A Functional Putative Phytochelatin Synthase from the Primitive Red Alga *Cyanidioschyzon merolae*

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**Key words:** cadmium; *Cyanidioschyzon merolae*; phytochelatin; phytochelatin synthase

Phytochelatin synthase (PCS) catalyzes the synthesis of phytochelatins (PCs), which play a detoxification role in higher plants. Heterologous expression of CmPCS, a product of a PCS-like gene from the genomic DNA of the red alga *Cyanidioschyzon merolae*, rescued Cd$^{2+}$-sensitive yeast from Cd$^{2+}$ toxicity. The fact that these transformed cells synthesized PCs demonstrates that CmPCS is functional.

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Phytochelatins (PCs) are nonproteinaceous peptides with the general structure ($\gamma$-Glu-Cys)$_n$-Gly, where $n \geq 2$. PCs play a role in heavy metal tolerance by forming complexes with toxic heavy metal ions. PC synthesis is mediated by phytochelatin synthase (PCS), the tripeptide glutathione ($\gamma$-Glu-Cys-Gly: GSH) and previously synthesized PCs acting as the substrate. The presence of a heavy metal is essential for synthesis to proceed. A range of metals belonging to groups 11 to 15 of the fourth, fifth, and sixth periods of the periodic table activate PCS, Cd$^{2+}$ being the most efficient activator.

Genes that encode PCS have been identified in wheat (*Triticum aestivum*), fission yeast (*Schizosaccharomyces pombe*), and Arabidopsis thaliana (*A. thaliana*). In addition to its identification in higher plants, PCS has also been identified in the animal kingdom. The worm Caenorhabditis elegans possesses a gene with high similarity to that of plant PCS, and the product is functional. The N-terminal portions of the PCSs (N domains) identified thus far are highly conserved. In contrast, the sequences of the C-terminal portions (C domains) are diverse, although they are rich in Cys residues, which form several motifs that are characteristic of metal-binding sites such as Cys-Xaa-Yaa-Cys, Cys-Xaa-Cys, and Cys-Cys, where Xaa and Yaa are noncysteine amino acids.

The cyanobacterium *Nostoc* sp. PCC 7122A has a PCS-like gene (NsPCS); the product is approximately half the size of most eukaryotic PCSs, has a primary sequence similar to that of the N domain in most eukaryotic PCSs, and very low, if any, PCS activity. A recent genome sequencing project detected the presence of a PCS-like sequence (CmPCS) in the red alga *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp/ or CM111C in KEGG/GenomeNet [http://www.genome.jp/]), a primitive photosynthetic eukaryote. Alignment of *A. thaliana* PCS (AtPCS1), CmPCS, and NsPCS showed that CmPCS is unique in that in addition to its N (residues 92–340) and C (residues 341–560) domains, which are common to eukaryotic PCSs (Fig. 1), the protein possesses an extra domain (X domain, residues 1–91) that extends outward from the N domain. It is therefore of interest to determine whether CmPCS is a functional PCS.

Strain 10D of *C. merolae* was obtained from the Microbial Culture Collection of the National Institute for Environmental Studies (Tsukuba, Japan). Modified Allen’s medium, containing 40 mM (NH$_4$)$_2$SO$_4$, 4 mM MgSO$_4$·7H$_2$O, 4 mM KH$_2$PO$_4$, 1 mM CaCl$_2$, 100 µM Fe-ethylenediaminetetraacetic acid, 100 µM H$_2$BO$_3$, 30 µM MnCl$_2$·4H$_2$O, 1.5 µM ZnCl$_2$, 4.5 µM Na$_2$MoO$_4$, 6.5 µM CoCl$_2$·6H$_2$O, and 0.6 µM CuCl$_2$, pH 2.8, was used. The cells were cultured phototrophically at 37°C under continuous illumination at 32 µmol photon m$^{-2}$ s$^{-1}$ with continuous shaking at 100 rpm.

We used the method of Sneller et al. to determine the types of PCSs present in the cells. After culture in medium containing 0, 10, 100, or 1,000 µM Cd$^{2+}$ for 3 d, algal cells were collected by centrifugation at 4,000 × g. A total of 1 ml of 0.1% trifluoracetic acid (TFA) containing 6.3 mM diethylthiainemethylenetricarboxylic acid (DTPA) was then added, followed by sonication to disrupt the cells. After centrifugation at 13,500 × g for 20 min, 450 µl of 200 mM HEPES/NaOH buffer, pH 8.2, containing 560 µM monobromobimane (mBBr), and 6.3 mM DTPA were added to 250 µl of the cell supernatant, and the thiol groups in the sample were derivatized with mBBr for 30 min at room temperature in the dark. The mBBr-derivatized PCs were then separated on an ODS-80T$_m$ C$_{18}$ column (4.6 mm ×
The column was equilibrated with 12% (v/v) methanol and 88% (v/v) water containing 0.1% (v/v) TFA. After injection of 20 μl of the derivatized sample, the mBBr-derivatized PCs were eluted using a linear gradient of 12–100% (v/v) methanol:water over 70 min. Emission at 470 nm was monitored using an excitation wavelength of 380 nm; the fluorescence intensities were calibrated using PC standards (Hayashi Kasei, Osaka, Japan). The cell density was based on OD$_{750}$, which was calibrated using a cell density determined using a hemocytometer.

PC synthesis in *C. merolae* was induced by Cd$^{2+}$, but no appreciable amount of PCs was found in cells cultured without Cd$^{2+}$ (Fig. 2). PC synthesis occurred in the presence of at least 10 μM Cd$^{2+}$, with a concomitant decrease in GSH in the cells, which is consistent with the synthesis of PCs at the expense of GSH, as observed in suspension cultures of the higher plant *Rauvolfia serpentina*. These results indicate that *C. merolae*

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**Fig. 1.** Comparison of the PCS Polypeptides from *Arabidopsis thaliana* (AtPCS1, AF135155) and *Cyanidioschyzon merolae* (CmPCS, CMI111C in KEGG/GenomeNet [http://www.genome.jp/]) and the PCS Derivatives from *Nostoc* sp. PCC 7122A (NsPCS, AP003584). Residues 1–91, 92–340, and 341–560 in CmPCS are referred to as the X, N, and C domains respectively. Asterisks indicate the residues in the catalytic triad. Residues that are conserved in at least two sequences are shown in white on a black background.

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**Fig. 2.** Glutathione and PC Concentrations in *Cyanidioschyzon merolae* Cells Cultured in the Presence of Various Concentrations of Cd$^{2+}$.
synthesizes, PCs, presumably in a manner similar to most other photosynthetic eukaryotes. Carboxypeptidase-mediated PC synthesis occurs in Saccharomyces cerevisiae. In C. merolae, however, the possibility that these enzymes are implicated in PC synthesis can be ruled out because trace levels of PC2 alone are synthesized in the yeast. Furthermore, the concentration ratio of PC2 to GSH is only <1% in S. cerevisiae, in contrast to PC synthesis in C. merolae, in which the ratio is >50%. The order (number of γ-Glu-Cys units) of the PCs synthesized was dependent on the concentration of Cd2+ in the medium. C. merolae cells cultured in 10 μM Cd2+ contained PC2 as the dominant species, followed by PC3 and PC4, whereas in cells cultured with 100 or 1,000 μM Cd2+, PC3 dominated, followed by PC2 and PC4. Given that higher-order PCs possess greater Cd2+-binding capacity, they can be synthesized to deal with higher levels of Cd2+.

Genomic DNA was extracted from a 30-ml culture of C. merolae cells grown for 7 d at 37°C. CmPCS was amplified from genomic DNA using a KODplus PCR kit (Toyobo, Osaka, Japan) and primers 5’-GAGAAAGCTTACCATGATCATTTGGCGTCCGTTGGTACGTTG-3’ (forward) and 5’-GAGAAATTCTACCTGTTGCTCCGTCTGTTGCTGGTGC-TTCCGCTTGTGCTGGCGCATCCA-3’ (reverse), which carry a HindIII and, an EcoRI site respectively. After the resulting fragment was ligated into pYES2 (Invitrogen, Carlsbad, CA) and transformed into the Escherichia coli XL-1 Blue strain (Stratagene, La Jolla, CA), the fidelity of the construct was confirmed by sequencing. The CmPCS gene was cloned into pYES2 vector and transformed into the yeast strain S. cerevisiae, which lacks the gene responsible for the transport of the biglutathionato-Cd2+ complex into the vacuole, was transformed with pYES2 + CmPCS. The transformed cells were precultured overnight, the cells were inoculated into 100 ml of SD medium containing Cd2+ solidified with 2% agarose, and the plates were incubated for 5 d at 30°C.

The ycf1∆ cells transformed with pYES2 + CmPCS showed no growth retardation at Cd2+ concentrations up to 100 μM (Fig. 3). This was in contrast with ycf1∆ cells transformed with only pYES2, which displayed severe growth inhibition at 50 μM Cd2+ and no apparent growth at 100 μM Cd2+. Hence we concluded that CmPCS is responsible for the amelioration of Cd2+ stress in ycf1∆ cells.

To determine whether the rescue of the Cd2+-hypersensitive mutant from Cd2+ stress resulted from the synthesis of PCs, the mutants were analyzed for PCs. ycf1∆ cells with and without CmPCS were cultured in SD medium at 30°C. After pre-culture overnight, the HPLC chromatogram indicates that the extract prepared from yeast cells transformed with pYES2 + CmPCS contained PCs. The mBBr-delivered PCs appeared at retention times of 30.3 min for PC2, 39.6 min for PC3, and 45.6 min for PC4 (upper chromatogram in Fig. 3B), but no mBBr-delivered PCs occurred in the cells were inoculated into 100 ml of SD medium to give an OD600 of 1.0, and were cultured for 24 h. Cd2+ was then added to the medium at a concentration of 100 μM, and the cells were cultured for a further 2 d. The cells were centrifuged and sonicated for 5 min, and the supernatant was obtained by centrifugation at 13,500 × g. The supernatant was analyzed for PC content, as described above.

The HPLC chromatogram indicates that the extract prepared from yeast cells transformed with pYES2 + CmPCS contained PCs. The mBBr-delivered PCs appeared at retention times of 30.3 min for PC2, 39.6 min for PC3, and 45.6 min for PC4 (upper chromatogram in Fig. 3B), but no mBBr-delivered PCs occurred in the
chromatogram of the extract prepared from yeast cells transformed with only pYES2 (lower chromatogram, Fig. 3B). Although confirmation of PCS activity is needed for purified CmPCS, these results strongly support the thesis that CmPCS encodes a functional PCS.

Detailed analyses of AtPCS1 have revealed the existence of a bisubstrate-substituted mechanism in which a γ-Glu-Cys acyl intermediate is formed in a metal-independent manner.19) A catalytic amino acid triad was identified that includes Cys-56, His-162, and Asp-180, the thiol group of Cys-56 acting as the acylation site. The γ-Glu-Cys dipeptide, which acylates the enzyme, is in turn transferred to GSH or to a previously formed PC. Also, it is likely that the C domain plays a role in sensing metal ions for the activation of AtPCS1, because partial truncation of the C domain reduces the activity of the enzyme 20 and alters its dependency on metal ions for activation.2) CmPCS has an equivalent catalytic triad (viz., Cys-164, His-283, and Asp-301; Fig. 1) and satisfies the enzyme acylation requirement, but it has a relatively small number of Cys residues in its C-terminal domain as compared with those of eukaryotic PCSs identified thus far. The X domain, which is specific to CmPCS and contains five scattered Cys residues (Fig. 1), might also be involved in metal sensing to activate the enzyme.

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References