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Phytochelatins: Peptides Involved in Heavy Metal Detoxification

Rama Pal · J. P. N. Rai

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Abstract Phytochelatins (PCs) are enzymatically synthesized peptides known to involve in heavy metal detoxification and accumulation, which have been measured in plants grown at high heavy metal concentrations, but few studies have examined the response of plants even at lower environmentally relevant metal concentrations. Recently, genes encoding the enzyme PC synthase have been identified in plants and other species enabling molecular biological studies to untangle the mechanisms underlying PC synthesis and its regulation. The present paper embodies review on recent advances in structure of PCs, their biosynthetic regulation, roles in heavy metal detoxification and/or accumulation, and PC synthase gene expression for better understanding of mechanism involved and to improve phytoremediation efficiency of plants for wider application.

Keywords Heavy metals · Phytochelatins · Vacuolar sequestration · Sulfide ions

Introduction

Heavy metals are among the most toxic inorganic substances which have contaminated large area of land due to use of sludge, pesticides, fertilizers, and emissions from municipal waste incinerators, car exhausts, residues from metalliferous mines, and smelting industries [1, 2]. Many metals which are essential for cells e.g. copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn)) are naturally available in the Earth's crust at various levels and become toxic at higher concentrations. Metal concentrations in soil typically range from less than one to as high as 1,00,000 mg kg^{-1}. Excessive levels of many metals can result in soil quality degradation, crop yield reduction, and poor quality of agricultural products [3], consequently posing significant hazards to human, animal, and ecosystem health [4]. Conventional engineering methods available for treating metal contaminated soils can be prohibitively expensive [5, 6]. However, plants capable of accumulating a

R. Pal · J. P. N. Rai (✉)
Ecotechnology Laboratory, Department of Environmental Science,
G.B.Pant. University of Agriculture and Technology, Pantnagar 263145, India
e-mail: jamesraionline@gmail.com

significant level of heavy metals from the soil offer an alternative and less expensive method to confiscate heavy metals directly from the soil. At present, at least 45 plant families with more than 400 plant species are known as metal accumulators [7, 8]. Many species belonging to *Brassicaceae* have been found to sequester reasonable amount of various metals in their tissues [9, 10, 11]. Plants have constitutive and adaptive mechanisms for accumulating or tolerating high concentrations of their rhizospheric contaminants. Several mechanisms for heavy metal tolerance and hyperaccumulation have been suggested, chiefly for Cd [12], Se, Ni, Zn [13], and As [14] in plants growing under metal stress. Plants accumulating Co [15], Pb [16], Cd, Cr, Ni, and As [17] have also been reported. However, the metal detoxification in plants may be accomplished through the distribution of metals to apoplast tissues like trichome and cell walls [18] and metal chelation by a ligand, followed by vacuolar sequestration of the metal–ligand complex [19].

Metals can be complexed with ligands extracellularly, e.g. the mechanism of Al tolerance by efflux of organic acids like malate and citrate from roots and intracellularly involving chelation by peptide ligands such as metallothioneins (MTs) and phytochelatins (PCs). MTs are cysteine-rich polypeptides, first identified in mammalian tissues as Cd-binding peptides. Later, a number of MT genes and proteins have been identified in plants [20, 21]. MTs are gene-encoded, whereas PCs are enzymatically synthesized, a family of peptides that were first identified in yeast, *Schizosaccharomyces pombe*, and termed cadystins [22]. PCs have also been identified in a wide variety of plant species including monocots, dicots, gymnosperms, and algae as heavy metal binding ligands [23], suggesting that the PC-producing pathway evolved very early in order to maintain the endurance of vascular plants growing under potentially hostile and toxic environments.

Molecular interactions of heavy metals and functional proteins regarding PC synthase activation by heavy metals such as Cd and consequent synthesis of PCs are of particular interest. Molecular biology underlying PC biosynthesis progressed after identification of PC synthase activity in cultured plant cells by Grill et al. [24] for the first time followed by identification and characterization of PC synthase genes in higher plants and fission yeast [25–27]. Moreover, recent enzymatic analyses of recombinant PC synthases have unraveled the molecular mechanisms underlying PC synthesis.

In this paper, the recent advances in understanding of the molecular mechanisms for PC biosynthesis and functions involving metal detoxification especially in plants have been reviewed to provide updated information about these unique peptides applicable to heavy metal detoxification and subsequently their sequestration.

Structure, Classification and Role of PCs in Metal Accumulation

PCs consist of only three amino acids, glutamine (Glu), cysteine (Cys), and glycine (Gly) with the Glu, and Cys residues linked through a γ-carboxylamide bond. They are structurally related to tripeptide glutathione (GSH; γ-GluCysGly) and are non-ribosomally synthesised [24, 25, 27]. PCs form a family of structures with increasing repetitions of the -Glu-Cys dipeptide units followed by a terminal Gly, (γ-Glu-Cys)*n*-Gly or (γ–EC)*n*-Gly, where *n* generally ranges from 2–5, but can be as high as 11 [21]. The chemical structures of PCs, GSH, and γ–EC are shown in Fig. 1.

On the basis of the number of -Glu-Cys units, PCs have been classified as PC_2, PC_3, PC_4, PC_5, and PC_6 etc. [28, 29]. PCs have been identified in a wide variety of plant species [30–33] and in some microorganisms [34, 35]. In addition, a number of structural variants,

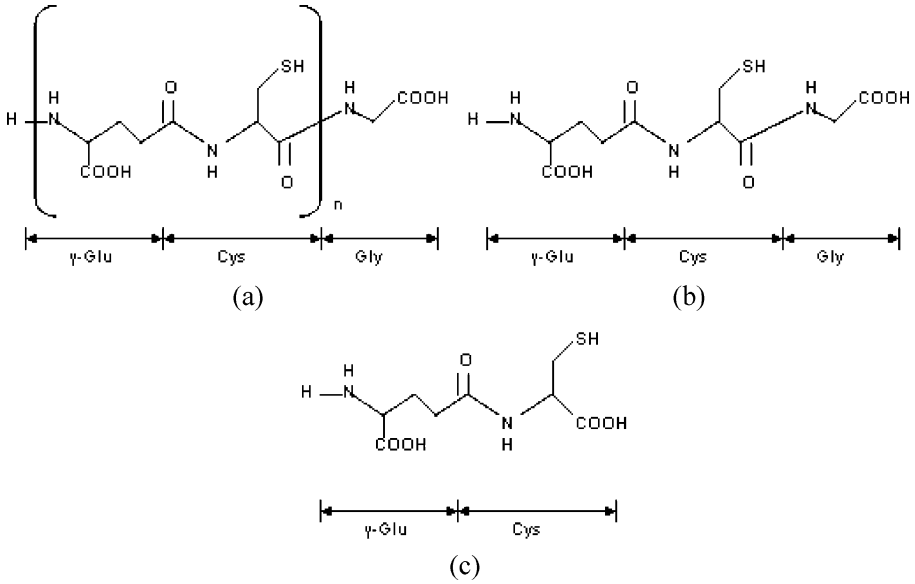

Fig. 1 Chemical structures of **a** phytochelatin, **b** glutathione, and **c** gamma-EC

for example, (γ-GluCys)*n*-β-Ala, (γ-GluCys)*n*-Ser, and (γ-GluCys)*n*-Glu, have been identified in some plant species [36].

Evidences supporting the role of PCs in the detoxification of various heavy metal ions are few and far between. PCs are immediately produced in cells and tissues after exposure to a range of heavy metal ions [29, 32, 37–39]. However, different metal ions have been found to induce PCs production to various levels. Maitani et al. [40] used post-column derivatization high-performance liquid chromatography (HPLC) in order to analyze PCs induction to various degrees in the roots of *Rubia tinctorum*, in the presence of a wide range of metal ions tested. The most potent persuasive were found to be Cd>Pb>Zn>Ag>Hg>As>Cu. Metal-induced PCs production was also proved by Morelli and Scarano [41] in a negative test where metal-stressed *Phaeodactylum tricornutum* cells, suspended in a metal-free medium, exhibited lower PC pool and a concomitant increase of glutathione, thus, also suggesting a mechanism of degradation and release of metal–phytochelatin complexes. Further confirmation for the induction of PCs under metal stress was provided by the fact that PC production was found to be positively correlated with the metal accumulation in the tissues. Iglesia-Turino et al. [42] studied Hg accumulation in rape (*Brassica napus*) plants grown under a Hg concentration gradient (0–1,000 μM) and found that Hg accumulation was strongly correlated with PC_2 concentration (r^2=0.98) (Table 1). Contrastingly, there was no correlation between PCs production and Cd accumulation in *Chlamydomonas spp*. which showed maintenance of high GSH level to be more important for Cd accumulation [43]. Another study by Figueroa et al. [44] demonstrated the effect of metal bioavailability on PCs production. Their results indicated that humic substance in soil restricted the availability of metal for creating stress and thereby resulted in reduced PC production in *Ricinus communis*. PCs synthesis is markedly influenced by the type of metal ion present. In support of this, Vestergaard et al. [45] established no PC synthesis in vitro by PCs in the presence of Mg^{2+}, Mn^{2+}, Ca^{2+}, and Na^{+}, whereas, Cd^{2+} and Zn^{2+} treatment led to appearance of PCs. Similarly, Zhang et al. [33] showed PC induction in vivo in the presence of Cd and Pb but not in the presence of Zn in *Sedum alfredii* Hance.

Table 1 Total Hg content (μg Hg g dry weight plant^{-1}) in root and shoot tissues extracts analyzed separately from rape plants treated with different $HgCl_2$ concentrations and total PC_2 content (μM PC_2 g fresh weight plant^{-1}) in roots.

Treatment	Shoot Hg content	Root Hg content	Root PC_2 content
μM $HgCl_2$	μg Hg g dry weight plant^{-1}	μg Hg g dry weight plant^{-1}	μM PC_2 g fresh weight plant^{-1}
0	13.37a±3.15	1.63a±0.25	0.132a±0.040
50	26.56a±8.64	59.47a±16.76	1.417ab±0.644
125	37.60a±6.66	242.72a±26.60	2.246ab±0.517
250	129.34b±37.74	339.09ab±31.12	3.245ab±0.412
375	129.34b±37.74	399.63ab±56.64	4.461bc±0.589
750	114.12b±14.26	1,012.43b±338.95	7.830cd±0.960
1,000	115.07b±12.73	2,351c±407.66	14.705e±3.126

Values are means of three replicates. Means followed by different letters are significantly different by Duncan test with $P<0.05$. Relationships of μM PC_2 g^{-1} fresh weight with μM $HgCl_2$ g^{-1} dry weight in rape showed linear correlation $r^2=0.98$, $P<0.05$ [42]

Phytochelatin synthesis was found to be catalyzed by PC synthase (EC 2.3.2.15] from the substrate like GSH and related thiol tripeptides in the presence of metal ions, such as Cd, Cu, Zn, Ag, Hg, and Pb in cultured cells of *Silene cucubalis* [24]. Moreover, several biochemical and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis. Particularly, genetic studies on GSH-deficient mutants of *S. pombe* confirmed that they were PC deficient and hypersensitive to Cd [46, 47]. Similarly, GSH-dependent activities of PC synthase were also identified in *Arabidopsis* [48], pea [49], and tomato [50]. Inouhe et al. [51] employed Cd hypersensitive *Vigna angularis* (Azuki beans) to prove the PC synthase activity in Cd tolerance. Cell suspension cultures of Azuki beans challenged with Cd stress were incompetent to tolerate Cd toxicity. These cells also lacked PC synthase activity as externally applied GSH failed to stimulate PC synthesis or confer Cd tolerance to the cells, which demonstrated the importance of PC synthesis in Cd tolerance by plants. Expression of a PC synthase gene of *Cynodon dactylon* L. in tobacco plants [52] also evidenced its role in enhancing phytochelatin production by 3.88-fold and subsequently enhancing Cd accumulation by 3.21-fold in transgenic tobacco. Furthermore, transgenic *Brassica juncea* plants expressing *Arabidopsis* PC synthase showed increased tolerance to and accumulation of Cd and As [53]. These studies suggest that GSH and PC concentrations can be manipulated to enhance heavy metals accumulation potential of plants. However, others reported that over-expression of PC synthase in plants paradoxically led to hypersensitivity to heavy metals. Lee et al. [54] studied few transgenic *Arabidopsis* lines generated following transformation with a construct containing AtPCS1 cDNA under the control of cauliflower mosaic virus (CeMV) 35 S promoter (35::AtPCS1). Among them two transgenic lines exhibited 14-fold increase in AtPCS1 transcript and 30% increase in PC level; however, these were more Cd-sensitive than wild type. While remaining transgenic lines were twofold Cd-tolerant and few showed slight increase in AtPCS1 expression and 15% increase in PC content. This suggested critical level of AtPCS1 was important in heavy metal detoxification. But whether AtPCS1 protein itself was responsible for Cd sensitivity or not, it was clarified without more ado by Lee et al. [55]. They observed that *Arabidopsis* plants overexpressing

Arabidopsis PC synthase (*AtPCS1*), designated PCs lines, accumulated 12–25-fold higher AtPCS1 mRNA and 1.3–2.1-fold increased PC concentration compared to wild type. They were more sensitive to Cd stress than a PC-deficient *Arabidopsis* mutant, *cad1-3* overexpressing AtPCS1 to similar levels as those of PCs lines, supplemented with low GSH levels. Cd hypersensitivity of PCs lines disappeared under increased GSH levels provided in the medium. Therefore, Cd hypersensitivity in PCs lines appeared not due to toxicity of AtPCS1 protein but due to toxicity of PCs as they have high Cys content and existed at supraoptimal levels when compared with GSH levels. Recently, Wojas et al. [56] also reported that overexpression of *AtPCS1* gene in tobacco led to Cd-hypersensitivity. PC synthase activity in transformants was around fivefold higher than in wild type. There was no substantial difference in Cd accumulation but a dramatic accumulation of γ-glutamylcysteine and strong depletion of glutathione with only a moderately and temporary increase in phytochelatin levels due to *AtPCS1* expression was observed. However, the marked change in non-protein thiols (NPT) composition decreased Cd detoxification capacity moderately, as reflected by lower SH–Cd ratios, and enhanced oxidative stress which explained higher Cd sensitivity. The results opposite to desired effect might be due to species-dependent activity of PC synthase. Besides combating heavy metal toxicity, PC synthase genes were also found to be involved in offering multiple abiotic stress tolerance as while analyzing the functional expression and characterization of PC synthase gene of *Anabaena sp.* PCC7120 in *Escherichia coli* using pGEX-5X-2 expression vector, Chaurasia et al. [35] observed better growth of transformed *E. coli* cells than control cells under temperature (47 °C), NaCl (6% *w*/*v*), carbofuran (0.025 mg/ml), $CdCl_2$ (4 mM), $CuCl_2$ (mM), and UV-B (10 min) exposure.

Chelation of heavy metals with PCs, a primary cellular mechanism for heavy metal detoxification, may work in synergism with secondary stress-defensive antioxidative system to combat heavy metal induced oxidative stress. Ranieri et al. [57] reported that the stress generated by low Cd accumulation in leaves of *Triticum aestivum* was counteracted by antioxidant response and by PC biosynthesis. On contrary, the excess of Cd caused a considerable H_2O_2 increase, despite the elevated presence of PCs and related thiol-peptide-compounds in roots. Mishra et al. [58] observed increase in antioxidant enzymes (viz., superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase) and PCs level in *Bacopa monnieri* at initial low Cd concentration which depleted with increase in exposure concentration and duration concomitant with increase in oxidative stress. Similarly, Chen et al. [11] reported rapid accumulation of PCs in roots and shoots with increasing Cd accumulation and sequestration of Cd by PCs in *Brassica chinensis* exposed to different concentrations of Cd. Furthermore, malondialdehyde formation, hydrogen peroxide content, and antioxidative enzyme activities were observed in shoots in response to Cd stress at lower concentrations. These resulted in reduced free Cd damage, enhanced Cd accumulation, and tolerance. Based on these, it can be concluded that antioxidant system is effective at lower metal concentrations depending upon the plant–metal system.

There are some plants which are naturally selected as heavy metal tolerant but produce less PCs than sensitive ecotypes [59, 60, 61]. Sun et al. [62] studied two geographically isolated populations of *Sedum alfredii*: one grew on an old Pb/Zn mine site, while other on non-mine site. The individuals of mine population exhibited strong heavy metal tolerance than those of non-mine population. Using a HPLC pre-column derivatization system, they detected the presence of PCs in plant tissues of non-mine population upon Cd exposure, whereas, no PC was found in mine population. However, Zhang et al. [33] suggested that PCs production could be induced in leaf, stem, and root tissues of mine population of *S.*

alfredii upon exposure to 400 μM Cd and only in the stem and root when exposed to 700 μM lead, whereas, no PCs were found in any plant part under Zn stress. This study strongly supports the wide inducement of PCs and its function in the detoxification of metals in many plants except for Cd sensitive Azuki bean.

Characterization of PCs and their Interaction with Metals

Several analytical methods, such as chromatographic separation (gel filtration or HPLC) coupled with UV detection, flame atomic absorption spectrometry, radio-active labeling, and inductively coupled plasma–mass spectrometry (ICP-MS), electrospray–MS (ESI-MS), or ESI-tandem MS have been used to analyse PCs and PC–metal complexes [28, 40, 63–66]. Navaza et al. [10] performed structural elucidation of PCs and other thiols, as well as their complexes with As and Cd, by electrospray-quadrupole-time-of-flight (ESI-Q-TOF) in *B. juncea* plants overexpressed with genes encoding for gamma-glutamyl cystein synthetase (γ-ECS) and glutathione synthetase (GS). In both the Cd and As exposed plants it was possible to observe the presence of oxidized PC_2 ($[M+H]^+$, *m*/*z* 538), GS-PC_2(-Glu) ($[M+H]^+$, *m*/*z* 716) as well as reduced GSH ($[M+H]^+$, *m*/*z* 308) and oxidized glutathione (GSSG) ($[M+H]^+$, *m*/*z* 613). Only GS plants exhibited the presence of $As(GS)_3$ complex ($[M+H]^+$, *m*/*z* 994) which was reported for the first time in *B. juncea* plant tissues. Similarly, Chen et al. [67] used SEC-ICP-MS and ESI-MS/MS for analyzing in vitro and in vivo Cd–phytochelatin complexes in a Cd hyperaccumulator *B. chinensis*. The PCs (n=1–5) obtained from Cd-stressed *B. chinensis* together with that from $CdCl_2$ used to synthesize in vitro Cd–PC complexes and the formation of $CdGS_{1-2}$, $(CdGS)_2$, $Cd_{1-2}PC_2$, $Cd_{1-3}PC_3$, $Cd_{1-3}PC_4$, and $Cd_{1-3}PC_5$ were observed. In addition, for the first time, in vivo $CdPC_3$ and $CdPC_4$ complexes, as well as Cd-free PCs (n=2–5) and desGlu-PC_3 were also detected in extracts of Cd-stressed *B. chinensis*.

PCs interact with the metals through thiol (-SH) group of cysteine. However, the degree of polymerization in PCs was observed with increasing intracellular metal concentration indicating increased binding stability of metal–PC_n complexes [68]. The metal–PC complex formation is governed by availability of ligand, kinetics of complex formation, and steric factor. The structural model of PC–Cd complex indicated binding stoichiometry of sulfur atoms either from single or multiple PC molecules to be 4 to 1, resulting in amorphous complexes (Fig 2a) [69, 70]. In contrast, Zn^{2+} complex consisted exclusively of a 1:1 complex [70]. In case of As^{3+}, Schmöger et al. [71] identified As^{3+}-$(PC_2)_2$ complex in vitro. However, cellular conditions did not favor formation of this complex and As^{3+} preferentially coordinated with three –SH Cys groups from PC_3 to form a more stable trihedral coordination than either As^{3+}-PC_2 or As^{3+}-$(PC_2)_2$ [72]. On the basis of these statements, the possible interaction between Zn^{2+} and PCs and As^{3+} and PCs could be visualized as Fig. 2b, c.

PC Synthase Genes

The enzyme catalyzing PC biosynthesis from GSH was first characterized by Grill et al. [24]. In spite the identification of PC synthase activity, the identification of a corresponding gene remained indefinable until the end of last decade. PC synthase genes were first isolated almost simultaneously by three research groups. Vatamaniuk et al. [27] identified an *Arabidopsis* cDNA, named *AtPCS1*. Expression of *AtPCS1* in *yap*1 and *ycf*1 mutants of

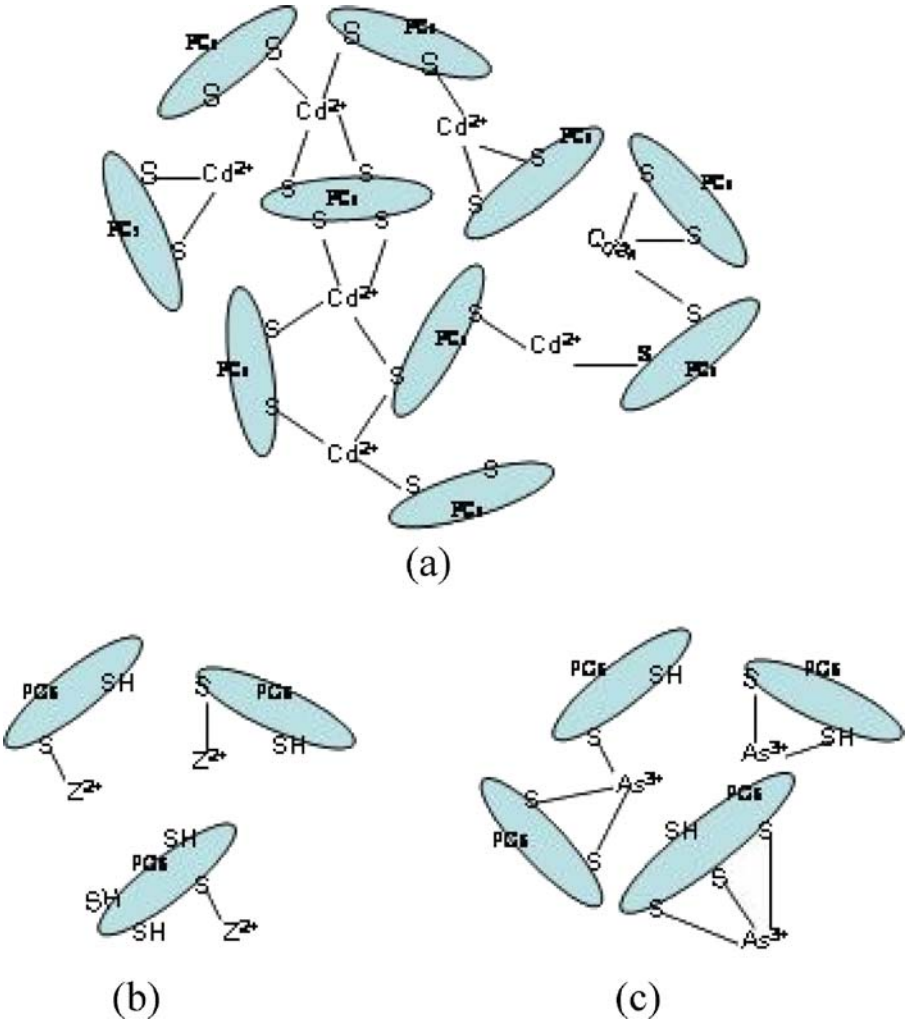

Fig. 2 Interaction of PCs with different heavy metals. Heavy metal ions **a** Cd^{2+}, **b** Zn^{2+}, and **c** As^{3+} coordinately binding with sulfur atoms of Cys residues from either single or multiple PC molecules, forming different complexes

Brewer's yeast suppressed the Cd-sensitive phenotype of both the mutants suggesting its role in Cd sequestration. *YAP1* encoded a transcription factor that was required for the expression of *YCF1*, a transporter responsible for the vacuolar sequestration of GSH–Cd complexes [19]. Clemens et al. [26] identified a wheat cDNA, *TaPCS1*, by carrying out cDNA library expression screening in *Saccharomyces cerevisiae*, which increased Cd resistance and accumulation just like *AtPCS1*. The third group identified *AtPCS1* through positional cloning of the *CAD1* gene of *Arabidopsis* [25]. The cad1 mutants were Cd sensitive and deficient in PC biosynthesis in vivo and PC synthase activity in vitro [48], indicating *CAD1* gene to encode a PC synthase. Database search by these groups also identified a yeast PC synthase gene (*SpPCS*) in *S. pombe* as a homologous to *AtPCS1*. Sequence analysis of the amino acids predicted to be encoded by *AtPCS1*, *TaPCS1*, and *SpPCS* indicated that the N-terminal region was well conserved, whereas the C-terminal

region was variable (Fig. 3) [73]. Later on also, many PCs genes were isolated from different plant species like *PvPCS1* from *Pteris vittata* [74] and *CdPCS1* from *C. dactylon* L. [52] by rapid amplification cDNA ends. Ramos et al. [39] identified *LjPCS2* and *LjPCS3* genes in *Lotus japonicus*, through screening of transformation-competent artificial chromosome libraries, encoding protein products viz. LjPCS2-7N and LjPCS3-7N which conferred Cd tolerance when expressed in *S. pombe*.

Animal genes in the genome of the nematodes *Caenorhabditis elegans* (ce-pcs-1) [75, 76] and the slime mould *Dictyostelium discoideum* [73] were identified as homologous to plant and fungal PC synthase genes. Recently, Brulle et al. [77] were able to clone and transcriptionally characterize a phytochelatin synthase in the earthworm *Eisenia fetida* belonging to the phylum Lophototrochozoa. The complete coding sequence of this enzyme was determined and the phylogenetic relationship to plant, yeast, and nematode enzymes was elucidated. Various prokaryotic PC synthase genes were also isolated from cyanobacteria *Nostoc* sp. PCC7120, *Anabaena variabilis*, *Prochlorococcus marinus*, *Trichodesmium erythraeum*, *Burkholderia fungorum*, and *Microbulbifer degradans* [78, 79]. The PC synthase encoded by these genes are approximately one-half the length of their cognates from eukaryotes because they lack more variable C-terminal domain. However, reports on PC synthesis in these organisms against heavy metal stress are awaited. Although Tsuji et al. [78] reported alr0975 gene in *Nostoc sp*. PCC 7120 encoding a PC-synthase-like protein, no PCs induction as well as its significant expression were observed even in Cd-treated cells; however, recombinant alr0975 protein strongly catalyzed PC synthesis when expressed in *E. coli* cells. These results suggested alr0975 protein might be a more primitive form of PC synthase found in eukaryotes. Recently, a study on *S. cerevisiae* has revealed the presence of another two enzymes i.e., vacuolar serine carboxypeptidases, CPY and CPC, possessing PC-like activity in vivo, thus, disclosing another route for PC biosynthesis in eukaryotes [80].

Molecular Mechanism of PC Synthesis

First, Grill et al. [24] proposed that in presence of free heavy metal ions PC synthase catalyses PC synthesis in two distinct reaction steps: step I, the Cys–Gly peptide bond of the donor i.e., GSH, (γ-EC)Gly, is cleaved to generate γ-Glu-Cys (γ-EC) unit; step II, γ-

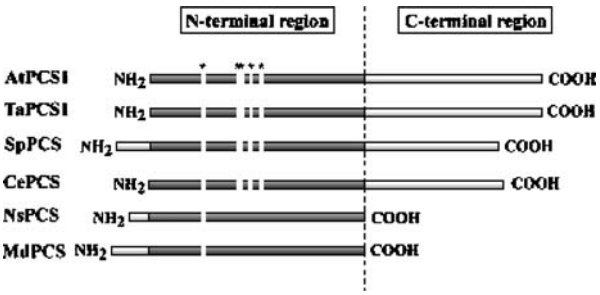

Fig. 3 Schematic alignment of phytochelatin synthases found in different organisms. The sequences are derived from *Arabidopsis thaliana* (AtPCS1, AF135155), *Triticum aestivum* (TaPCS, AF093252), *Schizosaccharomyces pombe* (SpPCS, Z68144), *Caenorhabditis elegans* (CePCS, AF299333), *Nostoc sp.* PCC7120 (NsPCS, AB162106), and *Microbulbifer degradans* 2–40 (MdPCS, ZP_00315875). The positions of five cysteine residues in the conserved N-terminal region are indicated by *vertical bars with asterisks* [73]

Glu-Cys (γ-EC) unit is transferred to an acceptor molecule, which is either GSH or an oligomeric PC peptide (PC_n), to generate PC_{n+1} via a transpeptidation reaction, as shown in the equation below:

Step I: γ-EC-Gly → γ-EC+Gly

Step II: γ-EC+$(\gamma\text{-EC})_n$-Gly → $(\gamma\text{-EC})_{n+1}$-Gly

in which chain extension proceeds from C to N terminus. Vatamaniuk et al. [81], while analyzing PC synthesis catalyzed by AtPCS1-*FLAG*, proposed that during step I of catalysis, PC synthesis involved acyl-enzyme intermediate formation with the concomitant release of glycine from blocked GSH. As exemplified by analysis of the immuno-purified recombinant enzyme from *Arabidopsis thaliana* (*AtPCS1-FLAG*), PC synthase found to catalyze PC synthetic reaction that approximated a bi-substrate substituted enzyme kinetics in which micromolar concentrations of heavy metal GSH thiolates (e.g. cadmium.GS_2) and millimolar concentrations of free GSH or millimolar concentration of S-alkylglutathiones served as cosubstrates [81]. Recently, Vatamaniuk et al. [82] confirmed that AtPCS1 is a dipeptidyltransferase, which undergoes γ-Glu-Cys acylation at two sites, with the release of Gly, during step I of catalysis that is necessary for net PC synthesis, but requirements for each acylation reaction are distinguishable. Free GSH alone was capable of acylating the first site, but not at second site unless Cd^{2+} was added to the reaction medium. Their results of mutagenesis experiments, in which each of five Cys (Cys^{56}, Cys^{90}, Cys^{91}, Cys^{109}, and Cys^{113}) and two Ser (Ser^{21} and Ser^{164}) of N-terminal half that were conserved between this protein and other PC synthase clones (*TaPCS1* and *SpPCS*) were singly substituted with Ser and Ala, respectively, showed that only Cys^{56} substitution abolished the capacity of heterologously expressed AtPCS1-FLAG to suppress the Cd^{2+} hypersensitivity of yeast strain DTY167 concomitant with the abolishment of both in vivo and in vitro PC synthetic activity and the amenability of enzyme to direct acylation by free GSH. In support, Tsuji et al. [83] also demonstrated indispensability of Cys^{56} residue in N-terminal region for PC synthesis. However, using site-directed mutagenesis studies Romanyuk et al. [84] demonstrated that not only Cys^{56} but also His^{162} and Asp^{180} are indeed required for net PC synthesis. Ruotolo et al. [85] established that two N-terminal fragments ending at positions 372 and 283 are competent in PC synthesis from GSH in presence of Cd^{2+} at rates only fivefold lower than those of full-length enzyme indicating sufficiency of N-terminal domain for catalysis. However, it remained unclear whether N-terminal and/or C-terminal domains participate in metal sensing and/or acylation at second site. Romanyuk et al. [84] demonstrated, using N-terminally hexahistidinyl-tagged full-length AtPCS1(HIS-AtPCS1) and C-terminally truncated enzyme (HIS-AtPCS1_221 tr) containing only 221 N-terminal amino acid residues, that N-terminal domain was activated by metal thiolates (Cd.GS_2), not by free metal ions, while C-terminal domain was not essential for catalysis, as it participated in auxiliary metal sensing to augment the catalytic process. Their results also showed that N-terminal domain of AtPCS1 was sufficient for Cd^{2+}-dependent synthesis of PCs from GSH, but also underwent Cd^{2+}-independent γ-Glu-Cys acylation at C^{56}. However, this domain was not sufficient for Cd^{2+}-dependent γ-Glu-Cys acylation at second site or for the stimulation of S-methyl-PC synthesis from S-methylglutathione by micromolar concentrations of free Cd^{2+}.These reactions required a contiguous C-terminal domain. Thus, C-terminal domain was required for augmentative metal activation and second site acylation of PC synthase, however, acylation position still remains obscure. Vestergaard et al [45] studied the interaction between Cd^{2+} and C-terminal region of PCs and showed that site-directed mutagenesis of Cys residues at $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif decreased the number of Cd^{2+} and other heavy metal ions interacting with the enzyme, which further supported the role of C-terminal region in binding to free metal ions.

Based on these studies, an advanced model explaining clearly how PC synthase is activated and PCs are synthesized, has been developed by us and shown in Fig. 4.

Regulation of PC Biosynthesis

Regulation of PC synthase activity is one of the most important regulatory mechanism of the PC biosynthetic pathway. PC synthase expression was observed to be independent of heavy metal exposure in *S. cucubalis* cell cultures [24], *Arabidopsis* [49], and tomato [50]. These observations imply that the enzyme activation by heavy metals is one of the targets for PC biosynthesis regulation.

The intracellular level of GSH has been found to regulate PC synthesis, in addition to the heavy-metals-induced activation of PC synthase [86, 11]. It is reported that GSH synthesis is regulated by oxidative stress [87, 88]. Xiang and Oliver [89] proposed that PC synthesis was regulated at multiple levels after exposure to Cd, wherein Cd induced the synthesis of PCs from GSH by activating PC synthase and promoted the synthesis of GSH not only through transcriptional activation of the GSH biosynthetic pathway but also by stimulating the endogenous generation of reactive oxygen species, ROS, such as H_2O_2. Similar regulatory mechanisms for the induction of PC synthesis by heavy metals exist in *Dunaliella tertiolecta* [73]. In this green alga, the production of PCs induced by Zn was significantly higher than those induced by Cd as shown in Fig. 5. This was probably due to both a large flux of GSH promoted by an increase in intracellular H_2O_2 levels caused by treatment with Zn and to a more intensive activation of PC synthase by Zn in comparison with higher plants.

In order to amplify PC biosynthesis and heavy metal tolerance, intracellular GSH pool can be improved through overexpression of enzymes of the GSH biosynthetic pathways. This suggestion is supported by coexpression of a variant γ-glutamylcysteine synthetase,

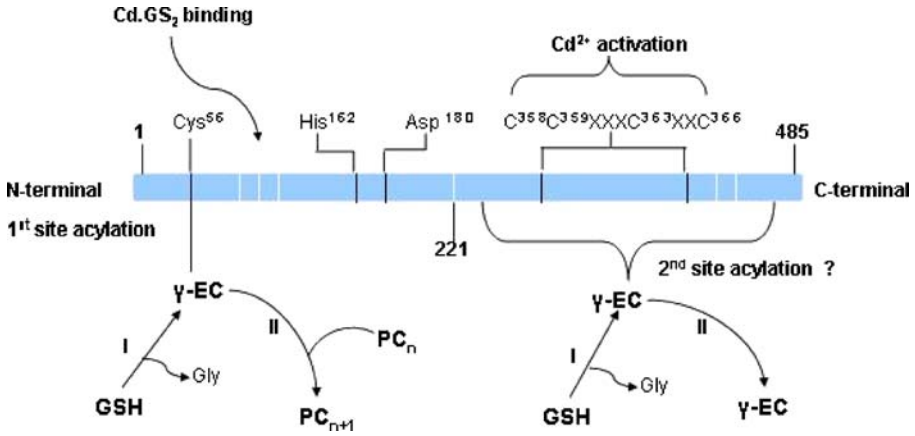

Fig. 4 Model for the function of PC synthase in eukaryotes. The residues Cys^{56}, His^{162}, and Asp^{180} in N-terminal region are required for catalysis. $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif in C-terminal region interact with free Cd^{2+} ions to accelerate the catalytic process. $Cd.GS_2$ binds at N-terminal region to activate the enzyme. In step I of catalysis, Cd^{2+}-independent first site acylation takes place at Cys^{56} with γ-EC transferred from GSH and Cd^{2+}-dependent second site acylation in the C-terminal region (position is unknown). In step II, γ-EC acylated at C^{56} is transferred to GSH (donor) or PCn to generate PCn+1, with the simultaneous release of γ-EC acylated at second site

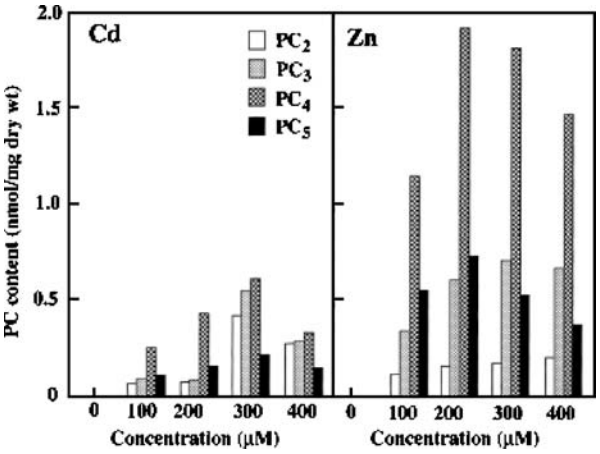

Fig. 5 Effect of Cd and Zn on PC synthesis in marine green alga, *Dunaliella tertiolecta*. Cells were treated with Cd or Zn at the indicated concentrations for 24 h. PCs were determined by HPLC [73]

first enzyme of GSH-biosynthetic pathway, in transgenic *E. coli* (overexpressed with *SpPCS*) which indicated enhanced PCs production and Cd accumulation by ten- and twofold, respectively [90]. Similarly, overexpression of *gsh1* (derived from baker's yeast) in *A. thaliana* had higher tolerance to and accumulated more Cd and As than wild-type due to higher PCs production [91].

In higher plants, PC synthase regarded as constitutive enzyme is expressed independent to heavy metal exposure [92]. This was supported by Northern and reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *AtPCS1*, which showed no transcriptional regulation of *AtPCS1* on exposure to heavy metals such as Cd, Zn, or Cu, or to oxidative stress, salt stress, jasmonic acid, or salicylic acid [25, 81, 93]. However, during early stages of plant development, the level of *AtPCS1* mRNA in *Arabidopsis* was found to increase twofold under Cd-stress as compared with controls [94]. Similarly, semi-quantitative reverse transcriptase-PCR revealed that AtPCS1 expression was increased significantly in roots of *Allium sativum* seedlings under Cd stress [95]. These studies indicate that expression of the PC synthase gene may be regulated in specific organs and at developmental stages, which require a strong defense to avoid heavy metals toxicity. However, a study by Ducruix et al. [96] paved a new insight in the regulation of PC biosynthesis from metabolic profiling analyses, where Cd treated *A. thaliana* cells on glycine supplementation showed lower γ-EC accumulation, enhanced iso-PCs concentration and limited impact on PCs and glutathione concentrations. Taken together, these results indicated that GSH was involved in the biosynthesis of the iso-PCs in vivo and biosynthesis of PC-related peptides was limited by the availability of glycine in the presence of high cadmium concentrations.

Long Distance Transport of PC–Metal Complexes

Recent studies have shown that PCs have the ability to undergo long-distance transport either from shoot to root or from root to shoot. Transgenic expression of wheat *TaPCS1* cDNA in *Arabidopsis* confirmed increased Cd^{2+} transport in stems and rosette leaves [97]. Li et al. [98] showed the long-distance movement of thiol-peptides from shoots down to roots by expressing a bacterial γ-glutamylcysteine synthetase (ECS) in the shoots of an *Arabidopsis* ECS-deficient mutant using a shoot-specific, light-induced regulatory cassette.

Expression of phytochelatin synthase (*TaPCS1*) gene under the control of a shoot-specific promoter (CAB2) in an *Arabidopsis* PC-deficient mutant cad1-3 (CAB2::TaPCS1/cad1-3). CAB2:: TaPCS1/cad1-3 plants exhibited higher Cd accumulation in roots and lower in shoot compared to wild type. Fluorescence HPLC coupled to mass spectrometry analysis directly detected PC_2 in the roots of CAB2::TaPCS1/cad1-3, but not in cad1-3 controls, suggesting that PC_2 was transported over long distance from shoot to root [99]. However, it remained obscure which tissue or vascular system mediated PC translocation and whether PC transport contributed to physiologically relevant long-distance transport of Cd between shoots and roots. To address these queries, Mendoza-Cozatl et al. [32] observed higher ratios of [PCs]/[Cd] and [glutathione]/[Cd] in phloem sap than xylem of *B. napus* in response to Cd exposure, suggesting that phloem was a major vascular system for long-distance source to sink transport of Cd as PC–Cd and glutathione–Cd complexes.

Vacuolar Sequestration of PC–Metal Complexes and Role of Sulfide Ions

The sequestered metals can be stored in subcellular compartments viz. vacuole by means of specialized transporters [100, 101]. The mesophyll protoplasts derived from tobacco plants when treated with Cd, almost all of both the Cd and PCs accumulation was confined to the vacuole [102]. As such, compartmentalization of metals in the vacuole has been regarded a part of tolerance mechanism in some metal hyperaccumulators [103, 104].

Within plant and yeast cells, PC–metal complexes are shuttled to the vacuole by an adenosine triphosphate (ATP)-binding cassette (ABC) type transporter protein in the tonoplast [19, 105]. In the case of Cd^{2+} exposure, two types of intracellular PC–metal complexes are produced: a low-molecular weight (LMW) PC–Cd complex and a more stable high-molecular weight (HMW) PC–CdS complex that contains acid-labile sulfide (S^{2-}). A gene, named *hmt1* (for heavy metal tolerance), encoding a vacuolar membrane protein (HMT1) that is a member of ABC type transporter family was isolated from LK 100, a mutant of *S. pombe* unable to form HMW PC–CdS complex [106]. Ortiz et al. [107] found that HMT1 protein mediated transport of cytoplasmic LMW PC–Cd complex into the vacuole in an ATP-dependent manner. Transport of PC–Cd complex was independent of the pH gradient as it remained unaffected by the vacuolar ATPase inhibitor bafilomycin or the H^+-K^+ ionophore nigericin. Similarly, an ATP-dependent and proton gradient-independent activity of HMT1 for transporting PCs and PC–Cd complexes was identified in oat roots [108]. Furthermore, Vatamaniuk et al. [109] demonstrated that heterologous expression of *hmt1*, from *C. elegans*, in *hmt*$^-$ mutants of *S. pombe* alleviated Cd^{2+} hypersensitivity concomitant with localization of CeHMT1 to the vacuolar membrane. Besides, multidrug resistance proteins (MRPs) were also considered likely candidates to transport phytochelatin–Cd or GS–Cd complexes across the tonoplast [110]. To date, the preeminent characterized ABC type vacuolar transporter and channel involved in metal tolerance is YCF1 of MRPs subfamily from *S. cerevisiae.* It is an Mg-ATP-dependent glutathione S-conjugate transporter responsible for vacuolar sequestration of GSH–metal complexes, that transport bis (glutathionato) cadmium (Cd-GS_2) into vacuoles [19, 111], as well as As-GS_3 [112] and Hg-GS_2 [113]. Recently, Mendoza-Cozatl et al. [114] showed the presence of PC–HMW complexes in chloroplasts of *Euglena gracilis*, suggesting role of chloroplast in Cd resistance mechanism in organisms lacking a plant-like vacuole (Fig. 6).

The sulfide ions were reported to affect the formation of PC–Cd complexes in some plants and in the yeasts *S. pombe* and *Candida glabrata*. The incorporation of sulfide ions into HMW complex increases the number of Cd ions per molecule and stability of complex.

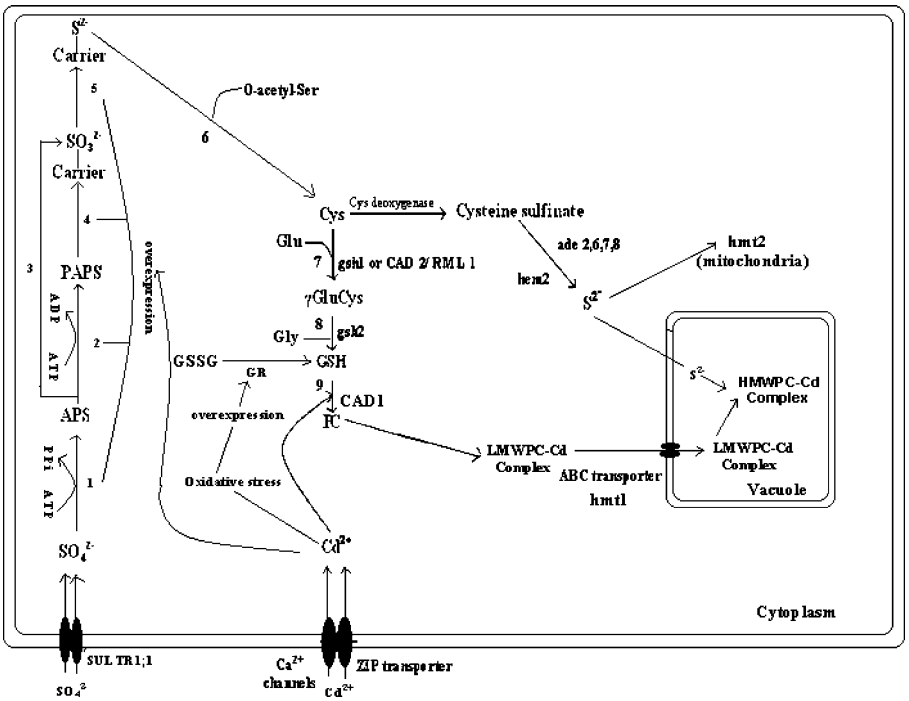

Fig. 6 A molecular model of Cd detoxification showing genes involved, in plants and fungi. Cd^{2+} ions are taken up into plant root cells by Fe^{2+}/Zn^{2+} transporters (belongs to ZIP: Zinc iron protease) and possibly by Ca^{2+} transporters/channels. In the cytosol, Cd^{2+} ion is chelated with PCs to form LMW complex which is transported into vacuole through ABC-like transporters where it forms HMW complex with the incorporation of sulfide ions. Oxidative stress created by Cd^{2+} activates overexpression of glutathione reductase (GR) which reduces oxidized glutathione. Cadmium stress also results in overexpression of enzymes involved in sulfur assimilation. SO_4^{2-} is taken up by sulphate transporters (e.g SULTR1;1 in *Arabidopsis*) and converted to cysteine through sulfur assimilatory pathway in plants [123] and fungi [124]. The enzymes involved in purine biosynthetic pathway carry out the conversion of cysteine sulfinate to sulfide compound to be incorporated in HMWPC–Cd complex. Genes ade2, 6, 7, and 8 encode adenylosuccinate synthetase, aminoimidazole ribonucleotide carboxylase, succinoaminoimidazole carboxamide ribonucleotide and adenylosuccinate lyase, respectively. Enzymes are designated as numericals: *1* ATP sulfurylase, *2* APS kinase, *3* APS reductase, *4* PAPS reductase, *5* sulfite reductase, *6* O-acetyl-Ser sulfhydrylase or O-acetyl-Ser (thiol)-lyase, *7* γ-Glu-Cys synthetase (gene: *gsh*1), *8* GSH synthetase (gene: *gsh2* or *CAD2*), and *9* phytochelatin synthase (gene: CAD1). Genes CAD1 and CAD2 are in *Arabidopsis*; hmt1, hmt2, ade2, ade6, ade7, and ade8 are in fission yeast; and hem2 is in *Candida glabrata*. Abbreviations used: *APS* adenosine 5'-phosphosulphate, *PAPS* 3'-phosphoadenosine 5'-phosphosulfate

Some complexes with a high ratio of S^{2-}/Cd consisting of aggregates of 20 Å-diameter particles appeared to be CdS crystallites surrounded by PC peptides [115, 116]. In the absence of sulfide, the Cys–Cd ratio has been estimated to be from 2:1 to 4:1, which drops to ~1:1 with the incorporation of sulfide ions [117]. Morelli et al. [118] identified increase in Cd/SCys ratio from 0.6 to 1.6 after incorporation of sulfide ions in Cd–PC complex in the Cd stressed *P. tricornutum* cells, which indicated increase in number of Cd ions per CdS crystallite.

During Cd stress, intracellular sulfide levels have been found to increase by five- to sevenfold in *S. pombe* [119]; therefore, the source of sulfide ion is an important issue to consider. The *S. pombe* mutants (carrying genetic lesions in *ade2* and *ade*6, encoding AMP-S synthase and AIR carboxylase, respectively, or *ade2* and *ade7*encoding SAICAR

synthetase) affected in the enzymes of adenine biosynthetic pathway were Cd-sensitive and provided evidence for the importance of sulfide ions in PCs function [120]. In spite of catalyzing the conversion of aspartate to intermediates in adenine biosynthesis, these enzymes could also utilize cysteine sulfinate (generated from cysteine by the action of cysteine dioxygenase) to generate other sulfur-containing compounds [121], which could act as source of sulfur to be incorporated into HMW complexes (Fig. 6). However, Harada et al. [122] reported increased transcription of genes encoding enzymes of sulfur assimilatory pathway (viz. adenosine phosphosulfate (APS) reductase, ATP sulfurylase, and sulfite reductase) and consequently increased cysteine concentrations in Cd stressed *Arabidopsis*. In higher plants, sulfate is reduced to sulfide and then converted to cysteine mainly in chloroplast via APS rather than phosphoadenosine phosphosulfate (PAPS) [123], whereas in other organisms like fungi and bacteria, sulfate is reduced via PAPS [124]. These studies conclude that sulfur assimilatory pathway is the ultimate and conversion of cysteine sulfinate to other sulfur-containing compounds is the direct source of intracellular sulfide ions. The role of sulfur in detoxification of other metals by PCs is still unknown.

The Cd-sensitive, *hem2* mutants of *C. glabrata*, also highlighted the importance of sulfide ions in the PCs function [125]. These mutants were deficient in porphobilinogen synthase, one of the enzymes involved in biosynthesis of siroheme—a cofactor of sulfite reductase for sulfide biosynthesis. This deficiency might lead to Cd-sensitivity in these mutants. However, in *hem*2 mutants of fission yeast, the role of this gene in Cd tolerance is uncertain, but was proposed to mediate detoxification of excess sulfide ions generated during the formation of HMW PC–Cd complex. HMT2, a mitochondrial enzyme, oxidizes sulfide ions. *hmt2*$^{-}$ mutants accumulated high sulfide ions to form CdS, and thereby reduced availability of free Cd ions for creating stress. This resulted in prevention of Cd-induced PC production, upregulation of glutathione production and formation of Cd–glutathione thiolate complex, which was required as a substrate for PCS [126]. Thus, although sulfide is required for PC-mediated metal tolerance, aberrantly high sulfide levels can inhibit this pathway.

Future Application of PC-Based Phytoremediation

The naturally occurring metal hyperaccumulators have gained importance recently due to immense potential of these plants in phytoremediation of heavy metal-contaminated sites. However, due to slow metal extraction coupled with low biomass production and metal recovery process, this technology bears limited scope. Since, it is an established fact that PCs are involved in metal detoxification and/or accumulation in plants, the above problem can be overcome by engineering PC synthase genes in common plants capable of growing fast and producing large biomass. The major targets of phytoremediation technology are larger metal-polluted natural-systems having multimetal conditions. Hence, to achieve its successful application at commercial scale, it is imperative to know the exact nature of PCs produced and their interaction with metals independently and in combinations. Among the heavy metals tested, Cd, which is generally toxic, is the most potent activator of PC synthase enzyme; however, in case of *D. tertiolecta* Zn was found to be much better activator than Cd [73]. Since, the binding capacity of PCs to Zn is much less than Cd, Hg, and As [73], it could be advantageous to incorporate PC synthase gene from *D. tertiolecta* into plants for practical applicability of phytoremediation of various toxic metals.

The isolation of PC synthase genes from a number of species will further clarify understanding of the mechanism of metal-induced PC biosynthesis and the catalytic

mechanism. Stacking of modified genes capable of increasing heavy metal tolerance and accumulation in transgenic lines represents a highly promising new tool for use in phytoremediation especially in nature where often multimetal conditions prevail. In this endeavor, investigations on: (1) three-dimensional characterization of PC synthase for better understanding of its activation by heavy metals (2) genetic regulation of PC synthase under various conditions (3) tissue-specific localization of PC synthase, and (4) determining origin and destination of PC–metal complex transport pathway, will provide additional insights for designing and/or improving phytoremediation technology for its success enabling wider application.

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