Ancestral function of the phytochelatin synthase C-terminal domain in inhibition of heavy metal-mediated enzyme overactivation

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Abstract

Phytochelatin synthases (PCSs) play essential roles in detoxification of a broad range of heavy metals in plants and other organisms. Until now, however, no PCS gene from liverworts, the earliest branch of land plants and possibly the first one to acquire a PCS with a C-terminal domain, has been characterized. In this study, we isolated and functionally characterized the first PCS gene from a liverwort, Marchantia polymorpha (MpPCS). MpPCS is constitutively expressed in all organs examined, with stronger expression in thallus midrib. The gene expression is repressed by Cd2+ and Zn2+. The ability of MpPCS to increase heavy metal resistance in yeast and to complement cad1-3 (the null mutant of the Arabidopsis ortholog AtPCS1) proves its function as the only PCS from M. polymorpha. Site-directed mutagenesis of the most conserved cysteines of the C-terminus of the enzyme further uncovered that two twin-cysteine motifs repress, to different extents, enzyme activation by heavy metal exposure. These results highlight an ancestral function of the PCS elusive C-terminus as a regulatory domain inhibiting enzyme overactivation by essential and non-essential heavy metals. The latter finding may be relevant for obtaining crops with decreased root to shoot mobility of cadmium, thus preventing its accumulation in the food chain.

Keywords: Cadmium, C-terminal domain, Marchantia polymorpha, overactivation, phytochelatin, phytochelatin synthase, site-directed mutagenesis, twin-cysteine motif, zinc.

Introduction

Plants are sessile organisms, thus they have evolved diverse defense mechanisms such as accumulation and detoxification of different metals to adapt to environmental stresses related to the mineral composition of soil. Some heavy metals such as zinc (Zn), copper (Cu), and iron (Fe) are essential for plant growth and development, as they are cofactors in protein structural and catalytic components, mediating ligand interactions and redox reactions (Giles et al., 2003; Olson et al., 2013; Schmidt and Husted, 2019). Other heavy metal(loids) such as cadmium (Cd), arsenic (As), and lead (Pb) are non-essential, as they have no biological function in plants. On the contrary, they are toxic even at micromolar concentrations,
because of their competition with endogenous metal cofactors for binding sites (Rea, 2012). Excess heavy metals can even lead to acute toxicity and plant death. Therefore, tight regulation of heavy metal accumulation in plants is an important mechanism to maintain plant fitness (Tennstedt et al., 2009). In plants, phytochelatins (PCn) are among the most important and studied chelators for heavy metal detoxification (Clemens, 2019).

PCn are non-ribosomally synthesized cysteine-containing peptides which have the general structure (γ-Glu-Cys)n-X (where n=2–5, and X is generally glycine) (Grill et al., 1985). PC biosynthesis starts from glutathione (GSH) in a transeptidase reaction catalyzed by phytochelatin synthase (PCS; EC 2.3.2.15) (Grill et al., 1989). PCn were discovered first in the fission yeast Schizosaccharomyces pombe (Kondo et al., 1984) and then in plants from cell cultures of Rauwolfa serpentina (Grill et al., 1985). Afterwards, PCn were identified in all plant species investigated as well as in algae, fungi, diatoms, and animals (Rea et al., 2004; Tsuji et al., 2004). PCS genes are constitutively expressed, and PC accumulation is activated by exposure to various physiological and non-physiological metal ions (Vatamaniuk et al., 2000) and sequestered into the vacuole through ATP-dependent transporters (Song et al., 2010). Vacuole sequestration terminates the complexation of PCn with heavy metals and prevents accumulation of heavy metal ions in the cytosol.

Cloning and functional characterization of PCS genes from Arabidopsis thaliana (AtPCS1), S. pombe (SpPCS), Triticum aestivum (TaPCS1), and Caenorhabditis elegans (CePCS1) (Glaeser et al., 1991; Howden et al., 1995; Clemens et al., 1999, 2001; Ha et al., 1999; Vatamaniuk et al., 1999, 2001) dramatically increased our knowledge of how PCn control heavy metal detoxification at the molecular level. In addition, the identification of PC-deficient mutants from Arabidopsis (cad1) and S. pombe further broadened our understanding of the roles of PCn in heavy metal accumulation and tolerance. Based on these pioneer studies, later research attempted to increase heavy metal accumulation and tolerance in plants. A series of PCS genes from different species were isolated and overexpressed in model species (Liu et al., 2011; Zhang et al., 2018; Li et al., 2019), but, surprisingly, these transgenic approaches resulted in diverse outcomes. For instance, transgenic plants, which were highly sensitive to Cd treatment, were obtained by overexpressing AtPCS1 in Arabidopsis (Lee et al., 2003a), even though there was only a small increase of PCn compared with wild-type (WT) plants. On the other hand, overexpression of the same gene in Brassica juncea resulted in high tolerance to Cd and Zn exposure, and the accumulations of Cd and Zn were significantly lower than in WT plants (Gasic and Korban, 2007). Overall, it was estimated that 33.3% of experiments with transgenic plants overexpressing PCS1 showed a positive relationship between Cd tolerance and accumulation, while 25% evidenced a negative relationship (Lee and Hwang, 2015). At present, the reasons underlying such contrasting effects are still not clear. The elegant works performed independently by different groups, however, indicate that the different response to Cd exposure of transgenic plants might be caused by several concurring factors, including differences of PCS activities, endogenous PC and GSH concentrations, as well as PC polymerization levels in transgenic plants (Wojas et al., 2008; Brunetti et al., 2011; De Benedictis et al., 2018). Another important aspect intensively addressed was the elucidation of the structural bases of PCS function. Analysis of AtPCS1 by limited proteolysis showed that the conserved N-terminal domain is necessary and sufficient for enzyme catalytic activity, while the evolutionarily divergent C-terminal domain is involved in responsiveness to a wide range of heavy metals (Ruo et al., 2004). The crystal structure of a cyanobacterial PCS homolog (lacking, like all cyanobacterial PCS enzymes, the C-terminal domain) suggested that the N-terminus is essential for core catalysis, and further implied the involvement of the C-terminal domain present in eukaryotes in sensing free heavy metals (Vivares et al., 2005; Rea, 2006). The different responsiveness to a set of heavy metals of LjPCS1 and LjPCS3, two different PCS enzymes in Lotus japonicus, indicated that the different patterns of heavy metal activation between these two proteins were mainly due to the differences in their C-terminal domains (Ramos et al., 2008). More recently, in Arabidopsis, the region of the AtPCS1 C-terminal domain responsible for Zn-dependent PC formation was identified through a set of C-terminal truncations (Kühnlenz et al., 2016), while As-specific activation of PC synthesis was demonstrated to occur in a smaller region of the C-terminal domain (Uraguchi et al., 2018).

Although the molecular mechanisms of heavy metal detoxification by PCn from higher plant species have been studied intensively during the last decades, very few studies on early diverging plant lineages have been carried out. The constitutive presence of PCSs in some species of bryophytes and lycophytes was demonstrated through HPLC and MS analyses (Petraglia et al., 2014; Bellini et al., 2020b), and PC-mediated heavy metal detoxification was confirmed to be compartmentalized in vacuoles in the liverwort Lunularia cruciata (L.) Dumort (Degola et al., 2014) and Leptodictyum riparium (Bellini et al., 2020a). However, no isolation and detailed functional characterization of PCS genes from bryophytes have been reported to date. Bryophytes, comprising liverworts, mosses, and hornworts, are the earliest diverging lineages of land plants (Qiu et al., 2006; Shimamura, 2016); so far, the phylogenetic relationship among these three bryophytes is still enigmatic (Shaw and Renzaglia, 2004), but liverworts are considered to be placed in a key phylogenetic position among the earliest land plants. The model species Marchantia polymorpha is a dioecious liverwort with separate female and male gametophytes that produce archegoniophores and antheridiophores, respectively. Due to its dominant haploid life cycle and easy asexual propagation through gemmae yielding isogenic experimental lines, M. polymorpha has well established molecular genetic tools ranging from mutant populations, genetic transformation, silencing, and genome editing (Ishizaki et al., 2016). Furthermore, its genome was fully sequenced recently (Bowman et al., 2017). Therefore, M. polymorpha has been widely used as a model species to elucidate the evolutionary processes of gene regulation mechanisms across land plants (Busch et al., 2019; Naramoto et al., 2019), but, to date, no systematic investigation has been performed on heavy metal detoxification in this species.
In this study, the putative PCS gene from *Marchantia polymorpha* (*MpPCS*) was isolated and functionally characterized *in vitro* through overexpression in yeast and Arabidopsis to address the question of whether it is functional and can complement higher plants PCS enzymes. With MpPCS being the most basal PCS with a C-terminal domain in land plants, we further asked whether analysis of the few highly conserved cysteines in this region affects metal responsiveness to elucidate the ancestral function of this enigmatic domain.

### Materials and methods

#### Plant materials, growth conditions, and stress treatments

*Marchantia polymorpha* L. Cam2 (UK Cambridge-2 WT) female gametophytes, *A. thaliana* Col-0 WT, and *Cad1-3* mutant and transgenic plants were used in this study. *Marchantia polymorpha* was propagated in Petri dishes containing half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose and 1% phytoagar under long-day conditions (16 h light/8 h dark) at 21°C with a light intensity of 60 µmol m⁻² s⁻¹ in the growth chamber. For heavy metal treatments in *M. polymorpha*, 2-week-old gemmae were transferred to fresh Petri dishes either without or with addition of 50 µM CdSO₄ or 200 µM ZnSO₄. The gemmae were independently collected before and after treatments for 1, 3, 6, 12, and 24 h, snap-frozen in liquid nitrogen, and stored at −80°C until used for real-time and thiol-peptide analyses. Three biological replicates at each sampling time point were applied for the entire treatment. For heavy metal treatments in Arabidopsis plants, sterilized seeds were germinated in half-strength MS agar medium and 2% (w/v) sucrose, supplemented either with 50 µM or 85 µM CdSO₄ or 200, 400, or 600 µM ZnSO₄ in 100×100×15 mm square plates. In total, 15 seeds for Col-0 and each transgenic line were sown in one plate. After stratification at 4°C for 3 d, the plates were grown vertically for 10 d under standard long-day conditions at 23°C with a light intensity of 100–120 µmol m⁻² s⁻¹ in the growth chamber. At least 80 plants per genotype were processed for this analysis. For thiol-peptide analyses of *cad1-3* complementation lines, plants grown for 10 d on the control plates were transferred either to plates containing 85 µM CdSO₄ or to fresh plates, and maintained for an additional 3 d. A total of 15–20 seedlings per genotype were pooled as a single biological sample, frozen in liquid nitrogen, and stored at −80°C.

#### Cloning, plasmid constructs, and transformation

For the analysis of the *MpPCS* expression pattern in *M. polymorpha*, a promoter region of 2.8 kb upstream of the *MpPCS* coding sequence (CDS) was amplified using primers *MpPCS_prom_For* and *MpPCS_prom_Rev* (see Supplementary Table S1 at JXB online) with Phusion High Fidelity DNA Polymerase (Thermo Scientific), cloned into pENTR/D TOPO vector (Invitrogen), and recombined into the destination vector pMPGW104 (Ishizaki et al., 2015) in front of the β-glucuronidase (GUS) CDS using LR clonase II (Invitrogen). The T-DNA was integrated into the *M. polymorpha* genome by Agrobacterium tumefaciens-mediated transformation as previously described (Kubota et al., 2013). T₅ transgenic plants were selected on half-strength solid Gamborg B5 medium supplemented with 10 mg l⁻¹ hygromycin. The isoegenic G₅ lines from the T₅ lines were obtained by subcultivating single gemmae, and gemmae generated from G₅ lines (G₅ generation) were used for experimental analyses.

For overexpression of *MpPCS* in *M. polymorpha*, the full-length *MpPCS* cDNA was amplified with primers *MpPCS_For* and *MpPCS_Rev* (Supplementary Table S1). The resulting PCR fragment was cloned into the pENTR/D TOPO vector (Invitrogen) and recombined into the destination vector pK7WG2 under the transcriptional control of the strong constitutive *Caflflower mosaic virus* (CaMV) 35S promoter (Karimi et al., 2002) in the same way as mentioned above to yield the final construct (p35S::*MpPCS*). This construct was transformed into *A. tumefaciens* strain GV3101-pMP90RK by electroporation and further transformed into the *A. thaliana* Col-0 ecotype by the floral dip method (Clough and Bent, 1998). T₅ transgenic lines were screened on solid MS medium supplemented with 50 mg l⁻¹ kanamycin. Homozygous single-copy T₅ seeds from two selected lines were used for all downstream analyses.

Mutational analysis of *MpPCS* was carried out as follows. In total, six mutations targeting three positions with highly conserved cysteines were introduced into the *MpPCS* WT CDS using the Quikchange Site Directed Mutagenesis Kit (Strategene) in *M. polymorpha* pENTR_MpPCS plasmid. All primers used for mutagenesis are listed in Supplementary Table S1. The resulting entry vectors, respectively named pENTR_MpPCS_M1, pENTR_MpPCS_M2, pENTR_MpPCS_M3, pENTR_MpPCS_M4, pENTR_MpPCS_M5, and pENTR_MpPCS_M6, and the cognate WT pENTR_MpPCS plasmid were further recombined into the pYES-DEST52 vector (Invitrogen) and transformed into Cd-sensitive *Saccharomyces cerevisiae* strain YK44 (ana3-52 his3-200, ΔZRCDCot1, mating type α) using the lithium acetate method (Gietz and Schiestl, 2007). All sequences used for any of the constructs described above were verified by sequencing with a 96-capillary 3730xl DNA Analyzer (Thermo Scientific).

#### Histochemical analysis of GUS expression in transgenic *Marchantia polymorpha*

About 10–15 gemmae of 17-day-old WT and transgenic *M. polymorpha* lines were incubated at 37°C overnight in GUS assay solution as previously described (Gazzani et al., 2009); the chlorophyll was cleared with a series of incubations in fresh 70% ethanol (v/v).

#### Total RNA extraction, cDNA synthesis, and real-time PCR (RT-PCR) analyses

Total RNA was extracted from 100 mg of frozen plant material using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) following the manufacturer’s instructions, and treated with Amplification-Grade DNase I (Sigma-Aldrich) for elimination of genomic DNA contamination. The integrity and quality analyses of extracted total RNA were performed in Bioanalyzer 2100 (Agilent Technologies), and cDNA was then synthesized with 1 µg of total RNA using SuperScript™III reverse transcriptase (Invitrogen). Semi-quantitative RT-PCR (qRT-PCR) was carried out for different organs of *M. polymorpha* using the *MpACT* gene as a reference (Saint-Marcoux et al., 2015) and using *ActinII* for Arabidopsis transgenic plants. For real-time analysis, qRT-PCR was conducted with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) using *MpAPT* and *MpACT* as reference genes for *M. polymorpha* (Saint-Marcoux et al., 2015) and *AtActII* and *AtEF1a* for Arabidopsis transgenic plants in a Bio-Rad C1000 Thermal Cycler detection system. Stability of reference genes was calculated with the RefFinder software (Xie et al., 2012). All reactions for qRT-PCR analyses were performed in triplets, and the 2^−ΔΔCT method was applied to calculate fold changes. Primer sequences are listed in Supplementary Table S1.

#### Phylogenetic reconstruction

Arabidopsis thaliana PCS1 protein was blasted against the *M. polymorpha* MarpolBase ‘primary’ and ‘alternative’ (version 3.1, November 2013) protein databases (https://marchantia.info/tools/bast/Plant/) using an E-value cut-off of 10⁻⁵. The *A. thaliana* PCS1 protein was further blasted against all angiosperm Phytozone 12 (Goodstein et al., 2012) proteomes. The resulting hits were downloaded and representative sequences were selected based on protein completeness and phylogenetic distance from each other, to provide a representative sample of PCSs in plants. Proteins were aligned using the MAFFT online server (Katoh et al., 2019), and regions with low homology were removed using the GBLOCKS server (Talavera and Castresana, 2007) with standard settings. The best-fitting model of protein evolution was selected with the online version of SMS (Lefort et al., 2017) and this model was directly applied for maximum likelihood phylogenetic
reconstruction using the PhyML online server (Guindon et al., 2010) and aBayes approximate support branch estimates. The resulting tree was visualized with FigTree v1.4.4.

Yeast complementation assay and induction for thiol-peptide analyses

A single colony from each transformant carrying either the WT construct (pYES52-MpPCS), one of the six mutations (pYES52-MpPCS_M1, pYES52-MpPCS_M2, pYES52-MpPCS_M3, pYES52-MpPCS_M4, pYES52-MpPCS_M5, pYES52-MpPCS_M6), or the pYES52 empty vector was cultured in YSGD-U liquid medium overnight at 30 °C. Culture aliquots normalized to OD₆₀₀=0.5 were pelleted and resuspended in 500 µl of YPGAL [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose] liquid medium and further diluted to 10⁻¹, 10⁻², and 10⁻³ and 5 µl from each aliquot were spotted on YPGAL solid medium supplemented with or without CdSO₄/ZnSO₄. Yeast growth was stopped after a 3 d incubation at 30 °C. For the in vivo assay of thiol-peptides, yeast cells at an OD₆₀₀ of 0.1 were shaken overnight at 30 °C in YSD-U liquid medium, and protein expression was induced at an OD₆₀₀ of 0.5 for 4 h by supplementing 2% galactose and 100 µM CdSO₄. Afterwards, cells were harvested by centrifugation, washed twice with distilled water, snap-frozen in liquid nitrogen, and stored at −80 °C. All experiments were independently repeated four times.

Generation of recombinant protein for MpPCS, its C-terminal mutations, and PCS activity assay

The CDSs of full-length MpPCS and the six mutations mentioned above were amplified from the corresponding pENTR clone used for plant transformation with primers listed in Supplementary Table S1 and were cloned into the expression vector pET28a in-frame with an N-terminal hexa-His-tag. The expression plasmids were transformed into Escherichia coli Rosetta™(DE3)pLysS cells, which were induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside with overnight culture at room temperature. The cells were harvested by centrifugation and the soluble fraction of recombinant protein was purified as previously described (Fischer et al., 2014), further desalted using a PD-10 desalting column (GE Healthcare), quantified with the Quant-iT Protein Assay Kit (Thermo Fisher Scientific), and assessed by 10% SDS-PAGE. The PCS activity assay was carried out as previously described (Ogawa et al., 2010; Uraguchi et al., 2017). In brief, the reaction mixture (100 µl) containing 200 mM HEPES–NaOH (pH 8.0), 10 mM 2-mercaptoethanol, 12.5 mM GSH, 100 µM Cd or 200 µM Zn, and 50 ng of recombinant PCS was incubated at 35 °C for 60 min, then terminated by the addition of 25 µl of 10% trichloroacetic acid (TFA). The terminated reactions were maintained at 10 °C in the autosampler tray and immediately analyzed by HPLC-ESI-MS-MS to identify and quantify PC₄ produced using the analytical method described in (Bellini et al., 2019). For an accurate quantification, terminated reactions were diluted by a factor of 100 only for PC₂.

Analyses of thiol-peptides

Marchantia polymorpha and A. thaliana samples, previously stored at −80 °C, were extracted according to Bellini et al. (2019), whereas yeast cells were extracted following the protocol described in Ramos et al. (2008) with some modifications. Briefly, yeast cells were resuspended in 300 µl of the extraction buffer containing 0.1% (v/v) TFA, 0.5 mM DTPA (diethylenetriaminepentacetic acid), and 200 ng ml⁻¹ (glycine-¹³C₂,¹⁵N)-labeled GSH and PC₂ internal standards. All the other analyses and quantification of thiol-peptides were performed following the procedures detailed in Bellini et al. (2019). System control, data acquisition, and processing were carried out using AB Sciex Analyst® version 1.6.3 software.

Cad1-3 complementation

Cad1-3 mutant plants were used for transformation by the floral dip method (Clough and Bent, 1998) using Agrobacterium tumefaciens GV3101-pMP90RK harboring a plant gene expression construct (p35S::MpPCS). Transformated seeds were selected on MS agar medium containing 50 mg l⁻¹ kanamycin, and T₃ homozygous seeds were used for complementation analyses.

Statistical analyses

Data with one independent factor were analyzed using one-way ANOVA. Data with two independent factors were analyzed using two-way ANOVA. Tukey’s multiple comparison and least significant difference (LSD) tests were used to identify significant differences. Differences were considered significant if P ≤ 0.05 in the two-sided test. Compact letter display was used to summarize the differences among means. All analyses were run in R version 4.0.0 (04.24; R Core Team, 2020) using the scripts provided in Mangiafico (2015). All experiments were performed with at least n=3 biological replicates.

Results

Phylogenetic reconstruction of MpPCS

Based on a homology search of AtPCS1 in the fully sequenced genome of M. polymorpha (Bowman et al., 2017), the full-length coding region of PCS of M. polymorpha (MpPCS) was identified and isolated (accession number Mapoly004600028.1). Only a single copy of PCS is present in the M. polymorpha genome, in contrast to the two copies found in Arabidopsis and many other higher plant species (Filiz et al., 2019). The MpPCS protein is, as expected, basal to all angiosperm PCSs (Fig. 1A). MpPCS encodes a 530 amino acid polypeptide with a predicted molecular mass of ~57 kDa. The protein sequence alignment among MpPCS and other PCSs from higher plant species indicated that it shares 46–52% overall sequence identity (Fig. 1B). Conservation of the N-terminal domain is higher than that of the more divergent C-terminal domain, and the N-terminal domain has the typical catalytic triad of PCS enzymes, namely Cys56, His162, and Asp180 (Romanyuk et al., 2006; Li et al., 2019) (Fig. 1B).

MpPCS is constitutively expressed and repressed by Cd²⁺ and Zn²⁺ treatment in vegetative organs of M. polymorpha

Semi-quantitative RT-PCR was performed to determine the expression patterns of MpPCS in M. polymorpha gemmae, entire plants, 4-week-old thalli, and rhizoids. This analysis indicated that MpPCS was expressed at similar levels in all four organs examined here (Fig. 2A). Furthermore, the expression pattern of MpPCS was visualized in transgenic M. polymorpha expressing the GUS gene under the control of the MpPCS promoter. The GUS staining analysis of 17-day-old gemmae showed that MpPCS was expressed mainly in the midrib region of the thallus and rhizoids; no expression was observed correspondingly in WT plants (Fig. 2B, C).

To assess the general responsiveness of MpPCS in M. polymorpha to heavy metal treatments, 2-week-old gemmae were treated with either 50 µM CdSO₄ or 200 µM ZnSO₄, and the entire gemmae were collected at different time points before and after heavy metal treatments and subjected to qRT-PCR analysis. The overall stabilities of the two reference genes...
Fig. 1. Phylogenetic reconstruction and multiple sequence alignment of PCS proteins. (A) Maximum likelihood tree of PCS proteins from representative plant taxa. Numbers are approximate Bayes (aBayes) support values calculated by PhyML. (B) Multiple sequence alignment of MpPCS (Mapoly0046s0028.1), SmoPCS (98967), OsaPCS (LOC_Os06g01260.1), and AtPCS (AT5G44070.1). Dashes (–) represent gaps; dots indicate residues identical to those of the first sequence. The amino acids of the catalytic triad (Cys56, His162, and Asp180 in A. thaliana, corresponding to the same positions in MpPCS) are highlighted with a yellow background. The boundary between the N-terminus (1–221 amino acids in A. thaliana) and the enzyme end is indicated with a black arrow. The color of the amino acids indicates the chemico-physical properties (e.g. red is used for negatively charged and blue for positively charged amino acids).
Li et al. used, MpAct and MpAPT, were 1.41 and 1.19, respectively. The expression levels of the MpPCS transcript under CdSO$_4$ and ZnSO$_4$ treatments gradually decreased, reaching the minimum after 12 h of treatment, and then increased after 24 h to a level similar to that at 1 h of treatment (see Fig. 2D). One-way ANOVA further indicated that the expression level of MpPCS after 3, 6, and 12 h of treatment with both heavy metals was significantly different from those of the other time points tested (Fig. 2D, E). Given the identical trends for Zn$^{2+}$ and Cd$^{2+}$ treatments, we assessed the amount of GSH and PC$n$ only for Cd. The means of the GSH amounts over time were significantly different from those of control gemmae, as the amount of GSH had a slight increase at 12 h after CdSO$_4$ treatment (one-way ANOVA, $F_{5,18}=12.992$, $P=1.898\times10^{-5}$; Fig. 2F). On the other hand, PC$_2$ content increased from 3 h on and reached a plateau at about double the amount of the control from 6 h to 24 h (one-way ANOVA, $F_{5,18}=40.267$, $P=3.713\times10^{-9}$; Fig. 2G). The amount of PC$_3$ did not significantly change as compared with the control, although significant differences could be found among time points (one-way ANOVA, $F_{5,18}=3.5281$, $P=0.0213$; Fig. 2H).

**MpPCS overexpression confers heavy metal tolerance to YK44 yeast and hypersensitivity to Arabidopsis plants**

Overexpression of candidate PCS genes in heterologous systems is a common method to assess their functionality and capacity to change responsiveness to heavy metals (Lee et al., 2003b). First of all, MpPCS was transformed into yeast strain YK44, which is hypersensitive to heavy metal treatment. Growth of yeast lines transformed with either the MpPCS CDS or the empty vector was examined on YPGAL medium supplemented without and with different concentrations of Cd$^{2+}$ and Zn$^{2+}$.

This analysis clearly showed that in the control medium (no heavy metals), growth of yeast transformed either with MpPCS or the empty vector was similar, while yeast expressing MpPCS grew more than the empty vector control.
line at concentrations of 100 µM CdSO₄ and 700 µM ZnSO₄ (Supplementary Fig. S1). Therefore, overexpression of MpPCS enhanced resistance of yeast strain YK44 to heavy metal stress.

In addition, transgenic Arabidopsis plants were generated to evaluate the ability of the 35S::MpPCS construct to affect tolerance to heavy metals in planta. Semi-quantitative RT-PCR was carried out first to estimate the relative expression of MpPCS in single-copy transgenic Arabidopsis lines (Supplementary Fig. S2), and the two lines with the highest relative expression were selected for phenotypic analyses. In control growth medium, both transgenic lines overexpressing MpPCS under the control of the strong 35S promoter and Col-0 WT plants grew comparably, as no statistical differences were detected in fresh weight and root length among genotypes (Fig. 3A).

However, when plants were grown in medium supplemented with 50 µM CdSO₄, the mean fresh weights of both transgenic lines were significantly lower than that of Col-0 plants (Fig. 3B), and root length was also much shorter compared with Col-0. The same growth trend, in a more severe manner, was also observed for both transgenic lines and Col-0 plants treated with 85 µM CdSO₄ (Fig. 3B). To assess whether the phenotypic variation observed for CdSO₄ treatment would be the same also for an excess of an essential heavy metal, both transgenic lines and Col-0 WT plants were grown on the same basal medium with different concentrations of ZnSO₄. Col-0 plants grew more than both transgenic lines under 200 µM ZnSO₄ treatment (Fig. 3A), and both fresh weight and root length were significantly different between the two transgenic lines and Col-0 WT plants grown under different concentrations of ZnSO₄.

Fig. 3. Phenotypic variations upon CdSO₄ or ZnSO₄ treatment of Arabidopsis transgenic plants overexpressing MpPCS from M. polymorpha. (A) Phenotypes of 10-day-old MpPCS transgenic and wild-type Col-0 plants under non-treated (top) and treated conditions with different concentrations of CdSO₄ (50 µM and 85 µM; middle) or ZnSO₄ (200 µM and 400 µM; bottom). Dashed lines indicate the separation of different genotypes. (B) Fresh weight of the corresponding plants shown in (A); bars represent the SD of three biological replicates, and two stars indicate statistically very significant differences compared with Col-0. At least 60 plants were used for each analysis.
lines and Col-0 (Fig. 3B). Significant differences were also observed under 400 µM ZnSO₄ treatment (Fig. 3B).

**Overexpressing MpPCS complements the Arabidopsis Cad1-3 mutant**

The cad1-3 mutant, a knockout mutation of Arabidopsis PCS1 (AtPCS1), is highly sensitive to treatment with heavy metals such as Cd²⁺ (Howden et al., 1995; Ha et al., 1999). Thus, to verify whether MpPCS was the functional *M. polymorpha* ortholog of *AtPCS1*, a complementation assay was performed by overexpressing MpPCS in the cad1-3 mutant. In total, 13 independent homozygous lines were used to assess growth under different concentrations of CdSO₄ treatment compared with Col-0 and the cad1-3 mutant. In control growth medium, seedlings of all transgenic lines grew similarly compared with those of Col-0 and the cad1-3 mutant, and no statistically significant differences in fresh weight were detected among all genotypes (Fig. 4). When the growth medium was supplemented with 50 µM or 85 µM CdSO₄, the fresh weights of all lines and Col-0 were very similar from each treatment; statistical analysis indicated that no significant differences for the majority of lines were detectable, but many were significantly more resistant to CdSO₄ treatment compared with the Cad1-3 mutant. We then assessed the amount of GSH and PC₄ for controls and plants treated with 85 µM CdSO₄. A significant interaction was found among Cd²⁺ concentration and genotypes with respect to GSH concentration (two-way ANOVA, $F_{3,24}=4.5085$, $P=0.01206$; Fig. 4B). The amount of GSH was significantly higher for the MpPCS-27 transgenic line compared with all other genotypes in control conditions, while both transgenic lines had a higher GSH amount under Cd²⁺

![Fig. 4](image-url). Functional complementation of the cad1-3 mutant by overexpressing MpPCS. (A) Fresh weight was measured to evaluate the recovery of Cd²⁺ hypersensitivity from thirteen 10-day-old independent transgenic T₃ lines compared with cad1-3 and Col-0 plants growing in a medium supplemented with 0 µM (top), 50 µM (middle), or 85 µM (bottom) CdSO₄. Bars correspond to the SD ($n=45$ plants). (B) GSH amount. (C) PC₂ amount. (D) PC₃ amount. For thiol-peptide analyses, 10-day old seedlings were treated with or without 85 µM CdSO₄ for an additional 3 d. Bars indicate the SE ($n=4$ biological replicates), and different letters represent statistically significant differences (two-way ANOVA test, $P<0.05$).
constructs displayed variable levels of resistance to Cd$^{2+}$. A significant interaction was found among Cd$^{2+}$ concentration and genotypes with respect to PC$_2$ concentration (two-way ANOVA, $F_{3,24}=99.83$, $P=1.082\times10^{-13}$; Fig. 4C). PC$_2$ content did not change for the $\text{cad}1-3$ genotype, but increased significantly for all other genotypes upon Cd$^{2+}$ treatment. Both transgenic lines had levels of PC$_2$ comparable with (MpPCS-50) or slightly higher (MpPCS-27) than Col-0 (Fig. 4C). A significant interaction was also found among Cd$^{2+}$ concentration and genotypes with respect to PC$_3$ concentration (two-way ANOVA, $F_{3,24}=308.66$, $P=2.2\times10^{-16}$; Fig. 4D), with a similar trend to that of PC$_2$, but in this case Col-0 produced more PC$_3$ than both transgenic lines (Fig. 4D). To confirm whether phenotypic complementation was due to overexpression of the MpPCS gene, the expression level was measured by qPCR for all tested lines. Even though variations of expression levels were detected, MpPCS was expressed in all 13 lines (Supplementary Fig. S3).

Diverse heavy metal responsiveness in C-terminal point mutations of MpPCS in yeast and PCS activity assay of recombinant protein

To pinpoint whether the amino acids in the C-terminal domain of MpPCS had a role in sensing of different heavy metals, six independent sets of mutations were constructed, targeting three different positions with evolutionarily conserved cysteines (mutants MpPCS-m1, MpPCS-m2, MpPCS-m3, MpPCS-m4, MpPCS-m5, and MpPCS-m6; Fig. 5). Upon transformation into yeast strain YK44, heavy metal responsiveness was first qualitatively evaluated on the growth medium following exposure to Cd$^{2+}$ and Zn$^{2+}$. In control YPGAL medium, yeast lines transformed with the empty vector either in the absence or in presence of 100 µM Cd$^{2+}$ caused any effect on yeast in the absence of excess heavy metals (Fig. 6). Upon exposure to 100 µM Cd$^{2+}$, the six constructs displayed variable levels of resistance to Cd$^{2+}$. The yeast line transformed with MpPCS-m3 showed the highest resistance to Cd$^{2+}$, being even more resistant than the line expressing WT MpPCS. On the other hand, the MpPCS-m4 line was less resistant than the MpPCS line, with growth levels similar to the empty vector line. The other mutations showed levels of resistance similar to the WT MpPCS line. Also in the case of Zn$^{2+}$ treatment, the mutant lines showed different resistance patterns. Again, the MpPCS-m3 mutation caused higher tolerance to Zn$^{2+}$ treatment than WT MpPCS. MpPCS-m1, MpPCS-m5, and Mpo-PCS-m6 lines were also resistant to Zn$^{2+}$, but less so than the MpPCS line. The other mutations caused an almost complete loss of resistance to Zn$^{2+}$, with growth rates very similar to that of the empty vector line. Given the identical trends for Zn and Cd treatments, we assessed the amount of GSH and PC$_n$ only for Cd in the yeast lines transformed with the empty vector, the WT MpPCS, and the MpPCS-m3 mutant. In the case of GSH concentration, no significant interaction was found between the amount of Cd$^{2+}$ and genotypes (two-way ANOVA, $F_{2,18}=1.380$, $P=0.277$; Fig. 6B), and no significant difference was found in GSH content between Cd$^{2+}$ concentrations and among genotypes (two-way ANOVA for Cd$^{2+}$ treatments, $F_{2,18}=2.846$, $P=0.084$). Thus, all the strains contained, under both control and treated conditions, the same amount of GSH (Fig. 6B). In the case of PC$_2$, we found an interaction among amount of Cd$^{2+}$ and genotypes (two-way ANOVA, $F_{2,18}=33.161$, $P=9.203\times10^{-7}$; Fig. 6C). As expected, only trace amounts of PC$_2$ were present in lines transformed with the empty vector either in the absence or in presence of 100 µM Cd$^{2+}$. Significantly larger amounts of PC$_2$ was present in lines transformed with either the WT MpPCS or the MpPCS-m3 constructs, with the latter containing less PC$_2$ than the former in both control and treated conditions (Fig. 6C). In the case of PC$_3$, the results were similar [interaction among amount of Cd$^{2+}$ and genotypes, two-way ANOVA, $F_{2,18}=235.95$, $P=1.22\times10^{-13}$ with the difference that the mean PC$_3$ amount in MpPCS-m3 did not differ from those in lines transformed with the empty vector (Fig. 6D). To confirm the qualitative differences observed in yeast in responses of the six mutations to Cd$^{2+}$ and Zn$^{2+}$ relative to wild-type MpPCS, we expressed all constructs as 6×His tag N-terminal fusions in E. coli. Soluble recombinant proteins could be purified only for three constructs (WT MpPCS, MpPCS-m3, and MpPCS-m5; Supplementary Fig. S4). For two other constructs (MpPCS-m2 and MpPCS-m4), proteins were expressed exclusively in inclusion bodies, while

![Fig. 5](6663). Scheme of C-terminal mutations in MpPCS proteins. The numbers above the wild-type sequence indicate the first and last positions of mutated amino acids, a dot indicates any amino acid identical to that of wild-type MpPCS, and the symbol ‘//’ marks the omission of a partial amino acid sequence due to space limitations.)
in the case of MpPCS-m6, no recombinant protein was expressed either in inclusion bodies or in the soluble supernatant in *E. coli*, suggesting that the mutations involving stretches of several amino acids could affect protein folding/stability. Thus, PCS activity could be measured exclusively for WT MpPCS, MpPCS-m3, and MpPCS-m5 enzymes upon Cd\(^{2+}\) and Zn\(^{2+}\) activation through quantification of the three major PC polymerization levels (PC\(_2\), PC\(_3\), and PC\(_4\)). In the presence of 100 µM CdSO\(_4\), the means of total PC\(_n\) formation were significantly heterogeneous across the three enzymes (one-way ANOVA, \(F_{2,12}=62.3, P=4.58\times10^{-7}\)), and it was significantly higher in both MpPCS-m3 and MpPCS-m5.
enzymes compared with WT MpPCS in the order MpPCS-m3>MpPCS-m5>MpPCS (Supplementary Fig. S5). In the presence of 200 µM ZnSO₄, the production of PCₙ was still significantly heterogeneous across the three enzymes (one-way ANOVA, $F_{2,12}=131.6, P=6.86\times10^{-9}$). Also in this case, the highest PCₙ production among them was from MpPCS-m3, while MpPCS did not differ significantly from MpPCS-m5 (Supplementary Fig. S5). In addition, to evaluate in more detail the pattern of individual PCₙ production, the comparison among different genotypes upon heavy metal induction was examined. For the 100 µM CdSO₄ treatment, the activity of MpPCS-m3 was significantly higher than those of MpPCS and MpPCS-m5 for the production of PC₂ and PC₃, as well as PC₄ (Fig. 7). The activity of MpPCS-m5 was also significantly higher than that of MpPCS for PC₂, but significantly lower than MpPCS for PC₃ and PC₄ production. The same pattern was also observed for MpPCS-m3 subjected to ZnSO₄ treatment, and also for MpPCS-m5 only for PC₃ and PC₄ formation; however, no significant difference between MpPCS and MpPCS-m5 was detected for PC₄ (Fig. 7). Thus, the MpPCS-m3 and MpPCS-m5 mutations significantly varied in the polymerization level of PCₙ as compared with WT MpPCS, with MpPCS-m3 having a higher polymerization level and MpPCS-m5 having a lower polymerization level than the WT in both heavy metal treatments.

**Discussion**

Decades of studies have shown that PCₙ are constitutively present in various plant lineages and play critical roles for detoxification/homeostasis of a wide range of heavy metals (Degola *et al.*, 2014; Kühnlenz *et al.*, 2016; Fontanini *et al.*, 2018). Till now, however, only the genes encoding PCS proteins in higher plant species have been isolated and functionally characterized (e.g. Loscos *et al.*, 2006; Li *et al.*, 2019). In contrast, no detailed molecular studies of PCSs of early diverging land plants such as liverworts have been conducted, despite the high relevance of this clade which is considered to encompass the earliest representatives of the radiation of plants on land (Bowman *et al.*, 2016). Thus, in this study, we addressed the functional characterization of MpPCS from *M. polymorpha* to shed new light on the evolution of molecular mechanisms for heavy metal detoxification in land plants and in particular on the role of the PCS C-terminal domain.

**MpPCS is the functional ortholog of angiosperm PCS genes**

Several lines of evidence indicate that *MpPCS* shares common features with the *PCS* genes characterized in angiosperms so far. *MpPCS* has the two domains found in all other PCS proteins, a typically highly conserved N-terminal domain and a more divergent C-terminal domain. *MpPCS* further displays in its N-terminal domain fully conserved amino acids of the catalytic triad (Fig. 1B; Romanyuk *et al.*, 2006). The *MpPCS* gene is constitutively expressed in different organs under control growing conditions (Fig. 2A), and its overexpression in yeast strain YK44 enhances heavy metal resistance upon exposure to Cd²⁺ or Zn²⁺ like many other functional PCSs (Supplementary Fig. S1; Liu *et al.*, 2011; Zhao *et al.*, 2014). Furthermore, overexpression of the *MpPCS* CDS fully complements the PC-deficient mutant *cad1-3* in Arabidopsis (Fig. 4). Additionally, the purified recombinant protein *MpPCS* was able to catalyze the synthesis of PCₙ using GSH as substrate following activation by Cd²⁺ or Zn²⁺ in vitro (Fig. 7; Supplementary Fig. S5). Taken together, these data clearly demonstrate that *MpPCS* is the ortholog in *M. polymorpha* and has the same function as *AtpCS1*.

Previous studies have shown transcriptional regulation of angiosperm *PCS* genes upon heavy metal exposures. For instance, the transcripts of *AtpCS1* in Arabidopsis young seedlings were induced upon heavy metal Cd²⁺ exposure (Lee and Korban, 2002). Also, a dramatic increase of *TaPCS1* expression in wheat root was observed after Cd²⁺ treatment (Clemens...
et al., 1999), and the time course analyses of MaPCS1 and MaPCS2 expression patterns in different organs of mulberry also indicated significant induction upon exposure to Cd\(^{2+}\) or Zn\(^{2+}\) (Fan et al., 2018). Interestingly, in contrast to these results, the expression of MpPCS was repressed upon Cd\(^{2+}\) and Zn\(^{2+}\) exposure. Analogously to higher plant PCS genes, however, MpPCS induction by Cd\(^{2+}\) was modest, attaining just a 2-fold change as compared with untreated controls (Lee and Korban, 2002; Li et al., 2019), confirming the overall limited transcriptional responsiveness of PCS in the course of evolution. This observation further suggests that the well-known heavy metal-dependent post-transcriptional regulation of PCS enzymatic activity has been playing a major role in the tight control of PC\(_n\) production since the early stages of evolution of this enzyme. Our results on both the differential transcriptional regulation and enzymatic activation by Cd\(^{2+}\) as compared with Zn\(^{2+}\) further confirm the highest induction capacity of the former to elicit PC\(_n\) biosynthesis, as previously suggested not only for higher plants but also for another liverwort, L. Dumort (Degola et al., 2014; Petraglia et al., 2014).

Many studies have attempted to increase the heavy metal tolerance of plants by overexpressing PCS genes from different species, but only in a minority of cases have enhanced tolerance to heavy metals and increased PC\(_n\) content been attained (Liu et al., 2012; Shukla et al., 2012; Fan et al., 2018). More commonly, overexpression of heterologous PCS genes resulted in hypersensitivity to heavy metals (Wojas et al., 2008; Wang et al., 2012; Li et al., 2019). In the case of transgenic plants overexpressing MpPCS in Arabidopsis, a substantial increase of sensitivity to Cd\(^{2+}\) or Zn\(^{2+}\) compared with WT Col-0 was obtained (Fig. 3A, B). Almost certainly this result can be explained by a depletion of the pool of GSH, the substrate of PCS enzymes, and the resulting disruption of the cellular redox balance (Wojas et al., 2008; Brunetti et al., 2011). Taken together, our results highlight how, despite hundreds of million of years of divergent evolution between extant liverworts and angiosperms, the involvement of PCS in heavy metal detoxification is fully functionally conserved between M. polymorpha and A. thaliana.

A conserved cysteine motif in the land plant PCS C-terminal domain prevents enzyme overactivation by heavy metals

The C-terminal domain of PCS has been demonstrated to be fundamental for the sustained PCS activity and stability necessary for plants to cope with elevated concentrations of the non-essential Cd\(^{2+}\) ion and proposed to be involved in heavy metal perception/specificity as well as metallochaperone and metallothionein functions (Ruotolo et al., 2004; Vestergaard et al., 2008). Identification of MpPCS as the functionally validated enzyme from the most basal land plant sequenced to date provides a unique opportunity to functionally test the ancestral role of this still enigmatic domain, as conserved cysteines have been proposed to be those most relevant for metal binding (Maier et al., 2003). Among the three sets of most conserved cysteines in the C-terminus of land plants PCSs, two (C355–C356 and C372–C373) were homologous to residues previously identified to bind Cd\(^{2+}\) in TaPCS1 (C351–C352 and C369–C370), while C231 did not bind any metal ion (Maier et al., 2003). While, in general, in our work the mutations involving multiple substitutions in a stretch of residues around the conserved cysteines (MpPCS-m2, MpPCS-m4, and MpPCS-m6) impaired protein solubility/stability too heavily to provide useful information, mutations of only the conserved cysteines (MpPCS-m1, MpPCS-m3, and MpPCS-m5) were highly informative. Consistent with a possible structural role, the C231A (MpPCS-m1) single mutation provided a very weak increase in heavy metal tolerance in yeast and could not be stably expressed in E. coli, suggesting that it is essential for correct folding/stability of the enzyme. In contrast, the other two double mutant proteins, C355A–C356A (MpPCS-m3) and C372A–C373A (MpPCS-m5), could be solubly expressed in E. coli, suggesting that they do not overly destabilize the enzyme. Indeed, the quadruple mutant of AtPCS1, where both cysteine homologs to C372A–C373A and an additional two cysteine residues in close proximity (not conserved in MpPCS) were mutated to alanines, was previously demonstrated to be fully stable (Vestergaard et al., 2008). In vivo yeast expression does not support that the MpPCS-m3 mutation enhances PC\(_n\) production as compared with the WT MpPCS, indicating that in this heterologous system the MpPCS-m3 mutant enzyme is less active than the WT enzyme in the conditions tested. Currently the reasons for this difference remain to be investigated. However, in vitro enzymatic assays demonstrate that MpPCS-m5 and especially MpPCS-m3 mutations enhance PC\(_n\) production as compared with the WT enzyme. To the best of our knowledge, this is the first time a mutation increasing the enzyme Cd\(^{2+}\)-responsive activity has been found. Recently, deletion of the last 10 amino acids of the AtPCS1 C-terminus was found to increase As\(^{3+}\)-dependent PC\(_n\) production, suggesting that some of the residues in this region may inhibit PCS activation by As\(^{3+}\) (Uraguchi et al., 2018). Our findings indicate that in addition to As\(^{3+}\), Cd\(^{2+}\)-dependent repressing mechanisms also possibly exist to prevent enzyme overactivation. Most importantly, these regulatory functions of the PCS C-terminus are evolutionarily conserved and can have different degrees of selectivity: while the MpPCS-m5 mutation selectively abolishes Cd\(^{2+}\)-dependent repression, MpPCS-m3 mutation abolishes both Cd\(^{2+}\)- and Zn\(^{2+}\)-dependent repression in vitro. We speculate that the cysteines at both sites may constitute low-affinity binding sites for Cd\(^{2+}\) and Zn\(^{2+}\) that, above a certain threshold concentration of these metal ions, act by inhibiting PCS activity. A protective function of the C-terminus had been previously proposed in cases where the free heavy metal ion concentration would exceed those required for formation of the metal–thiolate substrate (Romanyuk et al., 2006; Rea, 2012). AtPCS1 activity as a function of Cd\(^{2+}\) concentrations is indeed bell-shaped, with a maximum at ~1 µM (Rea, 2012), implying either heavy metal-mediated enzyme inactivation or the existence of feedback inhibition mechanisms to avoid enzyme overactivation in the presence of high heavy metal concentrations. Our results indicate that such mechanisms indeed exist, are conserved across the whole evolutionary history (hundreds of million of years of divergent evolution between extant liverworts and angiosperms; Rubinstein et al., 2010) of
land plants, and are mediated by metal-binding cysteines. This finding points to a likely role for the PCS C-terminal domain in maintaining homeostatic levels of essential ions (Steffens et al., 1986; Grill et al., 1987; Kühnlenz et al., 2016) and GSH (Lee et al., 2003a), while preventing Cd²⁺ toxicity. From an applied perspective, if confirmed in planta, the higher activity of PCS mutants with increased activity may be exploited to decrease the root to shoot transport of Cd²⁺, as previously suggested in the case of arsenic (Uraguchi et al., 2018). This, in turn, would contribute to reduce the amount of this highly toxic heavy metal in the human food chain.

**Supplementary data**

The following supplementary data are available at JXB online. Table S1. List of primers used in this study.

Fig. S1. Yeast growth of heavy metal-hypersensitive strain YK44 transformed with empty vector and wild-type MpPCS.

Fig. S2. Semi-quantitative RT-PCR of MpPCS transcription in 16 independent Arabidopsis transgenic lines.

Fig. S3. Relative expression of MpPCS by qRT-PCR, from 7-day-old seedlings of 13 transgenic Arabidopsis lines.

Fig. S4. Recombinant proteins MpPCS-WT, MpPCS-m3, and MpPCS5 purified from E. coli and electrophoresed by 10% SDS–PAGE.

Fig. S5. Total PC (PC₂, PC₃, and PC₄) production by recombinant proteins MpPCS, MpPCS-m3, and MpPCS-m5 in vitro.

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**Author contributions**

ML, LST, and CV planned and designed the research. ML performed experiments and analysed the data, with the help of EBe and EBa. ML and CV wrote the manuscript. ML, EBe, AS, LST, and CV corrected the manuscript.

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