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HEAVY METAL BINDING POLYPEPTIDES IN PLANTS AND THEIR ROLE IN METAL DETOXIFICATION

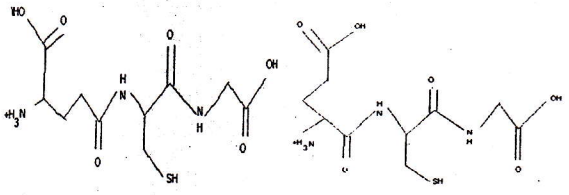
G. NAGENDRA BABU, R. RANJANI , G. FAREEDA and S .D .S. MURTHY
Department of Biochemistry, Sri Venkateswara University, Tirupati-517502, India.

Heavy metals are a very heterogenous group of elements varying in chemical properties and biological functions. In plants the heavy metal binding polypeptides are synthesized which are induced by heavy metals. These have distinct homology to animal metallothioneins and are called as phytochelatins. Glutathione serves as a precursor of phytochelatins. Phytochelatin synthase is the enzyme which is activated by the heavy metal cations Cd,Ag,Bi,Pb,Zn,Cu,Hg and Au, and is completely inactive in their absence. Its activity can be inhibited in the presence of buthionine sulfoximine. Phytochelatins are involved in metal homeostasis, detoxification and sulfur metabolism.

Keywords: Glutathione; Metallothionein; Metal toxicity; Phytochelatin.

Introduction and classification:

The heavy metal binding polypeptide synthesis is induced by heavy metals and has generalized structure (γ-Glu-Cys)n-Gly, n=2-7 (Fig. 1). They have trivial names Cadystins[1], Phytochelatins (PCs)[2], γ-Glutamyl peptide[3], poly- (γ-Glutamyl Cysteinyl) glycines[4] and cadmium peptide[5]. Cadystins holds priority and is restricted to yeasts. PCs suggest chelating agents and approximates phycogenitic distribution of peptides in nature.



γ-Glu-Cys- Gly α-Glu-Cys- Gly

Fig.1. Structures of Metal binding polypeptides.

Excess intra cellular heavy metals induce biosynthesis of proteins called metallothiones in animals[6]. Early efforts in understanding the detoxification of excess heavy metals in plants depended largely on models based on metallothioneins (MTs). The structural and regulatory conservation exhibited by metallothioneins from a broad array of organisms suggested that proteins similar in structure and function to metallothioneins would occur in plants. Studies of plants exposed to heavy metals revealed the apparent presence of metallothionein like proteins[7,8]. The metal inducibility of these proteins was demonstrated in cabbage, tobacco leaves[9,10] and in maize[11].

Biosynthesis and Primary sequence of PCs

In contrast to animal systems, metal-binding polypeptides in plants showed non-protein metal binding polypeptides that differ in structure and biosynthesis and are functionally analogous to metallothiones[2,9,12]. Glutathione serves as a precursor of PCs, which are composed of two or more repeating gamma-glutamylcysteine units with a terminal glycine residue (gamma-glutamylcysteine)$_n$-Gly[5,12], where n = 2 to 11. In comparison, GSH has the structure γ-Glu-Cys-Gly in which the peptide bond is formed between γ or side chain carboxylate of glutamic acid rather than the α-carboxylate utilized in peptide bonds of polypeptides whose synthesis is ribosome dependent. ATP dependent γ-glutamyl cysteine synthetase catalyses the reaction between Glu and Cys to form γ-Glu-Cys. In those plants that produce homo ß-Ala specific ATP dependent enzyme homoglutathione synthetase uses γ-Glu-Cys to produce γ-Glu-Cys- ß-Ala. Induction of phytochelatins by heavy metal ions is given in Table -1.

PC synthesis is inhibited by BSO[12]. Buthionine sulfoximine treated cells are unable to synthesize PCs and become susceptible to growth inhibition by heavy metals at concentrations lower than normal that inhibit in absence of BSO[12]. The sensitivity to metals conferred by BSO may be in part due to elimination of an initial GSH complexation step leading to decreased free metal concentrations in the time before significant phytochelatin accumulation occurs.

In the first hours after the metal ion exposure, the rate and extent of GSH disappearance is nearly equal to the rate of γ-glutamyl cysteine incorporation in to PC

Table 1. Induction of phytochelatins by heavy metal ions.

Salt formula	Concentration	Total γ-Glu-Cys in PC (μ mol g^{-1})
$Cd(NO_3)_2$	100	20.5
$Pb(NO_3)_2$	1000	11.4
$ZnSO_4$	1000	8.5
$SbCl_3$	200	8.5
$AgNO_3$	50	8.2
$Ni(NO_3)_2$	100	5.8
$Hg(NO_3)_2$	10	4.3
Na_2HAsO_4	20	3.8
$CuSO_4$	50	3.1
$SnSO_4$	100	2.8
$NaSeO_3$	100	2.4
AuCl	50	2.0
$Bi(NO_3)_3$	100	1.9
$TeCl_4$	10	1.8
WCl_6	100	1.1
None	--	0

and is not affected by buthionine sulfoximine[2] . PC synthase is activated by the heavy metal cations Cd, Ag, Bi, Pb, Zn, Cu, Hg and Au, and is completely inactive in their absence. Relative ability of the different metal ions to activate metal PC synthase *in vitro* was found closely mirror their ability *in vivo* to induce PC synthesis[2]. Ability of the *in vivo* anionic inducers selenate and arsenate to activate PC synthase was not reported[2]. PC synthesis ceases immediately after addition of EDTA or metal free PCs due to activation of the enzyme by metal ion. When sufficient PCs have been synthesized to complex free metal ions, activity ceases[2].

The peptide bond in the repeating Glu-Cys pairs is a γ -carboxyamide linkage, which is not synthesized on ribosomes. These come under class III metallothiones which are defined as atypical, non-translationally synthesized metal thiolate polypeptides[13]. These polypeptides were originally described by Murasugi *et al.*[14, 15] in fission yeast *Schizosaccharomyces pombe*, induced by cadmium and called them cadystins. Grill and co-workers[2] reported a similar series of metal binding peptides, $(\gamma\text{-Glu-Cys})_{3-7}$-Gly. The term PCs was proposed for this structure based on wide sequestration of metal ions[2]. In legumes that produced the homologous tripeptides homoglutathione or γ-Glu-Cys-β-Ala, a second family of γ-Glu-Cys peptides was anticipated. The cadmium binding complexes from those plants contained another set of peptides $(\gamma\text{-Glu-Cys})_n$-β-Ala named homoPCs. Cd induced γ-Glu-Cys peptides with a carboxy terminal serine were found in certain species of Poaceae[16]. The primary structures were γ-Glu-Cys-Ser. Since these peptides were related to the tri peptide hydroxyl methyl-GSH (γ-Glu-Cys-Ser), these polymers were named hydroxymethyl PCs. Recently in maize $(\gamma\text{-Glu-Cys})_n$-Glu, a novel tripeptide was isolated[17] . The enzymatic assembly of amino acids in to the tripeptide glutathione and homo glutathione is well characterized in plants.

Occurrence- The data on the occurrence of PCs among monocotyledons, dicotyledons through to the red, green and brown algae is well documented[18-20]. No other thiol rich, heavy metal binding constituents other than PCs were detectable in many plants assayed. The yeast *S.pombe* and *Candida glabrata* respond to cadmium producing $(\gamma\text{-Glu-Cys})_n$-Gly and $(\gamma\text{-Glu-Cys})_n$. GSH2 gene in *S. pombe* encodes a bifunctional enzyme to catalyse both synthesis of GSH and the synthesis of PCs[21]. PC synthase genes have been identified in *Arabidopsis* and other plant species as well as in number of animal species suggesting PCs play a wider role in metal detoxification than previously anticipated[22].When exposed to excess Cu, C. *glabrata* produces two MT like proteins possessing 30mol% Cys and two repeats of Cys-X-Cys motif typical of MTs.

Rice, wheat, rye, oats are graminaceous species that produce $(\gamma\text{-Glu-Cys})_n$-Gly, $(\gamma\text{-Glu-Cys})_n$ and $(\gamma\text{-Glu-Cys})_n$-Glu[17]. Mature wheat embryos were reported to contain MTs[21]. The protein present in the wheat germ embryos contain 59 amino acids where 10 cysteine residues showed broad homology with 19 cysteine residues of rat liver MT[20] .

The enzyme responsible for the synthesis of these peptides is known as phytochelatin synthase[19,23] . PCs play an important role in the detoxification of certain heavy metals (particularly cadmium) in plants[19,23-25]. These peptides appear upon induction of plants with metals of the transition and main groups (Ib-Va, Z = 29-83) of the periodic table of elements[26]. In *Rubia tinctorum* PCs (class III metallothionein) are induced by many metal ions, but only a few (Ag, Cd and Cu) were bound to the PCs that they induced[27]. These peptides are induced in all autotrophic plants so far analyzed, as well as in certain fungi[26]. Phytochelatin synthase (PC synthase) (glutathione gamma-glutamylcysteinyltransferase or gamma-glutamylcysteine dipeptidyl transpeptidase) [EC 2.3.2.15] is a constitutive enzyme that is activated by cadmium and other metal ions[23]. It catalyzes the following reaction: gamma-Glu-Cys-Gly + $(\text{gamma-Glu-Cys})_n$-Gly-->$(\text{gamma-Glu-Cys})_n$+1-Gly + Gly.

The isolation of a Cd^{2+}-sensitive *cadl* mutant of

Arabidopsis thaliana, that is deficient in PC synthase, demonstrates conclusively the importance of PC for heavy metal tolerance[24-26]. Over expression of the *E. coli* glutathione synthetase gene in Indian mustard leads to increased cadmium tolerance, in part due to increased production of PCs[28]. Cadmium tolerance and accumulation in Indian mustard is also enhanced by overexpressing gamma-glutamylcysteine synthetase[29]. In certain plants (notably legumes), which can synthesize homoglutathione, in which B-alanine is substituted for glycine as the terminal amino acid, homoPCs are synthesized along with PCs in response to Cd[16].

In maize and certain other members of the Poaceae, a third family of PCs has been found in which serine is the carboxy-terminal amino acid[30]. Some members of the family Poaceae synthesize PCs that contain glutamic acid at their C-terminal end[26]. The plant vacuole is the transient storage compartment for these peptides[26]. Cd detoxification may require transport of the Cd-phytochelatin complexes into the vacuole. A transport system has been recently described for these complexes[31]. Glutathione-S-conjugates are also transported into the vacuole in an ATP-dependent manner. The Cd-phytochelatin complexes probably dissociate, and the metal-free peptide is subsequently degraded[26].

In *Arabidopsis*, both Cd and Cu induce transcription of the genes for glutathione synthesis (gamma-glutamylcysteine synthetase and glutathione synthetase), as well as glutathione reductase[32]. Jasmonic acid also activates the same genes, but does not elevate glutathione content[32]. Although nucleic acid sequences and proteins are found in higher plants that have distant homology to animal metallothioneins, there is little evidence that these "plant metallothioneins" are involved in the detoxification of heavy metals[26].

PCs are distinctive in nature and are induced by certain chemicals. Among the common metals Cd seems to be strongest inducer where as Zn appears to be weaker (Table 1).

Metal binding - Complexes of $(\gamma\text{-Glu-Cys})_n$-Gly are extremely heterogonous because of multiple peptide components and many possible combinations of complexes that can arise from these mixtures. Thus stoichiometry is based on mols sulfhydryl rather than mols peptide. Ratio of Cys-SH to metal in phytochelatin complexes is 2:1 for Cd, Zn, and Pb. Copper present in $(\gamma\text{-Glu-Cys})_n$-Gly complex as Cu(I) is bound to sulfhydryls at a ratio of 1:1[33].

Ligands - The metal ligand in PC complexes is the cysteine thiol. There is no evidence of carboxylate and amino group participation in this function. Charge transfer transition exhibited at 254 nm in native PC complexes indicates the presence of Cd thiolate ligands. Cotton effect extrema at 254 nm in circular dichroism spectra are reminiscent of Cd MT spectra and indicative of similar Cd thiolate ligands[2]. UV excitation of *S. pombe* Cu complex results in luminescence at 619 nm. Binding of metals by PCs is pH dependent with low pH favoring protonation of thiolate ligands and displacement of metal ions. The Cu ion 50% displaced from PC complex at pH 1.3[3].

Molecular mass - PC complexes elute as broad peaks in gel permeation complexes. The M_r of Cu and Cd PC complexes varies from about 3000 to 10000 depending upon ionic strength[18]. The lower M_r observed at high ionic strengths suggests that complexes possess a trimeric or tetrameric peptide stoichiometry[18]. The high M_r observed at lower ionic strengths suggested to result from electrostatic repulsion of the negatively charged free Glu carboxylates of the polypeptides and form complex aggregation[34].

Labile Sulfide - Acidification of purified PC complex results in the evolution of H_2S, indicating the presence of labile sulfide ion[12] (Fig. 2). High-sulfide complexes of the yeasts *C. glabrata* and *S. pombe* possess properties of quantum semiconductor crystallites[35]. One effect of sulfide in the PC complex is the stabilization of the pH at which dissociation of metal occurs. Presence of sulfide in Cd complexes leads to a characteristic UV transition in the region 305 - 318 nm whose maximum is dependent on quantity of sulfide in the complex. Incorporation of sulfide ion and the resulting higher stability and metal binding capacity may increase the effectiveness of these peptide complexes as mechanism for sequestration of toxic metals.

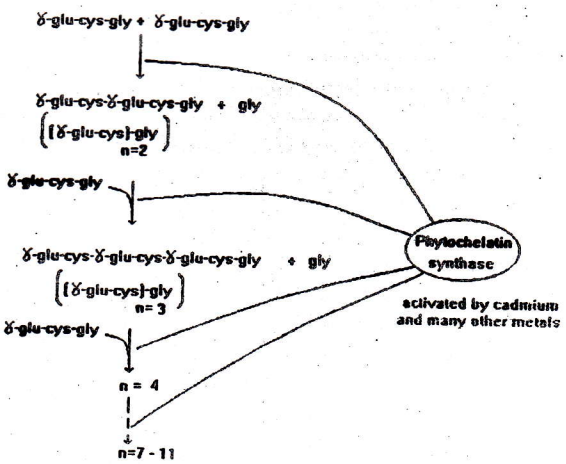


Fig. 2. Sulfate uptake and assimilation in PCs.

Labile Sulfie - An important aspect of labile sulfur in PC complexes is the presence of sulfur in oxidation states other than sulfide. In tomato cells labile sulfite is present at levels several times higher than labile sulfide. Like sulfide, sulfite ion is labile to both EDTA and acidification. PC synthesis, ATP sulfurylase and APS sulfotransferase activities are coordinately regulated by Cd exposure.

Functions of PCs - The synthesis of heavy metal binding polypeptides is one of the few examples in plant stress biology in which it can be readily demonstrated that the stress response (PCs synthesis) is truly an adaptive stress response. Plants possess a range of potential cellular mechanisms that are involved in the detoxification and tolerance to metal stress. These include reduced uptake or efflux pumping of metals at the plasma membrane, for chelation of metals in the cytosol by PCs, for the repair of stress damaged proteins, for the compartmentalization of metals in the vacuole by tonoplast located transporters[36].

Metal homeostasis and sulfur metabolism - Copper and Zinc are essential micronutrients, which interfere with sulfhydryl groups of proteins. As new Cu or Zn requiring apoenzymes are formed, the metal binding complexes would supply the necessary cofactors. To accept the view that PC participated in metal ion homeostasis requires the assumption that Cu and Zn were bound in vivo to PC. Sulfate reduction in leaves occurs in chloroplasts, which led to the suggestion that PC biosynthesis was localized in these organelles. However, roots of Cd treated maize seedlings showed more sulfate reduction than leaves[37]. Within one day, increasing Cd concentrations in roots raised the activity of ATP - sulfurylase and adenosine 5'phosphosulfate sulfotransferase. This is the aberrant sulfur and PC metabolism specifically triggered by the non-essential element Cd.

Metal detoxification and tolerance - Identification of differential metal tolerance in plants depends on assays of root growth, itself governed by an un specified number of characters. In *H. lanatus, Agrostis capillaries, Chlamydomonas reinhardtii, M. guttatus* and *S. vulgaris* one or two major genes give differential tolerance to arsenate, Cd, or Cu with another genes as modifiers[38]. No locus of differential tolerance has been isolated and cloned. Explanations regarding differential metal tolerance among naturally occurring selected ecotypes have been sought on the basis of γ-Glu-Cys peptides because of their propensity to bind metals. In the case of Cu and Cd the analysis were made on the apical portion of roots, the regions actually exhibiting differential growth. It was concluded that elevated production of PCs was not instrumental in producing differential metal tolerance. Cd binding complex in entire roots of Silene was a sink for excess Cd rather than the cause of differential tolerance. The retention of plant Cd by roots ranged from 10-97% for various species[39]. Molecular mechanisms of understanding the intracellular metal trafficking by chelators and chaperons were studied by Clemens[40].

Conclusions and future perspectives - PCs are the primary metal binding polypeptides of plants and enzyme catalyzing their biosynthesis PC synthase is constitutively expressed in plants. PCs are also involved in trace metal homeostasis and their participation in detoxification of excess metals may be a consequence of this homeostatic function. PC complexes are heterogenous in their peptide composition and contain labile sulfur in addition to heavy metals. Understanding the function and biosynthesis of labile sulfur in PC complexes may illuminate the evolution of this pathway in plants. Differential screening of Cu induced roots of Cu tolerant *Mimulus guttatus* showed a MT with the 3Cys-X-Cys sequences. Determination of its role in the evolution of metal tolerance in *Mimicus* and its occurrence and regulation in other species will be met with great interest.

There are two points of caution about PCs from a physiological view. First it is possible that the reactions of plants and certain fungi to excess Cd, a non-essential element, may be specific to Cd rather than general for other metals such as Cu, Ni or Zn. Perhaps it is fortuitous that Cd is the best apparent inducer of PCs and that it stimulates sulfate metabolism to the degree that sulfite and sulfide accumulate to participate in Cd complexation. Second, intact plants grown in metal polluted soils may utilize other mechanisms in addition to PCs to manage excess metals, which occur in the soils at concentrations much lower than those used in our usual experimental model systems.

Further development of techniques such as miniaturization and quantitation of specific complexes in various plant tissues will enhance progress towards evaluating the actual functional import of the γ-Glu-Cys peptides in cellular metal sequestration. Elucidating the nature of the gene for differential metal tolerance and the connection with the gene for differential metal tolerance and the connection with the gene for dipeptidyl transpeptidase may offer additional tools for amelioration of metal impacts in food production and bioremediation of contaminated soils.

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