Adaptive Engineering of Phytochelatin-based Heavy Metal Tolerance*

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Background: Plants synthesize phytochelatin peptides for protection against heavy metals.

Results: Metabolic engineering in yeast and plants using a phytochelatin synthase variant leads to improved cadmium tolerance.

Conclusion: Enhanced cadmium tolerance results from a balance between phytochelatin synthesis and redox state.

Significance: Our results emphasize the importance of metabolic context for pathway engineering and broaden the range of tools for environmental remediation.

Metabolic engineering approaches are increasingly employed for environmental applications. Because phytochelatins (PC) protect plants from heavy metal toxicity, strategies directed at manipulating the biosynthesis of these peptides hold promise for the remediation of soils and groundwaters contaminated with heavy metals. Directed evolution of Arabidopsis thaliana phytochelatin synthase (AtPCS1) yields mutants that confer levels of cadmium tolerance and accumulation greater than expression of the wild-type enzyme in Saccharomyces cerevisiae, Arabidopsis, or Brassica juncea. Surprisingly, the AtPCS1 mutants that enhance cadmium tolerance and accumulation are catalytically less efficient than wild-type enzyme. Metabolite analyses indicate that transformation with AtPCS1, but not with the mutant variants, decreases the levels of the PC precursors, glutathione and γ-glutamylcysteine, upon exposure to cadmium. Selection of AtPCS1 variants with diminished catalytic activity alleviates depletion of these metabolites, which maintains redox homeostasis while supporting PC synthesis during cadmium exposure. These results emphasize the importance of metabolic context for pathway engineering and broaden the range of tools available for environmental remediation.

Environmental heavy metal contamination, which is implicated in many diseases and agricultural losses (1–2), poses a challenge with a price tag for remediation estimated at upward of $200 billion in the United States alone (2). Heavy metals, such as cadmium (Cd2+), undergo aberrant capping reactions with the thiol groups of proteins and some coenzymes, displace endogenous metal cofactors from their cellular binding sites, and promote the formation of reactive oxygen species (2). Engineering plants and microbes for the detoxification of heavy metal-contaminated soils and waters traditionally rely on the modification of existing metabolic pathways and/or metal transport and sequestration systems (3–7). Although transgenic methods can enhance heavy metal tolerance and accumulation, the inherent biochemical properties of the proteins introduced often limit the extent of the enhancement. Engineering the proteins that are used for this purpose can offset inherent limitations on tolerance to and/or accumulation of heavy metals so as to broaden the range of molecular tools available for environmental clean-up (8).

Plants employ a combination of metabolic enzymes and transport systems for protection against toxic levels of heavy metals (9). Exposure of plants to heavy metals, such as cadmium, arsenic, mercury, or lead, promotes the synthesis of phytochelatin (PC)2 peptides from glutathione (GSH) by the enzyme PC synthase (PCS) (Fig. 1A) (10–13). PCs bind heavy metals with high affinity and, as first established by the isolation of PCS-deficient, cadmium-sensitive mutants of Arabidopsis thaliana, they confer significant protection from heavy metal toxicity (14, 15).

PCS catalyzes the heavy metal-activated transeptidation of a γ-glutamylcysteine (γEC) unit from one GSH molecule to a second GSH molecule to yield a PC (PC2) containing two γEC units and a C-terminal glycine residue to which further γEC units may be added to generate peptides containing up to 15 γEC repeats (Fig. 1B) (16–18). Vacuolar sequestration of PC-metal complexes finishes the detoxification mechanism (19–21). Comparison of PCS from different species reveals a conserved N-terminal cysteine protease-like catalytic domain followed by highly variable C-terminal region (22–26). Given the critical role of PCS for heavy metal tolerance, strategies for enhancing PC biosynthetic capacity offer the potential for generating plant stocks for the phytoremediation of heavy metal-contaminated soils and groundwaters. Toward this end, we targeted Arabidopsis 1 (AtPCS1) for directed evolution with the

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‡ The abbreviations used are: PC, phytochelatin; PCS, PC synthase; γEC, γ-glutamylcysteine; GSH, reduced glutathione; GSSG, oxidized glutathione.
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A
\[
\begin{align*}
\text{γ-glutamate} & \rightarrow \text{γ-glutamate} & \text{cysteine} & \rightarrow \text{cysteine} & \rightarrow \text{glutathione} \\
\text{H}_2\text{N} & \text{O} & \text{O} & \text{H}_2\text{N} & \text{O} & \text{OH}
\end{align*}
\]

B
\[
\begin{align*}
\text{glycine} & \rightarrow \text{glutathione (GSH)} & \text{2-unit phytochelatin} & \rightarrow \text{PCS} \\
\end{align*}
\]

FIGURE 1. Phytochelatin structure and synthesis. A, general structure of a representative PC. The example shown is PC2, which contains two γEC repeats. The core structures of PCs consist of 2–15 γEC repeats derived from glutathione (GSH) by the transfer of a γEC unit from one GSH molecule to another or by the transfer of γEC units from GSH to pre-existent PCs. PCs have the general structure (γEC)x, where X is usually Gly. B, the overall reaction catalyzed by PCS is a metal, for instance Cd^{2+}, activated dipeptidyl transfer reaction in which PC chain extension proceeds in the N to C direction.

aim of identifying variants that improve PC-based protection from and accumulation of cadmium in plants.

Experimental Procedures

Materials—Saccharomyces cerevisiae ycf1Δ strain DTY167 (MATα ura3–52 leu2–3,112 his−Δ200 trp1−Δ901 lys2−801 suc2−Δ9 ycf1::hisG), which is Cd^{2+} hypersensitive (27–28), pYES3 yeast-Escherichia coli expression vector (29), which is a derivative of pYES2 (Invitrogen) with the galactose-inducible promoter replaced by the constitutive 3-phosphoglycerate kinase promoter and engineered to encode a C-terminal FLAG epitope tag, and the pYES3-AtPCS1::FLAG vector (11) were used for the mutant screens and analyses of the effects of constitutively heterologously expressed AtPCS1. The pART27 vector (30) was provided by Dr. Edgar Cahoon. Arabidopsis thaliana ecotype Col-0 and Brassica juncea seeds (accession no. 173874) were obtained from the Arabidopsis Biological Research Center (Ohio State University, Columbus, OH) and the North Central Regional Plant Introduction Station (Ames, IA), respectively. The pET28a-AtPCS1 construct was previously described (26). For the generation of pET28a-AtPCS1 mutant constructs, AtPCS1 coding regions were PCR amplified from the appropriate vectors and subcloned into pET28a.

Mutant Library Generation and Screens—Random mutagenesis of AtPCS1 (GenBank™ AF085230) was performed directly on the pYES3-AtPCS1::FLAG construct (11) using the GeneMorph kit (Stratagene). Reactions were performed using template quantities estimated to give 1–4 base pair changes per gene copy. The gel-purified PCR amplification products were ligated into NotI/BamH1 double-digested pYES3 to generate an expression construct library. After transformation by electroporation into E. coli DH5α for amplification, plasmid DNA was isolated and transformed into S. cerevisiae ycf1Δ DTY167 cells using a Frozen EZ II yeast transformation kit (Zymo Research). The library of transformants was amplified by growth at 30°C on CSM/ura− medium supplemented with 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% dextrose. To screen for Cd^{2+}-tolerant Ura+ transformants, the library was plated on media supplemented with 0–1 mM CdCl₂.

Generation and Preliminary Characterization of Transgenic Plants—For Arabidopsis transformation, the wild-type and mutant AtPCS1::FLAG inserts from pYES3 were subcloned into pART27 (30), a modified binary vector with a cauliflower mosaic virus 35S promoter-driven expression cassette and a kanamycin-resistance marker. The pART27, pART27-AtPCS1::FLAG, and pART27-AtPCS1-Y186C::FLAG vectors were introduced into Agrobacterium tumefaciens by electroporation, and Arabidopsis plants were transformed by the floral dip method and grown to maturity. After selecting and harvesting the T1 seeds, multiple kanamycin-resistant lines were isolated for the generation of T2 seeds. T2 seeds exhibiting a 3:1 segregation ratio on kanamycin plates were used for the subsequent isolation of multiple independent homozygous lines whose identity was confirmed by PCR. To confirm expression of FLAG-tagged AtPCS1, the seedlings were grown in liquid nitrogen and extracted in 50 mM potassium phosphate buffer (pH 8). Proteins were separated by SDS-PAGE and electrophoresed to nitrocellulose membranes for Western analysis with anti-FLAG M2 antibody (Sigma). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences). An in vitro plant tissue culture method was used to transform B. juncea (31, 32). The pART27, pART27-AtPCS1::FLAG, and pART27-AtPCS1-Y186C::FLAG vectors were transformed into Agrobacterium and 200–300 B. juncea hypocotyl segments each for the vector control, AtPCS1 and AtPCS1-Y186C constructs were subjected to transformation. Stable kanamycin-resistant transformants were identified by PCR and used to generate T2 seeds for subsequent analysis. Expression of FLAG-tagged AtPCS1 was confirmed by Western analysis as described for the Arabidopsis transformants.

Yeast Heavy Metal Tolerance Assays—To assess the capacity of heterologously expressed wild-type and mutant AtPCS1::FLAG for conferring heavy metal tolerance, S. cerevisiae ycf1Δ strain DTY167 was transformed with pYES3 vector, pYES3 containing the AtPCS1::FLAG insert, or pYES3 containing the mutant AtPCS1::FLAG insert and grown at 30°C to an A_600 nm of 1.5–1.8 in AHC medium supplemented with glucose and tryptophan before inoculating aliquots into 4-ml of the same medium containing the concentrations of CdCl₂ indicated. After growth for 12–24 h, when the subcultures were in mid-
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Enhanced Cd\(^{2+}\) Tolerance and Increased PC Accumulation in Yeast Expressing Mutant AtPCS1—For characterization of the mutants isolated from the screen that conferred enhanced Cd\(^{2+}\) tolerance, individual clones were re-transformed into DTY167 cells and assayed for cadmium tolerance on agar plates (Fig. 2A) and in liquid media (Fig. 3; Table 1). Transformants of strain DTY167 displayed 4–8-fold increases in Cd\(^{2+}\) tolerance compared with transformants containing the wild-type construct based on the concentration of CdCl\(_2\) required to decrease cell density by 50% (IC\(_{50}\)) after growth for 24 h. Each mutant not only increased Cd\(^{2+}\) tolerance but also elicited PC accumulation by 3–6-fold compared with wild-type (Fig. 2B; Table 1). In agreement with previous studies (11), the principal PCs synthesized after exposure of the transformants to Cd\(^{2+}\) were PC\(_2\), PC\(_3\), PC\(_4\), and PC\(_5\) (7:1). Sample digests were analyzed using a PerkinElmer AAnalyst 300 atomic absorption spectrometer.

Results

Mutations in the N-terminal Catalytic Domain of AtPCS1—Random mutagenic PCR was employed to generate a library of AtPCS1 variants that were subcloned into the yeast-\textit{E. coli} shuttle vector pYES3 for constitutive heterologous expression in the Cd\(^{2+}\)-hypersensitive \textit{ycf}\textsubscript{D} \textit{S. cerevisiae} strain DTY167. All AtPCS1 variants were C terminally FLAG-tagged to facilitate immunodetection. DNA sequencing of 200 randomly selected clones from a total pool of \textasciitilde 30,000 randomly mutagenized pYES3-AtPCS1::FLAG constructs established that mutations were distributed throughout the coding sequence of AtPCS1.

To identify AtPCS1::FLAG mutants that conferred improved Cd\(^{2+}\) tolerance compared with wild-type AtPCS1::FLAG, yeast strain DTY167 transformed with empty pYES3 vector or vector containing either wild-type or mutant AtPCS1::FLAG was plated on agar plates containing 0–1 mM CdCl\(_2\). Although the majority of yeast transformants containing either wild-type or mutant pYES3-AtPCS1::FLAG grew on plates with 0 to 200 \textmu M Cd\(^{2+}\), there was a subset of transformants capable of growing on plates containing up to 800 \textmu M Cd\(^{2+}\). Sequence analysis of the pYES3-AtPCS1::FLAG constructs isolated from the colonies growing on plates containing 800 \textmu M CdCl\(_2\) identified 17 variants containing 1–4 amino acid substitutions largely in the N-terminal catalytic domain (residues 1–221) of the enzyme (Table 1).

Estimation of Cadmium Content of Yeast and Plant Extracts—The Cd\(^{2+}\) contents of yeast strain DTY167 after transformation with pYES3, pYES3-AtPCS1::FLAG, or mutant pYES3-AtPCS1::FLAG were estimated after growth at 30 °C for 24 h in media containing 0, 5, 50, or 250 \textmu M CdCl\(_2\). The cultures were pelleted by centrifugation and washed twice with 1.5 mM tartaric acid for 15 min before lyophilization. After weighing, the dry lyophilizates were exhaustively digested with 65% nitric acid at 200 °C for 6 h for metal analysis. For measurements of the Cd\(^{2+}\) content of plant tissues, stable transformants were selected by germination on Murashige-Skoog (MS) agar plates containing kanamycin (30 \textmu M) before the transfer of seedlings to plates containing 0 and 100 \textmu M CdCl\(_2\) and grown for 3 weeks. After harvesting, the shoots of the seedlings were thoroughly washed with deionized water, dried at 70 °C for 48 h, weighed, and digested in a mixture of nitric and perchloric acid (7:1). Sample digests were analyzed using a PerkinElmer AAnalyst 300 atomic absorption spectrometer.

Measurement of PCs, \gamma EC, and GSH—The cellular PC contents of yeast transformed with pYES3, wild-type pYES3-AtPCS1::FLAG, or mutant pYES3-AtPCS1::FLAG were estimated as described previously (11) by reversed-phase HPLC and spectrophotometric measurements of the thiol contents of the chromatographic fractions after reaction with Ellman’s reagent (5,5′-dithio-bis(nitrobenzoic acid)) at \textit{A}\textasciitilde124 nm. The PC contents of plant materials were estimated after the addition of 300 \textmu l of 1 M NaOH containing 1 \mu g \mu l\(^{-1}\) of NaBH\(_4\) to 100-\mu l aliquots of the tissue homogenates and centrifugation of the samples for 3 min at 13,000 \times g. Reversed-phase HPLC of the supernatants from centrifugation was performed on a Hypersil ODS C\(_18\) column (250 × 4.6 mm; 5 \mu m particle size) after acidification of the samples with 5% 5-sulfosalicylic acid. The column was developed with a 0–20% linear concentration gradient of acetonitrile, 0.1% formic acid at a flow rate of 0.5 ml min\(^{-1}\) and the thiol contents of the chromatographic fractions were estimated spectrophotometrically after reaction with Ellman’s reagent as described above. The \gamma EC and total glutathione contents of yeast and plant extracts were estimated fluorometrically after derivatization of the samples with monobromobimane as described previously (33). Levels of free glutathione (GSH) and glutathione disulfide (GSSG) were determined using glutathione reductase and 2-vinylpyridine (34).

Mass Spectrometry—The identities of the PCs isolated from extracts of the Cd\(^{2+}\)-grown yeast DTY167 pYES3-AtPCS1::FLAG transformants, which were subsequently used as standards for their identification in plant extracts, were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Samples (1 \mu l) of the HPLC-purified PC fractions were mixed with 10 \mu l of matrix solution (10 mg ml\(^{-1}\) of \textalpha- cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/acetonitrile + water (1:1)) and applied to a MALDI plate for laser desorption ionization and detection in negative ion mode in a Voyager-DE STR system (Applied Biosystems). The PCs identified in the yeast ex-tracts (with their monoisotopic [M-H] \textsuperscript{-} mass values in parenthesis) were PC\(_2\) (−538.82), PC\(_3\) (−769.31), PC\(_4\) (−1001.89), and PC\(_5\) (−1232.67).

Estimation of Cadmium Content of Yeast and Plant Extracts—The Cd\(^{2+}\) contents of yeast strain DTY167 after transformation with pYES3, pYES3-AtPCS1::FLAG, or mutant pYES3-AtPCS1::FLAG were estimated after growth at 30 °C for 24 h in media containing 0, 5, 50, or 250 \mu M CdCl\(_2\). The cultures were pelleted by centrifugation and washed twice with 1.5 mM tartaric acid for 15 min before lyophilization. After weighing, the dry lyophilizates were exhaustively digested with 65% nitric acid at 200 °C for 6 h for metal analysis. For measurements of the Cd\(^{2+}\) content of plant tissues, stable transformants were selected by germination on Murashige-Skoog (MS) agar plates containing kanamycin (30 \mu M) before the transfer of seedlings to plates containing 0 and 100 \mu M CdCl\(_2\) and grown for 3 weeks. After harvesting, the shoots of the seedlings were thoroughly washed with deionized water, dried at 70 °C for 48 h, weighed, and digested in a mixture of nitric and perchloric acid (7:1). Sample digests were analyzed using a PerkinElmer AAnalyst 300 atomic absorption spectrometer.

Measurement of PCS Activity—Wild-type and mutant AtPCS1 were overexpressed and purified from \textit{E. coli} BL21(DE3) for determination of enzymatic activity and kinetic parameters (26). Unless indicated to the contrary, the PCS activities of affinity-purified N terminally His-tagged wild-type and mutant AtPCS1 were assayed in media containing 3.3 mM GSH, 50 \mu M CdCl\(_2\), and 100 mM BTP-HEPES buffer (pH 8.0) at 30 °C for 10 min as described (23). For the quantitation of PCs, thios were estimated spectrophotometrically in the RP-HPLC-separated fractions after reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Rates of PC synthesis were expressed as thios (micro-mole) incorporated per min per mg of protein (\mu mol min\(^{-1}\) mg of protein\(^{-1}\)).

Results

Mutations in the N-terminal Catalytic Domain of AtPCS1—Random mutagenic PCR was employed to generate a library of AtPCS1 variants that were subcloned into the yeast-\textit{E. coli} shuttle vector pYES3 for constitutive heterologous expression in the Cd\(^{2+}\)-hypersensitive \textit{ycf}\textsubscript{D} \textit{S. cerevisiae} strain DTY167. All AtPCS1 variants were C terminally FLAG-tagged to facilitate immunodetection. DNA sequencing of 200 randomly selected clones from a total pool of \textasciitilde 30,000 randomly mutagenized pYES3-AtPCS1::FLAG constructs established that mutations were distributed throughout the coding sequence of AtPCS1. To identify AtPCS1::FLAG mutants that conferred improved Cd\(^{2+}\) tolerance compared with wild-type AtPCS1::FLAG, yeast strain DTY167 transformed with empty pYES3 vector or vector containing either wild-type or mutant AtPCS1::FLAG was plated on agar plates containing 0–1 mM CdCl\(_2\). Although the majority of yeast transformants containing either wild-type or mutant pYES3-AtPCS1::FLAG grew on plates with 0 to 200 \mu M Cd\(^{2+}\), there was a subset of transformants capable of growing on plates containing up to 800 \mu M Cd\(^{2+}\). Sequence analysis of the pYES3-AtPCS1::FLAG constructs isolated from the colonies growing on plates containing 800 \mu M CdCl\(_2\) identified 17 variants containing 1–4 amino acid substitutions largely in the N-terminal catalytic domain (residues 1–221) of the enzyme (Table 1).
**TABLE 1**

Summary of AtPCS1 mutants that enhance Cd\(^{2+}\) tolerance when heterologously expressed in *S. cerevisiae* ycf1Δ strain DTY167

| AtPCS1 variant       | IC\(_{50}\)^a | PC\(_2-PC5\) content\(^b\) | Specific activity\(^c\) |
|----------------------|---------------|----------------------------|-------------------------|
| Wild-type            | 0.15 ± 0.05   | 9 ± 1                      | 16.8 ± 1.4              |
| Q48R/C144Y/G168S/W280R | 0.55 ± 0.14   | 26 ± 6                     | 5.0 ± 0.4               |
| S51T/N143I/H220R     | 0.71 ± 0.13   | 38 ± 8                     | 3.3 ± 0.5               |
| E52K                 | 0.82 ± 0.18   | 32 ± 4                     | 3.2 ± 0.4               |
| A59V                 | 0.73 ± 0.10   | 34 ± 8                     | 2.9 ± 0.8               |
| S60C/S202I           | 0.58 ± 0.09   | 30 ± 9                     | 5.9 ± 0.3               |
| I371N                | 0.68 ± 0.14   | 35 ± 7                     | 5.1 ± 0.3               |
| F83C/N323C/1,250R    | 0.71 ± 0.15   | 40 ± 8                     | 3.7 ± 0.6               |
| C91S/A199S           | 0.69 ± 0.19   | 25 ± 5                     | 4.6 ± 0.4               |
| V97L                 | 0.64 ± 0.13   | 25 ± 9                     | 4.8 ± 0.5               |
| C109Y                | 0.81 ± 0.16   | 34 ± 9                     | 2.8 ± 0.1               |
| T123R/F163I          | 1.02 ± 0.18   | 50 ± 4                     | 1.8 ± 0.3               |
| T139P                | 0.70 ± 0.19   | 36 ± 7                     | 3.6 ± 0.3               |
| V181G                | 0.72 ± 0.15   | 37 ± 4                     | 2.6 ± 0.9               |
| A182G/A282V/G329S    | 0.86 ± 0.15   | 28 ± 8                     | 5.5 ± 0.7               |
| F83C/N170D           | 0.81 ± 0.27   | 35 ± 9                     | 3.1 ± 0.7               |
| C91S/A199S           | 0.69 ± 0.19   | 25 ± 5                     | 4.6 ± 0.4               |
| V97L                 | 0.64 ± 0.13   | 25 ± 9                     | 4.8 ± 0.5               |
| C109Y                | 0.81 ± 0.16   | 34 ± 9                     | 2.8 ± 0.1               |
| T123R/F163I          | 1.02 ± 0.18   | 50 ± 4                     | 1.8 ± 0.3               |
| T139P                | 0.70 ± 0.19   | 36 ± 7                     | 3.6 ± 0.3               |
| V181G                | 0.72 ± 0.15   | 37 ± 4                     | 2.6 ± 0.9               |
| A182G/A282V/G329S    | 0.86 ± 0.15   | 28 ± 8                     | 5.5 ± 0.7               |
| Y186C                | 1.18 ± 0.12   | 56 ± 9                     | 1.3 ± 0.2               |

\(^a\) IC\(_{50}\) values for yeast heterologously expressing AtPCS1 variants were determined in liquid medium (11).

\(^b\) Specific activity of AtPCS1 variants were determined after purification of their His-tagged derivatives from *E. coli* (11, 26). Values shown are mean ± S.E. (n = 3).

\(^c\) PC content, expressed as the sum total of PC\(_2-PC5\) was determined by RP-HPLC (11).

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**FIGURE 2.** Cd\(^{2+}\) tolerance, PC accumulation, and Cd\(^{2+}\) accumulation in yeast strain DTY167 heterologously expressing wild-type or mutant variants of AtPCS1::FLAG. A, Cd\(^{2+}\) sensitivity of *S. cerevisiae* ycf1Δ strain DTY167 transformed with pYES3 (Vec), pYES3-AtPCS1::FLAG (AtPCS1), or pYES3-AtPCS1::FLAG constructs carrying AtPCS1 mutations. B, RP-HPLC analysis of PCs extracted from yeast strain DTY167 transformed with pYES3-AtPCS1::FLAG (blue) or pYES3-AtPCS1::FLAG (red) after growth for 2 h in liquid media containing 250 \(\mu\)M CdCl\(_2\). Peaks labeled as PC\(_2-PC5\) were identified by MALDI-TOF MS. C, Western analysis of the FLAG tag in yeast strain DTY167 transformants expressing wild-type or mutant AtPCS1::FLAG. Aliquots (20 \(\mu\)g of protein) of the soluble fractions were separated by SDS-PAGE, electrotransferred to nitrocellulose membranes, and probed with anti-FLAG M2 antibody. The 55,000 AtPCS1::FLAG polypeptide was the major immunoreactive band. D, comparison of cellular Cd\(^{2+}\) contents of yeast strain DTY167 transformed with empty pYES3 vector (white), pYES3-AtPCS1::FLAG (black), pYES3-AtPCS1::FLAG (red), or pYES3-AtPCS1::FLAG (blue) after growth for 24 h in liquid media containing 0, 50, or 250 \(\mu\)M CdCl\(_2\). The histogram in the left-hand panel is Cd\(^{2+}\) content (\(\mu\)g per 10\(^7\) cells); the histogram in the right-hand panel is Cd\(^{2+}\) content of the total culture cell mass. Values shown are mean ± S.E. (n = 3).
strains transformed with pYES3 vector, pYES3-AtPCS1::FLAG, pYES3-AtPCS1-C109Y::FLAG, and pYES-AtPCS1-Y186C::FLAG were determined after growth for 24 h in liquid media containing 5, 50, or 250 μM CdCl₂. Yeast heterologously expressing either mutant established cellular Cd²⁺ contents greater than those achieved with empty vector or by overexpression of wild-type enzyme (Fig. 2D). Expression of AtPCS1::FLAG increased Cd²⁺ accumulation on a per cell basis versus vector controls, but the enhancements associated with the expression of AtPCS1-C109Y::FLAG and AtPCS1-Y186C::FLAG were 1.3–1.8-fold greater at 250 μM Cd²⁺ (Fig. 2D). Indeed, when cellular Cd²⁺ accumulation was normalized on the basis of total cell weight rather than cell number, the differences were even more pronounced. The total cellular Cd²⁺ accumulation in medium containing 250 μM CdCl₂ was 2–3-fold higher in cultures of AtPCS1-C109Y::FLAG and AtPCS1-Y186C::FLAG transformants than cultures of AtPCS1::FLAG transformants (Fig. 2D). This increase in total Cd²⁺ content reflects the enhanced accumulation per cell in combination with the greater cell densities achieved by the AtPCS1-C109Y::FLAG and AtPCS1-Y186C::FLAG transformants compared with the wild-type AtPCS1::FLAG transformants.

Ectopic Expression of Wild-type AtPCS1 and AtPCS1-Y186C in Arabidopsis—To test whether ectopic expression of an AtPCS1 variant would improve cadmium tolerance and/or accumulation in plants, we compared the effect of AtPCS1 and AtPCS1-Y186C overexpression in Arabidopsis. Multiple lines of Arabidopsis transformed with the empty pART27 vector, pART27-AtPCS1::FLAG, or pART27-AtPCS1-Y186C::FLAG were generated. One T2 line of the pART27 transformants and four independent T2 lines each of the pART27-AtPCS1::FLAG (lines PCS1–4) and pART27-AtPCS1-Y186C::FLAG transformants (lines Y186C1–4) were studied further. After confirming expression of FLAG-tagged wild-type and mutant AtPCS1 in the transformants by Western analysis (Fig. 4A), seeds were germinated on standard horizontal MS plates before transfer of the seedlings to vertical MS plates containing 0–200 μM CdCl₂ to assess tolerance in terms of the effects of Cd²⁺ on root growth.

As described by others (35, 36), overexpression of AtPCS1 in Arabidopsis yielded seedlings that showed little or no change in Cd²⁺ tolerance as determined from root growth assays compared with the pART27 controls. In contrast, overexpression of AtPCS1-Y186C::FLAG improved Cd²⁺ tolerance (Fig. 4B). All four lines expressing AtPCS1-Y186C::FLAG showed increased Cd²⁺ tolerance (Fig. 4C), as indicated by an up to 6-fold increase in root length over that of pART27 con-*
Ectopic Expression of Wild-type AtPCS1 and AtPCS1-Y186C in B. juncea—The encouraging results obtained from Arabidopsis ectopically expressing mutant AtPCS1, and knowing that the variable results obtained using wild-type PCS clones in this plant species apply also to Nicotiana tabacum (tobacco) and B. juncea (Indian mustard) (31, 32, 37–39), prompted expansion of the studies described here to another plant species to gauge the general applicability of our findings. As a fast growing genetically manipulable plant of greater biomass than Arabidopsis and with a high intrinsic capacity for trace metal accumulation (38–40), B. juncea, which for these reasons lends itself to heavy metal remediation, was the species of choice. Transgenic B. juncea lines for AtPCS1::FLAG and the AtPCS1-Y186C::FLAG mutant were generated by hypocotyl transformation and tissue culture. After confirming expression of FLAG-tagged protein in four lines each of the AtPCS1::FLAG and AtPCS1-Y186C::FLAG transgenic lines (Fig. 5A, B, C, D), they were subjected to Cd2+ tolerance screens in parallel with measurements of PC and Cd2+ accumulation. As determined for Arabidopsis, ectopic expression of AtPCS1::FLAG and AtPCS1-Y186C::FLAG resulted in pronounced differences in Cd2+ tolerance, PC accumulation, and Cd2+ accumulation (Fig. 5, B–E). Whereas expression of AtPCS1::FLAG conferred modest (<2-fold) increases in Cd2+ tolerance, PC accumulation, and Cd2+ accumulation compared with untransformed seedlings, expression of AtPCS1-Y186C::FLAG enhanced these three attributes by 4-, 2-, and 3-fold, respectively, compared with untransformed controls (Fig. 5, C–E).
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AtPCS1-dependent Cd\(^{2+}\) tolerance had 3–13-fold lower specific activity compared with wild-type enzyme (Fig. 6B). No differences between the PC product profiles or the kinetics of activation by Cd\(^{2+}\) of AtPCS1 and AtPCS1-Y186C were discernible.

Maintenance of Upstream PC Precursor Levels—In S. cerevisiae, A. thaliana, and B. juncea, overexpression of the kinetically less efficient AtPCS1-Y186C mutant led to higher levels of PCs, improved Cd\(^{2+}\) tolerance, and enhanced Cd\(^{2+}\) accumulation compared with expression of wild-type enzyme. Based on our observations, we speculated that the AtPCS1-Y186C mutant maintains PC synthesis without imposing a drain on cellular GSH and \(\gamma\)EC levels and/or changes in the ratio of reduced to oxidized glutathione (GSH/GSSG ratio). To explore this possibility we examined the levels of GSH and \(\gamma\)EC and the GSH/GSSG ratio before and after exposure to Cd\(^{2+}\) in yeast and plants expressing either AtPCS1 or AtPCS1-Y186C (Table 2). In all three of the organisms engineered for heterologous or ectopic expression, the same pattern was seen.

AtPCS1 when heterologously expressed in yeast or ectopically overexpressed in Arabidopsis or B. juncea was associated with a decrease in the levels of total glutathione and \(\gamma\)EC and changes in the GSH/GSSG ratio following exposure to 200 \(\mu\)M CdCl\(_2\). The decreases in GSH and \(\gamma\)EC after Cd\(^{2+}\) exposure versus controls ranged from about 5% to as much as 40–50% depending on the metabolite and/or organism (Table 2). For example, in yeast overexpressing AtPCS1, the pre-treatment levels of GSH and GSSG were 6,026 and 404 nmol g\(^{-1}\) dry weight, respectively. Following Cd\(^{2+}\) exposure, the total glutathione pool is reduced by ~20% and the levels of GSH and GSSG were 3,914 and 1,186 nmol g\(^{-1}\) dry weight, respectively. In contrast, expression of AtPCS1-Y186C in each organism generally maintained the levels of total glutathione and \(\gamma\)EC and the GSH/GSSG ratio (Table 2).

Discussion

Directed evolution and protein engineering strategies aim to improve the biochemical function of a protein. Typically, for an enzyme, the enhancement of catalytic efficiency for a new substrate or alteration in physiochemical properties, such as thermostability, lead to new functionality and applications. To improve the ability of PCS to protect yeast and plants against heavy metal toxicity, the use of random mutagenesis and screening for Cd\(^{2+}\) exposure led to the identification of mutants with the desired phenotype in multiple organisms (Figs. 2–5); however, the biochemical properties of the AtPCS1 mutants were all inferior to the wild-type enzyme (Table 1). The adaptive engineering of AtPCS1 provides an example of how a metabolic system can constrain biochemical function for an improved biological outcome.

In a variety of organisms, PCS plays a critical role in providing a base level of protection against a range of heavy metals (10–18, 22–26). Comparison of the PCS from different species shows that the N-terminal catalytic domain is more similar (10–18, 22–26). Comparison of the PCS from different species shows that the N-terminal catalytic domain is more similar (10–18, 22–26). Comparison of the PCS from different species shows that the N-terminal catalytic domain is more similar (10–18, 22–26). Comparison of the PCS from different species shows that the N-terminal catalytic domain is more similar (10–18, 22–26). In the specific case of AtPCS1-Y186C, which was kinetically characterized in detail because it conferred the greatest improvement in the AtPCS1-dependent Cd\(^{2+}\) tolerant phenotype in yeast, Arabidopsis, and B. juncea, the reduced activity was associated with a decrease in \(V_{\text{max}}\) (from 14.7 to 2.1 \(\mu\)mol min\(^{-1}\) mg of protein\(^{-1}\)) and an increase in \(K_{m}\)\(\text{GSH}\) (from 6.7 to 16.4 \(\mu\)M) versus wild-type to give an overall 17-fold decrease in catalytic efficiency (Fig. 6B).
strates that the N-terminal domain of AtPCS1 is sufficient for PC synthesis (24, 26). Second, Cys-56 is acylated by γEC during the reaction that extends the length of the PC chain and is an essential catalytic residue (26). Third, the identification of a protein from the cyanobacterium Nostoc, which is similar in sequence to the N-terminal domain of AtPCS1, catalyzes the hydrolysis of glycine from GSH (25). The specific role of the variable C-terminal region of the PCS in different organisms remains unclear.

Because of the domain architecture of AtPCS1, it is notable that of the 31 point mutations in the AtPCS1 variants isolated from the Cd²⁺ tolerance screen, 26 were in the N-terminal catalytic domain (Table 1; Fig. 6C). Random sequencing of the original random mutagenesis library showed a distribution of mutations across the length of the coding region; however, the pool of mutants isolated after selection for Cd²⁺ tolerance clearly implicates the N-terminal catalytic domain as a key feature for the improved phenotype.

Although the three-dimensional structure of a bona fide PCS remains to be determined, homology modeling of the catalytic domain of AtPCS1 based on the structure of the GSH hydrolase from Nostoc (25) (Fig. 6C) suggests that many of the amino acid substitutions identified in the screen localize to putative substrate interaction loops (Ser-51, Glu-52, Ala-59, Ser-60, Phe-163, Ala-182, and Tyr-186) (25, 41). For instance, recent structure-function studies and modeling of AtPCS1 indicates that Tyr-186 on loop B of the structure forms part of the binding site for the second substrate (41), which is probably GSH (17, 26) (Fig. 6C). This may explain why the impaired catalytic activity of AtPCS1-Y186C was associated with an increased $K_m^{GSH}$ concomitant with a decrease in $V_{max}$ with little or no change in the susceptibility of the enzyme to activation by Cd²⁺. The next question is how does a kinetically inferior mutant protein result in increased levels of PCs and a phenotype of Cd²⁺ improved tolerance and accumulation compared with expression of wild-type AtPCS1.

Knowing that the inherent reactivity of heavy metals toward thiol groups is not only a major factor in their toxicity but is also crucial for their detoxification by GSH through the removal of reactive active oxygen species, high-level ectopic expression of fully active AtPCS1 may impose conflicting demands on GSH and its immediate precursor γEC for alleviating oxidative stress associated with heavy metal toxicity (17, 38, 39). Normally, constitutive endogenous expression of PCS provides a basic level of protection against heavy metal toxicity (Fig. 7, black), as loss of PCS activity leads to sensitivity to a variety of heavy metals (11–15, 23). Although PCS is expressed in plants (and yeast like Schizosaccharomyces pombe), the enzymatic activity is only detected in the presence of heavy metals (11–13, 16–18). Moreover, sequestration of heavy metals by either PC or other chelating agents terminates PCS activity both in vitro and in vivo (42).

Overexpression of fully active AtPCS1 leads to enzymatic activation following metal exposure, which promotes PC synthesis from GSH (Fig. 7, orange). The higher levels of PCS begin to make PC from GSH, which can contribute to heavy metal detoxification; however, if the pathways supplying the substrate do not maintain metabolite levels in the presence of highly expressed and fully activated PCS, this exacerbates oxidative stress through consumption of cellular GSH and γEC reserves and changes in the GSH/GSSG ratio (Table 2). Because cellular redox potential is highly sensitive to small changes in both the GSH/GSSG ratio and total glutathione levels (43), this can lead to oxidative stress conditions. In fact, this is the molecular basis for the effect of buthionine sulfoximine, an inhibitor of γ-glutamylcysteine ligase, on rapidly growing tumor cells and for its effects on plant glutathione biosynthesis (44–47).

In contrast, analysis of GSH and γEC levels and the GSH/GSSG ratio in yeast, Arabidopsis, and B. juncea (Table 2) indicate that overexpression of the less active AtPCS1-Y186C maintains the reservoir of these metabolites following Cd²⁺ exposure (Fig. 7, green). These results underscore the importance of the cellular redox state for supporting metabolism linked to heavy metal tolerance. As suggested here, maintaining both total glutathione levels and the ratio of reduced:oxidized peptide is important for providing substrates for PCS but also for the activation of other enzymes in plant sulfur metabolism that support glutathione production (45, 56). Similar to earlier work showing that either lower level expression of AtPCS1 or supplementation of the growth medium with exogenous GSH

### TABLE 2

| Yeast    | 0 μM CdCl₂ | 200 μM CdCl₂ | 0 μM CdCl₂ | 200 μM CdCl₂ | 0 μM CdCl₂ | 200 μM CdCl₂ |
|----------|------------|--------------|------------|--------------|------------|--------------|
| γEC      | 53 ± 14    | 74 ± 16      | 42 ± 4     | 45 ± 6       | 65 ± 3     | 54 ± 5       |
| Total glutathione | 6,800 ± 430 | 5,280 ± 680 | 6,430 ± 110 | 5,100 ± 170 | 6,230 ± 290 | 7,980 ± 620 |
| GSH/GSSG ratio | 1.4 ± 1.4 | 1.1 ± 1.4   | 1.5 ± 1.5  | 1.6 ± 1.5   | 2.1 ± 2.4  | 1.5 ± 1.5   |
| Arabidopsis | 16 ± 4.5   | 15 ± 4.5     | 14.9 ± 3.5 | 3.3 ± 0.8    | 16.9 ± 2.4 | 15.5 ± 2.9 |
| γEC      | 20 ± 7     | 17 ± 10      | 17 ± 9     | 10 ± 5       | 25 ± 2     | 19 ± 6       |
| Total glutathione | 845 ± 93   | 751 ± 47     | 820 ± 12   | 684 ± 58     | 862 ± 100  | 989 ± 74     |
| GSH/GSSG ratio | 1.3 ± 1.2 | 1.4 ± 0.6    | 1.5 ± 0.5  | 1.6 ± 1.6    | 1.9 ± 1.5  | 1.5 ± 1.5   |
| B. juncea | 14 ± 8     | 122 ± 18     | 11 ± 3     | 138 ± 15     | 16 ± 7     | 167 ± 63     |
| Total glutathione | 930 ± 160  | 1,155 ± 120  | 920 ± 160  | 730 ± 67     | 965 ± 49   | 1,080 ± 90   |
| GSH/GSSG ratio | 10.5 ± 1.9 | 2.9 ± 0.5    | 11.8 ± 1.4 | 3.8 ± 1.9    | 10.7 ± 1.0 | 9.5 ± 1.4    |

Yeast, Arabidopsis, and B. juncea transformed with wild-type and mutant AtPCS1

Yeast, Arabidopsis, and B. juncea transformed with empty vector (control) or vector containing either AtPCS1 or AtPCS1-Y186C inserts were grown in 0 and 200 μM CdCl₂ for determination of γEC and total glutathione levels and the GSH/GSSG ratio (42, 43). For Arabidopsis, lines PCS3 and Y186C1 were used; for B. juncea, lines PCS2 and Y186C4 were used. Values shown are nmol g⁻¹ dry weight for yeast and nmol g⁻¹ FW for plants and are mean ± S.E. (n = 3).
alleviates Cd²⁺ hypersensitivity (38, 39), the selection of lower activity PCS variants appears to strike a balance between maintaining redox buffering capacity of cellular GSH and supporting sustained PC production, which leads to improved Cd²⁺ tolerance and enhanced Cd²⁺ accumulation. Moreover, the effect of overexpressing less active PCS variants suggests that tuning of wild-type protein expression could be another useful approach to modulate metal tolerance in different organisms. In addition, coupling of PC production with modifications in the vacuolar transporter that remove the chelated metals from the cell could also be tested (19–21, 57).

Ultimately, efforts to engineer different components of heavy metal detoxification systems in plants at the protein and pathway level offer tools for environmental remediation. Although directed evolution aims to improve proteins and pathways for optimized biochemical properties and/or biological phenotypes (3), our results emphasize the importance of the metabolic context of the target protein for engineering. Considering the connection of PCs to the major cellular redox buffer, GSH, our counterintuitive finding, kinetic inferiority leading to phenotypic superiority, demonstrates the need to approach metabolic engineering with a systems level perspective to identify key control points amenable to the adaptive and context-dependent engineering of plant and microbial metabolism.

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