J Ind Microbiol Biotechnol (2005) 32: 527–533
DOI 10.1007/s10295-005-0234-1

SPECIAL TOPIC: BIODIVERSITY

**Ruibin Dong · Elide Formentin · Carmen Losseso
Francesco Carimi · Piero Benedetti · Mario Terzi
Fiorella Lo Schiavo**

Molecular cloning and characterization of a phytochelatin synthase gene, *PvPCS1*, from *Pteris vittata* L.

Received: 25 August 2004 / Accepted: 15 April 2005 / Published online: 26 May 2005

Abstract *Pteris vittata* L. is a staggeringly efficient arsenic hyperaccumulator that has been shown to be capable of accumulating up to 23,000 μg arsenic g^{-1}, and thus represents a species that may fully exploit the adaptive potential of plants to toxic metals. However, the molecular mechanisms of adaptation to toxic metal tolerance and hyperaccumulation remain unknown, and *P. vittata* genes related to metal detoxification have not yet been identified. Here, we report the isolation of a full-length cDNA sequence encoding a phytochelatin synthase (PCS) from *P. vittata*. The cDNA, designated *PvPCS1*, predicts a protein of 512 amino acids with a molecular weight of 56.9 kDa. Homology analysis of the *PvPCS1* nucleotide sequence revealed that it has low identity with most known plant PCS genes except *AyPCS1*, and the homology is largely confined to two highly conserved regions near the 5′-end, where the similarity is as high as 85–95%. The amino acid sequence of *PvPCS1* contains two Cys-Cys motifs and 12 single Cys, only 4 of which (Cys-56, Cys-90/91, and Cys-109) in the N-terminal half of the protein are conserved in other known PCS polypeptides. When expressed in *Saccharomyces cerevisae*, *PvPCS1* mediated increased Cd tolerance. Cloning of the PCS gene from an arsenic hyperaccumulator may provide information that will help further our understanding of the genetic basis underlying toxic metal tolerance and hyperaccumulation.

R. Dong (✉) · E. Formentin · C. Losseso
F. Carimi · P. Benedetti · M. Terzi · F. L. Schiavo
Centro di Ricerca Interdipartimentale per le
Biotechnologie Innovative, Universita' di Padova,
Via G. Colombo 3, 35121 Padua, Italy
E-mail: ruibindong@hotmail.com

Present address: R. Dong
Department of Environmental Engineering,
College of Environmental Science and Engineering,
Nanchang University, No.339, East Beijing Rd.,
Nanchang 330029, P. R. China

Keywords *Pteris vittata* · Phytochelatin · Phytochelatin synthase · Arsenic hyperaccumulation · Heavy metal hypertolerance

Introduction

Pteris vittata L. is a fern recently identified as an arsenic hyperaccumulator because of its ability to accumulate arsenic [14] and its possession of three key features typical of plant hyperaccumulators: efficient root uptake, efficient root-to-shoot translocation, and a much-enhanced tolerance to arsenic within plant cells [30]. Inorganic arsenic species are generally highly phytotoxic, and plants exhibiting arsenic tolerance, and especially those with arsenic hyperaccumulation ability, are not common in nature. *P. vittata* plants can absorb exceptionally high quantities of arsenic without toxic effects. The plants transport 95% of the absorbed arsenic from roots to shoots, accumulating it in fronds in a staggeringly efficient way. This plant seems capable of fully exploiting the adaptive potential of plants to toxic metals. However, the molecular mechanisms underlying its toxic metal tolerance and metal detoxification ability remain unknown.

It is becoming increasingly clear that the general mechanism of heavy metal tolerance and homeostasis in plants is achieved by biosynthesis of heavy-metal-binding ligands and, in some cases, by subsequent compartmentalization of the ligand-metal complex [3]. Two types of metal-binding ligands have now been recognized in plants: extracellular chelation by organic acids such as citrate and malate, and cytoplasmic peptide ligands including metallothioneins (MTs) and phytochelatins (PCs) [5]. In eukaryotic cells, toxic ions appear to be removed from the cytosol mainly by chelation [7, 22]. PCs are small thiolate peptides with the general structure (r-Glu Cys) *n*-Gly (where $n = 2–11$), which are synthesized enzymatically from glutathione by a constitutively expressed enzyme named phytochelatin synthase (PCS; EC 2.3.2.15) [7, 31]. In phytochelatin biosynthetic

reactions, PCS is active only in the presence of metal ions—although the exact mechanism of enzyme activation by free metal ions has not been determined [15, 21, 28]—and reactions continue until the activating metal ions have been chelated [8, 13]. This provides a mechanism to autoregulate the biosynthesis of PCs in which the product of the reaction chelates the activating metal, thereby terminating the reaction [3]. The chelated metal ions, in the form of PC-metal complexes, are then sequestered to the vacuole [19, 20, 23, 29], or possibly a cytosolic location [2]. A range of metal ions (Cd^{2+}, Ag^{+}, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Sb^{3+}, As^{5+}, and Au^{+}) can activate the enzyme [31], but Cd^{2+} is by far the best [8]. Although the enzymatic action of PCS is activated by a range of metal ions, there is little evidence supporting a role for PCs in the detoxification of such a wide range of metal ions [3]. However, evidence for the role of PCs in cadmium- and arsenic-detoxification [2, 9, 16, 25, 26], is well established.

Genes encoding PCS were first isolated independently in three laboratories from Arabidopsis [9, 27], the yeast *Schizosaccharomyces pombe* [9], and wheat [2], and were subsequently also cloned from *Brassica juncea* [11] and the nickel hyperaccumulator *Thlaspi japonicum* [17]. However, no such gene has yet been identified from *P. vittata*. Here we report the isolation of a PCS gene (*PvPCS1*) from *P. vittata*. The cloning of a PCS gene from an arsenic hyperaccumulator may provide information helpful in further enhancing our understanding of the genetic basis of both hyperaccumulation and toxic metal tolerance.

Materials and methods

Plant material and culture

Spores of *P. vittata* were collected from the Botanical Garden of Padua (Padua, Italy), sterilized with 2% sodium hypochlorite, and sown on Murashige and Skoog (MS) solid medium [18] (Sigma, St. Louis, Mo.) with the addition of 3% sucrose and 0.8% pure agar (Sigma). After spore germination, and after gametophytes of the plant had developed, water was added periodically. When prothalli formed, the plantlets were fertilized and grown into sporelings with true leaves (fronds). Plants were then transferred into Magenta boxes (Micropoli, Cesano Boscone, Italy) containing solid MS medium, and cultured to the four- to six-frond stage. Fronds were then used for RNA and DNA extraction. All plants were cultured in an environmentally controlled chamber with 16 h light 22°C and 8 h dark 15°C periods.

DNA and RNA isolation

Genomic DNA was isolated using a CTAB strategy. Briefly, 0.5–2.0 g plant material ground in liquid nitrogen was extracted with DNA extraction buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% β-mercaptoethanol] at 60°C and then an equal volume of chloroform/isoamylalcohol (24:1) was added. The phases were separated by centrifugation for 15 min at 1,300 *g* at 4°C, and the aqueous phase was precipitated with 0.7 volumes of cold isopropanol at −20°C for at least 30 min. The pelleted DNA was washed with 70% ethanol, air-dried, resuspended in an appropriate volume of sterile water, then kept at −20°C before use. Total RNA was also isolated by a CTAB strategy. In brief, finely ground fern tissues were treated with RNA extraction buffer [2% CTAB, 2% PVP (MW 25,000), 2 M NaCl, 25 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% β-mercaptoethanol], and then submitted to chloroform/isoamylalcohol extraction. RNA was precipitated with 4 M LiCl, washed first with 3 M sodium acetate (pH 5.2) and then with 75% ethanol, air-dried, dissolved in diethylpyrocarbonate (DEPC)-treated sterile water, and stored at −80°C until use.

Gradient PCR and RT-PCR

Gradient PCR was performed using the following program: 30 s at 95°C (1 cycle), followed by 20 s at 94°C, 30 s at 45–60°C, and 1 min at 72°C (35 cycles), and a final 10 min 72°C extension step, with the following primers: primer pair one (PCSFOR1: 5′-AAGGTCCCT GGAGGTGGTTCGATGAATC-3′; PC SREV1: 5′-AA TGAGGGGGATACTTGAAACGAGC-3′) and primer pair two (PCSFOR2: 5′-TGGAGGTGGTTCGATGAA TCAATG-3′ and PCSREV2: 5′-GGGATACTTGAAA CGAGCAACATC-3′). RNA used for RT-PCR was treated with RNase-free DNase (30 min, 37°C). RT-PCR was performed by using 500 ng oligo$(dT)_{12\text{-}18}$ primer for first-strand synthesis under standard conditions. Negative controls with water in place of reverse transcriptase were prepared for all samples in order to control for possible genomic DNA contamination of the RNA samples.

5′- and 3′-rapid amplification of cDNA ends

To extend the PvPCS1 sequence in the 5′- and 3′-directions, we performed rapid amplification of cDNA ends (RACE) using a SMART 5′/3′-RACE kit according to the manufacturer's instructions (Clontech, Oxford, UK). The two sets of gene-specific primers used were: 5′-GCCATATCCTTGTGGCGATGATACCCACCCA CGG-3′ (first) and 5′-GTGGCGATGATACCCACCC ACGGGTGAGA-3′ (first nested), and 5′-AGGTGTCT TGTTTGGCACAATGTGCTGGGGC-3′ (second) and 5′-GGCACAATGTGCTGGGGCTTCAGTCCAAGC-3′ (second nested).

Bioinformatic analysis

Homologous sequences were identified by searching within the DDBJ/EMBL/GenBank database using

BLAST [1]. Alignments were performed using the CLUSTAL W multiple sequence alignment program.

DNA manipulation, sequencing and sequence data analysis

PCR-amplified DNA fragments were cloned, either directly or following purified from agarose gels using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), into PCR 2.1-TOPO plasmid vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.). The *Escherichia coli* strains XL1 blue (electrocompetent; Stratagene, La Jolla, Calif.) and TOPO10 (chemically competent; Invitrogen) were used as hosts for plasmid DNA. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium containing the appropriate concentration of antibiotic: 50 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin. Plasmid DNA was prepared using a NucleoSpin plasmid kit (Macherey-Nagel, Düren, Germany). About 500 ng double-stranded DNA template was sequenced using either the vector forward and reverse primers (M13) or gene-specific primers, using an ABI prism Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI cycle sequencer A3700 (sequencing service, University of Padua). Sequence data analysis was performed using Lasergene software (DNAstar, Madison, Wis.).

Yeast cultures, transformation and complementation assay

Saccharomyces cerevisiae strain YPH252−Δyap1 (MATα ura3-52 lys2-801 ade2-101 trp1-1Δ leu2-Δ 1 his3Δ::Δ yap1) was used in this study. Full-length PvPCS1 was amplified using primers carrying a *Bam*HI and an *Eco*RI site (5′-CGGGATCCGGCCCAAAGG CCC-3′ and 5′-CGGAATTCCTGAACCAAAATG-3′) at an annealing temperature of 68°C, and inserted into YCp and YEp yeast expression vectors; constructs were confirmed by automated sequencing. Both the YCp-PvPCS1 and YEp-PvPCS1 constructs and the empty vector were introduced into yeast by the lithium acetate method. Transformants were selected at 30°C for 3 days on selective ura^{-}-agar medium. Cells of yeast transformants grown to log phase were diluted to an OD_{600} of 0.3, and a series of dilutions of yeast cells were spotted on both YPD-glucose-agar and YPD-galactose-agar plates containing different Cd^{2+} concentrations (from 0 to 600 μM). Plating was performed twice to assess reproducibility.

Accession number

The nucleotide sequence of the PCS gene described in this paper has been submitted to GenBank under the accession number AY542894.

Results

Cloning and characterization of *PvPCS1* cDNA

Oligonucleotide primer pairs were designed based on the highly conserved PCS regions and used in gradient PCR with *P. vittata* genomic DNA as template. A fragment of about 800 bp was obtained with one of the primer pairs. As previous studies have shown that the PCS gene is constitutively expressed, this latter primer pair was then employed to perform PCR on RT-cDNA templates derived from both gametophytic and sporophytic *P. vittata* RNA. Only one cDNA fragment of about 400 bp was amplified from both these templates (Fig. 1). This cDNA fragment was then cloned and sequenced. DNA sequence analysis revealed that the amplified fragment showed only 86% identity to PCS sequence from *Athyrium yokoscense*, and 64% identity to that of *Typha latifolia*, and hence can be regarded as a partial PCS cDNA. This fragment was then used to design primers to amplify both the 5′- and 3′-end of PvPCS1 by RACE. After determination of the sequence of 5′/3′ ends, an independent RT-PCR was performed to isolate full-length cDNA. The nucleotide sequence of the full-length *PvPCS1*

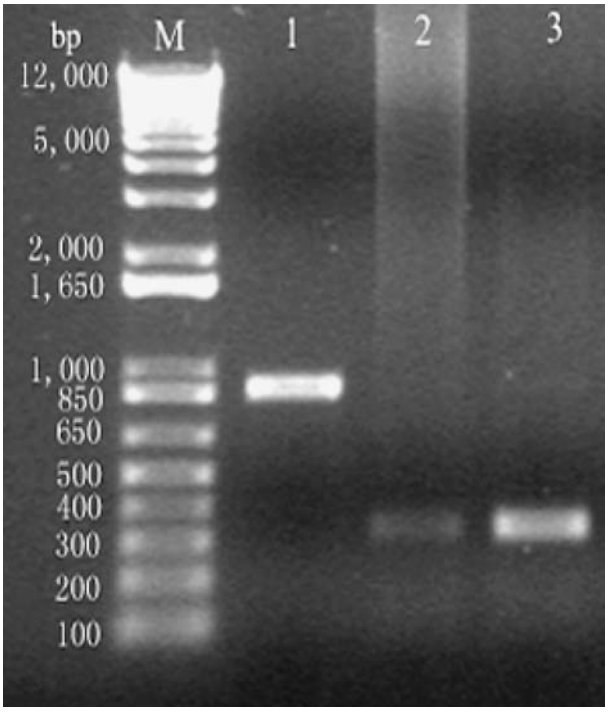

Fig. 1 Agarose gel electrophoretic analysis of RT-PCR-amplified cDNA of *Pteris vittata*. Using an oligonucleotide primer pair designed based on highly conserved regions of phytochelatin synthase (PCS), one cDNA fragment of about 400 bp was amplified from both gametophytic and sporophytic cDNA of *P. vittata*. Lanes: *M* 1.0 kb DNA marker; *1–3* RT-PCR amplicons from genomic DNA, gametophyte RNA and sporophyte RNA, respectively

cDNA comprises a 5′ untranslated region of 66 nucleotides, a single ORF of 1,536 nucleotides and a 3′ untranslated region of 367 nucleotides. A search in the NCBI database using the basic local alignment search tool (BLAST [1]) indicated that the nucleotide sequence homology between PvPCS1 and other PCS nucleotide sequences was very low, and largely confined to two highly conserved regions near the 5′-end of the sequence. In these two regions nucleotide similarity was as high as 85–95% (Fig. 2).

Comparison of the amino acid sequence of *PvPCS1* with other known PCS polypeptides

The amino acid sequence deduced from the DNA sequence of *PvPCS1* predicts a protein of 512 amino acids with a molecular weight of 56.9 kDa. The amino acid sequence of *PvPCS1* was compared with the sequences of other known PCS polypeptides by using the CLUSTAL W multiple sequence alignment program. The aligned scores were 75% with *AyPCS1* (*A. yokoscense*, GenBank accession number BAB64932), 48% with *NtPCS1* (*Nicotiana tabacum*, AY235426.1), 47% with *BjPCS1* (*B. juncea*, AJ278627.1), 47% with *AtPCS1* (*Arabidopsis thaliana*, AF085230), 47% with *TaPCS1* (*Triticum aestivum*, AF093752), 47% with *TjPCS* (*Thlaspi japonicum*, AB085626.1), 46% with *TcPCS* (*Thlaspi caerulescens*, AB085627.1), 46% with *StPCS1* (*Solanum tuberosum*, AJ548472.1), 45% with *AtPCS2* (*A. thaliana*, AY044049.1), 45% with *GmhomoPCS1* (*Glycine max* homo-PCS, AF411075.1), 30% with *CePCS1* (*Caenorhabditis elegans*, AF299333.1), 28% with *AsPCS*1 (*Allium sativum*, AF384110.1) and 28% with *SpPCS* (*S. pombe*, Z68144). Previous comparison of PCS polypeptides from four different organisms (*Arabidopsis*, *T. aestivum*, fission yeast *S. pombe*, and *Caenorhabdtis*) showed that only the N-terminal half of PCS was conserved; little apparent conservation of amino acid sequence was found in the C-terminal half [3]. Comparison of *PvPCS1* with other PCS polypeptides supports this conclusion, as the *PvPCS1* polypeptide shares about 60% identity with other PCS proteins in the N-terminal end, but has only limited similarity in the C-terminal portion.

Cys-Cys pairs and single Cys residues are known from MTs as metal-binding motifs [5, 12], and have proved to be the main determinants of the metal-binding sites in PCSs [15]. The alignment of *PvPCS1* with other PCS protein sequences revealed some characteristics of the Cys residues in these sequences. In *PvPCS1*, there are two Cys-Cys motifs and 12 single Cys, but only 4 of them (Cys-56, Cys-90/91, and Cys-109) are conserved in all known PCS peptides (Fig. 3). Another characteristic of the PvPCS1 polypeptide is that it has relatively more Cys residues in the N-terminal domain than other PCS polypeptides. Moreover, in the C-terminal region, PvPCS1 has less Cys-Cys pairs and C–X–X–C motifs, which occur more frequently in the C-terminal domains of other PCS polypeptides.

Expression of *PvPCS1* in *Saccharomyces cerevisae* mediates increased Cd-resistance

The *S. cerevisae* strain −Δyap1 was used to express *PvPCS1*. This strain is deficient in Δyap1, a transcription factor containing a bZip DNA-binding motif that belongs to the family of Jun transcriptional activators. Yap1 activates transcription of several target genes encoding ABC- and MFS-transport proteins as well as proteins involved in the oxidative stress response. Thus, −Δyap1 mutants are particularly sensitive to Cd, and thus suitable for PCS assay.

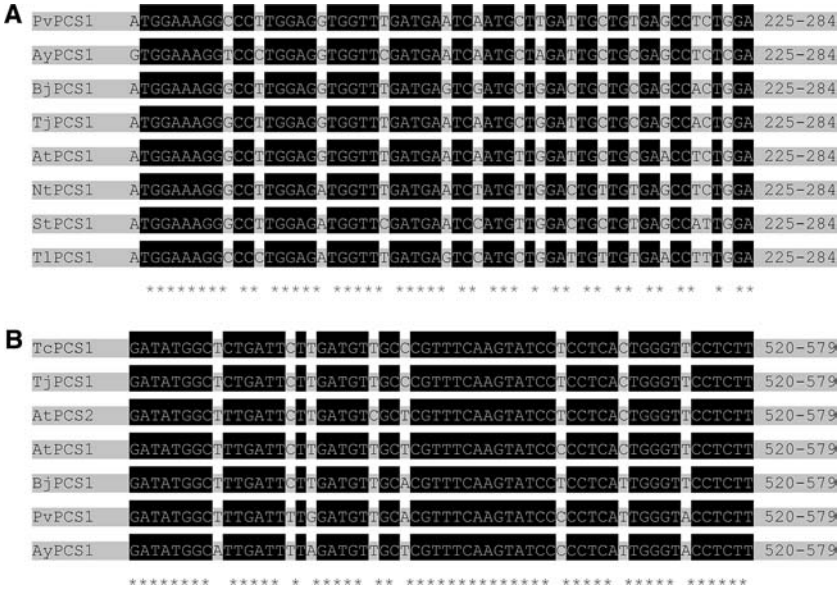

Fig. 2a,b Comparison of nucleotide sequences in the two highly conserved regions of most known PCS genes. **a** Region one (alignment score 85–93%), **b** region two (88–95%). Numbers on the right indicate the positions of the two regions in the open reading frame of each gene. Identical residues are *shaded*

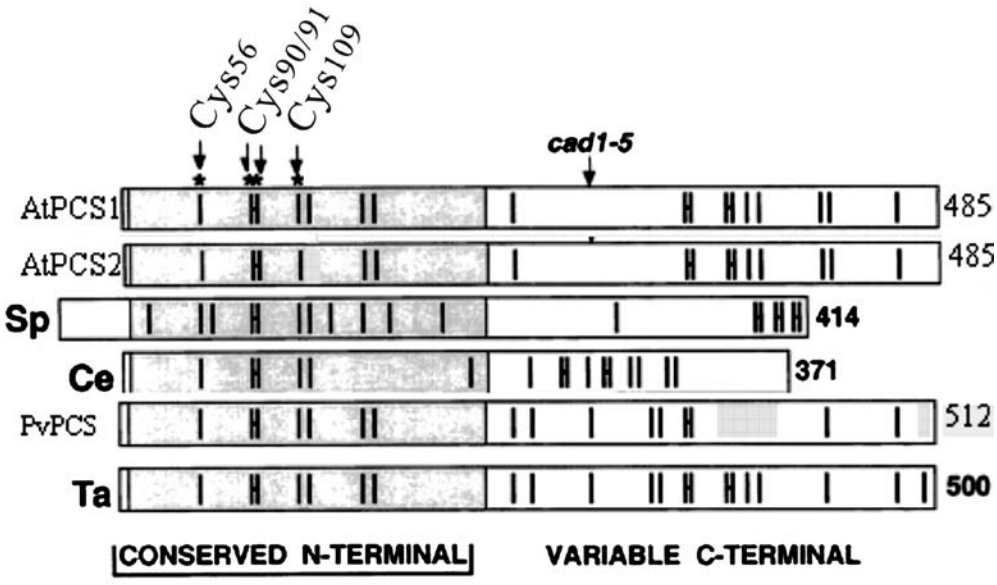

Fig. 3 PvPCS contains two Cys-Cys motifs and 12 single Cys residues, 4 of which (Cys-56, Cys-90/91, and Cys-109) are conserved in all known PCS peptides (*asterisks*). *Vertical lines* Cysteine residues. PCS sources: *AtPCS1*/*AtPCS2 Arabidopsis thaliana*, *Sp Schizosaccharomyces pombe*, *Ce Caenorhabditis elegans*, *PvPCS P. vittata*, Ta *Triticum aestivum*

Full-length *PvPCS1* was introduced into −Δyap1 yeast mutants under the control of the inducible pGAL1 promoter in the yeast expression vectors YCp and YEp. Yeast cells carrying either empty vector (YEp), the YCp-PvPCS1 construct, or the YEp-PvPCS1 construct were spotted onto YPD-agar plates containing a linear 0–600 μM gradient of $CdCl_2$. Growth assays showed that, under inducing conditions (+galactose), cells of *S. cerevisae* carrying either YCp-PvPCS1 or YEp-PvPCS1 grew in the presence of Cd^{2+} concentrations up to 200 μM (Fig. 4), while growth of cells carrying empty vector were inhibited even at 50 μM Cd. This is obviously a result of strong expression of PvPCS1 in galactose-containing medium. Under repressing conditions (+glucose), however, growth of cells carrying either empty vector or YCp-PvPCS1 was inhibited; only cells carrying YEp-PvPCS1 grew in the presence of Cd (Fig. 4). This could be explained as a result of minimal expression of PvPCS1 in the presence of glucose. As YCp is a single copy plasmid and YEp is multi-copy, it is reasonable to see minimal expression only with the YEp vector. The growth of YEp-PvPCS1-carrying cells in the presence of Cd indicated that this minimal expression of *PvPCS1* is enough to render the yeast cells Cd-resistance. Hence, we conclude that expression of *PvPCS1* mediates increased Cd-tolerance of *S. cerevisae*.

Discussion

Phytochelatins were first identified as a heavy-metal-binding peptides involved in the accumulation, detoxification, and metabolism of metal ions [7], and there is currently no direct evidence that PCs have any function

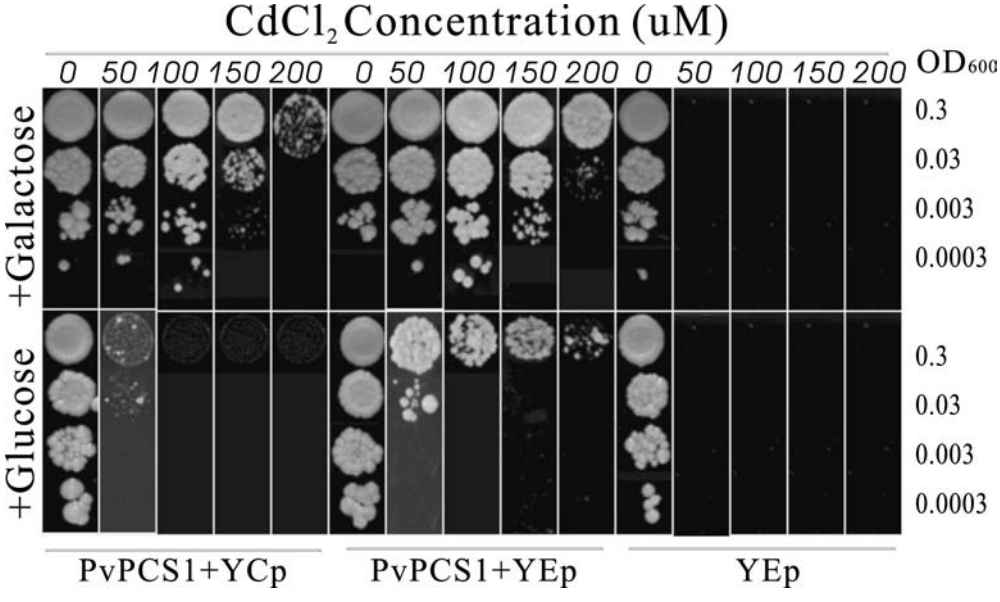

Fig. 4 Cd^{2+} tolerance of *Saccharomyces cerevisae* cells expressing PvPCS1. Yeast cells carrying empty vector (YEp), YCp + PvPCS1 constructs, or YEp + PvPCS1 constructs were grown on YPD-ura-agar plates containing a gradient of $CdCl_2$ (0–200 μM) under inducing (2% galactose) or repressing (2% glucose) conditions

other than in metal detoxification [3]. A wealth of evidence has been collected supporting the role of PC in arsenic detoxification [10, 16, 24–26]; however, a recent physiological study by Zhao [32] suggested that PCs play a limited role in hypertolerance of arsenic in *P. vittata*. Similar conclusions were drawn by Ebbs [6] and by Schat [24] in another hyperaccumulator, *T. caerulescens*. This implies that PCs may be responsible only for low levels of metal tolerance in nonhyperaccumulating plants, and may not be essential to the high amount of metal disposal in hyperaccumulators. If this is true, mechanisms for general metal tolerance and for metal hypertolerance seem to have a completely different basis [4]. However, in hyperaccumulators, PCS genes are constitutively expressed, and PC synthesis is indeed induced by metal exposure [6, 24, 32]. Thus, the exact role of PC in metal hypertolerance, and especially in metal hyperaccumulation, remains to be determined. The isolation of the PCS gene (*PvPCS1*) from the hyperaccumulator *P. vittata* reported here may provide alternatives to gather information for better understanding of the role of PCS in metal hyperaccumulation.

One model for the function of PCS enzymes is that the conserved N-terminal domains posses the catalytic activity, and that activation might arise from metal ions interacting with residues in this domain, possibly Cys or His residues [3]. Previous comparison of PCS polypeptide sequences identified five Cys residues (two of which are adjacent) that are highly conserved in their positions in the N-terminal domain of PCS sequences [3]. However, it now seems that there are only four instead of five, if we take into account the AtPCS2 protein sequence, which has a Ser instead of Cys in one of the five conserved Cys positions. Interestingly, these four conserved Cys residues are present in PvPCS1. Hence, if Cys residues are required for the function of the enzyme, these four Cys residues (Cys-56, Cys-90/91, and Cys-109) are likely to be functionally essential.

In conclusion, in this study, we describe the cloning, expression and characterization of *PvPCS1*, a PCS gene from the first known arsenic hyperaccumulator, *P. vittata*. Sequence analysis of *PvPCS1* predicts that it encodes a protein of about 56 kDa that shows high similarity to most known PCS genes, sharing with them four Cys residues that are probably functionally essential for enzyme activity. Expression of *PvPCS1* in *S. cerevisae* confers Cd-resistance on the yeast. Certainly, further investigation is needed to determine the functional significance and biological role of *PvPCS1*, especially its role in metal hyperaccumulators.

Acknowledgement We thank Mr. Robert Tacchetto (Botanical Garden of Padua) for assisting in plant growth

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