

Phytochelatins and Their Roles in Heavy Metal Detoxification

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Plants respond to heavy metal toxicity in a variety of different ways. Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms such as ethylene and stress proteins. These mechanisms have been reviewed comprehensively by Sanita di Toppi and Gabbrielli (1999) for plants exposed to Cd, the heavy metal for which there have been arguably the greatest number and most wide-ranging studies over many decades. Understanding the molecular and genetic basis for these mechanisms will be an important aspect of developing plants as agents for the phytoremediation of contaminated sites (Salt et al., 1998). One recurrent general mechanism for heavy metal detoxification in plants and other organisms is the chelation of the metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex.

A number of metal-binding ligands have now been recognized in plants. The roles of several ligands have been reviewed by Rauser (1999). Extracellular chelation by organic acids, such as citrate and malate, is important in mechanisms of aluminum tolerance. For example, malate efflux from root apices is stimulated by exposure to aluminum and is correlated with aluminum tolerance in wheat (Delhaize and Ryan, 1995). Some aluminum-resistant mutants of *Arabidopsis* also have increased organic acid efflux from roots (Larsen et al., 1998). Organic acids and some amino acids, particularly His, also have roles in the chelation of metal ions both within cells and in xylem sap (Kramer et al., 1996; Rauser, 1999).

Peptide ligands include the metallothioneins (MTs), small gene-encoded, Cys-rich polypeptides. Our current understanding of the functions and expression of MTs in plants, particularly *Arabidopsis*, have been reviewed elsewhere (Fordham-Skelton et al., 1998; Rauser, 1999). In contrast, the phytochelatins (PCs), the subject of this *Update*, are enzymatically synthesized Cys-rich peptides. The most recent review of PC structure, biosynthesis, and function in this journal was by Rauser (1995). Other more recent reviews are by Zenk (1996) and Rauser (1999). Recent advances in our understanding of aspects of PC biosynthesis and function are derived predominantly

from molecular genetic approaches using model organisms.

PLANTS MAKE TWO TYPES OF PEPTIDE METAL-BINDING LIGANDS: MTs AND PCs

The historical context of the identification of MTs and PCs in plants is worth discussing. MTs were first identified as Cd-binding proteins in mammalian tissues. Similar proteins have been identified in a large number of animal species (Kägi, 1991). Early reports of metal-binding proteins in plants were generally assumed to be MTs. However, in the absence of detailed characterization or primary amino acid sequences, many of these metal-binding complexes may have been comprised, at least in part, of PCs, particularly where they were identified in studies of plant responses to Cd.

After the structures of PCs had been elucidated and it was found that these peptides are distributed widely in the plant kingdom, it was proposed that PCs were the functional equivalent of MTs (Grill et al., 1987). Subsequently, numerous examples of MT-like genes, and in some cases MT proteins, have been isolated from a variety of plant species and it is now apparent that plants express both of these Cys-containing metal-binding ligands. Furthermore, it is likely that the two play relatively independent functions in metal detoxification and/or metabolism. However, the extent to which this is true is not yet clear and will not become apparent until a complete set of MT-deficient mutants have been identified in *Arabidopsis*. PCs have not been reported in an animal species, supporting the notion that in animals, MTs may well perform some of the functions normally contributed by PCs in plants. However, the isolation of the PC synthase gene from plants and the consequent identification of similar genes in animal species, described below, suggests that, at least in some animal species, both of these mechanisms contribute to metal detoxification and/or metabolism. Although PCs have also been classified as class III MTs and the use of the term "phyto" to designate these compounds appears to be increasingly inaccurate, I believe "phytochelatins" has become so entrenched in the literature that a more broadly encompassing designation is likely to cause only confusion.

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PCs HAVE THE GENERAL STRUCTURE $(\gamma\text{-Glu-Cys})_n\text{-Gly}$

Early analyses demonstrated PCs consisted of only the three amino acids: Glu, Cys and Gly with the Glu, and Cys residues linked through a γ -carboxylamide bond. PCs form a family of structures with increasing repetitions of the γ -Glu-Cys dipeptide followed by a terminal Gly; $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n has been reported as being as high as 11, but is generally in the range of 2 to 5. PCs have been identified in a wide variety of plant species and in some microorganisms. They are structurally related to glutathione (GSH; $\gamma\text{-Glu-Cys-Gly}$) and were presumed to be the products of a biosynthetic pathway. In addition, a number of structural variants, for example, $(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-Ala}$, $(\gamma\text{-Glu-Cys})_n\text{-Ser}$, and $(\gamma\text{-Glu-Cys})_n\text{-Glu}$, have been identified in some plant species (Rauser, 1995, 1999; Zenk, 1996).

PCs ARE SYNTHESIZED FROM GSH

Numerous physiological, biochemical, and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis (Rauser, 1995, 1999; Zenk, 1996). Such studies have used a variety of plant species, sometimes as intact plants but often in the form of in vitro cell cultures. Early studies with cell cultures demonstrated that induction of PCs in the presence of Cd coincided with a transient decrease in levels of GSH. Furthermore, the exposure of either cell cultures or intact plants to an inhibitor of GSH biosynthesis, buthionine sulfoximine, conferred increased sensitivity to Cd with a corresponding inhibition of PC biosynthesis. This could be reversed by the addition of GSH to the growth medium.

By far the most detailed characterization of the pathway of PC biosynthesis has come from studies in the fission yeast (*Schizosaccharomyces pombe*), and in Arabidopsis. Genetic studies have confirmed GSH-

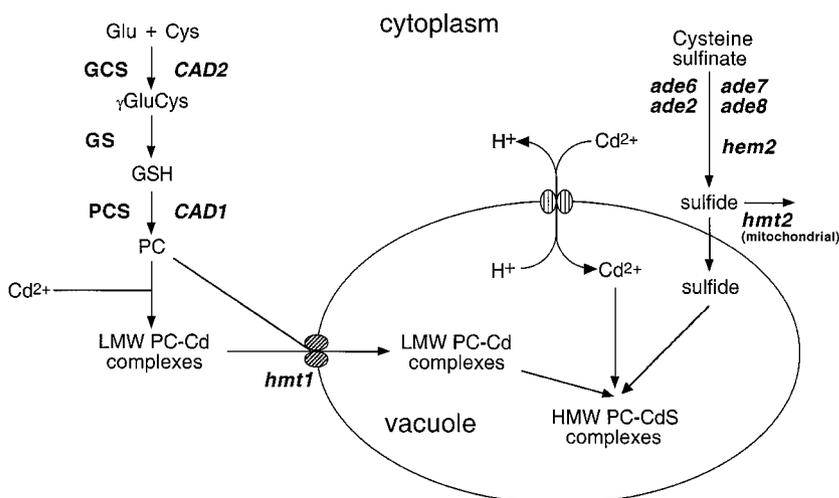
deficient mutants of the fission yeast and Arabidopsis are also PC deficient and hypersensitive to Cd. In particular, the *cad2-1* mutant of Arabidopsis is partially deficient in GSH and in γ -glutamyl-Cys synthetase (GCS) activity, the first of the two GSH biosynthetic enzymes. The *cad2-1* mutation is a 6-bp deletion within an exon of the GCS gene affecting residues in the vicinity of the presumed active site of the enzyme (Cobbett et al., 1998). The PC biosynthetic pathway is illustrated in Figure 1.

PC SYNTHASE IS ACTIVATED BY HEAVY METAL IONS

Grill et al. (1989) first identified an enzyme activity from cultured cells of *Silene cucubalis* that synthesized PCs from GSH by transferring a γ -Glu-Cys moiety from a donor to an acceptor molecule. The reaction involved the transpeptidation of the γ -Glu-Cys moiety of GSH onto initially a second GSH molecule to form PC_2 or, in later stages of the incubation, onto a PC molecule to produce an $n + 1$ oligomer. This γ -Glu-Cys dipeptididyl transpeptidase (EC 2.3.2.15) has been named PC synthase. The enzyme is a 95,000- M_r tetramer with a K_m of 6.7 mM for GSH. In vitro the activity of the partially purified enzyme was active only in the presence of metal ions. The best activator tested was Cd followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au cations. These metals also induce PC biosynthesis in vivo in plant cell cultures. In in vitro reactions, PC biosynthesis continued until the activating metal ions were chelated either by the PCs formed or by the addition of a metal chelator such as EDTA (Loeffler et al., 1989). This provides a mechanism to autoregulate the biosynthesis of PCs in which the product of the reaction chelates the activating metal, thereby terminating the reaction.

Similar PC synthase activities have been detected in pea (Klapheck et al., 1995), tomato (Chen et al., 1997), and Arabidopsis (Howden et al., 1995). Crude

Figure 1. Genes and functions contributing to Cd detoxification in plants and fungi. The figure is a composite of different functions described in different organisms. Enzyme abbreviations are shown in bold. GS, GSH synthetase; PCS, Phytochelatin synthase. Gene loci are shown in bold italics. *CAD1* and *CAD2* are in Arabidopsis; *hmt1*, *hmt2*, *ade2*, *ade6*, *ade7*, and *ade8* are in fission yeast; and *hem2* is in *Candida glabrata*. The details of sulfide metabolism on the right of the figure are not well understood.



enzyme preparations from the roots of pea, which normally contain both GSH and homo-GSH (γ -Glu-Cys- β -Ala), could use GSH efficiently, but homo-GSH or γ -Glu-Cys-Ser less efficiently, as substrates for PC synthesis. In the presence of both GSH and homo-GSH, synthesis of homo-PCs was enhanced (Klapheck et al., 1995). These observations were interpreted to indicate the enzyme had a γ -Glu-Cys donor site that was relatively specific for GSH but a less specific acceptor site able to use each of the three substrates. Little is known about the tissue specificity of PC synthase expression and/or PC biosynthesis. In the only study of tissue-specific PC synthase expression to date, the activity was detected in the roots and stems of tomato plants but not in leaves or fruits (Chen et al., 1997).

PC SYNTHASE GENES WERE ISOLATED INDEPENDENTLY IN THREE LABORATORIES

Despite the identification and purification of PC synthase a decade ago, the isolation of the corresponding gene and a consequent detailed understanding of the mechanism of PC biosynthesis have eluded us until recently. PC synthase genes have been isolated simultaneously by three research groups using different approaches. Two groups used expression of plant genes in Brewer's yeast (*Saccharomyces cerevisiae*) to identify genes involved in Cd resistance. One group identified an Arabidopsis cDNA (*AtPCS1*) that suppressed the Cd-sensitive phenotype of both Brewer's yeast *yap1* and *ycf1* mutants (Vatamaniuk et al., 1999). *YAP1* encodes a transcription factor that is required for the expression of *YCF1*, a transporter responsible for the vacuolar sequestration of GSH-Cd complexes (Li et al., 1997). This group was searching for functional plant homologs of *YAP1* by using a two-step screening procedure to identify Arabidopsis cDNAs that could suppress a *yap1*, but not a *ycf1*, mutant. In this screen they fortuitously identified *AtPCS1*. By expressing *AtPCS1* in various mutant strains, they demonstrated that the mechanism of *AtPCS1*-mediated Cd tolerance functioned in both *yap1* and *ycf1* mutants, and in the MT-deficient mutant, *cup1*. Thus the mechanism appeared to be distinct from other recognized Cd-detoxification mechanisms. Expression of *AtPCS1* mediated an increase in Cd accumulation indicating it was probably involved in chelation or sequestration. It also functioned in a vacuole-deficient mutant (*pep5*) demonstrating the *AtPCS1* gene product was not a vacuolar membrane transport function similar to *YCF1*, for example. Consistent with the cDNA encoding PC synthase, the mechanism was not expressed in a GSH-deficient mutant (*gsh2*) and mediated PC biosynthesis in vivo in Brewer's yeast.

A second group identified a wheat cDNA (*TaPCS1*) that conferred increased Cd resistance when expressed in a wild-type (for Cd tolerance) strain of

Brewer's yeast (Clemens et al., 1999). Similar to *AtPCS1*, the Cd resistance mediated by *TaPCS1* was associated with an increase in Cd accumulation, functioned in a vacuole-deficient mutant (*vps18*) and was GSH dependent, being diminished in the presence of an inhibitor of GSH biosynthesis. *TaPCS1* also mediated PC biosynthesis in vivo in Brewer's yeast.

AtPCS1 has also been isolated through the cloning of the *CAD1* gene of Arabidopsis. Mutants at the *cad1* locus in Arabidopsis, like the *cad2-1* mutant, are Cd sensitive and deficient in the formation of Cd-binding complexes and in PC biosynthesis. In particular, PCs are undetectable in the *cad1-3* mutant after prolonged exposure to Cd. In contrast to the *cad2-1* mutant, the *cad1* mutants have wild-type levels of GSH, suggesting a defect in PC synthase. Consistent with this, PC synthase activity in crude extracts from *cad1* mutants was less than 1% of the level in both the wild-type and the *cad2-1* mutant (Howden et al., 1995). Thus it was likely that *CAD1* is the structural gene for PC synthase (Fig. 1). The *CAD1* gene has been isolated using a positional cloning strategy (Ha et al., 1999). The position of the gene was mapped using molecular markers and a candidate gene identified from the Arabidopsis genome initiative genomic sequence. That this candidate was *CAD1* was confirmed by the identification of a different mutation in that gene in each of the *cad1* mutants and by complementation of the Cd-sensitive phenotype of *cad1-3* by a genomic clone spanning this gene.

A similar sequence (*SpPCS*) was identified in the genome of fission yeast and targeted deletions of that gene were constructed in two of these studies (Clemens et al., 1999; Ha et al., 1999). The resulting mutants were, like the Arabidopsis *cad1* mutants, Cd sensitive and PC deficient, confirming the analogous function of the two genes in the different organisms. Expression of the *CAD1*(*AtPCS1*) and *SpPCS* genes in *Escherichia coli* (Ha et al., 1999) or purification of epitope-tagged derivatives of *SpPCS* (Clemens et al., 1999) and *AtPCS1* (Vatamaniuk et al., 1999) expressed in Brewer's yeast was used to demonstrate that both were necessary and sufficient for GSH-dependent, metal ion-activated PC biosynthesis in vitro.

The Arabidopsis *CAD1*(*AtPCS1*) cDNA encodes a predicted 55-kD polypeptide of 485 amino acids. A comparison of the Arabidopsis and fission yeast amino acid sequences showed that the N-terminal regions of the two are very similar (45% identical), whereas the C-terminal sequences show little apparent conservation of amino acid sequence (Fig. 2). The most apparent common feature of the C-terminal regions is the occurrence of multiple Cys residues, often as pairs. The C-terminal regions of the Arabidopsis and fission yeast proteins have 10 and seven Cys residues, respectively, of which four and six, respectively, are as pairs. However, there is no apparent conservation of the positions of these Cys

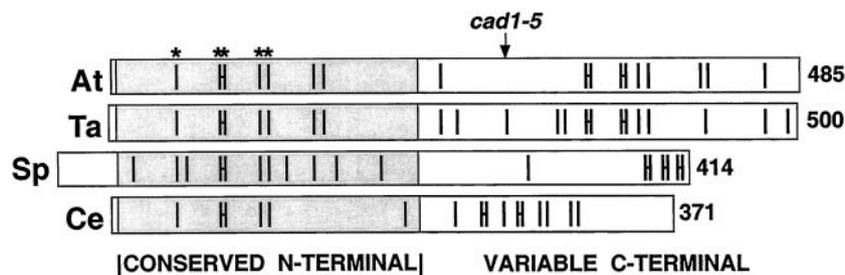


Figure 2. Schematic comparison of PC synthase polypeptides from different organisms. At, *Arabidopsis* (CAD1/AtPCS1; GenBank accession nos. AF135155 and AF085230); Ta, *Triticum aestivum* (TaPCS1; accession no. AF093252); Sp, fission yeast (SpPCS; accession no. Z68144); Ce, *Caenorhabditis elegans* (CePCS1; accession no. Z66513). The total number of amino acids in each is shown on the right. Approximate positions of all Cys residues are indicated by vertical bars; adjacent Cys residues are connected by a horizontal bar; and Cys residues conserved across the three sequences are highlighted with an asterisk. The conserved N-terminal domains exhibit at least 40% identical amino acids in pair-wise comparisons of the four sequences. An arrowhead indicates the position of the *Arabidopsis cad1-5* nonsense mutation.

residues relative to each other. The two plant PC synthase sequences, TaPCS and AtPCS, can be aligned across their entire length (55% identity) (Clemens et al., 1999). The former contains 14 Cys residues, including two pairs, in the C-terminal domain.

PC BIOSYNTHESIS MAY BE REGULATED IN SEVERAL WAYS

There are a number of mechanisms by which the PC biosynthetic pathway may be regulated. The first of these is likely to be regulation of GSH biosynthesis. Studies of transgenic Indian mustard (*Brassica juncea*) plants, in which the expression of the enzymes of the GSH biosynthetic pathway was increased have shown that PC biosynthesis and Cd tolerance can also be increased (Yong et al., 1999; Zhu et al., 1999). This supports the idea that regulation of GSH biosynthesis is a plausible endogenous mechanism by which PC expression might be modulated. In support of this is the observation that a Cd-tolerant tomato cell line has increased GCS activity, although a causal link between phenotype and increased enzyme activity was not established (Chen and Goldsbrough, 1994).

Wild-type Indian mustard plants respond to exposure to Cd with increased levels of a GCS transcript (Schafer et al., 1998). Similarly, exposure of *Arabidopsis* plants to Cd and Cu causes an increase in transcript levels of the two genes in the GSH biosynthetic pathway and of GSH reductase (Xiang and Oliver, 1998). The signal molecule, jasmonate, mediated a similar effect in the absence of heavy metal exposure although it has not been demonstrated that the effect of heavy metal stress on gene expression is mediated via jasmonate. There is also circumstantial evidence supporting post-transcriptional regulation of GCS expression in addition to the well-recognized regulation of GCS activity through GSH feedback inhibition (May et al., 1998; Noctor and Foyer, 1998).

Regulation of PC synthase activity is expected to be the primary point at which PC synthesis is regulated. Kinetic studies using plant cell cultures demon-

strated that PC biosynthesis occurs within minutes of exposure to Cd and is independent of de novo protein synthesis, consistent with the observation of enzyme activation in vitro. The enzyme appears to be expressed independently of heavy metal exposure. It has been detected in *S. cucubalis* cells grown in culture medium (Grill et al., 1989), and in tomato (Chen et al., 1997) and *Arabidopsis* (Howden et al., 1995) plants grown in soil or agar medium, in the presence of only trace levels of essential heavy metals. Together these observations indicate that PC synthase is primarily regulated by activation of the enzyme in the presence of heavy metals. In vivo studies have shown that PC synthesis can be induced by a range of metal ions in fission yeast and in both intact plants and plant cell cultures (Rauser, 1995). This has been mirrored by in vitro studies of PC synthase expressed in *E. coli* or in Brewer's yeast where the enzyme was activated to varying extents by Cd, Cu, Ag, Hg, Zn, and Pb ions (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999).

A PC SYNTHASE MUTANT GIVES INSIGHT INTO THE MECHANISM OF ENZYME ACTIVATION

The mechanism by which PC synthase is activated will undoubtedly prove an interesting one, particularly because it is relatively non-specific with respect to the activating metal ion, although some metals are more effective than others. One model for the function of PC synthase enzymes is that the conserved N-terminal domains possess the catalytic activity. Activation probably arises from metal ions interacting with residues in this domain, possibly Cys or His residues. Five Cys (two of which are adjacent) (Fig. 2) and a single His residue are conserved in this domain. This model is supported by the molecular characterization of mutant *cad1* alleles. One in particular, *cad1-5*, has a nonsense mutation that would result in premature termination of translation downstream of the conserved domain (Ha et al., 1999). The truncated polypeptide is predicted to lack nine of the 10 Cys

residues in the C-terminal domain (Fig. 2). That this mutant enzyme is the least affected (as measured by in vivo PC levels and sensitivity to Cd) and the mutant activity is expressed only in the presence of Cd (Howden et al., 1995) confirms that the C-terminal domain is not absolutely required for either catalysis or activation.

Since the truncation of the *cad1-5* mutant polypeptide produces a mutant phenotype, the C-terminal domain clearly has some role in activity. It is likely that this domain acts as a local sensor by binding heavy metal ions (presumably via the multiple Cys residues, but possibly also others) and bringing them into contact with the activation site in the catalytic domain. This model is consistent with biochemical studies using epitope-tagged AtPCS, which demonstrated it binds Cd ions at high affinity ($K_d = 0.54 \pm 0.20 \mu\text{M}$) and high capacity (stoichiometric ratio = 7.09 ± 0.94) (Vatamaniuk et al., 1999). A schematic illustration of this model is shown in Figure 3.

Previous studies indicated PC synthase is expressed constitutively and levels of enzyme are generally unaffected by exposure of cell cultures or intact plants to Cd. This suggests the induction of PC synthase gene expression is unlikely to play a significant role in regulating PC biosynthesis. This is supported by northern or reverse transcriptase-PCR analysis of the expression of *AtPCS1/CAD1* which showed that levels of mRNA were not influenced by exposure of plants to Cd, even under conditions of severe stress (S.-B. Ha and C.S. Cobbett, unpublished data), thus suggesting an absence of regulation at the level of transcription (Ha et al., 1999; Vatamaniuk et al., 1999). Interestingly, however, reverse transcriptase-PCR analysis of *TaPCS1* expression in roots indicated increased levels of mRNA on exposure to Cd (Clemens et al., 1999). This suggests that, in some species, PC synthase activity may be regulated at both the transcriptional and post-translational levels.

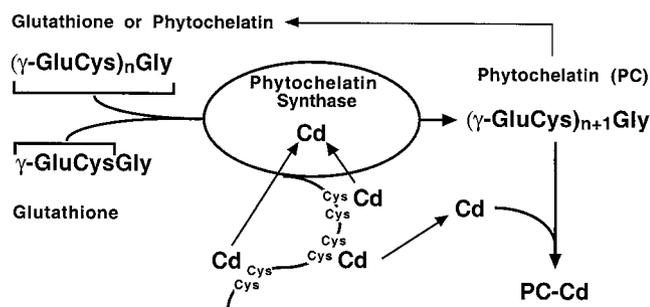


Figure 3. A model for the function of PC synthase. The C-terminal domain acts as a local sensor of heavy metal ions, such as Cd. The Cys residues bind Cd ions, bringing them into closer proximity and transferring them to the activation site in the N-terminal, catalytic domain. The activated N-terminal domain catalyzes the transfer of the γ -Glu-Cys moiety of a molecule of GSH onto a second molecule of GSH or an existing PC_n molecule to form a PC_{n+1} product.

PC SYNTHASE GENES IN ANIMAL SPECIES?

Database searches also identified a similar gene in the nematode, *C. elegans*. The predicted amino acid sequence of the N-terminal region shows 40% to 50% identical amino acids with the corresponding regions of the plant and yeast gene products. Furthermore, the C-terminal domain, although showing no obvious sequence similarity to the plant or yeast gene products, contains 10 Cys residues including two pairs (Fig. 2). An expressed sequence tag (GenBank accession no. AU061531) with similarity to PC synthase genes has also been identified in slime mold (*Dictyostelium discoideum*). In addition, using PCR similar sequences to the conserved N-terminal regions of the three genes have been identified from the aquatic midge, *Chironomus oppositus*, and earthworm species (W. Dietrich and C.S. Cobbett, unpublished data). As yet we have no evidence that these animal genes also encode a protein with PC synthase activity. However, in view of the high level of identity of the nematode gene product with the conserved N-terminal domains of the yeast and plant enzymes, as well as the presence of a variable domain containing multiple Cys residues, it seems likely that it too encodes PC synthase. This, then, would suggest that PCs play a wider role in heavy metal detoxification than previously expected. A superficial view of the limited selection of species in which such sequences have been identified might suggest that organisms with an aquatic or soil habitat are more likely to express PCs.

PC-Cd COMPLEXES ARE SEQUESTERED IN VACUOLES

PC-Cd complexes are sequestered to the vacuole. In fission yeast this process has been most clearly demonstrated through studies of the Cd-sensitive mutant, *hmt1*. In extracts of fission yeast two PC-Cd complexes (referred to as high M_r [HMW] and low M_r [LMW]) can be clearly resolved using gel-filtration chromatography. The *hmt1* mutant is unable to form HMW complexes on exposure to Cd. The *Hmt1* gene encodes a member of the family of ATP-binding cassette (ABC) membrane transport proteins that is located in the vacuolar membrane (Ortiz et al., 1992). Both HMT1 and ATP were required for the transport of PCs in the absence of Cd and LMW PC-Cd complexes into vacuolar membrane vesicles (Fig. 1). HMT1 did not transport Cd alone and the transport of PCs and PC-Cd complexes was not dependent on the proton gradient established across the vacuolar membrane by the vacuolar proton-ATPase (Ortiz et al., 1995). Interestingly, in *C. elegans*, various mutations affecting ABC transporter proteins also confer heavy metal sensitivity (Broeks et al., 1996). This would not be unexpected if PCs are expressed in *C. elegans* and are sequestered in a similar manner at the cellular level.

Sequestration of PCs to the vacuole has also been observed in plants. In mesophyll protoplasts derived

from tobacco plants exposed to Cd almost all of both the Cd and PCs accumulated was confined to the vacuole (Vogeli-Lange and Wagner, 1990) and an ATP-dependent, proton gradient-independent activity, similar to that of HMT1, capable of transporting both PCs and PC-Cd complexes into tonoplast vesicles derived from oat roots has been identified (Salt and Rauser, 1995).

Distinct from the PC transporter activity, an alternative mechanism, identified in both fission yeast vacuolar membrane and oat tonoplast vesicles, is a Cd/H⁺ antiporter activity dependent on the proton gradient (Salt and Wagner, 1993; Ortiz et al., 1995) (Fig. 1). In addition, in Brewer's yeast, YCF1, mentioned above, is also a member of the ABC family of transporters and transports both GSH conjugates and (GSH)₂Cd complexes to the vacuole (Li et al., 1997).

SULFIDE IONS PLAY A ROLE IN PC FUNCTION

In some plants and in the yeasts, fission yeast, and *C. glabrata*, HMW PC-Cd complexes contain both Cd and acid-labile sulfide. In general the ratio of S²⁻:Cd is higher in the HMW complex compared with the LMW complex. Those complexes with a comparatively high ratio of S²⁻:Cd consist of aggregates of 20-Å diameter particles which themselves consist of a CdS crystallite core coated with PCs (Dameron et al., 1989; Reese et al., 1992). The incorporation of sulfide into the HMW complexes increases both the amount of Cd per molecule and the stability of the complex.

Genetic evidence for the importance of sulfide in the function of PCs has been obtained from the analysis of Cd-sensitive mutants of fission yeast that are deficient in PC-Cd complexes. In one case the gene mutated in the Cd-sensitive derivative, when cloned, was identified as a gene involved in adenine biosynthesis. Subsequent genetic analysis demonstrated that different single or double mutants deficient in steps in the adenine biosynthetic pathway lacked HMW complexes (Speiser et al., 1992). Biochemical characterization of the enzymes encoded by these genes indicated that this pathway, in addition to catalyzing the conversion of Asp to intermediates in adenine biosynthesis, could also utilize Cys sulfinate, a sulfur-containing analog of Asp, to form other sulfur-containing compounds. These are believed to be intermediates or carriers in the pathway of sulfide incorporation into HMW complexes (Juang et al., 1993) (Fig. 1). Together these observations confirm the importance of sulfide in the mechanism of PC detoxification of Cd. Whether or not sulfide is involved in the detoxification of other metal ions by PCs is unknown.

More recently, Cd-sensitive mutants isolated in fission yeast and *Candida glabrata* have identified additional functions which are probably also important in sulfide metabolism. In fission yeast the *hmt2* mutant hyperaccumulates sulfide in both the presence and absence of Cd (Vande Weghe and Ow, 1999). The

HMT2 gene encodes a mitochondrial sulfide/quinone oxidoreductase, which was suggested to function in the detoxification of endogenous sulfide. The role of HMT2 in Cd tolerance is uncertain, but one possibility is to detoxify excess sulfide generated during the formation of HMW PC-Cd complexes after Cd exposure (Fig. 1). In *C. glabrata* the *hem2* mutant is deficient in porphobilinogen synthase involved in siroheme biosynthesis (Hunter and Mehra, 1998). Siroheme is a cofactor for sulfite reductase required for sulfide biosynthesis (Fig. 1). This deficiency may contribute to the Cd-sensitive phenotype. However, additional studies are required to establish the precise influence of this pathway on PC function.

DO PCs DETOXIFY METALS OTHER THAN Cd?

Although both induction of PCs in vivo and activation of PC synthase in vitro are conferred by a range of metal ions, there is little evidence supporting a role for PCs in the detoxification of such a wide range of metal ions. For metals other than Cd there are few studies demonstrating the formation of PC-metal complexes either in vitro or in vivo. PCs can form complexes with Pb, Ag, and Hg in vitro (for example, see Mehra et al., 1996; Rauser, 1999). Maitani et al. (1996) used inductively coupled plasma-atomic emission spectroscopy in combination with HPLC separation of native PC-metal complexes in the roots of *Rubia tinctorum*. PCs were induced to varying levels by a wide range of metal ions tested. The most effective appeared to be Ag, arsenate, Cd, Cu, Hg, and Pb ions. However, the only PC complexes identified in vivo were with Cd, Ag, and Cu ions. PC complexes formed in response to Pb and arsenate but these complexes contained copper ions and not the metal ion used for induction of synthesis.

The clearest evidence for the role of PCs in heavy metal detoxification comes from characterization of the PC synthase-deficient mutants of Arabidopsis and fission yeast. A comparison of the relative sensitivity of the Arabidopsis and fission yeast mutants to different heavy metals revealed a similar but not identical pattern (Ha et al., 1999). In both organisms PCs appeared to play an important role in Cd and arsenate detoxification and no apparent role in the detoxification of Zn, Ni, and selenite ions. Minor differences between the two organisms were observed with respect to Cu, Hg, and Ag.

From the preceding discussion it is apparent that the mechanism of metal detoxification is more complex than simply the chelation of the metal ion by PCs. The metal ion must activate PC synthase, be chelated by the PCs synthesized, and then presumably be transported to the vacuole and possibly form a more complex aggregation in the vacuole with, for example, sulfide or organic acids. For Cd, the inability of the organism to carry out any of these steps decreases the capacity of the organism for detoxification and

thereby confers a sensitive phenotype. Whether the same sequence of events occurs and is required for other metal ions is not clear. PCs appear to have a relatively insignificant role in the detoxification of metal ions such as Cu, Zn, Ni, and SeO₃ in *Arabidopsis*. Thus, for example, although PCs are induced by Cu in vivo, PC synthase is activated effectively by Cu in vitro, and PCs can chelate Cu in vitro, it is unknown whether PCs effectively chelate Cu in vivo or whether PC-Cu complexes, if formed, can be sequestered in the vacuole. Zn and Ni appear to be relatively ineffective activators of PC synthase in vitro. It may be that for these there are alternative, more effective detoxification mechanisms, such as MTs or His, respectively.

WHAT ARE THE ROLES OF PCs?

PCs can be detected in plant tissues and cell cultures exposed only to trace levels of essential metals and the level of PCs observed in cell cultures correlates with the depletion of metal ions from the medium. These observations have been interpreted to indicate a role for PCs in the homeostasis of essential metal ion metabolism (Rauser, 1995, 1999; Zenk, 1996). In addition, in vitro experiments have shown that PC-Cu and PC-Zn complexes could reactivate the apo forms of the copper-dependent enzyme diamino oxidase and the Zn-dependent enzyme carbonic anhydrase, respectively (Thumann et al., 1991). Although these experiments demonstrate that PC-metal complexes are capable of donating metal ions to metal-requiring enzymes, in each case the Cu or Zn complexes were no more effective than the free metal sulfate salt. In addition, roles for PCs in Fe or sulfur metabolism have also been proposed (Zenk, 1996; Sanita di Toppi and Gabbrielli, 1999). However, there is currently no direct evidence that PCs have functions other than in metal detoxification.

Although PCs clearly can have a role in Cd detoxification, for example, is this role of any physiological or ecological relevance? Whereas most experimental studies use Cd concentrations above 1 μM (Sanita di Toppi and Gabbrielli, 1999), it has been estimated that solutions of non-polluted soils contain Cd concentrations ranging up to 0.3 μM (Wagner, 1993). Wagner (1993) has also argued that, at low levels of Cd exposure, as represented by most soils, Cd would be largely complexed with vacuolar citrate and only at high levels of Cd exposure (not generally found in natural environments) might PCs play a role. Counter to this argument is the observation that a PC-deficient mutant of *Arabidopsis* is highly sensitive to concentrations of Cd as low as 0.6 μM (Howden et al., 1995). Even at concentrations of Cd where the mutant is not obviously sensitive, the wild type may nonetheless have a selective advantage. This suggests that PCs may have a role in heavy metal detoxification in an unpolluted environment. The absence of PC-deficient mutants of other species makes this question difficult to address.

FUTURE DIRECTIONS

The most significant recent advances in our understanding of PC biosynthesis and function have come from molecular genetic studies using a variety of model systems. These will continue to provide a wealth of mutants for biochemical, molecular, and physiological analysis. Interesting questions relating to the roles of PC synthase and PCs themselves in different organisms, possibly including animal species, remain to be answered. The isolation of PC synthase genes from a number of species will allow a considerably greater understanding of the mechanism of metal activation of PC biosynthesis and the catalytic mechanism itself. There is considerable potential for the application of that understanding to optimizing the process of phytoremediation.

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LITERATURE CITED

- Broeks A, Gerrard B, Allikmets R, Dean M, Plasterk RH (1996) Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. *EMBO J* 15: 6132–6143
- Chen J, Goldsbrough PB (1994) Increased activity of γ -glutamylcysteine synthetase in tomato cells selected for cadmium tolerance. *Plant Physiol* 106: 233–239
- Chen J, Zhou J, Goldsbrough PB (1997) Characterization of phytochelatin synthase from tomato. *Physiol Plant* 101: 165–172
- Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J* 18: 3325–3333
- Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ -glutamylcysteine synthetase. *Plant J* 16: 73–78
- Dameron CT, Reese RN, Mehra RK, Kortan AR, Carroll PJ, Steigerwald ML, Brus LE, Winge DR (1989) Biosynthesis of cadmium sulfide quantum semiconductor crystallites. *Nature* 338: 596–597
- Delhaize EP, Ryan R (1995) Aluminum toxicity and tolerance in plants. *Plant Physiol* 107: 315–321
- Fordham-Skelton AP, Robinson NJ, Goldsbrough PB (1998) Metallothionein-like genes and phytochelatin in higher plants. In S Silver, W Walden, eds, *Metal Ions in Gene Regulation*. Chapman & Hall, London, pp 398–431
- Grill E, Löffler S, Winnacker E-L, Zenk MH (1989) Phytochelatin synthase, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ -glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proc Natl Acad Sci USA* 86: 6838–6842
- Grill E, Winnacker E-L, Zenk MH (1987) Phytochelatin, a class of heavy-metal-binding peptides from plants are functionally analogous to metallothioneins. *Proc Natl Acad Sci USA* 84: 439–443

- Ha S-B, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS** (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast, *Schizosaccharomyces pombe*. *Plant Cell* **11**: 1153–1164
- Howden R, Goldsbrough PB, Andersen CR, Cobbett CS** (1995) Cadmium-sensitive, *cad1*, mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiol* **107**: 1059–1066
- Hunter TC, Mehra RK** (1998) A role for *HEM2* in cadmium tolerance. *J Inorg Biochem* **69**: 293–303
- Juang R-H, MacCue KF, Ow DW** (1993) Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. *Arch Biochem Biophys* **304**: 392–401
- Kägi JHR** (1991) Overview of metallothionein. *Methods Enzymol* **205**: 613–626
- Klapheck S, Schlunz S, Bergmann L** (1995) Synthesis of phytochelatin and homo-phytochelatin in *Pisum sativum* L. *Plant Physiol* **107**: 515–521
- Kramer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC** (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**: 635–638
- Larsen PB, Degenhardt J, Stenzler LM, Howell SH, Kochian LV** (1998) Aluminum-resistant *Arabidopsis* mutant that exhibit altered patterns of aluminum accumulation and organic acid release from roots. *Plant Physiol* **117**: 9–18
- Li Z-S, Lu Y-P, Zhen R-G, Szczypka M, Thiele DJ, Rea PA** (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato) cadmium. *Proc Natl Acad Sci USA* **94**: 42–47
- Loeffler S, Hochberger A, Grill E, Winnacker E-L, Zenk M-H** (1989) Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. *FEBS Lett* **258**: 42–46
- Maitani T, Kubota H, Sato K, Yamada T** (1996) The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. *Plant Physiol* **110**: 1145–1150
- May MJ, Vernoux T, Leaver C, Van Montague M, Inze D** (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. *J Exp Bot* **49**: 649–667
- Mehra RK, Tran K, Scott GW, Mulchandani P, Sani SS** (1996) Ag(I)-binding to phytochelatin. *J Inorg Biochem* **61**: 125–142
- Noctor G, Foyer CH** (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 249–279
- Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW** (1992) Heavy-metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO J* **11**: 3491–3499
- Ortiz DF, Ruscitti T, McCue KF, Ow DW** (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J Biol Chem* **270**: 4721–4728
- Rauser WE** (1995) Phytochelatin and related peptides: structure, biosynthesis, and function. *Plant Physiol* **109**: 1141–1149
- Rauser WE** (1999) Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin and metallothioneins. *Cell Biochem Biophys* **31**: 19–48
- Reese RN, White CA, Winge DR** (1992) Cadmium sulfide crystallites in Cd-(γ -EC)_nG peptide complexes from tomato. *Plant Physiol* **98**: 225–229
- Salt DE, Rauser WE** (1995) MgATP-dependent transport of phytochelatin across the tonoplast of oat roots. *Plant Physiol* **107**: 1293–1301
- Salt DE, Smith RD, Raskin I** (1998) Phytoremediation. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 643–668
- Salt DE, Wagner GJ** (1993) Cadmium transport across tonoplast of vesicles from oat roots: evidence for a Cd²⁺/H⁺ antiport activity. *J Biol Chem* **268**: 12297–12302
- Sanita di Toppi L, Gabbriellini R** (1999) Response to cadmium in higher plants. *Environ Exp Bot* **41**: 105–130
- Schafer HJ, Haag-Kerwer A, Rausch T** (1998) cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy metal accumulator *Brassica juncea* L.: evidence of Cd-induction of a putative mitochondrial γ -glutamylcysteine synthetase isoform. *Plant Mol Biol* **37**: 87–97
- Speiser DM, Ortiz DF, Kreppel L, Ow DW** (1992) Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. *Mol Cell Biol* **12**: 5301–5310
- Thumann J, Grill E, Winnacker E-L, Zenk MH** (1991) Reactivation of metal-requiring apoenzymes by phytochelatin-metal complexes. *FEBS Lett* **284**: 66–69
- Vande Weghe JG, Ow DW** (1999) A fission yeast gene for mitochondrial sulfide oxidation. *J Biol Chem* **274**: 13250–13257
- Vatamaniuk OK, Mari S, Lu Y-P, Rea PA** (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. *Proc Natl Acad Sci USA* **96**: 7110–7115
- Vogeli-Lange R, Wagner GJ** (1990) Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves: implication of a transport function for cadmium-binding peptides. *Plant Physiol* **92**: 1086–1093
- Wagner GJ** (1993) Accumulation of cadmium in crop plants and its consequences to human health. *Adv Agron* **51**: 173–212
- Xiang C, Oliver DJ** (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *Plant Cell* **10**: 1539–1550
- Yong LZ, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L, Terry N** (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ -glutamylcysteine synthetase. *Plant Physiol* **121**: 1169–1177
- Zenk MH** (1996) Heavy metal detoxification in higher plants: a review. *Gene* **179**: 21–30
- Zhu YL, Pilon-Smits EAH, Jouanin L, Terry N** (1999) Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiol* **119**: 73–79