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AtPCS1, a phytochelatin synthase from *Arabidopsis*: Isolation and *in vitro* reconstitution

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ABSTRACT **Phytochelatins, a class of posttranslationally synthesized peptides, play a pivotal role in heavy metal, primarily Cd^{2+}, tolerance in plants and fungi by chelating these substances and decreasing their free concentrations. Derived from glutathione and related thiols by the action of γ-glutamylcysteine dipeptidyl transpeptidases (phytochelatin synthases; EC 2.3.2.15), phytochelatins consist of repeating units of γ-glutamylcysteine followed by a C-terminal Gly, Ser, or β-Ala residue [poly-(γ-Glu-Cys)$_n$-Xaa]. Here we report the suppression cloning of a cDNA (*AtPCS1*) from *Arabidopsis thaliana* encoding a 55-kDa soluble protein that enhances heavy-metal tolerance and elicits Cd^{2+}-activated phytochelatin accumulation when expressed in *Saccharomyces cerevisiae*. On the basis of these properties and the sufficiency of immunoaffinity-purified epitope-tagged AtPCS1 polypeptide for high rates of Cd^{2+}-activated phytochelatin synthesis from glutathione *in vitro*, AtPCS1 is concluded to encode the enzyme phytochelatin synthase.**

Essential heavy metals, such as Cu and Zn, are required as cofactors in redox reactions and ligand interactions as well as for charge stabilization, charge shielding, and water ionization during biocatalysis (1). However, both essential and nonessential heavy metals can pose an acute problem for organisms. Supraoptimal concentrations of essential heavy metals and micromolar concentrations of nonessential heavy metals, such as As, Cd, Hg, and Pb, displace endogenous metal cofactors, heavy or otherwise, from their cellular binding sites, undergo aberrant reactions with the thiol groups of proteins and coenzymes, and promote the formation of active oxygen species (2).

Three classes of peptides, glutathione (GSH), metallothioneins (MTs), and phytochelatins (PCs), have been implicated in heavy-metal homeostasis in plants. The thiol peptide, GSH (γ-Glu-Cys-Gly), and in some species its variant homoglutathione (h-GSH, γ-Glu-Cys-β-Ala), is considered to influence the form and toxicity of heavy metals such as As, Cd, Cu, Hg, and Zn, in several ways. These include direct metal binding (3), promotion of the transfer of metals to other ligands, such as MTs and PCs (4), provision of reducing equivalents for the generation of metal oxidation states more amenable to binding by MTs and possibly PCs (4), removal of the active oxygen species formed as a result of exposure of cells to heavy metals (5), and/or the formation of transport-active metal complexes (6). MTs, small (4- to 8-kDa) Cys-rich metal-binding peptides containing multiple Cys-Xaa-Cys motifs, confer tolerance to a broad range of metals in mammals (7) but appear to be involved primarily in Cu homeostasis in plants (8). *Arabidopsis* MT1 and MT2 confer tolerance to high levels of Cu^{2+} but only low levels of Cd^{2+} when heterologously expressed in MT-deficient *cup1*Δ mutants of *Saccharomyces cerevisiae* (8), MT expression in *Arabidopsis* seedlings is strongly induced by Cu^{2+} but not by Cd^{2+} (8, 9), and comparisons between different *Arabidopsis* ecotypes show MT2 expression to be more closely correlated with Cu tolerance than tolerance to other metals (10). PCs, poly-(γ-Glu-Cys)$_n$-Xaa polymers, unlike MTs, are synthesized posttranslationally (11, 12). Found in some fungi and all plant species investigated (11, 12), PCs bind heavy metals, such as Cd^{2+}, with high affinity, localize together with Cd^{2+} to the vacuole of intact cells (13) and, as indicated by the hypersensitivity of PC-deficient *Arabidopsis cad1* mutants to Cd^{2+} but not Cu^{2+} (14), contribute most markedly to Cd^{2+} detoxification *in planta*. PC-dependent vacuolar Cd^{2+} sequestration is best understood in the fission yeast *Schizosaccharomyces pombe*, in which the *hmt1*$^+$ gene product, a PC-selective ATP-binding cassette transporter, pumps Cd·PCs and apo-PCs from the cytosol into the vacuole at the expense of ATP (15, 16).

Despite the importance of PCs for heavy-metal tolerance, the molecular identity of the enzyme(s) responsible for the elaboration of these peptides has eluded definition. Several investigators have described the partial purification of heavy-metal-, primarily Cd^{2+}-, activated enzymes (γ-glutamylcysteine dipeptidyl transpeptidases, trivial name PC synthases; EC 2.3.2.15) competent in the synthesis of PCs from GSH, homoglutathione, or related thiol peptides by transfer of a γ-glutamylcysteine unit from one thiol tripeptide to another or to a previously synthesized PC molecule (12, 17), but none have been able to identify or isolate the moiety or moieties concerned at either the protein or gene level.

Here we describe the isolation of a gene from *Arabidopsis* (*AtPCS1*) encoding a protein whose heterologous expression in *S. cerevisiae* confers heavy-metal tolerance by promoting the Cd^{2+}-dependent accumulation of PCs. *AtPCS1* is shown to encode a single polypeptide species that, when purified to homogeneity, is sufficient for Cd^{2+}-activated PC synthesis from GSH *in vitro* at rates far in excess of those reported previously for partially purified PC synthase preparations from plants and fungi.

MATERIALS AND METHODS

Yeast Strains and Plant Materials. The yeast strains used in these investigations were: the *yap1*Δ mutant SM12 (*MATα leu2–3,-112 ura3–52 his3-Δ200 trp1-Δ901 lys2–801 suc2-Δ9 Mel$^-$ yap1-Δ1*::*HIS3*) (18); the *ycf1*Δ mutants DTY167 (*MATα ura3–52 leu2–3,-112 his-Δ200 trp1-Δ901 lys2–801 suc2-Δ9 ycf1*::*hisG*) (19) and DTY168 (*MATα ura3–52 leu2–3-112 his6 ycf1*::*hisG*) (20); the *pep5*Δ mutant DTY214 (*MATα ura3–52 leu2–3,-112 his3-Δ200 trp1-Δ901 lys2–801 suc-Δ9 pep5*::*LEU2*) (a gift from D.J. Thiele, University of Michigan, Ann Arbor, MI); and the *cup1*Δ mutant DTY4 (*MATα ura3–52 leu2–3,-112 trp1–1 gal1 his$^-$ cup1*Δ::*URA3*) (21). *A. thaliana* cv Columbia

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Abbreviations: AtPCS1, *Arabidopsis thaliana* PC synthase 1; GSH, glutathione; MT, metallothionein; YAP1, yeast activation protein 1; PC, phytochelatin.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF085230).
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was the source of the RNA used for construction of the pFL61 cDNA library (22) and Northern analyses and the genomic DNA used for the Southern analyses.

Isolation of AtPCS1. To identify plant genes able to suppress Cd^{2+}-hypersensitivity, *S. cerevisiae yap1*Δ strain SM12 was transformed with an *Arabidopsis* cDNA library constructed in the yeast–*Escherichia coli* shuttle vector pFL61 (22). Stable Cd^{2+}-tolerant Ura^+ transformants were selected by first plating on synthetic complete Ura medium containing 100 μM $CdCl_2$ followed by replication to medium containing 200 μM $CdCl_2$. The pFL61 plasmids from all 105 Cd^{2+}-tolerant SM12 transformants identified in this step were rescued (23), transformed into *S. cerevisiae ycf1*Δ strain DTY168, and Cd^{2+}-tolerant transformants were selected by plating on AHC medium (24) containing 200 μM $CdCl_2$. Of a total of 104 pFL61 clones found to suppress the Cd^{2+}-hypersensitivity of both *yap1*Δ and *ycf1*Δ mutants, two, whose cDNA inserts were found to be identical, conferred a particularly high level of Cd^{2+} tolerance. One of these, pFL61-AtPCS1, was analyzed functionally.

Heterologous Expression of FLAG-Tagged AtPCS1. For constitutive expression of immunoreactive protein in *S. cerevisiae*, the cDNA insert of pFL61-AtPCS1 was subcloned into vector, pYES3, a derivative of pYES2 (Invitrogen) in which the galactose-inducible yeast *GAL1* gene promoter was replaced by the constitutive 3-phosphoglycerate kinase (*PGK*) gene promoter (25) and engineered to code AtPCS1 fused with a C-terminal FLAG (DYKDDDDK) epitope tag. After confirming the fidelity of the resulting construct, pYES3-AtPCS1::FLAG, by sequencing, *S. cerevisiae ycf1*Δ strain DTY167 was transformed with this or empty vector lacking the *AtPCS1*::*FLAG* insert by the LiOAc/polyethylene glycol method (26) and selected for uracil prototrophy by plating on AHC medium with or without tryptophan supplementation, respectively.

Cell Fractionation. For investigations of the localization of AtPCS1-FLAG, DTY167/pYES3-AtPCS1::FLAG or DTY167/pYES3 cells were subjected to cell wall digestion, disruption, and fractionation by differential centrifugation. Two hundred-milliliter volumes of stationary phase cultures were diluted into 1 liter of AHC medium containing tryptophan, grown for 16–18 h at 30°C to an OD_{600} of approximately 1.0, collected by centrifugation and converted to spheroplasts as described (25). After disruption of the spheroplasts by homogenization in homogenization medium (10 mM Tris·HCl, pH 7.6) containing 1 mM phenylmethyl sulfonylfluoride and 1 μg/ml each of leupeptin, pepstatin, and aprotinin, in a Dounce homogenizer, the crude lysate was cleared by centrifugation at 4,000 × *g* for 10 min before two final centrifugations at 100,000 × *g* for 1 h. The supernatant (soluble fraction) was frozen and reduced in volume by lyophilization, and the pellet (membrane fraction) was resuspended in 1–2 ml of homogenization buffer containing protease inhibitors before freezing in liquid nitrogen and storage at −85°C. For investigations of the PC contents and PC synthetic capacities of the soluble fractions and purification of AtPCS1-FLAG, the cells were fractionated in an identical manner except that the homogenization medium contained 10 mM 2-mercaptoethanol, 10% (vol/vol) glycerol, and 50 mM Tris·HCl (pH 8.0), in addition to protease inhibitors.

Purification of AtPCS1-FLAG. AtPCS1-FLAG was purified from the soluble fraction of DTY167/pYES3-AtPCS1-FLAG cells on an anti-FLAG M2 affinity gel column (Sigma) according to the manufacturer's recommendations, except that the wash and elution buffers contained 10% (vol/vol) glycerol in addition to TBS (150 mM NaCl, 50 mM Tris·HCl, pH 7.4) and 0.1 M glycine-HCl (pH 3.5), respectively.

Equilibrium Dialysis of AtPCS1-FLAG. Binding of Cd^{2+} was determined by equilibrium dialysis of a 400- to 800-μl (160 μg) sample of purified AtPCS1-FLAG against 80-ml volumes of 10 mM Tris·HCl buffer, pH 7.8, containing 0.05 to 20 μM $^{109}CdCl_2$ (specific activity 22 Ci/mol) for 12 h at 4°C in 2-ml mini-collodion membrane tubes (MWCO 25,000, Scheicher & Schuell). Protein-bound ^{109}Cd was estimated by measuring the radioactivity of the bulk medium outside the dialysis tube and that of the solution within the dialysis tube and determining the increase in ^{109}Cd radioactivity consequent on AtPCS1-FLAG. Binding constants and stoichiometries of binding were estimated by nonlinear least-squares analysis (27) by using the Ultrafit nonlinear curve fitting package from Biosoft (Milltown, NJ).

Gel-Filtration FPLC of Soluble Fractions and Purified AtPCS1-FLAG. Analysis of the distribution of ^{109}Cd in the soluble fractions extracted from DTY167/pYES3-AtPCS1-FLAG and DTY167/pYES3 cells after growth in media containing $^{109}CdCl_2$ was by FPLC of the extracts on a Superose 6 HR10/30 column (Amersham Pharmacia). One hundred milliliter aliquots of stationary phase cultures grown in AHC medium supplemented with glucose and tryptophan were diluted into 500-ml volumes of fresh medium containing 50 μM $^{109}CdCl_2$ (specific activity 2.3 Ci/mol) and grown at 30°C for a further 18 h. Samples of the soluble fractions (1 ml, 1.2–1.8 mg protein) prepared as described were incubated for 30 min on ice with 10 mM DTT and applied to the Superose-6 column. The column was developed with 50 mM Tris·HCl, pH 7.8, at a flowrate of 0.3 ml/min. Fractions of 0.5 ml were collected and ^{109}Cd was determined by counting aliquots (60 μl) in BCS liquid scintillation mixture (Amersham Pharmacia). To determine the distribution of AtPCS1-FLAG, aliquots of the column fractions were separated by SDS/PAGE and subjected to Western analysis as described. Immunoaffinity-purified AtPCS1-FLAG (1 ml, 140–180 μg protein), either before or after equilibrium dialysis against 5 μM $^{109}CdCl_2$ (specific activity 2.3 Ci/mol), was chromatographed under identical conditions.

Measurement of PCs and PC Synthase Activity. Cellular PC content was estimated by reverse-phase FPLC of the soluble fractions prepared from DTY167/pYES3-AtPCS1::FLAG or DTY167/pYES3 cells after growth in liquid media containing or lacking 50 μM $CdCl_2$. For reverse-phase FPLC, 1–2 ml volumes of the extracts (2.3–4.6 mg protein) were made 5% (wt/vol) with 5-sulfosalicylic acid and centrifuged before loading aliquots (500 μl) of the supernatant onto a PepRPC HR5/5 FPLC column (Amersham Pharmacia). The column was developed with a water/0.1% (vol/vol) phosphoric acid (solvent A)-acetonitrile/0.1% (vol/vol) phosphoric acid (solvent B) gradient at a flowrate of 0.4 ml/min. The program parameters were: 0–2 min, 0–2% solvent B; 2–5 min, 2% solvent B; 5–33 min, 2–30% solvent B. Thiols were estimated spectrophotometrically at 412 nm by reacting aliquots (100 μl) of the column fractions with 0.4 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (900 μl) dissolved in 50 mM phosphate buffer, pH 7.6 (28). PC standards were extracted from Cd^{2+}-grown *S. pombe* (ATCC 38390) (16).

PC synthase activity was assayed according to Grill *et al.* (17) in reaction media containing crude soluble fraction (50 μg protein), purified AtPCS1-FLAG (0.5 μg) or no protein, 3.3 mM GSH, 10 mM 2-mercaptoethanol, and 200 mM Tris·HCl buffer (pH 8.0), plus or minus 200 μM $CdCl_2$ at 37°C for 40 min. After the reactions were terminated with 5% (wt/vol) 5-sulfosalicylic acid, PCs were estimated in the supernatant by reverse-phase FPLC and reaction with 5,5′-dithio-bis(2-nitrobenzoic acid).

Amino Acid Analysis. The chain lengths of the PCs synthesized from GSH by AtPCS1-FLAG *in vitro* were determined by estimating their Glu/Gly ratios (ratio = *n* = number of Glu-Cys repeats per Gly) after acid hydrolysis of the appropriate fractions from reverse-phase FPLC. Aliquots of the fractions were taken to dryness in pyrolyzed glass tubes, hydrolyzed in gas-phase 6 N HCl for 20 h at 110°C before ion-exchange chromatography, post-column derivatization with *O*-phthalaldehyde, and fluorescence detection (29).

Northern and Southern Analyses. *Arabidopsis* seedlings were grown for 21 days in Gamborg's B-5 medium and total RNA and genomic DNA were extracted from roots and shoots in TRIzol R Reagent [GIBCO/BRL; Life Technologies (Grand Island, NY)], according to the manufacturer's recommendations. The RNA samples and restricted DNA samples were electrophoresed, blotted, and hybridized with ^{32}P-labeled random-primed 1.5 kb *Not*I/*Sma*I restriction fragment corresponding to the coding sequence of *AtPCS1,* by standard procedures (25).

SDS/PAGE and Western Analyses. Protein samples were dissolved in denaturation buffer and subjected to one-dimensional SDS/PAGE on 10% (wt/vol) slab gels in a Bio-Rad mini-gel apparatus (24). For direct protein detection, the gels were stained with Silver Stain Plus (Bio-Rad). For immunodetection of AtPCS1-FLAG, the separated samples were electrotransferred and probed with anti-FLAG M2 antibody (Sigma) by standard procedures. Immunoreactive bands were visualized by ECL [Amersham Pharmacia; Life Sciences (St. Petersburg, FL)].

Protein Estimations. Protein was estimated by the dye-binding method (31).

Chemicals. All of the general reagents were obtained from Fisher Scientific, Research Organics, or Sigma.

RESULTS

Suppression Cloning. The isolation of a plant cDNA encoding what proved to be a PC synthase was a byproduct of yeast suppression screens for *Arabidopsis* yeast activation protein 1 (YAP1)-like transcription factors. Knowing that expression of the yeast (*S. cerevisiae*) cadmium (resistance) factor gene *YCF1*, which encodes an ATP-binding cassette transporter responsible for the vacuolar sequestration of Cd·GS complexes (6), is transcriptionally activated by the bZIP DNA-binding protein YAP1 (18), the screens were in two steps: (*i*) By transformation of the *S. cerevisiae yap1*Δ disruptant SM12 with an *Arabidopsis* cDNA library constructed in vector pFL61 and selection for transformants capable of growth on YPD medium containing 100 μM $CdCl_2$; (*ii*) by rescue of plasmids pFL61 from the Cd^{2+}-resistant transformants identified in step *i*, their transformation into *S. cerevisiae ycf1*Δ disruptant strain DTY168 and selection for Ura^+ transformants capable of growth on AHC medium containing 200 μM $CdCl_2$. It was reasoned that pFL61-borne *Arabidopsis* cDNAs encoding YAP1-like factors would suppress *yap1*Δ but not *ycf1*Δ mutants, whereas cDNAs encoding Cd^{2+} tolerance factors acting through a pathway distinct from that of YAP1 would suppress not only *yap1*Δ but also *ycf1*Δ mutants.

Of a total of 105 Cd^{2+}-tolerant SM12 transformants identified in step *i*, only one harbored a pFL61-borne cDNA satisfying the minimum requirement of a YAP1 equivalent—an inability to suppress the Cd^{2+} hypersensitivity of strain DTY168 in step *ii*. All of the remaining SM12 transformants from step *i* harbored pFL61 plasmids able to suppress the Cd^{2+} hypersensitivity of strain DTY168 in step *ii*. Of these, two identical clones were of particular interest because of their ability to confer tolerance to Cd^{2+} concentrations well in excess of those tolerated by DTY168 cells transformed with any one of the other clones. After determining that the cDNA inserts of these clones were identical by sequencing their ends, one, pFL61-AtPCS1, was investigated further.

Sequence Characteristics. The ORF of the 1.7-kb cDNA insert of pFL61-AtPCS1 encoded a 55-kDa polypeptide sharing 33% identity (48% similarity) and 32% identity (45% similarity) to two hypothetical gene products in the GenBank database, one from *S. pombe* (accession no. Q10075) and another from *Caenorhabditis elegans* (accession no. Z66513), in overlaps of 368 and 367 aa residues, respectively (Fig. 1). Whereas AtPCS1 was distinguishable from the other two polypeptides by its possession of a 73–74 aa residue C-terminal extension, the conserved sequences corresponding to positions 49–68, 70–91, 152–165, and 177–191 of AtPCS1 (Fig. 1) were family-specific: BLAST searches of the GenBank database by using these sequences identified these three gene products but no others.

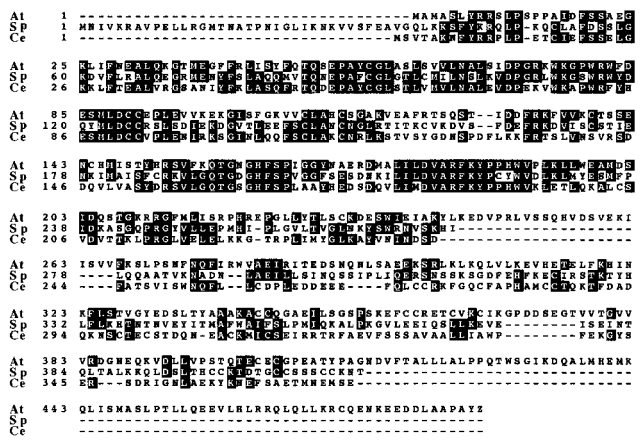

FIG. 1. Alignment of the deduced amino acid sequence of the polypeptide encoded by *AtPCS1* and its 46.7- and 40.8-kDa homologs from *S. pombe* (GenBank accession number Q10075) and *C. elegans* (Z66513), respectively. Sequences were aligned by the CLUSTAL method (30).

Searches of the *Arabidopsis* Genomic Database indicated at least two *AtPCS* genes in this organism: *AtPCS1,* which is located within P1 clone MRH10 and maps adjacent to marker mi83 on chromosome 5, and another gene, nominally *AtPCS2*, located within BAC clone F21M11, which maps to chromosome 1 and encodes a hypothetical polypeptide sharing greater than 70% sequence identity (85% similarity) with AtPCS1. Because Southern analyses of restricted *Arabidopsis* genomic DNA yielded hybridization patterns necessitating two, but no more, *AtPCS*-like genes when probed with the coding sequence of *AtPCS1* (data not shown), *AtPCS1* and *AtPCS2* are inferred to be the sole representatives of this gene family in *Arabidopsis*.

High-stringency Northern blot analysis detected a single 1.7-kb band after hybridization of random-primed ^{32}P-labeled *AtPCS1* cDNA with total RNA extracted from roots and shoots of 21-day-old *Arabidopsis* seedlings (Fig. 2), indicating that the cDNA insert of pFL61-AtPCS1 was full length and derived from *Arabidopsis* rather than a nonplant contaminant of the cDNA library.

Heavy-Metal Tolerance. To probe the functional capabilities of *AtPCS1* and render its translation product immunodetectable, *AtPCS1*::*FLAG* fusions were engineered into vector, pYES3, and transformed into *S. cerevisiae*. Because BLAST searches did not disclose any *AtPCS1*-like sequences in *S. cerevisiae*, this organism was considered suitable for examining the mode of action of AtPCS1 without interference from endogenous orthologs.

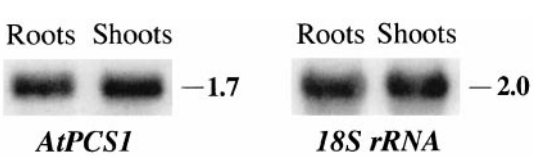

FIG. 2. Northern analysis of expression of *AtPCS1* transcript in roots and shoots of *Arabidopsis.* Total RNA was extracted from 21-day-old *Arabidopsis* seedlings and hybridized with ^{32}P-labeled random-primed *AtPCS1* cDNA. Ten μg of RNA was loaded on each lane. The 1.7-kb bands shown were the only ^{32}P-labeled bands detected after probing with *AtPCS1*. Also shown are the results of hybridizing the same blots with a probe directed against 18S rRNA to verify that similar amounts of RNA had been loaded on each lane.

After establishing that the resulting *AtPCS1*::*FLAG* construct (and its C-terminally FLAG epitope-tagged translation product) was as efficacious as pFL61-AtPCS1 and pYES3-AtPCS1 in suppressing the Cd^{2+} hypersensitivity of *S. cerevisiae ycf1*Δ strain DTY167, pYES3-AtPCS1::FLAG-dependent suppression of sensitivity to a broader range of metals or their oxides was screened in this strain and the metallothionein-deficient Cu^{2+}-hypersensitive *cup1*Δ strain DTY4 (21) to show that AtPCS1-FLAG was a multispecific heavy-metal resistance factor. Plasmid-borne AtPCS1-FLAG conferred not only marked resistance to Cd^{2+} but also moderate resistance to AsO_4^{3-}, AsO_2^-, Cu^{2+}, and Hg^{2+} with an overall rank order, based on the fold increases in the concentrations at which the metals exerted 50% inhibition of growth in liquid media vs. pYES3 vectors controls, of Cd^{2+} (24-fold) $\gg$ Cu^{2+} (2.4) > AsO_4^{3-} (1.8) > AsO_2^- (1.6) = Hg^{2+} (1.6) (Fig. 3).

Cellular Localization. Heterologously expressed AtPCS1-FLAG did not appear to confer tolerance by promoting the exclusion or extrusion of heavy metals from the cytosol through an association with the plasma or vacuolar membrane. Cells expressing AtPCS1-FLAG had an enhanced, rather than diminished, heavy-metal content (below) and the vacuole-deficient *pep5*Δ strain DTY214, whose Cd^{2+} hypersensitivity is attributable to the absence of a sizable vacuolar compartment for metal sequestration (6), was nearly as susceptible to suppression by pYES3-AtPCS1::FLAG as strain DTY167 (data not shown). AtPCS1-FLAG instead localized almost exclusively to the soluble fraction of DTY167/pYES3-AtPCS1::FLAG transformants.

Differential centrifugation of mechanically disrupted DTY167/pYES3-AtPCS1::FLAG spheroplasts resolved the total extracts into soluble (supernatant) and membrane-enriched (pelletable) fractions, of which only the former yielded appreciable M_r 58,000 anti-FLAG antibody-reactive polypeptide after SDS/PAGE and Western analysis (Fig. 4). Because the migration properties of the anti-FLAG antibody-reactive polypeptide were precisely those expected from the coding sequence of *AtPCS1*::*FLAG,* and fractionation of DTY167/pYES3 cells did not yield immunoreactive protein in either the soluble or membrane fraction (Fig. 4), the FLAG-bearing band in the soluble fraction from DTY167/pYES3-AtPCS1-FLAG cells was inferred to be derived solely from the cDNA insert of pYES3-AtPCS1::FLAG.

***In Vivo* Cd^{2+} Binding.** Having determined that heterologously expressed AtPCS1-FLAG was a soluble protein, its

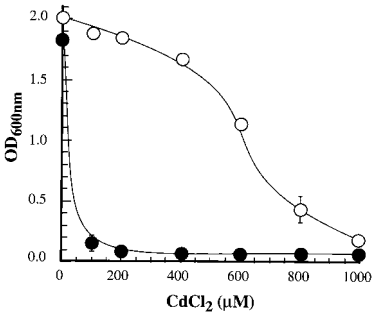

FIG. 3. Suppression of Cd^{2+} hypersensitivity of *S. cerevisiae ycf1*Δ mutant strain DTY167 by plasmid-borne *AtPCS1*. Yeast *ycf1*Δ strain DTY167 was transformed with pYES3-AtPCS::FLAG (O) or empty pYES3 vector (●). Cells were grown at 30°C to an OD_{600} of approximately 1.8 in AHC medium supplemented with glucose and tryptophan before aliquots were inoculated into 2-ml volumes of the same medium containing different concentrations of heavy-metal salts. OD_{600} was measured after growth for 36 h. Similar growth assays in liquid media containing AsO_4^{3-}, AsO_2^-, Cu^{2+}, or Hg^{2+} demonstrated that plasmid-borne *AtPCS1*::*FLAG* increased the concentrations of these metal ions or their oxides required for 50% attenuation of cell density from 300, 200, 250, and 4 μM to 550, 325, 600, and 6.3 μM, respectively.

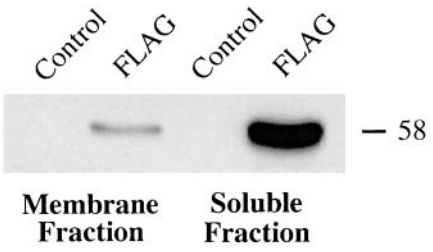

FIG. 4. SDS/PAGE and Western analysis of AtPCS1-FLAG in total soluble and membrane fractions from whole-cell extracts prepared from DTY167/pYES3-AtPCS1::FLAG (*FLAG*) and DTY167/pYES3 cells (*Control*). Protein (25 μg) was subjected to SDS/PAGE on 10% gels, electrotransferred, and probed with anti-FLAG M2 monoclonal antibody.

effects, if any, on the amount and distribution of bound heavy metal were assessed by comparing the chromatographic profiles of soluble extracts from DTY167/pYES3-AtPCS1::FLAG and DTY167/pYES3 cells after growth in liquid medium containing $^{109}CdCl_2$. The results of these analyses not only provided the first indication that AtPCS1-FLAG itself binds $^{109}Cd^{2+}$ but also implicated this polypeptide in increasing the amount of, and/or increasing the degree of binding of $^{109}Cd^{2+}$ to, another heavy-metal-binding factor.

FPLC of the soluble fraction from $^{109}CdCl_2$-grown DTY167/pYES3-AtPCS1::FLAG cells on a Superose-6 HR 10/30 column resolved two major peaks and one minor peak of ^{109}Cd radioactivity with maxima corresponding to fractions 34 (peak 1), 39 (peak 2), and 43 (peak 3) (Fig. 5). Equivalent ^{109}Cd radioactivity profiles were obtained for the soluble fraction from $^{109}CdCl_2$-grown DTY167/pYES3-AtPCS1 cells (data not shown), demonstrating that the FLAG epitope tag did not contribute to $^{109}Cd^{2+}$ binding. By contrast, the soluble fraction from DTY167/pYES3 cells grown under identical conditions lacked most of the ^{109}Cd radioactivity under peak 1, though peaks 2 and 3 had the same profiles and eluted at the same positions as the corresponding peaks in the DTY167/pYES3-AtPCS1::FLAG extracts (Fig. 5). Although superficially consistent with the notion that the peak unique to the DTY167/pYES3-AtPCS1::FLAG extracts (peak 1) might be wholly explicable by direct binding of ^{109}Cd to AtPCS1-FLAG, closer inspection of the column fractions and experi-

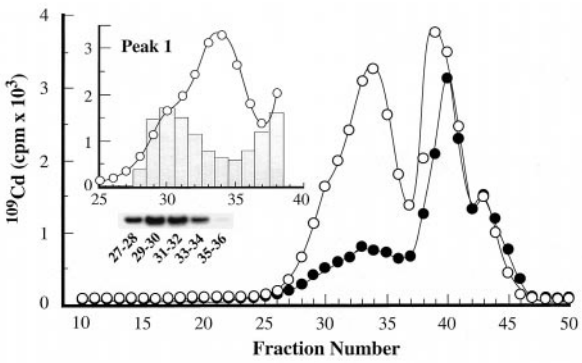

FIG. 5. Gel-filtration chromatography of soluble fractions extracted from DTY167/pYES3-AtPCS1::FLAG (O) and DTY167/pYES3 cells (●) after growth in media containing $^{109}CdCl_2$ (main figure). Direct comparison of the ^{109}Cd-radioactivity profile of peak 1 from the soluble fraction extracted from DTY167/pYES3-AtPCS1::FLAG cells (O) with the ^{109}Cd radioactivity profile obtained after Superose-6 chromatography of immunoaffinity-purified AtPCS1-FLAG after equilibrium dialysis against 5 μM $^{109}CdCl_2$ (histogram) (Peak 1). AtPCS1-FLAG was detected by SDS/PAGE and Western analysis of 20 μl aliquots of pooled pairs of fractions from chromatography of the DTY167/pYES3-AtPCS1::FLAG extracts.

ments on purified AtPCS1-FLAG demonstrated this not to be the case. AtPCS1-FLAG bound Cd^{2+} at high affinity and high capacity but this, alone, did not account for the peak profiles of the DTY167/pYES3-AtPCS1::FLAG cell extracts.

Immunoaffinity Purification. To define its chromatographic and metal-binding properties directly (and eventually for assaying its enzymic activity), AtPCS1-FLAG was purified from the soluble fraction of DTY167/pYES3-AtPCS1::FLAG cells by immunoaffinity chromatography on an anti-FLAG M2 affinity column. SDS/PAGE and Western analysis of the soluble fraction before and after chromatography demonstrated that this procedure yielded a single anti-FLAG antibody-reactive M_r 58,000 species retaining the electrophoretic and immunological properties of the polypeptide in the starting material (Fig. 6).

Intrinsic and Extrinsic Binding. Equilibrium dialysis of the immunoaffinity-purified protein against a range of $^{109}CdCl_2$ concentrations verified that AtPCS1-FLAG bound Cd^{2+} at high affinity (K_d = 0.54 ± 0.20 μM) and high capacity (stoichiometric ratio = 7.09 ± 0.94). However, though consistent with a contribution by AtPCS1-FLAG to the overall ^{109}Cd radioactivity profile, the intrinsic Cd^{2+}-binding activity of AtPCS1-FLAG, whether in whole extracts or in a purified state, was not sufficient to account fully for the ^{109}Cd-radioactivity profile of peak 1 in the DTY167/pYES3-AtPCS1::FLAG extracts. Whereas SDS/PAGE and Western analysis of the fractions from chromatography of the DTY167/pYES3-AtPCS1::FLAG cell extracts demonstrated a consistent displacement of the maximum for AtPCS1-FLAG polypeptide (fractions 29–32) from the maximum for ^{109}Cd radioactivity (fractions 33–35), this could not be simulated with pure ^{109}Cd-complexed AtPCS1-FLAG. Chromatography of purified AtPCS1-FLAG after equilibrium dialysis against $^{109}CdCl_2$ yielded strictly superimposable profiles for AtPCS1-FLAG polypeptide and ^{109}Cd radioactivity whose maxima (fractions 29–32) coincided with that of AtPCS1-FLAG in the DTY167/pYES3-AtPCS1::FLAG extracts (Fig. 5 *Inset*). On this basis, AtPCS1-FLAG was concluded not only to bind Cd^{2+} itself but also to elicit the formation or activation of a lower molecular-weight metal-binding factor or factors. This interpretation was reinforced by the finding that Cd^{2+}-complexed and free AtPCS1-FLAG coeluted during Superose-6 chromatography, so excluding a change in the elution properties of AtPCS1-FLAG consequent on Cd^{2+}-binding as a factor contributing to peak profile, and by our inability to verify a scheme in which displacement of the AtPCS1-FLAG and ^{109}Cd radioactivity maxima was the result of partial dissociation of bound ^{109}Cd from AtPCS1-FLAG and its transfer to a smaller

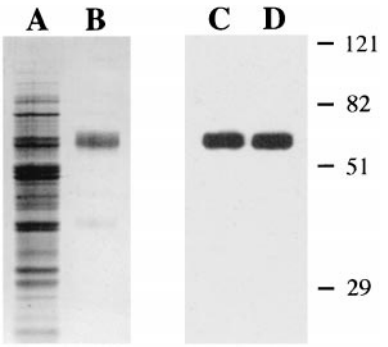

FIG. 6. Purification of AtPCS1-FLAG by immunoaffinity chromatography of the soluble fraction from DTY167/pYES4-AtPCS1::FLAG cells on an anti-FLAG M2 immunoaffinity column. Silver-stained SDS gel of soluble fraction before (5 μg protein) (*A*) and after (1 μg protein) (*B*) purification of AtPCS1-FLAG. Western analysis of FLAG epitope before (25 μg protein) (*C*) and after (1 μg protein) (*D*) purification of AtPCS1-FLAG. Positions of molecular-weight markers ($M_r \times 10^3$) are indicated.

preexistent factor common to both DTY167/pYES3-AtPCS1::FLAG and DTY167/pYES3 cells.

PC Biosynthesis. AtPCS1-FLAG's capacity for binding Cd^{2+} and stimulating the formation of lower molecular-weight metal ligands was telltale in that PC synthase ostensibly has the same property: the facility to catalyze PC synthesis when activated by Cd^{2+}. Other similarities between AtPCS1 and PC synthase that prompted investigations of whether AtPCS1 is a PC synthase or at least contributes to PC synthase activity were several-fold: (*i*) AtPCS1 has a computed mass of 55,000 (Fig. 1) and an electrophoretic mobility of 58,000 (Figs. 4 and 6), which is commensurate with an apparent subunit size of 50,000 for the activity of partially purified PC synthase preparations from plants (17). (*ii*) AtPCS1 and its homologs (Fig. 1) are found in both plants and *S. pombe* as is PC synthase (11, 12). (*iii*) AtPCS1, like the products of PC synthase activity, though capable of conferring greatest tolerance to Cd^{2+}, also confers tolerance to other heavy metals such as Cu^{2+} (11, 12). (*iv*) Transformation of GSH-deficient *S. cerevisiae gsh2*Δ mutants, in which the coding sequence for GSH synthetase is disrupted (32) with pYES3-AtPCS1::FLAG, does not confer Cd^{2+} tolerance (data not shown), suggesting that AtPCS1, like PC synthase, is ineffective unless GSH is available.

The involvement of AtPCS1 in PC biosynthesis was examined at three levels: (*i*) by determining whether $CdCl_2$-grown DTY167/pYES3-AtPCS1::FLAG cells contained elevated PC levels by comparison with $CdCl_2$-grown DTY167/pYES3 cells, and, if so, whether PC accumulation required growth in media containing Cd^{2+}; (*ii*) by determining whether the soluble fraction from DTY167/pYES3-AtPCS1::FLAG cells had an increased capacity for Cd^{2+}-dependent PC synthesis *in vitro* by comparison with the soluble fraction from DTY167/pYES3 cells; (c) by determining whether purified AtPCS1-FLAG catalyzed Cd^{2+}-dependent PC synthesis from GSH. At whatever level it was investigated, AtPCS1 exhibited all of the characteristics of a PC synthase.

DTY167/pYES3-AtPCS1::FLAG cells accumulated PCs in a Cd^{2+}-dependent manner. Reverse-phase FPLC analysis of nonprotein thiol compounds in the soluble fraction from DTY167/pYES3-AtPCS1::FLAG cells after growth in medium containing $CdCl_2$ revealed multiple peaks eluting after the GSH/2-mercaptoethanol injection peak whose total thiol content represented 51 nmol/mg extracted protein (Fig. 7*A*). The two most prominent of these peaks, peaks 1 and 2, contributed 4 nmol thiol/mg protein and eluted at the same positions as PC_2 and PC_3 standards prepared from *S. pombe* (Fig. 7*A*). The corresponding fractions from $CdCl_2$-grown DTY167/pYES3 cells and DTY167/pYES3-AtPCS1::FLAG cells after growth in medium lacking Cd^{2+} were devoid of nonprotein thiols other than those associated with the GSH/2-mercaptoethanol injection peak (data not shown).

Expression of plasmid-borne *AtPCS1*::*FLAG* was necessary for the generation of extractable PC synthase activity but, unlike intracellular PC accumulation, did not require exposure of the cells to Cd^{2+} before extraction. *In vitro* assays of the capacity of the soluble fractions from DTY167/pYES3-AtPCS1::FLAG cells grown in the presence and absence of Cd^{2+} for the incorporation of GSH into PCs demonstrated exclusive synthesis of PC_2 and PC_3 at aggregate rates of 0.5 and 0.34 nmol/mg/min when assayed in media containing 3.3 mM GSH and 200 μM $CdCl_2$. The corresponding fractions from DTY167/pYES3 cells yielded rates below the limits of detection (<0.01 nmol/mg/min) irrespective of whether the cells had been grown in the presence or absence of Cd^{2+}. In no case was PC synthase activity detectable when Cd^{2+} was omitted from the assay medium, implying an obligate requirement for heavy-metal ions for PC synthesis.

AtPCS1-FLAG was sufficient for the Cd^{2+}-dependent synthesis of PCs from GSH *in vitro*. The single M_r 58,000 polypeptide species purified by immunoaffinity chromatogra-

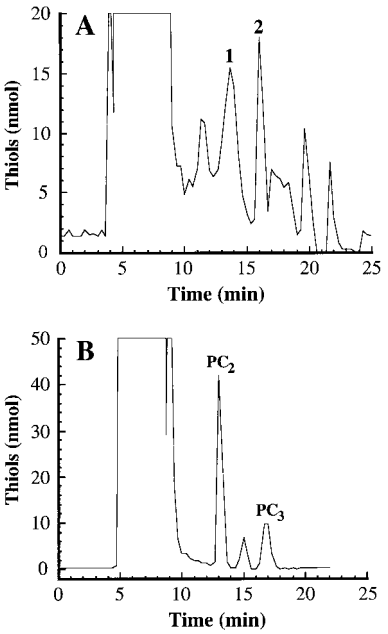

FIG. 7. AtPCS1-FLAG-dependent PC synthesis *in vivo* and *in vitro*. (*A*) Reverse-phase FPLC analysis of nonprotein thiols in the soluble fractions extracted from DTY167/pYES3-AtPCS1::FLAG cells after growth in liquid medium containing $CdCl_2$ (50 μM). Peaks 1 and 2 were found to comigrate with PC_2 and PC_3 standards, respectively, purified from *S. pombe*. The equivalent fractions extracted from DTY167/pYES3-AtPCS1::FLAG cells after growth in medium lacking $CdCl_2$ and from DTY167/pYES3 cells after growth in medium containing $CdCl_2$ (50 μM) were devoid of PC-like nonprotein thiols. (*B*) Reverse-phase FPLC analysis of the nonprotein thiols formed after incubation of GSH (3.3 mM) with immunoaffinity-purified AtPCS1-FLAG in the presence of Cd^{2+} (200 μM). The peaks designated "PC_2" and "PC_3" were identified on the basis of their Glu/Gly ratios (2.1 ± 0.1 and 2.9 ± 0.2, respectively) and comigration with *S. pombe* PC standards.

phy of the soluble fraction from DTY167/pYES3-AtPCS1::FLAG cells (Fig. 6) catalyzed the incorporation of GSH into PC_2 and PC_3 at an aggregate rate of 30–35 μmol/mg/min and <0.01 nmol/mg/min in the presence and absence of Cd^{2+}, respectively (Fig. 7*B*). Control reaction mixtures lacking AtPCS1-FLAG did not yield any PCs (data not shown). Given that AtPCS1-FLAG was the sole protein species in the reaction medium and the products of its reaction with GSH, PC_2 and PC_3, were determined by quantitative amino acid analysis to have Glu/Gly ratios of 2.1 ± 0.1 and 2.9 ± 0.2, respectively, AtPCS1 was evidently capable of catalyzing the transfer of a γ-glutamylcysteine unit from one GSH molecule to another to form PC_2, or from one GSH molecule to PC_2 to form PC_3. As would be expected for a *bona fide* enzyme-catalyzed reaction, the amounts of PC_2 and PC_3 synthesized increased linearly with time to yield strict proportionality between the rates of synthesis and the amounts of AtPCS1-FLAG added to the reaction medium.

CONCLUSIONS

The results presented establish that *AtPCS1*, a cDNA from *Arabidopsis*, and by implication its homologs from *S. pombe* and *C. elegans* as well as *AtPCS2*, encode PC synthases—Cd^{2+}-binding enzymes capable of high rates of Cd^{2+}-activated PC synthesis from GSH. As such, the cloning and *in vitro* reconstitution of AtPCS1 is of considerable strategic value. Enzymologically, the ease with which AtPCS1-FLAG can be purified to apparent homogeneity in a single step from pYES3-AtPCS1::FLAG-transformed yeast to yield PC synthase preparations with a catalytic activity exceeding that of previous preparations from plants (17) by more than 10^3-fold will enable detailed mechanistic and crystallographic studies of this enzyme and the facile production of PCs *in vitro* on a milligram-to-gram scale. Molecularly, the amenability of AtPCS1 to expression in yeast in an active state to confer a selectable phenotype provides a basis for probing the structural requirements of AtPCS1-catalyzed PC synthesis by the application of both site-directed and random mutagenic approaches. Evolutionarily, although the existence of an AtPCS1 homolog in the genome of *S. pombe* is, in retrospect, to be expected, given the extent to which PC-based metal tolerance has been researched in this organism (11, 12), the discovery of an *AtPCS1* homolog in the genome of *C. elegans* was surprising in that it invokes a role not only for GSH and MTs but also for PCs in metal homeostasis in at least some animals. Genetically, the now ready availability of isolated genes encoding PC synthases and the demonstrated sufficiency of a single gene product for reconstitution of core catalysis will expedite investigations into the mechanisms by which plants and other organisms detoxify heavy metals.

Note Added in Proof: Two other papers describing the molecular characterization of PC synthases are soon to be published. They are refs. 33 and 34.

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