Skoog medium supplemented with 50 μM $CdCl_2$ (A), 150 μM KH_2AsO_4 (B), 40 μM $CuSO_4$ (C), and 150 μM $ZnSO_4$ (D), and grown vertically for 4 weeks. W2-11 and W7-3 are independent lines transgenic for pBI121-35S/*SpHMT1*:*cmyc* in wild-type Col-0 background. Fresh weight (FW; E) and metal content (F) were determined in plants grown under conditions in A to D. Values are mean ± SD, $n = 12$ for E, or $n = 4$ for F, each containing greater than five plants. Asterisks indicate significant difference between wild type and the transgenic lines at $P < 0.05$ (*) and 0.01 (**) by *t* tests.

faciens vector to generate pBI121-35S/*SpHMT1*:*cmyc* for transformation of Arabidopsis. Reverse transcription-PCR analyses of transgenic plants derived from wild-type Columbia-0 (Col-0) and the PC-deficient mutant *cad1-3* showed that *SpHMT1* was expressed in both genetic backgrounds (Supplemental Fig. S2, A and B). Further subcellular localization analyses using Arabidopsis protoplasts showed that SpHMT1 is tonoplast localized (Fig. 1B), consistent with the results in fission yeast (Ortiz et al., 1992, 1995).

SpHMT1 Enhanced Metal Tolerance and Accumulation in Arabidopsis

Representative transgenic lines expressing *SpHMT1* were tested for tolerance to heavy metals. When germinated without heavy metals, transgenic lines W2-11 and W7-3 showed similar growth and biomass as the wild-type Col-0 (Supplemental Fig. S3; $P > 0.05$). However, when germinated on 50 μM Cd^{2+} (Fig. 2A), 150 μM As (Fig. 2B), 40 μM copper (Cu; Fig. 2C), or 150 μM zinc (Zn; Fig. 2D), lines W2-11 and W7-3 grew longer roots than did the wild-type control. Total biomass and metal content were also significantly higher than those of the control plants (Fig. 2, E and F; $P < 0.01$). For Cd^{2+}, 39% to 42% more of the metal were found in the transgenic lines. Adjusting for biomass differences, this translates to 1.7- to 1.9-fold more Cd^{2+} per fresh weight of tissue. Similar increases in accumulation of As, Cu^{2+}, or Zn^{2+} were also found (Fig. 2F; $P < 0.01$).

Cd Tolerance and Accumulation in Transgenic Plants Aborted by BSO

PCs and PC-Cd were described as substrates of SpHMT1 (Ortiz et al., 1992, 1995; Mendoza-Cózatl

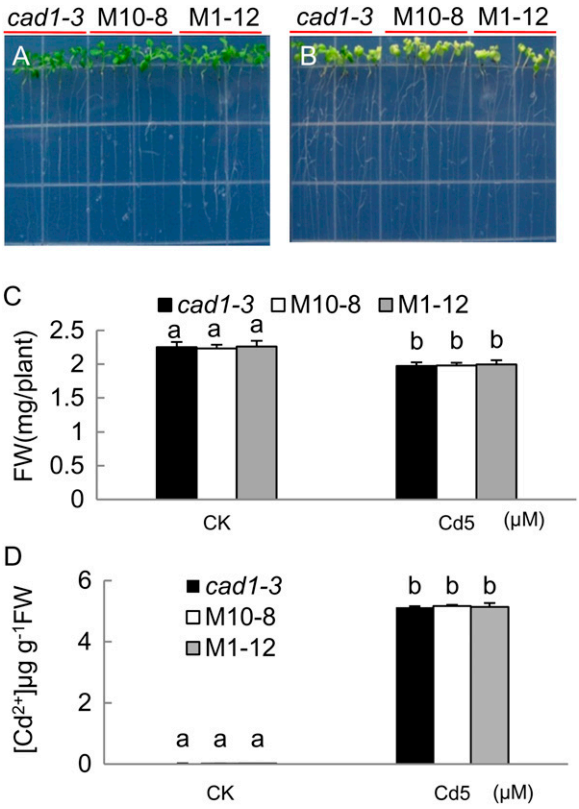

Figure 3. Cd^{2+} sensitivity and accumulation in *cad1-3* expressing *SpHMT1*. Plants were germinated and grown vertically on one-fourth Murashige and Skoog plates under control condition (A) or with 5 μM $CdCl_2$ (B). M10-8 and M1-12 represent *cad1-3* plants transformed with pBI121-35S/*SpHMT1*:*cmyc*. Photographs taken at day 7 (A) and day 20 (B) post germination. C, Fresh weights of plants grown under control condition (CK) in A or with 5 μM $CdCl_2$ (Cd5) in B. D, Cd^{2+} concentration in seedlings grown under conditions in A and B. Values are mean ± SD, $n = 12$ for C, or $n = 4$ for D, each containing greater than five plants. Lowercase letters indicate whether averages were statistically different at $P < 0.05$ by *t* tests. [See online article for color version of this figure.]

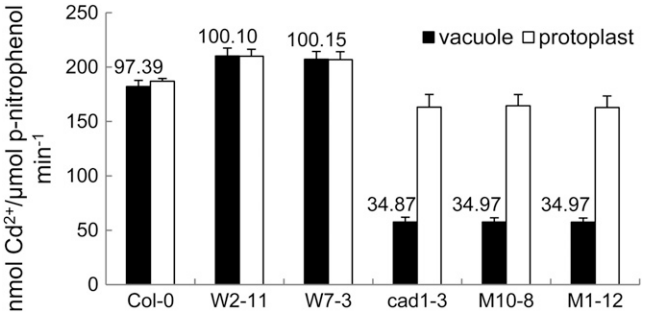

Figure 4. Subcellular localization of Cd^{2+}. Protoplasts and vacuoles isolated from leaves of soil-grown plants (Col-0, *cad1-3*, and transgenic W2-11, W7-3, M1-12, and M10-8) were measured for Cd^{2+} content. Values above the bars represent the percentage of vacuolar Cd^{2+} relative to the total in protoplasts. Acid phosphatase (ACP) activity specific to vacuole was determined and used to normalize Cd^{2+} accumulation; data are mean ± SE from three independent experiments.

et al., 2010). Subsequent studies from overexpression of *SpHMT1* also implicated GS2-Cd as a likely substrate when PCs were absent (Prévéral et al., 2009; Sooksa-Nguan et al., 2009). The production of both PCs and GSH can be abolished by BSO that inhibits a key enzyme in GSH biosynthesis (Howden et al., 1995a). Should the enhanced metal tolerance and accumulation phenotype in W2-11 and W7-3 be due to SpHMT1 recognition of PC-Cd and GS2-Cd, then BSO should indirectly block *SpHMT1* function. When germinated with 0.5 mM BSO, both transgenic and wild-type plants showed similar phenotype and biomass (Supplemental Fig. S4, A and C). When 10 μM $CdCl_2$ was included along with 0.5 mM BSO, reduced growth and chlorotic leaves were observed, and to the same degree between transgenic and wild-type plants (Supplemental Fig. S4, B and C). Cd^{2+} content in roots, rosette leaves, or shoots also failed to show a significant difference among transgenic and wild-type plants exposed to Cd^{2+} and BSO (Supplemental Fig. S4D; $P > 0.05$). We conclude that BSO neutralized the effect of *SpHMT1* for metal tolerance and accumulation.

SpHMT1 Failed to Increase Cd Tolerance and Accumulation in *cad1-3*

The mutant *cad1-3* harbors a defective PCS gene. While PC synthesis is impaired, GSH production is unaffected (Howden et al., 1995a). If GSH were necessary for SpHMT1 function, an expectation would be that overexpression of *SpHMT1* in the *cad1-3* mutant would still enhance Cd^{2+} tolerance and accumulation through recognition of GSH. However, this was not the case, as representative lines of transgenic *SpHMT1* in the *cad1-3* mutant background exhibited similar growth as the *cad1-3* mutant, either in the absence of metal stress (Fig. 3, A and C), or when germinated with 5 μM Cd^{2+} where both *cad1-3* and the transgenic derivatives M10-8 and M1-12 showed similar chlorosis and reduced growth (Fig. 3, B and C). Given that a relatively low Cd^{2+} concentration had to be used due to the metal hypersensitivity of *cad1-3* mutation, a root-bending assay that allows treatment with increased dosage was also conducted. When exposed to 20 μM Cd^{2+} (Supplemental Fig. S5A), growth hooks could be observed in both *cad1-3* and the transgenic plant roots (M10-8, M1-12, and M1-4), but not at 30 μM Cd^{2+} (Supplemental Fig. S5B). In contrast, significant growth hooks were observed in transgenic plants W2-11, W3-13, and W7-3, but not in the wild-type Col-0 when exposed to 150 μM Cd^{2+} (Supplemental Fig. S6). In sum, a difference between *cad1-3* and its *SpHMT1* derivatives was not observed with respect to Cd^{2+} tolerance and accumulation (Fig. 3D). As the *cad1-3* mutant is proficient in GSH production, the simplest interpretation is that SpHMT1 requires PCs rather than GSH to enhance tolerance and accumulation of Cd^{2+} in Arabidopsis.

PC-Assisted Cd^{2+} Distribution between Vacuoles and Cytosol

To test whether *SpHMT1* promotes vacuolar sequestration of Cd^{2+} in Arabidopsis, the distribution of Cd^{2+} was examined in isolated protoplasts and vacuoles (Supplemental Table S1). The *SpHMT1* transgenic lines W2-11 and W7-3 showed higher protoplast and vacuolar Cd^{2+} content than the wild-type control, but a difference was not observed among the PC-deficient lines *cad1-3*, M10-8, and M1-12 (Fig. 4). More Cd^{2+} accumulated in Col-0, W2-11, and W7-3 than in *cad1-3*, M10-8, and M1-12, especially in the vacuoles, and nearly 100% of the cellular Cd^{2+} in Col-0, W2-11, and W7-3 was vacuolar (Fig. 4). However, the lack of PCs did not abolish vacuolar sequestration entirely in *cad1-3*, M10-8, and M1-12, as approximately 35% of the cellular Cd^{2+} was in vacuoles of these PC-deficient lines. To assess whether this approximately 35% vacuolar accumulation of Cd^{2+} might be GSH mediated, the same analysis was extended to mutant *cad2-1* that produces substantially decreased amounts of GSH and PCs (Howden et al., 1995b). Even less Cd^{2+} accumulated in protoplasts and vacuoles of *cad2-1* compared to *cad1-3*, with only 24% of the protoplast Cd^{2+} localized in vacuoles (Supplemental Fig. S7). HPLC analyses showed that in Col-0, both GSH and PCs (PC_2, PC_3, and PC_4) were detected in protoplasts, while only PCs were detected in vacuoles (Fig. 5A). In *cad1-3*, PCs were not detected in protoplasts and vacuoles, but consistently, GSH was detected only in protoplasts (Fig. 5B). Although it appeared that a small amount of GSH might accumulate in *cad2-1* protoplasts, detectable GSH or PCs were not observed in vacuoles (Fig. 5C). Interestingly, in W2-11 and W7-3, more PCs, approximately 165% of the wild-type level, were detected in vacuoles (Fig. 6; $P < 0.05$). Taken together, the data indicate that PCs play a predominant role in vacuolar Cd^{2+} sequestration. GSH also contributes, but other Cd^{2+} cross-tonoplast transport mechanisms other

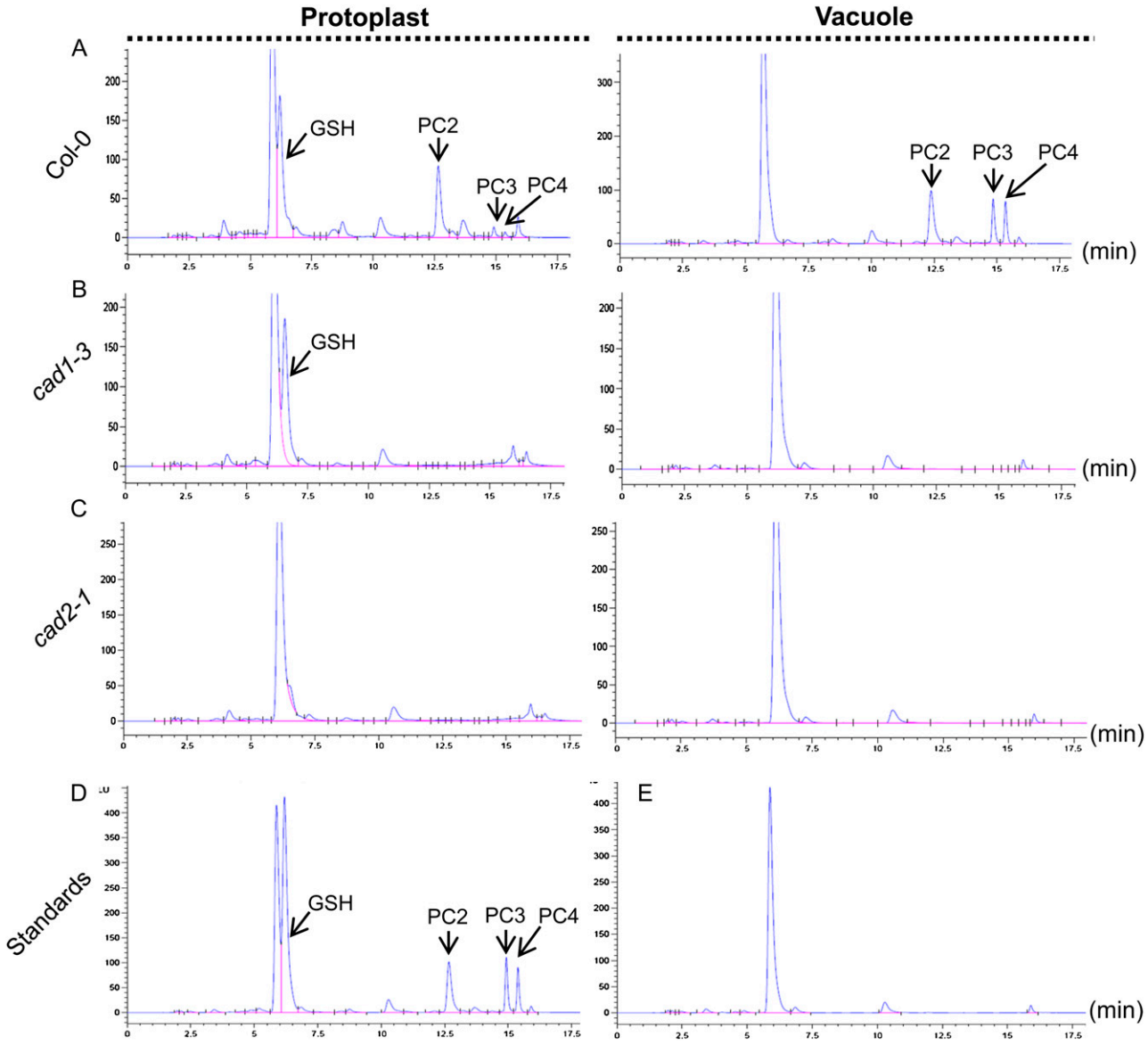

Figure 5. Distribution of GSH and PCs. Protoplasts and vacuoles were extracted from soil-grown plant leaves of Col-0 (A), *cad1-3* (B), and *cad2-1* (C). GSH and PCs measured by fluorescence HPLC. D, Synthesized GSH, PC2, PC3, and PC4 used as standards. E, Blank control. Peaks are indicated by arrows. [See online article for color version of this figure.]

than PC or GSH assisted are also involved. Additionally, the significantly higher Cd^{2+} and PC contents found in W2-11 and W7-3 vacuoles over those of the control are consistent with an interpretation that SpHMT1 promotes PC-Cd sequestration into the plant vacuoles.

Long-Distance Root-to-Shoot Transport of Cd^{2+} Was Delayed by *SpHMT1*

To examine if *SpHMT1* affects Cd^{2+} long-distance transport, Cd^{2+} contents in roots and shoots were measured. When exposed to 10 μM $CdCl_2$ for 24 h, more Cd^{2+} accumulated in the roots while less Cd^{2+} was detected in rosette leaves and stems of transgenic plants compared to the wild-type control (Fig. 7A; $P < 0.05$). By day 3, more Cd^{2+} accumulated in leaves as well as in the roots, but less in the stems of the transgenic plants (Fig. 7B; $P < 0.05$). After a week, the transgenic plants accumulated higher Cd^{2+} content in all three tissue types (Fig. 7C; $P < 0.01$). Consistently, delayed Cd^{2+} entry into xylem sap by SpHMT1 was also observed (Supplemental Fig. S8), where sap Cd^{2+} increase in transgenic plants was slower than in Col-0. Note that at day 3, Cd^{2+} concentration in wild-type sap began to decline, which possibly resulted from a more severely inhibited transpiration rate. Taken together, these data show that *SpHMT1* plants delayed long-distance transport of Cd^{2+} from roots to shoots.

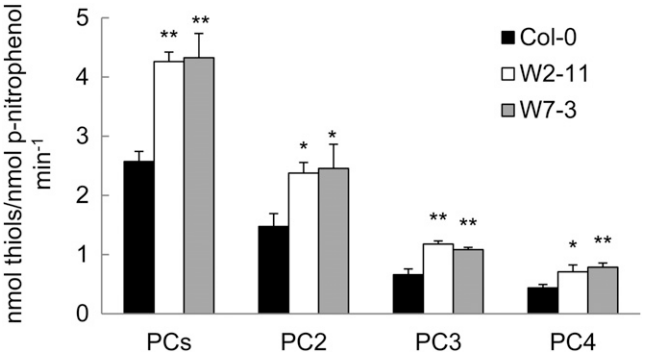

Figure 6. Increased vacuolar PCs in transgenic *SpHMT1* plants. Soil-grown plants were used for protoplasts and vacuoles isolation. PC contents in vacuoles from Col-0 and the transgenic plants W2-11 and W7-3 were determined as described in "Materials and Methods." Acid phosphatase (ACP) activity specific to vacuoles was determined and used to normalize PC accumulation. Data are mean ± SE from three independent experiments. Asterisks indicate significant difference between wild type and the transgenic lines at $P < 0.05$ (*) and 0.01 (**) by *t* tests.

Root-Specific Expression of *SpHMT1* Reduced Heavy Metal Accumulation in Plant Aerial Parts

Given that constitutive *SpHMT1* expression delayed Cd^{2+} transport to aerial parts (Fig. 7), we sought to test if root-specific expression of *SpHMT1* could further reduce Cd^{2+} accumulation in leaves and seeds, the edible parts of leafy vegetables, or grain crops. For this purpose, the *SpHMT1* fragment was placed under the control of the Arabidopsis *alcohol dehydrogenase* (*Adh*) promoter (Gong et al., 2003) in the construct pBI121-Adh/*SpHMT1*:*cmyc*, and root-specific expression of *SpHMT1* was found in transgenic lines A5-9 and A26-4 (Supplemental Fig. S2, C and D). When treated with 10 μM $CdCl_2$, 100 μM KH_2AsO_4, or 20 μM $CuSO_4$ for 3 d, more metals accumulated in the roots of these plants compared to the wild-type Col-0 (Fig. 8, A–C; $P < 0.05$). Conversely, significantly less toxic metals were detected in leaves (Fig. 8, A–C; $P < 0.01$). Furthermore, seeds derived from plants grown with 5 μM $CdCl_2$, KH_2AsO_4, or $CuSO_4$ accumulated less of these toxic metals. Compared to the wild-type control, approximately 40% less of Cd^{2+} (Fig. 8D), approximately 70% less of As (Fig. 8E), and approximately 45% less of Cu^{2+} (Fig. 8F), were found in A5-9 and A26-4 seeds. These results suggest that root-specific expression of SpHMT1 substantially decreases heavy metal accumulation in aerial tissues.

DISCUSSION

SpHMT1-Mediated Sequestration of Cd^{2+} Requires PCs

SpHMT1 was originally identified as a principal vacuolar PC-Cd transporter in fission yeast (Ortiz et al., 1992, 1995). In this study, ectopic expression of *SpHMT1* in Arabidopsis wild type enhanced both metal tolerance and accumulation (Fig. 2). However, application of BSO that inhibits GSH biosynthesis and thus inhibits PC synthesis, aborted both the increased metal tolerance and accumulation in transgenic plants (Supplemental Fig. S4), while in transgenic *cad1-3* plants, which are deficient in PCs but proficient for GSH production (Howden et al., 1995a; Ha et al., 1999), significant increase in either metal tolerance or accumulation was not observed (Fig. 3; Supplemental Fig. S5). Furthermore, transgenic *SpHMT1* significantly increased vacuolar Cd^{2+} and PC levels in Col-0 (Figs. 4 and 6). These data suggest that PCs are required for SpHMT1-mediated Cd^{2+} sequestration in Arabidopsis.

However, previous studies suggested that GSH instead of PCs is required for SpHMT1 function in yeast (Prévéral et al., 2009; Sooksa-Nguan et al., 2009), in contrast to the current result in Arabidopsis. The inconsistency possibly resulted from the unusual conditions used in yeast, where high abundance of SpHMT1 protein might recognize GSH that is devoid of competition from PCs. Alternatively, plants may have developed a mechanism that predominantly relies on PCs, thus lim-

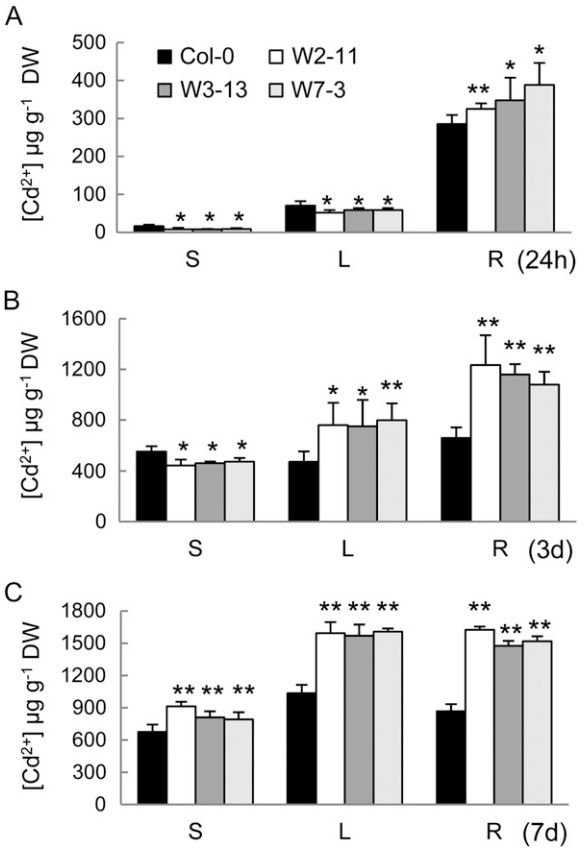

Figure 7. Long-distance Cd^{2+} transport from roots to shoots. Plants were grown hydroponically for 4 weeks before treatment with 10 μM $CdCl_2$ for 24 h (A), 3 d (B), and 7 d (C). Cd^{2+} concentrations in stems (S), rosette leaves (L), and roots (R) of wild-type Col-0 and transgenic lines (W2-11, W7-3, and W3-13) were determined. Values are mean ± SD, $n = 4$, each containing greater than eight plants. Asterisks indicate significant difference between wild type and the transgenic lines at $P < 0.05$ (*) and 0.01 (**) by *t* tests.

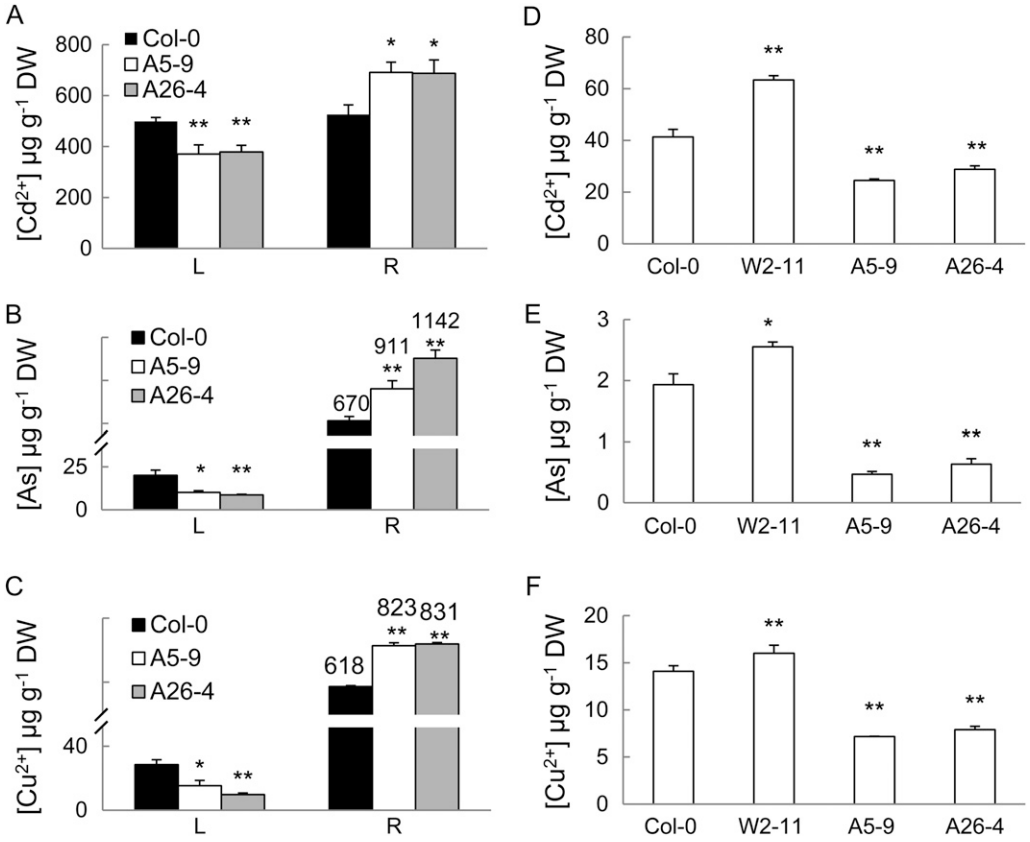

Figure 8. Targeted *SpHMT1* expression in roots decreased metal accumulation in leaves and seeds. Metal content in rosette leaves (L), roots (R), and seeds (D–F) measured in plants grown hydroponically to 4 weeks of age and exposed to 10 μM $CdCl_2$ (A), 100 μM KH_2AsO_4 (B), or 20 μM $CuSO_4$ (C) for 3 d before sampling, or in 2-week-old hydroponically grown plants supplemented with 5 μM $CdCl_2$ (D), KH_2AsO_4 (E), or $CuSO_4$ (F) until maturation of seeds. A5-9 and A26-4 represent independent pBI121-Adh/*SpHMT1*:*cmyc* transformed lines, while W2-11 represents a pBI121-35S/*SpHMT1*:*cmyc* transgenic line. Values are mean ± SD, $n = 4$, each containing greater than eight plants. Asterisks indicate significant difference between wild type and the transgenic lines at $P < 0.05$ (*) and 0.01 (**) by *t* tests.

iting substrate availability to ectopically expressed proteins. This latter hypothesis was supported by isolation and characterization of the Arabidopsis native PC transporters AtABCC1 and AtABCC2 (Song et al., 2010). In any case, the substrates recognized by SpHMT1 might be the reason that *SpHMT1* enhanced As tolerance in plants but not in yeast, as PCs are known to be more efficient than GSH in metal(loid)s detoxification (Howden et al., 1995a; Gong et al., 2003; Song et al., 2010). Further support for this model came from the result that SpHMT1 did effect As sensitivity in a dose-dependent manner in yeast (Prévéral et al., 2009; Sooksa-Nguan et al., 2009).

PCs Play a Primary Role in Metal Sequestration into Arabidopsis Vacuoles

This study also calls into question the physiological relevance of GSH for Cd sequestration by plant tonoplast transporters. Subcellular localization analyses showed that in wild-type Col-0, nearly 100% of the protoplast Cd^{2+} was localized in vacuoles, but in protoplasts of the PC-deficient mutant *cad1-3*, only 35% is present in vacuoles (Fig. 4), comparable to the result in *atabcc1 atabcc2* mutant in which PC and possibly GSH transport was disrupted (Park et al., 2012). These data consistently suggest that PCs play a primary role in vacuolar Cd^{2+} sequestration in Arabidopsis. In *cad2-1*, approximately 24% of the protoplast Cd^{2+} was localized in vacuoles (Supplemental Fig. S7), in contrast to approximately 35% in *cad1-3*, which further suggest that GSH contributes about 13% to 18% of the vacuolar sequestration of Cd^{2+}, given that a general 20% to 40% GSH of wild-type levels is present in *cad2-1* (Howden et al., 1995b; Cobbett et al., 1998). Note that the contribution by GSH might be indirect due to the lack of detectable GSH in vacuoles (Fig. 5).

Engineering of *SpHMT1* Is a Feasible and Efficient Way to Tackle Heavy-Metal-Related Food Safety Problems

Plant heavy metal uptake not only reduces crop yields, but also transits the pollutants to the human

diet, resulting in serious diseases including neurodegenerative conditions, dysfunction of vital organs, and cancers (Lee and White, 1980). Phytoextraction has been proposed to tackle this problem (Salt et al., 1998), though it seemed to be less efficient in situations with a wide range of multiple heavy metal pollutions (Cheng, 2003). Considering from this aspect, it might be more feasible to reduce toxic metal accumulation in plant edible parts.

Transgenic SpHMT1 led to 30% to 40% increase of Cd^{2+}, As, Cu, or Zn^{2+} accumulation in plants (Fig. 2F), which might not be sufficient for rapid cleanup of soil contamination. Even if this could translate to field applications, it would only shorten somewhat the estimated decades (century)-long process of soil cleanup by plants. For the purpose of reducing dietary intake of toxic metals, however, the root-specific expression of SpHMT1 reduced seed accumulation of Cd^{2+} and Cu by half, and of As to one-third of the wild-type level (Fig. 8, D–F). Decrease of metals in leafy tissue was also found though not as impressive (Fig. 8, A–C). Given that the consumption of grains such as rice represents the greatest proportion of daily calorie intake in many developing countries, especially in countries where significant toxic metal pollution has occurred, the development of grain crops with one-third to half of the normal amount of dietary toxic metals could lead to significant health benefits.

Previously, the engineering of the budding yeast YCF1 in Arabidopsis was reported to have enhanced Cd^{2+} and Pb^{2+} tolerance and accumulation (Song et al., 2003). However, the Cd^{2+} and Pb^{2+} accumulation was not in roots, but in the plant aerial parts. In this work, we found that the aerial portion could accumulate more Cd^{2+}, but the kinetics of Cd^{2+} accumulation suggested that there is a delay in root-to-shoot translocation (Fig. 7; Supplemental Fig. S8), as though the roots serve as an initial sink for the toxic metals. Root-specific expression of *SpHMT1* substantiated this deduction, with proportionally more metals in roots than shoots or seeds (Fig. 8), indicating that the roots can have high capacity for toxic metals storage. Given that root-targeted expression of the wheat (*Triticum aestivum*) PCS gene *TaPCS1* in Arabidopsis failed to trap Cd^{2+} in roots, but instead greatly enhanced long-distance Cd^{2+} transport to plant aerial parts (Gong et al., 2003), our data demonstrate that engineering vacuolar PC sequestration could overcome the plant overflow protection mechanism in which PC synthesis facilitates Cd^{2+} translocation and hence limits the metal concentration in a specific organelle (Gong et al., 2003), thus this sequestration strategy could be applied to targeting heavy metals to specific tissues.

In summary, PCs were found to play a primary role in Cd^{2+} sequestration into Arabidopsis vacuoles, and ectopic expression of *SpHMT1* enhanced this process. We further demonstrated that the engineering of *SpHMT1* could be a feasible and efficient way to tackle food safety concerns in countries with a wide range of heavy metal pollution.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Metal Sensitivity Analyses

Arabidopsis (*Arabidopsis thaliana*) plants were grown in quarter-strength hydroponics as described (Arteca and Arteca, 2000; Gong et al., 2003). At 4 weeks of age, plants were exposed to $CdCl_2$ or $CdCl_2$ BSO as indicated. Root, rosette leaf, and stem samples were harvested as described (Gong et al., 2003). Alternatively, Arabidopsis plants were grown in soil to 2 weeks old with a 16/8-h day/night period, and bottom flooded with 0.5 L of 40 μM $CdCl_2$ twice for 1 week prior to leaf tissue sampling. Seedling metal sensitivity analyses were performed on one-fourth Murashige and Skoog basal medium (Sigma-Aldrich) with 1 g/L MES, 1.5% Bacto agar, and heavy metals with or without BSO as indicated. Plants grew vertically for indicated time intervals prior to fresh weight and metal content measurements. Root-bending assay was performed as described (Howden and Cobbett, 1992) with minor modification: 6-d-old seedlings were transferred onto one-fourth Murashige and Skoog plates supplemented with indicated concentrations of $CdCl_2$, and the plates were then placed upside down and incubated for another 5 d.

DNA Constructs and Transformation into Yeast and Plants

The fission yeast (*Schizosaccharomyces pombe*) *SpHMT1* cDNA was amplified by reverse transcription-PCR, into which two restriction enzyme sites *Bam*HI and *Spe*I were introduced using forward primer 5′-AGGATCCAAATTAGCAATTGAAGCAGTAACAA-3′ and reverse primer 5′-AACTAGTGAATGAGTTTCAGCAGAAGTTTTT-3′. The *SpHMT1* cDNA fragment was sequenced prior to inserting into *Bam*HI and *Spe*I sites of binary vector pBI121-35S/*TaPCS1*:*cmyc* (Gong et al., 2003) to generate pBI121-35S/*SpHMT1*:*cmyc*. The same fragment was subsequently transferred to binary vector pBI121-Adh/*TaPCS1*:*cmyc* (Gong et al., 2003) to yield pBI121-Adh/*SpHMT1*:*cmyc*. Both constructs were used for Arabidopsis floral-dip transformation (Clough and Bent, 1998) into wild-type Col-0 and PC-deficient mutant *cad1-3* (Howden et al., 1995a) background. Transgenic lines with a segregation rate of 3:1 on kanamycin plates were used for further screening of homozygotes and strong alleles. The *SpHMT1* cDNA from pBI121-35S/*SpHMT1*:*cmyc* was also transferred as a *Bam*HI to *Sac*I fragment to fission yeast vector pART1 (Ortiz et al., 1992) to yield pART1-SpHMT1 for transformation by the lithium acetate method into fission yeast strain Sp223 (*h*$^-$ *ade6-216 leul-32 ura4-294*) and its isogenic *SpHMT1* mutant strain LK100 (*h*$^-$ *ade6-216 leul-32 ura4-294 hmt1*$^-$; Ortiz et al., 1992). Transformants were selected on Edinburgh minimal medium (EMM) plates without Leu (Okazaki et al., 1990) and cells were grown at 30°C in EMM supplemented with appropriate amino acids. For metal sensitivity assays, yeast (*Saccharomyces cerevisiae*) cells were grown to saturation, then diluted to an OD600 of 0.5, grown for approximately 4 h to log phase, and further diluted as indicated and incubated with different concentrations of $CdCl_2$. Cell density was determined after 16 to 20 h. Alternatively, the diluted cultures were spotted onto EMM plates containing $CdCl_2$ at indicated concentrations and grown for 3 to 5 d.

Isolation of Intact Protoplasts and Vacuoles and Determination of Metal and PC Levels

Rosette leaves from soil-grown Arabidopsis plants were used to isolate intact protoplasts and vacuoles as described (Robert et al., 2007) with minor modification: The purified protoplasts were divided into two equal aliquots, and one was used for further isolation of vacuoles. The purified protoplasts and vacuoles were then subsampled and subjected to determination of Cd^{2+} concentrations and marker enzyme activities as described (Vögeli-Lange and Wagner, 1990; Ma et al., 2005), respectively. Cd^{2+} contents in protoplasts and vacuoles or other metal contents in sampled plant tissues were measured by inductively coupled plasma-mass spectrometry (ELAN DRC-e, PerkinElmer) as described (Gong et al., 2003; Li et al., 2010) with corresponding modification. Protoplasts and vacuoles from Cd^{2+}-treated plants were analyzed by HPLC (Agilent 1200 series) using a reversed-phase C18 column (Agilent 300Extend-C18 4.6 × 150 mm, 3.5 μm particle size) as described (Minocha et al., 2008) with corresponding modification. Thiols were labeled by monobromobimane as described (Gong et al., 2003). PC standards, $(\gamma\text{-GluCys})_2\text{Gly}$ (PC_2), $(\gamma\text{-GluCys})_3\text{Gly}$ (PC_3), and $(\gamma\text{-GluCys})_4\text{Gly}$ (PC_4) were synthesized and purchased from AnaSpec. The activities of acid phosphatase and cytochrome c

oxidase were determined using plant ACP colorimetry assay kit and COX assay kit (GenMed Sci Inc.) following the manufacturer's instructions.

EYFP Fusion and Subcellular Localization

Two restriction sites *Bgl*II and *Xho*I were introduced into S*pHMT1* cDNA using forward primer 5′-AAGATCTATGGTTCTACGTTACAACAGCCC-3′ and reverse primer 5′-ACTCGAGTTAATGAGTTTCAGCAGAAGTTTTT-3′, by which the confirmed *SpHMT1* cDNA fragment was inserted into vector pMON530-35S/*EYFP* to make an *EYFP*:*SpHMT1* fusion construct. The resulted pMON530-35S/*EYFP*:*SpHMT1* construct together with the control pA7-35S/*EYFP* were transiently expressed in Arabidopsis protoplasts via polyethylene-glycol-mediated transformation as described (Yoo et al., 2007). The transformed protoplasts were treated and imaged as described (Li et al., 2010), and >100 protoplasts were analyzed from each of three replicates.

Statistical Analyses

Two-tailed Student's *t* tests were used to compare fresh weights and metal contents between transgenic plants and wild type. Differences were deemed significant at $P < 0.05$, and extremely significant at $P < 0.01$.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number CAA78419/Z14055.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Rescue of Cd^{2+} sensitivity in *hmt1*⁻ mutant LK100 by c-myc tagged *SpHMT1*.

Supplemental Figure S2. RT-PCR analysis of *SpHMT1* expression.

Supplemental Figure S3. Transgenic lines harboring *SpHMT1* showed similar growth with the wild type Col-0 under control condition.

Supplemental Figure S4. Enhanced Cd^{2+} tolerance and accumulation aborted by BSO.

Supplemental Figure S5. *SpHMT1* fails to rescue Cd^{2+} sensitivity in *cad1-3* mutant.

Supplemental Figure S6. *SpHMT1* enhanced cadmium tolerance in Col-0.

Supplemental Figure S7. Subcellular localization of Cd^{2+}.

Supplemental Figure S8. Cd^{2+} concentration in xylem sap.

Supplemental Table S1. Determination of vacuole purity.

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