Arabidopsis thaliana phytochelatin synthase 2 is constitutively active in vivo and can rescue the growth defect of the PCS1-deficient cad1-3 mutant on Cd-contaminated soil

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Abstract
Phytochelatins play a key role in the detoxification of metals in plants and many other eukaryotes. Their formation is catalysed by phytochelatin synthases (PCS) in the presence of metal excess. It appears to be common among higher plants to possess two PCS genes, even though in Arabidopsis thaliana only AtPCS1 has been demonstrated to confer metal tolerance. Employing a highly sensitive quantification method based on ultraperformance electrospray ionization quadrupole time-of-flight mass spectrometry, we detected AtPCS2-dependent phytochelatin formation. Overexpression of AtPCS2 resulted in constitutive phytochelatin accumulation, i.e. in the absence of metal excess, both in planta and in a heterologous system. This indicates distinct enzymatic differences between AtPCS1 and AtPCS2. Furthermore, AtPCS2 was able to partially rescue the Cd hypersensitivity of the AtPCS1-deficient cad1-3 mutant in a liquid seedling assay, and, more importantly, when plants were grown on soil spiked with Cd to a level that is close to what can be found in agricultural soils. No rescue was found in vertical-plate assays, the most commonly used method to assess metal tolerance. Constitutive AtPCS2-dependent phytochelatin synthesis suggests a physiological role of AtPCS2 other than metal detoxification. The differences observed between wild-type plants and cad1-3 on Cd soil demonstrated: (i) the essentiality of phytochelatin synthesis for tolerating levels of Cd contamination that can naturally be encountered by plants outside of metal-rich habitats, and (ii) a contribution to Cd accumulation under these conditions.

Key words: Arabidopsis, metal tolerance, Cd tolerance, Cd contamination, PCS overexpression, phytochelatins, LC-MS.

Introduction
Due to their sessile nature, plants have to cope with various biotic and abiotic stress conditions. Changing availabilities of heavy metals are among the latter. They pose a threefold challenge for plants. First, essential micronutrients such as Fe, Zn, Cu, Mn, and Mo have to be acquired in sufficient amounts. Secondly, their toxic effects have to be limited when present in supraoptimal concentrations. Thirdly, the co-occurrence of non-essential heavy metals or metalloids such as Cd and As, which are simply toxic and taken up as a result of their chemical similarity to essential nutrients, i.e. through transport proteins with a limited substrate specificity, necessitates an array of rapid and flexible but also specific detoxification mechanisms.

Abbreviations: ANOVA, analysis of variance; CAPS, cleaved amplified polymorphic sequence; DTPA, diethylenetriaminepentaacetic acid; EMM, Edinburgh’s minimal medium; EPPS, 4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid; HA, haemagglutinin; LLOD, lower limit of detection; LLOQ, lower limit of quantification; mBrB, monobromobimane; MES, 2-(N-morpholino)-ethanesulfonic acid; NAC, N-acetylcysteine; OD, optical density; PC, phytochelatin; PCS, phytochelatin synthase; SD, standard deviation; TCEP, Tris-(2-carboxyethyl)-phosphine, UPLC-ESI-QTOF-MS, ultraperformance electrospray ionization quadrupole time-of-flight mass spectrometry.

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One key component of the response to at least challenges two and three in all higher plants is the metal-activated synthesis of phytochelatins (PCs). These are small cysteine-rich peptides of the general structure (γ-Glu-Cys)n-Gly with chain lengths from \( n=2\)–7 (usually referred to as PC2–PC7) in vivo (Grill et al., 1985). Their synthesis is catalysed by the enzyme phytochelatin synthase (PCS) in the presence of a range of heavy-metal ions, which are then complexed by the free thiol groups of the formed PCs (Grill et al., 1987, 1989). In Arabidopsis thaliana, this metal-dependent transpeptidation reaction is carried out by AtPCS1, encoded by the CAD1 locus (Ha et al., 1999). Loss of function of this gene results in a highly Cd-hypersensitive mutant, namely cad1-3. Later, it was found that cad1-3 is also Zn hypersensitive (Tennstedt et al., 2009). The cad1-3 mutant was characterized as PC deficient (Howden et al., 1995b). Therefore, it was unexpected when a second PCS gene, AtPCS2, was found (Cazalé and Clemens, 2001).

AtPCS2 shares 84% identity at the amino acid level with AtPCS1 and the exon–intronic structure of the respective genes is similar. However, the intron sequences differ by 60–80%, suggesting a longer coexistence of both genes. The AtPCS2 transcript can be detected in shoots and roots, albeit at much lower levels than for AtPCS1 (Ha et al., 1999; Cazalé and Clemens, 2001). Fusion proteins of AtPCS1 and enhanced green fluorescent protein (AtPCS1:eGFP) expressed under control of the endogenous promoter have been detected in the cytoplasm throughout the whole plant, while the respective AtPCS2:eGFP fusion showed a signal only in the root tip (Blum et al., 2010).

To date, no function has been ascribed to AtPCS2 in planta. While heterologous expression of AtPCS2 conferred Cd tolerance to Schizosaccharomyces pombe as well as Saccharomyces cerevisiae (Cazalé and Clemens, 2001) and thereby indicated that this gene does encode a functional PCS, overexpression in cad1-3 led to at most a partial complementation upon Cd treatment (Lee and Kang, 2005). Moreover, the presence of a functional PCS2 gene in cad1-3 is not sufficient to confer Cd and Zn tolerance, as demonstrated by the pronounced hypersensitivity of this mutant. Finally, no mutant phenotype has yet been reported for an atpcs2 mutant (Blum et al., 2007).

More recently, AtPCS1 has been implicated not only in Zn tolerance (Tennstedt et al., 2009) but also in pathogen defence. A typical A. thaliana innate immune response—callose deposition activated by exposure to the microbe-associated molecular pattern flagellin (flg22)—was reported to be dependent on AtPCS1 (Clay et al., 2009). Both functions suggest PCS activities in the absence of toxic metal stress and a physiological role also of constitutive low-level PC synthesis. Therefore, the ability to detect PCs with high sensitivity is necessary. Furthermore, it is important to elucidate the possible contribution of AtPCS2 to PC synthesis in A. thaliana.

In this study, a highly sensitive method for PC2 and PC3 detection using ultraperformance electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS) is reported. This analytical approach enabled the detection of background levels of PC2 in cad1-3, which were absent in the generated cad1-3 atpcs2 double mutant. This strongly suggests AtPCS2-dependent constitutive PC synthesis. Furthermore, it was found that AtPCS2 was able to rescue the Cd hypersensitivity of cad1-3 when tested on Cd-contaminated soil. Interestingly, AtPCS2-dependent PC synthesis was not enhanced by the presence of Cd\(^{2+}\), which is in contrast to what was observed for AtPCS1.

Materials and methods

Plant lines

An A. thaliana T-DNA line with an insertion in an exon of the AtPCS2 gene was available from public repositories in the Wassilewskija (Ws-0) background only (Alonso et al., 2003; http://signal.salk.edu/cgi-bin/dnaexpress, last accessed 22 April 2014). Homozygous atpcs2 lines (FLAG_146G12 (Samson et al., 2002)) were identified via PCR using the T-DNA border primer TAG5 (5′-CTCAAAATGCGCTTTCTATGCAC-3′) and the gene-specific primer PCS2_rev (5′-GCGAGTTGT CTTCGTACACAGAG-3′). For detection of the wild-type fragment, PCS2_rev was combined with primer PCS2_fw (5′-GATGAATCTGTAAGAGTGGCTAGATGG-3′) (Supplementary Fig. S1 at JXB online). The PCS single mutants cad1-3 (Howden et al., 1995b) and atpcs2 were crossed to obtain the double mutant cad1-3 atpcs2. Homozygous lines were identified with the above-mentioned primer combinations for the AtPCS2 locus. The G/C nucleotide exchange in the cad1-3 mutant was scored by PCR with PCR primer pair AtPCS1_fw (5′-CCCGAAATTTGTGCTCAAAATG-3′) and cad1-3_mut_rev (5′-CCCAGAAGGTGTTAAGAGACG-3′) (annealing at 62 °C), which contained in addition to the mutated C nucleotide at the 3′ end a second mismatched base at position 3 from the 3′ end. The wild-type allele was identified using the primer combination cad1-3_wt_fw (5′-CAAGTTATCCCCTACCCG-3′) and AtPCS1_rev (5′-CTGAAGCCCTGGACCAACACAGA-3′) (annealing at 61 °C). For a quality check of the genomic DNA, the primer pair AtPCS1_fw/AtPCS1_rev was used.

The genetic backgrounds of cad1-3 and atpcs2 are Col-0 and Ws-0, respectively. Col-0 and Ws-0 are known to differ in a gene related to Cd transport. Col-0 carries a single base-pair deletion in AtHMA3, a P_{in}APase gene that influences shoot Cd accumulation (Chao et al., 2012). As the cad1-3 atpcs2 double mutant was obtained by crossing of the respective single mutants in the Col-0 or Ws-0 background, the genotype of AtHMA3 in the double mutant was verified by a cleaved amplified polymorphic sequence (CAPS) marker based on the single-nucleotide deletion in the Col-0 allele. An AtHMA3 fragment was amplified by PCR using the primer pair AtHMA3_CAPS_fw (5′-AGAGAGCTGAGTTACACAGTC-3′) and AtHMA3_CAPS_rev (5′-TACCATATTGGGTCGCGTTG-3′) and followed by DdeI (NEB) digestion. The double mutant used in this study was homozygous for the Col-0 allele of AtHMA3 (Supplementary Fig. S2 at JXB online).

Generation of lines overexpressing AtPCS1, driven by the cauliflower mosaic virus 35S promoter in the cad1-3 background, has been described previously (Tennstedt et al., 2009). AtPCS2-overexpression lines were obtained correspondingly. Both gene constructs add a hae-3′ end and a second mismatched base at position 3′ end a second mismatched base at position 3′ end (annealing at 62 °C), which contained in addition to the mutated C nucleotide at the 3′ end a second mismatched base at position 3. The wild-type allele was identified using the primer combination cad1-3_wt_fw (5′-CAAGTTATCCCCTACCCG-3′) and AtPCS1_rev (5′-CTGAAGCCCTGGACCAACACAGA-3′) (annealing at 61 °C). For a quality check of the genomic DNA, the primer pair AtPCS1_fw/AtPCS1_rev was used.

Plant growth

Seeds of A. thaliana were surface sterilized by exposure to chlorine gas (produced by adding 5 ml of 32% HCl to 10 ml of sodium hypochlorite solution) in a desiccator for 35 min.

The plant growth medium used was based on one-tenth-strength modified Hoagland’s solution No. 2 (0.28 mM Ca(NO\(_3\))\(_2\), 0.6 mM KNO\(_3\), 0.1 mM NH\(_4\)H\(_2\)PO\(_4\), 0.2 mM MgSO\(_4\), 4.63 mM H\(_2\)BO\(_3\), 32 mM
CuSO₄, 915 nM MnCl₂, 77 nM ZnSO₄, 11 nM MoO₃] (Hoagland and Arnon, 1950). Fe was supplied as N,N′-di-(2-hydroxybenzoyl)-ethylenediamine-N,N′-diacetic acid according to the method of Chaney (1988) to a final concentration of 5 µM. Tolerance assays were performed without microelements, except for iron, either in a liquid seedling assay or on agar plates in medium containing 1% (w/v) sucrose, 0.05% (w/v) MES at pH 5.7. Cd²⁺ was added as the chloride salt. Liquid seedling assays were performed in six-well tissue culture plates with six seeds per well in 5 ml of medium. For agar plate assays, the medium was solidified with 1% (w/v) Agar (Sigma-Aldrich). Plates of both assay types were sealed with Leucopore tape (Duchefa). Following stratification for 2 d at 4 °C, the seedlings were incubated under long-day conditions (16 h light/8 h dark) at 22 °C. The agar plates were placed vertically and the liquid assay was shaken gently.

For PC analysis, plants were grown hydroponically for 6.5 weeks under short-day conditions (8 h light, 22 °C/16 h dark, 18 °C) in one-tenth-strength modified Hoagland’s solution including all microelements without sucrose and modified concentrations of Ca(NO₃)₂ (0.4 mM) and (NH₄)₂HPO₄ (0.0871 mM). Cultivation started in agar-filled PCR tubes in pipette tip boxes for 3 weeks followed by transfer into 50 ml tubes (Greiner Bio-One) for another 3 weeks before addition of 0.5 µM or 5 µM CdCl₂. No additional heavy-metal ions were added to the respective controls. To guarantee a sufficient oxygen and mineral supply, the medium was changed weekly throughout cultivation.

Experiments on artificially contaminated soil were conducted under short-day conditions (8 h light, 22 °C/16 h dark, 18 °C) using a mineral soil type that was prepared as follows. After drying the soil at 70 °C, organic compounds were removed to the greatest possible extent by sieving. Five hundred grams of soil (dry weight) in plastic bottles and 250 ml of water containing 10 mg CdCl₂ monohydrate were mixed thoroughly for 1 h in an overhead shaker (Reax 20/12; Heidolph) until a homogeneous soil solution was obtained. Control soil was mixed with water only. Afterwards, the soil was dried, sieved again, and mixed to one-third with sand featuring a low ion-binding capacity. In order to achieve a high level of comparability, pots were filled with equal amounts of the prepared soil and moistened with the same volume of water. After loosening the humid soil, seedlings were transferred into this soil 10 d after germination on solid one-tenth-strength Hoagland medium. Plants were grown in a randomized manner for 24 d and all plants were irrigated daily with equal volumes of water. Furthermore, in order to minimize desiccation gradients that could influence metal availability and uptake, experimental plants were surrounded by a belt of control plants that were excluded from data acquisition. At the end of the experiment, two sample pools per plant line and condition were formed in the same volume of water. After removal of soil particles by washing with Millipore water, the pooled leaf material was frozen in liquid nitrogen.

Quantification of leaf area

The growth of plants on artificially contaminated soil was tracked by quantification of the leaf area. Single plants were photographed every 7 d after the transfer to soil and at the end of the experiment. Leaf area was measured using Adobe Photoshop CS2 version 9.0 by counting the number of pixels representing the leaf shape and normalizing to the number of pixels per pot.

Elemental analysis via inductively coupled plasma optical emission spectroscopy

One part of the pooled leaf material from soil-grown plants was freeze dried and digested in 4 ml of 65% HNO₃ and 2 ml of 30% H₂O₂ using a microwave (START 1500; MLS GmbH). Cd content was measured with an iCAP 6500 (Thermo Scientific) at a wavelength 226.5 nm. For the determination of extractable and exchangeable soil metal contents, 3 g of dried and sieved soil was extracted with 25 ml of the respective solution using a rotator (SB2, Stuart). The extractable metal content was analysed in extracts with 0.1 M HCl (30 min, 23 °C) (Kuo et al., 2006), whereas the exchangeable portion was measured after incubation for 2 h at 23 °C with either 10 mM CaCl₂ (Houbá et al., 2000) or 5 mM diethylenetriaminepentaacetic acid (DTPA) containing 0.1 M triethanolamine and 10 mM CaCl₂ (pH 7.3) (Lindsay and Norvell, 1978). If required, samples were diluted in 2% HNO₃ for the metal analysis.

PC analysis

PC concentrations were measured in plant material obtained from three different experimental setups: (i) whole seedlings after 11 d of growth in liquid one-tenth-strength Hoagland medium, control or exposed to 0.5 µM CdCl₂; (ii) leaf and root material from 6.5-week-old plants grown in hydroponic culture, untreated or treated for 3 d with 0.5 µM or 5 µM CdCl₂; and (iii) leaf material after 24 d of growth on soil with or without 7.5 mg Cd²⁺ kg⁻¹ of soil. All plant material was frozen in liquid nitrogen and ground to a homogenous powder. One hundred milligrams (fresh weight) was extracted with 300 µl of 0.1% (v/v) trifluoroacetic acid containing 6.3 mM DTPA (Sneller et al., 2000) and 40 µM N-acetylcysteine (NAC) as an internal standard. The plant material was suspended completely by exhaustive mixing. The homogenate was chilled on ice for 15 min and mixed occasionally. Following centrifugation (16 000g, 15 min, 4 °C), the PC-containing supernatant was reduced and derivatized based on the methods of Rijstenbil and Wijnholds (1996) and Sneller et al. (2000), as described by Thangavel et al. (2007) and Minocha et al. (2008). Derivatization was performed to achieve a better retention on reversed-phase column material. Briefly, 62.5 µl of the extract or a mix of the respective standards was added to 154 µl 200 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid (EPPS) (6.3 mM DTPA, pH 8.2) and 6.25 µl 20 mM Tris-(2-carboxyethyl)-phosphine (TCEP; prepared freshly in 200 mM EPPS, pH 8.2) and incubated for 10 min at 45 °C. Afterwards, 5 µl of 50 mM monobromobinane (mBrB; in acetonitrile) started the labelling reaction, which was performed for 30 min at 45 °C. The reaction was stopped by the addition of 25 µl of 1 M methanesulfonic acid. In contrast to former methods (Rijstenbil and Wijnholds, 1996; Sneller et al., 2000; Thangavel et al., 2007; Minocha et al., 2008), the internal standard NAC was already present in the extraction buffer in order to minimize variation between samples. Samples were centrifuged at 16 000g for 4 °C prior to analysis. A Waters Aquity UPLC system equipped with an HSS T3 column (1.8 µm, 2.1 × 100 mm; Waters Corporation, Milford, MA) was used for the separation of the mBrB-labelled thiols. The injection volume was 5 µl. A 15 min linear binary gradient of water (A) and acetonitrile (B), both acidified with 0.1% (v/v) formic acid, at a flow of 0.5 ml min⁻¹ was employed: 99.5% A, 0.5% B for 1 min, a linear gradient to 60.5% B at 10 min, gradient to 99.5% B at 12 min, flushing with 99.5% B for 1 min, a gradient back to initial conditions in 1 min and an additional re-equilibration for 1 min. The column temperature was set to 40 °C. Thiols were detected with a Q-TOF Premier mass spectrometer equipped with an ESI-source (Waters Corporation) operated in the V+ mode. The mass spectrometer was tuned for optimal sensitivity using leucine enkephalin. Basic parameters were: capillary 0.6 kV, sampling cone 30V, extraction cone 30V, ion guide 3.3 V, source temperature 120 °C, cone gas flow 10 l h⁻¹, desolvation gas flow 1000 l h⁻¹, collision energy 4.0 V. Data were acquired from m/z 300–2000 with a scan time of 0.3 s and an inter-scan delay of 0.05 s. For quantification the QuanLynx module of the MarkerLynx software was used. PC2 and PC3 were quantified by integration of the reconstructed ion traces of the protonated ions [M+H]+ m/z 354.1 ± 0.5 at 4.39 ± 0.3 min for mBrB–NAC (internal standard), m/z 920.3 ± 0.5 at 4.57 ± 0.3 min for mBrB–PC2 and the added ion traces of m/z 1342.4 + [M+2H]²⁺, and m/z 671.7 ± 0.5 at 5.06 ± 0.3 min for mBrB–PC3. Using a linear calibration forced through the origin (125, 250, and 500 nM and 2.5, 5, and 12.5 µM PC), the average R² was 0.98 for PC2 and 0.96 for PC3. All samples were measured in three technical replicates.
Western blotting

For detection of HA-tagged PCS proteins, leaf material was extracted with 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM dithiothreitol, 0.1% protease inhibitor mix (Roche), 0.1% (w/v) SDS and 0.1% (v/v) Triton-X-100. Total protein contents were determined by the bicinchoninic acid method (ThermoScientific). Protein extracts were separated on a denaturing 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (Protran134; Whatman). The PCS proteins were detected with monoclonal anti-HA antibody (Sigma-Aldrich; 1:3000 diluted) and stained using anti-mouse antibody (Sigma-Aldrich, 1:10 000 diluted) coupled to horseradish peroxidase and using the enhanced chemiluminescence method.

Transcript analysis

Total RNA extraction from whole seedlings and quantitative real-time RT-PCR were performed as described previously (Deinlein et al., 2012). cDNA synthesis was performed using a first-strand cDNA synthesis kit (Thermo Scientific) followed by quantitative real-time PCR using iQ SYBR Green supermix (BioRad). In order to evaluate the Zn and Cu status of seedlings grown in the modified Hoagland solution without microelements other than Fe, Zn9 (At4g33020), and CCH (At3g56240) and COX5b-1 (At3g15640) were used as established molecular markers for Zn and Cu deficiency, respectively (Talke et al., 2006; Bernal et al., 2012). Elongation factor 1α (At5g60390) served as a reference gene. For primer sequences, see Supplementary Table S2 at JXB online.

Heterologous expression of AtPCS1 and AtPCS2 in Schizosaccharomyces pombe

The Schizosaccharomyces pombe PCS knockout strain Δpcs carrying the vector constructs pSGP72-AtPCS1-HA or pSGP72-AtPCS2-HA were used for heterologous expression of PCS proteins (Clemens et al., 1999; Cazalé and Clemens, 2001). Cells carrying the empty vector served as negative control. Yeast cultivation was carried out at 30 °C in Edinburgh’s minimal medium (EMM; Moreno et al., 1991) containing 20 µM thiamine to suppress PCS expression. To monitor thiol profiles, pre-cultured cells were inoculated to an optical density of 600 nm (OD600) of 0.05 in EMM supplemented with 20 µM thiamine and grown overnight. The cells were then washed twice in thiamine-free EMM and inoculated at an OD600 of 0.4 in thiamine-free EMM in the presence or absence of extra metal ions. After 6 h incubation, the cells were harvested and lyophilized. Extraction, labelling and quantification of PCs were carried out as described above for plant samples.

For detection of the HA-tagged PCS proteins, protein extracts were prepared from cells before and after 6 h cultivation in thiamine-free EMM in the absence of Cd2+. Western blot analysis was performed as described above.

Statistical analysis

Statistical analyses were performed with SigmaPlot 11.0.

Results

Analysis of PCS2 presence in the plant kingdom

Few studies to date have reported evidence for the existence of a second PCS gene in species other than A. thaliana. Legumes such as Lotus japonicus were found to carry three PCS genes probably originating from two duplication events (Ramos et al., 2007). Metal-hyperaccumulating Brassicaceae Arabidopsis halleri and Noccaea caerulescens both possess two PCS genes like their relative A. thaliana (Meyer et al., 2011). Therefore, a search for PCS2-like genes among sequenced plant genomes was performed. Analysis of derived amino acid sequences revealed separate clusters of PCS1-like and PCS2-like proteins in Brassicaceae and Fabaceae (Fig. 1). Two distinct PCS genes were also found in monocot genomes. Thus, the presence of at least two PCS genes appears to be a general feature of plant genomes. This reinforces the need to functionally understand the role of PCS2 genes.

Development of a sensitive method for PC detection

The contribution of AtPCS2 to metal homeostasis and tolerance is to date unclear. No evidence for in planta transpeptidase activity, which should be discernible as PC production in cad1-3, has been reported yet. In order to evaluate if and how AtPCS2 contributes to the PC pool and thereby potentially to the buffering of free metal ions within the cell, it was necessary to establish a highly sensitive method for the detection of trace amounts of PCs. Coupling of reversed-phase LC to ESI-QTOF-MS enabled such PC detection (Bräutigam et al., 2009; Simmons et al., 2009; Tennstedt et al., 2009).

Fig. 1. A second PCS is encoded in the genome of monocot and dicot species. PCS1 and PCS2 protein sequences available for species representing the Poaceae, Brassicaceae, and Fabaceae were subjected to hierarchical cluster analysis. The dendrogram was generated on the basis of a ClustalW+ protein sequence alignment using the protein maximum-likelihood (PhyML) algorithm (http://www.trex.ucam.ca, last accessed 22 April 2014; Boc et al., 2012). Sequences included in the analysis are listed as follows (GenBank accession numbers in brackets): Cynodon dactylon (AAO13810, AAS48642), Sorghum bicolor (XP_002454970, XP_002454971), Oryza sativa (EEE64936, NP_001055554), Arabidopsis lyrata (XP_002865384, XP_002865385), Arabidopsis halleri (AAS45236, ADZ24787), Arabidopsis thaliana (NP_199220, AAK94671), Noccaea caerulescens (AAT0746, ABIY9866), Lotus japonicus (AAT80342, AAT80341), and Glycine max (NP_001235576, XP_003537353). Bar, 0.1 is equal to 10% sequence divergence. Numbers indicate bootstrap values (%).
In this study, mBrB-derivatized thiols, reduced with TCEP, were analysed via UPLC-ESI-QTOF-MS. The detection and quantification limits of the system were determined by examining the recovery rates of PC2–PC5 over a broad concentration range from 7.8 nM to 50 µM in extraction buffer and in matrix (Col-0 leaf extracts). Fig. 2 shows the correlations between the adjusted final concentrations of PC2 and PC3 injected, and the detector response relative to the internal standard NAC. Broken lines indicate the linear ranges with an $R^2 > 0.99$. They extended well into the nanomolar range (see insets in Fig. 2) and were not influenced by leaf extracts. Matrix effects were apparent, however, when comparing recovery rates, i.e. slopes of the response curves in the linear range. Leaf matrix slightly reduced the PC2 response (by about 9%) and had a more pronounced effect on PC3 (reduction of about 37%). The saturation that the response curves for PC2 and PC3 showed at concentrations $>25$ µM was negligible because concentrations above $13$ µM were never observed, even in extracts derived from Cd$^{2+}$-treated plants.

Lower limits of detection (LLOD; signal:noise ratio $>3$) of 50 and 100 fmol for PC2 and PC3, respectively, were determined. This translates into about a 4000-fold gain in sensitivity compared with the LLLOD for PC2 obtained by conventional HPLC analysis as reported by Howden et al. (1995b) and applied for the initial characterization of the cad1-3 mutant. Compared with studies employing HPLC of mBrB-derivatized PCs or LC-MS analyses of non-derivatized PCs, a gain of between about 20-fold (Simmons et al., 2009) and about 2-fold (Minocha et al., 2008) was achieved.

PC4 and PC5, which were part of the standard mix, were not included in later PC quantification due to the high $m/z$ ratio of their protonated ions of [M+H]$^+$ 1764.5 and 2186.7, respectively. The necessary expansion of the routinely used calibration range ($m/z$ 300–2000) and scan time would have resulted in a loss of sensitivity for PC2 and PC3. Moreover, the summed ion traces of [M+H]$^+$ and [M+2H]$^{2+}$ for PC4 and PC5 constituted maximally a small proportion of the total PC concentrations found.

Detection of PCs in cad1-3

Equipped with this highly sensitive method for PC detection, the contribution of AtPCS2 to PC accumulation was re-examined. A T-DNA line in the Ws-0 background with an insertion in exon 6 of AtPCS2 was obtained (Supplementary Fig. S1). Also, a double mutant was generated by crossing this atpcs2 mutant line with cad1-3. The genetic backgrounds of cad1-3 and atpcs2 are Col-0 and Ws-0, respectively. Because Col-0 in contrast to Ws-0 does not carry a functional allele of AtHMA3, a P1B-ATPase gene that influences shoot Cd accumulation (Chao et al., 2012), we selected a double mutant that was homozygous for the Col-0 allele of AtHMA3 (Supplementary Fig. S2).
Single mutants, the double mutant, and the respective wild types Col-0 and Ws-0 were grown in hydroponic culture and treated for 3 d with 0.5 or 5 µM CdCl₂ in addition to the regular microelement concentrations. Leaf and root material was analysed separately for PC accumulation via UPLC-ESI-QTOF-MS (Fig. 3). In roots of plants cultivated under control conditions, PC2 was detectable in both wild types and the single mutants, thus demonstrating PC formation even in the absence of any metal excess (Fig. 3A). PC3 was not detected in root extracts. No PCs above the lower limit of quantification (LLOQ; signal:noise ratio > 10) were found in leaves of plants grown in the absence of Cd²⁺. As expected, Cd²⁺-exposed wild-type plants showed a strong increase in PC accumulation. Following addition of 0.5 µM Cd²⁺, root PC levels were 3-fold (PC2) and 6-fold (PC3) higher than in leaves. At the external Cd²⁺ concentration of 5 µM, this difference disappeared and PC levels in the roots and shoots were similar.

Interestingly, while no growth defect of atpcs2 relative to Ws-0 was detected (data not shown), significant differences in PC2 and PC3 accumulation between the mutant and its corresponding wild type were found. In the presence of an external Cd²⁺ concentration of 5 µM, atpcs2 accumulated PC2 in roots to levels comparable to Col-0 but less than Ws-0. PC3 concentrations were lower than in both wild types. The same trend was visible at 0.5 µM Cd²⁺, which suggested a measurable contribution of AtPCS2 to PC accumulation. In leaves,

Fig. 3. Detection of PC2 formation in cad1-3. The A. thaliana AtPCS1 and AtPCS2 single mutants cad1-3 and atpcs2 as well as their respective wild types Col-0 and Ws-0 and the double mutant cad1-3 atpcs2 were grown hydroponically in one-tenth-strength Hoagland medium with all micronutrients for 6.5 weeks. PC accumulation was analysed by UPLC-ESI-QTOF-MS. (A) PC2 concentrations for plants grown in control medium. Note that, except for PC2 in roots, all values measured under control conditions were below the lower limit of quantification. (B, C) Plants were exposed to 0.5 or 5 µM CdCl₂ for 3 d. Leaves (B) and roots (C) were analysed separately. Data represent means±SD of two independent experiments with six samples in total per plant line and condition. For each sample, material from three plants was pooled. Data were statistically analysed via two-way analysis of variance (ANOVA) and grouped with Tukey's 95% confidence interval. f.w., Fresh weight.
no differences in PC accumulation between \textit{atpcs2} and \textit{Ws-0} were apparent.

More direct evidence for AtPCS2-dependent PC synthesis in roots came from the observation that PC2 accumulated in the leaves and roots of Cd\textsuperscript{2+}-treated \textit{cad1-3} plants to levels above the LLOQ (signal:noise ratio >10) (Fig. 3A–C). In contrast, no PC2 was detectable in any of the root samples derived from the double mutant. Leaf PC2 concentrations in the double mutant were below the LLOQ for plants exposed to 0.5 µM Cd\textsuperscript{2+}. In the presence of 5 µM Cd\textsuperscript{2+}, low amounts of PC2 could be detected in leaves. These were reduced compared with those found in \textit{cad1-3}. No PC3 was detectable in \textit{cad1-3} and \textit{cad1-3 atpcs2}. Only in leaves in the presence of 0.5 µM Cd\textsuperscript{2+} were trace amounts of PC3 found, but these were below the LLOQ.

Comparison of different metal tolerance assays reveals distinct phenotypes

The evidence suggesting AtPCS2-dependent PC accumulation in planta prompted the investigation of a possible rescue of \textit{cad1-3} through \textit{AtPCS2} overexpression. Respective lines expressing \textit{AtPCS2} under the control of the 35S promoter were generated and compared with \textit{cad1-3} lines transformed with a corresponding construct carrying \textit{AtPCS1}. Proteins were expressed with a C-terminal HA tag. Two lines each were selected that differed in protein expression as assessed by western blotting and immunostaining (Fig. 4). When analysed in vertical-plate assays, which clearly reveal the phenotypes of \textit{cad1-3} seedlings (Tennstedt et al., 2009), rescue of \textit{cad1-3} was observed only in 35S::\textit{AtPCS1} lines (Supplementary Fig. S3 at \textit{JXB} online). For lines expressing \textit{AtPCS2}, no more than a slight beneficial effect on shoot development was discernible.

It is now well documented that growth medium and conditions can strongly influence the emergence of metal-related phenotypes (Tennstedt et al., 2009; Gruber et al., 2013). Therefore, the \textit{cad1-3} complementation was tested in additional assays. First, a newly established liquid seedling assay was used. In accordance with the medium conditions of the plate assays (Tennstedt et al., 2009), seedlings were grown in one-tenth-strength Hoagland medium without micronutrients except for Fe. Quantitative real-time PCR tests with established markers for Zn (ZIP9; Talke et al., 2006) and Cu deficiency (\textit{CCH}, \textit{COX5b-1}; Bernal et al., 2012) showed that no micronutrient deficiency developed in Col-0 or \textit{cad1-3} seedlings during the 7 d of cultivation in this assay. Relative transcript levels were only marginally different (Supplementary Table S3 at \textit{JXB} online).

Indeed, the change from solidified to liquid medium indicated partial rescue of \textit{cad1-3} by \textit{AtPCS2} overexpression (Fig. 5B). Due to differences between the lines under control conditions (Fig. 5A), it was necessary to compare their growth in the presence of Cd\textsuperscript{2+} as relative values, as the percentage of growth under control conditions (Fig. 5C). The primary root length of \textit{cad1-3} following Cd\textsuperscript{2+} treatment reached 18% (±8%) of the root growth under control conditions, while roots of the 35S::\textit{AtPCS2} lines 3|4|2 and 11|2|1 grew to 30 and 23%, respectively. The difference was significant for the 35S::\textit{AtPCS2} line 3|4|2 (\(P\ <0.001\)). The second line, 11|2|1, with only weak \textit{AtPCS2} expression, was not significantly different from \textit{cad1-3} (\(P\ =0.106\) but nevertheless tended to have longer roots than \textit{cad1-3} in the presence of Cd\textsuperscript{2+}.
Cd^2+. Mutant lines overexpressing AtPCS1 showed a growth behaviour comparable to that of wild-type seedlings.

To test whether this partial complementation of cadl-3 by 35S::AtPCS2 overexpression was associated with a higher production of PCs, seedlings were analysed for their PC content after growth in the absence or presence of Cd^2+ (Supplementary Fig. S4 at JXB online). AtPCS1-overexpressing lines 3|3 and 9|4 contained similar amounts of PC2 and PC3 and showed a slightly increased PC accumulation compared with the wild-type Col-0 following Cd^2+ exposure. In addition, the 35S::AtPCS2 lines 3|2 and 11|2 were found to produce PC2 and PC3, while in cadl-3 only trace amounts of PC2 were detected. PC2 levels in 35S::AtPCS2 3|2 were comparable to Col-0, while the weakly expressing line 11|2 contained only 40% of the wild-type PC2 level. Both 35S::AtPCS2 lines were strongly impaired in the production of the longer-chain PC3 relative to the wild-type and the 35S::AtPCS1 lines. Interestingly, 35S::AtPCS2 lines showed a 6-fold or 3-fold higher PC2 production in the absence of additional Cd^2+ compared with Col-0. Constitutive PC2 production was also higher than in 35S::AtPCS1 seedlings.

Next, the growth behaviour of representative lines complementing cadl-3 at least partially was tested under conditions that resembled a natural habitat far more closely than in vitro assays. For this, a mineral soil with a low organic content was artificially contaminated with Cd to a final concentration of 7.5 mg kg^{-1} of soil. Extractable Cd as determined via the HCl method was 6.3 ± 0.3 mg Cd kg^{-1} of soil. The bioavailable Cd was estimated through extraction with DTPA or exchange with CaCl_2 which yielded a value of 4.3 ± 0.3 mg and 0.12 ± 0.02 mg Cd kg^{-1} of soil, respectively. In control soil, only traces of Cd below the limit of quantification (0.01 mg kg^{-1} of soil) were detectable. Concentrations of other metals are listed in Supplementary Table S1 at JXB online.

Wild-type Col-0, the cadl-3 mutant and the transgenic lines with stronger expression, 35S::AtPCS1 3|3 and 35S::AtPCS2 3|2, were cultivated on control mineral soil and soil spiked with Cd. After 24 d of growth on this contaminated soil, the Cd hypersensitivity of the cadl-3 mutant was obvious from the strong growth reduction and the leaf chlorosis (Fig. 6A). Plant growth was quantified based on the monitoring of leaf area throughout the course of the experiment (Fig. 6B). In order to account for the variation in the plant size between the two independent experiments, leaf area of plants grown on Cd-contaminated soil was expressed relative to the growth of control plants on soil without additional heavy metals. In this way, a growth reduction of up to 65% was determined for cadl-3, while Col-0 showed an even better growth on the Cd-contaminated soil than on the control soil. Remarkably, overexpression of AtPCS1 and AtPCS2 had an almost equally strong positive effect on growth of cadl-3 plants on Cd soil. Plants were clearly less chlorotic, and growth was impaired by only 15 and 25%, respectively, after d 24. Differences between the two transgenic lines were not significant, whereas already at d 21 the differences between wild-type, cadl-3 mutant, and the overexpression lines were highly significant with P<0.001 for all comparisons. Thus, complementation by AtPCS1 and AtPCS2 was not complete.

PCS activity is well known to influence Cd accumulation in plant tissues (Howden and Cobbett, 1992; Howden et al., 1995a; Pomponi et al., 2006; Li et al., 2007; Tennstedt et al., 2009). Cd concentrations in leaf material of the soil-grown
AtPCS2 encodes a constitutively active PCS and confers Cd tolerance

plants were determined using inductively coupled plasma optical emission spectroscopy (Fig. 6D). In plants cultivated on control soil, no Cd was detected. On Cd-contaminated soil, cad1-3 accumulated about 35% less Cd than Col-0. Both AtPCS1 and AtPCS2 overexpression resulted in an increase in leaf Cd levels. Full reversion to wild-type levels, however, was found only in the 35S::AtPCS1 line.

AtPCS2 shows higher activity than AtPCS1 in the absence of the activating metal Cd

When PC accumulation was analysed, PC2 was again detected even in wild-type plants not exposed to metal stress (Fig. 6C). In contrast, neither PC2 nor PC3 above the LLOD was found in cad1-3 leaves, regardless of soil conditions. 35S::AtPCS1 3|3|3 exhibited higher PC2 and PC3 levels than Col-0 after growth on Cd-contaminated soil, even though it did not show full complementation of the Cd-hypersensitive phenotype of cad1-3. Plants overexpressing AtPCS2 accumulated nearly as much PC2 as Col-0 when grown on Cd-contaminated soil. In contrast, PC3 levels were significantly lower. Interestingly, among plants on control soil, the 35S::AtPCS2 line showed the strongest accumulation of PC2 and PC3. In fact, concentrations were equally high under both conditions. This was very different from Col-0 and 35S::AtPCS1 3|3|3, where growth in the presence of Cd caused 30-fold or 40-fold increases in PC2 production, respectively.

Elevated PC production in AtPCS2-overexpression lines in the absence of additional Cd has been detected previously (Supplementary Fig. S4). These observations suggested that AtPCS2 activity is less responsive to Cd than AtPCS1 activity. In order to test this more directly, i.e. in the absence of plant factors potentially influencing enzyme activity, an attempt was made to characterize recombinant purified AtPCS2 was made. However, the protein could never be obtained in an active form. Therefore, heterologous expression of AtPCS1 and AtPCS2 in Schizosaccharomyces pombe ApES mutant cells was compared instead (Supplementary Fig. S5 at JXB online). Again, AtPCS2-dependent accumulation of PC2 and PC3 in the absence of Cd was observed that was stronger than the respective AtPCS1-dependent accumulation (Fig. 7). Levels of PC2 and PC3 were 50-fold and 8-fold higher, respectively, in AtPCS2-expressing cells than in AtPCS1-expressing cells under control conditions. Furthermore, a lower efficiency of AtPCS2 with respect to catalysing PC3 synthesis was also confirmed. The PC2:PC3 ratio in Cd2+-treated cells was about 1.1 for AtPCS1 and about 8.7 for AtPCS2. Because constitutive AtPCS2-dependent PC2 formation was observed in the liquid seedling assay with Fe being the only micronutrient present, possible activation of AtPCS2 by Fe was tested. However, no increase in PC levels in cells treated with an excess of Fe (20 and 100 µM) was detected. PC2 concentrations remained at around 0.2 nmol mg⁻¹ of dry weight.

Discussion

The synthesis of PCs is well established as the major detoxification mechanism for Cd, As, and Hg (Cobbett and Goldsborough, 2002). Given this function, however, it has been mysterious as to why PCS genes are so widespread among higher plants and expressed constitutively even in organs such as leaves, which are rarely if at all exposed to toxic non-essential metals (Rea et al., 2004). In fact, complex formation with PCs and reduced glutathione in the roots has been shown to efficiently restrict movement of toxic metals to the shoot (Chen et al., 2006; Li et al., 2006). The question can be extended to the existence of at least two PCS genes, which appears to be common in the genomes of higher plants (Fig. 1). Available evidence suggests two additional functions of PCS enzymes that could explain their wide occurrence and constitutive expression (Clemens and Peršoh, 2009; Rea, 2012). First, PC synthesis has been implicated in the homeostasis of the essential micronutrient Zn (Tennstedt et al., 2009; Adams et al., 2011). Secondly, AtPCS1 was shown to catalyse the deglycylation of glutathione S-conjugates (Grzam et al., 2006; Blum et al., 2007, 2010). This second activity is possibly underlying the role of AtPCS1 in mounting innate immune responses in A. thaliana seedlings (Clay et al., 2009).

With the aim of further elucidating the physiological functions of PCSs, the activities of the protein encoded by the second PCS gene in A. thaliana, AtPCS2, were analysed. The data presented in this study showed that it contributes to PC synthesis in A. thaliana, especially in the absence of metal excess, is able to rescue the Cd hypersensitivity of mutants lacking functional AtPCS1, and appears to possess enzymatic properties distinct from AtPCS1.

The mutant cad1-3 was initially characterized as devoid of PC accumulation (Howden et al., 1995b). This was later confirmed (Cazalé and Clemens, 2001), prompting the question as to the physiological role of the second PCS gene in A. thaliana, AtPCS2, which was found to encode a functional PCS when expressed in yeast. Therefore, a possible AtPCS2-dependent PC synthesis in cad1-3 was re-evaluated employing a more sensitive PC detection method than previously available. In recent years, the use of TCEP as a reductant (Rijstenbil and Wijnholds, 1996; Thangavel et al., 2007; Minocha et al.,
and of UPLC separation, coupled to ESI-QTOF-MS, as a method for PC detection have been reported (Bräutigam et al., 2010). Assessment of the UPLC-ESI-QTOF-MS platform employed and possible matrix effects (Fig. 2) yielded LLODs for PC2 and PC3 that were about three orders of magnitude lower than those initially reported for HPLC-based analysis of A. thaliana extracts (Howden et al., 1995b). When such values were reported in more recent studies, they were between approximately 2-fold (Minocha et al., 2008) and 20-fold (Simmons et al., 2009) higher. In order to achieve maximum sensitivity, the mass range was restricted to common values of m/z 300–2000 and the analysis was focused on PC2 and PC3, which are by far the most abundant PCs.

Thiol analysis of derivatized extracts of plants cultivated hydroponically and supplied with all required micronutrients, but in the absence of any potentially toxic metal excess, confirmed constitutive PC synthesis in roots and to a much lesser extent in leaves, which is in accordance with the physiological roles of PC synthesis beyond metal detoxification (Rea, 2012). Furthermore, these experiments yielded unequivocal detection of PC2 in the roots of cad1-3 (Fig. 3A). This could be due either to residual AtPCS1 activity in cad1-3 or to AtPCS2 activity. The latter interpretation is consistent with the cad1-3 mutation affecting an amino acid that is 100% conserved in PCS proteins across kingdoms, as well as the absence of the AtPCS1 transcript in cad1-3 (Ha et al., 1999). Furthermore, it is supported by two observations. First, an atpcs2 exon insertion line showed lower PC2 levels than the parental ecotype Ws. Secondly, and most importantly, no PC2 was detectable in the roots of the cad1-3 atpcs2 double mutant that was generated. These observations were confirmed for roots of Cd2+-treated plants, which as expected showed strong increases in PC accumulation dependent on the Cd2+ dose. In contrast, no evidence was found for AtPCS2-dependent PC3 synthesis or leaf PC2 synthesis in cad1-3. The latter is consistent with the finding that an AtPCS2–GFP fusion protein expressed under the control of the endogenous AtPCS2 promoter yielded signals only in the root tip (Blum et al., 2010). The surprising detection of trace amounts of PC2 in the leaves of the cad1-3 atpcs2 double mutant upon Cd2+ treatment might indicate the existence of another as-yet-unknown enzymatic activity that results in PC formation. This would also explain the similar trace amounts of PC2 in the leaves of Cd-treated cad1-3 plants (Fig. 3B).

The evidence for AtPCS2-dependent PC synthesis in vivo prompted testing for a possible complementation of the cad1-3 mutant phenotype by AtPCS2 overexpression. Hence, transgenic lines with different levels of AtPCS2 protein were generated and compared with lines overexpressing AtPCS1. The most commonly used metal tolerance system, cultivation on vertical agar plates, did not yield any indication for rescue by AtPCS2, while expression of AtPCS1 fully complemented cad1-3 (Supplementary Fig. S3). In contrast, partial rescue at least in the more strongly expressing AtPCS2 line was indicated when Cd tolerance was assayed in an alternative system: growth of seedlings in liquid medium (Fig. 5). As a result of this finding, a tolerance assay that was far closer to natural conditions was established, i.e. growth in mineral soil spiked with Cd. The concentration of 7.5 mg kg⁻¹ is only three times higher than the upper limit of the range of background levels in topsoils (EFSA, 2009) and is within the reported span for agricultural soils in some countries (McLaughlin et al., 1999; Pan et al., 2010). Col-0 plants were not affected by this Cd level in the soil. In fact, leaf area as an indicator for growth was even slightly elevated when compared with plants cultivated on control mineral soil (Fig. 6A, B). In contrast, cad1-3 plants developed chlorotic leaves and growth was severely impaired. This difference between Col-0 and cad1-3 demonstrated the essential role of PC synthesis for tolerating levels of Cd contamination that can naturally be encountered by plants outside metal-rich habitats. Furthermore, the lower Cd accumulation in cad1-3 leaves relative to Col-0 (Fig. 6D) provided evidence for the contribution of PC synthesis to Cd accumulation under such conditions. This is highly relevant in light of the widespread background contamination of agricultural soils with Cd and the health risks associated with human intake of Cd through plant-derived food (Nawrot et al., 2010; Clemens et al., 2013; Uraguchi and Fujiiwara, 2013).

Interestingly, AtPCS1 and AtPCS2 expression rescued cad1-3 growth on Cd soil to the same extent. After 21 and 24 d of growth, the leaf areas of the two tested transgenic lines were significantly higher than the leaf areas of cad1-3. Lack of full complementation, i.e. slower growth than wild type on Cd soil, is in line with earlier observations suggesting that PCS overexpression does not result in a gain of Cd tolerance and in some cases there is even in a reduction (Lee et al., 2003a,b; Wojas et al., 2008). The latter was found not only on Cd soil but also in the liquid seedling assay (Fig. 5).

Contrasting PC accumulation patterns of plants overexpressing either AtPCS1 or AtPCS2 suggested at least two distinct differences in enzymatic properties of the proteins. AtPCS2 appeared to be less efficient in synthesizing PC3, possibly due to a reduced efficiency in accepting PC2 as a substrate in place of reduced glutathione. Moreover, AtPCS2-expressing lines accumulated higher levels of PC2 in the absence of any metal excess both in the liquid seedling assay and on soil (Fig. 6C and Supplementary Fig. S4). This does not correspond with what is considered a hallmark of PCSs, namely activation of the constitutively expressed proteins by metal excess (Grill et al., 1989; Vatamaniuk et al., 2000). In fact, 35S::AtPCS2 plants did not show any increase in PC2 or PC3 levels in leaves of plants grown on Cd soil (Fig. 6C), even though these plants clearly accumulated Cd. Cd2+ ions are among the most potent activators of AtPCS1 (Grill et al., 1987; Ha et al., 1999; Vatamaniuk et al., 2000). This could be seen in the strongly elevated PC2 and PC3 levels of 35S::AtPCS1 plants when grown on Cd-spiked soil. Thus, according to data obtained in different experimental systems, AtPCS2 appeared to show a PCS activity that, in contrast to the well-established knowledge for AtPCS1, was not stimulated by Cd in planta. Lack of AtPCS2 activation in Schizosaccharomyces pombe cells exposed to an excess of Fe, the only micronutrient present in the liquid seedling assay, argues against activation by other metals.
Attempts to further elucidate these differences in enzyme properties with recombinant purified proteins were not successful. While purification of active AtPCS1 was possible as reported by other groups (Vatamanuik et al., 2000), trials to obtain active soluble AtPCS2 failed even when using the same tags, vectors, cloning strategies, and Escherichia coli strains (data not shown). As the C-terminal part of the PCS protein has been shown to influence the enzyme stability (Ruohto et al., 2004), the insolubility of the AtPCS2 protein might be a consequence of the 90 bp deletion in exon 8, which causes a major difference in the AtPCS1 protein (Ha et al., 1999). Thus, AtPCS1 and AtPCS2 activities were instead compared following expression in Schizosaccharomyces pombe Δpcs cells. The results again confirmed higher PC2 accumulation in the absence of Cd²⁺ treatment due to stronger constitutive activity of AtPCS2 relative to AtPCS1 (Fig. 7). Using this heterologous expression system, it should be possible to dissect the structure–function relationships responsible for the differences in metal activation between AtPCS1 and AtPCS2.

Taken together, the data presented in this study establish constitutive PCS activity, i.e. catalysis of PC formation in the absence of any potentially toxic metal excess, for AtPCS2 in planta. When overexpressed, this activity is sufficient to at least partially rescue the severe Cd hypersensitivity of the cad1-3 mutant. However, AtPCS2-dependent constitutive PC2 formation in cad1-3 is as relative to the impact of AtPCS1, which is apparent from the PC2 accumulation in the roots of atpcs2 mutant plants under control conditions (Fig. 3A). Most likely, the small AtPCS2 contribution is due to globally restricted weak expression. It is not sufficient to confer Cd tolerance, as is obvious from the cad1-3 phenotype in the presence of Cd (Figs 5 and 6A). In addition, this phenotype might in part be attributable to the reduced PC3 formation by AtPCS2, because a rising chain length leads to a gain in pH stability of PC–Cd complexes (Satofuka et al., 1989) and accordingly to an increased binding affinity of PCs to metal ions (Loeffler et al., 1989). Furthermore, apart from slightly lower PC accumulation, no discernible differences between atpcs2 and Ws-0 were detected. This and the constitutive activity suggest a physiological role of AtPCS2 unrelated to metal detoxification. In accordance with recently demonstrated AtPCS1 functions (Clay et al., 2009), future experiments will have to test AtPCS2-dependent effects on glutathione S-conjugate metabolism or plant defence.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Isolation of the homozygous T-DNA insertion line atpcs2 (FLAG_146G12).

Supplementary Fig. S2. Genotype of A. thaliana wild-type lines, AtPCS1 and AtPCS2 mutants, and the PCS double mutant at the HMA3 locus.

Supplementary Fig. S3. Rescue of cad1-3 Cd hypersensitivity in vertical agar plate assays by AtPCS1 but not AtPCS2 expression.

Supplementary Fig. S4. PC accumulation in seedlings of AtPCS1- and AtPCS2-overexpressing lines after 11 d of growth in the presence or absence of Cd²⁺.

Supplementary Fig. S5. Western blot analysis for the detection of AtPCS1 and AtPCS2 expression in Schizosaccharomyces pombe.

Supplementary Table S1. Metal contents in the mineral soil type used as control soil for growth experiments.

Supplementary Table S2. Primer sequences used for quantitative real-time PCR.

Supplementary Table S3. Evaluation of the liquid seedling assay.

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