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Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast

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Phytochelatins play major roles in metal detoxification in plants and fungi. However, genes encoding phytochelatin synthases have not yet been identified. By screening for plant genes mediating metal tolerance we identified a wheat cDNA, *TaPCS1*, whose expression in *Saccharomyces cerevisiae* results in a dramatic increase in cadmium tolerance. *TaPCS1* encodes a protein of ~55 kDa with no similarity to proteins of known function. We identified homologs of this new gene family from *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and interestingly also *Caenorhabditis elegans*. The *Arabidopsis* and *S.pombe* genes were also demonstrated to confer substantial increases in metal tolerance in yeast. *PCS*-expressing cells accumulate more Cd^{2+} than controls. *PCS* expression mediates Cd^{2+} tolerance even in yeast mutants that are either deficient in vacuolar acidification or impaired in vacuolar biogenesis. PCS-induced metal resistance is lost upon exposure to an inhibitor of glutathione biosynthesis, a process necessary for phytochelatin formation. *Schizosaccharomyces pombe* cells disrupted in the *PCS* gene exhibit hypersensitivity to Cd^{2+} and Cu^{2+} and are unable to synthesize phytochelatins upon Cd^{2+} exposure as determined by HPLC analysis. *Saccharomyces cerevisiae* cells expressing *PCS* produce phytochelatins. Moreover, the recombinant purified *S.pombe* PCS protein displays phytochelatin synthase activity. These data demonstrate that *PCS* genes encode phytochelatin synthases and mediate metal detoxification in eukaryotes.

Keywords: bioremediation/metal tolerance/metal toxicity/phytochelatins/phytoremediation

Introduction

Heavy metal toxicity poses major environmental and health problems. Cadmium, for example, is a non-essential heavy metal which is toxic to living cells at very low concentrations. Cd^{2+} ions displace Ca^{2+} or Zn^{2+} in proteins and can cause oxidative stress (Stohs and Bagchi, 1995; Goyer, 1997). In humans, Cd^{2+} is a suspected carcinogen (Lemen *et al*., 1976). Furthermore the concentration of essential, but at high concentrations toxic, metals such as Cu^{2+}, Zn^{2+} and Fe^{2+} is tightly controlled. Several mechanisms are known that allow plants and other organisms to tolerate the presence of toxic non-essential metal ions inside the cell. In bacteria a wide range of efflux pumps, predominantly P-type ATPases, have been shown to mediate metal detoxification (Silver and Phung, 1996). In eukaryotic cells, toxic ions appear to be removed from the cytosol mainly by chelation and sequestration. *Schizosaccharomyces pombe* and other fungi as well as plants synthesize phytochelatins which chelate Cd^{2+}, Cu^{2+} and other heavy metal ions (Grill *et al*., 1985; Rauser, 1995). Phytochelatins are thiolate peptides with the primary structure $(\gamma\text{-Glu-Cys})_n$-Gly, which are non-translationally synthesized from glutathione (Grill *et al*., 1989). However, genes encoding phytochelatin synthases have not yet been identified. Low molecular weight phytochelatin–metal complexes are transported into vacuoles by ATP-binding cassette transporters, as shown for *S.pombe* (Ortiz *et al*., 1992, 1995). Phytochelatin-deficient *Arabidopsis* and *S.pombe* mutants are hypersensitive to Cd^{2+} (Mutoh and Hayashi, 1988; Howden *et al*., 1995), thereby demonstrating the importance of phytochelatins for plant and fungal metal tolerance. Furthermore, physiological studies indicate that heavy metal tolerance is one of the prerequisites of heavy metal hyperaccumulation in plants (Krämer *et al*., 1997; Raskin *et al*., 1997).

With the goal of identifying plant genes involved in Cd^{2+} resistance we pursued an expression cloning approach in *Saccharomyces cerevisiae*. Here we report on the cloning and characterization of a family of genes from plants and yeast which encode phytochelatin synthases, enzymes that play a crucial role in metal tolerance.

Results

Cloning of TaPCS1

A screen was pursued to identify plant genes that confer cellular Cd^{2+} tolerance. Yeast cells were transformed with a size-selected (>1.5 kb) wheat root library (Schachtman and Schroeder, 1994) and 2×10^7 cells representing ~1×10^6 independent transformants were added to 50 ml of arginine-phosphate liquid medium containing either 20 or 50 μM Cd^{2+}. These Cd^{2+} concentrations are growth-inhibiting for yeast cells in arginine-phosphate medium. However, the liquid cultures became saturated within 2–4 days. Following saturation, surviving yeast cell aliquots were taken, DNA was extracted and after *Escherichia coli* transformation the pYES2 inserts were analyzed. All inserts from the same culture showed identical restriction patterns indicating that the liquid cultures grew to saturation starting from one or a small number of yeast cells containing the same wheat cDNA. In six liquid cultures of this screen (which included two repetitions with newly

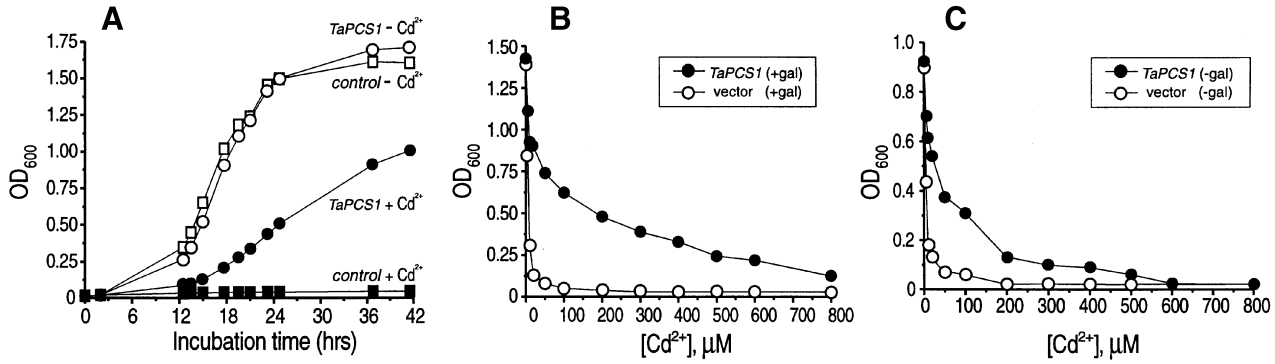

Fig. 1. *TaPCS1* expression renders yeast cells more Cd^{2+} tolerant. (**A**) Control cells [INVSc1 cells (Invitrogen, Carlsbad, CA) carrying the empty pYES2 plasmid] (squares) and *TaPCS1* expressing cells (circles) were grown in YNB (1% sucrose/1% galactose) containing either no (open symbols) or 200 µM Cd^{2+} (filled symbols). (**B**) Growth in YNB (1% sucrose/1% galactose) of control cells (open circles) and *TaPCS1* expressing cells (filled circles) at different Cd^{2+} concentrations. OD_{600} after 24 h is shown. (**C**) Growth in YNB (2% raffinose) of control cells (open circles) and *TaPCS1* expressing cells (filled circles) at different Cd^{2+} concentrations. OD_{600} of cultures after 40 h is shown.

transformed cells) a single cDNA, differing only in the length of the 5′ untranslated regions, was cloned. The cDNA was initially named *CdR* (for Cd^{2+} resistance) but after further characterization it was named *TaPCS1* for its function in *Triticum aestivum* phytochelatin synthesis.

TaPCS1 expression makes S.cerevisiae more Cd^{2+} tolerant

TaPCS1 expression mediated a dramatic increase in Cd^{2+} tolerance of *S.cerevisiae* cells. *TaPCS1*-expressing cells grew to saturation in the presence of Cd^{2+} concentrations which completely inhibited growth of control cells harboring the empty pYES2 plasmid (Figure 1A). Dose–response analyses showed that *TaPCS1*-expressing cells tolerate 15-fold higher Cd^{2+} concentrations than control cells (Figure 1B) [$K_{0.5}$(*TaPCS1*) = 90 µM Cd^{2+}; $K_{0.5}$(control) = 6 µM Cd^{2+}]. *TaPCS1*-expressing cells grown in the absence of the inducer galactose also displayed a strong degree of Cd^{2+} tolerance, suggesting that low levels of *TaPCS1* are sufficient for Cd^{2+} resistance and indicating a catalytic role for *TaPCS1* in mediating Cd^{2+} tolerance (Figure 1C), rather than tolerance by direct binding of metals to *TaPCS1*. Even with glucose as the carbon source, which represses the GAL1 promoter, a slight enhancement of growth was seen in *TaPCS1*-containing cells (data not shown), further suggesting a catalytic activity of *TaPCS1*.

Sequence analysis and TaPCS1 homologs

The *TaPCS1* open reading frame (ORF) (DDBJ/EMBL/GenBank accession No. AF093752) encodes a polypeptide with a predicted mass of 55 kDa. The deduced amino acid sequence shows no homology to any protein of known function. However, homology was found for the *Arabidopsis thaliana* expressed sequence tag (EST) G11G3T7 (DDBJ/EMBL/GenBank accession No. W43439), a *S.pombe* hypothetical 46.7 kDa protein C3H1.10 (DDBJ/EMBL/GenBank accession No. Z68144), and the *Caenorhabditis elegans* ORF F54D5.1 (DDBJ/EMBL/GenBank accession No. Z66513). These sequences are 55, 28 and 32% identical at the amino acid level, respectively (Figure 2A). *TaPCS1* and its homologs thus constitute a new gene family. The homologs from *Arabidopsis* (*AtPCS1*) and *S.pombe* (*SpPCS*) also confer strong Cd^{2+} tolerance when expressed in *S.cerevisiae* (Figure 2B). Low-stringency DNA gel blot analysis in *Arabidopsis* suggested the presence of more than one *AtPCS* homolog (data not shown). Meanwhile, a second *Arabidopsis* gene (*AtPCS2*, DDBJ/EMBL/GenBank accession No. AC003027) and a second *C.elegans* gene (DDBJ/EMBL/GenBank accession No. AL023633) with homology to the *PCS* genes have been sequenced through genome sequencing efforts.

TaPCS1 mediates an increase in Cd^{2+} accumulation

One possible mechanism underlying Cd^{2+} tolerance is an efflux of Cd^{2+} ions as observed in many bacteria (Silver and Phung, 1996). This possibility was investigated by measuring the accumulation of Cd^{2+} by control and *TaPCS1* expressing cells grown at Cd^{2+} concentrations that do not significantly affect the growth of even the control cells. As shown in Figure 3, *TaPCS1* expression led to an increase in Cd^{2+} accumulation by ~30–50% during a 24 h culture period (n = 3). Similar results were found for the *Arabidopsis* homolog (n = 2, data not shown). We interpreted this finding as evidence in support of *PCS1*-dependent Cd^{2+} chelation or sequestration.

TaPCS1 confers Cd^{2+} tolerance even in vacuolar mutants

Sequestration of Cd^{2+} and other heavy metal ions into vacuoles is a well-characterized mechanism of detoxification (Rea *et al*., 1998). One postulated pathway for plants that would function in parallel to the transport of Cd–phytochelatin complexes into vacuoles is a Cd^{2+}/H^{+} exchanger (Salt and Wagner, 1993). To determine whether *TaPCS1* is involved in this process we expressed *TaPCS1* in a *Δvma4* strain, which lacks a functional vacuolar ATPase and therefore cannot establish a pH gradient (Ho *et al*., 1993), required for Cd^{2+} uptake via the Cd^{2+}/H^{+} exchanger. Growth assays with the *Δvma4* and the parental strain showed that *TaPCS1* still confers Cd^{2+} tolerance (n = 3, data not shown). Furthermore, we expressed *TaPCS1* in the yeast strain *Δvps18*, which fails to form any structures morphologically resembling normal vacuoles (Robinson *et al*., 1991). As expected, this strain is significantly more sensitive to Cd^{2+} than the parental strain (Figure 4, open circles). Interestingly, *TaPCS1* expression again led to a strong increase in Cd^{2+} tolerance (Figure 4, filled circles).

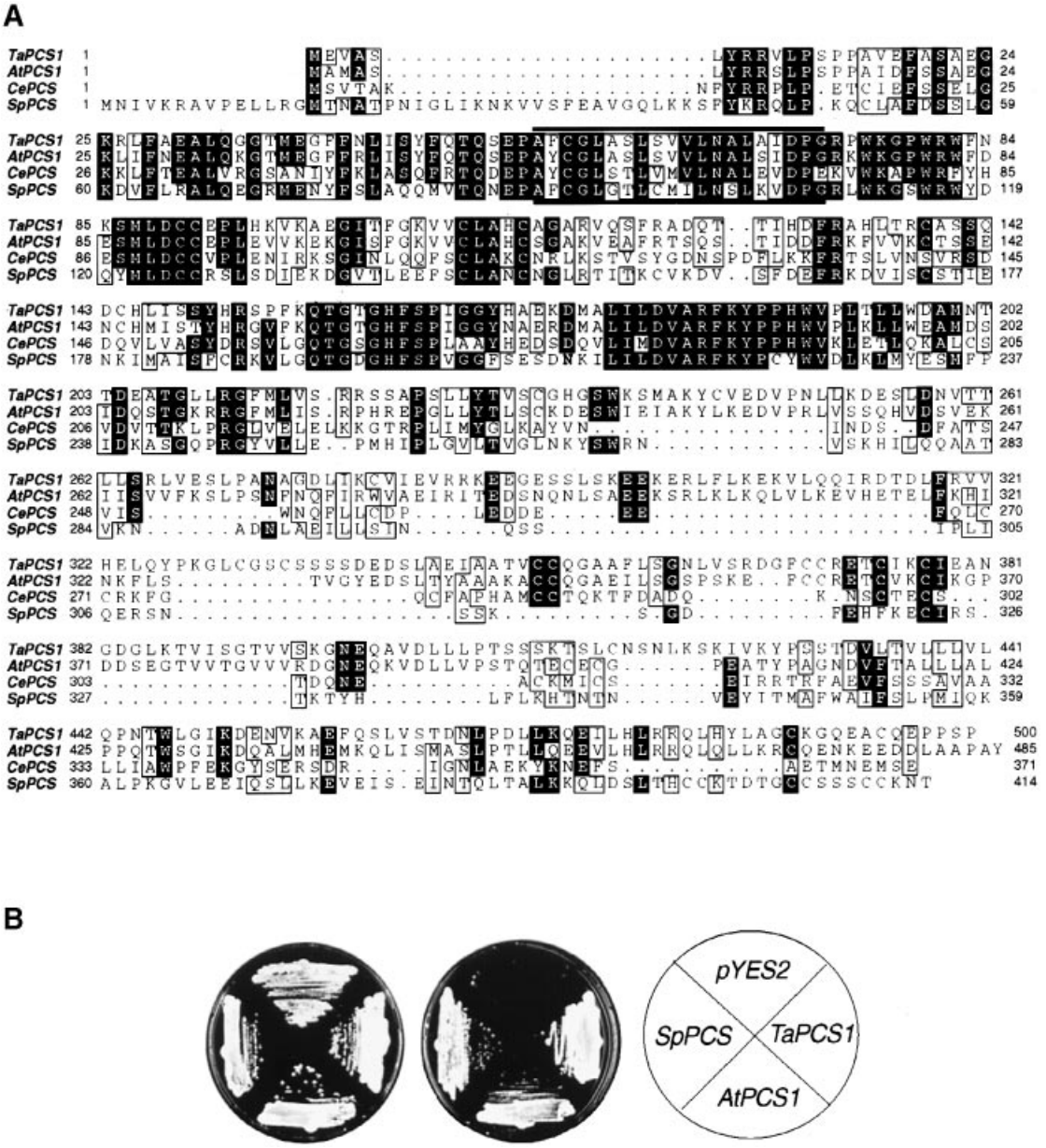

Fig. 2. *PCS* gene family. (**A**) CLUSTAL W alignment (Thompson *et al*., 1994) of the amino acid sequences of wheat (*TaPCS1*), *Arabidopsis* (*AtPCS1*), *S.pombe* (*SpPCS*) and *C.elegans* (*CePCS1*). Identical amino acid residues are in black boxes, similar amino acid residues in light boxes. (**B**) Growth of control cells (carrying the empty pYES2 plasmid) and cells expressing either *TaPCS1*, *AtPCS1* or *SpPCS* on YNB medium without (left) and with 100 μM Cd^{2+} (middle).

SpPCS deletion results in metal sensitivity

To investigate the physiological role of the *PCS* genes more directly, we generated a deletion mutant of *SpPCS* in *S.pombe*. First, Southern analysis of *S.pombe* genomic DNA was performed to search for additional *PCS* homologs. Under low-stringency conditions no indication of sequences homologous to *SpPCS* could be detected (Figure 5A), demonstrating that *SpPCS* is a single-copy gene in this organism. Subsequently, the *SpPCS* gene was deleted by a one-step gene disruption using the ura4 marker. The Cd^{2+} sensitivity of *S.pombe* with a disruption of the *SpPCS* gene (*ΔSpPCS*) was tested in media containing various Cd^{2+} concentrations. For controls a strain transformed with the empty plasmid pTZura4 and a transformant with a non-homologous integration of the knockout construct (Sp3) were analyzed. In the absence of Cd^{2+}, the knockout strain grew normally (Figure 5B, top). Growth of the knockout strain was more strongly inhibited by Cd^{2+} than that of the two different control strains (Figure 5B). Growth of the knockout strain was also more sensitive than control strains to copper (Figure 5C), showing a role for *SpPCS* in resistance to Cu^{2+}.

PCS genes are involved in phytochelatin synthesis

The findings indicating a catalytic role of the *PCS* genes in Cd^{2+} sequestration (Figure 1C) indicated a possible role for the *PCS* gene family in phytochelatin synthesis. Glutathione is a precursor required for phytochelatin synthesis (Grill *et al*., 1989). Pre-treatment of *TaPCS1*-expressing *S.cerevisiae* with the glutathione biosynthesis inhibitor BSO (L-buthionine sulfoxime) reduced the *TaPCS1*-mediated Cd^{2+} tolerance in a dose-dependent

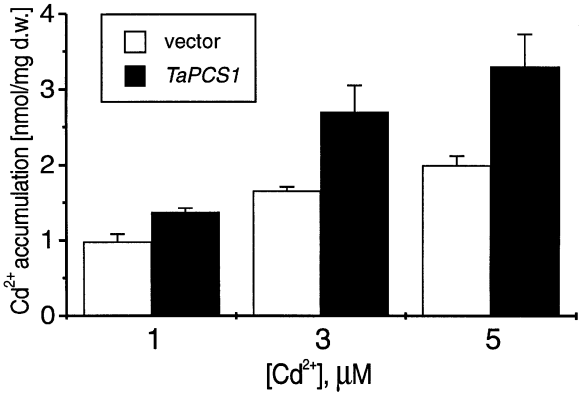

Fig. 3. *TaPCS1*-expressing cells accumulate more Cd^{2+} than control cells. Yeast cells carrying the empty pYES2 plasmid (white bars) and *TaPCS1*-expressing cells (black bars) were grown in arginine-phosphate medium containing different non-inhibitory Cd^{2+} concentrations. After 24 h cells were harvested, washed and the amount of Cd^{2+} accumulated inside the cells was determined using $^{109}Cd^{2+}$. Error bars indicate SE, $n = 3$.

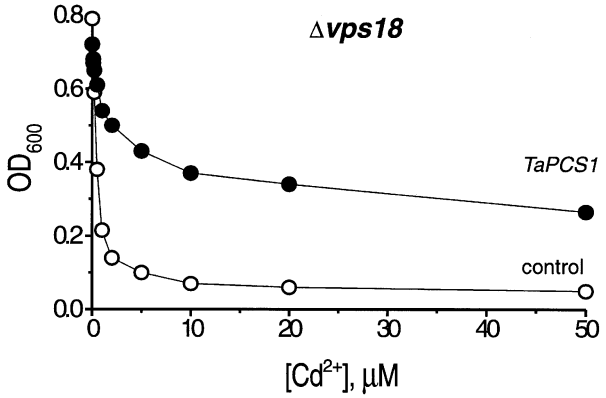

Fig. 4. Expression of *TaPCS1* in *Δvps18*, a *S.cerevisiae* strain lacking morphologically visible vacuoles (Robinson *et al*., 1991), still confers a Cd^{2+} tolerance phenotype. Cells carrying the empty pYES2 plasmid (open circles) and cells expressing *TaPCS1* (closed circles) were grown in YNB medium in the presence of different Cd^{2+} concentrations.

manner (Figure 6A, circles). In controls, expression of an *Arabidopsis* metallothionein (S.Clemens and J.I.Schroeder, unpublished results) showed no effect of BSO on metallothionein-dependent Cd^{2+} tolerance (Figure 6A, filled squares). Note that *TaPCS1* expressing cells showed a significantly higher growth rate at 200 µM Cd^{2+} than that of metallothionein-expressing cells at only 60 µM Cd^{2+} (Figure 6A).

Wild-type *S.pombe* cells (control) and the *ΔSpPCS* strain were analyzed for phytochelatin synthesis upon Cd^{2+} exposure by fluorescence HPLC. Peaks showing retention times identical to the synthesized phytochelatin standards, PC2 and PC3, were found in extracts from wild-type cells (Figure 6B, top, peaks 1 and 2) but were absent in extracts from *ΔSpPCS* cells (Figure 6B, bottom). Thus, the increased Cd^{2+} sensitivity of the *S.pombe* knockout is correlated with a deficiency in phytochelatin synthesis. Furthermore, *TaPCS1*-expressing *S.cerevisiae* cells synthesized compounds following Cd^{2+} treatment (Figure 6C, top) that were not formed in wild-type cells (Figure 6C, bottom). Those compounds showed retention times identical to PC2 and PC3 which were not observed in the *S.cerevisiae* controls, demonstrating that *TaPCS1* expression is sufficient to elicit the synthesis of phytochelatins in an organism proposed to not normally form phytochelatins (Rauser, 1995). Phytochelatin synthesis in *TaPCS1*-expressing cells can also be elicited by treatment with Cu^{2+} or Zn^{2+} (data not shown).

PCS proteins catalyze phytochelatin synthesis

PCS enzyme assays with crude extracts from *S.pombe* marker-transformed cells and *ΔSpPCS* cells showed that the knockout strain lacks PCS activity (Figure 7A, bottom). In extracts from control cells, PC2 formation from glutathione was clearly detectable (Figure 7A, top, peak 2), similar to previous reports using purified enzyme preparations (Grill *et al*., 1989; Hayashi *et al*., 1991). A purified recombinant *Arabidopsis* PCS protein showed phytochelatin synthase activity *in vitro* (P.Rea, University of Pennsylvania, personal communication). Thus, the *ΔSpPCS* strain provided a suitable background for the expression of a tagged SpPCS protein in order to test directly whether the PCS proteins catalyze phytochelatin synthesis. We expressed SpPCS with an N-terminal hemagglutinin (HA)-tag (SpPCS-HA) in the knockout strain and found that this construct restores metal tolerance, PCS enzyme activity and the ability to form PC2 and PC3 (data not shown). Immunoblots of extracts from cells expressing the HA-tagged SpPCS protein shows a band at ~46 kDa, corresponding to the predicted molecular weight of SpPCS, which is recognized by a monoclonal anti-HA antibody (BAbCo, Berkeley, CA) (Figure 7B, left). No signal was detected in extracts from control *ΔSpPCS* cells containing the empty pSGP73 plasmid (Figure 7B, right).

SpPCS-HA was purified using the monoclonal HA-antibody coupled to Sepharose (BAbCo, Berkeley, CA). The fraction eluted with synthesized HA peptide (YPYDVPDYA) was analyzed by SDS–polyacrylamide gel electrophoresis. Silver staining of the gel showed two bands, one at ~46 kDa, the same as found for the anti-HA-immunoreactive band detected from extracts of SpPCS-HA expressing cells, and a lower molecular weight band of ~30 kDa (Figure 7C, left). Western blot analysis of the eluate fraction demonstrated cross-reactivity of both polypeptides to anti-HA antibodies (Figure 7C, right). Recognition of the 30 kDa protein by anti-HA antibodies, combined with the fact that this band was not detected from lysates of SpPCS-HA expressing and control cells (Figure 7B), suggest that the polypeptide might represent a proteolytic degradation product of the 46 kDa protein which may have arisen during purification. Aliquots of the peptide eluate were further analyzed for PCS enzyme activity. As shown in Figure 7D, HPLC analysis of the monobromobimane-derivatized reaction products shows formation of a peak corresponding to PC2 from glutathione and Cd^{2+}, thereby demonstrating PCS enzyme activity in the fraction containing SpPCS-HA.

TaPCS1 expression is constitutive and enhanced by Cd^{2+}

TaPCS1 mRNA could not be detected by RNA blot analysis of wheat root mRNA. To determine whether the *TaPCS1* gene is transcribed in wheat seedlings, we performed RT–PCR experiments with *TaPCS1* specific primers. Fragments of the expected size were detectable

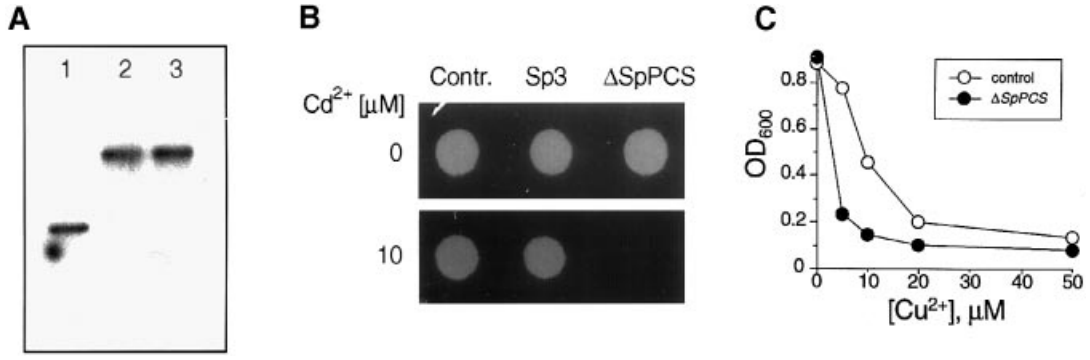

Fig. 5. A *S.pombe* strain with a disruption of *SpPCS* shows increased metal sensitivity. (**A**) Low-stringency Southern blot of *S.pombe* genomic DNA probed with *SpPCS*. Digests: lane 1, *Bam*HI; lane 2, *Hin*dIII; lane 3, *Eco*RI. (**B**) A *SpPCS* knockout strain (*ΔSpPCS*) and as controls a strain transformed with the empty plasmid pTZura4 (Contr.) and a transformant with a non-homologous integration of the knockout construct (Sp3) were grown on EMM-ura containing either 0 or 10 μM Cd^{2+}. (**C**) The marker-transformed control strain (open circles) and the *ΔSpPCS* strain (filled circles) were grown in liquid EMM-ura in the presence of different Cu^{2+} concentrations. OD was measured after 24 h of growth.

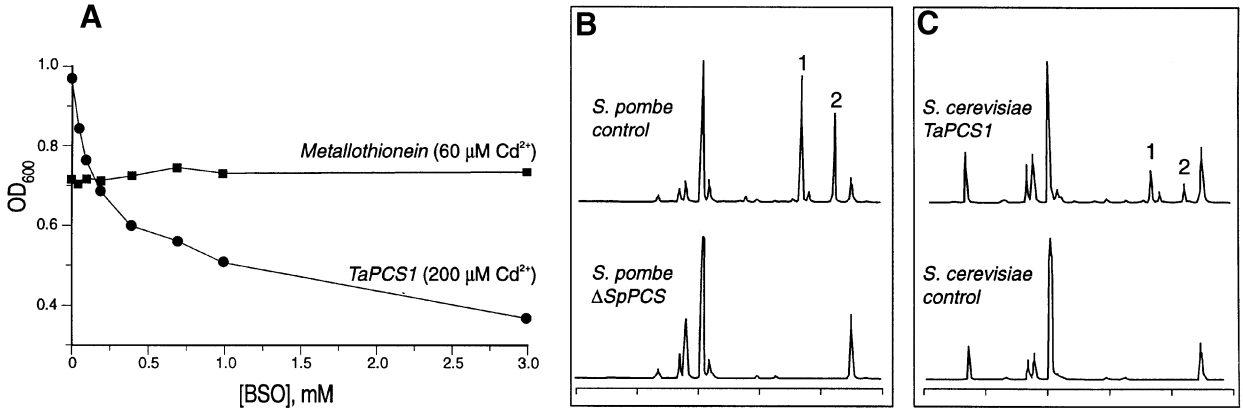

Fig. 6. Phytochelatin synthesis in cells expressing PCS. (**A**) Growth of *S.cerevisiae* cells expressing either *TaPCS1* (circles) or an *Arabidopsis* metallothionein (S.Clemens and J.I.Schroeder, unpublished data) (squares) in the presence of Cd^{2+} (200 μM for *TaPCS1*, 60 μM for metallothionein) following a 6 h preincubation with different concentrations of BSO (L-buthionine sulfoxime), a glutathione synthesis inhibitor. OD was measured 18 h after addition of Cd^{2+}. (B and C) Extracts of Cd^{2+}-treated *S.pombe* and *S.cerevisiae* cells were labeled with monobromobimane and analyzed by HPLC using a reversed-phase column and fluorescence detection. (**B**) *Schizosaccharomyces pombe* wild-type (top) and *S.pombe* knockout (bottom), (**C**) *S.cerevisiae* expressing *TaPCS1* (top) and *S.cerevisiae* wild-type (bottom). The peaks labeled 1 and 2 in B and C are identical based on co-injection experiments. They represent PC2 and PC3 as shown by comparison of retention times with standards synthesized on an Abimed peptide synthesizer (as described in Materials and methods).

for both root (Figure 8, top) and shoot samples (not shown). Expression of *AtPCS1* in *Arabidopsis* was also analyzed by RT–PCR. The results in *Arabidopsis* were the same as found for *TaPCS1* (data not shown), showing that both *TaPCS1* and *AtPCS1* are transcribed *in vivo*. To determine whether exposure to Cd^{2+} treatment affected *TaPCS1* expression in wheat we used wheat cDNA together with different amounts of competitor DNA in PCRs. Comparison of the band intensity indicated a 5- to 10-fold higher concentration of *TaPCS1* message in wheat roots treated with 100 μM Cd^{2+} (Figure 8).

Discussion

We have isolated and functionally characterized a novel family of genes in several different organisms that mediate a dramatic increase in Cd^{2+} tolerance when expressed in *S.cerevisiae*. Cadmium accumulation experiments, *TaPCS1* induction in roots, glutathione inhibitor studies and analysis in several yeast mutant backgrounds, together with the Cd^{2+} and Cu^{2+} sensitivity of a *SpPCS* disruption mutant in *S.pombe* show a central physiological role of the *PCS* gene family for metal tolerance. The PC deficiency of a *S.pombe* knockout strain, the phytochelatin synthesis observed in *TaPCS1*-expressing *S.cerevisiae* cells upon Cd^{2+} exposure and the phytochelatin synthase activity of purified recombinant SpPCS suggest that these genes mediate phytochelatin synthesis.

TaPCS1 was identified through an expression cloning strategy in *S.cerevisiae* by searching for clones mediating high tolerance to Cd^{2+} in the growth medium. *TaPCS1* expression enabled yeast cells to grow at >15-fold higher Cd^{2+} concentrations than control cells. Identification of *TaPCS1* using this approach was greatly enhanced by the fact that the screening was restricted to a >1.5 kb fraction of a cDNA library (Schachtman and Schroeder, 1994), which helped eliminate the cloning of cDNAs encoding small and abundant heavy metal-binding peptides such as metallothioneins. Selection of transformants exhibiting elevated tolerance to Cd^{2+} was performed in liquid medium in order to provide extremely homogeneous screening conditions, while easily permitting the screen to be performed in parallel under different levels of selective pressure. This screening method also allowed the isolation of those cDNAs most effective in conferring Cd^{2+} tolerance. The efficacy of this approach is demon-

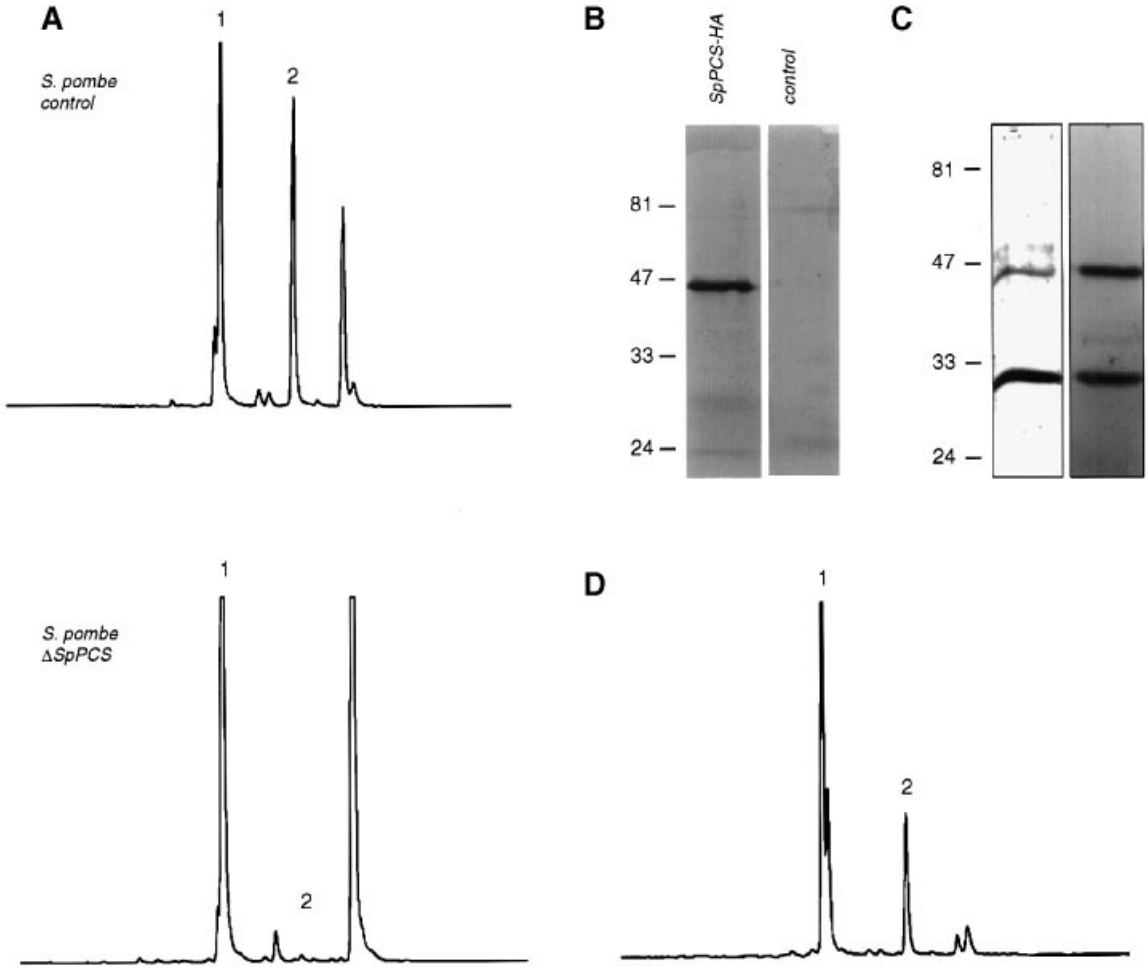

Fig. 7. PCS mediates phytochelatin synthesis. (**A**) Crude extracts of *S.pombe* control (top) or *ΔSpPCS* (bottom) cells were incubated in 200 mM Tris–Cl (pH 8.0), 1 mM DTT, 1 mM GSH and 0.1 mM $CdCl_2$ at 30°C for 30–120 min, and reaction products were monobromobimane-labeled and analyzed by HPLC. Peak 1 corresponds to free GSH, and peak 2 represents PC2. (**B**) Western blot analysis of extracts from *ΔSpPCS* cells harboring SpPCS-HA (left) or empty vector (right) using anti-HA monoclonal antibody (BAbCo, Berkeley, CA). Sizes of molecular mass standards run in parallel are indicated. (**C**) SpPCS-HA was purified from crude extracts of cells expressing SpPCS-HA using anti-HA antibody affinity column. Protein was eluted with 5 mg HA peptide (YPYDVPDYA) and the eluted fraction analyzed by SDS–PAGE (left) and Western blotting (right). A second band, possibly a proteolytic fragment of the 46 kDa SpPCS-HA, is detected at ~30 kDa. (**D**) Phytochelatin synthesis by purified SpPCS-HA. Affinity purified SpPCS-HA was assayed for phytochelatin synthase activity, and the products labeled and analyzed by HPLC as described in (A). Peaks 1 and 2 represent free GSH and PC2, respectively.

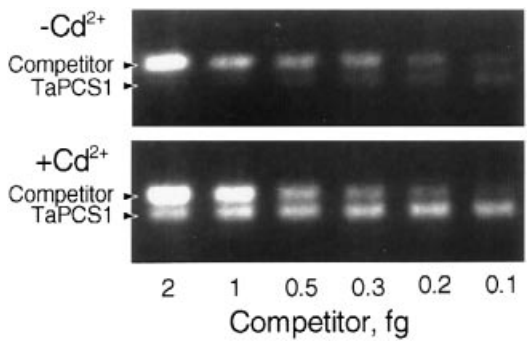

Fig. 8. *TaPCS1* expression in roots is induced by Cd^{2+} as shown by competitive PCR. RNA was isolated from 4-day-old wheat roots that were either untreated or treated with 100 μM Cd^{2+} for 6 h. First-strand cDNA was made and 10 ng were used as a template in PCRs. Competitor DNA used in this study was a PCR fragment amplified from genomic DNA which was ~100 bp longer than the cDNA-amplified fragment because of the presence of an intron. The indicated amounts of competitor DNA were added. Aliquots were analyzed by agarose gel electrophoresis. The experiment was repeated twice with similar results.

strated by the fact that, in independent experiments, *TaPCS1* was the only cDNA isolated.

A role in metal tolerance

A number of findings suggest a catalytic role of the PCS gene products in metal detoxification. The observed increase in Cd^{2+} accumulation (Figure 3) upon *TaPCS1* expression in *S.cerevisiae* showed that the tolerance phenotype is not based on the exclusion of the toxic metal, the dominant mechanism of metal detoxification in bacteria (Silver and Phung, 1996). On the contrary, the *TaPCS1*-dependent increase in Cd^{2+} accumulation is consistent with the hypothesis that *TaPCS1* is involved in Cd^{2+} sequestration as, for instance, *S.pombe* cells overexpressing the ABC-type transporter *hmt1* accumulate more Cd^{2+} (Ortiz *et al.*, 1992). Furthermore, *TaPCS1* can confer strong Cd^{2+} tolerance even when expressed at low levels under non-inducing conditions (Figure 1C).

Because most toxic materials inside plant cells are sequestered in vacuoles we performed Cd^{2+} sensitivity assays with yeast vacuolar mutants. The *TaPCS1*-mediated

increase in Cd^{2+} tolerance which was still observed in yeast strains that lack either a functional V-ATPase (Ho *et al*., 1993) or morphologically typical discernible vacuoles (Robinson *et al*., 1991; Figure 4) led us to conclude that a direct role for *TaPCS1* in vacuolar transport of Cd^{2+} ions appears unlikely, although an indirect involvement or early reaction preceding vacuolar uptake cannot be ruled out.

PCS genes mediate phytochelatin synthesis

Synthesis of phytochelatins from glutathione upon metal exposure has been shown to be directly involved in plant metal tolerance (Zenk, 1996). Phytochelatin synthase was proposed to catalyze the first step in the sequestration of Cd^{2+} as Cd–phytochelatin complexes in vacuoles. Because a cDNA encoding a phytochelatin synthase has not been isolated yet and our data suggested a *PCS*-mediated sequestration of Cd^{2+} we tested the hypothesis that the *PCS* genes are involved in phytochelatin synthesis.

Consistent with the aforementioned observations in plants, Cd^{2+} hypersensitive mutants of *S.pombe* have been isolated which show reduced phytochelatin levels (Mutoh and Hayashi, 1988). Thus, the observed Cd^{2+} hypersensitivity of the *ΔSpPCS* strain (Figure 6B) provided further indication for the hypothesis that *PCS* genes directly mediate phytochelatin synthesis. Cu^{2+} sensitivity is also consistent with a phytochelatin deficiency as phytochelatins form complexes with several toxic metals and with copper ions as well (Rauser, 1995).

To test more directly the hypothesis that *PCS* genes mediate phytochelatin synthesis, we first studied the effect of BSO, a potent, specific inhibitor of glutathione biosynthesis, on *TaPCS1*-mediated Cd^{2+} tolerance. BSO has been previously shown to reduce synthesis of phytochelatins and phytochelatin-associated Cd^{2+} tolerance in plant cell cultures (Steffens, 1990). The BSO-dependent reduction in Cd^{2+} tolerance of *TaPCS1* expressing *S.cerevisiae* cells (Figure 6A) demonstrated a role for glutathione biosynthesis on *TaPCS1*-mediated Cd^{2+} resistance. In contrast, control cells overexpressing metallothioneins showed no BSO sensitivity and less Cd^{2+} resistance (Figure 6A).

Subsequently, HPLC analysis of monobromobimane-labeled extracts from Cd^{2+}-treated wild-type *S.pombe* showed the expected peaks for PC2 and PC3, the dominant phytochelatins of fission yeast (Kondo *et al*., 1985) (Figure 6B, top, peaks 1 and 2). PC2 and PC3 were undetectable in extracts of Cd^{2+}-treated *ΔSpPCS* cells (Figure 6B, bottom). Correspondingly, no PCS enzyme activity was detectable in protein extracts of the knockout strain (Figure 7A, bottom), thereby establishing a role for *SpPCS* in phytochelatin synthesis. Furthermore, extracts of *S.cerevisiae* control cells did not show formation of phytochelatin peaks in the present study (Figure 6C, bottom). Note that the *S.cerevisiae* genome contains no PCS homologs (Mewes *et al*., 1997). In contrast, *TaPCS1* expressing *S.cerevisiae* cells formed PC2 and PC3 upon Cd^{2+} exposure (Figure 6C, top). Thus, *TaPCS1* expression is concluded to be sufficient for phytochelatin synthesis from glutathione in an organism whose genome does not contain a *PCS* homolog (Mewes *et al*., 1997). *Saccharomyces cerevisiae* has been reported to express only limited quantities of exclusively PC2 (Kneer *et al*., 1992), an activity which clearly differs from that observed in *TaPCS1*-expressing cells, and which mediates PC2 and PC3 synthesis. To obtain more evidence for a direct catalysis of phytochelatin synthesis by the PCS proteins, we used the *ΔSpPCS* strain as a null background for the expression and purification of a tagged version of SpPCS. Phytochelatin synthesis from glutathione was detectable in fractions eluted from a HA-antibody affinity matrix that contained no detectable protein other than the HA-tagged SpPCS and an apparent degradation product (Figure 7). These data show the direct catalysis of phytochelatin synthesis by the PCS proteins. On the basis of these results, we conclude that the *PCS* genes encode phytochelatin synthases.

Phytochelatin synthase was previously reported by Grill *et al*. (1989) to be a 95 kDa tetramer. The predicted molecular mass of the PCS proteins described here lies in range of 46–55 kDa. We cannot rule out that the PCS genes isolated here encode catalytic subunits of a multimeric phytochelatin synthase.

Phytochelatins are involved in metal tolerance in vivo

Our data on the metal sensitivity of the *ΔSpPCS* strain provide molecular evidence for the model that phytochelatins play a central role in metal detoxification in plants and *S.pombe*. Furthermore, we show that lack of phytochelatin synthesis also leads to Cu hypersensitivity. This provides evidence for a more general role of phytochelatins in metal homeostasis, as was suggested earlier (Rauser, 1990) and indicated by the finding that phytochelatin–metal complexes can activate metal-depleted apoenzymes *in vitro* (Thumann *et al*., 1991).

Consistent with the reported constitutive activity of phytochelatin synthase (Grill *et al*., 1989) in roots and stems (Chen *et al*., 1997) and the suggested requirement for organisms to express metal tolerance genes constitutively (Zenk, 1996), *TaPCS1* and *AtPCS1* message were detected in roots and shoots of non-metal-stressed wheat and *Arabidopsis* plants, respectively. Furthermore, competitive PCR experiments using Cd^{2+}-treated wheat roots and *TaPCS1* show metal-induced up-regulation of *PCS* mRNA levels (Figure 8). Cd^{2+}-induced increases in PCS activity have been previously reported by Chen *et al*. (1997) for tomato cell lines.

Heterologous expression of PCS genes is sufficient to enhance metal tolerance

In the models proposed for phytochelatin-mediated Cd^{2+} complexation (Ortiz *et al*., 1995; Rauser, 1995), phytochelatins function as cytosolic chelators and carriers of Cd^{2+} ions by forming low molecular weight complexes which are then transported into the vacuole by transporters such as the ABC-type transporter HMT1 in *S.pombe* (Ortiz *et al*., 1992). Inside the vacuole more Cd^{2+} and sulfide are added to the complex to produce the high molecular weight complexes which are believed to represent the sequestered form of Cd^{2+}.

The cloning of *TaPCS1* and growth assays with *S.cerevisiae* cells expressing *TaPCS1*, *AtPCS1* and *SpPCS* demonstrated that phytochelatin synthesis alone can significantly increase cellular Cd^{2+} tolerance (Figures 1 and 3). Taken together with the evidence for vacuole-independent

TaPCS1-mediated Cd^{2+} tolerance obtained from experiments with the *Δvps18* mutant (Figure 4), this indicates that phytochelatins represent a significant cytosolic buffer for metal ions. These data and the unexpected finding of two PCS homologs in the *C.elegans* genome raise the possibility that phytochelatin synthase overexpression could be successfully used to increase the metal tolerance of diverse organisms. Furthermore, transgenic PCS expression could be useful for enhancing the removal of toxic metals by plants and other organisms for bioremediation.

In conclusion, we have isolated a new family of metal tolerance genes from different organisms that encode phytochelatin synthases, as was demonstrated by glutathione dependence and by showing phytochelatin synthesis deficiency in a *ΔSpPCS* strain, phytochelatin synthesis in *S.cerevisiae* cells expressing *TaPCS1* and phytochelatin synthase activity of purified recombinant SpPCS. The presented data provide molecular evidence for the model that phytochelatins play a crucial role in metal tolerance (Grill *et al*., 1985; Howden *et al*., 1995; Zenk, 1996). The effects of *TaPCS1* expression in *S.cerevisiae* on Cd^{2+} tolerance show that heterologous expression of *PCS* genes can dramatically enhance metal tolerance. Future research in transgenic plants and other organisms will allow testing of the potential of *PCS* genes for toxic metal sequestration, metal detoxification and bioremediation.

Materials and methods

Yeast cultures, transformation and growth assays

The *S.cerevisiae* strains CY162 (*MATα ura3-52 trk1Δ his3Δ200 his4-15 trk2Δ1::pCK64*) (Anderson *et al*., 1992), INVSc1 (*MATα his3Δ1 leu2 trp1-289 ura3-62*), SEY6210 (*MATα leu2-3, 112 Ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*), Δvps-18 (Robinson *et al.*, 1991) and Δvma4 (Ho *et al.*, 1993), and the *S.pombe* strains FY254 (*h⁻ ade6-M210 leu1-32 ura4-Δ18 can1-1*) and FY261 (*h⁺ ade6-M216 leu1-32 ura4-Δ18 can1-1*), were used in this study. *Saccharomyces cerevisiae* cells were grown in yeast nitrogen base (YNB) or arginine-phosphate medium (Rodriguez-Navarro and Ramos, 1984) supplemented with the appropriate amino acids, *S.pombe* cells were grown in yeast extract medium (YE) or Edinburgh's minimal medium (EMM) (Nurse, 1975; Moreno *et al*., 1991), supplemented appropriately. Growth assays with *S.cerevisiae* were performed as described previously (Clemens *et al*., 1998). Growth of *S.pombe* in the presence of different Cd^{2+} concentrations was assayed on EMM plates and in EMM liquid medium.

Library screening

CY162 cells (Anderson *et al*., 1992) were transformed with a size-selected (>1.5 kb) fraction of a yeast expression library constructed using mRNA from root tips of wheat seedlings (Schachtman and Schroeder, 1994) following the lithium acetate method (Gietz and Schiestl, 1996). Transformants were first selected for uracil prototrophy on YNB-ura, then were transferred to arginine-phosphate liquid medium containing either 20 or 50 μM $CdCl_2$. After 2–4 days DNA was extracted from the saturated cultures and *E.coli* cells were transformed. Per flask, several colonies were analyzed by restriction digests and sequencing.

DNA manipulations

Escherichia coli strain DH5α was used for all DNA manipulations. Genes were expressed in *S.cerevisiae* using the inducible expression vector pYES2 (Invitrogen, Carlsbad, CA) or the constitutive expression vector pYX132 (R & D Systems, Abingdon, UK). DNA sequencing was performed on an ABI 370 automatic sequencer or using Sequenase (USB). PCR and Southern analysis were performed following established procedures (Ausubel *et al*., 1987). Homologous sequences were identified by searching within the DDBJ/EMBL/GenBank database using BLAST (Altschul *et al*., 1990). Amino acid sequences were analyzed with TMPred (Hofmann and Stoffel, 1993) for the presence of putative transmembrane spans. Alignments were performed using the CLUSTAL W multiple sequence alignment program (Thompson *et al*., 1994).

Expression analysis

Northern analysis of RNA from wheat and *Arabidopsis* plants cells grown in the presence and absence of Cd^{2+} was performed according to established procedures (Ausubel *et al*., 1987). For RT–PCR RNA was isolated from 4-day-old wheat plants that were either untreated or treated with 100 μM Cd^{2+} for 6 h. RNA was isolated from roots and shoots separately. First-strand cDNA was made from these RNA samples using the cDNA Cycle Kit (Invitrogen). Ten nanograms of cDNA were used per PCR. For competitive PCR we cloned a PCR fragment amplified from wheat genomic DNA using the same primer pair as for the RT–PCR. Due to the presence of an intron this fragment is ~100 bp longer than the fragment amplified from cDNA and was used as a competitor in PCRs. Competitor DNA was added to the PCR in varying amounts between 0.1 and 5 fg. The PCR was performed in 32 cycles of 30 s 94°C, 2 min 55°C, 1 min 72°C. PCR products were analyzed by agarose gel electrophoresis.

Cd^{2+} accumulation

Saccharomyces cerevisiae cells were grown in arginine-phosphate medium for 24 h in the presence of different amounts of $CdCl_2$ containing 0.5 μCi $^{109}Cd^{2+}$. Cells were harvested and washed and radioactivity was determined as described previously (Clemens *et al*., 1998).

Schizosaccharomyces pombe knockout

The internal *Xba*I–*Hin*dIII fragment of *SpPCS* was subcloned into pYES2. By site-directed mutagenesis a *Bam*HI site and a *Sac*I site were introduced into this construct. A *Bam*HI–*Sac*I fragment of the ura4 marker in pTZura was cloned into the mutated *SpPCS* construct. *Schizosaccharomyces pombe* strain FY254 was transformed with 0.5 μg of the linearized knockout construct using the LiAc procedure (Okazaki *et al*., 1990). Transformants were selected on EMM with all the required supplements omitting ura. Twenty-five transformants were selected and analyzed by Southern blotting for a disruption of *SpPCS*. Transformants with a disruption of the *SpPCS* gene were identified by the appearance of a second band due to the *Eco*RV site in the *ura4* gene. Non-homologous insertion of the knockout construct led to a third band hybridizing with the *SpPCS* probe.

Phytochelatin assay

Phytochelatins were assayed essentially as described (Fahey and Newton, 1987). Briefly, *S.pombe* and *S.cerevisiae* cells were grown to mid-log phase (in EMM-ura, YNB-ura, respectively) and treated with 100 μM Cd^{2+}. Six hours after Cd^{2+} addition, cells were harvested and lyophilized. One to five milligrams of the lyophilized material were extracted in 0.1% trifluoroacetic acid, centrifuged and the supernatant derivatized with monobromobimane at 45°C in the dark. Extracts were separated by HPLC on a C18 column (3 μM, 150 mm) using an acetonitrile gradient. SH-containing compounds were detected fluorimetrically. For the identification of phytochelatins, $(\gamma\text{-EC})_2G$ (=PC2), $(\gamma\text{-EC})_3G$ (=PC3) and $(\gamma\text{-EC})_4$ (=PC4) standards were synthesized on an Abimed (Langenfeld, Germany) Economy Peptide Synthesizer EPS 211 using N-α-Fmoc-L-glutamic acid α-butyl ester (Novabiochem, Läufelfingen, Switzerland).

Protein extraction from S.pombe

Protein was extracted from *S.pombe* cultures grown to mid-log phase in EMM essentially as described (Hayashi *et al*., 1991). In brief, cells were harvested by centrifugation. The cell pellet was frozen in liquid N_2 and ground in a chilled mortar with three volumes of quartz sand. Following extraction with 50 mM Tris–Cl pH 8.0, 10% glycerol, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin and centrifugation at 10 000 *g* for 15 min, ammonium sulfate was added to the supernatant to 75% saturation. After 30 min the precipitate was collected by centrifugation at 18 000 *g* for 15 min and dissolved in 25 mM Tris–Cl (pH 8.0), 10% glycerol and 1 mM DTT.

Phytochelatin synthase assay

Aliquots of crude extracts or column fractions were incubated in 200 mM Tris–Cl (pH 8.0), 1 mM DTT, 1 mM glutathione (total volume 100 μl). The assay mixtures were kept on ice for 5 min. $CdCl_2$ was added to a final concentration of 0.1 mM and the samples were incubated at 35°C for 30–120 min. At the end of the incubation 50 μl aliquots were taken and TFA was added to a concentration of 5%. Following a 10 min incubation on ice and 10 min centrifugation at 13 000 *g* aliquots of the supernatant were derivatized with monobromobimane and analyzed by HPLC as described above.

Purification of HA-tagged SpPCS

SpPCS was subcloned into pSGP73 to express SpPCS protein with an N-terminal HA-tag in the knockout strain. The crude extract from a 200 ml culture of SpPCS-HA-expressing cells grown to mid-log phase in EMM without leucine and uracil was incubated with 600 µl of HA-monoclonal antibody affinity matrix slurry (BAbCo, Berkeley, CA) at 4°C and under gentle shaking. After 3 h the mix was transferred to a column and the matrix allowed to settle. Subsequently, the column was washed with 20 ml 50 mM Tris–Cl pH 8.0, 10% glycerol, 150 mM NaCl, 1 mM DTT. HA-tagged protein was eluted at 30°C with 5 mg HA peptide dissolved in 5 ml wash buffer. Protein fractions were analyzed by SDS–PAGE and silver staining, and Western blotting following established procedures (Ausubel *et al*., 1987).

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Independent of the findings reported here, two other papers describing the molecular characterization of the PC synthases are soon to be published: Ha *et al*. (*Plant Cell* 1999, in press) and Vatamaniuk *et al*. (*Proc. Natl Acad. Sci. USA* 1999, in press).