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Expression of Tobacco cDNA Encoding Phytochelatin Synthase Promotes Tolerance to and Accumulation of Cd and As in *Saccharomyces cerevisiae*

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The first tobacco cDNA encoding phytochelatin synthase (NtPCS1) has been cloned by complementing the YCF1 (vacuolar ABC type transporter)-depleting yeast mutant DTY167 with an expression library from *Nicotiana tabacum*. When NtPCS1 was over-expressed in DTY165 (WT) and DTY167 (mutant), tolerance to and the accumulation of cadmium (Cd) were enhanced. Interestingly, its expression promoted these responses as well to arsenic (As), but only in DTY167. We conclude that *NtPCS1* plays a role in tolerance to and the accumulation of both toxic metals in *Saccharomyces cerevisiae*.

Keywords: accumulation, arsenite, cadmium, *Nicotiana tabacum* phytochelatin synthase, *Saccharomyces cerevisiae*, tolerance

Phytochelatins (PCs) constitute a family of peptides with the general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n=2 to 11. PCs contain a large percentage of Cys sulfhydryl residues, which bind and sequester heavy-metal ions, such as Cd^{2+}, with high affinity in stable complexes. PCs localize together with Cd^{2+} to the vacuoles of intact cells and contribute most markedly to cadmium detoxification in plants (Vogeli-Lange et al., 1990). They are structurally related to glutathione (GSH). Induction of PCs in the presence of Cd coincides with a transient decrease in GSH levels. Glutathione is synthesized from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions catalyzed by γ-glutamyl-Cys synthetase (γ-GCS) and glutathione synthetase (GS). PC synthase (γ-glutamylcysteine dipeptidyl transpeptidases; EC 2.3.2.15) subsequently catalyzes the elongation of $(\gamma\text{-Glu-Cys})_n$ by transferring a γ-glu-Cys group to glutathione or to the PCs (Zenk, 1996; Chen et al., 1997).

Arsenic (As) is a toxic element ubiquitous in the environment and in organisms (Cullen and Reimer, 1989). In bacteria, one widespread tolerance mechanism for arsenate ($As^{V}O_4^{3-}$) is based on an efflux system that exports arsenic specifically and ATP-dependently as arsenite ($As^{III}O_2^{-}$) from the cells, a process generated by cytosolic reduction (Silver, 1996). Plants are exposed to arsenical compounds. mainly in the form of arsenite or arsenate, which are readily taken up. Both anions trigger the formation of PCs (Grill et al., 1987; Maitani et al., 1996). Identification of As-induced PCs and the formation of As-PC complexes indicate that PCs play a role in detoxifying arsenic in plants (Schmöger et al., 2000). Tolerance to As is elevated in WT plants and the DTY167 mutant of wheat (*Triticum aestivum*) when PCS1 (*TaPCS1*) is expressed (Wysocki et al., 2003). In addition, the expression of *Arabidopsis thaliana PCS1* (*AtPCS1*) increases As tolerance in DTY167 (Vatamaniuk et al., 1999). Arsenic levels also are elevated in *AtPCS*-expressing *Escherichia coli* grown in 20 M arsenate (Sauge-Merle et al., 2003).

Cadmium tolerance is also improved by the expression of PCS1 genes from *Arabidopsis thaliana* (Vatamaniuk et al., 1999; Ha et al., 1999) and *T. aestivum* (Clemens et al., 1999) in yeast cells. In addition, the expression of *AtPCS1* confers Cd tolerance to *E. coli* (Sauge-Merle et al., 2003). Likewise, expression of *TaPCS1* (Clemens et al., 1999) and *AtPCS1* (Sauge-Merle et al., 2003) promotes the accumulation of Cd in *Saccharomyces cerevisiae* and *E. coli*, respectively.

With the goal of identifying the plant genes involved in resistance to heavy metals, we prepared a *Nicotiana tabacum* cDNA library in a yeast expression vector and isolated a gene from tobacco that encodes a protein whose heterologous expression in *S. cerevisiae* confers Cd tolerance. Here, we report the clo-

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ning of a cDNA (*NtPCS1*) from *N. tabacum* encoding a 55-kDa protein with homology to PCS genes from soybean, *Arabidopsis*, and wheat. Its expression enhances tolerance to and the accumulation of cadmium and arsenic in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast Strains and Plant Materials

Our *S. cerevisiae* strains included the wild-type DTY165 (MATα ura3-52 leu2-3,-112 his-Δ200 trp1-Δ901 lys2-801 suc2-Δ9) and the Δycf1 mutant DTY167 (MATα ura3-52 leu2-3,-112 his-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ycf1::hisG) defective in vacuolar Cd^{2+} sequestration (Li et al., 1996). *N. tabacum* suspension callus cells (BY2) were the RNA source for constructing our tobacco cDNA expression library. Genomic DNA from *N. tabacum* cv. SR1 was used for the Southern blot analyses.

Construction of the Yeast Expression Vector and the Expression Library

To determine the constitutive expression of plant genes in *S. cerevisiae*, we constructed a tobacco cDNA library in the yeast-*E. coli* shuttle vector pYES5. In that derivative of pYES2 (Invitrogen, USA), the galactose-inducible yeast GAL1 gene promoter is replaced by the constitutive 3-phosphoglycelate kinase (PGK) gene promoter (Lu et al., 1997).

Total RNA was extracted from tobacco suspension culture cells treated for 30 min with 20 μM $CdSO_4$, following the TRI-reagent (Molecular Research Center, USA) extraction method. Tobacco cDNA was synthesized with a cDNA synthesis kit (Stratagene, USA). The cDNAs were then separated by size via Sepharose CL-2B gel filtration; only RNA larger than 0.4 kb was recovered. The synthetic cDNA (1 μg) was ligated with an approximately stoichiometric amount of the pYES5 vector that was double-digested with *Eco*RI and *Xho*I, using T4 DNA ligase (Takara, Japan). After transforming the *E. coli* ($RecA^-$ strain XL-1-Blue MRF') with our tobacco cDNA library via electroporation, we obtained 1.1×10^6 clones, which were then pooled together. Aliquots were frozen at -80°C and the remainder was used to prepare plasmid DNA.

Screening for Selection of Cd^{2+}-Tolerant Colonies

DTY167 cells (Li et al., 1996) were transformed with a yeast expression library, according to the LiOAc/polyethylene glycol method (Gietz and Schiestl, 1995). Transformants were first selected for uracil prototrophy by growing them on complete minimal (CM) media that comprised a uracil-lacking, complete supplement mixture (CSM) (QBiogene, Canada) and a yeast nitrogen base (YNB) (QBiogene). Cd^{2+}-tolerant transformants were selected by growing them for 3 to 4 d at 30°C on CM agar media containing 200 μM $CdSO_4$.

DNA Manipulations

E. coli strain DH5α was used for plasmid manipulations. The DNA was sequenced with either an ABI 377 automatic sequencer or Sequenase (USB, USA). Homologous sequences were identified by searching the DDBJ/EMBL/GenBank database with BLAST. Alignment was performed using the CLUSTAL W multiple sequence alignment program (Thompson et al., 1994).

Southern Blot Analysis

Genomic DNA was prepared from tobacco leaves by the CTAB method (Scott and Arnold, 1988). Ten μg was digested with restriction enzymes, separated on a 0.8% agarose gel, and transferred to a positive nylon membrane (QBiogene). The membrane was hybridized with a ^{32}P-labeled 0.7-kb *Bst*X1 restriction fragment that corresponded to the coding sequence of *NtPCS1*, using the random priming method (Roche, Switzerland). After hybridization, the membrane was washed twice, at 50°C, in 2.0× SSC, 0.1% SDS and 0.2× SSC, 0.1% SDS before being autoradiographed.

Measurement of Metal Tolerance

S. cerevisiae cells were grown for 16 h (up to the late-log phase) in CM liquid medium, and cell density was adjusted to OD_{600} 0.1 with CM medium. These adjusted cells were then serially diluted with CM medium and grown on metal-containing CM agar medium for 4 d.

Assessment of Heavy Metals

Cells of *S. cerevisiae* were grown in a liquid CM medium for 24 h (initial OD_{600} of 0.1) in the presence of either 20 μM $CdSO_4$ or 10 μM $NaAsO_2$. They were then harvested, washed three times with the CM medium, and dried for 3 d at 60°C. Afterward, 1.0 g of the dried cells was digested with concen-

trated HNO_3 and $HClO_4$ in a Teflon Digestion Vessel (Savillex, USA). Metal contents were measured three times by ICP-AES (Perkin Elmer Optima 4300 DV, USA) at wavelengths of 228.80 nm (for Cd) and 188.98 nm (for As), at the Korean Basic Science Institute (KBSI). Three independent cultures were used for calculating the average metal concentrations.

RESULTS AND DISCUSSION

Cloning and Characterization of Tobacco cDNA Encoding PCS

To clone the plant genes that function in cadmium tolerance, cells of the Cd-sensitive *S. cerevisiae* Δycf1 mutant DTY167 were transformed with a tobacco

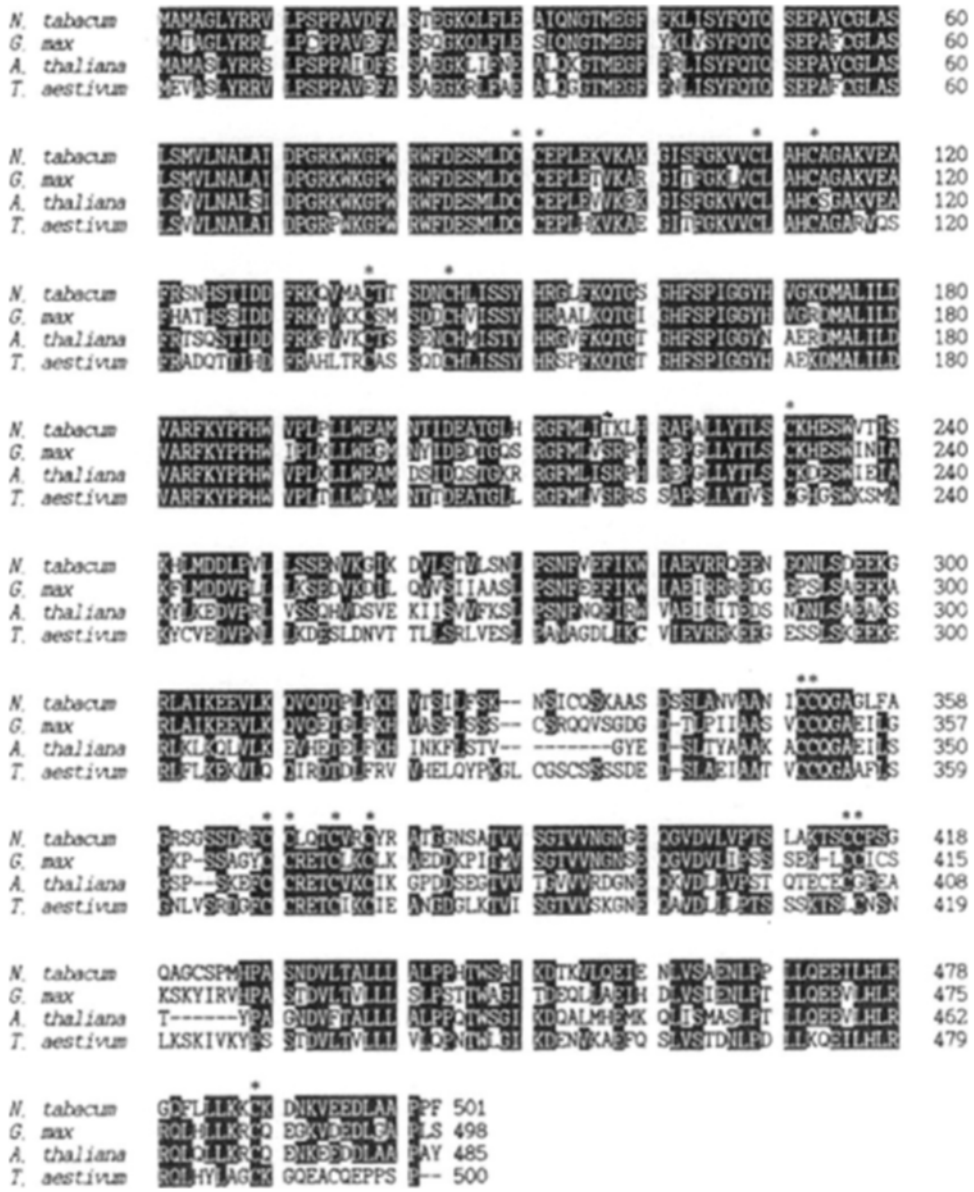

Figure 1. Comparison of tobacco PCS (*N. tabacum*, Accession No. AY235426) amino acid sequence with those of PCS genes from soybean (*Glycine max*, Accession No. AF411075), *A. thaliana* (Accession No. AF085230), and wheat (*T. aestivum*, Accession No. AF093752). Sequences were aligned by CLUSTAL W method (Thompson et al., 1994). Numbers indicate amino acid residues in sequence. Gaps in alignment are designated by dashes. Cys residues conserved across four sequences are highlighted with asterisk.

cDNA expression library constructed in the yeast vector pYES5. The yeast cadmium (resistance) factor gene *YCF1* encodes an ATP-binding cassette transporter responsible for vacuolar sequestration of the Cd-GS complex (Li et al., 1997). After screening the transformants on uracil-lacking CM media containing 200 μM $CdSO_4$, on which DTY167 normally cannot survive, we identified three growing colonies. Plasmid DNA was isolated from each of the surviving yeast groups, and their cDNA inserts were sequenced. All were identical, differing only in the lengths of their 5′ untranslated regions. One of the cDNA sequences was 2145 b long, and consisted of 23 b of a 5′-untranslated region, 1503 b of an open reading frame, and 619 b of a 3′-untranslated region. Because this ORF has 64% homology to the PCS1 gene that encodes a phytochelatin synthase (PCS) from soybean, we named it *NtPCS1* (DDBJ/EMBL/GenBank Accession No. AY235426).

The expected molecular mass of the NtPCS1 protein was about 55.0 kDa, based on the size of the ORF. Its deduced amino acid sequence was similar to PCS1 from soybean (Accession No. AF411075) (Oven et al., 2002), *Arabidopsis* (Accession No. AF085230) (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999), and wheat (Accession No. AF093752), showing homologies of 64, 59, and 56%, respectively (Fig. 1). Comparison of the plant PCS amino acid sequences revealed that their N-terminal regions were very similar, whereas the C-terminals were more varied (Fig. 1). One model for the functioning of the PCS enzyme is that the conserved N-terminal domains possess catalytic activity. Activation probably arises from metal ions that interact with residues, possibly of Cys and His, in this domain. In fact, five Cys (two of which were adjacent) and four His residues were conserved in the N-terminal domain (Fig. 1). This model is also supported by the molecular characterization of mutant cad1-5 alleles (Ha et al., 1999). The C-terminal domain is not absolutely required for either catalysis or activation (Howden et al., 1995).

Our tobacco genomic DNA was single-digested with three restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII), which do not have digestion sites in the *NtPCS1* fragment used as a template in preparing the ^{32}P-labeled probe. When they were hybridized with the *NtPCS1* cDNA probe (0.7 kb), two bands were visible for each digestion (Fig. 2). This implies that two gene copies or homologues exist per tobacco genome. Low-stringency DNA gel blot analysis by Vatamaniuk et al. (1999) in *Arabidopsis* also suggests the presence of more than one *AtPCS* homolog,

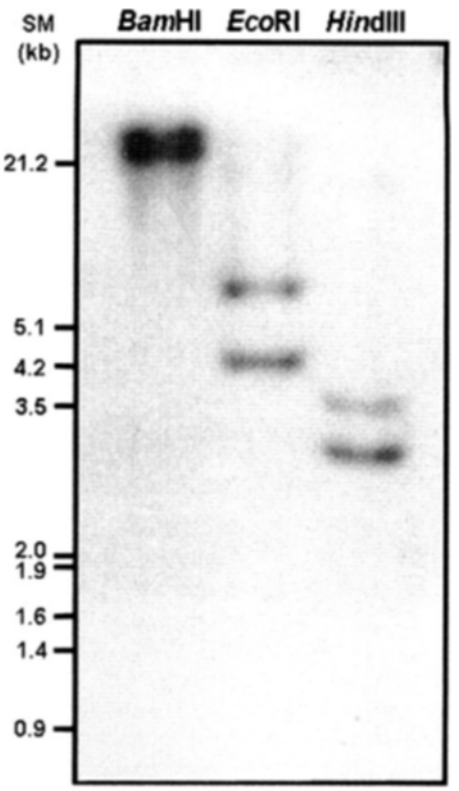

Figure 2. Southern blot analysis of PCS genes in tobacco genome. Genomic DNA (10 ug) was digested with indicated restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII), then separated on 0.8% agarose gel, transferred to nylon membrane, hybridized with radio-labeled, 682-bp internal *NtPCS1* cDNA fragment, and washed under high stringency conditions. Size markers are indicated in kbp.

based on probing with the coding sequence for *AtPCS1*. A second *Arabidopsis* gene (*AtPCS2*, GenBank Accession No. AC003027) has now been sequenced as well (Cazale and Clemens, 2001).

Expression of NtPCS1 Increases the Capacity to Tolerate and Accumulate Cadmium in *S. cerevisiae*

To ascertain the function of NtPCS1 in tolerating and accumulating heavy metals, *NtPCS1* was overexpressed in wild type DTY165 and the ycf-deficient mutant DTY167 of *S. cerevisiae*. Its expression in the former increased tolerance to Cd when transformed cells were grown on the agar medium containing 200 μM $CdSO_4$ (Fig. 4A). In addition, Cd tolerance was conferred to the cadmium-sensitive mutant DTY167 (Fig. 4B). Cd tolerance was observed even on the medium that included 1 mM Cd^{2+} (data not shown).

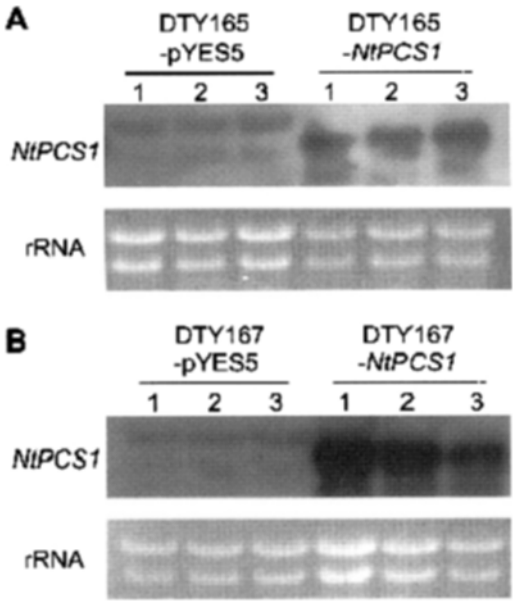

Figure 3. Expression of *NtPCS1* at mRNA level in NtPCS1-expressing DTY165 and DTY167 cells. Twenty μg of total RNA from DTY165/pYES5 or DTY167/pYES5, and DTY165/NtPCS1 or DTY167/NtPCS1, were electrophoresed, blotted, and hybridized with *NtPCS1* cDNA probe. Numbers 1, 2, and 3 indicate different yeast lines introduced with pYES5 or pYES5-NtPCS1.

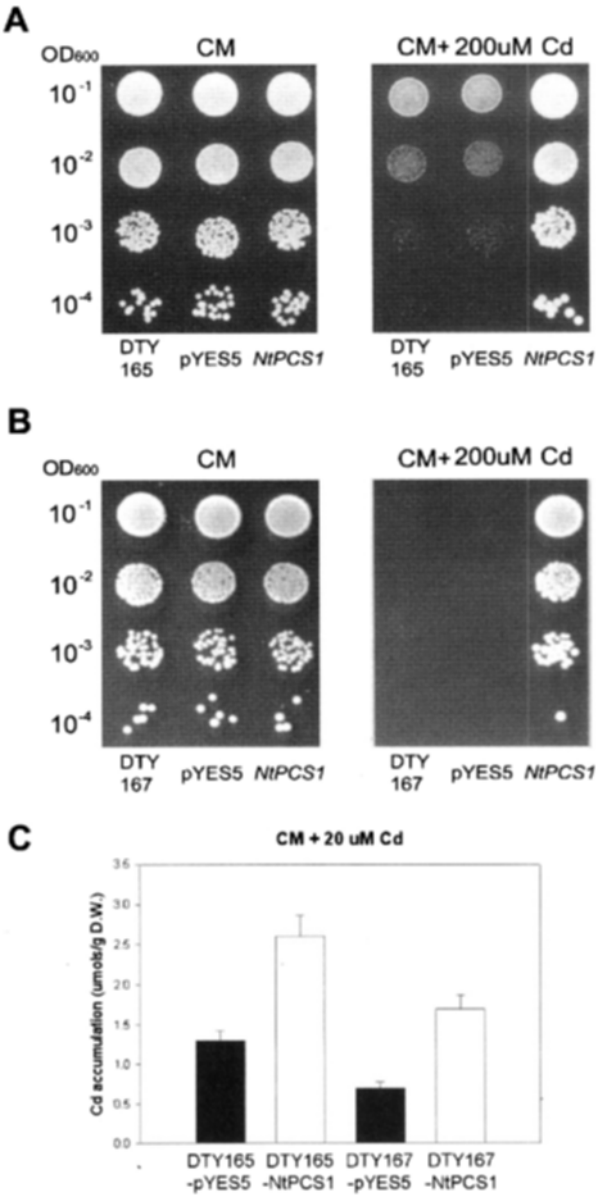

Figure 4. NtPCS1 increases Cd tolerance and accumulation in DTY165 (WT) and DTY167 yeasts. Growth was compared among control cells (DTY165 and DTY167), cells carrying empty pYES5 plasmid, and cells expressing NtPCS1 on CM media without (left) or with (right) 200 μM Cd^{2+} (**A** and **B**). Cells were diluted serially and cultured for 4 d at 30°C. NtPCS1-expressing cells accumulated more Cd^{2+} than control cells (**C**). Cells were grown for 24 h in liquid CM media with 20 μM $CdSO_4$, and their Cd concentrations were determined by ICP-AES. Three independent cultures were used for determining Cd concentration. Error bars indicate SE, n=3.

Tolerance to this metal is also enhanced by the expression of PCS1 genes from *A. thaliana* (Ha et al., 1999; Vatamaniuk et al., 1999) and *T. aestivum* (Clemens et al., 1999) in yeasts. In addition, the expression of *AtPCS1* from *A. thaliana* confers Cd tolerance to *E. coli* (Sauge-Merle et al., 2003).

We also examined the effect of *NtPCS1* expression on cadmium accumulation. When DTY165 and DTY167, both of which express this gene, were cultured in a liquid medium containing 20 μM $CdSO_4$, each showed an increase of greater than two-fold in their levels of cellular Cd compared with DTY165 and DTY167 that contained the control vector (Fig. 4C). DTY165 accumulated more Cd than did DTY167 (1.3 versus 0.9 μmol g^{-1} DW) because the latter lacks the vacuolar ABC transporter involved in sequestering Cd-glutathione complexes. Previous research has shown that expression of *TaPCS1* (Clemens et al., 1999) and *AtPCS1* (Sauge-Merle et al., 2003) also promote Cd accumulations in *S. cerevisiae* and *E. coli*, respectively.

Therefore, all our results strongly suggest that NtPCS1 plays a role in promoting Cd^{2+} tolerance and accumulation in *S. cerevisiae*. These increases, induced by *NtPCS1* expression, imply that the tolerance phenotype is not due to the exclusion of the toxic metal, i.e., the dominant mechanism for metal detoxification in bacteria (Silver and Phung, 1996), but rather

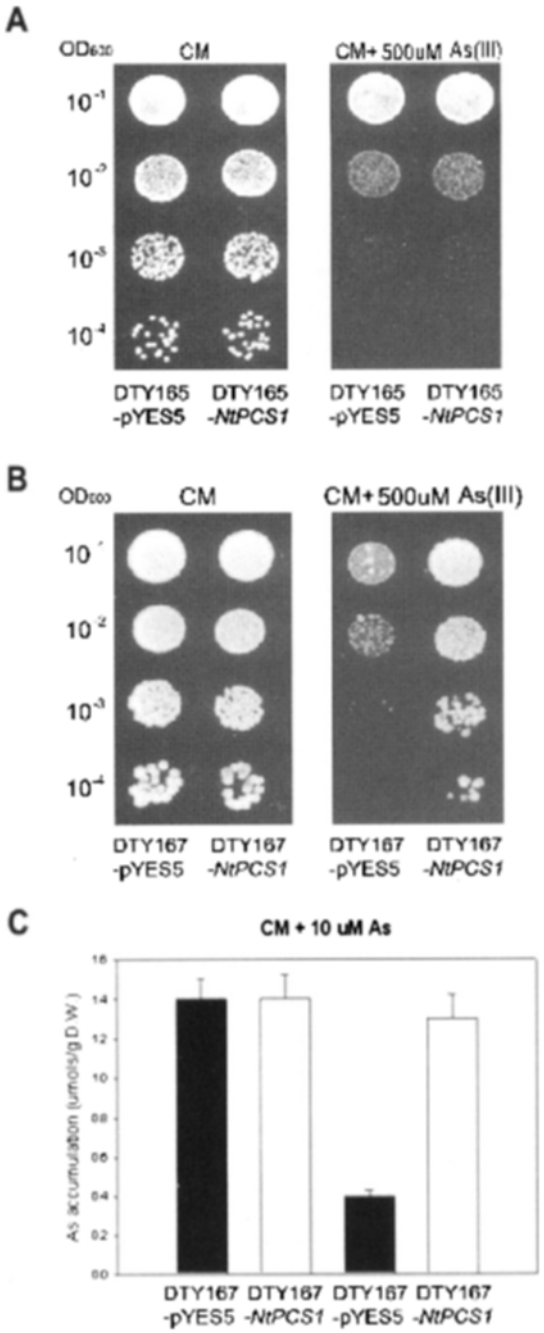

Figure 5. NtPCS1 expression elevates As tolerance and accumulation in yeast mutant DTY167. Growth was compared among control cells (DTY165 and DTY167), cells carrying empty pYES5 plasmid, and cells expressing NtPCS1 on CM media without (left) or with 500 μM arsenite (As^{III}) (right) (**A** and **B**). Cells were diluted serially and cultured for 4 d at 30°C. NtPCS1-expressing DTY167 cells accumulated more As than DTY167 cells (**C**). To measure As level in yeasts, cells were grown for 24 h in liquid CM media with 10 μM arsenite, and their As concentrations were determined by ICP-AES. Three independent cultures were used for determining As concentration. Error bars indicate SE, n=3.

because of increased detoxification. We can infer that expressed NtPCS1 plays a role in detoxifying Cd in the cytoplasm or sequestering Cd into the vacuoles. Although PC has been shown to sequester Cd^{2+} into the vacuoles via the ABC transporter hmt1 in *Schizosaccharomyces pombe* (Ortiz et al., 1992), this has not been proven in *S. cerevisiae*. Our observations of increases in both Cd accumulation and tolerance strongly suggest that the phytochelatin, synthesized through the expression of *NtPCS1*, functions in detoxifying cadmium, although this was not analyzed here. Therefore, the precise mechanism for this should be elucidated, with one candidate being the sequestration of the Cd-PC complex into vacuoles. Even though we examined *NtPCS1* expression at the mRNA level (Fig. 3A, B), it should still be confirmed that PC levels really are enhanced by such expression in yeast cells.

Expression of NtPCS1 Increases Tolerance to and the Accumulation of Arsenic in *S. cerevisiae* DTY167

Phytochelatin is involved in detoxifying arsenic in plants (Schmöger et al., 2000). Therefore, we also examined the role of NtPCS1 in tolerating and accumulating As in yeast cells. When *NtPCS1* was overexpressed in DTY 165, the tolerance level for arsenite was unaltered (Fig. 5A), whereas it was increased in the YCF1-lacking DTY167 (Fig. 5B). Arsenite tolerance is also elevated in both DTY165 and DTY167 when the PCS1 of *T. aestivum* (*TaPCS1*) is expressed (Wysocki et al., 2003). In addition, *AtPCS1* increases tolerance in DTY167 (Vatamaniuk et al., 1999).

When we over-expressed NtPCS1 in DTY165 and 167, the following patterns of As accumulation were found in cells grown in a liquid medium containing 10 μM arsenite (As^{III}). First, the YCF1-lacking DTY167 exhibited a much lower level of As than did DTY165 (0.4 versus 1.4 μmol g^{-1} DW), a result that reflected the hypersensitive phenotype of DTY167 on the agar medium that included 500 μM arsenite (Fig. 5B, C). Second, the arsenic level in DTY165 was not altered by *NtPCS1* expression (Fig. 5C), resulting in no change in its tolerance (Fig. 5A). Third, *NtPCS1*-expressing DTY167 showed elevated As tolerance (Fig. 5B) and accumulation, at levels up to that measured in the WT (Fig. 5C). Expression of *NtPCS1* did not change either the tolerance or the ability to accumulate As by DTY165; perhaps these yeast strains had reached their maximum capacities for arsenic accumulation because DTY165-pYES5, -NtPCS1, and

DTY167-NtPCS1 all showed similar levels (i.e., 1.3~1.4 umol g^{-1} DW). It is possible that the arsenite export pump Acr3p is involved in limiting the amount of As in *S. cerevisiae* (Wysocki et al., 1997). Although ours is the first report on the expression of plant PCS and enhanced As accumulation in yeasts, arsenic concentrations have been shown to rise in AtPCS-expressing *E. coli* when grown with 20 μM arsenate (Sauge-Merle et al., 2003). Therefore, even though we measured *NtPCS1* expression at the mRNA level (Fig. 3A, B), it still remains to be confirmed that the level of PC really is enhanced by the expression of *NtPCS1* in yeast cells.

In conclusion, we have isolated the first tobacco cDNA that encodes PCS1 (phytochelatin synthase) and increases tolerance to and the accumulation of cadmium and arsenic in yeast cells. Although we believe that the enhanced PCs play a role in these responses, their levels must still be measured. In addition, the mechanism by which PC functions in this way must be elucidated. Our findings, which are the first to report that plant PCS1 expression enhances the level of arsenic in yeasts, are also applicable for research to generate metal-tolerant plants or good phytoremediators.

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