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Structure and Function of Metal Chelators Produced by Plants

The Case for Organic Acids, Amino Acids, Phytin, and Metallothioneins

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ABSTRACT

Plants produce a range of ligands for cadmium (Cd), copper (Cu), nickel (Ni), and zinc (Zn). Cd- and Zn-citrate complexes are prevalent in leaves, even though malate is more abundant. In the xylem sap moving from roots to leaves, citrate and histidine are the principal ligands for Cu, Ni, and Zn. Phosphorus-rich globular bodies in young roots are probably Zn-phytate. Metallothioneins (MTs) are cysteine (Cys)-rich ligands. Plants produce class II MTs (MT-IIs) which differ from the archetypal mammalian MT-I in the location and number of Cys. The E_c protein from wheat embryos has Cys in three domains, binds Zn, and disappears with seedling development. The first 59 amino acids have been sequenced for the protein. Fifty-eight genes for MT-IIs, from a range of plants and tissues, predict proteins with Cys in two domains. Most of the predicted proteins have not been isolated, and their metal binding is poorly documented. Three protein bands, corresponding to six MT genes, have been isolated from *Arabidopsis*, and the amino acids sequenced for nine fragments. The MT-IIIs are atypical, nontranslationally synthesized polypeptides with variously repeating γ-glutamylcysteine units. Of the five families known, those with carboxy-terminal glycine are the most widespread among plants, algae, and certain yeasts. A heterogeneous grouping of these molecules form Cd-binding complexes with tetrahedral coordination and a Cd–sulfur interatomic distance of 2.52 Å. One complex is cytosolic, the dominant one is vacuolar. Together, they can bind a large proportion of cellular Cd; other ligands may also function. Little is known about the counterpart situation for Cu and Zn.

Index Entries: Cadmium; zinc; chelation; citrate; malate; histidine; phytin; plant metallothionein; phytochelatin.

INTRODUCTION

Terrestrial and aquatic environments provide plants with an array of essential and nonessential elements. Among these are metals, including the micronutrients copper (Cu), nickel (Ni), and zinc (Zn), and others, such as cadmium (Cd), lead (Pb), and mercury (Hg), for which no essential role is known in plants. These metals exist at low to high concentrations, depending on the origin of the soil and the history of anthropogenic disturbances. Sessile plants normally live in fluctuating environments, where bioavailability of metals

may change, leading to shifting internal concentrations. Normal plant growth and development depends on mechanisms that maintain internal concentrations of essential metals between limits of deficiency and toxicity, and of nonessential metals below their toxicity thresholds. The emphasis of this review is on the chelators of Cd, Cu, Ni, and Zn, which plants produce to accomplish cellular metal homeostasis, and, particularly, detoxification. The range of specific ligands for iron (Fe) is not considered.

The metals mentioned previously are known as class B and borderline ions *(1)*. The class B metals, Hg, Pb(IV), and Cu(I), seek out sulfur (S) and nitrogen (N) centers in biological systems, and may become irreversibly bound. The borderline metals, Cu(II), Cd, Ni, and Zn, form stable complexes with S, N, and oxygen (O) as the donor atom, as is the preference of class A metals (e.g., aluminum [Al]). The relative availability of different ligands in plants will affect the nature of the complexes found. Interest in Cu, Cd, Ni, Pb, and Zn is founded in the study of metal tolerance in plants, in which ecotypes of wild plants have the ability to grow in the presence of otherwise toxic concentrations of the metals *(2,3)*. Plants able to grow in disturbed habitats of mines and smelters offer classic examples of adaptation to metals and microevolution *(4)*. Comparisons between metal tolerant and nontolerant ecotypes of the same species have been used to determine whether the concentrations of an assumed chelator are sufficiently large to account for the tolerance. Because plants absorb essential and nonessential metals, the domesticated species containing nonphytotoxic levels become sources of metals potentially problematic to animals and humans. Since plant foods contribute at least 70% of the Cd intake in humans, an understanding of how plants deal with this metal, and partition it between organs, is a major interest *(5)*. The largest body of detailed information is for Cd; Cu and Zn are considered mostly from the perspective of metal tolerance, and Ni in relation to long-distance transport in hyperaccumulators. Vascular plants are the major focus of the review, but important evidence is also drawn from certain yeasts.

This review is selective, rather than exhaustive, and aims to raise issues pertinent to metal chelators inside plants. The selection of works reflects this reviewer's perspectives and limitations.

ORGANS AND COMPARTMENTS FOR PARTITIONING METALS

The accumulation of metals by vascular plants is principally from soil, where factors such as pH, redox potential, soil type, cation exchange capacity, metal concentration, competing ions, microbial flora, and mycorrhizal status influence metal bioavailability and absorption by roots. The metal absorbed is partitioned between the root system and the shoots. The degree to which roots retain metal, rather than pass it to the shoots, varies greatly with plant species, the metal, and the concentrations supplied. As an example of variations within a species, of 19 maize inbred lines, 12 were found to be shoot Cd excluders, three had high-shoot Cd concentrations, and four were intermediate between the extremes *(6)*. The genotypic differences in internal distribution were not caused by differences in Cd uptake or the loss of water through transpiration. High retention by roots, with low transfer to the shoots, is desirable in the case of Cd in domesticated plants, where the shoot and grain are foodstuffs. High transfer to the shoot is desirable for phytoremediation of polluted soil, where sequential harvesting of shoots of metal-accumulating plants would lower the metal content in the soil *(7)*. Demands for metal chelation and detoxification within root cells may differ in the two instances.

At the cellular level, the chief compartments are the cell wall, the cytosol, and the vacuoles. Plant cell walls are a continuous matrix, which acts as a cation exchanger, holding variable quantities of metal and providing for some metal exclusion. In roots of maize approx 4–7% of the tissue Cd was associated with cell walls; for the grass *Agrostis gigantea*,

it was 11–15% *(8)*. In Al-tolerant plants, a growing body of evidence shows that Al stimulates secretion of phosphate *(9)*, organic acids *(9,10)*, and mucilage *(11)* from root apices. These ligands complex Al, resulting in diminished absorption by the cells. Comparable evidence is not available for the borderline and class B metals considered here. Relatively little attention has been given to the role of the plasma membrane in minimizing the intracellular load of metal, through control of uptake or of stimulated efflux. The fact that metals accumulate in roots and shoots could mask differences in plasma membrane integrity and function, which may occur between metal tolerant and nontolerant ecotypes (*see* ref. *12* for a review). Examples of different influx systems, and of effective efflux, to account for tolerance of arensic (As), Cd, cobalt, and Ni, are known for bacteria, cyanobacteria, and an alga. The ability to maintain plasma membrane integrity is a specific feature of Cu-tolerant ecotypes of *Agrostis capillaris, Mimulus guttatus* and *Silene vulgaris (= cucubalus) (12)*. An important aspect is that the plasma membrane, the first site of contact of root cells with metals that permeate the wall, will not be protected by the intracellular chelators considered here.

This review is restricted to the intracellular chelators in the cytosol, the vacuoles, and the long-distance conducting cells of the vascular system. The possibility exists that metal chelators vary in these different compartments. In the meristematic cells of root apices, the cytosol occupies a large portion of the cell interior, with vacuoles occupying 1.6–5.6% of the tissue volume *(13)*. In these cells, it might be anticipated that cytosolic chelators would predominate in metal detoxification, rather than those in vacuoles. However, the majority of cells in root systems are mature cells, in which the cytosol occupies a small proportion of the cell volume, and the vacuoles the major part. In onion root cortical parenchyma cells, the cytoplasm occupies 8.2% and the vacuoles 78% of the root volume; the remainder is occupied by cell walls and intercellular spaces *(14)*. In these cells, it might be anticipated that vacuolar chelators would predominate in metal detoxification over those in the cytosol. Such differences in cellular compartments become particularly important in comparisons between metal-tolerant and nontolerant ecotypes. Metal tolerance is usually based on root elongation growth *(3,4)*, which is a result of activities in meristematic cells and their progeny during enlargement. However, identification and measurements of chelators are mostly for entire root systems, where mature cells predominate. The influence of the metal status in mature cells on meristematic cells, and vice versa, is poorly understood.

ORGANIC ACIDS AND AMINO ACIDS

Differences in concentrations of organic acids in leaves of various ecotypes of metal-tolerant plants in their natural habitat fostered consideration of these substances as cellular chelators. Higher concentrations of malic acid correlated with tolerance of Zn, but not of Cu or Ni *(15)*. Citric and oxalic acid contents did not correlate with Zn tolerance. In controlled experiments with various ecotypes of *S. vulgaris*, malic acid contents in leaves were elevated 4–7-fold in Zn-tolerant plants, compared to nontolerant plants, even when treated with excess Zn *(16)*. Oxalic acid levels were generally 10× higher than malic acid in all ecotypes, and remained unchanged with Zn treatment. Roots were not analyzed extensively, but both acids were at lower concentrations than in leaves, and oxalic acid again was more abundant. Mathys *(16)* proposed that malate chelated Zn in the cytosol and the complex was moved into the vacuole, where the uniformly abundant oxalate chelated the Zn, to free malate for return to the cytosol. In this early study, the reaction of roots to excess Zn, and the concentrations of Zn and citric acid in either roots or leaves, were not reported. The distribution of organic acids between the cytosol and vacuoles was not addressed.

In roots of the grass *Deschampsia caespitosa*, citric acid was in 5–10-fold excess over malic acid, and citric acid concentrations increased when Zn-tolerant ecotypes were exposed to

excess Zn *(17,18)*. In the sap expressed from roots (a fluid assumed to be mostly vacuolar contents), Zn:citrate molar ratios rose steadily from unity, after 1 d to 1.5–2 after 7 d *(18)*. To assess the nature of the Zn complex, these workers chromatographed root sap in a background of 2 m*M* Zn, and found two Zn peaks: a Zn–citrate–malate peak, with Zn:citrate near unity; and free Zn. Both groups concluded that organic acids could not complex all the Zn in the tissues, and that accumulation of citrate in itself could not confer the observed Zn tolerance.

The role of organic acids as ligands for Cd and Zn can be assessed by simulating the chemical thermodynamic state of the vacuole *(19,20)*. Vacuolar contents of potassium (K), magnesium (Mg), calcium (Ca), Cd, Zn, nitrate, chloride, sulfate, phosphate, malic, citric and oxalic acids, and Zn- and -Cd-binding peptides were estimated for tobacco leaf suspension cells *(21)*. Exposures to 45 μ*M* Cd, and to 300 or 2000 μ*M* Zn, were for only 4 h. In these situations, Cd induced a small amount of Cd-binding peptide (*see* Peptide Families), but Zn did not. Vacuolar speciation was simulated from pH 4.0 through 7.0 with the GEOCHEM-PC (Department of Soil and Environmental Science, University of California, Riverside, CA [21*a*]) computer model; the most likely pH in vivo was 5.0. In the analysis for Zn *(20)*, the K-, Ca-, Mg-, and Zn-malate species accounted for less than 2.3% of all malate species, irrespective of the moderate or high Zn supplied. The low potential for vacuolar malate to complex Zn was despite a nearly threefold greater concentration of malic acid than citric acid. Unless other stabilizing factors were involved, Wang et al. *(20)* discounted the likelihood that malate would complex Zn in the cytosol and act as its carrier into the vacuole, as proposed by Mathys *(16)*. Citrate dominated the complexation of Zn, compared to malate and oxalate, over the pH range of 4.0 to 7.0, and at both 300 and 2000 μ*M* Zn exposures. At pH 5.0, the dominant complex was predicted to be $Zn(CH_2)_2COH(COO)_3^-$ followed by $Zn(CH_2)_2COH(COO)_3H$. Only approx 7% of the citrate participated in complexing Zn at pH 5.0 for the 300 μ*M* treated cells; another 25% complexed Mg, Ca, and K. The predicted complexation of Zn by citrate supported the earlier interpretation with Zn-tolerant ecotypes of *D. caespitosa (18)*, and led to the speculation that cytosolic citrate was the more likely to be the shuttle for Zn to the vacuole than was malate *(20)*. The calculations showed oxalate to be an effective complexor of Zn, but, because of the low concentration of oxalate and the abundance of Mg and Ca relative to Zn, little of the total Zn was complexed by this acid. In high-oxalate-containing plants, this acid was predicted to play a significant role in Zn complexation, in accord with the hypothesis of Mathys *(16)*.

In plant cells and tissues, Cd induces the formation of specific sulfhydryl-rich peptides (*see* Peptide Families). For protoplasts isolated from leaves of tobacco seedlings grown in the presence of 20 μ*M* Cd for 7 d, all of the Cd and Cd-binding peptide were recovered in isolated vacuoles *(22)*. After exposure to 45 μ*M* Cd for 4 h, about 10% of the Cd in tobacco suspension cells was present as Cd-binding peptide *(21)*. Simulation of Cd speciation in such vacuoles showed that, at pH 5.0, the 17 m*M* malate, 0.5 m*M* oxalate, and 6 m*M* citrate complexed about 15, 3, and 60% of the total Cd, respectively; Cd-peptide bound 10%, and 6% was free Cd *(19)*. A rise in pH would enhance the proportion of Cd complexed by citrate, and decrease the influence of malate and oxalate. The dominant citrate complex at pH 5.0, the likely pH in vivo, was predicted to be $Cd(CH_2)_2COH(COO)_3^-$ followed by a small proportion of $Cd(CH_2)_2COH(COO)_3H$. Cd-binding peptides were calculated to have a high potential for forming soluble complexes in the vacuolar environment. At high concentrations of peptides, saturation of the ligands was predicted; at low peptide concentrations, citrate would effectively compete for Cd *(19)*. The amount of Cd-binding peptide in any tissue can be highly variable, because induction of Cd-binding peptides depends on the time of exposure, the concentration of Cd used, and the organ in a plant (*see* Peptide Induction by Metals). Wagner et al. *(5,19)* have argued consistently that, at the low levels of Cd found in soils used for agricultural production, where little

or no Cd-peptides would be induced, vacuolar citrate would effectively complex the cellular Cd. Plant growth at high Cd concentrations would induce Cd-binding peptides that would effectively complex high vacuolar Cd. These two speciation studies illustrate the conditions and preferences of metals for chelators. Zn and Cd both seek oxygen centers, particularly of citric acid; however, when sulfhydryl-rich peptides are available, Cd seeks the sulfur centers in these molecules. Applying the simulation technique to other metals, plant systems and roots, under various growth conditions, would clarify the importance and function of the various metal chelators produced by plants.

Mono- and bivalent ions are moved through plants in the relatively dilute xylem sap within dead xylem tracheids and vessels, and as the more concentrated phloem sap within phloem sieve tube members. Stable complexes found in these saps would prevent precipitation of metals from solution or adsorption of metals to cell walls. Mullins et al. *(23)* used the GEOCHEM computer model to predict metal speciation in xylem sap of soybean and tomato, and in phloem sap of *Yucca*. Those authors cautioned on the limitations of the predictions, because monovalent ion contents were not available, and the prominent ureides of soybeans, allantoin, and allantoic acid were not measured, nor were their stability constants known. For the purposes of this review, the predictions for Cu and Zn have been singled out, and Cd and Ni were not considered as constituents of the saps. Free Cu^{2+} was less than 1% of the total Cu in all soybean and tomato xylem saps. At normal Zn supply, asparagine (Asn), histidine (His), and citrate were predicted as the major ligands for Cu. Increased Zn supply favored more Cu-His at the expense of Cu-Asn, with Cu-citrate unchanged. Appreciable free Zn^{2+} was predicted; 17% at 0.5 μ*M* and 28% at 8 μ*M* supply. The organic acids citrate and malate were the ligands predicted to bind Zn to different degrees, at both normal and high Zn supply. For tomato, Cu-His and Cu-citrate predominated at normal Zn supply, but their prevalence was reversed at 80 μ*M* Zn supply. Considerable free Zn^{2+} was predicted for the tomato xylem sap (42 and 37% at normal and high Zn supply, respectively).

The ligands, malate and citrate, accounted for much of the remaining Zn under both Zn supply regimes *(23)*. Where these putative complexes are formed is unclear. They could arise in the living parenchyma cells, and perhaps be transported as such, across the plasma membrane of the xylem parenchyma, into the adjacent dead xylem tracheids and vessels, or the ligands and metals could be moved separately across the plasma membrane of the xylem parenchyma, into the dead xylem tracheids and vessels, where the complexes form in the xylem sap. The phloem sap of *Yucca* was predicted to contain essentially no free Cu^{2+} or Zn^{2+} with the amino acids, aspartate, glutamine, and tyrosine, being the principal ligands. About one-half of the oxalate present in the phloem sap was predicted to be the free ion, with the remainder being the ligand for Ca, Mg, and Fe *(23)*. Cd K-absorption near-edge spectra of xylem sap from *Brassica juncea* plants, exposed to 5 μ*M* Cd for 10 h or 7 d, showed interactions with O or N ligands, with a bond length of 2.3 Å *(24)*. Although the Cd ligand was not identified, it clearly differed from the S ligand found in the roots of the same plants (*see* Cd-Binding Complexes). These examples illustrate the variety of potential metal ligand species in plant saps, and that they can change with available nutrients. The composition of mineral ions in the saps feeding developing seeds and fruits is virtually unknown. This is an important aspect in any endeavors to limit metal entry into the food chain of animals and man.

Hyperaccumulators are those plants with metal concentrations exceeding 0.1% in their dry shoot biomass. Of some 400 hyperaccumulators, about 75% hyperaccumulate Ni. The leaf contents of citric acid correlate with increasing levels of Ni in 18 species, including Ni hyperaccumulators *(25)*. The anionic citrate-nickelate(II) complex was extractable from leaves, but $Ni(aquo)^{2+}$ was present as the

major cationic constituent *(26)*. For two species of Ni hyperaccumulators, approx 80% of the Ni occurred as the citrate and malate complexes; five other species had only the Ni-citrate complex; and the cationic aquo complex was the remaining form *(27)*. In previous studies, the form of Ni was determined by making aqueous extracts of leaves, and separating complexes by gel filtration or electrophoresis. Analysis of latex, from the Ni hyperaccumulating tree *Sebertia acuminata*, does not require aqueous extraction, only dilution. The latex contained Ni-citrate complex, citrate being confirmed by mass spectrometry, enzymatic degradation, and by ^{13}C NMR *(26,28)*. Nickel citrate complex accounted for approx 37% of the Ni in the latex, with nitrate being a likely counterion *(28)*. No metal-binding sulfhydryl-rich peptides (*see* Peptide Families) were detected in the latex. Energy-dispersive X-ray analysis of cryofixed stems showed the prominent localization of Ni in the laticifers within the bark *(28)*. Comparisons between Ni-hyperaccumulating and -non-hyperaccumulating species of *Alyssum* showed that, for a wide range of exposures, Ni elicited a large and proportional increase in His in the xylem sap *(29)*. The speciation of Ni in the xylem sap, using GEOCHEM-PC and a limited number of ions and ligands, indicated that Ni was chelated mostly by His (19%), glutamine (15%), citrate (9%), and malate (3%), with up to 48% predicted to be anionic Ni hydrate *(29)*. The extended X-ray absorption fine structure (EXAFS) spectrum of xylem sap was best fitted by octahedral coordination of Ni, with an imidazole N of His at 1.98 Å, and five light atoms at 2.05 Å. Almost identical spectra were obtained for whole leaves and roots from the same plants in which His levels were high because of Ni exposure. The in vivo spectra gave no indication for Ni coordination by S, thus excluding involvement of sulfhydryl-rich peptides (*see* Peptide Families) in chelating Ni.

PHYTIN

Protein bodies in mature seeds are special vacuoles in which phosphorus (P), Mg, K, Ca, and other mineral nutrients are stored *(30)*. The chief storage form is phytin or phytate, a mixed salt of myoinositol hexaphosphoric acid or phytic acid. Phytate may be dispersed throughout the protein matrix, or localized into dense aggregates called globoids. Energy-dispersive X-ray analysis (EDXA) of globoids generally show P, Mg, and K *(30)*, and, on occasion, other elements, such as Ca, manganese (Mn), Zn, barium (Ba), and Fe *(31)*. The elements found in smaller amounts often occur in protein bodies of certain cells, depending on the species *(30)*. The EDXA itself cannot identify the actual compounds involved in storing the minerals. Through extraction, the Zn-binding entity in fava bean cotyledons was found to be Zn phytate *(32)*.

The presence of Zn phytate in vegetative tissues was evaluated by Van Steveninck, using EDXA *(33–37)*. Analysis of globular bodies up to 1 μm in diameter, within small vacuoles in freeze-substituted young roots, showed the presence of Zn, K, Mg, and P *(33)*. Comparison to standard compounds suggested the presence of Zn phytate. The globules occurred mostly in cortical cells in the elongating zone of the root tips. A Zn-tolerant ecotype of *D. caespitosa*, grown on 1000 μ*M* Zn, produced larger and more frequent globules, with nearly three atoms of Zn per phytate, than did a nontolerant ecotype exposed to 100 μ*M* Zn. Using a quench freezing procedure, and examination of fracture faces, showed that hydrated leaf cells of *Lemna minor* fronds also had globular deposits of Zn, K, and P after growth on 300 μ*M* Zn *(34)*. The globular deposits in mature fronds exposed to 30 μ*M* Cd contained Cd, K, and P, but, in the immature cells, sheet-like deposits occurred, with Cd and S as the principal constituents *(35)*. For a number of crop species, but not all, the typical globular Zn, Mg, Ca, and P deposits were most common in cells of the endodermis and pericycle of root tips *(36,37)*. Exposure of the same species to Cd, or to Zn and Cd, did not induce Cd-containing globular deposits *(37)*. There is a clear need to isolate and characterize the putative Zn and Cd phytate from mature and immature vegetative

tissues, and to evaluate phytin as a ligand of other metals. Quantitative measurements of the Zn complexed with phytin are required, to assess the impact of this chelator on the overall speciation of Zn in roots and leaves of plants exposed to a range of Zn concentrations, and to evaluate this chelator's relevance to metal tolerance.

METALLOTHIONEINS

Metallothioneins (MTs) are a group of Cys-rich molecules, which, in their reduced state, provide thiols for metal chelation. Since their discovery in equine kidney, MTs have been found broadly distributed among animals, eukaryotic microorganisms, certain prokaryotes, and plants. The wealth of information, particularly on animal and microbial MTs, is available in one of several reviews *(38)*, and from the latest in a series of three international conferences *(39)*. A variety of methods appear in a dedicated volume of *Methods in Enzymology (40)*, and several reviews focus on plants *(41–45)*.

Mammalian MTs contain 20 Cys among a total of 61 amino acids, and bind a maximum of seven equivalents of bivalent metal. The metal ions are coordinated through mercaptide bonds, in arrangements typical of metal–thiolate clusters *(46)*. A variety of techniques established that the archetypal MT had a two-domain structure: a $metal_3Cys_9$ cluster in the amino terminal region, and a $metal_4Cys_{11}$ cluster in the carboxy-terminal region. The two domains were linked by 2–4 amino acids. Based on structural relationships, MTs are subdivided into three classes. MT class I (MT-I) are those molecules in which the Cys alignment along the chain is similar to the invariant mammalian MTs. The Cys occur as Cys-Cys, Cys-x-Cys, and Cys-xx-Cys, where x stands for any amino acid other than Cys. MT-Is occur in a variety of mammals, certain fish, crabs, oysters, and mussels *(46)*. The MT-IIs are those in which the position of the Cys residues lack obvious homology to the archetypal mammalian MT, and among each other. They are found in *Drosophila*, sea urchins, a nematode, fungi, cyanobacteria, and plants. A MT-II protein has been isolated from wheat embryos; 58 genes from a variety of plants predict MT-like proteins, of which only six are partly characterized. The MT-IIIs are atypical, nontranslationally synthesized polypeptides based on repeating units of γ-glutamylcysteine (γ-Glu-Cys). MT-IIIs are widely distributed in the plant kingdom, including algae and certain fungi.

MT-IIs in Plants

An early Cys-labeled (E_c) protein, encoded by mRNA conserved in mature wheat embryos *(47)*, was isolated in an alkylated form, and the first 59 amino acids sequenced *(48,49)*. This region contained four pairs of Cys-x-Cys and two lone Cys *(48)*, but the positions of the Cys did not match those of MT-Is. The unalkylated E_c protein bound Zn at approx 5 mol/mol protein *(49)*. Displacement of Zn by Cd created the characteristic Cd-mercaptide spectrum, as though the Zn had been bound similarly. The E_c protein is the first MT-II protein characterized for plants. Three genes for the E_c protein from wheat and one from maize are known (Table 1; *50,51*). The cDNA sequences predict proteins with 17 Cys arranged into three groups of 6, 6, and 5 Cys, with 12–15 amino acids separating the three groups (Table 1). The partially sequenced E_c protein *(48)* essentially corresponds to the first group, and most of the second group of Cys predicted from the cDNA. The predicted arrangement of Cys in the full-length sequence is not homologous with MT-Is. The wheat E_c protein bound approx 5% of the total Zn in the embryo, indicating an unlikely role for the protein in detoxification *(50)*. It was suggested that E_c protein was involved in Zn^{2+} homeostasis, such as interaction with Zn-dependent DNA and RNA polymerases and *trans*-acting Zn-finger proteins. Such functions would be accentuated during embryogenesis *(50,52)*. The E_c protein declined after germination, as did the mRNA for the third gene (Table 1; *50*). The upregulation of E_c transcript by abscisic

Table 1
MT-II Genes in Plants: Predicted Number of Cysteine Motifs, Distribution, and Regulation of the E_c Type

		Cys motif		Transcript abundance				
Plant	Gene	CxC	Other	Root	Leaf	Seed	Regulation	Ref.
Type E_c								
Wheat E_c	cDNA I	7	3 lone C	–	–	Embryo		*(50)*
	cDNA II	7	3 lone C	–	–	Embryo		*(50)*
	Sequence corroborated						Microspore embryogenesis	*(52)*
	cDNA III	7	3 lone C	–	–	Embryo	Not by Zn, up by abscisic acid, disappears on imbibition	*(50)*
Maize		7	3 lone C	–	none	Embryo	Up by abscisic acid, osmoticum	*(51)*
	Arrangement of Cys						Cases	*(50,51)*
							4	
E_cMT	2xCxxxCxCxxxCxxxx(x)CxC 12,14 CxCxxxCxCxxCxC 15 CxCxxxCxCxxC5x							*(50,51)*

The number and type of cysteine motifs are shown, with x denoting any amino acid other than cysteine. In the summarizing arrangement of Cys, the numbers denote larger stretches of Cys-deficient regions, an (x) indicates a deletion in one case.

acid and osmoticum (Table 1; *50,51*), during embryogenesis, may be related to a sequence similar to the abscisic-acid-responsive element in other plant genes, which occurs on the 5′ flank of the E_c MT gene *(50)*. No Zn-responsive element was evident; indeed, Zn treatment of wheat did not induce transcripts for E_c mRNA.

The first MT-like gene in plants was described in 1990 for a Cu-tolerant ecotype of *M. guttatus (53)*. In their 1993 review, Robinson et al. *(43)* considered nine plant MT-like genes. That list has grown to 58 genes from a range of plants and tissues (Table 2). The genes are discussed here in terms of the predicted amino acid sequences, but it is emphasized that, by early 1998, only one group had published amino acid sequences of nine tryptic acid fragments from six *MT* genes in *Arabidopsis* (see below).

The plant *MT*-like genes predict that the Cys are grouped into two domains, as is the case with MT-Is, but unlike the three domains found in the E_c MT-IIs (Table 1). One major distinction, however, is that, in most plant MT-IIs, 30–45 amino acids link the two Cys-rich domains (Table 2); 2–4 amino acids do so in MT-Is. The lowest numbers in the linker region, 7 and 19, are found among members of the Brassicaceae family. Based on the predicted location of Cys residues, Robinson et al. *(43)* proposed two categories of MT-like proteins. Type 1 contained Cys-x-Cys motifs exclusively, and type 2 contained a Cys-Cys and a Cys-x-x-Cys pair in the N-terminal domain 1. This grouping has been maintained and extended in Tables 2 and 3. Of the 14 type 1 genes characterized with four independent corroborations (Table 2), the three belonging to the family Brassicaceae stand out as a subtype, because only seven amino acids link domains 1 and 2, and a seventh Cys is predicted in domain 2 (Table 3). The 27 genes of type 2 class MT-IIs (Table 2) uniformly predict a cluster of eight Cys in domain 1 and six Cys in domain 2 (Table 3). The type 3 genes, recently described for rice, share an identical Cys arrangement in domain 1 with the type 2 genes, but the Cys in domain 2 have been increased to 9 (Tables 2 and 3). The type 4 genes

from a variety of fruit and rice (Table 2) have a truncated domain 1, with four Cys, and a domain 2 with Cys arranged as in types 1 and 2, subtype Brassicaceae excluded (Table 3).

Genes that do not fit any of these patterns are grouped under others (Tables 2 and 3). The *Arabidopsis MT1b* sequence in domain 1 is like that of the subtype Brassicaceae, but domain 2 is truncated by three Cys. This gene was deemed inactive *(62)*. The *MT3* gene from *Arabidopsis* has a unique domain 1, and a domain 2 much like that of the subtype Brassicaceae, except for an extra Cys. The tomato gene predicts a unique arrangement of six Cys in domain 1, with domain 2 much like that of the subtype Brassicaceae. The *Brassica campestris* sequence has the same Cys arrangement as the type 2 genes, except that domain 1 has one less Cys. The gene from strawberry has a domain 1 like the type 2 genes, and domain 2 like the subtype Brassicaceae. Domain 1 in the banana clone pBAN 3-23 resembles the type 2 genes, except for eight amino acids separating the first two Cys, domain 2 is like that of type 1 or 2 genes (Table 3). Douglas fir, the only nonangiospermous plant reported, has a MT-II gene, with a unique arrangement of three and two Cys in domains 1 and 2, respectively (Tables 2 and 3).

Limited attention has been given to the isolation of the protein products predicted for the MT-II genes listed in Table 2. An initial success was achieved for six proteins from *Arabidopsis (96)*. The 4.5 kDa MT1 protein, digested with trypsin, gave a 16-amino-acid sequence specific to *MT1a*, which corresponded to the six penultimate Cys predicted for domain 2 (type 1 subtype Brassicaceae, Table 3). Two sequences of three and four amino acids corresponded to common regions of *MT1b* and *MT1c*. A 16-amino-acid sequence represented about one-third of the 47 residues predicted for *MT2a* to link domains 1 and 2, and included the first Cys of domain 2 (type 2, Table 3). Of two shorter fragments, one corresponded to three of the six Cys predicted for domain 2 by *MT2b*, the other was part of the linker region predicted for the same gene. For the 7 kDa MT3 protein, an eight-amino-acid fragment corresponded to the beginning of domain 1, and a five-amino-acid sequence was part of the linker region close to domain 2. A 17-amino-acid fragment corresponded to most of domain 2, including the C-terminal Asn and seven of the eight Cys predicted from *MT3 (96)*.

Transcripts of MT-IIs are detected in roots, stems, leaves, flowers, fruits, and seeds, and become evident under different conditions (Table 2). When expressed in leaves, transcript levels increased with leaf senescence *(64,65,77,85)*. In kiwi fruit, specific transcripts increased early or late during fruit development *(74)*. In banana, one transcript increased during fruit ripening, but another decreased *(94)*. Transcripts of type 4 MT-II in apple increased during cold storage *(92)*. Transcription of both MT-II genes in shoot apices of *B. campestris* increased during vernalization *(81)*. The function of such MT-II gene products in stems, leaves, flowers, fruits, and seeds remains unclear. The MT could scavenge metal ions released from protein degradation during leaf senescence, or the Cys-rich proteins would protect DNA from oxidative damage caused by free radicals *(85)*.

In nine cases, the type 1 MT-II genes were highly expressed in roots (*see* Table 3; *Mimulus*, pea, barley, maize, wheat, *Arabidopsis MT1a*, rice, cotton, fava bean); six type 2 genes were highly expressed in shoot tissues (soybean, *Arabidopsis MT2a*, fava bean, rice, tomato). Transcription of type 2 genes in leaves was enhanced by Cu *(61,62,79)*, was not affected by metal *(73)*, or was downregulated by Cu *(70,82)*. In roots, type 1 transcripts were increased by Fe deficiency *(55)*; Al, not Cd *(59)*; particularly by Cu, and less so by Zn and Cd *(61,62,65)*; not changed by metal *(68)*; or downregulated by Cu, Zn, and Cd in Cu-tolerant *M. guttatus (53)* (Table 2). Metal regulatory elements were sought in the promoter region of the *Arabidopsis MT1* and *MT2* genes without success *(62)*. Putative metal and ethylene regulatory elements were found in the 5′-flanking region of $LeMT_B$ *(84)*, a type 2 MT gene in leaves. Regulation of this gene by metals was not examined. The product of the

Table 2
MT-II Genes in Plants: Predicted Number of Cys Motifs, Distribution, and Regulation

Plant	Gene	Cys motif			Residues between domains	Transcript abundance				Regulation	Ref.
		CxC	CxxC	Other		Root	Leaf	Seed	Other		
Type 1											
Mimulus		6	0	–	39	High	Low	–	–	Down by Cu, Zn, Cd	*(53)*
Pea	$PsMT_A$	6	0	–	42	High	Low	–	–	–	*(54)*
Barley	*ids-1*	6	0	–	43	High	–	–	–	Up with Fe deficiency	*(55)*
		Sequence corroborated									*(56)*
Maize		6	0	–	45	High	Low	Low	Pith	–	*(57)*
		Sequence corroborated				Yes	–	–	–	Up with root tip excision, Up with glucose starvation	*(58)*
Wheat	*wali1*	6	0	–	44	High	High	–	–	Up by Al in roots, not by Cd, constitutively high in leaves	*(59)*
White clover		6	0	–	42	–	–	–	–	–	*(60)*
Arabidopsis	*MT1/MT1a*	6	0	1 lone C	7	High	Low	–	–	Up by Cu > Zn, Cd	*(61,62)*
		Sequence corroborated as *AtMT-q*									*(63)*
	MT1c	6	0	1 lone C	7	Yes	Yes	–	Siliques	–	*(62)*
Brassica napus		6	0	1 lone C	7	None	High	–	Flower	Up with leaf senescence	*(64)*
Rice	*OsMT-1*	6	0	–	43	High	Some	–	Cultured cells	Up by Cu, heat stress, sucrose starvation, leaf senescence, down with ABA	*(65)*
		Sequence corroborated									*(66)*
Cotton	*MT1-A*	6	0	–	42	High	Low/nil	–	–	Evidence for two further *MT1* genes	*(67)*
Fava bean	*MT1a*	6	0	–	45	High	High	–	Stem	Not by Cu, Cd, Zn, Fe	*(68)*
	MT1b	6	0	–	43	–	–	–	–	–	*(68)*
Red fescue		6	0	–	39	–	–	–	–	–	*(69)*
Type 2											
Soybean		5	1	1 CC	41	Low	High	–	–	Down by Cu	*(70)*
Arabidopsis		5	1	1 CC	43	–	–	–	–	–	*(71)*
	MT2/MT2a	Sequence corroborated				Low	High	–	–	Up by Cu > Zn, Cd	*(61,62)*
		Sequence corroborated as *AtMT-k*									*(63)*
	MT2b	5	1	1 CC	39	Yes	Yes	–	Siliques	Slightly up by Cu	*(62)*
Castor bean		5	1	1 CC	42	–	–	–	–	–	*(72)*
Fava bean		5	1	1 CC	39	Least	High	–	Flower Trichomes	Down by cold, salt, salicylic acid not by Cu, Zn, Cd	*(73)*
Kiwi fruit	*pKIWI504*	5	1	1 CC	40	None	–	–	Fruit	Up early in fruit development	*(74)*

Coffee		5	1	1 CC	42	–	From young leaves			–	*(75)*
Chinese cabbage		5	1	1 CC	42	–	From the inflorescence			–	*(76)*
Sambucus		5	1	1 CC	39	–	Yes		Abscission zone	Up with ethylene, leaflet senescence	*(72)*
Tobacco		5	1	1 CC	37	–	–	–	–	Modulated by cytokinin	*(78)*
		5	1	1 CC	42	–	Yes	–	–	Up with Cu, virus, wounding	*(79)*
White clover		5	1	1 CC	39	–	–	–	–	–	*(80)*
Brassica campestris		5	1	1 CC	42	–	–	–	Shoot apex	Up with vernalization	*(81)*
Rice	*OsMT-2*	5	1	1 CC	43	Low	High		Cultured cells	Up by heat shock, sucrose starvation Down by Cu, Cd	*(82)*
Tomato (three sequences)		5	1	1 CC	35,36,43	Yes	–	–	–	–	*(83)*
	$LeMT_A$	5	1	1 CC	35	Low	High	–	–	–	*(84)*
	$LeMT_B$	5	1	1 CC	43	Low	High	–	–	–	*(84)*
Brassica napus *Brassica juncea* (5 sequences)		5	1	1 CC	19	–	Yes	–	–	Up with senescence	*(85)*
		5	1	1 CC	42,43	Yes	–	–	–	–	*(86)*
Apricot		5	1	1 CC	40	–	–	–	Fruit	–	*(87)*
Common ice plant		5	1	1 CC	42	–	–	–	–	Confers Cu tolerance	*(88)*
Type 3											
Rice		4	2	2 CC 1 lone C	40	–	–	–	–	–	(89)
		5	1	2 CC 1 lone C	37	–	–	–	Stem	–	*(90)*
		5	1	2 CC 1 lone C	37	–	–	–	–	–	*(91)*
Type 4											
Kiwi fruit	clone 503	4	0	2 lone C	32	None	–	–	Fruit	Up late in fruit development	*(74)*
Apple		4	0	2 lone C	34	–	–	–	–	Up with cold storage	*(92)*
Papaya		4	0	2 lone C	33	–	–	–	–	–	*(93)*
Banana	clone 3-6	4	0	2 lone C	34	No	Yes	–	Fruit	Up with fruit ripening	*(94)*
Rice (two sequences)		4	0	2 lone C	30,33	–	–	–	–	–	*(95)*
Sweet cherry		4	0	2 lone C	34	–	–	–	Fruit	–	*(95a)*
Others											
Arabidopsis	*MT1b*	4	0	2 lone C	7	–	–	–	–	Inactive gene	*(62)*
Arabidopsis	*MT3*	4	0	4 lone C	34	–	–	–	Seedlings	Up by Cu	*(96)*
Tomato		5	–	3 lone C	40	Yes	–	–	–	–	*(97)*
Brassica campestris		4	1	1 CC 1 lone C	42	–	–	–	Shoot apex	Up with vernalization	*(81)*
Douglas fir	*PM 2.1*	1	0	3 lone C	38	–	–	Low	–	Maximal at midembryogenesis Up by osmoticum, ABA, Zn	*(98)*
Strawberry	*FMET1*	5	1	1 CC 1 lone C	40	–	–	–	Fruit	–	*(99)*
Banana	clone 3–23	5	1	2 lone C	41	No	Low	–	Fruit, corm	Down with fruit ripening	*(94)*

Number and type of cysteine motifs are shown with x denoting any amino acid other than Cys. The Cys-rich domains 1 and 2 are separated by a Cys-deficient region varying in the number of amino acid residues. The listing for a type of Cys motif is chronological.

Table 3
Arrangement of Cys in Amino- and Carboxy-terminal Domains Predicted for Plant MT-like Genes

	Domain 1	Linker region	Domain 2	Number of cysteines	Cases	First ref.
Type 1	2-4xCxCxxxCxCxxxCxC	----------	CxCxxxCxCxxCxC0-2x	6 + 6	11	*(53)*
Subtype *Brassicaceae*						
	5xCxCxxxCxCxxxCxC	----------	CxxCxCxxxCxCxx(x)CxC	6 + 7	3	*(61)*
Type 2	xxCCxxxCxCxxxCxCxx(x)CxxC	----------	CxCxxxCxCxxCxC0-2x	8 + 6	27	*(70)*
Type 3	xxCCxxxCxCxxxCxCxxxCxxC	----------	CxCxxCxCxxxCx(x)CxCCxCx	8 + 9	3	*(89)*
Type 4	2-4xCxxCxCxxxxxC	----------	CxCxxxCxCxxCxC0-2x	4 + 6	7	*(74)*
Others						
Arabidopsis MT1b	11xCxCxxxCxCxxxCxC	----------	CxxCxCxxCxx	6 + 4	1	*(62)*
MT3	4xCxxCxxCxxxxxC	----------	CxCxxCxxxCxCxxCxCC2x	4 + 8	1	*(96)*
Tomato	3xCxxxCxCxxxCxxxxxC	----------	CxCxxxCxCxxCxCC7x	6 + 7	1	*(97)*
B. campestris	xxCCxxxCxCxxxCxxxxxCxxC	----------	CxCxxxCxCxxCxCxx	7 + 6	1	*(81)*
Douglas fir	7xCxCxxxxxC	----------	CxxxxxxxxxxxCx	3 + 2	1	*(98)*
Strawberry	xxCCxxxCxCxxxCxCxxxCxxC	----------	CxxCxCxxCxCxxCxCx	8 + 7	1	*(99)*
Banana clone 3-23	3xC8xCxxxxCxCxxxCxCxxxCxxC	----------	CxCxxxCxCxxCxCx	8 + 6	1	*(94)*

Amino-terminus is in domain 1, the carboxy-terminus in domain 2, the linker region between the domains is a variable number of amino acids. The x denotes any amino acid other than Cys, an (x) indicates a deletion in one case, the numbers at the amino- and carboxy-termini indicate the range known.

auxin-regulated gene *parA* is localized primarily in the nucleus, where it may regulate transcription of certain genes. Of all the plant hormones, only auxin caused expression of *parA*, as did Cd *(100)*. The *parA* promoter contained the *cis*-acting, Cd-responsive sequence *pas*, which shared DNA-binding proteins with the nuclear factor AFS-1. The *pas* sequence responded to Cd, but not to Cu. Whether this Cd-responsive sequence, or homologs to other metals, participates in plant MT regulation, is open to investigation.

The anticipated function of metal binding by the variety of gene products described in Table 2, as demonstrated for wheat E_c protein *(49)* and other MT-Is and -IIs *(38,46)*, has received some attention. One difficulty has been the isolation of nondegraded native proteins that may be in low abundance, particularly when plants react to exogenous metal by producing MT-IIIs (*see* Peptide Families). An elaborate protocol was used to isolate and purify the low-abundance MT1, MT2, and MT3 proteins from *Arabidopsis (96)*. The native proteins were freed of Cu, to allow their purification through Cu- and thiol-affinity chromatography. Incubation of the purified apoproteins with Cu produced Cu:protein ratios of 8.4, 7.3, and 5.5 for MT1, MT2, and MT3, respectively *(96)*. How closely these ratios reflect the situation in vivo remains unclear.

An alternative approach has been to express a plant MT gene in *Escherichia coli* or MT-deficient yeast or cyanobacterium. The type 1 gene $PsMT_A$, from pea, was coupled to a glutathione-S-transferase (GST) fusion-protein expression vector, and inserted into *E. coli (101)*. The $PsMT_A$-GST fusion protein, isolated from cells grown with Zn, Cd, or Cu, contained more of the respective metal than did the GST protein alone. The pH at which 50% of the metal was dissociated from the fusion protein was estimated at 5.35, 3.95, and 1.45 for Zn, Cd, and Cu, respectively. *E. coli* cells expressing the $PsMT_A$ protein accumulated about 8× more Cu than did transformed cells without the plant gene; Zn and Cd were not accumulated *(102)*. The type 1 *MT1a* and *MT1b* and type 2 *MT* genes from fava bean were also expressed as the GST-fusion protein in *E. coli (68)*. The cells exposed to a mixture of Cu, Cd, Fe, and Zn produced fusion protein that bound Cu and Cd. Only when cells were exposed to Zn alone was Zn bound. Fe was not bound. These results, and the fact that Zn is released at a higher pH than are Cu or Cd *(101)*, suggest that plant class MT-II ligands coordinate Zn more weakly than Cu or Cd. The *MT1* and *MT2* genes of *Arabidopsis* have been expressed in MT-deficient mutants of yeast and *Synechococus (61,103)*. In both cases, the plant genes complemented the mutations, so that the cells could resist concentrations of Cu *(61)* and Zn *(103)* that were otherwise harmful.

Using an independent isolate of $PsMT_A$, Kille et al. *(104)* inserted the cDNA into *E. coli* via a heat-inducible expression vector. Only transformed cells expressing $PsMT_A$-accumulated Cd. Cd-binding protein from the transformed cells was separated into three overlapping regions by anion exchange chromatography. Each pool had an amino acid composition comparable to that predicted for the $PsMT_A$ gene product. The pools contained 5.6, 5.8, and 6.1 g atoms Cd/mol protein; Zn and Cu were absent. Material in pools 1 and 2, which was digested separately with proteinase K and acidified to pH 2.0, to dissociate metal, resulted in the same two residual peptides. The amino acid composition of both peptides matched that predicted for domains 1 and 2, plus the first three and the last five amino acids of the linker region of $PsMT_A$. In this case, the act of Cd-binding protected the local regions of the protein chain from proteolytic attack, a well-known feature for a wide range of MTs *(105)*. The favored interpretation was that the Cys-rich domains 1 and 2 were folded together to participate in binding Cd. Whether this is a Cd_6Cys_{12} cluster warrants a detailed structural investigation. The long linker region was vulnerable to endogenous proteolysis, thus forming the three pools of apparently identical Cd-binding protein *(104)*. Because most of the genes in Table 2 predict products with 30–45 amino acids linking the

two Cys-rich domains, these products would be vulnerable to endogenous plant proteinases. Kille et al. *(104)* suggested that this was one explanation for the difficulties encountered in detecting MT-IIs in plant cell extracts in which complete proteins were expected otherwise. It would seem that those plants with the shortest predicted linker region, those from the subtype Brassicaceae (Table 2), would be least vulnerable to proteolytic attack. This conjecture is supported by the fact that proteinase K digestion of the linker region of GST-PsMT$_a$ fusion protein was incomplete, leaving three and five amino acids adjacent to the terminal and beginning Cys, respectively, of domains 1 and 2 *(104)*. The partial success in characterizing the amino acid sequences of MT1, MT2, and MT3 proteins in *Arabidopsis* (*see* above; *96*) may not be coincidental.

The relationship between MT-IIs and metal tolerance was assessed in *Arabidopsis* (106). Transcription of *MT2a* in seedlings increased in those ecotypes whose root elongation growth was increasingly possible in 40 μ*M* Cu. The correlation was particularly high for MT2a mRNA (r = 0.998) and lower for MT1a mRNA (r = 0.89). The range of root elongation growth in the 10 ecotypes correlated poorly (r = 0.77) with their thiol contents, which represented MT-IIIs (*see* Peptide Families). In the three ecotypes most tolerant of Cu, MT1a mRNA was constitutively present, and not affected when seedlings were exposed to excess Cu, silver (Ag), Cd, Zn, Ni, or Al. These same metals, however, induced the appearance of MT2a mRNA. In this study, the transcripts for MT1a and MT2a were measured in whole seedlings, and not specifically in root tips, which is the tissue directly responsible for the root growth assay of Cu tolerance. Antibodies to the fusion proteins GST-MT1a and GST-MT2a were used to assess the amount of MT1a and MT2a protein in the most Cu-tolerant ecotype *(96)*. Like the MT1a mRNA, the MT1a protein was present equally in control and Cu-treated seedlings. Also, MT2a protein was low in seedlings, and increased with Cu treatment, as was the case for the MT2a mRNA. Both proteins were detected in greater quantity in the root tips, compared to the basal portion of the root system. The type 1 MT genes in maize *(58)* and cotton *(67)* were both highly expressed in root tips. One interpretation of these findings is that the constitutive MT functions in a homeostatic capacity in the embryonic cells abundant in the root apex; the Cu-inducible MT is generated as a metal-protective mechanism. It is unfortunate that the antibodies used by Murphy et al. *(96)* did not work in crude extracts, but required an elaborate purification protocol, not conducive, for example, to comparisons of root tips from ecotypes of varying Cu tolerance.

In the yeast *Saccharomyces cerevisiae*, three soluble proteins participate in the cellular trafficking of Cu to specific sites. These factors, called Cu chaperones, differ from MTs. ATX1 and LYS7 had a single Cys-xx-Cys motif *(106a,106b)*; the gene for COX17 predicted four lone Cys and a Cys-Cys-x-Cys region *(106c)*. It was proposed that these chaperones received Cu from the Cu transporter in the plasma membrane *(106b,106c)*, but whether they could obtain Cu from a storage protein, such as Cu-MT, is unclear. MT-IIs in plants could perhaps serve their putative role in homeostasis as stores of Cu and Zn for metal chaperones, or, analogous to the Cys-rich COX17, the MTs may themselves function as metal chaperones to specific metalloenzymes. Such possibilities remain to be explored.

MT-IIIs in Plants

The MT-IIIs are polypeptides with repeating γ-Glu-Cys units. Common features are that Glu is the N-terminal amino acid, that the next residue is Cys with the peptide bond to the γ-carboxyl of Glu, and that γ-Glu-Cys units are repeated two or more times. The collective designation γ-Glu-Cys peptides is used here. These peptides are atypical, because the γ-carboxyamide bonds in the molecules are not known to be synthesized on ribosomes. No genes specify the primary structures of the γ-Glu-Cys peptides, but, rather, nonribosomal enzyme(s) produce the γ-Glu-Cys pep-

tides. Much of the work on these peptides relates to Cd, with fewer observations for Cu and Zn. The MT-Is occur as individual metal-binding proteins, and, presumably, the same applies to the MT-IIs in plants. In contrast to this, an ill-defined number of heterogeneous γ-Glu-Cys peptides bind Cd, and combine to form Cd-binding complexes.

Peptide Families

Five families of γ-Glu-Cys peptides are known. These groupings are based on the C-terminal amino acid, which can be either glycine (Gly), β-alanine (β-Ala), Cys, serine (Ser), or Glu. In each family, the γ-Glu-Cys pair is repeated 2–7× depending on the organism and medium. Nondefined repeats are denoted by the subscript n. The first MT-IIIs to be characterized were the $(\gamma\text{-Glu-Cys})_n$-Gly family. Cd stimulated their appearance in the fission yeast *Schizosaccharomyces pombe*; the peptides were named cadystins *(107)*. The name "phytochelatin" (PCs) was proposed for the same peptides, independently characterized for cultured cells from four plant species *(108)*. There is no consensus on the trivial name. An unfortunate tendency is emerging to call all types of MT-IIIs "phytochelatins" (PCs) when this name really refers only to the glycyl family of γ-Glu-Cys peptides. PCs occurred in all plants examined, following Cd exposure, ranging from vascular and nonvascular plants to mosses and algae *(109–111)*. They also occurred in plants from natural habitats *(112,113)*, and in an alga in a marine estuary *(114)*. Among yeasts, *S. pombe* responded to Cu and Cd by producing $(\gamma\text{-Glu-Cys})_n$-Gly *(115,116)*; however, *Candida glabrata* produced PCs in response to Cd and MT-IIs, on exposure to Cu *(117)*. *S. cerevisiae* and *Neurospora crassa* produce MT-IIs *(46)*, and also PCs, particularly with Cd *(118)*. The resemblance of PCs to the ubiquitous monothiol glutathione, γ-Glu-Cys-Gly, suggested its involvement in chain extension for PC biosynthesis (*see* Biosynthesis).

The β-alanyl family of peptides, with the general structure $(\gamma\text{-Glu-Cys})_n$-β-Ala, was discovered in those plants of the family Fabaceae, or the legumes, in which the monothiol homoglutathione, γ-Glu-Cys-β-Ala, replaced glutathione *(119)*. Thirteen species in the Fabaceae produced only $(\gamma\text{-Glu-Cys})_n$-β-Ala peptides or homo-PC; 22 species able to synthesize both glutathione and homoglutathione produced the glycyl and the β-alanyl peptides, on exposure to Cd. Seven species of the Fabaceae produced only PCs because they contained glutathione exclusively *(119)*.

The $(\gamma\text{-Glu-Cys})_n$ peptides are the third family of MT-IIIs. These peptides were lesser components in Cu-treated *S. pombe (120)*, Cd-treated *C. glabrata (117,121)*, and cultured roots of *Rubia tinctorum* exposed to Cd *(122)*, and major components in Cd-exposed graminaceous species *(123–125)*. The $(\gamma\text{-Glu-Cys})_n$ peptides are related to γ-Glu-Cys, one of the substrates for glutathione biosynthesis.

The fourth family is the $(\gamma\text{-Glu-Cys})_n$-Ser group *(123)*. These seryl peptides were found in certain members of the family Poaceae (= Gramineae), including rice, wheat, rye, and oats. These plants synthesized the monothiol γ-Glu-Cys-Ser, in addition to glutathione *(126)*. Maize did not produce the seryl peptides.

The $(\gamma\text{-Glu-Cys})_n$-Glu peptides are the fifth and most recent MT-IIIs *(124)*. They were isolated from Cd-treated roots of maize. The monothiol γ-Glu-Cys-Glu was almost absent from control roots and shoots *(127)*, but the other monothiols were reasonably abundant in the respective species in control tissues.

Another consistent nomenclature for the MT-IIIs has been proposed on the basis of the same function, i.e., metal chelation for the homologous peptides *(128)*. The names and abbreviations are, in the order of those discussed above, phytochelatin, PC; homophytochelatin (β-alanine), *iso*-PC (β-Ala); desglycine phytochelatin, desGly-PC; hydroxymethyl-phytochelatin (serine), *iso*-PC (Ser); and *iso*-phytochelatin (glutamic acid), *iso*-PC (Glu). This terminology would be expandable, should new *iso*-PCs be discovered.

Which family or families of γ-Glu-Cys peptides occur in an individual plant, and the number of repeat units, n, vary with the species and the metal used. Most plants, if they

reacted to the metal, produced phytochelatins (e.g., Cd)(*109–111*). The exceptions were those legumes that relied solely on homoglutathione, and thus produced only the $(\gamma\text{-Glu-Cys})_n$-β-Ala family of peptides. Those legumes that produced both glutathione and homoglutathione produced the glycyl and β-alanyl peptides *(119)*. More recent reports indicate that the $(\gamma\text{-Glu-Cys})_n$ peptides, sometimes in large amounts, accompanied the phytochelatins *(122–125)*. A wider distribution of these cysteinyl peptides is suspected, because they are discernible as small unidentified peaks in some published high pressure liquid chromatography profiles. The peptide loads applied to the analytical columns may have been restrictive, thus diminishing the impact of lesser components. The seryl peptides *(123)* and the glutamyl peptides *(124,125)* accompanied both phytochelatins and the cysteinyl peptides in the respective plants.

Peptide Induction by Metals

The first comprehensive test for which metals induced MT-IIIs was reported for cell suspension cultures of *Rauvolfia serpentina* grown on Zn- and Cu-free medium *(109)*. Salts of the following metals or metalloids induced the formation of PCs n_2 and n_3: Cd, Pb, Zn, antimony (Sb), Ag, Ni, Hg, arsenate, Cu, tin (Sn), selenate, gold (Au), bismuth (Bi), tellurium (Te), and tungsten (W). Recently, it was noted that induction by Ni, selenate, Te, and W could not be repeated *(128)*. No PCs were detected with Al, Ca, Co, Chromium (Cr), caesium (Cs), K, Mg, Mn, molybdate, or Vanadium (Va). The most active metals were Cd, Zn, Pb, Ag, and Sb, but they were presented at varying concentrations, ranging from 50 to 1000 μ*M (109)*. At that time, only Cd was known to induce phytochelatins, and to be bound by the ligands as Cd-binding complex *(108,109)*. Maitani et al. *(129)* used root cultures of *R. tinctorum*, and confirmed that Cd, Pb, Zn, Hg, arsenate, and Cu induced PCs as did Ni, selenate, gallium (Ga), indium (In), and palladium (Pd). All the metals and metalloids, except Ni, Pb, selenate, and Zn, also induced the $(\gamma\text{-Glu-Cys})_{2,3}$ peptides. The best induction of MT-IIIs in the *Rubia* roots was, in descending order, with Ag, Cd, Pb, Hg, and arsenate (concentrations ranging from 10 to 1000 μ*M*). An alkaline extract, which maintained metal-binding complexes, was separated by gel filtration, with continuous simultaneous detection of several elements in an inductively coupled plasma *(129)*. Roots induced with Ag showed a Ag-binding component that also contained Cu and Fe. A Cd-induced sample contained a peak with both Cd and Cu, a Cu-induced sample had a similarly eluting Cu-binding component. For an arsenate induced sample a typical Cu-binding peak was evident, but no As was coincident. The peptide compositions of the metal-binding peaks were not determined, as has been done for Cd-binding complexes (*see* Cd-Binding Complexes) (*108,109*). There is a clear need to test whether a metal that induces γ-Glu-Cys peptides is itself bound by the peptides. Without such evidence, it may be fallacious to equate peptide induction with metal chelation.

Biosynthesis

Knowledge of the primary structures of the various γ-Glu-Cys peptides required a paradigm shift away from searches for the genes that defined the molecules to enzymatic pathways for peptide biosynthesis. Transcriptional, translational, and kinetic regulation of the biosynthetic enzyme(s) become paramount to understanding how γ-Glu-Cys peptide appearance is controlled at the cellular, tissue, and physiological levels.

Grill et al. *(130)* purified an enzyme from cultured cells of *S. vulgaris*, which performed a transpeptidation of the γ-Glu-Cys moiety of glutathione onto another glutathione, to form $(\gamma\text{-Glu-Cys})_2$-Gly and Gly, or onto another $(\gamma\text{-Glu-Cys})_n$-Gly to form the n + 1 oligomer and Gly. The enzyme was named γ-glutamylcysteine dipeptidyl transpeptidase (EC 2.3.2.15) or phytochelatin synthase. For the partly purified enzyme, the reaction proceeded as long as free Cd was available. When sufficient ligand was formed to bind all Cd at a molar ratio of 1 Cd:2 Cys, the enzyme stopped *(131)*. Based on the optimum concentration for enzyme activation,

the order of decreasing efficacy was Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au, arsenate, In, thallium (Tl), germanium (Ge), bismuth (Bi), and Ga *(128)*. The presence of constitutive PC synthase was confirmed with crude extracts from fission yeast *(132,133)* and plants *(134–136)*. Activation by Cd, Ag, Pb, Cu, Zn, Au, and Hg was confirmed for the partly purified enzyme from tomato, in which Fe was also effective; without Cd, no catalysis was evident *(136)*. The enzyme in crude extract of fission yeast did not show a strict regulation by Cd *(132,133)*. Establishing Cd regulation of enzyme activity in crude extracts is problematic, because endogenous Cu and Zn may still be present, to give a basal activity. Hayashi et al. *(133)* presented evidence for an additional pathway, one involving polymerization of γ-Glu-Cys into $(\gamma\text{-Glu-Cys})_n$, and later addition of Gly by glutathione synthetase, to form $(\gamma\text{-Glu-Cys})_n$-Gly. This is the only work suggesting a biosynthetic origin for the $(\gamma\text{-Glu-Cys})_n$ peptides. A possible catabolic source would be the suggested action of carboxydase on $(\gamma\text{-Glu-Cys})_n$-Gly *(123)*. The crude PC synthase preparation from pea *(134)* used glutathione, in a Cd- and Cu-dependent manner, to produce PC. The same preparation, when given glutathione and γ-Glu-Cys-β-Ala, produced $(\gamma\text{-Glu-Cys})_2$-β-Ala, or, when given glutathione and γ-Glu-Cys-Ser, produced $(\gamma\text{-Glu-Cys})_2$-Ser, a tripeptide not endogenous to pea. The glutamyl peptides in maize may arise through an unknown biosynthesis or degradation of other γ-Glu-Cys peptides *(124,125)*. The latter view was prompted by two facts: The monothiol γ-Glu-Cys-Glu was barely apparent in the absence of Cd; and the glutamyl peptides were consistently shorter and at lower concentrations than the other γ-Glu-Cys peptides. For degradation, action of a γ-glutamyl transpeptidase cleaving intramolecular γ-Glu-Cys linkages would be required (S. Klapheck, personal communication). Biosynthesis of γ-Glu-Cys peptides places demands on the pools of glutathione, Glu, Cys, and Gly. In maize roots, glutathione pools declined rapidly with 3 μ*M* Cd, and did not recover completely by 7 d; however, the pool of Cys was maintained, and γ-Glu-Cys increased substantially *(137)*. Stimulated demand for Glu in Cd-treated maize roots resulted in increased amounts of enzyme protein for phosphoenolpyruvate carboxylase and glutamine synthetase, without any change in glutamate dehydrogenase and glutamate synthase *(138)*. These data are consistent with the findings for lupin roots, in which 0.1– 10 μ*M* Cd increased the contents of Glu, Cys, and Gly after 15–17 d of exposure *(139)*. In leaves of poplar under oxidative stress, the demand for glutathione was alleviated by addition of Gly *(140)*. Without knowledge of the Gly pool size in roots of maize in the early phase of Cd exposure, it can be speculated that carboxydase action on PCs might be a mechanism to recycle Gly for glutathione biosynthesis, while keeping the γ-Glu-Cys portion of the ligand intact for Cd chelation.

More certainty about γ-Glu-Cys peptide biosynthesis may be gained from mutant strains like MN70 and MN72 of fission yeast *(141)* and *cad1* of *Arabidopsis (142)*. These mutants contained normal γ-Glu-Cys synthetase and glutathione synthetase, but lacked the ability to produce γ-Glu-Cys peptides. Selection of tomato cell lines increasingly resistant of Cd resulted in cells with a higher specific activity of γ-Glu-Cys synthetase *(143)*. This feature provided enhanced capacity for glutathione synthesis, and, along with increased PC synthase activity *(143)*, maintained production of γ-Glu-Cys peptides with Cd stress. Glutathione production in *B. juncea* plants was elevated particularly by Cu, and only later by Cd *(144)*. The mRNA for γ-Glu-Cys synthetase was increased in roots and shoots by Cu, transiently by Zn, and was not changed by Cd. Transcripts for MT2 mRNA were decreased more in shoots than in roots by Cu; Zn caused a transient increase, and Cd no change. Studies that consider both MT-IIs and -IIIs and their respective regulation will enhance understanding of the intricate reactions plants have toward metals.

Cd-Binding Complexes

The metal-binding complexes formed by MT-IIIs and Cd provide the most definitive

information, at present. The nonessential element, Cd, does not elicit formation of any other prominent ligands within plant cells; the abundance of proteins that normally bind the essential elements Cu and Zn tend to obscure specific reactions to excess Cu and Zn. There is a dearth of information on the ligand–metal interactions of the remaining metals known to induce γ-Glu-Cys peptides.

The evidence for two Cd-binding complexes came first from fission yeast *(145)*. Alkaline extracts of cells exposed to 1 m*M* Cd for 5 h, and separated by gel filtration, showed that most Cd eluted in two peaks, nearly baseline-resolved, named Cd-binding peptides 1 and 2. The purified complexes had apparent mol wt of 4000 and 1800, respectively. The current names for these complexes are the high-mol-wt (HMW) and low-mol-wt (LMW) complexes. Both complexes were induced by Cd. After 9.5 h of Cd exposure, the amount of HMW complex had more than doubled, while the LMW complex remained nearly constant, but was less well resolved on the trailing edge of the prominent HMW peak. The HMW complex bound mostly Cd, and a little Zn and Cu *(145)*. Such a complex was later found to contain $(\gamma\text{-Glu-Cys})_2$-Gly and $(\gamma\text{-Glu-Cys})_3$-Gly *(107)*. The HMW complex continues to be studied nearly exclusively, because it is abundant compared to the LMW complex, which has been ignored as a minor component, or missed entirely. Recently, an intermediate form, a medium-mol-wt (MMW) complex, was isolated from *R. serpentina*, in addition to the HMW and LMW complexes, but *S. vulgaris* yielded only the HMW complex *(146)*.

The apparent mol wt of the HMW complex changed markedly with ionic strength, from approx 8000 at 10 m*M*, to 3600 at 300 m*M* and greater *(109)*. Whether the ionic strength of 300 m*M*, and greater, applies to HMW complexes from sources other than *R. serpentina* remains untested. Another difficulty is that not all constituent peptides can occur in the same entity, to conform to the apparent mol wt. Assuming that one molecule of the least-predominant peptide occurs in the HMW complex of *R. serpentina (147)* or maize *(125)*, and then calculating the others proportionate to their abundance, gives theoretical masses far in excess of that suggested by gel filtration. The HMW Cd-binding complex in plants, which appears as a Gaussian peak in gel filtration, must be a collection of subcomplexes that differ in their individual complements of γ-Glu-Cys peptides. Whether anion exchange chromatography could separate the putative subcomplexes remains to be explored. Anion exchange chromatography of Cd-binding complexes from *S. pombe*, *C. glabrata*, and tomato gave only partial resolution of what have become known as the HMW and LMW complexes, without an indication of subcomplexes *(116,117,148)*.

Examination of the HMW complex from *R. serpentina*, by EXAFS, showed a Cd-S interatomic distance of 2.52 ± 0.02 Å, typical of $[Cd(SR)_4]$ thiolate complexes *(147)*. The carboxylate groups were not involved in Cd coordination. *In situ* EXAFS of relatively mature roots and shoots of *B. juncea* suggested $Cd–S_4$ coordination at a Cd–S interatomic distance of 2.53 Å *(24)*. The best overall fit was to purified HMW Cd-binding complex from maize *(149)*.

Murasugi et al. *(150)* first showed that an appreciable quantity of acid-labile sulfide was part of the HMW complex, with much less in the LMW complex. A similar situation was found for the two complexes from *B. juncea (151)* and maize *(152)*. The three complexes from *R. serpentina* contained acid-labile sulfide, most being in the HMW complex *(147)*. The yeasts *S. pombe* and *C. glabrata*, grown in 1 m*M* Cd, produced complexes with CdS crystallites *(153)*. The 20 Å crystallite of *C. glabrata* consisted of ~85 CdS units surrounded by ~30 $(\gamma\text{-Glu-Cys})_2$-Gly and $(\gamma\text{-Glu-Cys})_2$ molecules. The high sulfide fractions of the tomato complex had optical characteristics of small CdS crystallites *(148)*. Addition of sulfide to partially purified low-sulfide LMW Cd-binding complex from *S. pombe* imparted greater pH stability, an increased apparent mol wt, and an enhanced capacity to bind Cd *(154)*. From these in vitro data has come the idea that the HMW

Table 4
Peptides and Molar Ratios of Cd and Acid-labile Sulfide in Cd-binding Complexes

	Rauvolfia serpentina (146)			Maize *(152)*	
	LMW	MMW	HMW	LMW	HMW
Peptide					
$(\gamma\text{-Glu-Cys})_n$-Gly	n_2 to n_4	n_2 to n_7	n_2 to n_7	n_1 to n_5	n_1 to n_5
$(\gamma\text{-Glu-Cys})_n$				n_1 to n_5	n_1 to n_5
$(\gamma\text{-Glu-Cys})_n$-Glu				n_1 to n_5	n_1 to n_5
Molar ratio					
Cd:peptide SH	0.34	0.43	0.69	0.82 ± 0.08	1.61 ± 0.06[a]
S^{2-}:peptide SH	0.01	0.01	0.28	0.27 ± 0.02	0.29 ± 0.02
Cd:S^{2-}	30.20	43.67	2.45	3.12 ± 0.53	5.49 ± 0.22[a]

LMW, MMW, and HMW complexes were isolated from cultured cells of *Rauvolfia serpentina* and roots of maize. For maize, the ratios are the mean ± SE of four replicates.

[a]Significantly higher than the ratio for LMW complex at $p = 0.01$.

complex is the LMW complex with added sulfide and Cd. This notion is to be questioned for the situation in vivo, because the complement of peptides differed between LMW and HMW complexes isolated from two plants (Table 4). The LMW complex from *R. serpentina* was composed of the n_2 to n_4 PCs, and the MMW and HMW complexes contained a preponderance of the larger, up to n_7, PCs *(146)*. For maize, the proportions of mono- and dithiols of the three types of γ-Glu-Cys peptides were greater in the LMW complex than the HMW form; the proportions of the other 10 peptides were the same *(152)*. In both examples (Table 4), the HMW complexes contained more Cd per peptide thiol, but only for *R. serpentina* was there added acid-labile sulfide in the HMW complex.

A few of the many isolates of Cd-binding complexes are reported to contain small amounts of Zn, and sometimes Cu *(145,109,129)*. The Ag-induced complex from *R. tinctorum* contained some Cu and Fe *(129)*. Fe co-purified with the Cd-binding complex from *Datura innoxia*; however, it was uncertain whether the association was of physiological relevance, or fortuitous binding to the abundant carboxyl groups of Glu in the constituent γ-Glu-Cys peptides *(155)*. Binding stoichiometries and metal transfers in vitro were reported for individual γ-Glu-Cys peptides *(156–159)*. The peptides used were the n_2, n_3, and n_4 molecules of $(\gamma\text{-Glu-Cys})_n$-Gly. The binding stoichiometries for Cu(I) were 1.5, 2.0, and 2.5 atoms/mol of n_2, n_3, and n_4 peptides, respectively *(156)*. One atom of Pb was bound to the n_2 and n_3 peptides; the n_4 peptide formed complexes with one and two atoms of Pb *(157)*. At pH 7.0, Ag(I) binding was 1, 1.5, and 4 atoms/n_2, n_3, and n_4 peptide, respectively. Two and three Ag atoms were bound at pH 5.0 by the n_2 and n_3 peptides, respectively *(158)*. One atom of Hg(II) was bound/n_2 peptide, but 1.25 and 2 atoms by both n_3 and n_4 peptides *(159)*. The final binding capacities for Hg were not influenced by dropping the pH from neutrality to 2.0. Transfer of Cu, Pb, and Hg was demonstrated from the metal–glutathione complex to each of the three γ-Glu-Cys peptides individually, and also from a shorter γ-Glu-Cys peptide to a longer one *(156,157,159)*. Because both Pb and Hg induced the appearance of γ-Glu-Cys peptides *(109,129)*, and these ligands bound the metals in vitro *(157,159)*, one would anticipate that Pb- and Hg-binding complexes, analogous to Cd-binding complexes, might be isolated from plants. Whether this actually happens *in planta* remains to be tested. Because glutathione transferred metal to PCs, Mehra et al. *(157)* suggested that glutathione

was probably the in vivo donor of metals to PCs. Comparison of the synthetic analog (Glu-Cys)$_2$-Gly to natural (γ-Glu-Cys)$_2$-Gly showed that both ligands bound Cd, Hg, and Pb, even when donated by glutathione, and that CdS particles formed with each *(159a)*. Synthetic genes for the entirely α-linked analogs to the PC family can be produced: Their suitability for developing transgenic plants for phytoremediation is now testable *(159a)*.

Cellular Location of Cd-Binding Complexes

Two Cd-hypersensitive mutants of *S. pombe* were able to produce LMW Cd-binding complex, but not the HMW form *(141)*. The HMW complex was essential for growth in Cd: The LMW complex alone was insufficient. A gene, designated *hmt1*, isolated from *S. pombe*, complemented a Cd-hypersensitive mutant unable to produce the sulfide-rich HMW complex *(160)*. The deduced protein for the *hmt1* gene was an adenosine triphosphate (ATP)-binding cassette-type transporter associated with the vacuolar membrane. In vitro transport studies with vacuolar membrane vesicles showed that HMT1 was an ATP-dependent transporter of apo-PCs and LMW Cd-binding complex, which worked poorly with the HMW complex containing sulfide *(161)*. Those authors proposed the cellular model, in which PCs were synthesized in the cytosol in response to Cd entry, and formed the LMW Cd-binding complex. The cytosolic LMW complex was moved into the vacuole by the ATP-binding cassette-type transporter. Within the vacuole, more Cd, entering by a Cd^{2+}/H^+ antiporter, and sulfide were added, to produce the HMW sulfide-rich Cd-binding complex. Transport of protons into plant vacuoles by the vacuolar-ATPase is well established; the Cd^{2+}/H^+ antiporter was described for oat vacuolar vesicles *(162)*. Transport of apo-PCs n_2 and n_3 and the complex, Cd-[(γ-Glu-Cys)$_3$-Gly], was demonstrated for oat root vacuolar vesicles, and had characteristics of an ATP-binding cassette-type transporter *(163)*. The different complements of peptides comprising the Cd-binding complexes from *R. serpentina* (Table 4) (*146*) do not entirely support the model of Ortiz et al. *(161)*, assuming that the LMW complex is cytosolic and the MMW and HMW forms are vacuolar. The addition of Cd and acid-labile sulfide to the HMW complex of *R. serpentina* is according to the model. For maize, the LMW complex, containing a preponderance of mono- and dithiol γ-Glu-Cys peptides, was the nascent complex, but it already contained acid-labile sulfide (Table 4) (*152*). In this case, the ATP-binding cassette-type transporter would move the LMW sulfide-rich Cd-binding complex into the vacuole, where further Cd would be available to form the HMW sulfide-rich Cd-binding complex. The HMW complex contained more Cd per unit peptide thiol than did the LMW form (Table 4) (*152*). There is little knowledge for any other metal regarding the types of complexes, compartmentation, and specialized membrane transport. To accept the Cd model for other metals could be fallacious: Much experimentation is required to ascertain the relevant details for other metals.

Biological Function

The MT-IIIs are involved in metal homeostasis. During growth of *R. serpentina* cells in fresh medium, Cu and Zn disappeared biphasically, and PCs accumulated maximally before the stationary growth phase *(164)*. Once both metals were depleted, the PC content declined during the stationary growth phase at a half-life of about 3.4 d. If Cu and Zn were to be complexed by γ-Glu-Cys peptides in vivo, they could be sources of metal for metal-requiring apoenzymes. The apo form of diamino oxidase prepared from pea seedlings was activated by reconstituted Cu–PCs *(165)*. At the same addition of Cu, the shortest PC, n_2, was nearly as efficient as $CuSO_4$ in activating diamino oxidase, and more so than the longer n_4 and n_5 Cu complexes. After removal of Zn from commercial bovine carbonic anhydrase, the apoenzyme was activated by reconstituted Zn–PC complexes. At the same Zn supply, Zn–PC n_2 was nearly as effective as $ZnSO_4$, and more effective than the n_7 Zn–PC complex. Kneer and Zenk *(166)*

investigated the protective effect of Zn– or Cd–PC complexes on the metal-sensitive enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase, nitrate reductase, glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, and urease. The concentrations of Zn or Cd salt that inhibited enzyme activity by 50% could be exceeded by 10–150-fold, when the Zn or Cd was supplied as the metal–PC complex. Addition of apo-PC n_2 was more effective than glutathione or citrate in restoring activity of Cd-inhibited nitrate reductase *(166)*.

To assess the importance of γ-Glu-Cys peptides as Cd ligands, one can determine what proportion of intracellular Cd occurs as Cd-binding complexes. Many reports state that a high percentage of Cd in gel filtration occurs as Cd-binding complex, without giving information on the Cd concentrations in the tissue. Recalculation of reported data shows that Cd-binding complexes account for 52% of the Cd in water-rinsed roots of *S. vulgaris (167)*, and for 62% of the total ^{109}Cd in washed cells of *R. serpentina (166)*. These proportions depend on the efficacy of removing Cd external to the plasma membrane, to give the intracellular Cd content, and the degree to which intracellular Cd is subsequently solubilized prior to analysis. The two prior reports used the initial extract of roots or cells for analysis of complexes by gel filtration. In suitably desorbed roots of maize, the initial extract yielded 63–74% of the Cd in the roots; five additional extractions of the root debris yielded a total of 92–94% of the root Cd *(125)*. The six extracts were concentrated by anion exchange chromatography prior to gel filtration. The HMW sulfide-rich Cd-binding complex accounted for 19, 39, 51, and 59% of the root Cd at d 1, 2, 5, and 7, respectively, of seedlings exposed to 3 μ*M* Cd. No LMW complex was found, probably because the particular anion exchange procedure did not capture this form. Using an extraction buffer that did not chelate metal and lyophilizing the six extracts that together represented 93–96% of the root Cd, yielded both LMW and HMW Cd-binding complexes *(152)*. In two cultivars of maize, these two complexes together bound 82% of the Cd in the roots after 5 and 7 d exposures to 3 μ*M* Cd. The ligands for the remaining Cd were not assessed, but they may have been organic acids (*see* above). Five-d-old *B. juncea* seedlings, immersed in growth solution, were exposed to 1 μ*M* Cd for 6–24 h, and then the Cd environment was examined by EXAFS *(149)*. At 6 h of exposure, approx 64% of the total intracellular Cd was coordinated octahedrally, probably to oxygen atoms; 25% was coordinated tetrahedrally, with sulfur typical of Cd-binding complexes, based on γ-Glu-Cys peptides. By 24 h, and thereafter, the oxygen ligands accounted for 42–45% of the Cd, and the sulfur ligands for 56–60% of the intracellular Cd. The octahedral Cd–O coordination at 2.28 Å was best-fitted by $Cd(NO_3)_2$, and less well by Cd–citrate complex. This coordination was previously found for the xylem sap *(24)*. The Cd–S coordination was at 2.53 Å *(149)*. Because whole plants were examined, the source of Cd–O coordination could be organic acids inside cells, and those in the xylem sap. These various studies show that the γ-Glu-Cys peptides function to bind some of the intracellular Cd from small to large proportions, depending on the duration of exposure to Cd, and that other ligands participate to chelate some of the remaining Cd.

Loss of PCs after arresting Cd exposure was examined in roots of *S. vulgaris (135)*. For both Cd-sensitive and Cd-tolerant clones, the Cd concentration in the roots was stable from 4 to 10 d after Cd removal. During this time, the PC to Cd ratio declined, with half lost in about 5 d. Concomitantly, the amount of unidentified thiols increased, particularly in the Cd-tolerant clone. Whether the unidentified thiols participated in binding Cd is unclear. The authors *(135)* suggested that Cd was not finally stored as a PC complex, much like an earlier suggestion *(22)*. In both studies, PC-based Cd-binding complexes were not measured *(22,135)*. Accumulation of the HMW complex with time, and continued exposure to Cd *(125)*, contradicts the idea that these complexes are temporary storage forms. The turnover of γ-Glu-Cys peptides in Cd-binding complexes, in tissues continuously supplied with low Cd, remains to be explored.

Involvement of γ-Glu-Cys peptides in the phenomenon of metal tolerance in plants is highly attractive, because a variety of metals stimulate biosynthesis of these ligands, and the metals may be bound by them. The simplest expectation is that the metal-tolerant ecotypes produce more metal-binding γ-Glu-Cys peptides than do the nontolerant ecotypes. Because metal tolerance is mostly based on root growth assays *(3,4)*, the expectation should apply to root apices. For Cu- and Cd-tolerant ecotypes of *S. vulgaris*, the expectation was not met *(168,169)*. The root apices of metal-tolerant ecotypes contained lower (or the same) amounts of nonprotein thiols than the sensitive ecotypes, at external metal concentrations that gave equivalent root growth inhibitions. The measure of nonprotein thiols included γ-Glu-Cys peptides and other acid-soluble thiols, with glutathione subtracted. A similar situation was found for entire root systems of Zn-tolerant and nontolerant *S. vulgaris (170)*. The correlation between nonprotein thiols in root systems and the degree of Cu tolerance in *Arabidopsis thaliana* was less strong (r = 0.77) than with either MT2a mRNA (r = 0.998) and MT1a mRNA (r = 0.98) *(106)*. The Cd-binding complexes based on γ-Glu-Cys peptides were seen as sinks for excess Cd, rather than as the cause of differential tolerance of Cd *(169)*. Progress in defining the role of γ-Glu-Cys peptides in metal tolerance requires simultaneous measurements in root apices of complexes based on MT-IIs and MT-IIIs, and probably organic acids and phytin. The variety of ligands may chelate metal to achieve detoxification, but other metal-induced stresses may cause cellular damage, despite metal chelation. Depletion of glutathione *(137,171)*, required for γ-Glu-Cys peptide production, resulted in oxidative stress *(171)* that influenced membrane function. Restricted synthesis of phytochelatins in root cells of Cu-tolerant *S. vulgaris (168)* would have placed a lesser demand on glutathione, and thus would have enhanced cell membrane mechanisms, to maintain low cellular Cu and display Cu tolerance *(171)*.

CONCLUSION

Most work on MT-IIIs deals with Cd-exposed cells and plants. Experimental concentrations used to obtain the results reviewed here vary tremendously, ranging from 1 to 3 μ*M* Cd, used with *B. juncea (149)* and maize *(124)*, to 1000 μ*M* Cd for yeasts to produce CdS crystallites *(153)*. Soils used for agricultural production contain low levels of Cd (0.04 to 0.32 μ*M* Cd in soil solution), which may rise to 3.2 μ*M* Cd in highly polluted soil *(5)*. Until further data are available at lower experimental Cd exposures, which better reflect Cd-polluted soils, it is best to use caution before invoking some of the effects reviewed here. For instance, it is quite possible that, in Cd-polluted soils, organic acids produced by the plant are more prominent ligands than the γ-Glu-Cys peptides *(5,19)*.

Attempts to change plants genetically, so that little Cd is transferred into edible portions, have not been possible through overproduction of γ-Glu-Cys peptides in roots, because the genes for their biosynthesis (e.g., PC synthase) are lacking. Use of synthetic genes for the totally α-linked analogs of PCs is a novel prospect *(159a)*. The genetic basis for metal tolerance is still not clear *(3)*. Incorporation of mammalian MT genes into plants has provided some promise (e.g., *172–174*), but this area has not been considered in this review.

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