HEAVY METAL-BINDING PEPTIDES AND PROTEINS IN PLANTS. A REVIEW

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In plants, two kinds of specific metal-binding peptides or proteins are synthesized. Plant metallothioneins (MTs) and MT-like proteins are cysteine-rich translation products of genes inducible in tissue-specific manner during embryogenesis and plant development. In addition, differential expression of MT-like protein genes could be due to variation of external heavy metal concentrations (especially of Cu^{2+} and Fe^{2+}), influence of various stress factors (heat shock, sucrose starvation, oxidative stress, wounding, plant pathogens). The principal role of plant MTs and MT-like proteins seems to be in homeostasis of essential transition metals rather than in metal detoxification. Phytochelatins (PCs) have general structure $(\gamma\text{-Glu-Cys})_n$-Xaa, where n = 2–11 and Xaa amino acids Gly, β-Ala, Ser, and Glu which depend on the species; the des-Xaa forms of PC also exist. PCs are synthesized in plants and some yeasts by a constitutive enzyme phytochelatin synthase (active only in the presence of

free heavy metal ion) from glutathione or its anologue. Despite the PC capability of forming complexes with transition metal ions (their role in metal homeostasis could not be excluded) and virtually prominent role in Cd^{2+} detoxification within plant cell, there is no evidence that elevated production of PCs may contribute to differential tolerance and/or could be responsible for the resistance to toxic metals. A review with 172 references.
Key words: Metallothioneins; Metallothionein-like proteins; Phytochelatins, Metal binding; Metal tolerance; Metal homeostasis; Metalloproteins.

1. INTRODUCTION

Heavy metals are bound in many metalloproteins by electrostatic forces and coordination bonds. Essential heavy metals act in catalytic sites of metalloenzymes and enable specific folding of many other metalloproteins. Examples of their importance for metalloprotein folding are many DNA binding proteins. The conformation of these proteins is stabilized by tetrahedral coordination of Zn^{2+} in "zinc finger" DNA binding domains *via* cysteine and histidine residues. Cadmium or other nonessential transition metals as well as the essential metal ions in excess levels can act as poisons because of their high affinity to amino acid residues involved in important biological functions.

The poisonous levels of free heavy metal ions are detoxified in many organisms *via* binding to intracellular ligands. A group of such ligands of particular importance was named metallothioneins[1–3]. The name metallothionein (MT) was first used by Margoshes and Vallee[2] for a cadmium-binding protein from mammalian kidney. Metallothioneins, isolated subsequently from other sources, were subdivided into classes on the basis of structural similarities. Class-I MTs are defined as polypeptides whose primary structure is related to mammalian MTs, while those of class II display none or only a very distant sequence similarity to mammalian MTs (ref.[3], Fig. 1). Mammalian MTs are usually composed of 61 amino acids, lacking aromatic amino acids and histidine. Their molecular weight is 6–7 kDa. Two distinct domains of MTs co-ordinate 7 divalent or 12 monovalent metal ions by 20 Cys residues, which are shared along the sequence. The Cys-X-Cys (where X is an amino acid residue other than Cys) and Cys-Cys sequence motifs are characteristic of and invariant for both class I and class II MTs. Divalent transition metals are in mammalian MTs bound in tetrahedral tetrathiolate clusters[4].

Expression of mammalian MTs is induced not only by metal ions, but also by certain hormones, cytokines, growth factors, tumor inducers and

MT of class I

Human, *Homo sapiens sapiens* (MT-2)[3]

```
MDPNCSCAAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASDKCSCCA
    * *     * *   * *  * *  *   ** **   *  *   * *      * **
```

MT of class II

Yeast, *Saccharomyces cerevisiae* (CUP1)[85]

```
MFSELINFQNEGHCQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSCCSGK
              * * *  *     *   * *   *     * *          **
```

Wheat, *Tricium aestivum* (E_C)[17]

```
MGCNDKCGCAVPCPGGTGCRCTSARSDAAAGEHTTCGCGEHCGCNPCACGREGTPSGRANRRANCSCGAACNCASCGSTTA
```

Maize, *Zea mays* (similar to E_C)[19]

```
MGCDDKCGCAVPCPGGKDCRCTSGSGGQR..EHTTCGCGEHCECSPCTCGRATMPSGRENRRANCSCGASCNCASCASA
  *   * *   *     * *              * *   * *  * *                * *   * *  *
```

MT-like protein of type 1

Pea, *Pisum sativum* (PsMTA)[20]

```
....MSGCGCGSSCNCGDSCKCNKRSSGLSYS.EMETTETVILGVGPA.KIQFEGAEMSAASEDG.GCKCGDNCTC.DPCNCK
```

Mimulus guttatus[21]

```
...MSSGCSCGSGSKCGDNCSC.SMYPDM....ETNTTVTMIEGVAP.LKMYSEGSEKSFGAEGGNGCKCGSNCKC.DPCNC
```

Arabidopsis thaliana (MT1a)[29,30]

```
..MADSNCGCGSSCKCGDSCSCEKNY.................................NKECD.NCSCGSNCSCGSNCNC
       * *   * *   * *                                        (*)  * *   * *   * *
```

MT-like protein of type 2

Tomato, *Lysopersicon esculentum* (LeMTB)[43]

```
MSCCGGNCGCGSSCKCGNGCGGCKMYPDMSYTESSTTTETLVLGVGPEKTSFGAMEMGESPVAEN.GCKCGSDCKCNPCTCSK
```

Arabidopsis thaliana (MT2a)[29,30]

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MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNAESD.ACKCGSDCKCDPCTCK
```

Arabidopsis thaliana (MT2b)[30]

```
MSCCGGSCGCGSACKCGNGCGGCKRYPDL....ENTATETLVLGVAPAMNSQYEASGETFVAEND.ACKCGSDCKCNPCTCK
  **   * *   * *   * *                                          * *   * *  * *
```

MT-like proteins different from type 1 and 2

Douglas-fir, *Pseudothsuga menziesii*[39]

```
MSSDGKDCGCADPTQCDKKGNSLGVEMVETSYDYNMNMSFGFEYEMETVA............AEN.GCKSGASSKYSN.RCN
```

Kiwi fruit, *Actinidia deliciosa*[28]

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MSDKCGNCDCADSSQCVKKGNSIDIVETDKSYIEDVVMGVPAAESGGK...................CKCGTSCPCVNCTCD
   (*) * *     *                                                    *(*   * *  *)*
```

MT of class III – phytochelatins (PCs)

PC_n are abundant in plants and some yeasts

PC_3:	γECγECγECG;	*iso*PC_3 (βAla):	γECγECγECβA	*iso*PC_3 (Glu):	γECγECγECE
des-Gly-PC_3:	γECγECγEC;	*iso*PC_3 (Ser):	γECγECγECS;		

FIG. 1

The amino acid sequence of metallothioneins (MTs) and MT-like proteins. The class-III MTs named phytochelatins (PCs) are also included. The dots are included for optimum alignment of homologous sequences and asterisks emphasize the cysteine distribution. The first eight amino acids which are cleaved from CUP1 after translation are italicized. The PC_n are generally of n = 2–11. Note that glutamic acid of glutamylcysteinyl repeats provides γ-carboxyl for the peptide bond (for details see the text and Fig. 2)

chemical and physical stress[3,5]. Induction of MTs is transcriptionally regulated and *cis*-acting metal regulatory elements and *trans*-acting factors are known in animals, yeasts and cyanobacteria[6]. A large number of factors inducing synthesis of MTs and variation of their levels during organism development, regeneration and reproduction, suggest that they act not only in detoxification of harmful chemicals[5] but have other, likely primary, roles in metal homeostasis and formation of pool of essential heavy metals.

Metallothioneins isolated from other than animal sources such as yeasts (*Saccharomyces cerevisiae*, *Candida glabrata*, *Candida albicans* – ref.[7]), algae (ref.[8]) or cyanobacteria (*Synechococcus sp.*[9,10]) are of class II. The only species of lower organisms synthesizing MT of class I reported so far is ascomycete *Neurospora crassa*[11]. The geometry of metal coordination by class-II MTs shows a close similarity to that of mammalian MTs.

The class III of metallothioneins was established as well. As MTIII were classified polypeptides called cadystins, γ-glutamyl peptides, poly(γ-glutamylcysteinyl)$_n$ glycine, γ(EC)$_n$G or phytochelatins. These peptides of nontranslation origin posessing γ-Glu-Cys repetitions are in this review referred as phytochelatins (PCs) since this name has become most widely accepted, indicating the general abundance of PCs in the plant kingdom (*i.e.*, in higher plants and fungi) and the fact that glycine is not the only C-terminal amino acid of PCs. In addition to PCs, plants produce also metallothioneins of class II and proteins showing in some features similarity to MTs of class I and II in both primary structure and predicted function – metallothionein-like (MT-like) proteins.

Here we present the overview of current understanding in the developing field of plant MTs and MT-like proteins of plants and summarize knowledge of synthesis and function of PCs in plants. We would also like to turn the reader's attention to previous reviews[6,12–15] on the two topics published in 1990–1996.

2. METALLOTHIONEINS AND METALLOTHIONEIN-LIKE PROTEINS OF PLANTS

2.1. Plant Metallothioneins

The only well-characterized plant MT of class II, designated E_c protein, has been isolated from wheat (*Tricum aestivum*) germ[16,17]. In contrast to mammalian MT genes, which are organized in multigene clusters, E_C genes are in single copies localized on chromosomes 1A, 1B and 1D of hexaploid wheat[17]. E_C genes are expressed during embryogenesis in developmental-stage-dependent manner. The highest levels are detected at the earliest

stages when rapid cell division and differentiation occurs. Upstream the sequence coding the E_C protein, which binds Zn^{2+} with stoichiometry of 5 metal ions to 1 E_C molecule[18], sequences homologous to abscisic acid-(ABA)-responsive elements were identified but there was no homology with known metal-responsive elements[17]. Indeed, on addition of ABA but not of Zn^{2+} to germination media increased the level of E_C mRNA. The role of E_C protein in homeostasis of Zn^{2+} and regulation of zinc-dependent metalloproteins during development but not for storage of Zn^{2+} in mature wheat embryos (only 5% of Zn^{2+} associated with E_C) has been thus proposed[6,17].

The cDNA of gene encoding a protein showing high similarity to wheat E_C protein (Fig. 1) was isolated from maize[19] (*Zea mays*). To our knowledge, these peptides from wheat and maize are the only plant MTs of class II described so far.

2.2. Metallothionein-Like Proteins

Although the E_C protein of wheat and its maize homologue are the only plant proteins classified as MTs (of class II) the occurrence of "metallothionein-like" (MT-like) proteins showing high similarity to mammalian MTs is reported since the early 90's. According to classic definition, MT-like proteins are rather peptides because their relative molecular weights are lower than 10 kDa (see below). However, as they are referred as MT-like proteins in literature, this name will be used here either. Discovery of this family of proteins originally resulted from screening of cDNA libraries in search for ethylene-regulated genes, root-specific mRNA's, transcripts repressed by elevated Cu^{2+} or induced by iron depletion[6]. The cDNA sequences corresponding to MT-like proteins have been found in many plants of both monocots and dicots[20–46]. The name "metallothionein-like" (MT-like) is due to the fact that these proteins do show (at least on the primary structure level) some features distant from that of MTs (Fig. 1 and the text below).

A computerized comparison of amino acid sequences predicted from the earliest discovered cDNA sequences corresponding to MT-like proteins from *Pisum sativum*[20] and *Mimulus guttatus*[21] revealed their high similarity to MT I and II which is due to Cys-rich domains at the N- and C-termini of MT-like proteins (Fig. 1). Analysis of cDNAs isolated subsequently from other sources revealed that bulk of plant MT-like proteins exhibit more than 50% sequence homology and could be sorted on the basis of predicted location of Cys residues into two groups[6]. In type 1, there is exclusively Cys-X-Cys

motif whereas in type 2 there are Cys-Cys and Cys-X-X-Cys motifs abundant within N-terminal domain. However, some recently described sequences could not be grouped with either type as Cys distribution at N-terminal domain (mRNAs isolated from kiwi fruit *Actinidia deliciosa*[28], papaya fruit *Carica papaya*[38], apple *Malus domestica*[42], banana *Musa acuminata*[40], citrus fruit *Citrus unshiu*[45]) or that of both C- and N-terminal domains (sequence found in Douglas-fir *Pseudotsuga menziesii*[39]) differs from the Cys arrangement of types 1 or 2 (Fig. 1).

Most of plant MT-like proteins consist of about 63–83 amino acids. Terminal Cys-rich domains of plant MT-like proteins are separated by a central region without any Cys residues. The presence of this region is the principal difference from mammalian and fungal metallothioneins as well as from wheat E_C protein and its homologue from maize (Fig. 1). The size of the central region usually varies around 40 amino acids (*e.g. A. deliciosa*: 32 amino acids[28], *M. guttatus*: 39 amino acids[21], *Vicia faba*: 45 amino acids[41]) and contains also aromatic amino acids, which are "forbidden" in MTs. The importance of central region for protein folding or its targeting was hypothesized[6,47] in light of the fact that the 42-amino-acid central region of a recombinant MT-like protein from pea *P. sativum* (product of $PsMT_A$ gene) was cleaved both in *Escherichia coli* and *in vitro* by proteinase K. The metal binding ability of both cysteine-rich cleavage products was retained. However, similar proteolysis of this protein has not yet been proved in plants. Moreover, the recombinant MT-like protein of *Arabidopsis thaliana* (type 2 named MT2a), possessing the central region which is of 50% homologous to that of pea (Fig. 1), was shown to remain intact in cyanobacteria *Synechococcus* PC 7942 as well as in *E. coli*[48]. Later, Murphy *et al.*[49] isolated MT2a from various tissues of *A. thaliana* as a fraction of 8 kDa corresponding to full-size protein as should be predicted from its mRNA sequence. Besides "long" MT-like proteins, also short MT-like proteins (45 amino acids) are abundant in plants (with lower frequency). These MT-like proteins (Fig. 1) possess "full-size" Cys-rich domains, but the "central spacer" containing one aromatic amino acid (usually Tyr) is reduced to 7 amino acids (*A. thaliana*[29,30], *Brasica napus*[26]). The general role of the large central region in MT-like protein folding or targeting thus seems to be unlikely at present.

2.2.1. Regulation of Expression of MT-Like Genes and Metal Homeostasis

The information on the expression of MT-like genes are based mostly on detected levels of mRNA's as the corresponding proteins are difficult to de-

tect with the comparable sensitivity in plant tissue at present. However, it was shown that the level of translated product in Arabidopsis reflected the extent of MT-like protein mRNA transcription[49]. No general conclusions on expression of various MT-like proteins even within given plant species could be ruled but the transcription of type 1 of MT-like proteins is considerably stronger (but not exclusive) in roots than in aerial portions of the normally growing plants[20–22,24,29,30,36,41,49,50]. The opposite pattern could be followed with type 2 MT-like proteins[29,30,41,43].

The organization of structural genes of MT-like proteins is not unique: they are either clustered[29,36] similarly to mammalian MTs, or appear at different genetic *loci* even on different chromosomes[29,44]. The structural genes of MT-like proteins usually contain one or two introns. The enhancer function of intron from rice (*Oriza sativa*) MT-like protein of type 1 gene was demonstrated by *in vitro* experiment[51]. The identification of upstream ABA as well as ethylene-responsive elements from the genomic clones of MT-like protein genes[43,50–52] confirmed a direct connection between the plant germination, development and/or tissue type and MT-like protein gene transcription observed *in vivo*[32,39,53,54]. With developing Arabidopsis seeds, the level of type 2 MT-like protein (MT2) transcript remained unchanged whereas temporal variations indicating possible regulation of metal pool showed the type 1 MT-like protein (MT1) mRNA (ref.[55]). In transgenic *A. thaliana* bearing GUS (β-glucuronidase) gene from *E. coli* fused with upstream promoter region of pea $PsMT_A$ gene, a high level GUS activity was detected in the roots as it could be expected for type 1 MT-like protein[50]. In-soil grown plants, where the *GUS* expression was generally low, the GUS activity was absent in the more mature root regions indicating putative role of $PsMT_A$ during cell differentiation. The 298 bp region of $PsMT_A$ upstream sequence bearing ethylene-responsive element appeared to be crucial for gene transcription in roots. The specific accumulation of MTs in animals was reported to be linked with the differentiation of embryos[56] when a variety of metal (Zn^{2+})-dependent in *trans*-acting DNA binding factors is required. A similar role of MT-like proteins in homeostasis of essential heavy metal ions is in light of the aforementioned findings thought to be *ad dispute*. However, their ability to modulate the action of metal-dependent factors must be demonstrated.

The transcription of MT-like genes by plants grown in excess of heavy metals is organism- and metal-specific. The most pronounced effect has Cu^{2+}. For example, in response to elevated external Cu^{2+}, the level of type 2 MT-like mRNA was decreased in *Brassica juncea* whereas Zn^{2+} had a reverse effect[57]. In some cases, the exposure of plants to increased environmental

concentrations of heavy metals showed a very small or no effect on the levels of particular MT-like protein transcripts[21,32,41]. A complex metal-induction pattern was observed with MT-like gene family of *A. thaliana*. In original study[29], Zhou and Goldsbrough reported that there were two MT-like protein mRNAs found in *A. thaliana*. The MT1 transcripts were present predominantly in roots and dark-grown seedlings of Arabidopsis, whereas mRNA of MT2 accumulated more in leaves, as it is characteristic of type-1 and -2 MT-like proteins. On exposure to Cu^{2+}, the MT2 mRNA level increased strongly but Cd^{2+} and Zn^{2+} had only a slight effect. The effect of external Cu^{2+} on MT1 mRNA levels was not observed in roots and seedlings, where it was abundant at high levels, but the MT1 transcript level increased in leaf discs submerged to media supplemented with Cu^{2+}. A detailed study showed that Arabidopsis genome contains at least 6 different MT-like genes[30,49] (1 of them inactive) which are expressed in tissue-dependent manner being specifically influenced by Cu^{2+}. The transcription of one of type-1 genes, of MT1a, normally occurred in roots, germinating embryos, leaf vascular tissue and trichomes and flowers. The MT1a mRNA accumulated also in leaf mesophyl and in vascular tissue of developing siliques and seeds of Cu^{2+}-treated plants[55]. The MT2a but not MT2b mRNAs synthesis in seedlings was shown to be strongly induced by Cu^{2+} (ref.[30]). Differential transcription due to exposure to heavy metal ions was reported with rice seedlings where the MT-like protein of type 1 mRNA was enhanced in shoots by an addition of Cu^{2+}, Zn^{2+}, Cd^{2+}, Fe^{2+}, Pb^{2+}, and Al^{3+}, whereas decreased in roots by metals other than Cu^{2+}. The enhanced transcription of pea $PsMT_A$ gene as a consequence of elevated copper accumulation due to iron starvation[6,20] is another argument for proposed metalloregulatory function of this class of proteins and their role in metal homeostasis. However, of the known relevant genomic clones, the only (putative) element showing homology to the known metal-responsive element was identified in tomato (*Lysopersicon esculentum*)[43]. The relation between MT-like protein induction and heavy metals at the molecular level thus remains to be clarified.

The MT-like protein gene transcripts were also detected in response to various stress conditions. The cDNA encoding MT-like protein of type 2 responding to sucrose starvation and heat shock, but not to excess metals was isolated from rice[32]. The same rice cultivar transcribes type-1 MT-like protein gene in response to Cu^{2+} (ref.[31]). Besides the metal-influenced transcription pattern, the MT-like protein genes of tomato are induced also by oxidative stress[44]. Such induction pattern parallels the proposed antioxidant role of animal MTs (ref.[5]). For example, the evidence that DNA dam-

age by oxidative stress is reduced in the presence of MTs but enhanced if MTs are suppressed in Chinese hamster cells[58] supports such protective ability of thiol-rich peptides or proteins.

The finding that transcription of tobacco (*Nicotiana glutinosa*) MT-like gene would be exclusively induced by wounding and tobacco virus infection[37] enhances the number of known factors increasing expression of genes of this family. The question which should be answered is whether or not a relatively large number of factors affecting expression of MT-like protein genes, the presence of quite a large number of different MT-like protein genes in genome of particular plant as well as the ability of proteins of this family to bind metals (see below) correspond to their role in regulation of genes dependent on metal-requiring DNA binding proteins.

2.2.2. Metal-Binding Properties of MT-Like Proteins and Their Role in Tolerance to Heavy Metals

The indirect evidence of metal-binding abilities of type-1 MT-like proteins came from increased Cd^{2+} and copper (as Cu^{2+} or Cu^{+}; ref.[6]) accumulation due to expression of recombinant pea $PsMT_A$ gene in *E. coli*[47,59]. Over-accumulation of copper by transgenic *A. thaliana* in which constitutive synthesis of $PsMT_A$ was driven by CaMV 35S promoter was also reported[59]. The Cd^{2+}-to-$PsMT_A$ stoichiometry was estimated to range from 5.6 to 6.1 Cd^{2+} ions per mole of purified recombinant protein. The extent of metal ion displacement by H^{+} could be used as a measure of the binding constant[60]. The pH of 50% Zn^{2+}, Cd^{2+}, and Cu^{+} dissociation was estimated to be 5.25, 3.95, and 1.45, respectively, *i.e.*, the values comparable with that of equine renal MT (ref.[61]).

The ability of MT2 of Arabidopsis to complement the *CUP1* knock-out in *Saccharomyces cerevisiae* provided the evidence supporting the potential of type-2 MT-like proteins to bind heavy metal ions *in vivo*[29]. Yeasts unable to synthesize CUP1 protein, a MT of class II (Fig. 1) that is responsible for copper tolerance, were capable of tolerating 10 times higher concentrations of Cu^{2+} and 20 times higher levels of Cd^{2+} as a consequence of expression of gene encoding the MT2a of Arabidopsis. Synthesis of MT1a in mutant *S. cerevisiae* had the same effect on copper tolerance whereas Cd^{2+} tolerance increased only twice. Furthermore, a partial complementation of Zn^{2+} hypersensitivity of cyanobacteria *Synechococcus* PCC 7942 mutant deficient in its MT gene *smtA* was achieved by expression of Arabidopsis MT2a (ref.[48]). The recombinant MT2 was shown to bind Zn^{2+} *in vitro* and the pH of half Zn^{2+} dissociation was estimated to be 5.05. The Cd^{2+}, $Cu^{+/2+}$, and Zn^{2+}

binding *in vitro* was also shown with recombinant MT-like proteins (types 1 and 2) of *V. faba*[41].

The evidence that MT-like proteins may protect plants from heavy metal poisoning comes from tobacco (*Nicotiana tabacum*) overexpressing the type-2 MT-like protein of *N. glutinosa*[62]. The transgenic plant constitutively expressing MT-like gene was able to develope on media contaning 200 μM concentration of Cd^{2+} whereas non-transgenic plants underwent chlorosis at such a metal concentration. These data are in agreement with previously described contribution of various mammalian MTs or yeast CUP1 to the cadmium tolerance of the corresponding transgenic plants[63–67]. However, the issue of heavy metal tolerance in plants is more complex as discussed below.

3. PHYTOCHELATINS

In contrast to MTs of class I and II or plant MT-like proteins, phytochelatins (PCs) are not directly encoded by genes. However, PCs exhibit some features characteristic of MTs and they are thus sometimes referred as class-III metallothioneins (Fig. 1).

The most frequently reported amino acid sequence of PCs is $(\gamma\text{-Glu-Cys})_n\text{Gly}$, abbreviated as PC_n, where *n* ranges from 2 to 11 depending on the source. The primary sequence of these peptides, originally isolated from fission yeast *Schizosaccharomyces pombe*[68], was determined by Kondo *et al.*[69] as $(\gamma\text{-Glu-Cys})_2\text{Gly}$ and $(\gamma\text{-Glu-Cys})_3\text{Gly}$. The peptides with the same amino acid composition were subsequently found by Grill *et al.*[70] in *Rauvolfia serpentina* cell cultures. The number of γ-Glu-Cys unit repeats in PCs from this plant ranged from 2 to 7. Peptides with general sequence $(\gamma\text{-Glu-Cys})_n\beta\text{-Ala}$ (n = 2–7) originally named homo-phytochelatins (h-PCs) were found in plants of the order Fabalceae[71,72]. Another PC homologue with a C-terminal serine and n = 2–4 was identified in several species of the family Poaceae[73] and was named hydroxymethyl-phytochelatin (hm-PC). Peptides of structure $(\gamma\text{-Glu-Cys})_n\text{Glu}$ (n = 2 or 3) were identified in roots of maize (*Zea mays*) seedlings[74–76]. The occurrence of Gly-deficient forms of PC_n (n = 2) was noted both in yeasts *S. pombe*[77] and/or *Candida glabrata*[78–80] and in plants *Z. mays* (n = 2–4; refs[75,76,81]) and/or *Oryza sativa* (n = 2 or 3; ref.[73]).

The new terminology establishing the prefix *iso* for PC_n homologues having C-terminal residues other than Gly was proposed by Zenk[15]. The C-terminal amino acid of the "*iso*-PC" should be given parentetically. Accordingly, the $(\gamma\text{-Glu-Cys})_3\beta\text{-Ala}$ peptide should be expressed as *iso*-PC_3

(β-Ala). The chemical structures and corresponding names of PC and known *iso*-PC molecules are summarized in Fig. 2.

The presence of γ-Glu linkage offers a higher degree of structural flexibility to the PCs than α-Glu-linked peptides. However, the unusual γ-Glu linkage in PC molecule probably does not have critical functional importance

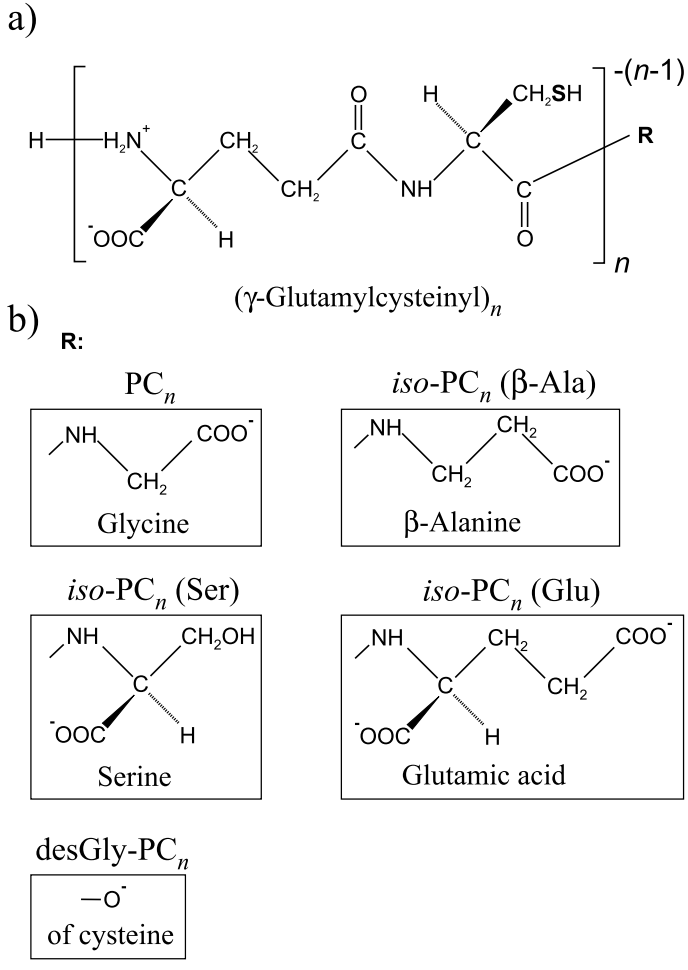

FIG. 2

a The chemical structure of the γ-glutamylcysteinyl repeat that is invariant in both phytochelatins (PCs) and *iso*-PCs. The number of repeats (*n*) vary from 2 to 11, depending on the source. b The basic structural difference between *iso*-PCs and PCs is in the C-terminal amino acid residue (R) that is other than glycine. The names proposed by Zenk[15] for *iso*-PCs are indicated. The desGly form of PCs is also shown

(including an accommodation of acid-labile sulfide; see the text below) as was shown using synthetic α-Glu-linked PC analogues[82–84]. The presence of -Cys-X-Cys- motif, analogous to that found in metallothioneins of class I and II or plant MT-like proteins (for review see refs[3,6,85] and text above) has probably greater importance for metal binding properties of PCs.

3.1. Phytochelatin Induction

Mammalian MTs are induced not only by heavy metal ions but also by hormones, second messengers, growth factors, inflammatory agents, cytokines, tumor promoters, vitamins, antibiotics, cytotoxic agents, and various stress conditions[3]. The plant metallothionein of class II (E_C protein) appeared in germinating seeds and its abundance increased following an addition of abscisic acid[17]. In contrast, there have not been found any stress factors inducing PCs other than metals and metalloids. Neither heat shock nor cold-acclimation-induced PC production[86]. Oxidative stress, return from anoxia, ultraviolet radiation, fungal cell walls and alteration of hormonal levels did not induce PCs (ref.[12]).

PCs were detected in cell cultures of *R. serpentina* as a response to Cd^{2+}, Pb^{2+}, Zn^{2+}, Sb^{3+}, Ag^{+}, Ni^{2+}, Hg^{2+}, AsO_4^{3-}, Cu^{2+}, Sn^{2+}, SeO_3^{2-}, Au^{+}, Bi^{3+}, Te^{4+}, and W^{6+} (ref.[86]). An excess of K^{+}, Na^{+}, Cs^{+}, Ca^{2+}, Mg^{2+}, Al^{2+}, Cr^{2+}, MoO_4^{2-}, Mn^{2+}, Fe^{2+}, Co^{2+}, VO^{2+} or UO^{2+} did not appear to induce PC synthesis in this culture. In addition, an induction of *iso*-PC (β-Ala) with Ga^{3+} was observed in plants of order Fabalaes whereas Ba^{2+}, B^{3+}, and Ce^{3+} were reported as non-inducers[71]. Fission yeast *S. pombe* produced PCs under elevated concentrations of Cd^{2+}, Bi^{3+}, AsO_4^{3-}, Cu^{2+}, Pb^{2+}, Zn^{2+} or Ag^{+} present in media[87]. Cd^{2+} but not Cu^{2+} (see below) induced PCs in *C. glabrata*[78].

It should be stressed that Cd^{2+} is by far the most potent inducer of PC synthesis. Cd^{2+} has been found to be a 6 times stronger PC inducer than Cu^{2+} in *R. serpentina* cell culture[86]. The 40 times lower Cu^{2+}-induced PC_n levels in comparison with those induced by corresponding external concentration of Cd^{2+} were detected in *S. pombe*[87]. Scheller *et al.* demonstrated a positive correlation between the external Cd^{2+} concentration ranging from 50 to 400 μM and accumulation of PCs in tomato (*L. esculentum*) cell culture[88]. Exposure to Cd^{2+} stimulated PC synthesis during 5–15 min in both plant cell cultures of *R. serpentina*[86] or *Datura innoxia*[89,90] and *S. pombe*[87]. Zinc was found to induce similar PC levels at concentrations 250 times higher (900–1 800 μM) in comparison with Cd^{2+} in cell culture of *Nicotiana tabacum*[91]. This finding is in agreement with the data published for *S. pombe*[87]. However, *R. serpentina* cell culture produced significant level of

PCs when transferred from Cu^{2+}- and Zn^{2+}-depleted media into Cu^{2+}-free media with optimum concentration (35 μM) of Zn^{2+}, ref.[92]. This is in agreement with the fact that small quantities of PCs can be detected in plant cell cultures growing in standard media[89,93,94].

3.2. Biosynthesis of Phytochelatins

The presence of unusual γ-carboxamide linkage of glutamic acid in PC molecules implied a possible role of glutathione (GSH) and its homologues as precursors of these peptides. On the other hand, *in vivo* deamination of proteins that may introduce γ-carboxamide linkages was considered as well. However, the synthetic oligonucleotide probes encoding $(Glu\text{-}Cys)_3Gly$ did not hybridise with mRNAs of *D. innoxia* cell culture synthesizing PCs (ref.[89]).

The GSH levels in plant cell cultures, intact plants and fission yeast declined following induction of PCs with heavy metal ions. This decrease correlated with external metal concentration[86,88,95–101]. Further evidence for GSH as PC precursor was provided by experiments with buthionine sulfoximine (BSO). BSO is a potent inhibitor of plant γ-glutamylcysteine synthetase[102] (E.C.6.3.2.2), an enzyme which is involved in GSH biosynthesis. Plant cell cultures treated with BSO lost their ability to produce PCs in response to heavy metals, when the initial GSH pool disappeared[91,93], and addition of external GSH restored PC synthesis[88]. Experiments with plants treated with cycloheximide revealed that the initial induction of PCs was not prevented in plants with inhibited *de novo* protein synthesis[88,89]. This finding and the fact that PCs appear rapidly after heavy metal administration implies that the enzyme(s) involved in the biosynthesis of PCs in plants are constitutive. Indeed, the enzyme capable of PC synthesis was isolated from *Silene cucubalus* and characterized by Grill *et al.*[103]. This γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase) activity is believed to be abundant as a constitutive generally in all PC and *iso*-PC producing species.

The phytochelatin synthase (E.C.2.3.2.15) from cell *S. cucubalus* was reported having molecular weight 95 kDa being tetramer of 25 kDa subunits and retaining its synthetic activity in dimeric form (50 kDa). The enzyme activity was found to be metal dependent (Fig. 3). The Cd^{2+} was the strongest activator of PC synthase *in vitro*. The PC_2 appeared without noticeable lag period following Cd^{2+} administration and peptides of $n = 3$ and 4 were detected after 15 and 35 min, respectively. Sequestering all Cd^{2+} from reaction mixtures by PCs resulted in termination of reaction until further Cd^{2+}

was added. Lower induction of phytochelatin synthase activity in comparison to Cd^{2+} was reported for Ag^{+}, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, and $AuCl_4^-$ and no activity was induced by Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Al^{3+} or Fe^{2+} (ref.[103]). These results correspond with data obtained *in vivo*[71,86,87] (see above).

The tetrameric form of the enzyme activated by Cd^{2+} exhibited a specific activity of 453 pkat/mg of protein, a wide temperature range of action

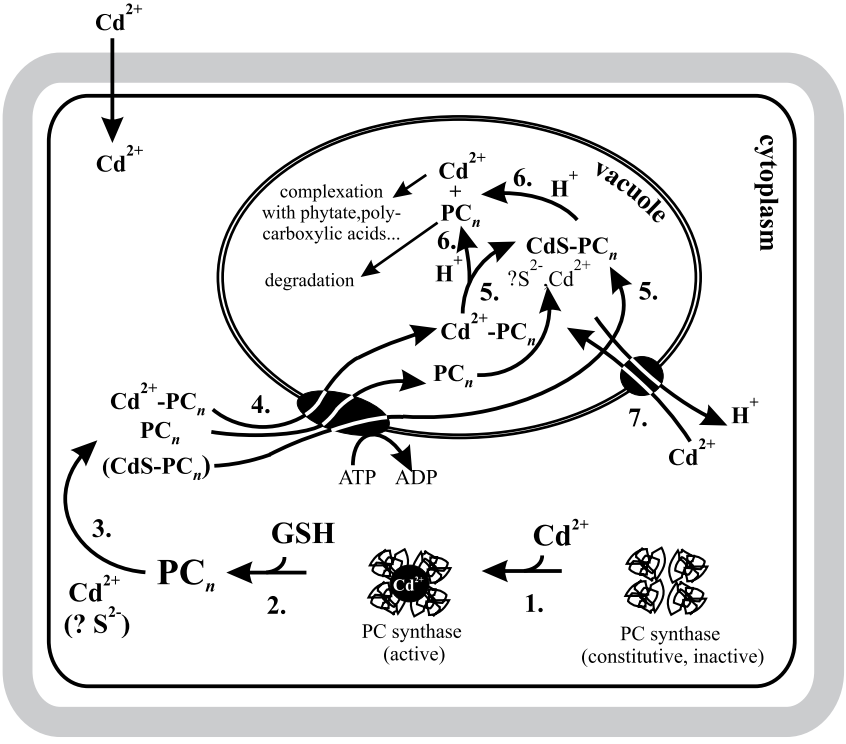

FIG. 3

The general model describing synthesis of phytochelatins (PCs) in response to Cd^{2+} and intracellular localization of the Cd^{2+}–PC complex. The Cd^{2+} entering cytoplasm (1) activates PC synthase which (2) build up PCs. Subsequently, PCs (3) sequester Cd^{2+} and form LMW Cd^{2+}–PC complex (an HMW complex of CdS–PC could be also formed in the cytoplasm). The Cd^{2+}–PC complex and apo-PCs are (4) transported in ATP-dependent manner through tonoplast (CdS–PC complex is also translocated to vacuole, but with a much lower efficiency). In the vacuole, the Cd^{2+}–PC (5) accommodates S^{2-} and forms HMW CdS–PC, which may reside in vacuole. Alternatively, Cd^{2+}–PC and/or CdS–PC complexes (6) dissociate in acidic vacuolar sap, Cd^{2+} can be bound by vacuolar ligands and PC are degraded. The mechanism of S^{2-} accommodation *in vivo* is not clear, but seems to be linked to purine biosynthesis. The Cd^{2+}/H^{+} antiporter (7)

(with maximum at 35 °C) and pH optimum 7.9. GSH served as a primary substrate with K_m value 6.7 mM, but higher PC homologues were synthesized from $(\gamma\text{-Glu-Cys})_2\text{Gly}$ in its absence. The fact that *S*-substituted GSH analogue (*S*-Monobromobimaneglutathione) is a substrate for phytochelatin synthase[103] (with even lower K_m = 1.5 mM) suggests that the GSH: Cd^{2+} complex is not necessary for peptide synthesis. Optimizing the reaction parameters, Friederich *et al.* accomplished *in vitro* an enzymatic synthesis of 35 g of PCs within 55-day period[104].

Grill *et al.*[103] described the PC synthesis by the equation:

$(\gamma\text{-Glu-Cys})_n\text{Gly} + (\gamma\text{-Glu-Cys})_n\text{Gly} \rightarrow (\gamma\text{-Glu-Cys})_{n+1}\text{Gly} + (\gamma\text{-Glu-Cys})_{n-1}\text{Gly}$, where n = 1, 2, 3, . . . The (γ-Glu-Cys) unit is primarily provided by GSH; however, PC synthase is able to use also PCs as a (γ-Glu-Cys) donor. Despite this fact, peptides with lower n are not expected to be degradation products of PCs of higher number of (γ-Glu-Cys) repeats. It is noteworthy, that the (γ-Glu-Cys) is not a substrate for PC synthesis as demonstrated with the PC synthase from tomato (*L. esculentum*)[94].

Studies on *iso*-PC synthesis provided some insight into enzymology of reaction in terms of substrate specifity. Use of homo-glutathione (h-GSH) or hydroxymethyl-glutathione (hm-GSH) as sole substrates for PC synthase of *Pisum sativum* led to only slight synthesis of *iso*-PC(β-Ala) or *iso*-PC(Ser), respectively[72]. However, in the presence of GSH and h-GSH or hm-GSH, the corresponding *iso*-PCs were synthesized. PC synthesis was inhibited by increasing concentration of GSH homologues. Klapheck *et al.*[69] thus concluded that PC synthase (of pea) has a (γ-Glu-Cys) donor binding site, which is very specific to GSH, and (γ-Glu-Cys) acceptor binding site, which is less specific and accepts GSH as well as its homologues. The *Pisum* PC synthase is capable of building up *iso*-PC_n(Ser) even if the hm-GSH is not abundant in pea. In contrast, PC synthase of *S. cucubalus* does not accept h-GSH as a substrate for synthesis of *iso*-PC_n(β-Ala) (ref.[15]). This suggests that different plant species possess specific [*iso*-] PC synthases. To our knowledge, there has not been any plant cDNA of PC synthase cloned and sequenced yet.

An appropriate metal activates the enzyme and probably protects the formed PCs from becoming a dipeptidyl donor. Phytochelatin synthase, heavy metals and PCs form a self-regulating loop at the enzymatic level in plants. To our knowledge, there is no evidence of regulation at the level of *de novo* PC synthase expression. On the other hand, the expression of genes involved in GSH biosynthetic pathway was shown to respond to Cd^{2+} administration. The transcription of both γ-glutamylcysteine synthase and glutathione synthase in *Arabidopsis thaliana* was increased in response to

Cd^{2+} and/or Cu^{2+} (ref.[105]). The Zn^{2+}- or Cu^{2+}-induced increase in γ-glutamylcysteine synthase mRNA level was also observed with *Brasica juncea*[57,106]. Such flexibility in GSH synthesis explains the fact that the GSH levels in plants synthesizing large amounts of PCs are (at "steady state" established after the initial drop in is overcome) only moderately decreased.

Hayashi *et al.* described two ATP-independent pathways of PC synthesis in cell-free extracts of *S. pombe*[107]. The first one closely resembled that found in *S. cucubalus* where PCs are formed from γ-Glu-Cys units which are added to GSH and/or to $(\gamma\text{-Glu-Cys})_n$Gly. Interestingly, no increase in activity was detected using Cd^{2+}-induced cells. Assuming that intracellular Zn^{2+} and $Cu^{+/2+}$ concentration of *S. pombe* was much higher than that used by Grill *et al.*[103] for activation of plant phytochelatin synthase, Hayashi *et al.*[107] concluded that the regulation of PC synthesis in *S. pombe* should be different from that in plants. The question remaining is the form and intracellular distribution of both Zn^{2+} and $Cu^{+/2+}$, but the fact that addition of Cd^{2+} into cell free extract did not improve phytochelatin synthesis supports this hypothesis. The other described pathway of PC synthesis is based on (γ-Glu-Cys) addition to $(\gamma\text{-Glu-Cys})_n$ followed by Gly addition most likely by glutathione synthetase. The possibility that *gsh2* gene of *S. pombe* encodes bifunctional enzyme possessing both glutathione synthetase activity (E.C.6.3.2.3) and PC synthetase activity was confirmed[108]. Despite the *S. pombe* mutant defective in glutathione synthetase (*gsh2*) gene retained 44% of the wild-type GSH level, it lost its ability to produce PCs.

3.3. Metal–Phytochelatin Complexes

The propensity of metal ion to bind with particular ligand is based on the theory of hard and soft acids and bases[109] (HSAB). Metals and ligands are classified as hard, intermediate or soft with respect to their electron mobility or polarizability. Heavy metals ions are classified as soft (*e.g.*, Cu^{+}, Cd^{2+}, Ag^{+}, Hg^{2+}) or intermediate (*e.g.*, Cu^{2+}, Pb^{2+}, Zn^{2+}, Co^{2+}) acids (tend to have high polarizability, low electropositivity and are large in size). According to the HSAB theory, the hard (soft) acids form strong bond with hard (soft) bases. The sulfhydryl group of cysteine residue, the sole donor in the native metal–PC complex, is referred to be the soft base in biological systems (having relatively high polarizability, low electronegativity and large size). Possible participation of nitrogen or oxygen atoms in Cd^{2+} binding was excluded[83]. The only report on potential binding of metal ion by residue other than cysteine comes from Jackson *et al.* who found large amount of Fe^{2+} to be associated as uncharacterized complex with Cd^{2+}–PC of *D.*

innoxia[90]. The Authors suggested the electrostatic interaction of Fe-complex with negatively charged Cd^{2+}–PC complex as a binding force.

Two different species of Cd^{2+}–PC complexes could be recognized in plants and yeasts on the molecular weight basis determined by gel filtration. These are thus referred as low-molecular-weight (LMW) and high-molecular-weight (HMW) and basically differ in the amount of accommodated sulfide ion, which could be liberated at acidic environment (acid-labile sulfide). The S^{2-} : Cd^{2+} molar ratio of the HMW complex may vary up to 1 in plants[110,111]. The HMW Cd^{2+}-binding complexes containing high amount of acid-labile sulfide (CdS–PC) were originally described in *S. pombe* by Murasugi *et al.*[112]. Similar complexes were subsequently described in yeast *C. glabrata*[79,113] and various plants[75,93,110,111,114,115].

Metal–PC complexes possess some spectroscopic characteristics similar to those found for MTs of class I and II. Ultraviolet spectrum of metal–PC complexes showed absorption maxima between 250 and 290 nm (refs[93,110,116–119]) characteristic of metal–thiolate bonds. CdS–PC complexes showed specific absorption spectra in the UV region with transition at 270–320 nm. The absorption maximum was more red-shifted for larger complexes with higher S^{2-} : Cd^{2+} ratios[79,110,112,117,120]. The Cu^{+}–PC complex from *S. pombe* exhibited, in addition, ultraviolet-excited luminiscence with maximum at 619 nm (ref.[116]). CD spectroscopy revealed a positive Cotton band at 275–280 nm and negative one at 253–257 nm for Cd^{2+}–PC, *i.e.*, a pattern similar to that found for vertebrate metallothioneins[13]. Purified HMW CdS–PC complexes from *C. glabrata* and *S. pombe* are analogous to quantum semiconductors[79]. CdS crystallites from *C. glabrata* coated by GSH also showed these properties[115,121].

The apparent molecular weight of native LMW Cd^{2+}–PC complexes ranges from 2 to 4 kDa (refs[86,122–124]). The native complexes are frequently composed of two or three isopeptides of n = 2, 3, 4, and 5 (refs[89,107,110,116,122,124]). Using extended X-ray fine structure spectroscopy (EXAFS), Strasdeit *et al.*[125] demonstrated that the Cd^{2+}–PC complex (S^{2-} : Cd^{2+} = 0.01) isolated from *R. serpentina* has Cd coordinated by four Cys residues with the Cd–S bond length of 2.52 ± 0.02 Å. This value (2.54 Å) as also reported for the Cd–S distance of Cd^{2+}–PC complex of maize. The presence of polynuclear Cd^{2+} clusters in the natural maize Cd^{2+}–PC complex, similar to that of mammalian MTs (see above), was suggested by EXAFS data[83]. The same arrangement of Cd^{2+} coordination was also observed with complexes made of synthetic PC_3 and $(\alpha\text{-Glu-Cys})_3Gly$, indicating that such geometry is not attributable to S^{2-} accommodation in the complex.

Half displacement of bound Cd^{2+} from equine renal MT and pea metallothionein-like protein ($PsMT_A$) appears at pH 3 and 3.95, respectively[61] (see above), whereas 50% metal dissociation of LMW Cd^{2+}–PC complex was observed at pH 5–5.4 (refs[116,117,122]).

An HMW CdS–PC complex with an apparent molecular weight ranging from 6 to 9 kDa was isolated from *S. pombe* by Murasugi *et al.*[112]. The half-displacement of metal of CdS–PC complex isolated from *S. pombe* occurred at pH 3.8–4.0, *i.e.*, at value lower than that of Cd^{2+}–PC indicating a higher stability of the CdS–PC (refs[116,117]). The CdS–PC (S^{2-} : Cd^{2+} < 0.4) complex from *C. glabrata* was characterized as 20-Å crystallites composed of 85 CdS units coated by 30 PC molecules (predominantly PC_2 and des-Gly PC_2) (ref.[79]). Bae *et al.*[121] predicted the maximum radius of PC-capped CdS crystallites as 11.8 Å. On the other hand, the radius of GSH-capped crystallites ranged from 10.8 to 17.3 Å, indicating that only PCs are able to terminate and thus control the crystallite size. The peptide-born sulfhydryl-to-Cd^{2+} ratio of this complex was lower than 1 and PCs appear to act in controlling this biomineralization process by termination of the crystallite growth. The HMW CdS–PC (PC, *iso*-PC_n(Glu), and des-Gly PC_n of predominant n = 3 and 4) complex isolated from maize showed PC sulfhydryl-to-Cd^{2+} ratio 1.01 ± 0.07 and S^{2-} : Cd^{2+} ratio 0.18 (ref.[75]). Plocke and Kägi[120] isolated from *S. pombe* two HMW CdS–PC complexes of the stoichiometric formulas $[(\gamma\text{-Glu-Cys})_2\text{Gly}]_6\cdot[(\gamma\text{-Glu-Cys})_3\text{Gly}]_2\cdot[S^{2-}]_1\cdot[Cd^{2+}]_6$ (m.w. 5 464 Da) and $[(\gamma\text{-Glu-Cys})_2\text{Gly}]_2\cdot[(\gamma\text{-Glu-Cys})_3\text{Gly}]_3\cdot[(\gamma\text{-Glu-Cys})_4\text{Gly}]_1\cdot[S^{2-}]_1\cdot[Cd^{2+}]_6$ (m.w. 5 086 Da). The CD and MCD spectra of CdS–PC complexes revealed that co-ordination of the metal with sulfur atoms yields a regular structure made up of tetrahedral CdS_4 units, similar to the coordination pattern in MT I and II.

In comprehensive studies by Mehra *et al.*[119,126,127], the formation of Pb^{2+}, Hg^{2+}, and Ag^+, respectively, complexes with PCs of n = 2–4 was followed *in vitro*. Both PC_2 and PC_3 bound one Pb^{2+} per peptide molecule, whereas two distinct species with stoichiometries of one and two Pb^{2+} ions per PC_4 molecule were formed[126]. Two species of complexes also formed Hg^{2+} with PC_3 and PC_4 (ref.[119]). The corresponding stoichiometries are 1.25 or 2.0 and 1.25 or 2.5 Hg^{2+} ions per PC_n molecule, respectively. PC_2 binds Hg^{2+} as well, with stoichiometry exclusively 1 : 1. At neutral pH, the Ag^+-to-PC_2, -PC_3 or -PC_4 stoichiometry was 1.0, 1.5 or 4.0, respectively, but it reached 1.0 with all n = 2–4 peptides at pH below 5.0 (ref.[127]). Such pH-dependent reassembly of the complex did not occur with Hg–PC_n (ref.[119]). Despite all these three metals are inducers of PC synthesis as demonstrated both *in vivo*[71,86] and *in vitro*[103], the native complexes (if they are of physiological relevance) remains to be isolated.

Very poor data are available on production of PCs as detoxification tools for an excess of intracellular $Cu^{+/2+}$ and Zn^{2+} (see below). The Cu^{+}-to-ligand ratio was reported to vary up to 1 : 6, but this variation is assumed to be due to instability of Cu^{+} oxidation state[13]. The Cu^{+}-to-cysteine sulfhydryl ratio in natural the Cu^{+}–PC complex was estimated[128] to be between 1 : 1 and 1 : 2, *i.e.*, similar to mammalian[3] and yeast[85] metallothioneins. Mehra and Mulchandani demonstrated *in vitro* the Cu^{+}-to-peptide stoichiometries of 1.25, 2.0, and 2.5 for complexes of the metal with PC_2, PC_3, and PC_4, respectively[129]. The 50% of metal dissociated from native Cu^{+}–PC of *S. pombe* at pH 1.3, *i.e.*, at value lower than that found for equine renal MT and $PsMT_A$ (1.8 and 1.5, respectively[61]). The Zn^{2+}-to-sulfhydryl ratio of Zn^{2+}–PC complexes is 1 : 2 (ref.[128]); however, it is conceivable that PCs binds little Zn^{2+} *in vivo* because of the apparently low affinity of PCs for Zn^{2+} ions[100,122,128]. To our knowledge, no clear data are available on the presence of labile sulfide in Cu^{+}– or Zn^{2+}–PC complexes.

3.4. Intracellular Localization of Metal–Phytochelatin Complexes

The enzymes involved in synthesis of GSH are localized in cytoplasm, chloroplasts or mitochondria and PC synthase is abundant in cytoplasm. However, in tobacco cell suspension cultures, the major part of intracellular Cd^{2+} and Zn^{2+} was found to be vacuolar[130]. With intact tobacco, virtually all of the intracellular Cd^{2+} and PCs in leaves were localized in vacuolar sap[131]. The vacuole is thus supposed to be the final sink for sequestered Cd^{2+}. In oat *Avena sativa*, the apo-PC and Cd^{2+}–PCs were demonstrated to be transported into vacuoles in MgATP-dependent, ΔpH-independent manner[132] (Fig. 3). However, the transporter from *A. sativa* has not yet been identified. Cd^{2+} alone is transported into vacuoles *via* Cd^{2+}/H^{+} antiport which is not attributable to Cd^{2+}–PC transporter[133].

Considerably more details are known about the tonoplast-associated protein of *S. pombe* designated HMT1 (Heavy Metal Tolerance) which resembles close functional similarity to transporter expected in *A. sativa*. The *hmt1* ORF shows sequence similarity to prokaryotic and eukaryotic ATP-binding cassette-type transporters[134]. The ability of HMT1 to transport apo-PCs and Cd^{2+}–PCs (LMW) in an ATP-dependent manner has been proved with vacuolar vesicles of *S. pombe*[135]. The HMT1 function is supposed to be closely related to the formation of HMW as increased expression of *hmt1* resulted in a higher CdS–PC level and, on the contrary, the Cd^{2+}–PC was the only Cd^{2+}-binding component in mutant of *S. pombe* lacking *hmt1* gene. The CdS–PC complex is also taken up by HMT1, albeit with much lower ef-

ficiency[135]. Thus the transport of LMW complex to vacuole, accumulation of S^{2-} and formation of HMW CdS–PC complex in the compartment are now assumed to be the true mechanism of Cd^{2+} disposal within cell[14] (Fig. 3). However, Mehra *et al.* showed that CdS–PC complexes may originate in the cytosol of *C. glabrata* and these are subsequently slowly accumulated in vacuoles[136].

The mechanism by which the S^{2-} is accommodated is not known yet. It was demonstrated *in vitro* that LMW Cd^{2+}–PC complex could be converted to HMW CdS–PC by the addition of inorganic sulfide[110,117]. However, no vacuolar transporter translocating S^{2-} through tonoplast was shown to be contributory in HMW complex formation. Some insight into possible *in vivo* accommodation of sulfide into HMW complex is provided by a mutant of *S. pombe* defective in two genes involved in purine biosynthetic pathway[129]. Mutants lacking adenylosuccinate synthetase and succinoaminoimidazole carboxamide ribonuclease (SAICAR) were unable to form an HMW CdS–PC complex. The suggested mechanism[137,138] anticipates that a sulfur analogue of aspartate, cysteine sulfinate, might be utilized by these enzymes and that sulfide of the HMW complex originate in sulfite of cysteine sulfinate which is converted to S^{2-} by pathway involving adenylosuccinate synthetase and SAICAR. The association of both sulfide and sulfite with PCs was reported earlier[12,139] and it was hypothesized to be a consequence of the role of PCs as a carrier of sulfite for assimilatory sulfur reduction pathway, which has not been proved yet.

The further fate of the complex and form of intravacuolar Cd^{2+} could be the point of discussion. The HMW may (i) reside in vacuole as it could be stabilized by additional sulfide in acidic vacuolar sap or (ii) the complex could dissociate and Cd^{2+} could be bound by by ligands normally abundant in vacuoles. Polycarboxylic acids and phytate could be taken into account as potential Cd^{2+}-binding compounds in vacuole[130,140–142]. The turnover of (liberated) PC peptides is indicated by decrease in intracellular PC levels after arresting exposure to toxic metal ion[143] (Fig. 3). On the contrary, following the transfer to Cd^{2+}-free media, the PC content of alga *Kappaphycus alvarezii* showed no significant change, while the Cd^{2+} content was decreased[144].

3.5. The Relevance of PC Synthesis and Localization to Metal Tolerance and Homeostasis

The terms sensitive and tolerant have been used by different authors in various, inconsistent ways leading to confusion. This difficulty originates in

establishing of "base line", *i.e.*, wild-type response to particular element or compound. Rauser suggested to use terms sensitive or hypersensitive for mutants unable to survive at metal concentrations which are otherwise non-lethal for wild-type organism of the same species[14]. These terms apply to both excess of metal in the environment and deficiency in the essential elements. The tolerant mutant is that one capable of sustaining growth at concentrations of metal which are outside the limits of adequate metal doses. On the other hand the wild-type could be viewed as tolerant when compared with (hyper)sensitive mutant. In this case, the term "differential metal tolerance/sensitivity" should be used.

The role of PCs in cadmium detoxification seems to be *ad dispute*. Howden *et al.* described two mutants of *A. thaliana* deficient in PC synthase[115] (*cad1*) and glutathione synthesis[145] (*cad2*) and thus unable to synthesize sufficient levels of PCs. An allelic series of *A. thaliana* mutants deficient in *CAD1* gene, which is believed to be PC synthase structural gene, showed sensitivity to Cd^{2+}. These mutants lost their ability to build up HMW complex and only small amounts of LMW complex or none was formed[115]. The degree of sensitivity of the Arabidopsis *cad1* mutants correlated with the amount of accumulated PCs. A valuable information on the role of HMW and its translocation-coupled formation in Cd^{2+} detoxification was obtained with *S. pombe*[134,135,146]. The *hmt1* mutant of this yeast showing cadmium-sensitive phenotype was unable to build up HMW CdS–PC complex, but it still formed LMW Cd^{2+}–PC complex. On the other hand, overexpression of *hmt1* gene in *S. pombe* followed by increased accumulation of HMW complex resulted in more cadmium-tolerant phenotype[134].

It has been shown *in vitro* that PCs are capable of protecting metal-sensitive enzymes from poisoning by heavy metals[147]. The Cd^{2+}–PC complex exhibited a 10–1 000 lower inhibitory effect to Rubisco enzyme, nitrate reductase, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and/or urease than corresponding concentration of free Cd^{2+}. In addition, PC_3 was capable of reactivating Cd^{2+}-poisoned nitrate reductase.

Despite the cadmium-tolerant tomato (*L. esculentum*) cell line accumulated significantly higher amounts of PCs than wild-type line[93,148], the conclusion that the overaccumulation of PCs is a general way to reach resistance to Cd^{2+} was rather contraindicated by many workers. Comparison of wild-type and tolerant *D. innoxia* did not show any difference in PC concentrations but the formation of Cd^{2+}–PC complex was delayed in the non-tolerant cell line[91]. Moreover, 2–3 times higher levels of PCs were found in roots of non-tolerant *Silene vulgaris* than in the roots of the tolerant

plant[149]. The increased translocation of Cd^{2+} from roots to shoots was observed in the wild-type plant. PCs of $n = 3$, which are assumed to have higher affinity for metal ion, were the most abundant form in sensitive plants whereas PCs of $n = 2$ in tolerant ones[150]. The activity of PC synthase isolated from the wild-type and tolerant *S. vulgaris* did not differ and the relative decrease in PC concentration (turnover rate) after arresting Cd^{2+} exposure was in both ecotypes also the same[143].

Despite the formation of Ag^{+}, Cu^{+}, Hg^{2+} or Pb^{2+} complexes with PCs demonstrated *in vitro* (referenced above) and many metals have been shown to induce PC synthesis (see above) a direct link of PCs to detoxification of heavy metal ions other than Cd^{2+} is not clear yet. The data reported in literature are rather against such a role of PCs: Addition of buthionine sulfoximide (inhibitor of GSH and hence PC synthesis) sensitized tobacco cell cultures to Cd^{2+}, but no differential tolerance was observed with Zn^{2+} or Cu^{2+} (ref.[91]). Huang *et al.* showed that cadmium-tolerant tomato cells exhibit only a slight increase in copper tolerance and they are not cross-tolerant to lead, zinc, mercury and silver[123]. Neither wild-type or copper-tolerant *Lotus purshianus* accumulated PCs and $Cu^{+/2+}$ appeared to be associated with protein rich in aspartate and glutamate (36 mole %) and poor in cysteine (2.5 mole %) (ref.[151]). To bring more confusion, PC synthesis in response to Cu^{2+} (but not Zn^{2+}) was reported for *Polyglonum cuspidatum*[152]. Only 30% of the total intracellular $Cu^{+/2+}$ was found to be bound by PCs in *S. cucubalus* exposed to this metal[153]. Moreover, de Vos *et al.* suggested that copper tolerance of *S. cucubalus* is due to lowered synthesis of PCs as the glutathione depletion due to enhanced PC synthesis in non-tolerant line of *S. cucubalus* caused oxidative stress[98].

The amount of PCs was found to be too low to sequester significant proportion of the intracellular metal in zinc-tolerant line of *S. vulgaris*[100]. Low-molecular-weight organic acids such as malic, citric, oxalic or phytic acids are believed to be involved in intravacuolar Zn^{2+} binding rather than PCs (refs[130,140–142,154]). On the other hand, the enzyme Rubisco, which is more sensitive to Zn^{2+} than Cd^{2+}, is efficiently protected by Zn^{2+}–PC complex *in vitro*[147] (even low affinity of PCs to Zn^{2+} is advised). As zinc and copper are essential elements, possible contribution of PCs in metal homeostasis could be hypothesized (see below).

An indirect evidence suggesting a protective effect of PCs against Hg^{2+} or CH_3HgCl was demonstrated for *Hordeum vulgare*[155] and *Allium cepa*[156]. However, the PC synthesis had to be induced by preadministration to Cd^{2+} indicating that PC production is not instrumental in natural mechanism of Hg^{2+} detoxification.

Despite the well documented importance of PCs in Cd^{2+} detoxification, viewing high diversity of data on PC levels induced in wild-type and tolerant ecotypes as well as poor or even no PC synthesis in response to other metals, it could be concluded that overproduction of PCs is not a general mechanism for detoxification of or increased tolerance to heavy metal ions in both plants and yeasts. The production of various intracellular metal binding compounds[157] but also alterations of metal compartmentation patterns, cellular metabolism, membrane structure, and exclusion of metal from plant body or the avoidance of metal entry into plant cells represent the general mechanisms which might lead to increased metal resistance in plants[158,159]. Such resistance is usually highly metal specific[142,158] but the cross-resistance to excessive amounts of another metal has also been reported[158,160]. However, there is no evidence that resistance to different metals will indeed depend on the same genes. The number of genes involved is unknown but often assumed to be high[158,161]. The feasible complexity of response to heavy metals was demonstrated by the differential response of various yeasts to heavy metal administration, which could be followed in the literature. On the exposure to excess Cd^{2+} the synthesis of PCs was detected in *Saccharomyces cerevisiae*[162], *Schizosaccharomyces octosporus*[163] as well as in *S. pombe* (referenced above throughout the text). MTs were formed in response to Cd^{2+} in *S. cerevisiae*[164], *Pichia farinosa* and *Torulaspora delbruecki*[163]. The level of tolerated Cd^{2+} concentration similar to that found in MT-producing *S. cerevisiae* was observed in *Saccharomyces exiguus* that retained most Cd^{2+} in the cell wall and no intracellular Cd^{2+}-binding complex was detected[163]. Interestingly, as mentioned above, *C. glabrata* produced exclusively PCs in response to Cd^{2+}, but in response to elevated external concentrations of Cu^{2+}, two MTs but no PCs were formed[78]. Moreover, Yu *et al.* demostrated inability of one of these two MTs designated MTII to bind Cd^{2+} *in vivo*[165]. The MTII of *C. glabrata* thus appears to be the only MT known at present that forms an unstable complex with Cd^{2+}.

The possible role of PCs in metabolism (homeostasis) of essential heavy metals such as zinc or copper indicates small quantities of PCs that could be detected in normally grown plant cell cultures[89,93]. The levels of PCs increased 100-fold in cells of *R. serpentina* after their transfer from spent medium into fresh one containing optimum (35 μM) concentration of Zn^{2+} (ref.[92]). Thumann *et al.* showed that Zn^{2+}–PC and Cu^{2+}–PC complexes activate *in vitro* apoforms of carbonic anhydrase and diamino oxidase, respectively[166]. The activity of PC synthase was in tomato cell culture found to be cell-cycle-dependent and the differential activity was also observed within the plant body[94]. Clearly, these experiments do not conclusively establish

the role of PCs in metal homeostasis but they pinpoint such capability of PCs and they make it possible to hypothesize the cooperation of PCs and MT-like proteins in the metal homeostasis and activation of variety of genes. As the *cad1* knock-out in *A. thaliana* leading to lack of PC synthase is not lethal under normal conditions[115], PCs do not play a critical role in the plant development and reproduction.

4. CONCLUDING REMARKS

Plants developed two specific families of metal binding proteins and peptides – metallothioneins and metallothionein-like proteins and phytochelatins. The transcription pattern of the former two metal-binding proteins was reported to vary during embryogenesis, plant development, and in tissue-specific manner. In addition, differential expression of MT-like genes might be also a consequence of variation of heavy metal concentrations (especially of $Cu^{+/2+}$ and $Fe^{2+/3+}$) in the environment, influence of stressing agents (heat shock, sucrose starvation, oxidative stress) and also wounding and plant pathogens. The principal role of plant MTs and MT-like proteins seems to be in homeostasis of essential transition metals rather than in metal detoxification which, however could not be excluded. The molecular events leading to specific expression of particular MT or MT-like gene should be clarified as well as the action of these proteins in the "buffering" of available metal ion by donation to or removing from other metalloproteins. The metal coordination geometry should be resolved for this family of plant proteins. A possible link to PCs with respect to homeostasis of heavy metals should be examined either.

Not counteracting the indisputable importance of PCs in heavy metal detoxification, it could be concluded that PCs provide a specific and potent ligand, but still one of several possible, enabling plants or yeasts to buffer metal concentration and to protect intracellular processes from their poisoning effects. An example of substitution of PCs in detoxification process by other metal-binding peptide is provided by inhibition of PC synthesis in response to Cd^{2+} and Cu^{2+} observed with *C. glabrata* and *S. pombe* constitutively expressing the metallothionein gene *CUP1* (ref.[165]). Despite the ability of (overexpressed) MT-like protein to protect plant from cadmium toxicity[62], the specific reason why plants developed PCs, but not MTs as a detoxification tool (as it could be concluded in light of present data) is not known. Further research spent on metal detoxification within the plant body and evolution of tolerant genotype should be directed toward understanding of a link between genes to be identified involved in (differential)

metal tolerance and the role PCs in these processes. Evaluation of such connections would allow constructing (transgenic) plants such as hyperaccumulators suitable for bioremediation, hypertolerant plants to green highly contaminated areas and/or plants with a reduced metal uptake in consumable parts.

The importance of this approach is underlined by the fact that many previous attempts to obtain transgenic plants suitable for bioremediation by introduction of MT genes failed. Expression of either mammalian[64,167–169] or yeast[170] MTs in plants driven by constitutive 35S promoter of cauliflower mosaic virus did not lead to a significantly increased accumulation of Cd^{2+} in upper parts of plant, which could be beneficial to removal of metals from contaminated soil. Moreover, MT expression resulted in some cases in a decrease of up to 70% in Cd^{2+} translocation from roots to shoots in comparison with a wild-type plant[168,169]. To our knowledge, the only successful attempt to enhance both tolerance to and accumulation of Cd^{2+} was with *B. juncea* that overexpressed *E. coli*-borne glutathion synthetase gene *gshII* (ref.[171]). Transgenic plant accumulated in shoots up to 3-fold higher amount of the metal that the wild-type plant. The tolerance to Cd^{2+} and the metal accumulation correlated with the *gshII* expression level.

In order to elucidate possible role of PCs in homeostasis of essential transition metals (in cooperation with plant MTs and MT-like proteins?), more details should be evaluated on their tissue partitioning. The levels of PCs should be followed during plant germination and development of seeds and in differentially growing cells (such as in root meristem) of normally grown plants. The valuable information on developmental stage of particular importance could provide the known differential expression pattern of MT-like proteins. Highly sensitive techniques for determination of potential metal–PC complexes should be developed for this purpose.

Note Added at Editing Proof

The novel family of genes (phytochelatin synthases; *PCS*) was isolated and cloned from *T. aestivum*, *A. thaliana*, *S. pombe* and also from nematode *Caenorhabditis elegans*[172]. It opens an exceptional way for understanding and exploitation of PC synthases in the circumstances depicted above.

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