

Metallomics

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MINI-REVIEW

Metabolites and metals in Metazoa – what role do phytochelatins play in animals?

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Phytochelatin synthesis is one of the key mechanisms by which plants protect themselves against toxic soft metal ions such as cadmium. It has been known for a while now that some invertebrates also possess functional phytochelatin synthase (PCS) enzymes, and that at least one species, the nematode *Caenorhabditis elegans*, produces phytochelatins to help detoxify cadmium, and probably also other metal and metalloid ions including arsenic, zinc, selenium, silver, and copper. Here, we review recent studies on the occurrence, utilization, and regulation of phytochelatin synthesis in invertebrates. The phytochelatin synthase gene has a wide phylogenetic distribution, and can be found in species that cover almost all of the animal tree of life. The evidence to date, though, suggests that the occurrence is patchy, and even though some members of particular taxonomic groups may contain PCS genes, there are also many species without these genes. For animal species that do possess PCS genes, some of them (e.g. earthworms) do synthesize phytochelatins in response to potentially toxic elements, whereas others (e.g. *Schistosoma mansoni*, a parasitic helminth) do not appear to do so. Just how (and if) phytochelatins in invertebrates complement the function of metallothioneins remains to be elucidated, and the temporal, spatial, and metal specificity of the two systems is still unknown.

What are phytochelatins?

Phytochelatins (PCs) are cysteine-rich non-ribosomal peptides, typically with the structure $(\gamma\text{-GluCys})_n\text{Gly}$ where n is between 2 to 4, although some species also have a different terminal amino acid than glycine.¹ They are widely found in plants, where they play a key role in metal ion detoxification. PCs chelate metals through the free thiols on the cysteine residues, and hence bind to soft metal ions; prototypically cadmium, but also to a range of other elements, including As, Hg, Ag, Zn, and Cu.¹

Phytochelatins are synthesized from glutathione by the enzyme phytochelatin synthase (PCS). The PCS enzymes belong to the papain-like cysteine protease superfamily; they comprise a conserved N-terminal domain, which contains the active catalytic site (including a conserved cysteine/histidine/aspartate triad), as well as a more variable C-terminal domain, which contributes to the regulation of the enzymatic activity.^{2,3} In general, the substrate for PCS is the glutathione-chelated metal ion complex, and so PCS does not need external regulatory mechanisms to induce its activity – an increase in cytosolic metal ion concentrations is sufficient.²

Because glutathione is usually present at high cytosolic concentrations in cells, any influx of metal ions will lead to GSH-complexed species, which are then rapidly converted into PCs by constitutively-expressed PCS. The multidentate phytochelatins bind metal ions such as cadmium much more strongly than the unidentate GSH molecules.⁴

The other key metal-handling and -detoxification system in animals is metallothioneins (MTs). These, like PCs, are cysteine-rich peptides; the key difference is that the sequence of MTs is genetically encoded. This has a number of consequences: one is that, because there can be differences in the peptide chain, animals can (and generally do) have more than one MT isoform. This also means that different MT isoforms can have preferences for different metal ions, and that these can differ between species. In contrast, PCs have the same binding affinities for metals no matter what species they are found in – clearly the situation is more complex for MTs. However, excellent and exhaustive reviews of MTs already exist,^{1,5,6} including reviews of MTs in invertebrates^{7,8} (most relevant to our current work) as well as mammals,^{9,10} plus more specific reviews focussing on aspects such as the biochemistry

of metal ion binding.¹¹ There are also excellent and comprehensive reviews of PCs that deal largely with their occurrence in plants.^{1,2,12,13} Because of this, we have here decided to focus solely on PCs in invertebrates.

Phytochelatinins are found in animals

A few years after the discovery of PCs, homologues of the PCS gene were identified in metazoans, including the nematode *Caenorhabditis elegans*. In 2001, two independent studies showed that the *Caenorhabditis elegans* PCS (CePCS; we will throughout refer to 'XyPCS' for different species, where X and y are the first letters of the genus and species, respectively) could synthesize PCs when recombinantly expressed in an appropriate host (either *Saccharomyces cerevisiae*, which does not possess a PCS gene, or *Schizosaccharomyces pombe*, with its own PCS gene deleted).^{14,15} What's more, knocking down the CePCS gene made the nematodes more sensitive to the canonical PC-binding metal cadmium – the first direct evidence that PCs have a functional role in response to metals in a metazoan.¹⁵ Several studies have since measured PCs by direct biochemical analysis of *C. elegans* tissue extracts, and found that cadmium exposure did indeed increase PC levels in *C. elegans*:¹⁶⁻¹⁸ PC₂, PC₃, and PC₄ have all been found, with PC₂ the highest concentration. Subsequent studies have shown that PCs are presumably protective against more elements than just cadmium in *C. elegans*, as the CePCS knockout strain also has increased sensitivity to arsenic, zinc, selenium, silver, and copper – although PC concentrations have not yet been measured directly in response to these.¹⁸⁻²¹

Phytochelatin production has been studied in a number of other animal species. Still within the phylum Nematoda, Rigouin et al. looked at the parasitic species *Ancylostoma ceylanicum*, and showed that the AcPCS also produced PCs when expressed in *S. cerevisiae*.²² Furthermore, they reported close homologues of PCS in other parasitic nematode species, including *Brugia malayi*, *Loa loa*, and *Ascaris suum*. Ray et al. extended the knowledge of animal PCs to a second phylum, Platyhelminthes, when they expressed the *Schistosoma mansoni* PCS in *S. cerevisiae*, and showed that the recombinant enzyme made PCs in response to cadmium.²³ Unlike the situation for *C. elegans*, though, exposing the flukes to cadmium didn't result in any detectable PCs in tissue extracts.²⁴ In contrast, the earthworm *Lumbricus rubellus* (Annelida), which possesses two PCS orthologues, has not yet had recombinant LrPCS characterized, but had clear *in vivo* increases in PC₂ and PC₃ in response to arsenic exposure in a laboratory experiment.²⁵ PC levels were also increased in native *L. rubellus* worms taken from polluted sites with mixed arsenic, copper, and cadmium contamination compared to *L. rubellus* from relatively clean sites, although the PC levels were generally lower than those seen in the acute-exposure laboratory experiment.²⁵ Franchi et al.²⁶ detected PC₂ in the tunicate *Ciona intestinalis*, although they did not test changes in concentration after metal treatment.

Distribution of the PCS gene in metazoans

Given this evidence for PCS enzymes in multiple phyla, the question of the distribution of the PCS gene in animals is intriguing. To date, PCS homologues have been identified in only a very small number of species (and biochemical characterization of recombinant PCS so far restricted to three: *C. elegans*, *S. mansoni*, and *A. ceylanicum*). This might sound like the gene has limited spread, and phytochelatin production

in animals would therefore be more of a curiosity than of general importance, but this is belied by the very wide taxonomic distribution of the species with PCS homologues described so far. In fact, PCS-containing species are found across the deepest divisions of the Metazoa. Clemens and Persoh¹² listed putative PCS genes in species from seven metazoan phyla: Nematoda, Platyhelminthes, Annelida, Mollusca, Chordata, Cnidaria, and Echinodermata. In addition, the NCBI protein database currently (February 2014) also lists two sequences from the acorn worm *Saccoglossus kowalevskii* (phylum Hemichordata), although these may not represent 'standard' animal PCS enzymes, as they are shorter than most PCS proteins, being restricted to the more conserved N-terminal region (accession numbers XP_002730374.1 and XP_002730372.1). The exact organization of the animal tree of life at the phylum level is still controversial;²⁷ here, we follow Jones and Blaxter²⁸ in assuming a superphyletic organization into three major groups, Lophotrochozoa, Ecdysozoa, and Deuterostomia, with additional outgroups including Ctenophora and Cnidaria. The phyla listed by Clemens and Persoh contain examples from all three of these superphyletic groups, as well as the outlying phylum Cnidaria – although PCS is not present in the sole ctenophoran sequenced to date, *Mnemiopsis leidyi*.²⁹ Interestingly, *Tetrahymena thermophila* (a single-celled eukaryote, but not an animal) contains a PCS-like protein, or "pseudo-phytochelatin synthase". This TtPCS is transcriptionally upregulated in response to cadmium, but does not appear to make full-length phytochelatin: rather, it possesses the PCS hydrolase activity by converting glutathione to γ -glutamylcysteine, but is unable to then combine this with a second glutathione molecule.³⁰ The authors pointed out that this makes the TtPCS more similar to bacterial PCS genes (or pseudo-PCS) than to higher eukaryote PCS, and suggested that it might be an example of an intermediate evolutionary form. We present a phylogenetic tree of selected PCS sequences from both metazoans and single-celled eukaryotes (Figure 1). There is some differentiation of the sequences according to the taxonomic groupings, with a separate group of nematode sequences, also the only ecdysozoans; the lophotrochozoan species (molluscs, annelids, and platyhelminths) also cluster closely together. The deuterostome sequences (echinoderms, hemichordates, and chordates) form a widely spaced group together with the cnidarian sequences. Surprisingly, the single-celled eukaryotic PCS sequences do not form a separate cluster, but separate groups are embedded within the metazoan sequence clusters. The sequence alignment shows a number of conserved residues, including the key Cys/Asp/His catalytic triad (supplementary information; the aspartate residue has been replaced by glutamate in a small number of species, but this still allows synthesis of PCs²⁶).

This wide taxonomic distribution of PCS raises a key question: how many animal species *do* contain PCS genes?³¹ PCS genes may turn out to be rare occurrences, found here and there in the occasional species. Alternatively, they may be common within certain taxa. We decided to examine a single taxon as an exemplar, and chose Nematoda, because it already has a number of species with known PCS orthologues, and because the CePCS is the best-characterized animal PCS enzyme. We used the NemaBLAST tool at nematode.net to carry out a search across 45 nematode species from a number of different ecotypes (5 free-living species, 7 human pathogens, 17 vertebrate pathogens, 14 plant pathogens, and one entomopathogenic species)³² against the *C. elegans* PCS-1a sequence. The database contains species from four out of the

five major clades within Nematoda, as defined by Blaxter et al.³³ The results were intriguing. None of the four species in clade I, five of the seven species in clade III, none of the twenty species in clade IV, and eight of the thirteen species in clade V had a recognizable PCS gene, i.e. about 30% of the species had a PCS homologue. All of the ecotypes were represented by at least one species with a PCS gene, except for the plant pathogenic nematodes. However, not all of the species in the database contain full genome sequence data, and so negative results cannot be taken as definitively indicating the absence of PCS – for example, the search failed to find the known AcPCS from *A. ceylanicum* – and so the true percentage may turn out to be much higher.

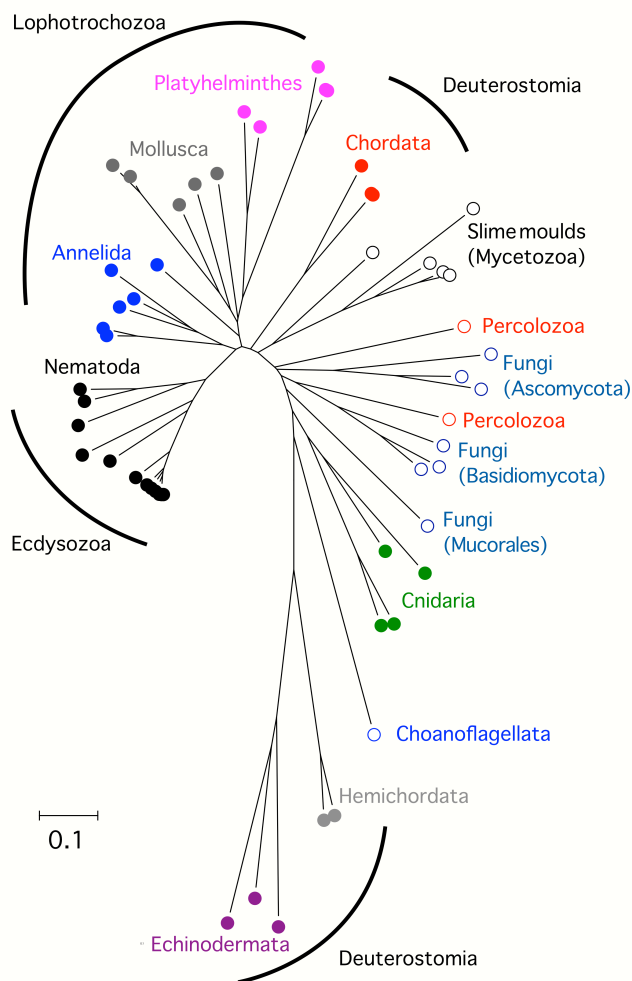


Figure 1. Phylogenetic tree (nearest-neighbour joining, 10,000 bootstraps) of PCS protein sequences, created using previously published settings.²⁶ Solid symbols: animal sequences. Open symbols: single-celled eukaryotes. A key to this tree with all species individually labelled is given as supplementary information.

As well as asking which animal species possess PCS genes, an equally interesting question is which species do *not* have PCS genes? Clearly a majority of phyla have no species with described PCS genes (so far). However, which phyla really do lack species with this gene, as opposed to ones where the data

just don't yet exist? The phylum Arthropoda is a key example, as it contains the majority of all animal species.³⁴ No PCS gene has yet been described in any arthropod, despite the availability of genome sequences for a wide range of species. This is, of course, a long way from demonstrating that PCS genes are really absent from all arthropods, but can be regarded as an accumulation of negative evidence. The sole indication (of which we are aware) of a possible arthropod PCS gene is a description of a match to a PCR product from the midge *Chironomus oppositus*.³⁵ However, as other sequenced culicomorph dipterans such as mosquitos do not contain any PCS homologues, and the evidence from the *C. oppositus* PCR data is not conclusive (Cobbett, pers. comm., 2014), we conclude that the weight of the evidence so far is that this gene is systematically lacking in arthropods.

Another interesting phylum is Chordata, not least because it is our own. Chordata is divided into three major sub-phyletic groups, Craniata (animals with skulls), Tunicata (sea squirts), and Cephalochordata (lancelets). At least some tunicates do have PCS genes, such as the sea squirts *C. intestinalis*^{36,37} and *C. savignyi* (Ensembl), and the metabolite PC₂ can be detected in *C. intestinalis* intestinal homogenates.²⁶ However, no vertebrate species is known to possess PCS. In addition, although lancelets are thought to be the most basal sub-phyletic group within Chordata, i.e. the split from lancelets occurred before the remaining chordates split into tunicates and craniates, surprisingly, the sequenced species *Branchiostoma floridae* (Amphioxus) does not have a PCS gene.³⁸ It may well be the case that this gene was lost early on during the evolutionary history of Chordata,³⁶ but it is still unexpected that it turns out to be found in Tunicata but not in Cephalochordata – was it lost twice?

Another superphyletic group that has members with PCS genes is the Lophotrochozoa, although it is difficult to estimate the PCS distribution, as there are relatively few sequenced lophotrochozoan species. Earthworms possess PCS genes, as already described. Species from other lophotrochozoan taxa that possess PCS genes include a leech and a marine polychaete (both annelids, like earthworms), and a gastropod mollusc.³⁹ Bivalve molluscs also possess PCS homologues, including the oysters *Crassostrea gigas* and *Pinctada fucata*.⁴⁰ It is, therefore, slightly surprising that a BLAST search against *Enchytraeus albidus* (EnchyBASE) gives no apparent hits to PCS genes, even though enchytraeids are oligochaete annelids closely related to earthworms, and EnchyBASE contains expressed sequence tags isolated after metal treatment.⁴¹ Further genomic information would be valuable here.

In summary, we do not yet have genome sequence data from enough species to be able to draw accurate conclusions about the true distribution of PCS in animals. However, the current data do paint a very interesting picture. As far as we can tell at the moment, PCS genes are widely but sparsely found across the animal tree of life: they are not found in all phyla; and major sub-groups within phyla may be lacking PCS homologues. Genome sequencing technology has rapidly decreased in cost and increased in throughput over the last few years, and we are certain to see many novel invertebrate genomes released soon. This will help to answer the question of the true distribution of PCS in Metazoa. New data may also help address functional questions, such as why has this gene apparently been lost from the majority of animal species, but is so commonly found in plants?

The contribution of phytochelatins to metal detoxification in animals

As well as their phylogenetic distribution, it is interesting to know what functional role PCS genes play in animals – do they synthesize phytochelatins *in vivo*, and if so, does this contribute to metal ion detoxification?³¹ We currently only have data for three species, from three different phyla – *C. elegans*, *S. mansoni*, and *L. rubellus*. Two have had the PCS enzyme biochemically characterized: the CePCS and SmPCS both synthesize PCs when cloned into an appropriate host.^{14,15,23} We have the greatest amount of knowledge for *C. elegans*, as phenotypes have been tested by genetic manipulation: interfering with CePCS activity makes the nematode hypersensitive to cadmium,¹⁵ and PCs are increased in *C. elegans* tissue extracts after exposure to cadmium.^{16,17} We can firmly conclude that phytochelatin production plays a major role in protecting *C. elegans* against cadmium toxicity. The story is different, though, for *S. mansoni*. Treating them with 100 μ M cadmium for 6 hours did not lead to any detectable increase in phytochelatins in *S. mansoni* tissue extracts,²⁴ and so the most parsimonious conclusion is that phytochelatin synthase does not participate in metal detoxification in this species. (It is still of course possible that PCs might yet turn out to be responsive at different timescales, concentrations, or to different metal ions.) Recombinant earthworm LrPCS, on the other hand, has not yet been biochemically characterized, but both PC₂ and PC₃ were increased in whole-worm tissue extracts in a clearly dose-responsive manner following 28 days of exposure to arsenic. Cadmium-exposed *C. elegans* whole-organism extracts were reported to have 17 nmol aggregate PC thiols per mg protein,¹⁸ this was not measured for *L. rubellus*, but assuming a protein content of 11% of tissue wet weight,⁴² then this gives an approximate value of 0.33 nmol PC₂ mg⁻¹ protein for arsenic-exposed worms.²⁵ This is less than for *C. elegans* following Cd exposure, but was still greater than the amount of As(III) in the tissue samples. Taken together with the good alignment of the LrPCS sequence to validated functional PCS proteins, such as those from *C. elegans* and *S. pombe*, it strongly argues that phytochelatins play a functional role in toxic element handling in *L. rubellus*. PC₂ and PC₃ were also increased in autochthonous *L. rubellus* populations sampled from contaminated sites, which presumably represents much longer-term exposure. However, as is common for contaminated field sites, because the sites contained a mixture of potentially inducing metal ions (cadmium, arsenic, copper, zinc), it was not possible to identify exactly which metal or metals had caused PC production in these worms.²⁵ Hence, two out of the three species (representing three different phyla) for which we so far have direct evidence for functional PCS enzymes probably use them for metal ion detoxification. Perhaps the SmPCS has a different role because *S. mansoni* is an internal parasite, and so less likely to be exposed to localized hotspots of high levels of metal ions than free-living organisms. It will be interesting in the future to see if PCs are metal-responsive in parasitic nematodes, and thus whether PC production is more driven by phylogeny or ecology.

What happens downstream of metal ion complexation by phytochelatins? The yeast *S. pombe* possesses an ATP-binding cassette (ABC) transporter, Hmt1, which was originally thought to play a possible role in translocation of PC-metal complexes to the vacuole. However, while knocking out the *C. elegans* HMT-1 (CeHMT-1) does increase sensitivity to cadmium, the increase is greater than could be explained by a lack of PCS alone. In addition, there are other physiological differences

between worms treated with RNAi against *pcs-1* (intestinal cells undergo necrosis) or against *hmt-1* (intestinal cells develop inclusions), and double RNAi against both *pcs-1* and *hmt-1* showed both of these phenotypes, implying that CeHMT-1 acts independently of phytochelatins.⁴³ Sooksa-Nguan et al. confirmed that Hmt1 cannot be the sole PC transporter, as Hmt1 is also required for metal tolerance in *Drosophila melanogaster*, even though this species does not make phytochelatins, and that vacuolar cadmium and PC uptake occurs in *S. pombe* even in Hmt1 knockouts.⁴⁴ Furthermore, the CePCS and CeHMT-1 proteins are largely expressed in different cells in *C. elegans*.¹⁸ A bacterial Hmt1 orthologue had very high activities for GSH-complexed metal ions – much higher than for GSH alone – although the activity against PC-metal ion complexes was not measured.⁴⁵ How, then, do animals detoxify PC-metal ion complexes? Recently, the vacuolar ABC transporters Abc2 (in *S. pombe*) and ABCC1 and ABCC2 (in *Arabidopsis thaliana*) have been identified as the primary phytochelatin transporters,⁴⁶ and shown to be required for *A. thaliana* tolerance to the classic phytochelatin binding substrates arsenic, cadmium, and mercury.^{47,48} *C. elegans* has a set of *mrp* (multidrug resistance-associated protein) genes, with *mrp-1* being the closest homologue to *S. pombe* Abc2. It is therefore extremely relevant that a *C. elegans* MRP-1 deletion strain has increased sensitivity to both cadmium and arsenic (III), exactly as would be expected for a protein involved in PC-metal ion transport.⁴⁹ Of course, animal cells do not contain vacuoles, but perhaps it mediates transport to a lysosomal compartment.

A recent study by Franchi et al. of *C. intestinalis* also shows some very interesting results.²⁶ The CiPCS gene expression had a complex response to 10 μ M Cd, with an early increase in expression compared to controls, followed by a decrease and then another increase. An interesting observation is that these changes in CiPCS expression were mirrored by changes in the expression of the proliferating cell nuclear antigen (PCNA) gene, considered a marker of cell proliferation, which the authors interpreted as a result of an increase in a specific granular amoebocyte cell type. Excitingly, the same was true for *C. intestinalis* MT⁵⁰ – indicating that this circulating cell type may be part of a detoxification mechanism. The authors proposed a mechanism in which granular amoebocytes accumulated cadmium, and, when transported to an appropriate storage site, then undergo apoptosis.²⁶ It is, therefore, particularly noteworthy that the CePCS is also found in coelomocytes in *C. elegans*, which are required for metal tolerance.¹⁸ It will be interesting in the future to see if animal PCS enzymes are generally associated with circulatory or otherwise mobile cell types.

Finally, it is important to note that MTs are widely established as a key metal detoxification system in animals, including in the development of tolerant populations,⁵¹ even though they certainly have many other biological functions as well. As yet, there is very little known about how (or if) MTs and PCs may complement each other for dealing with toxic metals. The sole evidence to date (that we are aware of) is for the nematode *C. elegans*, where PCs are more important than MTs in protecting against cadmium in *C. elegans*: the PCS knockout strain is even more sensitive to cadmium than the *mtl-1;mtl-2* double knockout, as judged by phenotypic endpoints such as growth, survival, and reproduction.^{16,17} However, there is clearly an additive effect on Cd sensitivity, as a triple *pcs-1;mtl-1;mtl-2* mutant is more sensitive still. We do not yet know what the situation will be for other metal ions, or for other

organisms, but, for animal species that can make phytochelatins, a full understanding of toxic metal handling will likely need to consider both MTs and PCs.

Regulation of phytochelatin synthesis

Phytochelatin synthases make phytochelatins *in vitro* when challenged with appropriate heavy metal ions. The glutathione-complexed metal ions are the enzyme substrate⁵², and so direct regulation of the enzyme activity is not necessarily needed beyond that: simple accumulation of cytosolic metal ions will lead to phytochelatin synthesis, so long as PCS is present. Indeed, PCS enzymes are often not transcriptionally regulated, and so perhaps PC production forms a biochemical 'rapid response' to metal ions.¹ The regulation of PCS enzymes, though, is generally complex,⁵³ and there are a number of examples in plants where PCS transcription does appear to be metal-dependent. Given this, what is the situation in animals with PCS enzymes? Again, and perhaps unsurprisingly, it appears to be complex. Transcriptomic studies have shown no evidence of induction of PCS transcription in response to cadmium in either *C. elegans* or *L. rubellus*.^{54,55} Targetted qPCR analysis of PCS expression also did not show any changes in response to arsenic in *L. rubellus*, even though PCs were synthesized.²⁵ The results are particularly interesting for the earthworm *Eisenia fetida*, because of evidence of potential coordination with metallothioneins: Brulle et al. cloned a PCS orthologue from *E. fetida*, and showed that its expression was cadmium-responsive in some situations only, in that it was increased at low (8 mg/kg soil) but not high (80 or 800 mg/kg soil) cadmium concentrations. Intriguingly, an *E. fetida* metallothionein showed the opposite pattern, with very high increases in expression at high cadmium, but only a small change at the low Cd level and during a short time course.⁵⁶ Additional experiments have exposed *E. fetida* to authentically contaminated soils, but these have tended to show either small or no effect on EfPCS gene transcription.⁵⁷⁻⁶⁰ Experiments done with *S. mansoni* also demonstrate the potential complexity of PCS regulation. The transcription of SmPCS increased about sixfold in response to cadmium, even though there was no apparent synthesis of phytochelatins. This does not appear to be a metal-specific effect, though, as there were also transcriptional increases (ranging from about two- to tenfold) in response to a number of different stress treatments, including oxidative stress, organic compounds, an anthelmintic drug, and iron (which one would not normally expect to be chelated by thiol ligands).²⁴ A comparison of *S. mansoni* gene expression across different developmental parasitic stages showed that SmPCS was also upregulated in the undifferentiated 'germ ball' stage inside its snail host, compared to later stages involved in infection and adaptation to a human host.⁶¹ It has been suggested that phytochelatins may contribute to the homeostasis of essential metals, and particularly zinc, in plants,^{12,62} although this remains to be fully elucidated. The exact functional role of SmPCS similarly remains not yet completely understood, but perhaps it is involved in normal metal biology rather than as a response to a toxic concentration of metal ions.

Conclusions

While the role played by PCS enzymes and phytochelatins in animals still remains to be fully explored, there is increasing evidence that PCS genes are likely to be found in many important animal groups – earthworms, nematodes (both parasitic and free-living), platyhelminths, tunicates, and molluscs – and that phytochelatins may well turn out to be important players in metal ion detoxification in many of these species. Direct analysis of phytochelatins will be essential for future studies, as gene expression data alone are not sufficient to demonstrate whether PCs are produced or not. Measuring absolute concentrations of PCs in tissues (i.e. not just relative changes) and reporting limits of detection will be valuable in the future, as it will allow better comparison between studies, and improve understanding of whether PCs are involved in metal detoxification in a particular organism or not. Understanding how (or if) there is an interplay between phytochelatins and metallothioneins for metal detoxification is also likely to prove of particular importance: phytochelatins are well established as the main detoxifiers of cadmium in plants, but plant metallothioneins generally do not bind cadmium strongly in the same way that animal metallothioneins do.⁶³ It will definitely be of interest in the future to see whether different animal species coordinate phytochelatin and metallothionein responses to potentially toxic elements, and if this is different for different metal ions.

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