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Synthetic phytochelatins complement a phytochelatin-deficient *Arabidopsis* mutant and enhance the accumulation of heavy metal(loid)s

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ABSTRACT

Phytochelatins (PCs) are naturally occurring thiol-rich peptides containing gamma (γ) peptide bonds and are well known for their metal-binding and detoxification capabilities. Whether synthetic phytochelatins (ECs) can be used as an alternative approach for enhancing the metal-binding capacity of plants has been investigated in this study. The metal-binding potential of ECs has been demonstrated in bacteria; however, no report has investigated the expression of ECs in plants. We have expressed three synthetic genes encoding ECs of different lengths in wild type (WT) *Arabidopsis* (Col-0 background) and a phytochelatin-deficient *Arabidopsis* mutant (*cad1-3*). After exposure to different heavy metals, the transgenic plants were examined for phenotypic changes, and metal accumulation was evaluated. The expression of *EC* genes rescued the sensitive phenotype of the *cad1-3* mutant under heavy metal(loid) stress. Transgenic *Arabidopsis* plants expressing EC genes accumulated a significantly enhanced level of heavy metal(loid)s in comparison with the WT plant. The mutant complementation and enhanced heavy metal(loid) accumulation in the transgenic *Arabidopsis* plants suggest that ECs work in a manner similar to that of PCs in plants and that ECs could be used as an alternative for phytoremediation of heavy metal(loid) exposure.

1. Introduction

Naturally occurring metal-binding peptides, such as metallothioneins (MTs) and phytochelatins (PCs), are the major metal-binding molecules used by cells to immobilise, sequester, and detoxify metal ions. PCs have a general structure of $(\gamma\text{-Glu-Cys})_n$-Xaa, where *n* is 2–11, and Xaa is usually Gly [1,2]. PCs are preferred over MTs because of their unique structural characteristics, particularly the continuously repeating γ-Glu-Cys unit. These peptides contain a γ bond between the glutamic acid and cysteine and are synthesised enzymatically by the phytochelatin synthase (PCS) enzyme using reduced glutathione (GSH) or related thiols [1,3]. Previously, different groups overexpressed PCS to enhance the biosynthesis of PCs for developing heavy metal tolerance and accumulation in plants. However, in some of the studies, transgenic plants developed sensitivity towards metal stress despite expression of the PCS gene [4,5]. A few studies suggested that these paradoxical results were dependent on the availability or limitation of GSH at the cellular level due to enhanced demand for PC biosynthesis [4–7].

An alternative strategy to produce PCs is to develop organisms expressing synthetic genes encoding peptide analogues of PCs with a general structure of $\text{Met(Glu-Cys)}_n\text{Gly}$ (synthetic phytochelatins, or ECs). These peptides (ECs) differ from PCs at the peptide bond (Fig. 1A and B) between the glutamic acid and cysteine. ECs contain an α peptide bond rather than the γ peptide found in enzymatically synthesised PCs. Previously, the difference between α- and γ-bonding was thought to influence the metal-binding affinity between ECs and PCs. However, detailed experiments demonstrated that these peptides bind various metals in a similar manner as PCs [8–10].

The wide application of ECs, including expression on bacterial surfaces, the construction of capacitance biosensors, coating of nanocrystals with *EC*20, and formation of elastin-like polypeptides, has been demonstrated [11–16]. The ECs strongly bind various heavy metal(loid)s and can be effectively used for remediation. However, no study has reported the use of this strategy in plants. In the present study, we have examined the effect of EC expression in plants for the first time and studied its role in metal accumulation. We expressed different lengths of *EC* genes encoding *EC*14, *EC*16, and *EC*20 in WT (Col-0) and *cad1-3* (PC-deficient) mutant *Arabidopsis* plants. Our study suggested that ECs could complement the PC-deficient *Arabidopsis* (*cad1-3*) mutant and demonstrated

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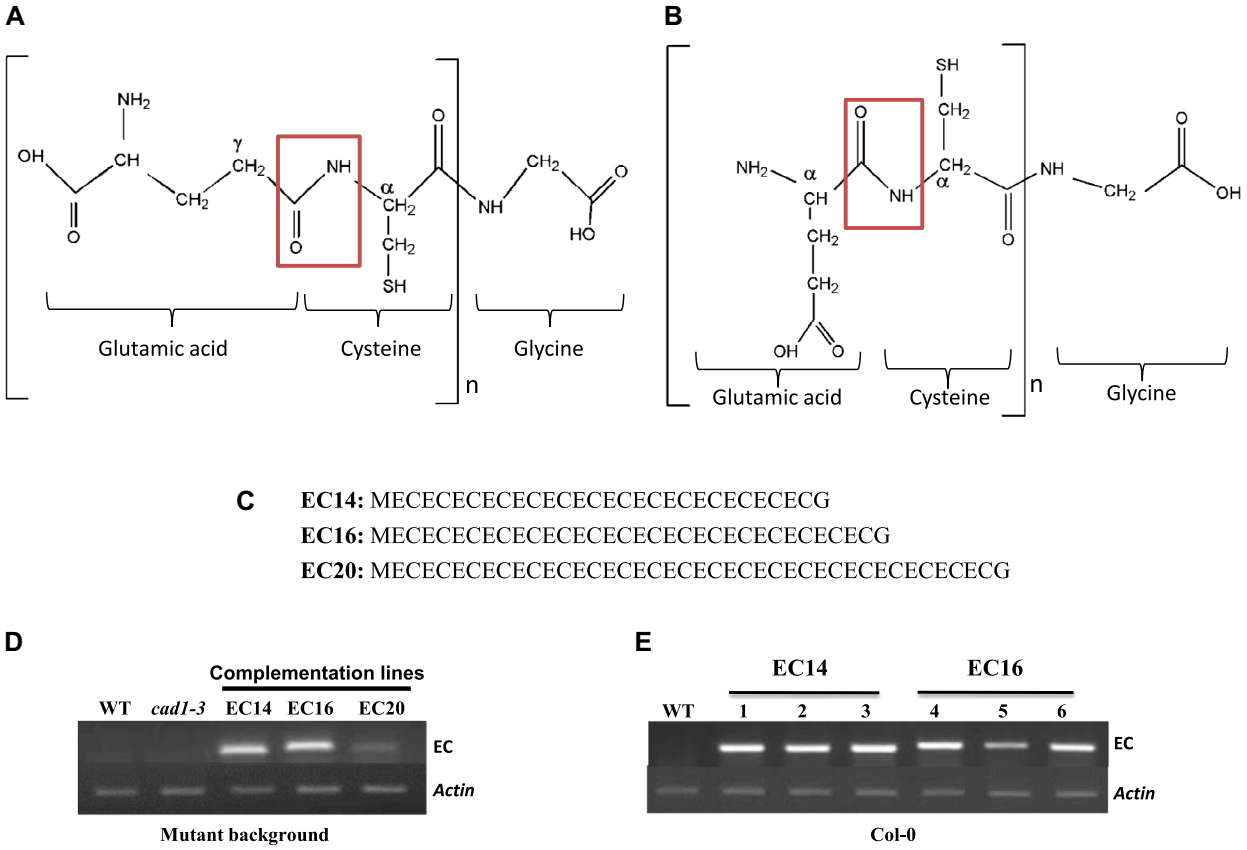

Fig. 1. Schematic representation of the synthetic phytochelatin structure and transgene confirmation through RT-PCR in *Arabidopsis*. (A) The general structure of PCs with a γ peptide bond between the glutamic acid and cysteine. (B) The chemical structure of ECs with an α peptide bond between the glutamic acid and cysteine. (C) The amino acid sequences of different ECs. (D) The expression of the *EC14*, *EC16*, and *EC20* genes in the transgenic lines (*cad1-3* background). RT-PCR of total RNA extracted from the leaves of mature *Arabidopsis* plants grown in the absence of heavy metals. (E) The expression of the synthetic phytochelatin genes *EC14* and *EC16* in transgenic *Arabidopsis* (Col-0). RT-PCR of total RNA extracted from the leaves of mature *Arabidopsis* plants grown in the absence of heavy metals. Numbers 1, 2, 3 and 4, 5, 6 represent independent transgenic lines expressing *EC14* and *EC16*, respectively.

that the accumulation of heavy metal(loid)s was significantly enhanced compared to the respective controls.

2. Materials and methods

2.1. Plant materials, growth conditions, and treatment

Arabidopsis (Col-0 wild type and *cad1-3* mutant) plants were grown under a 16 h light and 8 h dark photoperiod at a light intensity of 120 μmol m^{-2} s^{-1} with 50% relative humidity at 23 °C. The seeds of *Arabidopsis* (Col-0 and *cad1-3*) controls and *EC*-expressing transgenic lines were germinated on half-strength MS medium supplemented with 1.5% (w/v) sucrose (pH 5.8) in 135 mm round plates. The plates were supplemented with $CdCl_2$ (10 or 20 μM), Na_2HAsO_4 [As(V); 30 μM], or Na_2HAsO_2 [As(III); 2.5 μM] to study the effect of these metals on the growth parameters. A transgenic *Arabidopsis* line (CdPCS1C1) expressing the *PCS* gene from *Ceratophyllum demersum* in the *cad1-3* mutant that had been developed in a previous study by Shukla et al. [5] was used here as a positive control in one of the complementation experiments. For the analysis of heavy metal(loid) accumulation, the seeds were germinated in plastic dishes containing Soilrite (Keltech Energies Limited, India) and grown for 20 days. These plants were grown for 10 more days in the presence of $CdCl_2$ (100 μM), As(V) (300 μM), or As(III) (25 μM) within the nutrient medium. The plants were irrigated every 3 days. Aerial tissues were harvested, washed, oven dried, and used for measurements of metal accumulation.

2.2. Synthesis and cloning of synthetic PC (EC) genes

To synthesise and clone the synthetic genes (*EC14*, *EC16*, and *EC20*) encoding Met(Glu-Cys)$_{14}$Gly, Met(Glu-Cys)$_{16}$Gly, and Met(Glu-Cys)$_{20}$Gly, respectively, forward and reverse overlapping primers were designed as previously described by Bae et al. [11]. The nucleotide sequences of the forward and reverse primers are given in Supplementary Table S1. The three artificial genes were synthesised and cloned into pTZ57R/T by T-A cloning. The resulting clones were sequenced to confirm the presence of the correct EC fragments.

2.3. Preparation of EC gene constructs and transformation of *Arabidopsis*

The synthetic genes were digested with the restriction enzymes *BamHI* and *SacI*, and the resulting fragments were cloned into the plant transformation binary vector pBI121, which was previously digested with the same restriction enzymes. The resultant clones were sequenced to confirm the presence of the correct *EC*14, *EC*16, and *EC*20 fragments. For transformation, the plants were grown for 6 weeks in 10 cm pots containing Soilrite and maintained in a growth chamber under the same growth conditions as described previously. These plants were used for transformation by the floral-dip method [17] using *Agrobacterium tumefaciens* strain GV3101 harbouring the EC gene constructs. The transformed seeds were selected on 0.5× MS medium containing kanamycin

(50 mg l^{-1}). The homozygous seeds were collected and used in further experiments.

2.4. Expression analysis

Total RNA was extracted from 100 mg of frozen leaf tissue using an RNeasy Plant Mini-Kit with on-column DNase digestion (QIAGEN, USA). RT-PCR was performed using 20 μl of the cDNA in a reaction containing 2× PCR Master mix (Fermantas, Life Sciences, Ontario, Canada). The RT-PCR product for the actin gene was used as a control to confirm that equal amounts of RNA were used in each reaction. PCR was performed with 3 μl of the RT reaction product using the specific primers CaMV 35 S 5′ UTR and NosT 3′ UTR for recombinant *EC* genes as described in Supplementary Table S1.

2.5. Plant growth during Cd, As(V), and As(III) exposure

WT and transgenic lines expressing EC14, EC16, and EC20 were germinated and grown on Petri plates containing 0.5× MS medium with 1.5% sucrose and different concentrations of Cd (10 μM, 20 μM), As(V) (30 μM), or As(III) (2.5 μM). The plates were maintained at 4 °C for 2 days in the dark for stratification, and then the plates were placed vertically and grown in a culture room at 23 °C on a 16 h day/8 h night cycle for 10 days. The root lengths of the seedlings were recorded. For estimation of the metal accumulation, the seeds were germinated in plastic dishes containing Soilrite and grown for 20 days. The plants were irrigated with a nutrient medium containing 100 μM $CdCl_2$, 300 μM As(V), and 25 μM As(III) every 3 days. After 10 days, the plant samples were dried and digested. The digested samples were used for metal estimation by atomic absorption spectrometry (AAS, Perkin–Elmer, AAnalyst 600) fitted with a graphite furnace. The reference standards used for calibration of the AAS were purchased from Accustandard (USA).

2.6. Statistical analysis

Each independent experiment was performed using a completely randomised design and at least three replicates for each set. The data were analysed by Student's paired *t*-test, and the mean values for each treatment were compared at $P \leqslant 0.05$–0.001.

3. Results

3.1. Expression of EC genes in Arabidopsis

A primer extension strategy was adopted to develop synthetic genes. Three synthetic *EC* genes encoding $M(EC)_{14}G$, $M(EC)_{16}G$, and $M(EC)_{20}G$ polypeptides (Fig. 1C) were generated using overlapping primers. The sizes of the synthetic *EC14*, *EC16*, and *EC20* genes were 105, 117, and 134 bp, respectively. To compare the functional role of different polypeptides encoded by the *EC* genes, the constructs used the CaMV35S promoter, and *Arabidopsis* (*cad1-3* and Col-0 background) plants were transformed with these constructs. No significant phenotypic changes were observed in transgenic lines expressing different *EC* genes compared with their respective controls (Supplementary Fig. S1).

Three independent lines for each *EC* gene were selected for further analysis based on their growth response under heavy metal exposure. The expression of the *EC* genes in transgenic *cad1-3* and Col-0 lines was confirmed through semi-quantitative RT-PCR analysis. The expression of the transgene was detected in transgenic lines, but expression was absent in untransformed WT and mutant plants (Fig. 1D and E).

3.2. EC genes complement the cad1–3 mutant

To test our hypothesis that *EC* genes function in a similar manner to the natural PC, three constructs of EC (EC14, EC16, and EC20) and *CdPCS1*[5] were transformed into the PC-deficient *Arabidopsis* mutant (*cad1-3*) and grown under Cd (10 μM) stress. The *cad1-3 Arabidopsis* mutant is deficient in PC biosynthesis and showed a hypersensitive response toward heavy metals. The growth of *cad1-3* was highly inhibited in our experiments (Fig. 2A and B); however, the expression of the *EC* genes in *cad1-3* rescued the sensitive phenotype of the mutant. The rescue was either complete or partial, depending on the chain length of the synthetic EC peptides. The rescue efficiency of EC14 was similar to that of CdPCS1 in the *cad1-3* plants. An increase in the chain length (EC16 and EC20) resulted in a decrease in the percent gain of function in *cad1-3* mutants, as indicated by root length data (Fig. 2A and B). Therefore, we conclude that an increase in the EC chain length diminishes the complementation ability of the EC in the *cad1-3 Arabidopsis* mutant. *EC20*-expressing plants were excluded from further studies because these plants were least able to rescue the sensitive phenotype of the *cad1-3* mutant, compared to the plants expression *EC14* or *EC16*. Our results suggested that the ECs function in a similar manner to the PCs *in vivo*, although the complementation ability of ECs in the *cad1-3* mutant vary substantially with EC chain length.

3.3. Heavy metal response of Arabidopsis plants expressing EC14 and EC16

To study the effect of Cd on *EC*-expressing lines in both Col-0 and *cad1-3* backgrounds, the seeds of each of the transgenic lines were germinated on 20 μM Cd(II) and grown for 10 days. The root lengths of *EC14*- and *EC16*-expressing Col-0 transgenic lines decreased significantly by >35% relative to WT (Fig. 2C and D). The root lengths of *cad1-3* lines complemented with *EC14* and *EC16* increased by 211% and 125%, respectively, relative to *cad1-3*, indicating a gain of function in the mutant. Overall, our results suggested that the expression of *ECs* restores function in the mutant under different concentrations of Cd(II) exposure; however, the expression of *ECs* in transgenic plants (Col-0 background) results in growth sensitivity (Fig. 2C and D).

Similar to Cd, sensitivity in terms of root length was also observed in transgenic lines expressing the *EC14* and *EC16* genes grown on 30 μM As(V) for 10 days (Fig. 3A and B). The root lengths of *EC14*- and *EC16*-expressing *cad1-3* lines increased by 37% and 60%, respectively, relative to the mutant *cad1-3* (Fig. 3A and B), indicating that EC expression complemented the mutant. These results suggested that the expression of *ECs* enables the restoration of function of the mutant under As(V) exposure. However, Col-0 plants expressing EC genes had significant sensitivity towards As(V) in comparison with the non-transgenic plants. The effect of As(III), a more toxic form of As compared to As(V) [18], was also evaluated. Interestingly, concentrations as low as 2.5 μM As(III) were found to be highly lethal to both mutant and *EC*-expressing plants because their growth was significantly inhibited. However, EC14-expressing *cad1-3* mutant plants were more tolerant of As(III) exposure than the non-transformed mutant plants. These results suggested that the expression of *ECs* in transgenic plants resulted in sensitivity to As(III) exposure; however, the root lengths of EC14- and EC16-complemented lines only increased slightly relative to the *cad1-3* mutant (Fig. 3C and D).

3.4. Heavy metal(loid) accumulation in Arabidopsis

To investigate the accumulation of heavy metal(loid)s in *Arabidopsis* transgenic plants expressing EC14 and EC16, the metal(loid)

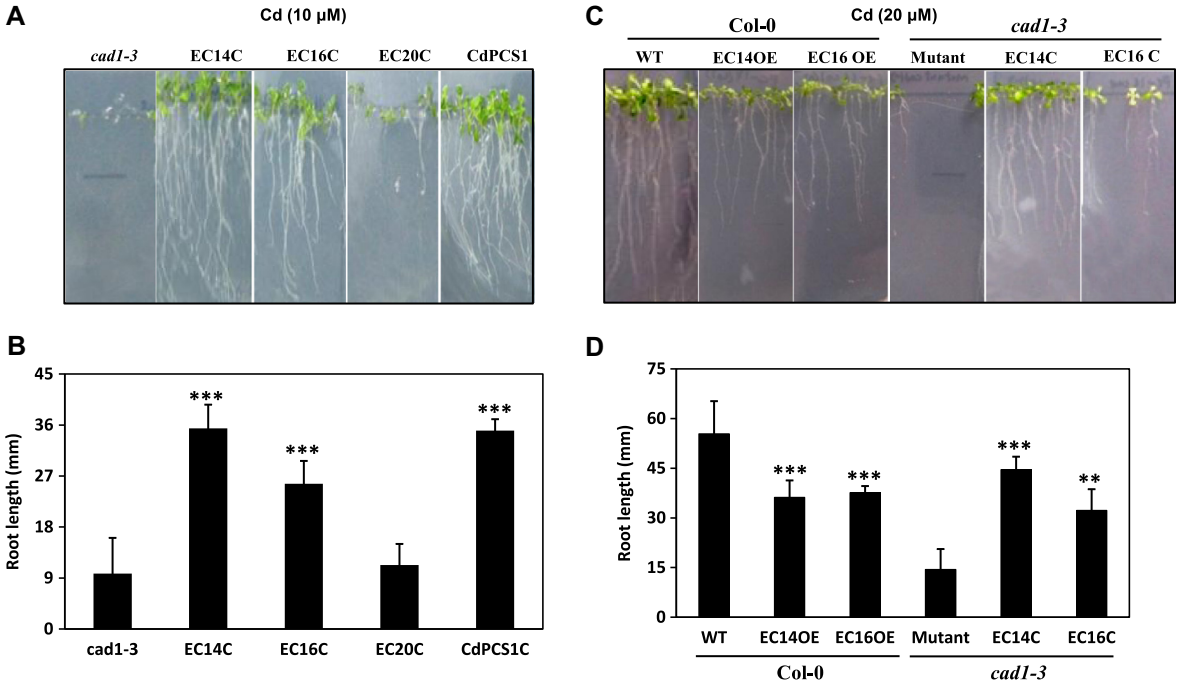

Fig. 2. The effect of Cd stress on transgenic lines expressing *EC* genes in *Arabidopsis*. (A) The phenotype of *cad1-3* mutant and various lines complemented with *EC14*, *EC16*, *EC20*, and *CdPCS1* grown on Cd (10 μM). *EC14*- and *CdPCS1*-expressing lines were equally capable of complementing the *cad1-3* mutant. (B) The root lengths of the *cad1-3* mutant and various lines complemented with *EC14*, *EC16*, *EC20*, and *CdPCS1* grown on Cd (10 μM). (C) The effect of 20 μM Cd on the growth of WT, *cad1-3*, and transgenic plants expressing *EC14* and *EC16* in Col-0 and *cad1-3* backgrounds. EC14OE and EC16OE showed significant growth retardation compared with WT. EC14C and EC16C complemented *cad1-3* less effectively. (D) The root lengths of Col-0 and *cad1-3* mutants expressing *EC14* and *EC16* grown on Cd (20 μM). The data shown are the average ± SD of three independent experiments, with each experiment consisting of 10 individual plants. ** and *** indicate values that differ significantly from their respective controls at $P < 0.01$ and $P < 0.001$, respectively, according to Student's paired *t*-test.

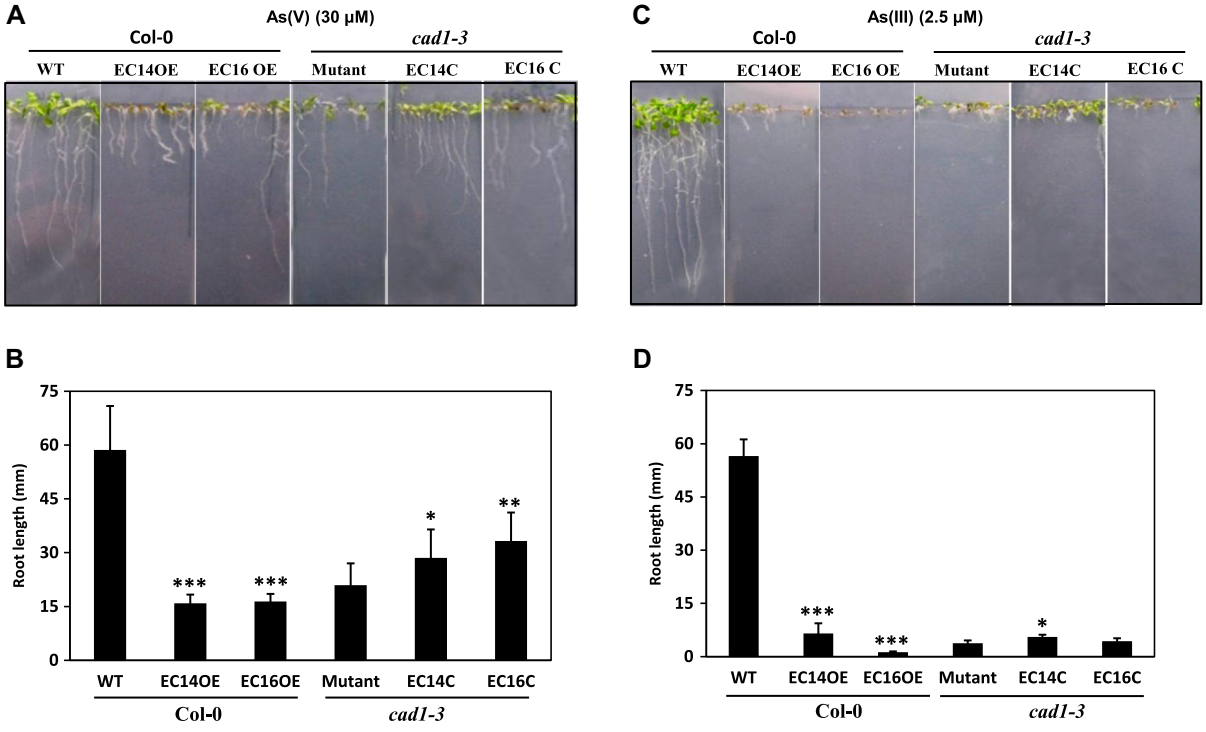

Fig. 3. The effect of arsenic stress on transgenic, *cad1-3* mutant, and WT plants. (A) The effect of 30 μM As(V) on the growth of WT, *cad1-3*, and transgenic plants expressing *EC14* and *EC16* in Col-0 and *cad1-3* backgrounds. EC14OE and EC16OE showed significant growth retardation compared with WT. EC14C and EC16C complemented *cad1-3* less effectively. (B) The root lengths of Col-0 and *cad1-3* mutant plants expressing *EC14* and *EC16* grown on As(V) (30 μM). (C) The effect of 2.5 μM As(III) on the growth of WT, *cad1-3*, and transgenic plants expressing *EC14* and *EC16* in Col-0 and *cad1-3* backgrounds. (D) The root lengths of Col-0 and *cad1-3* mutants expressing *EC14* and *EC16* grown on As(III) (2.5 μM). The data shown are the average ± SD of three independent experiments ($n = 3$), with each experiment consisting of 10–12 individual plants. *, **, and *** indicate values that differ significantly from their respective controls at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to Student's paired *t*-test.

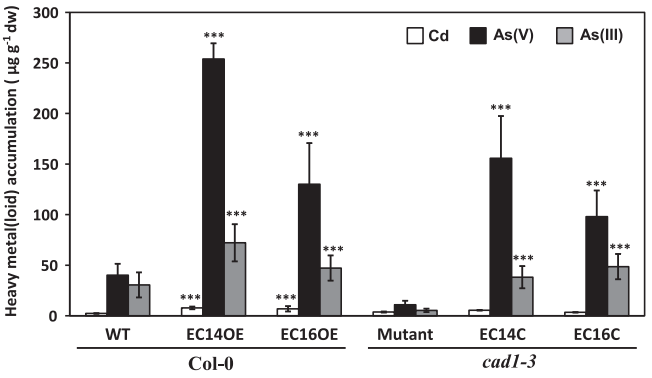

Fig. 4. Heavy metal(loid) accumulation in *Arabidopsis* expressing ECs exposed to heavy metal(loid)s. Sterilised homozygous seeds were germinated in plastic dishes containing Soilrite, irrigated with nutrient medium, and grown for 20 days. The plants were allowed to grow for 10 more days in the presence of 100 μM $CdCl_2$, 300 μM As(V), and 25 μM As(III) within the nutrient medium and were irrigated every 3 days. The plant samples were dried, digested, and subjected to AAS with a graphite furnace to determine metal(loid) concentrations. The data shown are the average ± SD of three independent experiments normalised to μg of metal(loid) s g^{-1} dry plant tissue. ** and *** indicate values that differ significantly from their respective controls at $P < 0.01$ and $P < 0.001$, respectively, according to Student's paired *t*-test.

content was estimated after growing plants under heavy metal exposure. Three-week-old WT and transgenic lines were grown in the presence of $CdCl_2$ (100 μM), As(V) (300 μM), or As(III) (25 μM) for 10 days. The aerial tissues were harvested, dried, and used for metal accumulation experiments. A 1.55- to 6.5-fold higher accumulation was observed in transgenic *Arabidopsis* (Col-0) plants expressing ECs compared with WT plants (Fig. 4). A 3.45-, 6.5-, and 2.37-fold higher metal accumulation was observed for *EC14*-expressing transgenic *Arabidopsis* plants compared with WT plants grown under Cd, As(V), and As(III) exposure, respectively (Fig. 4). The accumulation capacity of EC14-expressing plants was higher than that of EC16-expressing plants grown under heavy metal(loid) stress (Fig. 4). An estimation of metal(loid) accumulation was also performed in mutant and *cad1-3* complemented lines, and a similar metal-accumulation pattern was obtained in these lines. A 1.5-, 14.2-, and 7.0-fold higher metal(loid) accumulation was observed in EC14-expressing transgenic lines relative to the *cad1-3* mutant in plants grown under Cd, As(V), and As(III) stress, respectively (Fig. 4). In general, the *EC14*-complementing line showed a higher accumulation of As(V) and Cd(II) than the *EC16*-complementing line. However, under As(III) exposure, metalloid accumulation was not significantly different among the EC-complementing lines. These results clearly suggest that the expression of EC enhances the heavy metal(loid) accumulation potential of *Arabidopsis* plants.

4. Discussion

ECs can be used as an alternative to PCs because of sequence similarity and may offer high affinity binding for heavy metals [10–13]. Previously, researchers have speculated that the metal-binding affinity between ECs and PCs may differ because of the difference in the peptide bond. However, some studies have demonstrated that ECs can bind to heavy metals. The binding of ECs to heavy metals was more effective than the binding of MTs to heavy metals [10,11]. These observations were obtained by the expression of different lengths of ECs in bacteria. However, the use of ECs for metal accumulation in plants has not been studied. In the present study, we designed artificial genes encoding ECs of varying lengths with plant-specific codon optimisation (Supplementary Table S1) and expressed these genes in *Arabidopsis*. Our study suggests that ECs can work in a similar manner to PCs because the expression of the EC genes *EC14*, *EC16*, and *EC20* complemented and rescued the sensitive phenotype of an *Arabidopsis* mutant (*cad1-3*) grown in the presence of different heavy metal(loid)s.

The complementation ability of EC genes (*EC14*, *EC16*, and *EC20*) was evaluated by expression of these genes in the *cad1-3* mutant grown under heavy metal(loid) exposure. Similar to *CdPCS1*, complementation of the *cad1-3* mutant was observed with expression of different EC genes. However, the strongest complementation was observed with *EC14*. This result suggested that ECs might work in a similar manner as PCs (Fig. 2). Our results also suggested that the small chain length peptide (EC14) was more effective than the longer chain EC (EC16 or EC20). One possible explanation is that longer EC metal complexes cannot be easily transported across the tonoplast.

The expression of *EC genes* in *Arabidopsis* (Col-0) led to a sensitive phenotype, which was more pronounced for As than for Cd exposure. After entering the cell, metalloids become chelated with non-protein thiols and are subsequently sequestered into the vacuole. Recently, some vacuolar transporters of PCs have been identified, suggesting an ATP-dependent process [19–21]. The *de novo* synthesis of ECs may lead to high concentrations of EC–metal complexes in the cytosol. The subsequent sequestration of this large quantity of complexes into vacuoles might be limiting. In such a case, a supraoptimal concentration of EC-Cd/As complexes in the cytosol would cause toxicity, resulting in a sensitive phenotype [4,21,22]. Similar results have been obtained with overexpression of different *PCS* genes, leading to enhanced synthesis of PCs [4,6,21]. A significantly higher metal(loid) accumulation was also observed in the aerial part of soil-grown mature EC-expressing *Arabidopsis* plants, suggesting that ECs can be used for enhancing the metal accumulation potential of plants. Previous studies suggested that PCs have a role in the long distance transport of heavy metal(loid)s through either the xylem or phloem [23–25]. We suggest that a similar mechanism of long distance transport of heavy metal(loid)s might also be operating for ECs. This mechanism might be one of the reasons for enhanced heavy metal(loid) accumulation in the aerial tissue observed in this study.

In conclusion, *EC* genes are capable of rescuing the heavy metal(loid)-sensitive phenotype of the PC-deficient *cad1-3* mutant. The sensitive phenotype of transgenic *Arabidopsis* plants expressing *EC* genes to heavy metal(loid) exposure is similar to that observed for enhanced synthesis of PCs by overexpressing the *PCS* gene. ECs might work in a similar manner to PCs. Additionally, a significantly higher heavy metal(loid) accumulation observed in EC-expressing *Arabidopsis* plants suggests that ECs can be used in the phytoremediation of heavy metal(loid)s.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.138.

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