# Phytochelatins

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Phytochelatins (PCs) were first discovered as Cd-binding "Cadystins A and B" in a fission yeast and then in many plants as the major components of Cd-binding complexes. PCs have the general structure of (γ-glutamyl-cysteinyl)n-glycine (n=2-11) and the variants with the repeated γ-glutamyl-cysteinyl units are formed in some plants and yeast. They are capable of binding to various metals including Cd, Cu, Zn or As via the sulfhydryl and carboxyl residues, but their biosyntheses are controlled preferentially by the metal Cd or metalloid As. PCs are synthesized from glutathione (γ-glutamyl-cysteinyl-glycine) in steps mediated by PC synthase. Genes (*CAD1*, *PCS1*) of the enzyme have been isolated from plants, fission yeast and some animals. Inhibition studies of PC biosynthesis via glutathione have demonstrated their fundamental roles in the metal detoxification in yeast and fungi, green algae and some aquatic plants, and also in the suspension-cultured cells and intact tissues in higher plants. Over-expression of PC synthase genes increases the Cd-tolerance in yeast and bacteria efficiently but not always in higher plant tissues especially in metal-accumulating species. "Hyperaccumulators" of Cd, Zn, Ni or As in terrestrial plants have a common feature where massive metal transport to shoots prevails, besides the ability of their roots to form PCs. This suggests that PC-based metal detoxification might be an ancient type of defense mechanism established in micro-algae or micro-fungi, and the additional PC-independent mechanism via vascular transport system became established later in higher plants. Readjustment of the PC-dependent and independent mechanisms at the metal-binding sites in the symplast and apoplast of shoots can be effective for further improvement of the metal detoxification activities and the tolerance characteristics of higher plants under various conditions.

**Key words:** arsenic, cadmium-binding peptides, heavy metal-tolerance, phytochelatin, phytoremediation.

**Fitoquelatinas:** Fitoquelatinas (PCs) foram inicialmente descobertas como sendo ligantes de Cd do tipo "Cadystins A e B" em uma levedura e, em seguida, em várias plantas, como componentes principais dos complexos de ligação ao metal Cd. As PCs têm como estrutura geral (γ-glutamil-cisteina)n-glicina (n=2-11), assim como variantes com unidades repetidas de γ-glutamil-cyteinil, que são formadas em plantas e levedura. As PCs são capazes de se ligar a vários metais, incluindo Cd, Cu, Zn ou As, mediante os resíduos sulfidrila e carboxila, porém sua síntese é controlada, preferencialmente, pelo metal Cd ou pelo metalóide As. As PCs são sintetizadas a partir da glutationa (γ-glutamil-cisteinil-glicina) em passos catalisados pela enzima PC sintase. Os genes (*CAD1*, *PCS1*), que codificam a enzima, foram isolados de plantas, levedura e alguns animais. Estudos de inibição da síntese de PC via glutationa demonstraram seu papel fundamental na destoxificação de metais em levedura e fungos, algas verdes e algumas plantas aquáticas, assim como em células em suspensão e tecidos intactos de plantas superiores. A superexpressão dos genes da PC sintase levou ao aumento da tolerância ao Cd em leveduras e bactérias, mas não em todas as plantas superiores, particularmente nas espécies acumuladoras de metais. As espécies de plantas denominadas de "hiperacumuladoras" de Cd, Zn, Ni e As apresentam como característica comum, o transporte maciço para as partes aéreas, além da capacidade do sistema radicular de formar as PCs. Esses dados sugerem que a destoxificação de metais por PCs pode ser um tipo de mecanismo de defesa evolutivamente antigo, estabelecido em microalgas ou microfungos, tendo o mecanismo adicional independente de PCs via sistema vascular de transporte se estabelecido, posteriormente, em plantas superiores. Reajustes de ambos os mecanismos, dependente e não dependente de PCs, ao nível dos sítios de ligação de metais no simplasto e apoplasto das partes aéreas das plantas, podem ser efetivos para posterior melhoria dos sistemas de destoxificação de metais e para as características de tolerância de plantas superiores sob as mais variadas condições.

**Palavras-chaves:** arsênico, fitorremediação, metal pesado, peptídeos ligantes de cádmio, tolerância.

## INTRODUCTION

Several hundred million years ago plants evolved from water to terrestrial habitats. Consequently, they could increase both their photosynthetic activities and "suction pump" powers to increase their biomass and compensate the water- and nutrition-deficient status of the land. However, at the same time, these characteristics of terrestrial plants would be a disadvantage under conditions of excess levels of toxic heavy metals like cadmium, copper, mercury and arsenic and hence they would have to accumulate them inside the plants. The plants might have to evolve novel tolerance characteristics to cope with such a negative effect caused by the toxic metals or metalloids in terrestrial habitats. Nevertheless, higher plants have survived in terrestrial habitats and thus it can be assumed they were able to retain various tolerance characteristics towards heavy metals, in so far as they have not lost them during evolution. Some, but not all, are homologous to their aquatic ancestral plants and microorganisms because of evolution. Debates on heavy metal tolerance mechanisms now known at the molecular level are especially interesting if combined with speculations on evolutionary aspects.

The tolerance characteristics of plants to heavy metal ions are diverse among the metal ions involved (Foy et al., 1978; Woolhouse, 1983; Verkleij and Schat, 1990). This is especially true of a group of metals called "borderline class metals", including $Cd^{2+}$, $Cu^{2+}$ and $Zn^{2+}$, that are capable of binding to multiple types of naturally occurring chemicals or components in plants (Woolhouse, 1983). Nevertheless, the tolerance towards these toxic ions can be expressed in a highly specific manner for each metal and/or metalloid in plants in general, and co-tolerance or cross-resistance appears relatively rarely (Woolhouse, 1983; Mehra and Winge, 1991; Hall, 2002). The reason is not yet well understood but the fundamental basis of the mechanisms can be related to either the alteration of metal-sensitive metabolism and structure or to the development of new metal-sequestering principles within some cellular compartments (Kishinami and Widholm, 1987; Huang et al., 1987; Verkleij and Schat, 1990; Mehra and Winge, 1991). So far, as for the latter, various types of metal-binding complexes have been identified from plants and microorganisms. Among them the best characterized are phytochelatins (PCs) and metallothioneins (MTs). MTs are the subject of a separate review in this special issue. In this review, we will therefore focus our attention on the physiological roles of PC peptides in heavy metal tolerance in various plants and other organisms. Details of structures,

biosynthesis, analytical methods, related genes and the many other aspects of PC peptides are available in previous reviews (Rauser, 1995; Zenk, 1996; Mehra and Tripathi, 2000; Cobbett, 2000; Cobbett and Goldsbrough, 2002). Here, we have tried to provide more details of the occurrence of PCs and their variations between microorganisms and higher plants to help the reader speculate on the evolutionary aspects. The results using suspension-cultured cells are also emphasized here to some extent because of the importance of this model system in understanding the metal-tolerance mechanism at cellular levels and to bridge the gap between single cells and integrated cells of higher plants.

**Research history of PCs – a survey**

Before discovery of PCs, pioneers of the research for plant metal-binding complexes had extensively examined the possible involvement of metallothioneins (MTs) in metal-detoxification in plants, since MTs had been recognized as the major heavy metal-binding complexes in animals and fungi (Kägi, 1991). MTs are cysteine-rich proteins synthesized on ribosomes according to the mRNA information (Rauser, 1990,1995). It was just about 20 years ago that the PCs were identified as non-protein cysteine-rich oligopeptides entirely different from MTs in structure and biosynthetic pathways. Some key topics for research on PCs are listed in table 1. Hayashi and his group first discovered the peptides in the Cd-binding complexes produced in fission yeast, *Schizosaccharomyces pombe*, exposed to $Cd^{2+}$ ions and named them "cadystins" (Murasugi et al., 1981). Two types of cadystins, A and B were then identified as (γ-Glu-Cys)n-Gly with n = 2 and 3, respectively (Kondo et al. 1984). Independently, Grill et al. (1985) have found the ubiquitous occurrence of the same peptides and those with higher degrees of polymerization (n = 2-11) in various cells of plants exposed to $Cd^{2+}$ ions and termed them as "phytochelatins". The term "phyto-meaning plants" and "chelatin" the metal chelating properties. Thereafter, much evidence has been put forward suggesting the importance of the PC peptides in sequestering the toxic ions of the borderline class metals in plants as well as some yeast and microorganisms (Robinson, 1989; Rauser, 1990; Steffens, 1990; for reviews). The two standard characteristics attributed to the PCs are that (1) PC synthesis can be stimulated in cells exposed to various other metal ions such as $Cu^{2+}$, $Zn^{2+}$, $Pb^{2+}$ and $Ag^+$, and (2) the formed PCs are capable of binding to multiple types of metals and metalloids (Grill et al., 1987; Gekeler et al., 1989). The former, however, is controversial because it does not always fit for

all cases in plants as will be described later, but the latter is accepted with increasing evidence. Direct evidence for enzymes involved in the synthesis of PCs from GSH was presented in *Silene cucubalus* (Grill et al., 1989) and *S. pombe* (Hayashi et al., 1991), independently, while there was some difference in the catalytic modes. Cadmium appeared to be the best activator for PC synthase among various metals and metalloids tested (Grill et al., 1989). Buthionine sulfoximine (BSO), an inhibitor of GSH biosynthesis, has been successfully used to verify the relationships between the levels of PCs and heavy metal tolerance in many plant cells (Griffith and Meister, 1979; Steffens et al., 1986; Grill et al., 1987; Scheller et al., 1987). Correspondingly, PC-deficient Cd-hypersensitive mutants were obtained from *S. pombe* and *Arabidopsis thaliana* in 1988 and 1995, respectively (Mutoh and Hayashi, 1988; Howden et al., 1995a). Both biochemical and genetic approaches for modifying PC synthesis have contributed to our general understanding of the important role of PC synthase in heavy metal tolerance in plants and fungi. For example, recent sensational findings have come from three different laboratories where the genes (*CAD1*, *PCS1*) encoding PC synthase proteins were successfully isolated from plants, *S. pombe* and a worm *Caenorhabditis elegans* (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). Thereafter it became possible for many investigators to understand the genetic background for the expression and function of PC synthase genes in various types of plants

Table 1. Some important topics in PC research between 1981 to 1999.

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(1) Discovery of Phytochelatins (PCs)
   1981. <u>Cadystins</u> from Fission Yeast (*S. pombe*)
      *Murasugi et al., J. Biochem. 90: 1561-64.*
   1985. <u>Phytochelatins</u> from Higher Plants
      *Grill et al., Science 230: 674-76.*
(2) PC Synthase <u>Enzyme</u>
   1989. *S.cucubalus*
      *Grill et al., Proc. Natl. Acad. Sci. USA 86: 6838-42.*
   1991. *S. pombe*
      *Hayashi et al., Biochem. Cell Biol. 69: 115-21.*
(3) PC-deficient Cd-hypersensitive <u>Mutants</u>
   1988. Fission Yeast
      *Mutoh and Hayashi, BBRC 151:32-39.*
   1995. Arabidopsis
      *Howden et al., Plant Physiol. 107: 1059-66.*
(4) PC Synthase <u>Genes</u>
   1999. Plants, *S. pombe* and *C. elegance.*
      *Clemens et al., EMBO J. 18: 3325-33.*
      *Ha et al., Plant Cell, 11: 1153-63.*
      *Vatamaniuk et al., Proc. Natl. Acad. Sci. USA 96: 7110-15.*

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as well as in microorganisms (Rauser, 1999; Cobbett and Goldsbrough, 2002; for review). More recently, various gene structures related to *CAD1* or *PCS* have been researched with a genomic DNA data bank, not only in plants but also in the other living organisms (Beck et al., 2003; Tsuji et al., 2004). Functional assessments of the respective genes await further detailed research.

**PC biosynthesis**

Figure 1 illustrates the biosynthetic pathways of PCs from their common precursors, glutamine (Glu, E), cysteine (Cys, C), and glycine (Gly, E). The pathway is completely overlapped with that of GSH biosynthesis because PCs are synthesized from GSH as the direct substrate (Noctor and Foyer, 1998). The biosynthesis of GSH consists of two sequential reactions mediated by γEC synthetase (EC 6.3.2.2) and GSH synthetase (EC 6.3.2.3). Both the reactions require ATP as substrate. The former is a rate limiting enzyme for GSH synthesis but not the latter (Noctor and Foyer, 1998). The γEC synthetase activity is enhanced by $Cd^{2+}$ ions and specifically inhibited by the treatment with BSO (Steffens et al., 1986; Grill et al., 1987; Scheller et al., 1987). Similarly, the PC-deficient and Cd-hypersensitive mutants of *S. pombe* (Mutoh and Hayashi, 1988) and *Arabidopsis thaliana* (Howden et al., 1995b; Cobbett et al., 1998) are lacking either γEC synthetase or GSH synthetase. Isolated genes encoding the key enzymes for PC biosynthesis are also shown at the bottom of figure 1.

PC synthase, which mediates the synthesis of PCs from GSH, has been reported in suspension cultures of *Silene cucubalus* (Grill et al., 1989). It was characterized as the γEC dipeptidyl transpeptidase (EC 2.3.2.15) that sequentially adds a γEC-unit of GSH to another GSH or PCs *in vitro* (Grill et al., 1989; Loeffler et al., 1989). This enzyme is constitutively expressed but requires $Cd^{2+}$ ions, which are the most efficient activators among the metal ions (Grill et al., 1989). Similar
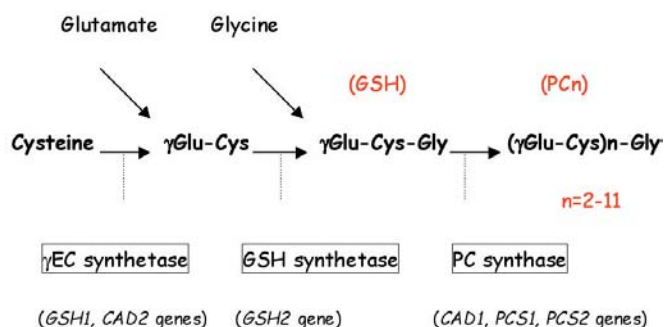


**Figure 1.** Biosynthesis of PCs in higher plants.

enzyme activities have been reported in the other plants (Klapheck et al., 1995; Howden et al., 1995; Chen et al., 1997; Inouhe et al. 2000). More recently, PC synthase genes (*PCS1*, *CAD1*) were isolated in *A. thaliana* (Ha et al., 1999; Vatamaniuk et al., 1999) and *Triticum aestivum* (Clemens et al., 1999). Homologous genes are found in *S. pombe* and *C. elegans*. These findings suggest that the PC synthase (gene) may be more widespread and have more general functions in organisms. These genes were expressed to proteins in *Escherichia coli* and *S. cerevisiae* or other translation systems and the synthesized PCS proteins were compared with the native ones (Matsumoto et al., 2004). There were some discrepancies in the structural properties, expression manner, and catalytic patterns (Grill et al., 1989; Matsumoto et al., 2004). For example, some PCS genes are constitutively expressing while others are inducible with the enhancement by Cd and other metal ions (Lee and Korban, 2002; Heiss et al., 2003; Matsumoto et al., 2004). These differences will be clarified if further information of the native CAD1/PCS proteins from various plants and other sources can be obtained. Furthermore, finding novel genes participating in the regulation of PC synthesis during accommodation and adaptation to increasing levels of toxic metals will be of great importance in the future.

**Variation of PC peptides in plants**

PCs and structurally PC-related peptides have been described in various plants and other organisms (table 2). Grill and his groups have found PCs with different degrees of polymerization from more than 300 species of plants and other organisms (Grill et al., 1985; Gekeler et al., 1989). In Angiosperms, more than 23 species of monocotyledonous plants and 90 species of dicotyledonous plants were tested and all of the plants were shown to produce PCs after Cd-treatments (Gekeler et al., 1989). The synthesis of PCs was also confirmed either in suspension cultures or differentiated plant seedlings of several Gymnosperms as well as in the lower plants that belong to Bryophyta (mosses) or Pteridophyta (ferns) (Gekeler et al., 1989; Zenk, 1996). Such a ubiquitous occurrence of the PC peptides with the same structures throughout the plant kingdom strongly suggests they play significant roles as primary metabolites in plants. However, their physiological role in the absence of heavy metals is totally unknown at the present time.

Some exceptional cases for the ubiquity of PCs in a restricted number of plants and yeast are as follows. PC peptides generally have Gly at the C-terminal end. The presence of some des-Gly variants of PCs in Cd-binding complexes has been reported for *S. pombe* (Hayashi et al., 1991) and *Candida glabrata* (Mehra et al., 1988). Such peptides were not abundant in many higher plants but substantial quantities could be found in *Zea mays* roots treated with $Cd^{2+}$ ions (Bernhard and Kägi, 1987). Furthermore, three other PC-related peptides were discovered from different plant sources. They differ in the amino acid residues found at the C-terminal end of (γ-Glu-Cys)n peptides, namely, Ala, Ser, Glu, or Gln (Grill et al., 1986a; Klapheck et al., 1992; Meuwly et al., 1993; Kubota et al., 2000). The (γ-Glu-Cys)-Ala peptides first isolated from plants belonging to Fabaceae (Phaseoleae) are called homo-phytochelatins (hPCs) because they are synthesized from homo-glutathione (hGSH) whose structure is γ-Glu-Cys-Ala (Price, 1957; Carnegie, 1963; Grill et al., 1986a). Some other variants of these peptides have been also detected in maize and other plants (Chassaigne et al., 2001; Rauser, 2003). Biological roles of these variant peptides are not well understood at present but should be elucidated in the future in both biochemical and evolutionary aspects.

The overall survey for the occurrence of PCs in various plants described above give us a strong enough background for the use of the term "phytochelatins" to describe the heavy-metal binding peptides in plants, as pointed out by previous reviewers (Rauser, 1995; Zenk, 1996; Cobbett, 2000). However, it has been shown that the PC synthase gene is not only restricted to plants, as was once thought, but also has an essential role in the model nematode *C. elegans* and other invertebrates including pathogenic nematodes, schistosomes and roundworms (Vatamaniuk et al., 2002). These studies will develop a new field in PC research and consequently affect the terminology.

**Table 2.** Various PC-like peptides produced by plants and yeast.

| PC-related γ(EC) peptides | Structure | Occurrence |
|---|---|---|
| Phytochelatin (Cadystin) | (γGlu-Cys)n-Gly | Plantae, yeast |
| Homophytochelatin | (γGlu-Cys)n-Ala | Leguminosae |
| Desglycine phytochelatin | (γGlu-Cys)n | Maize, yeast |
| Hydroxymethyl-PC | (γGlu-Cys)n-Ser | Gramineae |
| iso-Phytochelatin (Glu) | (γGlu-Cys)n-Glu | Maize |
| iso-Phytochelatin (Gln) | (γGlu-Cys)n-Gln | Horse radish |

**PCs and MTs in yeast and other microorganisms**

As shown in table 3 various metal-binding substances have been isolated from yeast and fungi. They include the Cd- and Cu-MTs from *S. cerevisiae* (Winge et al., 1985;

Inouhe et al., 1989), animal-like Cu-MTs from *Neurospora crassa* (Lerch, 1980) and *Agaricus bisporus* (Münger and Lerch, 1985), and also Cd-PCs (cadystins) from *S. pombe* (Murasugi et al., 1984) and *Candida glabrata* (Mehra et al., 1988). *C. glabrata* produces PCs in response to Cd but MT in response to Cu (Mehra et al., 1988). No direct evidence has been proposed for the presence of a native Cd-binding MT in eucaryotic fungi or plants, with the exception of a strain 301N of *S. cerevisiae* (Inouhe et al., 1989, 1991a). No direct evidence has been presented that supports the presence of PCS genes in *S. cerevisiae* or other yeast. These data provide important information relevant to studies of the evolutionary diversity of MTs and heavy-metal resistance among fungi and plants. It is not clear why the Cd-binding peptides produced by *S. pombe* are $PC_2$ and $PC_3$. This may be related to the catalytic properties of the PC synthase of the yeast member (genus) or the strains. The presence of PC peptides with a higher degree of polymerization was suggested to occur in the same yeast but the prevalent ones were n=2 and 3 (Grill et al., 1986b). Inouhe et al. (1996) have examined seven different species of yeast for Cd and Cu binding and growth responses. Only one of these capable of producing PCs was the same genus, *Schizosaccharomyces octosporus.* The predominant PCs produced by this yeast are also $PC_2$ and $PC_3$. No PC-like peptides were detected in this yeast exposed to $Cu^{2+}$ ions or in another yeast exposed to $Cd^{2+}$ or $Cu^{2+}$ ions (Inouhe et al., 1996). Little information on the occurrence of PCs in fungi other than the various yeast species mentioned above is available except for a few reports. PCs were found in the industrial "white-rod" fungi, *Funalia trogii* exposed to $Cd^{2+}$ ions (Yurekli et al., 2004). More extensive studies for

**Table 3.** Comparison of structures of fungal metal-binding peptides.

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       1            10           20
A: QNEGHECQCQCGSCKNNEQCQKS---
B: QNEGHECQCQCGSCKNNEQCQKS---
C: GDCGCSGASSCNCGSGCSCSNCGSK
D: GDCGCSGASSCTCASGQCTCSGCGK
E: (γEC)₃G (cadystin A), (γEC)₂G (cadystin B)
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A, Cd-MT from *S. cerevisiae* (Inouhe et al., 1991); B, Cu-MT from *S. cerevisiae* (Winge et al., 1985); C, Cu-MT from *N. crassa* (Lerch, 1980); D, Cu-MT from *A. bisporus* (Münger and Lerch, 1985); E, Cd-cadystins from *S. pombe* (Murasugi et al., 1981). The N-terminal amino-acid sequences from analysis of proteins or as predicted from DNA sequences are cited.

the heavy metal-binding peptides and proteins in other fungi should give us to a greater chance to discover novel types of metal-binding complexes containing various minor metallic elements.

Bacteria and some other prokaryotic cells can produce MT similar to mammalian MTs, as reported in *Synechococcus* sp. cyanobacterium (Olafson et al., 1988), *Pseudomonas putida* and *Thiobacillus* sp. (Higham et al., 1984; Yoshida et al., 1993). However there are few reports of the production of PCs by prokaryotes. GSH is the most abundant low molecular weight thiol in most organisms (Meister and Anderson, 1983; Rennenberg and Lamoureux, 1990), while some anaerobic bacteria contain very low amounts of GSH (Fahey et al., 1978). This suggests that most prokaryotes can biosynthesize GSH and GSH-related peptides but they generally lack the "functional" PCS genes. This idea is supported by the fact that transformed *E. coli* with plant PCS genes are capable of producing PCs thereby increasing their tolerance to several heavy metal ions (Matsumoto et al., 2004). More recently Tsuji et al. (2004) have identified a gene encoding a PC-like protein, termed alr0975, from a prokaryote (*Nostoc* sp. PCC 7120). The recombinant alr0975 protein strongly catalyzed the first step of PC synthesis from GSH but only weakly the second step, suggesting that the prokaryote retains a more primitive form of functional PCs than eukaryotes. Therefore, we need more information on prokaryotes before reaching a conclusion as to the general lack of PC synthesis or its presence in prokaryotes. Here, it can be expected that unknown prokaryotes or their DNA remain in deep seas and other harsh environments in the biosphere of the earth's surface, or perhaps in some microhabitats as in a fossil. Ivanova et al. (2002) have found that of 178 bacteria collected in The Sea of Japan and The Sea of Okhotsk, *Pseudoalteromonas citrea* and *Marinobacter* sp. have the chromosomal Cd-resistant systems mediated by PCs.

Cyanobacteria can be considered as an ancestor of the chloroplasts in green plants, mainly because the organisms and organelle perform a similar photosynthetic pattern evolving oxygen. GSH is an effective antioxidant against oxygen toxicity and is abundantly and commonly produced in the prokaryotic and eukaryotic algae as well as in higher plants (Rennenberg and Lamoureux, 1990). It is well characterized that more than 50-70 % of GSH is found in chloroplasts and less than 10 % in the cytoplasm of plant cells (Rennennberg, 1982). Little has been reported on the possible conversion of GSH to PCs in chloroplasts or the presence of a *PCS*-like gene in the plasmid DNA. Further comparative studies will

be very important to understand the evolutionary relationships between the organelles and organisms and also distinguish the evolved functions of PCs from GSH in different organelles (cytosol and chloroplast) and/or under different oxidative stresses (heavy metals or oxygen). Here, *Archaea* as a hyperthermophile and sulfur-metabolizing archaebacteria would be an interesting biological tool to assess the possible existence of PCS genes and PC-like peptides, especially when one considers that the cells might provide the origin of the cytoplasm of eukaryotic cells according to an endosymbiosis theory.

### PCs in green algae and some aquatic plants

PCs were isolated in 1988 from several "eukaryote" green algae exposed to heavy metal ions (Gekeler et al., 1988). Since then many studies have been carried out on PC synthesis in phytoplanktonic algae, including the marine diatom *Phaeodactylum tricornutum* (Morelli and Scarano, 1995; Scarano and Morelli, 2002), *Dunaliella tertiolecta* (Hirata et al., 2001; Tsuji et al., 2003), and *Chlamydomonas reinhardtii* (Howe and Merchant, 1992; Dominguez et al., 2003). Substantial levels of PCs are also found in *Euglena gracilis*, *Fragilaria crotonesis*, *Sargassum mutium* and *Porphyridium cruentum* (Gekeler et al., 1988; Reedy and Prasad, 1990; Mehra and Tripathi, 2000), and in the marine macroalgae *Kappaphycus alvarezzi* (Hu and Wu, 1998). The synthesis of PCs was compared for several marine algae species as affected by various metals (Ahner et al., 1995; Ahner and Morel, 1995). PCs are induced in the aquatic plants, such as the water fern *Salvinia minima* (Hoffmann et al., 2004), the duckweed *Lemna paucicostata* (Li and Xiong, 2004) and *Hydrilla verticillata* (Gupta et al., 1995), and the emergent aquatic plant *Phragmites australis* (Ederli et al., 2004). The most effective metal to induce PC synthesis in all these plants was Cd, while other metal species were effective to varying extents. It is interesting to note that Zn caused the strongest induction of PC synthesis in *Dunalliella tertiolecta* (Hirata et al., 2001), while Cd and As were generally the more effective for several other plants. Zn was ineffective for PC formation in some higher plants, as described later.

### PCs and heavy metal tolerances

Physiological roles of PCs and PC-related peptides widely distributed in the plant and other kingdoms have not been fully elucidated, as mentioned earlier. However, their potential roles in heavy metal detoxification in cells have been elucidated (Rauser, 1990; Mehra and Winge,

1991). It may be useful here to review their roles in heavy metal tolerance according to the path of evolution from primitive singular cell systems to the more complicated and integrated cell systems in higher plants. This relationship can be partially mimicked by that between undifferentiated and differentiated cells in many organisms, and again in dedifferentiated singular cells such as suspension-cultures of higher plants. Beside this endeavor, suspension cells have been frequently used as a model system to understand the cellular mechanisms of adaptation/tolerance to heavy metal ions in higher plants. In the following section, possible roles of PCs in terms of heavy metal-tolerance are described first for suspension cultures and then for various intact plants. Heavy metal ions or elements discussed here are centered on Cd, Cu, Zn, Ni and As, because these have been most extensively investigated or debated over the years regarding their influence on PC production and other tolerance mechanisms.

### Roles of PCs in suspension-cultured cells

Studies on heavy metal tolerance in suspension-cultured cells have been performed using common plants such as tomato (Bennetzen and Adams, 1984; Huang et al., 1987; Scheller et al., 1987; Inouhe et al., 1991b) and tobacco (Kishinami and Widholm, 1987; Reese and Wagner, 1987; Krotz et al., 1989). Some other metal-tolerant species have been used as suspension cells for similar studies, including *Rauvolfia serpentina*, *Agrostis tenius*, *Silene cucubalus* (Grill et al., 1987; Gekeler et al., 1989) and *Datura innoxa* (Jackson et al., 1984; Delhaize et al., 1989). In the most culture systems, both the PCs and Cd contents in the cells increase as a function of Cd concentrations in the media. In these cells, the Cd-binding PC complexes are detected as the major fraction containing $Cd^{2+}$ while free Cd ions are present in small amounts (Scheller et al., 1987; Delhaize et al., 1989; Inouhe et al., 1991b; Gupta and Goldsbrough, 1991). Furthermore, after subculturing in the presence of $Cd^{2+}$ ions, the cells exhibit further tolerance to $Cd^{2+}$ ions at 1 mM or more in view of the over-production of PC peptides (Inouhe et al., 1991b). Such characteristics are largely reduced by interference of PC synthesis with the specific inhibitor BSO (Steffens et al., 1986; Scheller et al., 1987; Grill et al., 1989). Thus the evidence on hand for a role for PCs in Cd-tolerance is very strong. These results for suspension cells are in good agreement with those of the fission yeast *S. pombe* (Mutoh and Hayashi, 1988) and some of eukaryotic micro algae (Morelli and Scarano, 1995; Howe and Merchant, 1992; Hirata et al.,

2001). The common features regarding these cells are their unicellular nature and their natural habitats are certain liquid media or seawaters. The cells interact with the environmental ions directly through their membrane and cell walls. If toxic ions penetrate into the cells across the barriers then the cells must detoxify the cytoplasm. Here it should be emphasized that PCs are peptides synthesized from GSH in the cytoplasm and/or symplast of the cells. Another appreciable homology is the growth pattern of the cells. They increase their biomass mainly by cell proliferation. In other words, PC formation might be required for cell division under the influence of Cd and other toxic ions. In fact, tomato cells contain their highest levels of PC activity at an exponential growth stage (Chen et al., 1997; Inouhe et al., 2000). Similar roles for PC peptides in Cd-detoxification can be partially applied to the cases of macro algae and other aquatic plants growing under water. However their functions in intact land plants might be restricted to some symplastic locations in the root or shoot tissues, as discussed later.

Inorganic arsenic (As) has been shown to induce the synthesis of PCs in cell cultures of *R. serpentina* and *S. vulgaris* (Grill et al., 1987; Schmöger et al., 2000) as well as in root cultures of *Rubia tinctorum* (Maitani et al., 1996). BSO causes a strong decrease in the levels of PCs and As-tolerance in a parallel manner (Schmöger et al., 2000). Therefore PCs appear to be involved in As-tolerance and detoxification of suspension-cultured cells in many plants (Meharg and Hartley-Whitaker, 2002). As compared with the abundant evidence for As and Cd tolerance, the roles of PCs in Cu or Zn tolerance in suspension cells are still controversial. Grill and co-workers have reported that many kinds of metal ions including $Cu^{2+}$ and $Zn^{2+}$ are capable of inducing PCs in many plant cells (Grill et al., 1985,1987; Gekeler et al., 1989). On the other hand, Wagner and co-workers have claimed that the tolerance of tobacco cells to $Cu^{2+}$ and $Zn^{2+}$ ions is not explained by formation of PCs (Reese and Wagner, 1987; Wagner and Krotz, 1989; Krotz et al., 1989). In some yeast $Cu^{2+}$ can be an inducing factor for MT but not for PCs (Mehra and Winge, 1991). A comparative study showed that tomato cells readily develop tolerance to $Cd^{2+}$ but not to $Cu^{2+}$ ions after repeated subculturings in the presence of the respective ions (Inouhe et al., 1991b). The cells accumulate $Cd^{2+}$ preferentially into the cytoplasmic PC fraction but $Cu^{2+}$ into the cell-wall fraction, suggesting that tomato cells have no or very low capacity of producing PCs under the stress of $Cu^{2+}$ ions. Metal binding assay has shown that synthetic PCs can bind to both ions $Cd^{2+}$ and $Cu^{2+}$, in vitro (Matsumoto et

al., 1990). Enzyme experiments *in vitro* have shown that the PC synthase activity of tomato is strongly stimulated by $Cd^{2+}$ (Grill et al., 1989; Ruegsegger and Brunald, 1992), but this is not confirmed for $Cu^{2+}$ ions (Inouhe et al., unpublished data). Thus, some side effects of $Cu^{2+}$ ions appear to be involved in the inhibition of the GSH and/or PCs biosynthesis in tomato cells (figure 2). Actually $Cu^{2+}$ ions enhance oxidation and/or consumption of thiols in roots (De Vos et al., 1992; Lappartient and Touraine, 1997) but this is not well established in suspension cells. Heavy metal ions affect various processes in plants, including membrane function (De Vos et al., 1989) and enzymatic activities (Sheoran et al., 1990). $Cu^{2+}$ ions cause damage of the plasma membrane at lower concentrations in plants (De Vos et al., 1989) and yeast (Joho et al., 1984) than do $Cd^{2+}$ ions. The apparent discrepancy for the metal-specific aspects of the activation of PC synthesis cannot be resolved at present, but it may contribute to disclose diversity of the regulation mechanisms in plants.
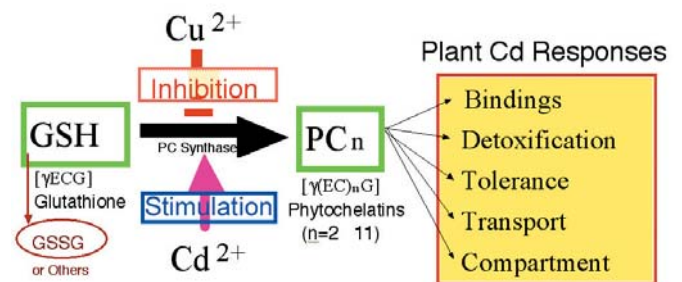


**Figure 2.** Possible effects of Cd and Cu in tomato cells.

The following is an exceptional case for the effectiveness of Cd on PC formation (Inouhe et al., 2000). Suspension-cultured cells of azuki bean (*Vigna angularis*) as well as the original root tissues are naturally hypersensitive to Cd (<10 μM). Repeated subculturings of the suspension cells with sub-lethal levels of Cd (1-10 μM) do not affect the subsequent response of cells to inhibitory levels of Cd (10-100 μM). The azuki bean cells challenged with Cd do not contain PC peptides and the protein extracts have no activity converting GSH to PCs, unlike tomato (Inouhe et al., 2000). These results suggest that azuki bean cells are lacking in the PC synthase activity *per se*, hence being Cd-hypersensitive. Azuki bean cells have been reported to contain hGSH and thus produce hPCs to some extent (Oven et al., 2001). While the role of these thiols in the Cd-hypersensitive plant have not been fully examined, it can be generally concluded that PC synthase has a more important role in Cd-tolerance of suspension-cultured cells in general.

**Roles of PCs in intact land plants**

The stimulation of PC formation by $Cd^{2+}$ has been reported in roots of various plant species (Fujita and Kawanishi, 1987; Gekeler et al., 1989; Rauser et al., 1990). A possible relationship between Cd-tolerance and Cd-accumulation has been reported at the different family level of plants (Kuboi et al., 1987; Obata and Umebayashi, 1993). Furthermore, positive relationships between levels of PCs and Cd-tolerance in intact plants have been reported in maize (Nussbaum et. al., 1988; Rüegsegger and Brunold, 1992; Rauser and Meuwly, 1995), *Brassica juncea* (Haag-Kerwer et al., 1999), *Pisum sativum* (Rüegsegger and Brunold, 1989), *A. thaliana* (Howden et al., 1995a,b), and other plants (Inouhe et al., 1994). Effects of $Cd^{2+}$ on growth and the synthesis of the Cd-binding complex were comparatively examined with 4 mono- and 7 dicotyledonous species under the same conditions (Inouhe et al., 1994). The results clearly showed that mono- and dicotyledonous roots exhibited a substantially different Cd-tolerance, depending on their abilities to produce the Cd-binding PC complexes in the cytoplasmic fractions. Among them, azuki bean roots are most sensitive to Cd at 1-3 µM and lacking in PC synthase activity *in vitro* (Inouhe et al., 2000). More direct evidence for the role of PC in Cd-tolerance in intact plants was obtained using PC-deficient *cad1* mutants of *Arabidopsis* (Howden et al., 1995a; Ha et al., 1999). The lowest Cd concentration to which the *cad1* mutants (*cad1-3*) were sensitive was 0.3 µM, suggesting an essential role of the *CAD1* gene for PC synthase in Cd-tolerance in roots.

Some investigators have shown that PCs have an important role in As-tolerance in intact plants (Grill et al., 1987; Ha et al., 1999; Schmöger et al., 2000; Hartley-Whitaker et al., 2002). Here we shall briefly survey the mechanisms for the transport and tolerance for As. Plants and microorganisms take up As in the form of the anion ($AsO_4^{3-}$) via a transporter for phosphate ($PO_4^{3-}$) in the cell membranes (Willsky and Malamy, 1980; Fujiwara et al., 2000; Meharg and Hartley-Whitaker, 2002; Lee et al., 2003). Two major types of As-tolerance/resistance mechanisms are conceivable in plants and microorganisms. One is the restriction of As uptake due to low phosphate uptake activity (Rosenberg et al., 1977; Meharg and Macnair, 1990; Silver, 1996; Fujiwara et al., 2000; Kobayashi et al., 2003) and the other is via its intracellular detoxification (Edmonds and Francesconi, 1981; Kaise et al., 1988). The inorganic As anions have already been shown to be capable of inducing or binding to PCs *in vivo* in roots of many plants as well as suspension cells (Grill et al.,

1987; Schmöger et al., 2000). Next are two interesting historical turning points in recent research on the subject. First, the As-tolerance plant *Holcus lanatus* isolated from an As-Cu contaminated site was considered to be due to the suppression of As uptake (Meharg and Macnair, 1992). However, this idea was recently revised since the As-tolerant plants were found to contain much higher levels of PCs and As than the wild-type plants when grown in the presence of high levels of As (Hartley-Whitaker et al., 2001). Second concerns the PCn species (n=2 and 3) involved in the As-bindings *in vivo* and *in vitro*. It has been shown through *in vitro* experiments that As preferentially binds to $PC_3$ forming the As (III)-$PC_3$ complex rather than to GSH or $PC_2$ (Raab et al., 2004). The As -$PC_3$ complex is the dominant complex formed in *Holcus lanatus*, while GSH, $PC_2$ and $PC_3$ are found in the extracts (Raab et al., 2004). However, *R. serpentina* produces the $PC_2$-As -$PC_2$ as a major form of As-complex (Schmöger et al., 2000). The bracken fern *Pteris cretica* synthesizes $PC_2$ and forms the GSH-As -$PC_2$ complexes predominantly (Raab et al., 2004), while the other species *P. vittata*, induces an unidentified thiol specifically by As treatment (Cai et al., 2004). Chickpea plants are capable of forming both PCn and hPCn in response to Cd and As, where $PC_2$ and $hPC_2$ show preference for Cd while $PC_3$ and $hPC_3$ show preference for As (Gupta et al., 2004). Roles of specific PCs, hPCs and other novel thiols in the As-tolerance mechanism can be expected to be clarified in the near future.

There is, however, some confirmed evidence against the role of PCs in heavy metal tolerance in higher plants, since some exceptions have been found as in the case of the suspension cells mentioned earlier. Ernst and his group first noticed that the Cd-tolerant and Cd-sensitive plants of *Silene vulgaris* are both capable of producing equal levels of PC peptides in response to Cd (Verkleij et al., 1990; de Knecht et al., 1994). They found that greater amounts of Cd and acid-labile sulfides are associated more so with the PCs in the tolerant plants than in the sensitive ones, suggesting a more important role of sulfides rather than PC itself in the Cd-detoxification process in the plants. Actually the general importance of the incorporation of acid-labile sulfides in the formation of the more stable and functional Cd-binding complexes has been demonstrated in the other plants and microorganisms (Murasugi et al., 1984; Mehra et al., 1988; Reese et al., 1992). The Cd-treatments are also known to stimulate sulfur assimilation required for sulfide formation in plants (Nussbaum et al., 1988; Robinson 1989). Further evidence against direct roles of PCs for Cu- or Zn-tolerances

have been presented in *S. vulgaris* (De Vos et al., 1992; Schat and Kalff, 1992; Harmens, 1993). More recently it has been shown that higher levels of expression of the PC synthase gene (*AtPCS1*) enhance the Cd-tolerance of cells in *E. coli* or *S. cerevisiae* (Matsumoto et al., 2004). However, such over expression of *AtPCS1* does not lead to any increased Cd-tolerance and accumulation in *A. thaliana* (Lee et al., 2003a), but paradoxically leads to hypersensitivity to Cd and Zn ions though not for Cu (Lee et al., 2003). Therefore it must be concluded that a variety of factors other than PC synthase are involved in the tolerance phenotypes of intact plants to heavy metals.

**Roles of PCs in the heavy metal-hyperaccumulators**

So far various plants (more than a hundred species) have been recognized as the heavy-metal hyper-accumulators that accumulate Zn, Cd, Ni, Cu, and As etc preferentially in the shoot organs. Their suction powers of metals from soils or waters are also believed to contribute to phytoremediation of contaminated sites with various heavy metals. It is one reasonable approach to understand the possible roles of PCs for the metal accumulator phenotypes and hence for phytoremediation process (Kräemer, 2003). However at present such evidence supporting a role for PC in metal hyperaccumulation and tolerance are not so strong. Ebbs et al. (2002) reported that PC synthesis was not responsible for Cd-tolerance of the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Further roles of PC in constitutive and adaptive heavy metal-tolerances were studied in hyperaccumulator and non-hyperaccumulator metallophytes, *S. vulgaris*, *C. caurulescens*, *H. lanatus* and *A. castellana* (Schat et al., 2002). It has been generally found that BSO treatments consistently did not affect the sensitivities to Cu, Zn, Ni, and Co. BSO also caused an increase in Cd-sensitivity in non-metallicolous plants but did not in hypertolerant plants. From this and other evidence (Salt et al., 1989; Davies et al., 1991; Schat and Kalff, 1992), the primary function of PC synthesis in hyperaccumulators is still elusive. Long-distance root-to-shoot transports of PCs and Cd are shown in *Arabidopsis* (Gong et al., 2003) but this is not fully established in the other major hyperaccumulator species. Although it would be interesting to discuss the mechanisms for the PC-independent metal-accumulation and transport to shoots in the hyperaccumulators, this is beyond the scope of the present review. One of the most well-known properties of the hyperaccumulator plants concerns their long-distance transport systems mainly governed by the transpiration stream. This apoplastic route which carries the stream of heavy metal-containing water transported from roots to shoots (leaves) can be a collective proof that higher land plants evolved from their ancestor plants or microbes living in water as described earlier. In the future, relationships between symplast and apoplast for metal binding and movement will be of great importance in research projects in both the pure and applied fields of science.

**PCs as biochemical indicators for heavy metal contamination**

Finally, the possible importance of the formation PCs and PC-related peptides in plant tissues as the biochemical indicators for heavy metal contamination is briefly described. As described earlier, PC formation in plant tissues occurs in function of external metal concentrations in general, irrespective of whether they are specific or non-specific to metals. Thus quantitative biochemical assays for the metal adjustments are also possible. For example, as shown in figure 3, chickpea provides very capable indicators for heavy metal contamination (Gupta et al., 2002, 2004). The roots are very sensitive in producing PCs and hPCs in a specific manner in response to Cd and As, and the shoots, as well as the roots of the plant, are sensitive to various metals in terms of the rapid increases in the levels of GSH, hGSH and cysteine. These changes can be used in the biochemical evaluation of some specific or overall heavy metal contamination in various habitats. Furthermore, the signaling and transport systems between root and shoot in terms of heavy-metal response will be a matter of great importance in the future. However, as for such a possible utilization of PC peptides as biochemical signals for contamination, Rauser (1990) has already cautioned that PC formation is not very sensitive at lower concentrations of Cd. He claimed that it should be recognized that many other mechanisms may prevail in plants growing in Cd-polluted soils where internal Cd concentrations are much lower than those used in most experimental model systems such as suspension cultures (Rauser, 1990). Nevertheless, there is a recent positive report showing that very low free ionic Cd ($10^{-9.6}$ M) increased PC production and GSH concentrations in romaine lettuce (*Lactuca sativa* L. var. *longifolia* Lam. var. Parris Island). Here, the plants were grown in a flow-through hydroponic (FTH) system (Maier et al., 2003) under a specific culture condition using an EDTA-buffered nutrient medium. The results bring a new insight to the field and point the direction of further research.

Morphological changes in metal hypersensitive plants can be used as a biological indicator for similar assessments for the metal contamination by counting the number of plants affected by the phyto-toxic effects of different metals. However this kind of evaluation may have some limitations for actual application because the justifications are made based upon the survival of the plants and thus need a large number of individuals and will only respond at higher concentrations of the contaminating metals. On the other hand, the biochemical indices of PC formation can be used for more sensitive and more specific assessments of environmental contamination by different heavy metal ions.
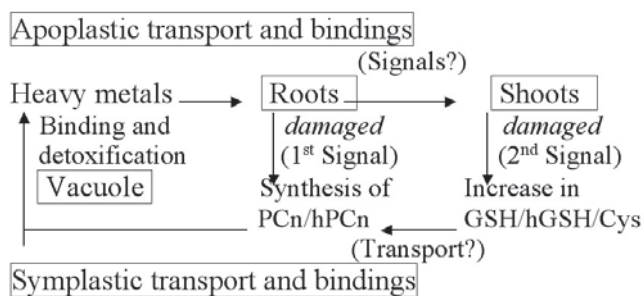


**Figure 3.** Possible effects of heavy metal ions on the synthesis of PCn and hPCs in roots and the levels of GSH, hGSH and cysteine in shoots of chickpea plants (Gupta et al., 2004).

**Concluding remarks**

Biochemical and genetic bases of the Cd-tolerance/ resistance phenotypes of plants may involve both PC-dependent and PC-independent processes. The former involves several different sub-processes: the activation of PC synthase, GSH biosynthesis, the accumulation of acid-labile sulfides, sulfur assimilation and transport of the Cd-PC complexes to the vacuole. All these would be required for the formation of stable and nontoxic Cd-complexes in the vacuole or other symplastic sites in the cells of most plants and some fungi and worms, where PC synthase is the key factor for the tolerance phenotypes to Cd and other ions. The PC-independent mechanisms are apparently present in more differentiated higher plants that inhabit terrestrial environments. Their hyperaccumulation phenotype of metal/ metalloids from soil and water can be attributed to the highly developed apoplastic transport systems. Readjustment of both the symplastic and apoplastic activities of metal-binding in shoots may work more effectively for the required phytoremediation processes of contaminated soil and water environments.

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