Annu. Rev. Plant Biol. 2002. 53:159–82
DOI: 10.1146/annurev.arplant.53.100301.135154

PHYTOCHELATINS AND METALLOTHIONEINS: Roles in Heavy Metal Detoxification and Homeostasis

Christopher Cobbett
Department of Genetics, University of Melbourne, Parkville, Australia 3052; e-mail: ccobbett@unimelb.edu.au

Peter Goldsbrough
Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, Indiana 47907-1165; e-mail: goldsbrough@hort.purdue.edu

Key Words heavy metals, detoxification, homeostasis, phytochelatin synthase

■ **Abstract** Among the heavy metal-binding ligands in plant cells the phytochelatins (PCs) and metallothioneins (MTs) are the best characterized. PCs and MTs are different classes of cysteine-rich, heavy metal-binding protein molecules. PCs are enzymatically synthesized peptides, whereas MTs are gene-encoded polypeptides. Recently, genes encoding the enzyme PC synthase have been identified in plants and other species while the completion of the Arabidopsis genome sequence has allowed the identification of the entire suite of MT genes in a higher plant. Recent advances in understanding the regulation of PC biosynthesis and MT gene expression and the possible roles of PCs and MTs in heavy metal detoxification and homeostasis are reviewed.

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INTRODUCTION

Some heavy metals, particularly copper and zinc, are essential micronutrients for a range of plant physiological processes via the action of Cu- and Zn-dependent enzymes. These and other nonessential heavy metal ions, such as cadmium, lead, and mercury, are highly reactive and consequently can be toxic to living cells. Thus plants, like all living organisms, have evolved a suite of mechanisms that control and respond to the uptake and accumulation of both essential and nonessential heavy metals. These mechanisms include the chelation and sequestration of heavy metals by particular ligands. The two best-characterized heavy metal-binding ligands in plant cells are the phytochelatins (PCs) and metallothioneins (MTs).

MTs are cysteine-rich polypeptides encoded by a family of genes. In contrast, PCs are a family of enzymatically synthesized cysteine-rich peptides. The history of studies of PCs and MTs in plants and other species provides some salutary examples of the development of unsubstantiated dogmas that have been overturned by recent studies. In the search for MTs similar to those that had been characterized in animal species, early studies in plants repeatedly identified PCs. Like MTs in animals, PCs in plants are heavy metal-inducible, heavy metal-binding, cysteine-rich polypeptides, and in the absence of evidence for MTs in plants, it was initially suggested that PCs might be functionally analogous to MTs. The dichotomy that MTs were animal-specific ligands and PCs were plant specific became entrenched over time. Even when MT genes (repeatedly referred to as MT-like genes) were described in plants and it became clear that some microorganisms expressed both MTs and PCs, the notion that if PCs were not plant-specific ligands then at least they were "nonanimal" was maintained. Only with the isolation of PC synthase genes from plants and the demonstration that functional homologues exist in at least some animal species has this artificial dichotomy been discarded. It is also satisfying to describe an example where studies, largely in plant systems, have informed our understanding of extensively studied mechanisms in animal species.

The now apparent breadth of PC function across the plant and animal kingdoms leads to questions about nomenclature. PCs have been given various alternative names (including Class III MTs) over the years, but "phytochelatins" is the name that has been most widely adopted. The term phytochelatins has, however, never been truly accurate, particularly as PCs were first discovered in the yeast *S. pombe*, and is even less so because PC synthase genes have now been identified in animals. Nonetheless, it serves to distinguish one broad class of heavy metal-binding compounds from another and has become so entrenched in the literature that there seems little reason to change it. Its use also encompasses something of the history of PC and MT research.

This review describes recent advances in our understanding of the expression and function of both PCs and MTs in plants, with reference to other organisms where appropriate. PCs and MTs are discussed separately because at present there is no evidence that their spheres of function in plant cells even overlap, although it is likely that this would be the case. Much of the recent work has involved

molecular genetic studies in Arabidopsis, which also provides a focus for this review. The isolation and characterization of PC-deficient mutants of Arabidopsis has provided considerable impetus to research into PC biosynthesis and function in plants. A similar set of mutants is required to illuminate the function of MTs.

PHYTOCHELATINS

Structure and Biosynthetic Pathway

PCs form a family of structures with increasing repetitions of the γ-GluCys dipeptide followed by a terminal Gly; $(\gamma\text{-GluCys})_n$-Gly, where *n* is generally in the range of 2 to 5. PCs have been identified in a wide variety of plant species and in some microorganisms. In addition, a number of structural variants, for example, $(\gamma\text{-GluCys})_n$-β-Ala, $(\gamma\text{-GluCys})_n$-Ser and $(\gamma\text{-GluCys})_n$-Glu, have been identified in some plant species. The reader is directed to previous reviews for a more detailed coverage of these early studies (14, 58, 59, 84).

PCs are structurally related to glutathione (GSH; γ-GluCysGly), and numerous physiological, biochemical, and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis (14, 58, 59, 84). In particular, genetic studies have confirmed that GSH-deficient mutants of *S. pombe* as well as *Arabidopsis* are PC deficient and hypersensitive to Cd. A list of mutants that identify a role for particular genes in PC biosynthesis or function is shown in Table 1. In addition, a schematic including the PC biosynthetic pathway is illustrated in Figure 1.

Identification of PC Synthase Genes

The enzyme catalyzing the biosynthesis of PCs from GSH, phytochelatin synthase, was first characterized by Grill et al. (24) in 1989. However, it was not until 1999 that the cloning of PC synthase genes was described. The PC synthase gene was first identified genetically in Arabidopsis. Cd-sensitive, *cad1*, mutants are PC-deficient but have wild-type levels of GSH. They also lack PC synthase activity, suggesting a defect in the PC synthase gene (28).

PC synthase genes were isolated simultaneously by three research groups using different approaches. Two groups used expression of Arabidopsis and wheat cDNA libraries in *S. cerevisiae* to identify genes [*AtPCS1* (78) and *TaPCS1* (11), respectively] conferring increased Cd resistance. The third group identified *AtPCS1* through the positional cloning of the *CAD1* gene of Arabidopsis (25). A similar sequence was identified in the genome of *S. pombe*, and targeted deletion mutants of that gene are, like Arabidopsis *cad1* mutants, Cd sensitive and PC deficient, confirming the analogous function of the two genes in the different organisms. Heterologous expression of the *CAD1*/*AtPCS1* and *SpPCS* genes (25) or purification of epitope-tagged derivatives of SpPCS (11) and AtPCS1 (78) was used to demonstrate both were necessary and sufficient for GSH-dependent PC

TABLE 1 Mutants affected in phytochelatin biosynthesis and function

Organism[a]	Gene/locus	Activity/function	Reference
PC biosynthesis			
Sp	*Gsh1*	γ-glutamylcysteine synthetase/ GSH biosynthesis	(23, 51)
At	*CAD2/RML1*	γ-glutamylcysteine synthetase/ GSH biosynthesis	(14, 80)
Sp	*Gsh2*	glutathione synthetase/ GSH biosynthesis	(23, 51)
At	*CAD1*	PC synthase/PC biosynthesis	(28)
Sp	*Pcs1*	PC synthase/PC biosynthesis	(11, 25)
Ce	*Pcs1*	PC synthase/PC biosynthesis	(79)
PC function			
Sp	*Hmt1*	PC-Cd vacuolar membrane ABC-type transporter	(53, 54)
Sp	*Ade2, 6, 7, 8*	Metabolism of cysteine sulfinate to products involved in sulphide biosynthesis; also required for adenine biosynthesis	(32, 71)
Sp	*Hmt2*	Mitochondrial sulfide: quinone oxidoreductase/detoxification of sulphide	(74)
Ca	*Hem2*	Porphobilinogen synthase/ siroheme biosynthesis (cofactor for sulfite reductase)	(31)

[a]*At, A. thaliana*; *Sp. S. pombe*; *Ca, Candida albicans*; *Ce*; *C. elegans.*

biosynthesis in vitro. This combination of genetic, molecular, and biochemical data was a conclusive demonstration that these genes encode PC synthase.

There is a second PC synthase gene, *AtPCS2*, in Arabidopsis with significant identity to *CAD1/AtPCS1* (25). This was an unexpected finding because PCs were not detected in a *cad1* mutant after prolonged exposure to Cd, suggesting the presence of only a single active PC synthase in wildtype (28). *AtPCS2* is transcribed, and expression experiments have demonstrated it encodes a functional PC synthase enzyme (C. Cobbett & A. Savage, unpublished data). The physiological function of this gene remains to be determined. In most tissues *AtPCS2* is expressed at a relatively low level compared with *AtPCS1*. However, because AtPCS2 has been preserved as a functional PC synthase through evolution, it must presumably be the predominant PC synthase in some tissue(s) or environmental conditions, thereby conferring a selective advantage. Full-length or partial cDNA clones encoding presumptive PC synthases have also been isolated from other plant species, including *Brassica juncea* and rice (Table 2).

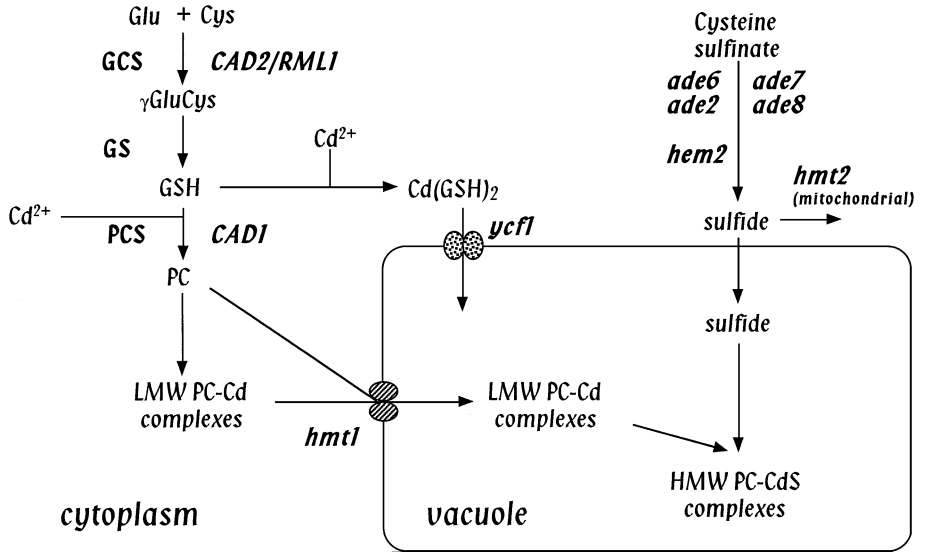

Figure 1 Genes and functions contributing to Cd detoxification in plants and fungi as a composite of various functions identified through the isolation of Cd-sensitive mutants of different organisms that express PCs. Refer to text and Table 1 for a more detailed description of the various functions. Gene loci are shown in italics: *CAD1* and *CAD2*/*RML1* are in *Arabidopsis*; *hmt1*, *hmt2*, *ade2*, *ade6*, *ade7*, and *ade8* are in *S. pombe*; *ycf1* is in *S. cerevisiae*; and *hem2* is in *C. glabrata*. Enzyme abbreviations: GCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; PCS, phytochelatin synthase.

Some Animals Express a PC Synthase

Through the history of studies of heavy metal detoxification in animals there has been no evidence for the presence of PCs. Thus, it came as a surprise when database searches identified similar genes in the nematodes *Caenorhabditis elegans* and *C. briggsae* and in the slime mould *Dictyostelium discoideum*. In addition, using polymerase chain reaction, investigators have identified partial sequences with homology to the plant and yeast PC synthase genes from the aquatic midge, *Chironomus*, and earthworm species (C. Cobbett & W. Dietrich, unpublished data). Recent work has demonstrated that the *C. elegans* and *D. discoideum* genes encode PC synthase activity. *CePCS1* was able to rescue either a Cd-sensitive *ycf1* mutant of *S. cerevisiae* or a PC synthase-deficient mutant of *S. pombe* and catalysed PC biosynthesis in vivo in both heterologous hosts and in vitro (12, 77). Similarly, expression of the *D. discoidium* PC synthase in *S. cerevisiae* is also able to catalyze PC biosynthesis in vivo and confer increased Cd-resistance (C. Cobbett & A. Savage, unpublished data). Significantly, the suppression of *CePCS1* expression by using the double-stranded RNA interference technique resulted in Cd sensitivity, thereby demonstrating an essential role for PCs in heavy metal detoxification

TABLE 2 PC synthase enzymes predicted from DNA sequences

Organism	Protein	Predicted protein: N + Con + Var = aa (kD)[a]	Predicted protein: Arrangement of Cys residues in Var (number)[b]	Accession No.[c]
Plants				
Arabidopsis thaliana	AtPCS1	4 + 208 + 263 = 485 (54.4)	C CC CC3C2C C1C C (10)	AF135155
	AtPCS2	4 + 207 + 241 = 452 (51.5)	C C CC3C2C C (7)	AC003027
Brassica juncea	BjPCS1	4 + 208 + 263 = 485 (54.3)	C CC C CC3C2C C1C C (11)	AJ278627
Wheat	TaPCS1	4 + 208 + 288 = 500 (55.0)	C C C C2C CC CC3C2C C C5C (14)	AF093752
Rice	OsPCS1	2 + 208 + 289 = 499 (55.6)	C C C2C CC CC3C2C C C (12)	(C. Cobbett, unpublished)
Others				
Schizosaccaromyces pombe	SpPCS1	40 + 207 + 167 = 414 (46.7)	C CC4CC3CC (6)	Z68144
Caenorhabditis elegans	CePCS1	6 + 209 + 156 = 371 (42.1)	C CC4CC6CC C2C7C3C (11)	AF299332
Dictyostelium discoideum	DdPCS1	133 + 208 + 285 = 626 (70.5)	[C3C1C C2C] C C CC3C C C1C7C [5](9)	(C. Cobbett, unpublished)

[a]From an alignment, the amino acid sequences have been divided into a conserved domain (Con), a variable (Var) C-terminal domain, and an N-terminal (N) extension. The conserved domain corresponds to amino acids 5 to 212 of the AtPCS1 sequence and is arbitrarily based on the level of conservation accross the plant and nonplant sequences. The total number of amino acid residues (aa) and predicted MW (kD) are indicated.

[b]The arrangement of Cys (C) residues in the variable C-terminal domain is indicated. The number of amino acids separating Cys residues is indicated where that number is less than 8. Otherwise, a gap indicates an unspecified number of amino acid residues. Because the C-terminal regions of plant PCS proteins can be aligned, corresponding Cys resides are vertically aligned in the table. The C-terminal Cys residues in the nonplant sequences cannot be aligned, and this is not implied in the table. The DdPCS1 sequence has an extended N-terminal region that also contains Cys residues indicated by brackets. Total number of Cys residues in the variable C-terminal domains is indicated in parentheses.

[c]A single accession number is shown for each sequence, although there may be multiple entries in the sequence databases.

in *C. elegans* (77). This shows clearly that PCs play a wider role in heavy metal detoxification in biology than previously expected. In contrast, it appears that some organisms do not (or probably do not) express a PC synthase. There is, for example, no evidence for PC synthase–homologous sequences in the *S. cerevisiae*, *Drosophila melanogaster*, or mouse and human genomes. One view of the limited selection of species in which such sequences have been identified is that organisms with an aquatic or soil habitat are more likely to express PCs. However, the recent report of partial PC synthase–homologous ESTs in, for example, the mosquito-borne parasitic nematode *Brugia malayi* (77) undermines this simplistic categorization.

PC Synthase Enzymes and Their Regulation

The predicted molecular weights of various PC synthase enzymes deduced from DNA sequences are given in Table 2 and range from 42 kD to 70 kD. A comparison of the deduced amino acid sequences shows that the N-terminal regions of the plant, yeast, and animal PC synthases are very similar (40–50% identical), whereas the C-terminal sequences show little apparent conservation of amino acid sequence. The most apparent common feature of the C-terminal regions is the occurrence of multiple Cys residues, often as adjacent pairs or near pairs (Table 2). The arrangements of Cys residues is reminiscent of those found in MTs (see below). The C-terminal regions of the *Arabidopsis* and *S. pombe* PC synthase proteins, for example, have 10 and 7 Cys residues, respectively, of which 4 and 6, respectively, are as adjacent pairs. However, there is no apparent conservation of the positions of these Cys residues relative to each other. In contrast, monocot (TaPCS1) and dicot (AtPCS1) plant PC synthase sequences can be aligned across their entire length (55% identity) (11). The former contains 14 Cys residues, including two pairs, in the C-terminal domain. The *S. pombe* and *D. discoideum* sequences also contain N-terminal extensions, which in the latter also contains clusters of Cys residues that may play a role similar to the C-terminal Cys clusters.

When a PC synthase activity was first identified (from cultured cells of *Silene cucubalis*) it was characterized as a γ-GluCys dipeptididyl transpeptidase (EC 2.3.2.15) (24). It catalyzed the transpeptidation of the γ-GluCys moiety of GSH onto a second GSH molecule to form PC_2 or onto a PC molecule to produce an $n + 1$ oligomer. The enzyme was described as a tetramer of Mr 95,000 with a Km for GSH of 6.7 mM. The MW of this purified enzyme seems inconsistent with the MWs of the PC synthase sequences deduced from both dicot and monocot plant genes (Table 2). Furthermore, there is no evidence that cloned plant PC synthase enzymes are multimeric. This suggests a protein mixture may have been purified from *S. cucubalis* and that the PC synthase activity was not the major component detected in MW determinations. PC synthase activities have also been detected in pea (36), tomato (8), and *Arabidopsis* (28).

In vivo studies have shown that PC synthesis can be induced by a range of metal ions in *S. pombe* and in both intact plants and plant cell cultures (see 58). Kinetic studies using plant cell cultures demonstrated that PC biosynthesis occurs within

minutes of exposure to Cd and is independent of de novo protein synthesis. The enzyme appears to be expressed independently of heavy metal exposure. It has been detected in extracts of plant cell cultures or tissues grown 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 vitro, the partially purified enzyme from *S. cucubalis* was active only in the presence of a range of metal ions. The best activator tested was Cd, followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au cations. This result has been mirrored by in vitro studies of PC synthase expressed in *E. coli* or in *S. cereviseae*, where the enzyme was activated to varying extents by Cd, Cu, Ag, Hg, Zn, and Pb ions (11, 25, 78).

Early models for the activation of PC synthase assumed a direct interaction between metal ions and the enzyme but raised the question of how the enzyme might be activated by such a wide range of metals. A significant recent study has provided evidence for an alternative model that provides a solution to this dilemma (79). With the cloning of PC synthase genes, the expression and purification of tagged recombinant derivatives of the enzyme has led to a more comprehensive understanding of the mechanisms of enzyme activation and catalysis. Vatamaniuk et al. (79) demonstrated that, in contrast to earlier models of activation, metal binding to the enzyme, per se, is not responsible for catalytic activation. Although AtPCS1 binds Cd ions at high affinity (Kd $= 0.54 \pm 0.20\ \mu$M) and high capacity (stoichiometric ratio $= 7.09 \pm 0.94$) (78), it has a much lower affinity for other metal ions, such as Cu, which are equally effective activators.

The kinetics of PC synthesis are consistent with a mechanism in which heavy metal glutathione thiolate (e.g., $Cd.GS_2$) and free GSH act as γ-Glu-Cys acceptor and donor. First, modeling using the known binding constants of GSH and Cd showed that, in the presence of physiological concentrations of GSH and μM concentrations of Cd, essentially all of the Cd would be in the form of a GSH thiolate. Second, S-alkylglutathiones can participate in PC biosynthesis in the absence of heavy metals. These observations are consistent with a model in which blocked glutathione molecules (metal thiolates or alkyl substituted) are the substrates for PC biosynthesis. Thus the role of metal ions in enzyme activation is as an integral part of the substrate, rather than interacting directly with the enzyme itself. In this way, any metal ions that form thiolate bonds with GSH may have the capacity to activate PC biosynthesis, subject to possible steric constraints in binding at the active site of the enzyme. Early work suggested that PC biosynthesis in vitro was ultimately terminated by the PC products chelating the activating metal ions (or could be prematurely terminated by the addition of a metal chelator such as EDTA) (43), which provides a mechanism to autoregulate the biosynthesis of PCs. Viewed from a perspective where the metal ion forms part of the substrate, termination of the reaction results simply from exhaustion of substrate.

The conserved N-terminal domain of PC synthase is presumed to be the catalytic domain. The *cad1-5* mutation of Arabidopsis is a nonsense mutation that would result in premature termination of translation downstream of the conserved

domain (25). The truncated polypeptide would lack 9 of the 10 Cys residues in the C-terminal domain. Of all the *cad1* mutants characterized, *cad1-5* is the least sensitive to Cd and makes the highest residual level of PCs on exposure to Cd (28). This suggests that the C-terminal domain is not absolutely required for catalysis. Because the work of Vatamaniuk et al. (79) suggests that heavy metal "activation" of PC synthase is in fact an integral component of catalysis, what then is the role of the multiple Cys residues in the variable C-terminal domain? Because the truncation of the *cad1-5* mutant polypeptide produces a mutant phenotype, the C-terminal domain clearly has some role in activity. This domain probably acts to enhance activity by binding metal glutathione complexes, bringing them into closer proximity to the catalytic domain.

Studies indicating PC synthase is expressed constitutively and levels of enzyme are generally unaffected by exposure of cell cultures or intact plants to Cd suggest the induction of PC synthase gene expression is unlikely to play a significant role in regulating PC biosynthesis. This is supported by analysis of the expression of *AtPCS1*/*CAD1* that showed that levels of mRNA were not influenced by exposure of plants to Cd and other metals, thus suggesting an absence of regulation at the level of transcription (25, 79). In contrast, analysis of *TaPCS1* expression in roots indicated increased levels of mRNA on exposure to Cd (11). This suggests that, in some species, PC synthase activity may be regulated at both the transcriptional and posttranslational levels. Little is known about the tissue specificity of PC synthase expression and/or PC biosynthesis. In a study in tomato, activity was detected in the roots and stems of tomato plants but not in leaves or fruits (8).

Sequestration to the Vacuole

In both plant and yeast, PC-Cd complexes are sequestered to the vacuole. In *S. pombe*, this process has been most clearly demonstrated through studies of the Cd-sensitive mutant *hmt1*. In extracts of *S. pombe*, two PC-Cd complexes (referred to as HMW and LMW) can be clearly resolved using gel-filtration chromatography. The *hmt1* mutant is unable to form the HMW complexes. The *Hmt1* gene encodes a member of the family of ATP-binding cassette (ABC) membrane transport proteins that is located in the vacuolar membrane (53). Both HMT1 and ATP are required for the transport of LMW PC-Cd complexes into vacuolar membrane vesicles (54) (Figure 1). In *S. cerevisiae*, which appears not to express a PC synthase, YCF1 is also a member of the ABC family of transporters and carries $(GSH)_2Cd$ complexes to the vacuole (41). Interestingly, in *C. elegans*, various mutations affecting ABC transporter proteins also confer heavy metal sensitivity (4). It is possible that these transporters are involved in the sequestration of PC-metal complexes in *C. elegans*. The site of such sequestration is still unidentified.

In plants, sequestration of PCs to the vacuole has also been observed. In mesophyll protoplasts derived from tobacco plants exposed to Cd, almost all of both the Cd and PCs accumulated was confined to the vacuole (81). An ATP-dependent, proton gradient-independent activity, similar to that of HMT1, capable of transporting

both PCs and PC-Cd complexes has been identified in oat roots (65). Nonetheless, a plant gene encoding this function has not yet been identified. A recent inventory (66) of the ABC transporter protein genes in the Arabidopsis genome has not revealed a clearly identifiable homologue of HMT1.

Sulfide Ions and PC Function

In some plants and in the yeasts *S. pombe* and *Candida glabrata*, sulphide ions play an important role in the efficacy of Cd detoxification by PCs. HMW PC-Cd complexes contain both Cd and acid-labile sulfide. The incorporation of sulfide into the HMW complexes increases both the amount of Cd per molecule and the stability of the complex. Some complexes with a comparatively high ratio of S^{2-}:Cd consist of aggregates of 20Å-diameter particles, which themselves consist of a CdS crystallite core coated with PCs (18, 60).

The analysis of Cd-sensitive mutants of *S. pombe* deficient in PC-Cd complexes has provided evidence for the importance of sulfide in the function of PCs. These include some mutants affected in steps in the adenine biosynthetic pathway (71). Juang et al. (32) have shown that these enzymes, in addition to catalyzing the conversion of aspartate to intermediates in adenine biosynthesis, could also utilize cysteine sulfinate, a sulfur-containing analog of aspartate, to form other sulfur-containing compounds, which may be intermediates or carriers in the pathway of sulfide incorporation into HMW complexes (Figure 1). Whether sulfide is involved in the detoxification of other metal ions by PCs is unknown.

Using other Cd-sensitive mutants of *S. pombe* and *Candida glabrata*, investigators have identified additional functions important in sulfide metabolism. In *C. glabrata*, the *hem2* mutant is deficient in porphobilinogen synthase, which is involved in siroheme biosynthesis (31). Siroheme is a cofactor for sulfite reductase required for sulfide biosynthesis (Figure 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. In *S. pombe*, the *hmt2* mutant hyperaccumulates sulfide in both the presence and absence of Cd (74). 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 that it detoxifies excess sulfide generated during the formation of HMW PC-Cd complexes after Cd exposure (Figure 1).

Metals Other than Cd

Although both induction of PCs in vivo and activation of PC synthase in vitro is 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 47, 59). Maitani et al. (45) 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. This seems to conflict with the model for PC synthase activity whereby a metal-GSH thiolate is the substrate for PC-metal biosynthesis. It may indicate that some metals in complexes with PCs can be exchanged for others. In contrast, however, Schmöger et al. (68) have clearly demonstrated the formation of PC-As complexes in vivo and in vitro.

The clearest evidence for the role of PCs in heavy metal detoxification comes from characterization of the PC synthase–deficient mutants of *Arabidopsis* and *S. pombe*. A comparison of the relative sensitivity of the *Arabidopsis* and *S. pombe* mutants to different heavy metals revealed a similar but not identical pattern (25). In both organisms, PC-deficient mutants are highly sensitive to Cd and arsenate. For other metals, including Cu, Hg, Ag, Zn, Ni, and selenite ions, the mutants showed little or no sensitivity. Suppression of PC synthase in *C. elegans* also resulted in a Cd-sensitive phenotype, but the effect on responses to other metals has not been reported (77). Thus, PCs play a clear role in Cd and arsenate detoxification. Cu, for example, is a strong activator of PC biosynthesis both in vivo and in vitro, yet PC-deficient mutants show relatively little sensitivity to Cu. PCs also form complexes with Cu in vivo. It is possible, nonetheless, that PC-Cu complexes are relatively poorly sequestered to the vacuole, that they are comparatively transient, or that there is an alternative, more effective, mechanism for Cu detoxification.

The Roles of PCs

Although PCs clearly can have an important role in metal detoxification, alternative primary roles of PCs in plant physiology have also been proposed. These have included roles in essential metal ion homeostasis and in Fe or sulphur metabolism (see 67, 84). However, there is currently no direct evidence that PCs have functions outside of metal detoxification. These proposals stem from the expectation that the levels of Cd and As, for example, to which most organisms would be exposed in the natural, nonpolluted environment would not be sufficiently high to select for such a detoxification mechanism. Most experimental studies in plants have used Cd concentrations above 1 μM (67). In contrast, it has been estimated that solutions of nonpolluted soils contain Cd concentrations ranging up to 0.3 μM (82). Wagner (82) has argued that only at high levels of Cd exposure (not generally found in natural environments) might PCs play a role. Counter to this is the observation that a PC-deficient mutant of Arabidopsis is highly sensitive to concentrations of Cd as low as 0.6 μM in agar medium (28). 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 existence of a PC-deficient mutant of Arabidopsis and the isolation of, for example, a PC-deficient insertion mutant of *C. elegans* may allow the role of PCs in organisms in unpolluted environments to be assessed.

METALLOTHIONEINS

Structure

Metallothionein proteins, products of mRNA translation, are characterized as low molecular weight, cysteine-rich, metal-binding proteins (33). Although PCs conform to many of these characteristics, the enzymatic synthesis of PCs distinguishes them from MT proteins. Since their discovery more than 40 years ago as Cd-binding proteins present in horse kidney, MT proteins and genes have been found throughout the animal and plant kingdoms as well as in the prokaryote Synechococcus. The large number of cysteine residues in MTs bind a variety of metals by mercaptide bonds. MTs typically contain two metal-binding, cysteine-rich domains that give these metalloproteins a dumbbell conformation. MT proteins are classified based on the arrangement of Cys residues (9). Class I MTs contain 20 highly conserved Cys residues based on mammalian MTs and are widespread in vertebrates. MTs without this strict arrangement of cysteines are referred to as Class II MTs and include all those from plants and fungi as well as nonvertebrate animals. In this MT classification system, PCs are, somewhat confusingly, described as Class III MTs.

Shortly after the discovery of PCs as an important metal ligand required for tolerance of plants to Cd, a MT protein was identified in wheat (39), and a number of MT genes were isolated from plants. The plant Class II MT proteins can be further classified based on amino acid sequence. Robinson et al. (62) first identified two plant MT types based on the position of cysteine residues in the predicted proteins. Since then, the number of characterized plant MT genes has increased dramatically, and because many do not conform to these two groups, additional categories have been added. Other classifications have been proposed (2, 42, 59). However, the system presented below builds on the one developed by Robinson et al. (62). and is able to place almost all of the known plant MT genes into four categories based on amino acid sequence (Figure 2). Type 1 and Type 2 MTs follow the classification of Robinson et al. (62), Type 3 includes many MTs expressed during fruit ripening, and Type 4 is exemplified by the wheat Ec protein, the first characterized plant MT protein.

Type 1 MTs contain a total of six Cys-Xaa-Cys motifs (where Xaa represents another amino acid) that are distributed equally among two domains. In the majority of Type 1 MTs, the two domains are separated by approximately 40 amino acids that include aromatic amino acids. This large spacer is a common feature of plant MTs and contrasts with most other MTs in which cysteine-rich domains are separated by a spacer of less than 10 amino acids that do not include aromatic residues. Within the Type 1 MTs, those from various Brassicaceae (Arabidopsis and Brassica species) have a number of distinguishing features, including a much shorter spacer between the cysteine-rich domains and an additional Cys residue (5, 85).

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Type 1
          * *    * *   * *                                                  * *   *  *   * *
AtMT1a  MADSNCGCGS SCKCGDSCSC EKNY..... .......... .......... ......NKEC DNCSCGSNCS CGSNCNC
AtMT1c  MAGSNCGCGS SCKCGDSCSC EKNY..... .......... .......... ......NKEC DNCSCGSNCS CGSSCNC
BnMT1   MAGSNCGCGS GCKCGDSCSC EKNY..... .......... .......... ......NTEC DSCSCGSNCS CGDSCSC
OsMT1a  MS...CSCGS SCSCGSNCSC GKKYPDLEEK SSSTKATVVL GVAPEKKQQF EAAAESGETA HGCSCGSSCR CNP.CNC
PsMT1   MSG..CGCGS SCNCGDSCKC NKRSSGLSYS EMETTETVIL GVGPAKIQFE GAEMSAASED GGCKCGDNCT CDP.CNCK
MsMT1   MSG..CNCGS SCNCGDNCKC NSRSSGLGYL EGETTETVIL GVGPAKIHFE GAEMGVAAED GGCKCGDSCT CDP.CNCK

Type 2
          **   * *    * *   *   *                                          * *    * *  * *
AtMT2a  MSCCGGNCGC GSGCKCGNGC GGCKMYPDLG FSGETTTTET FVLGVAPAMK NQYEASGESN NAENDACKCG SDCKCDPCTC K
BoMT2   MSCCGGNCGC GSGCKCGNGC GGCKMYPDLG FSGELTTTET FVFGVAPTMK NQHEASGEGV .AENDACKCG SDCKCDPCTC E
AtMT2b  MSCCGGSCGC GSACKCGNGC GGCKRYPDL. ...ENTATET LVLGVAPAMN SQYEASGETF VAENDACKCG SDCKCNPCTC K
PhMT2   MSCCGGNCGC GSGCKCGNGC GGCKMYPDFS YT.ESTTTET LILGVGPEKT SFGSMEMGES PAEN.GCKCG SDCKCDPCTC SK
SvMT2   MSCCNGNCGC GSACKCGSGC GGCKMFPDFA E..GSSGSAS LVLGVAP.MA SYFDAEMEMG VATENGCKCG DNCQCNPCTC K
OsMT2   MSCCGGNCGC GSSCQCGNGC GGCK.YSEVE PTTTTTFLAD ATNKGSGAAS GGSEMGAENG SCGCNTCKCG TSCGCSCCNC N

Type 3
          *  * *       *                                    * *   *  *  * *
AtMT3   MSSNCGSCDC ADKTQCVKKG TSYTFDIVET QESYKEAMIM DVGAEENNAN CKCKCGSSCS CVNCTCCPN
MaMT3   MS.TCGNCDC VDKSQCVKKG NSYGIDIVET EKSYVDEVIV AAEAAEHDG. .KCKCGAACA CTDCKCGN
AdMT3   MSDKCGNCDC ADSSQCVKKG NS..IDIVET DKSYIEDVVM GVPAAESGG. .KCKCGTSCP CVNCTCD
OsMT3   MSDKCGNCDC ADKSQCVKKG TSYGVVIVEA EKSHFEEV.. .AAGEENGG. ..CKCGTSCS CTDCKCGK
GhMT3   MSDRCGNCDC ADRSQCTK.G NSNTM.IIET EKSYINTAVM DAPAENDG.. .KCKCGTGCS CTDCTCGH
PgMT3   MSSDCGNCDC ADKSQCTKKG FQID.GIVET SYEMGHGGD. ..VSLEND.. ..CKCGPNCQ CGTCTCHT

Type 4
                  *   * *    *     *  *                  *  *   * *  *  *                 * *    * *  *
AtMT4a  MADTGKGSSV AGCNDSCGCP SPCPGGNSCR CRM..R.EAS AGDQGHMVCP CGEHCGCNPC NCPKTQTQTS AKG....CTC GEGCTCASCA T
AtMT4b  MADTGKGSAS ASCNDRCGCP SPCPGGESCR CKM..MSEAS GGDQEHNTCP CGEHCGCNPC NCPKTQTQTS AKG....CTC GEGCTCATCA A
PhMT4   MADL.RGSS. AICDERCGCP SPCPGGVACR CASGGAATAG GGDMEHKKCP CGEHCGCNPC TCPKSEGTTA GSGK.AHCKC GPGCTCVQCA S
ZmMT4   MG........ ...DDKCGCA VPCPGGKDCR CTS...G..S GGQREHTTCG CGEHCECSPC TCGRATMPSG RENRRANCSC GASCNCASCA SA
TaMT4   MG........ ..CDDKCGCA VPCPGGTGCR CTS...ARSG AAAGEHTTCG CGEHCGCNPC ACGREGTPSG RANRRANCSC GAACNCASCG SATA
OsMT4   MG........ ..CDDKCGCA VPCPGGTGCR CAS...S.AR SGGGDHTTCS CGDHCGCNPC RCGRESOPTG RENRRAGCSC GDSCTCASCG STTTTAPAAT T
```

Figure 2 Alignment of plant MT amino acid sequences. Examples of the four types of plant MTs are shown. Cysteine residues are in bold, and conserved cysteines in each type are indicated by a star. The protein sequences are predicted from gene sequences in Arabidopsis (At), *Brassica napus* (Bn), rice (Os), pea (Ps), alfalfa (Ms), *Brassica oleracea* (Bo), petunia (Ph), *Silene vulgaris* (Sv), banana (Ma), kiwifruit (Ad), cotton (Gh), *Picea glauca* (Pg), maize (Zm), and wheat (Ta).

Type 2 MTs also contain two cysteine-rich domains separated by a spacer of approximately 40 amino acid residues. However, the first pair of cysteines is present as a Cys-Cys motif in amino acid positions 3 and 4 of these proteins. A Cys-Gly-Gly-Cys motif is present at the end of the N-terminal cysteine-rich domain. Overall, the sequences of the N-terminal domain of Type 2 MTs are highly conserved (MSCCGGNCGCS). The C-terminal domain contains three Cys-Xaa-Cys motifs. By contrast, the spacer region separating these domains in Type 2 MTs is much more variable between species.

Type 3 MTs contain only four Cys residues in the N-terminal domain. The consensus sequence for the first three is Cys-Gly-Asn-Cys-Asp-Cys. The fourth cysteine is not part of a pair of cysteines but is contained within a highly conserved motif, Gln-Cys-Xaa-Lys-Lys-Gly. The six Cys residues in the C-terminal cysteine-rich domain are arranged in Cys-Xaa-Cys motifs. As with the majority of Type 1 and Type 2 plant MTs, the two domains are separated from each other by approximately 40 amino acid residues.

Type 4 MTs differ from other plant MTs by having three cysteine-rich domains, each containing 5 or 6 conserved cysteine residues, which are separated by 10 to 15 residues. Most of the cysteines are present as Cys-Xaa-Cys motifs. Although a large number of Type 4 MTs have not been identified, compared to those from monocots, Type 4 MTs from dicots contain an additional 8 to 10 amino acids in the N-terminal domain before the first cysteine residue.

The vast majority of plant MT genes have been identified in the angiosperms. A number of species, including Arabidopsis, rice, and sugarcane (A. Figueira, personal communication), contain genes encoding all four types of MTs. This indicates that evolution of the four plant MT types predates the separation of monocots and dicots, and it is likely that the majority of flowering plants also contain the four different MT types. The presence of four types of MTs in plants with distinct arrangements of cysteines contrasts with the situation in animals. For example, the four mouse MTs all contain the same conserved cysteines, although they do differ in tissue expression (35). The diversity of the plant MT gene family suggests that these may differ not only in sequence but also in function. There is little information about MT genes in nonflowering plant species. However, genes encoding Type 3 MTs have been cloned from several gymnosperms (7). A MT-encoding gene has also been isolated from *Fucus vesiculocus*, a brown alga (48). This MT does not fit readily into any of the four plant types described above but, primarily on the basis of the cysteine residues, is equally similar to Arabidopsis MT1a and an oyster MT. Further studies are needed to determine if the diverse MT gene family present in angiosperms is also found in other divisions of the plant kingdom.

Gene Structure

Genomic DNA sequences have been determined for a small number of MT genes, and these provide some additional support for the classification system described above. Almost all plant MT genes contain an intron located close to the end of the N-terminal cysteine-rich domain. However, the position of this intron varies

in genes encoding different MT types. The single intron in Type 1 MT genes from monocots disrupts the codon after the last cysteine codon in the N-terminal cysteine-rich domain. All Type 3 MT genes that have been characterized contain two introns, and the first lies in the same relative position after the end of the N-terminal cysteine-rich domain. However, the first intron in genes encoding Type 1 MTs from dicots and Type 4 MTs lies in the codon preceding the last cysteine codon of the first domain. This is the same position as the single intron in Arabidopsis Type 2 MT genes and the first of two introns in Type 2 MT genes in rice. Interestingly, another classification of MTs has identified the Brassica MTs as variant forms of Type 2 plant MTs (2). Overall, apart from the difference between Type 1 MT genes from monocots and dicots, the position of the first intron in plant MT genes supports the classification of plant MTs into four types based on amino acid sequence.

The Arabidopsis genome sequence has provided information on how the seven members of the MT gene family are organized. The *MT1a* and *MT1c* genes lie within 4 kb as an inverted repeat on chromosome 1; *MT2a* and *MT3* are at distinct positions on chromosome 3; both *MT4a* and *MT4b* lie on chromosome 2 but are not closely linked; finally, *MT2b* is positioned on chromosome 5. One pseudogene, *MT1b*, has been identified in Arabidopsis and is also found on chromosome 5. Mapping and genome sequencing have demonstrated that MT genes are also distributed across different chromosomes in the tomato (22) and rice genomes. However, evidence of MT gene clustering has been found in cotton where three MT genes were identified within a 10-kb fragment of genomic DNA (30).

MT Proteins

The wheat Ec Type 4 MT protein was purified from embryos as a zinc binding protein and provided the first evidence that plants contained not only PCs but also MTs as cysteine-based metal ligands (39). The ensuing flood of information about plant genes and cDNAs encoding MT proteins has not been accompanied by a corresponding increase in knowledge about the expression or distribution of MT proteins. Consequently, there has been a trend to describe these as "metallothionein-like genes," for fear that they were in fact not translated into bona fide metal-binding proteins. However, it would seem quite perverse if these genes were not translated into proteins in plants, given that many are highly expressed and encode proteins with known metal-binding motifs and similarity to proteins required for specific functions in animals and fungi. Indeed, evidence for the occurrence of several MT proteins has been obtained in Arabidopsis (50). Peptide fragments derived from MT1a, MT2a, MT2b, and MT3 were identified after purification of the proteins under anaerobic conditions using several chromatographic separations, including Zn affinity chromatography. Immunoblot analysis also demonstrated that expression of MT1 and MT2 proteins corresponded to observed RNA levels in terms of tissue specificity and induction by copper treatment. Difficulties in identifying MTs in plants may arise from instability of these proteins in the presence of oxygen. There is, however, a critical need for more information about the distribution and form of MTs in plants, including the metals that are bound to these proteins in vivo. This

search would be assisted by the development of simpler purification procedures and isoform-specific antibodies for MTs as well as the application of protein-tagging procedures (e.g. myc or GFP) for in situ localization of these proteins.

Although it has been difficult to study MT proteins in planta, several plant MTs have been expressed in microbial hosts to examine the metal-binding properties of these proteins and their ability to provide metal tolerance. When expressed in *E. coli*, the pea Type 1 MT, PsMTa, bound Cu, Cd, and Zn, with the highest affinity for Cu (73). Similarly, a recombinant Fucus MT fusion protein showed a greater affinity for Cu than Cd, and the pH required for dissociation of Cd from Fucus MT was approximately 2 pH units higher than for a recombinant human MT. Arabidopsis MTs have been expressed in MT-deficient strains of yeast (85) and Synechococcus (63) and were able to complement these mutations in terms of restoring tolerance to copper and zinc, respectively. These studies provide important evidence that plant MTs are capable of providing a biological function—metal tolerance—albeit in nonplant systems. A number of studies have examined the effects of ectopic expression of MT proteins from various sources on metal tolerance in plants. Although some of these studies have resulted in increased metal tolerance or altered distribution of metals in plants, they have not been informative regarding the function of endogenous plant MTs.

MT Gene Expression

In attempting to shed light on their function, investigators have relied primarily on RNA blot hybridization to study the expression of MT genes during development and in response to various environmental factors. More detailed localization of MT mRNAs or MT gene promoter activity has been obtained in a small number of cases through in situ hybridization and reporter gene expression studies. Many MT genes are expressed at very high levels in plant tissues, at least in terms of transcript abundance. Direct evidence comes from gene profiling experiments in rice using the serial analysis of gene expression (SAGE) protocol (46). Transcripts from four MT genes comprised almost 3% of the transcripts in two-week-old seedlings. A Type 2 MT gene contributed 1.26% of all transcripts, the single most abundantly expressed gene in this tissue. Transcripts of two Type 3 MT genes accounted for an additional 1.25% of the mRNAs. Furthermore, ESTs for MT genes are among the most prevalent in randomly sequenced cDNA libraries from a number of plants. For example, a Type 2 MT gene accounted for 0.4% of the tomato ESTs, and 0.5% of maize ESTs were derived from a Type 1 MT gene (72). The large number of MT genes that have been identified by differential screening of cDNA libraries also indicates that RNAs encoding MTs are abundant in many other plant species.

Expression of Type 4 MTs, such as the wheat Ec MT, is restricted to developing seeds. Type 4 MT genes contain promoter sequences with homology to ABA-response elements, and their expression is regulated by ABA (83). These genes follow the same regulatory program as a large number of other genes that are expressed during the maturation of embryos and whose RNAs persist until imbibition and germination. Kawashima et al. (34) proposed that this embryo-specific

MT provides a mechanism for storing zinc that is required during germination. Expression of MT genes from other types has also been observed in developing seeds (A. Figueira, personal communication). If these MTs are involved in accumulation and storage of metals in seeds, they may play an important role in determining the concentrations of metals in grains. As more attention is paid to the nutritional composition of foods as opposed to simple calorific value, MTs may provide one mechanism to manipulate metal concentration in seeds (44).

Expression of other plant MT genes is not restricted to a single organ and cannot be categorized simply as that of Type 4 MTs (59). However, a number of general observations can be made about the expression of these genes. RNA expression of Type 1 MT genes tends to be higher in roots than shoots, whereas the reverse is observed generally for Type 2 MTs. In plants that produce fleshy fruits, e.g., banana (13), apple (61), and kiwi (40), Type 3 MT RNAs are highly expressed in the fruits as they ripen. Type 3 MTs are also expressed at high levels in leaves of plants that do not produce fleshy fruits, such as Arabidopsis (W. Bundithya & P. Goldsbrough, unpublished observation).

A small number of studies have examined the expression of MT genes at a more detailed level, using in situ hybridization and reporter gene expression. In both Arabidopsis and *Vicia faba*, MT RNA expression in leaves was shown by in situ hybridization to be highest in trichomes (19, 20). GUS reporter genes driven by the Arabidopsis *MT1a* and *MT2a* promoters also direct GUS expression preferentially in trichomes under some conditions (W. J. Guo, W. Bundithya, & P. Goldsbrough, unpublished observations). There are a number of possible explanations for high levels of MT gene expression in trichomes. Toxic metals such as Cd accumulate in trichomes (64). Although the metals that bind to MTs in most plant tissues are not known, MTs may be required for detoxification of metals that are deposited in trichomes. Foley & Singh (19) have suggested that specific metal-binding enzymes are highly expressed in trichomes and that MT expression may be involved in the delivery of metals into these specialized cells. Expression of Arabidopsis MT genes has also been localized to the phloem in a number of tissues (20; W. Bundithya, W. J. Guo, & P. Goldsbrough, unpublished observations), raising the possibility that MTs could play a role in metal ion transport. However, as yet, MTs have not been identified among the phloem exudate proteins characterized from various plants, but the MT proteins have been difficult to purify by standard methods.

The expression of some MT genes changes during development. One interesting example is the dramatic increase in MT RNA levels in senescing leaves. This was first reported for a Type 1 MT gene in *Brassica napus* (5) and has been confirmed in Arabidopsis and rice (20, 29). MT RNA expression in senescing leaves appears to be localized primarily to phloem tissue (6). At least two other genes that are specifically involved in copper homeostasis, one encoding a metal chaperone (AtCCH) and the other a copper transporter (AtRAN1), are also expressed in senescing leaves (26, 27). The homologous proteins in yeast, Atx1 and Ccc2, are involved in the delivery of copper to the trans-Golgi network for incorporation into copper-requiring proteins. They are components of an integrated system for the regulated uptake and distribution of copper (56). This system is able to maintain

the level of free copper ions below one ion per yeast cell, thereby preventing the damage that reactive copper ions can cause through the production of reactive oxygen species to membranes, proteins, and nucleic acids (57). Why are plant homologues of these genes expressed in senescing leaves? One possibility is that MTs are required to chelate copper released from metalloproteins that are being catabolized in senescing leaves. In the absence of MTs, or another ligand, free copper ions would precipitate a cascade of oxidative damage and disrupt the controlled senescence program. Expression of MTs in phloem during leaf senescence also suggests that MTs might serve as a chaperone for long-distance transport of copper. The other plant homologs of this copper homeostasis system, AtCCH and AtRAN1, may play a role in the export of copper from leaves to sinks such as developing seeds. The human homolog of AtCCH is thought to deliver copper to a transporter that is responsible for copper efflux from intestinal epithelial cells (56). This copper transporter is defective in Menkes disease patients, resulting in accumulation of copper in the intestine and consequent copper deficiencies in other tissues. AtRAN1, the Arabidopsis homolog of the Menkes copper transporter, may therefore participate in copper efflux from senescing leaves, and this could require the partner copper chaperone protein, AtCCH. It is of interest that MT gene expression has also been observed in other processes that involve apoptosis, including leaf abscission (17) and the hypersensitive response to pathogens (6).

A large number of reports have described the effects of various environmental factors on MT RNA expression in plants, and these have been tabulated by Rauser (59). Overall, these experiments show little in the way of consistent trends for conditions that modulate expression of specific MT types across species. For example, various metals including copper had either no effect or repressed MT gene expression in many species. However, copper induced expression of a Type 1 MT gene in Arabidopsis, rice, wheat, and tobacco (10, 29, 70, 85) as well as MT genes in Fucus (48) and *P. oceanica* (21). Type 1 MT genes are also induced by a variety of other stresses, including aluminum, cadmium, nutrient deprivation, and heat shock, in wheat and rice (29, 70), suggesting that MTs may be expressed as part of a general stress response, although an indirect connection to metal ion status could exist. It has been proposed that iron deficiency, which induces MT gene expression in barley and pea, mediates this response by increasing copper uptake (52, 62). That expression of MT genes in animals is also affected by a tremendous variety of conditions (33) is worth noting. Currently there is no information about the mechanisms that regulate transcription of plant MT genes in vegetative tissues, in contrast to the detailed knowledge of metal regulation of MT gene expression in yeast and mammals. The yeast transcription factor Ace1 is activated by copper and binds to elements in the CUP1 promoter to stimulate transcription of this MT gene (38). In mammals, MTF1 activates transcription of MT genes in response to metals by binding to metal response elements in MT gene promoters (1). This transcription factor is essential because an MTF1-null mutation is lethal in mice, even though MT-deficient mice are normal unless they are subjected to cadmium or zinc toxicity.

Function of Metallothioneins

What are the functions of MT genes in plants? In animals, MTs protect against cadmium toxicity (35), but this function in plants is clearly provided by PCs. Reconciling all the available data on plant MTs into a simple model may be impossible and may also be unrealistic given the diverse family of MT genes in plants. However, there is evidence to support the hypothesis that MTs are involved in copper tolerance and homeostasis in plants: Some plant MTs are functional copper-binding proteins; expression of some MT genes is induced by copper; MT gene expression in senescing leaves is coordinated with a set of genes involved in copper homeostasis; the level of expression of a Type 2 MT gene correlated closely with copper tolerance in a group of Arabidopsis ecotypes (49); expression of a Type 2 MT gene is elevated in a copper-sensitive mutant that accumulates copper (76); more recently, copper tolerant populations of *S. vulgaris* have been shown to have higher RNA expression and gene copy number of a Type 2 MT gene (75). In addition, PCs do not provide tolerance to copper in Arabidopsis, indicating that another mechanism, perhaps involving MTs, must be involved. While supporting a role for MTs in copper tolerance, this evidence is not conclusive.

The most direct approach to answering this and other questions about the function of MTs is to identify and analyze plants with defined MT-null genotypes. T-DNA insertional mutagenesis is well developed for this objective (37). However, MT genes present very small targets for this approach (less than 1 kb), and the probability of finding insertions even in populations of 50,000 lines is not high. Targeted gene disruption strategies using transposable element "launch pads" inserted close to specific MT loci may be more efficient (69), whereas RNA interference may provide an alternative that is not dependent on identifying DNA insertions into MT genes (16). It may be necessary to combine null mutants for more than one MT gene and test these plants under a variety of conditions in order to observe any phenotype (3). Use of this approach in a variety of model plants is necessary and overdue in order to provide definitive answers about MT function.

In spite of the availability of such experimental tools to study animal MTs, the function of these proteins remains somewhat of an enigma (55). Mammalian MTs have a highly conserved sequence, are expressed in many tissues, and respond to a wide variety of regulatory factors. Although these observations hint at an important function for MTs in mammals, the only role that has been established unequivocally is in protection against cadmium and zinc toxicity (55). Therefore, although MTs are expressed ubiquitously and conserved in plants, determining their function remains a future challenge.

FUTURE PROSPECTS

The use of model systems to study the biosynthesis, expression, regulation, and function of both PCs and MTs has offered significant advances in recent years. For PCs, the characterization of Cd-sensitive mutants of *S. pombe*, the organism in

which PCs were first recognized, has identified, with the remarkable exception of the PC synthase gene, various genes involved in PC biosynthesis or function. Thus far, in Arabidopsis, only mutants and genes in the PC biosynthetic pathway have been isolated. The parallel studies in *S. pombe* point to a number of additional functions still not discovered. There is clear evidence that Cd, in plants as in *S. pombe*, is sequestered to the vacuole in complexes with sulfide. Yet, for example, there is no apparent homologue of the vacuolar transporter, HMT1, in Arabidopsis. Clearly, further studies in plants are required to identify these additional functions. Also, as yet, no MT-deficient mutants in Arabidopsis have been characterized, and in view of the possibility of redundancy among the members of the MT gene family, the full suite of MT-deficient mutants is likely to be required to adequately determine the functions of the various genes.

The potential for the use of plants for the detoxification or "phytoremediation" of polluted environments is being increasingly examined. The manipulation of PC expression is one potential mechanism for increasing the capacity of plants for phytoremediation. Understanding the effect of the overexpression, possibly in a tissue-specific manner, of the genes of the GSH/PC biosynthetic pathway on metal tolerance and accumulation will soon lead to indications as to their usefulness in this endeavor. Here too, genes controlling other aspects of PC function may be required.

ACKNOWLEDGMENTS

The authors wish to thank present and former members of their laboratories for their contributions to aspects of the work reviewed here. Thanks to Metha Meetam and Woei-Jiun Guo for helpful comments. Research in the authors' laboratories is supported by Australian Research Council (C.C.) and USDA-NRI (P.G.).

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Annual Review of Plant Biology
Volume 53, 2002

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