Update on Environmental Stress

Phytochelatins and Related Peptides[1]

Structure, Biosynthesis, and Function

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Plants obtain micronutrients such as Cu and Zn from aquatic and terrestrial environments that may also provide the metals Cd, Pb, and Hg. These essential and nonessential metals exist at low to high concentrations depending on natural and manmade disturbances. In a fluctuating environment the plants may experience shifting internal concentrations of these bioreactive metals. Therefore, it is beneficial for plants to have mechanisms that (a) maintain internal concentrations of essential metals between deficient and toxic limits and (b) keep nonessential metals below their toxicity thresholds. The proteins and peptides known as MTs sequester metals and thereby may accomplish cellular metal homeostasis and detoxification. These molecules are rich in Cys's that provide thiols for binding the metals mentioned so far.

The early work on plants was modeled after equine renal MT, a metal-induced protein with 20 Cys's distributed uniquely within the sequence of 60 amino acids and M_r of approximately 9000 (Robinson et al., 1993). Studies of yeasts, algae, and plants relied heavily on isolating the metal-induced components of M_r of approximately 9000 that bound Cd or Cu. In many of these preparations Cys, Glu, and Gly accounted for 45 to 97% of the amino acids, which is inconsistent with a close relationship to the archetypal form, equine renal MT. Two independent groups, one working with the fission yeast *Schizosaccharomyces pombe* (Kondo et al., 1984) and the other working with cultured cells of *Rauvolfia serpentina* (Grill et al., 1985), showed that the molecules binding Cd were a family of peptides with the primary structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where $n = 2$ to 7 depending on the organism. The peptide bond in the repeating Glu-Cys pairs is a γ-carboxyamide linkage not synthesized on ribosomes. Consequently, these and related peptides are now designated class III MTs, which are defined as atypical, nontranslationally synthesized metal thiolate polypeptides (Robinson et al., 1993). No consensus has been reached concerning a trivial name for the peptides. The name cadystin holds priority (Kondo et al., 1984), with phytochelatin being popularly used for algae and plants (Grill et al., 1985). Recent progress on class III MTs

is presented here and builds on two reviews (Rauser, 1990; Steffens, 1990); hence, most reports prior to 1989 and found in these reviews are not referenced.

Identification of phytochelatins in plants arose from the search for archetypal MT. Finding that much of the cellular Cd apparently is complexed by phytochelatins has lessened the search for archetypal MTs in plants. Mature wheat embryos contain a Cys-rich Zn-binding protein with considerable amino acid sequence homology to class II MTs, proteins distantly related to archetypal mammalian MTs (Robinson et al., 1993). This protein disappears with seedling development. Searches for DNA sequences homologous to class II MT genes have been successful with *Mimulus guttatus*, pea, soybean, maize, barley, wheat, castor bean, *Brassica napus*, and *Arabidopsis thaliana*. The specific gene products in plants require isolation. Perhaps the putative archetypal plant MTs are expressed particularly in embryos and highly regenerative parts such as meristems, whereas rapid synthesis of γ-Glu-Cys peptides, as with Cd, is the dominant response of mature cells in the bulk of plant tissues.

FIVE FAMILIES OF γ-GLU-CYS PEPTIDES
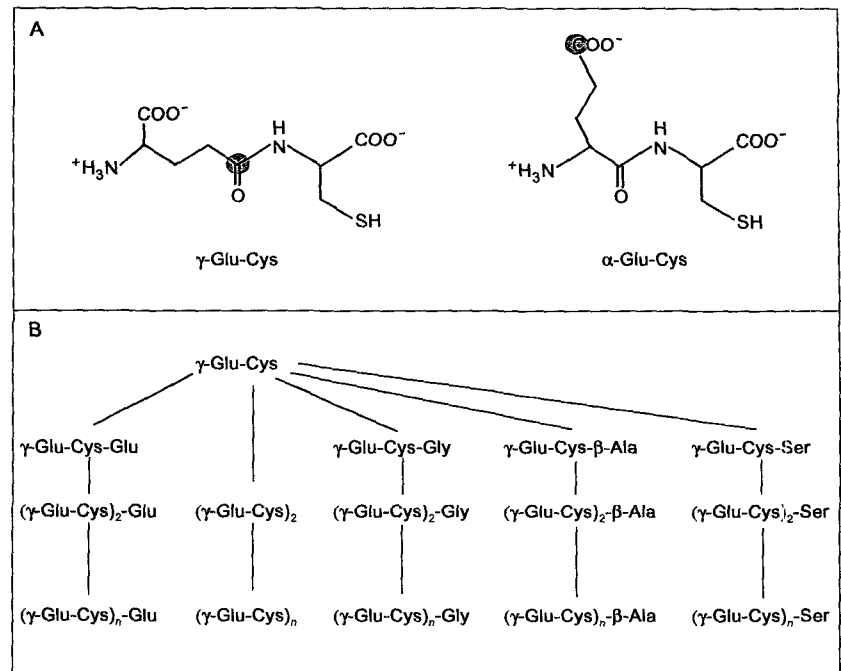
Primary Structures

The primary structures of molecules are known for five families of peptides in class III MTs. The common features are that (a) Glu occupies the amino-terminal position, (b) the next residue is Cys with the peptide bond to the γ-carboxyl of Glu, and (c) γ-Glu-Cys pairs are repeated two or more times, with the subscript n signifying the exact number of repeats. These commonalities between peptides lead to their collective designation as γ-Glu-Cys peptides while acknowledging the fact that the carboxy-terminal amino acid varies and divides the class III MTs into five families. During mRNA translation on ribosomes only the α-carboxyl of Glu is used to produce the α-carboxyamide bond and not the γ-carboxyamide bond (Fig. 1A). Documentation of the primary structures of the γ-Glu-Cys peptides dictated a paradigm shift away from searches for genes defining the molecules to pathways of biosynthesis.

Abbreviations: HMW, high molecular weight; LMW, low molecular weight; MT, metallothionein.

Figure 1. The structure of various γ-Glu-Cys peptides. The γ-carboxy C of Glu is highlighted in A to differentiate between α- and γ-carboxy-amide linkages. B is a model summarizing the five families of γ-Glu-Cys peptides involved in metal sequestration in plants and certain yeasts. The lines connecting peptides refer to family relationships and do not necessarily specify bio-synthetic sequences. Adapted from Meuwly et al. (1995).

The primary structures first determined were for the phytochelatins or cadystins, the prominent peptides iso-lated from prepurified Cd-binding complexes from fission yeast and plant cell cultures. A variety of chemical and physical techniques gave the structure (γ-Glu-Cys)$_n$-Gly with $n = 2$ to 4. These peptides resembled the ubiquitous tripeptide glutathione, γ-Glu-Cys-Gly, suggestive of the involvement of glutathione in phytochelatin biosynthesis. For those legumes that produced the homologous tripep-tide homoglutathione or γ-Glu-Cys-β-Ala, a second family of γ-Glu-Cys peptides was anticipated. The Cd-binding complex from such plants contained the peptides (γ-Glu-Cys)$_n$-β-Ala named homophytochelatins. The third family of γ-Glu-Cys peptides was first noticed as minor constitu-ents of Cd-binding complexes in the fission yeast *S. pombe* and later as major constituents in maize (Meuwly et al., 1995). MS verified the structures as (γ-Glu-Cys)$_n$. Cd-in-duced γ-Glu-Cys peptides with a carboxy-terminal Ser were detected in certain species of the Poaceae (=Gra-mineae) (Klapheck et al., 1994). The primary structures were (γ-Glu-Cys)$_n$-Ser. Since these peptides were related to the tripeptide hydroxymethyl-glutathione (γ-Glu-Cys-Ser), the polymers were named hydroxymethyl-phytochelatins. The most recent addition is the fifth family with the struc-ture (γ-Glu-Cys)$_n$-Glu related to the novel tripeptide γ-Glu-Cys-Glu isolated from maize (Meuwly et al., 1995). The five families of γ-Glu-Cys peptides and their structural rela-tionships to di- or tripeptides are summarized in Figure 1B. the relationships do not necessarily signify enzymatic path-ways. Phytochelatins are but one specific family of γ-Glu-Cys peptides. Use of the trivial name phytochelatins for all thiols belonging to class III MTs is inconsistent with the original definition of the molecules and should be avoided.

Occurrence

The wide distribution of phytochelatins among monocoty-ledonous and dicotyledonous species through to the red, green, and brown algae is well documented (Grill et al., 1987; Rauser, 1990; Steffens, 1990). The responses followed expo-sure of cultured cells or plants to Cd. In some instances in which phytochelatins have been reported in the past and the HPLC separations of peptides show minor thiols, future re-visions may recognize one or more of the four other families of γ-Glu-Cys peptides. Peptide loadings and resolution can markedly influence what is detected. The complement of γ-Glu-Cys peptides from the five families (Fig. 1B) varies according to species and sometimes according to the metal. The yeasts *S. pombe* and *Candida glabrata* respond to Cd by producing (γ-Glu-Cys)$_n$-Gly and (γ-Glu-Cys)$_n$; however, only *C. glabrata* exposed to excess Cu produces class II MT rather than γ-Glu-Cys peptides. For legumes the type of tripeptide produced by the species dictates the type of γ-Glu-Cys pep-tide formed. Seven species that produced only glutathione made (γ-Glu-Cys)$_n$-Gly, 13 that produced only homogluta-thione made (γ-Glu-Cys)$_n$-β-Ala, and 23 species containing both tripeptides made both families of γ-Glu-Cys peptides when exposed to Cd. Rice, wheat, rye, and oats are among the graminaceous species that produce (γ-Glu-Cys)$_n$-Gly, (γ-Glu-Cys)$_n$, and (γ-Glu-Cys)$_n$-Ser (Klapheck et al., 1994); however, maize produces (γ-Glu-Cys)$_n$-Gly, (γ-Glu-Cys)$_n$, and (γ-Glu-Cys)$_n$-Glu (Meuwly et al., 1995).

BIOSYNTHESIS

Induction by Metals

Plants and cultured cells grown in normal inorganic salts contain a species-dependent abundance of the glycyl,

β-alanyl, and seryl tripeptides shown in Figure 1B, low concentrations of γ-Glu-Cys, and even lower concentrations of $(\gamma$-Glu-Cys$)_{2-3}$-Gly if detected at all. Exposure to 3 to 500 μM Cd causes the rapid appearance or increase (within 10–15 min) of $(\gamma$-Glu-Cys$)_2$-Gly, followed by the larger oligomers of the various γ-Glu-Cys peptides (Grill et al., 1987; Klapheck et al., 1994; Meuwly et al., 1995). In maize roots the tripeptide γ-Glu-Cys-Glu appears within 2 h of Cd exposure, followed closely by $(\gamma$-Glu-Cys$)_{2-3}$-Glu (Meuwly et al., 1995). In cell cultures of R. serpentina $(\gamma$-Glu-Cys$)_{2-4}$-Gly were induced to various degrees by nitrate or sulfate salts of Ni, Cu, Zn, Ag, Sn, Sb, Te, W, Au, Hg, Pb, and Bi and the anions arsenate and selenate (Grill et al., 1987). No induction of phytochelatins was observed with inorganic salts of Na, Mg, Al, Ca, V, Cr, Mn, Fe, Co, molybdate, and Cs. These listings are according to the increasing position of the elements in the periodic table. The claim that Cd is the most potent inducer of phytochelatins should be regarded with caution because the R. serpentina cells were exposed to unequal external concentrations of the various elements. A further caution is that even though various metals induce phytochelatins their function in sequestering the metal in vivo has been demonstrated extensively only with Cd and infrequently with Cu.

The prevailing method for quantitating γ-Glu-Cys peptides uses the thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (Grill et al., 1987) with a detection limit of about 0.2 nmol thiol. The sulfhydryl reagent monobromobimane with fluorescence detection gives 100-fold greater sensitivity (Steffens, 1990). By this means Ahner et al. (1994) demonstrated that induction of $(\gamma$-Glu-Cys$)_{2-4}$-Gly in a marine diatom depended on free ionic Cd^{2+}, Cu^{2+}, Ni^{2+}, or Pb^{2+} rather than the total metal concentration, with free metal concentrations of 0.01 to 1 nM being effective. Measurements of phytochelatins in natural phytoplankton populations at environmentally relevant concentrations supported the idea that phytochelatins might be good quantitative indicators of metal exposure.

Enzymology

The enzymatic assembly of amino acids into the tripeptides glutathione and homoglutathione is well characterized for plants. Glu and Cys are joined by ATP-dependent γ-glutamylcysteine synthetase to form γ-Glu-Cys. Addition of Gly to the dipeptide by ATP-dependent glutathione synthetase produces γ-Glu-Cys-Gly. In those plants that produce homoglutathione, the β-Ala-specific ATP-dependent enzyme homoglutathione synthetase uses γ-Glu-Cys to produce γ-Glu-Cys-β-Ala. These enzymes are cytosolic and chloroplastic. The biosynthesis of γ-Glu-Cys-Ser and γ-Glu-Cys-Glu (Fig. 1B) has not been elucidated.

The view that phytochelatins arise from chain extension of glutathione is based on (a) the structural resemblance between phytochelatins and glutathione, (b) the appearance of phytochelatins with concomitant disappearance of glutathione, (c) reduced or no phytochelatin synthesis in mutants deficient in or lacking glutathione synthetase or γ-glutamylcysteine synthetase, and (d) inhibition of phy-

tochelatin synthesis by buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase. Cultured cells of Silene cucubalus (= vulgaris) yielded an enzyme that used glutathione to first synthesize $(\gamma$-Glu-Cys$)_2$-Gly, followed in about 15 min by the appearance of $(\gamma$-Glu-Cys$)_3$-Gly and after a further 20 min by $(\gamma$-Glu-Cys$)_4$-Gly. This enzyme caused the transpeptidation of the γ-Glu-Cys moiety of glutathione onto another glutathione molecule, forming $(\gamma$-Glu-Cys$)_2$-Gly, or onto another $(\gamma$-Glu-Cys$)_n$-Gly molecule to produce the $n + 1$ oligomer. The enzyme was named γ-glutamylcysteine dipeptidyl transpeptidase and given the trivial name phytochelatin synthase (Grill et al., 1989). The highly purified enzyme was a tetramer of M_r 95,000 (an active dimer at M_r 50,000 appeared later in purification) with a K_m for glutathione of 6.7 mM. A less-pure preparation of the Silene enzyme was activated best by Cd and also by Ag, Bi, Pb, Zn, Cu, Hg, and Au, all inducers of phytochelatins in R. serpentina. Once enough $(\gamma$-Glu-Cys$)_{2-4}$-Gly was present to sequester the available Cd at a thiol:Cd ratio of 2:1, enzyme catalysis ceased. Phytochelatin synthase appears to be a self-regulated enzyme in that the product of the reaction chelates the enzyme-activating metal, thereby terminating the enzyme reaction. To our knowledge, this important aspect has not been tested in other systems. The enzyme is constitutive and its formation is not noticeably altered by exposing the cells to Cd.

Constitutive dipeptidyl transpeptidase activity was also found in the fission yeast S. pombe (Hayashi et al., 1991). The crude preparation differed from the Silene enzyme in two respects: Cd was not necessary for catalysis and some $(\gamma$-Glu-Cys$)_2$ appeared along with the cadystins (=phytochelatins). Incubations of glutathione with γ-Glu-Cys, $(\gamma$-Glu-Cys$)_2$, or $(\gamma$-Glu-Cys$)_3$ produced the $n + 1$ oligomer of the $(\gamma$-Glu-Cys$)_n$ provided. The preparation also polymerized γ-Glu-Cys into $(\gamma$-Glu-Cys$)_{2,3}$. This is the only work suggesting a biosynthetic origin for $(\gamma$-Glu-Cys$)_n$ peptides (Fig. 1B). Glutathione synthetase added Gly to $(\gamma$-Glu-Cys$)_{2,3}$, giving the n_2 and n_3 oligomers of phytochelatin, respectively. Hayashi et al. (1991) proposed that polymerization of γ-Glu-Cys to $(\gamma$-Glu-Cys$)_n$ followed by glutathione synthetase adding Gly was a second pathway for phytochelatin biosynthesis.

Pea roots that normally produce both glutathione and homoglutathione also contain a constitutive phytochelatin synthase activity (Klapheck et al., 1995). A crude enzyme preparation was used. Given only glutathione, the extract produced $(\gamma$-Glu-Cys$)_2$-Gly; yet when presented only γ-Glu-Cys-β-Ala or γ-Glu-Cys-Ser, the rate of production of the respective n_2 oligomer was much less. In the presence of both glutathione and γ-Glu-Cys-β-Ala or γ-Glu-Cys-Ser the synthesis of the respective β-alanyl or seryl n_2 oligomer was greatly enhanced, whereas $(\gamma$-Glu-Cys$)_2$-Gly synthesis suffered. Klapheck et al. (1995) explained their results by assuming that the dipeptidyl transpeptidase had a γ-Glu-Cys donor-binding site specific for glutathione and a less specific γ-Glu-Cys acceptor-binding site able to use several tripeptides, namely γ-Glu-Cys-Gly, γ-Glu-Cys-β-Ala, or γ-Glu-Cys-Ser. The dipeptide γ-Glu-Cys was, how-

ever, not a substrate in pea. The partly purified pea enzyme was activated by Cd and Cu and activated poorly by Zn. Parallel studies are required with maize and rice to determine whether these plants produce (γ-Glu-Cys)$_n$-Glu and (γ-Glu-Cys)$_n$-Ser, respectively, by dipeptidyl transpeptidase as in pea simply because the glutamyl or seryl tripeptide and glutathione are available.

The origin of (γ-Glu-Cys)$_n$, particularly abundant in Cd-exposed maize roots (Meuwly et al., 1995; Rauser and Meuwly, 1995), is unresolved. The dipeptide γ-Glu-Cys accumulates at the time that glutathione declines rapidly and would serve as precursor to (γ-Glu-Cys)$_n$ as found for S. pombe (Hayashi et al., 1991). A possible catabolic source would be action of carboxypeptidase on (γ-Glu-Cys)$_n$-Gly (Klapheck et al., 1994). The tripeptide γ-Glu-Cys-Glu is found in maize only after the Cd-induced appearance of (γ-Glu-Cys)$_n$-Gly and (γ-Glu-Cys)$_n$, offering the possibility that the family of γ-Glu-Cys peptides with amino-terminal Glu are degradation products of other thiol peptides. Action of a γ-glutamyl transpeptidase cleaving intramolecular γ-Glu linkages would be required (S. Klapheck, personal communication). These uncertainties accentuate the limitations of the presentation in Figure 1B: the structural relationships among the five families of γ-Glu-Cys peptides do not necessarily describe enzymatic pathways.

Genetic studies of A. thaliana have yielded four cad1 mutants that differ in hypersensitivity of Cd, the ability to form (γ-Glu-Cys)$_n$-Gly, and their deficiency in phytochelatin synthase (Howden et al., 1995b). These workers suggested that CAD1 is the structural gene for phytochelatin synthase. The cad2 mutant of Arabidopsis is deficient in glutathione synthetase and thus produces few phytochelatins and becomes Cd hypersensitive (Howden et al., 1995a). Cd-hypersensitive mutants of S. pombe enabled isolation of the gene for the large subunit of glutathione synthetase (Mutoh et al., 1991) and that of γ-glutamylcysteine synthetase (Mutoh et al., 1995).

PEPTIDE FUNCTION

Homeostasis

Metal homeostasis requires intracellular complexation of metals when there is a cellular surplus and later release of metals to metal-requiring apoproteins and perhaps to final storage sites within cells. Some support for the involvement of phytochelatins in this process was obtained from various cultured plant cells on transfer to a variety of fresh media containing micronutrient concentrations of Cu and Zn (Grill et al., 1988). The concentrations of phytochelatins increased as the medium was depleted of Cu and Zn, reaching a maximum just prior to the stationary phase and coinciding with total disappearance of the metals from the medium. The stationary phase cells then gradually lost phytochelatins presumably to peptide degradation. The phytochelatins behaved as transient metabolites with considerable turnover. The extent to which cellular Cu and Zn actually occurred as a metal-phytochelatin complex during the culture cycle was, however, not evaluated. Exchange of metal with [109]Cd in a crude extract from Rauvolfia cells

grown in micronutrient concentrations of Cu and Zn indicated a small amount of endogenous metal-phytochelatin complex (Kneer and Zenk, 1992).

One prerequisite for homeostasis is that γ-Glu-Cys peptides release bound metals to apo forms of metal-requiring enzymes. This was tested in vitro with phytochelatins (Thumann et al., 1991). Cu complexed with individual phytochelatins or in a native complex from a plant reactivated the apo form of Cu-requiring diamino oxidase nearly as well as CuSO$_4$. Apocarbonic anhydrase was reactivated best by Zn-[(γ-Glu-Cys)$_2$-Gly] and less effectively by Zn-[(γ-Glu-Cys)$_7$-Gly], both complexes being less efficient than ZnSO$_4$. The ability of Cu- and Zn-complexes with the n_2 oligomer to most effectively reconstitute the apoenzymes probably reflects the increasing strength whereby the larger oligomers bind metal.

The role of γ-Glu-Cys peptides in protecting metal-sensitive enzymes is another aspect of homeostasis. Cd in the form of Cd-binding complex was 10 to 1000 times less inhibitory in vitro than the same concentration of Cd as Cd(NO$_3$)$_2$ for the enzymes Rubisco, nitrate reductase, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and urease (Kneer and Zenk, 1992). Even Rubisco, which is more sensitive to Zn than Cd, was effectively protected in the presence of Zn-binding complex. The activity of nitrate reductase poisoned by Cd-acetate was fully regained when (γ-Glu-Cys)$_3$-Gly was provided in a peptide:Cd molar excess of 1:4. Glutathione was at least 1000-fold less effective than the phytochelatin in attenuating inhibition by Cd, whereas citrate was ineffective.

The in vitro experiments support the premise that γ-Glu-Cys peptides participate in metal homeostasis. Whether the demonstrated attributes operate collectively within the entire plant remains unsettled. For instance, the cad1-3 mutant of Arabidopsis has no detectable phytochelatins or phytochelatin synthase activity; yet growth is normal in the presence of Cu and Zn in micronutrient concentrations (Howden et al., 1995b). Here phytochelatins do not play an essential role in metal homeostasis. How this mutant grows in the absence of Cu and Zn remains to be established.

Cd Sequestration in Complexes

The prominent function of γ-Glu-Cys peptides is sequestration of certain metals. The results in vivo are metal-binding complexes that are accretions of multiple peptides of various lengths with metal atoms. Cd-binding complexes have been studied extensively and in greatest detail because Cd is an effective inducer of γ-Glu-Cys peptides, and plant foods are the major source of Cd intake by humans. Cu-binding complexes are less well characterized; indeed, the ease with which Cu(I) is oxidized presents considerable technical challenges. It is unclear whether native Zn-binding complexes occur in plants, and no information is available concerning peptide complexes with the other metals and metalloids that induce γ-Glu-Cys peptides. Caution is advisable in the tendency to generalize to other metals the knowledge gained for the nonessential element Cd. Further considerations are here limited to Cd-binding complexes in certain yeasts and plants.

Two complexes differing in molecular weight can be found. They are referred to as HMW and LMW complexes based largely on their migration during gel-filtration chromatography. The true molecular weights are not clear because the ionic strength of the buffer markedly influences M_r. For instance the dominant Cd-binding complex from *Rauvolfia*, presumably the HMW complex, has an M_r of 8000 at low ionic strength and decreases gradually to 3600 at ionic strengths greater than 0.3 M (Grill et al., 1987). Methods other than gel filtration need be used to determine the molecular weight of the HMW and LMW complexes. Certainty of molecular weight combined with peptide composition and metal concentration will enable better structural and spatial formulations of the complexes.

The earliest separations of Cd-binding complexes from fission yeast showed good resolution of HMW and LMW complexes. Several situations are found with plants, ranging from good resolution (Kneer and Zenk, 1992), partial resolution of the LMW complex on the trailing shoulder of an abundant HMW complex (Howden et al., 1995b), to no evidence for a LMW complex (Rauser and Meuwly, 1995). Such variations are attributed to a combination of differences between organisms, type of nutrient medium for growth, concentration of Cd, exposure time, preparation of the extract, the gel-filtration matrix, and column bed dimensions. In plants the HMW complex is more abundant at the times examined and therefore has been the component extensively characterized. Because of the potential that HMW and LMW complexes are located in different cell compartments (see below), it becomes imperative to evaluate the abundance and characteristics of both complexes as plants react to excess Cd. In the case of Cd-hypersensitive *cad1* mutants of Arabidopsis the plants had no ability to make HMW complex and only small amounts of LMW complex or none at all (Howden et al., 1995b).

A variety of γ-Glu-Cys peptides participate in binding Cd to form accretions of soluble HMW complex. In the Cd-binding complexes from the yeasts *S. pombe* and *C. glabrata* the n_{2-4} oligomers of (γ-Glu-Cys)$_n$-Gly predominated with lesser concentrations of (γ-Glu-Cys)$_{2,3}$. In addition, the HMW complex from *S. pombe* contained acid-labile sulfide. In *Brassica juncea* and tomato plants grown in 100 μM Cd acid-labile sulfide predominated in the HMW complex (S^{2-}:Cd molar ratio 0.15–1) with less in the LMW complex (Reese et al., 1992; Speiser et al., 1992). Sulfide accumulation in the HMW complex is not just a manifestation of plants grown in high concentrations of Cd. Maize roots exposed to 3 μM Cd produced HMW complex with acid-labile sulfide (S^{2-}:Cd molar ratio 0.18) at all times during 1- to 7-d exposures (Rauser and Meuwly, 1995). Those HMW complexes from yeasts with S^{2-}:Cd molar ratios greater than 0.4 appeared as dense aggregates of 20-Å-diameter particles. The x-ray diffraction patterns indicated the presence of a CdS crystallite, where about 80 CdS units were coated by about 30 molecules of γ-Glu-Cys-Gly, (γ-Glu-Cys)$_2$-Gly, and (γ-Glu-Cys)$_2$ (Dameron et al., 1989). Those fractions of tomato Cd-binding complex with high acid-labile sulfide contained small CdS crystallites (Reese et al., 1992). This formation of crystallites is an example of biomineralization where biopolymers (i.e. γ-Glu-Cys peptides) provide an ordered structure to one component (i.e. Cd) so that another constituent (i.e. sulfide) can be added to generate a specific crystal. Structural analysis of an abundant *Rauvolfia* Cd-binding complex containing various (γ-Glu-Cys)$_n$-Gly peptides and low acid-labile sulfide (S^{2-}: Cd molar ratio 0.01) showed that a Cd atom was in an immediate environment of four sulfur atoms (Strasdeit et al., 1991). The coordination consisted of Cd(SCys)$_4$ centers with a Cd-S bond length of 2.52 $\pm$ 0.02 Å. The peptide sulfhydryl:Cd molar ratio in the soluble complex was 3.78. When the complex was intentionally saturated with CdSO$_4$ the material precipitated, the peptide sulfhydryl:Cd molar ratio changed to 1.01, and a Cd atom was coordinated by one sulfur and three to four O and N atoms. The sulfide-containing HMW complex from 1- to 7-d exposures of maize roots had peptide sulfhydryl:Cd molar ratios of 1.01 $\pm$ 0.07, as if the maximal amount of Cd was bound by the minimal amount of peptide thiol while remaining soluble (Rauser and Meuwly, 1995). Detailed studies, perhaps by extended x-ray absorption fine structure, are required to determine the interactions among Cd, sulfide, and γ-Glu-Cys peptides in native HMW complexes from maize and other sources.

The Cd-binding complexes from several plant sources are composed of (γ-Glu-Cys)$_n$-Gly, the n_3 and n_4 oligomers being most abundant, with additional unidentified thiols present in some. In soybean (*Glycine max*) the $n_{1,2,3,4}$ oligomers of (γ-Glu-Cys)$_n$-β-Ala formed the Cd-binding complex. The HMW complex in maize was formed by peptides from three families: (γ-Glu-Cys)$_n$-Gly, (γ-Glu-Cys)$_n$, and (γ-Glu-Cys)$_n$-Glu (Rauser and Meuwly, 1995). The quantities of individual γ-Glu-Cys peptides in the maize complex were compared to the total quantities of the same peptides obtained by acid extraction of roots. At pH less than 3.5 Cd-binding complexes break apart and γ-Glu-Cys peptides are completely solubilized while extensive protein denaturation occurs. The comparisons revealed that during the 1- to 7-d exposure the γ-Glu-Cys peptides in the HMW complex were a subset of the total peptides in maize roots. For instance, the n_2 oligomers of any of the three γ-Glu-Cys peptides were sparse in the complexes, whereas increasing percentages, although never 100, of the n_3 and n_4 oligomers formed the HMW complex. If these circumstances are found to hold in other tissues and plants, it means that measurement of total γ-Glu-Cys peptides in acid extracts is an unreliable measure of their participation in binding Cd, even if corrections are made for glutathione and other monothiols not involved in complexes. The same concern applies even if nonprotein thiols are the measure of choice. Equating total γ-Glu-Cys peptide analyses with binding of Cd or other metals is common. The only way to assess the function of metal binding by the metal-induced γ-Glu-Cys peptides seems to be quantitative isolation of the complexes and then determination of the constituent peptides. Clarification is required about those γ-Glu-Cys peptides that do not occur in the HMW complex of maize roots. Are they peptides in the process of becoming larger oligomers

or do they participate in forming the putative LMW complex not yet demonstrated in maize?

Cellular Localization

The enzymes producing γ-Glu-Cys, γ-Glu-Cys-Gly, and γ-Glu-Cys-β-Ala operate in the cytosol and chloroplasts. The enzyme γ-glutamylcysteine dipeptidyl transpeptidase causing chain elongation of γ-Glu-Cys peptides is readily solubilized (Grill et al., 1989), as are the HMW and LMW Cd-binding complexes from all sources. The cytosol and vacuoles are therefore the prime cellular compartments where Cd-binding complexes would be located. Vögeli-Lange and Wagner (1990) isolated mesophyll protoplasts from tobacco exposed to Cd and showed that the vacuoles contained 110 ± 8% of the protoplast Cd and 104 ± 8% of the protoplast (γ-Glu-Cys)$_n$-Gly. These workers envisaged synthesis of (γ-Glu-Cys)$_n$-Gly in the cytosol with transfer of Cd and peptides, perhaps as a complex, across the tonoplast into the vacuole where peptides and organic acids chelated Cd.

Insight into the cellular location of HMW complex comes from studies with *S. pombe*. A mutant defective in producing HMW complex was more sensitive to 100 μM Cd than wild-type strains (Mutoh and Hayashi, 1988). The gene *hmt1* complementing a mutant *S. pombe*, also deficient in HMW complex and yet able to produce phytochelatins, was isolated and found to share sequence identity with the family of ATP-binding cassette-type transport proteins (Ortiz et al., 1992). Yeast strains harboring an *hmt1*-expressing multicopy plasmid exhibited enhanced resistance to Cd compared to wild-type strains and accumulated more Cd intracellularly while HMW complex was formed. The native HMT1 polypeptide was part of the vacuolar membrane (Ortiz et al., 1995). This protein transported apophytochelatins and LMW and HMW Cd-binding complexes in an ATP-dependent manner into vacuolar vesicles. The sulfide-poor LMW complex was transported more efficiently than the sulfide-rich HMW complex; however, the HMT1 polypeptide did not transport Cd^{2+}. The electrochemical potential generated by the vacuolar ATPase did not drive transport of peptides or complexes. The model shown in Figure 2 incorporates what these workers envisaged. Phytochelatins synthesized in the cytosol combine with Cd to form the LMW complex that is moved across the tonoplast by an ATP-binding cassette-type transporter. Once inside the vacuole more Cd, transported by a Cd^{2+}/H^+ antiporter, along with sulfide are added to the complex to produce HMW complex. The sulfide-rich HMW complex would be more stable in the acidic vacuole and have a higher Cd-binding capacity than the LMW complex. In this model the LMW complex functions as a cytosolic carrier and the vacuolar HMW complex is the major storage form of cellular Cd. The source of sulfide for the HMW complex (Fig. 2) has been attributed to purine metabolism (Juang et al., 1993).

The model in Figure 2 is supported by data from oats in two crucial ways. Tonoplast vesicles from oat roots have a Cd^{2+}/H^+ antiporter (Salt and Wagner, 1993) and show MgATP-dependent transport of phytochelatins and Cd-
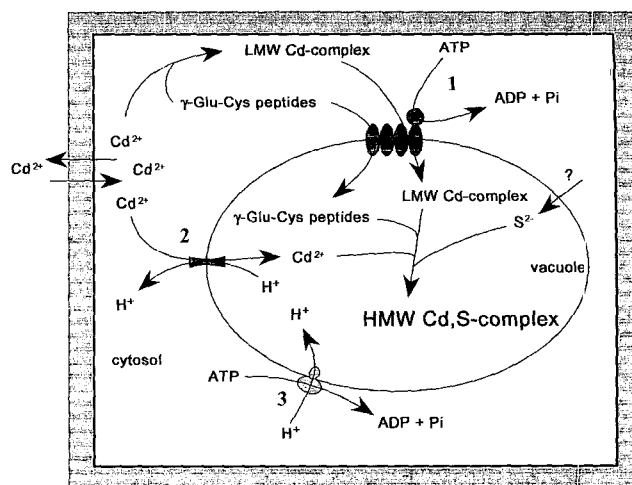
Figure 2. A model of the intracellular location of Cd-binding complexes and associated transport mechanisms. The model is adapted from Ortiz et al. (1995) and augmented with the observed transport of apo-γ-Glu-Cys peptides. Numbers 1, The ATP-binding cassette-type transport activity at the tonoplast (Ortiz et al., 1995; Salt and Rauser, 1995); 2, the Cd^{2+}/H^+ antiport (Salt and Wagner, 1993); and 3, the vacuolar-type ATPase generating a proton gradient. Mechanisms involved in the arrival of sulfide and the traffic of Cd across the plasmalemma are unclear.

phytochelatin complex (Salt and Rauser, 1995). The latter shared properties with the superfamily of ATP-binding cassette-type transporters. Peptide transport was not driven by the electrochemical potential generated by the vacuolar ATPase. Figure 2 shows an alternate route where γ-Glu-Cys peptides formed in the cytosol are transported into the vacuole as apopeptides (Ortiz et al., 1995; Salt and Rauser, 1995) and there combine with Cd and sulfide to form the HMW complex. Whether LMW and HMW complexes in plants are compartmentalized as depicted in Figure 2 and are of the same peptide composition await direct evaluation. Movement of the presumed Cd^{2+} species across the plasmalemma requires study.

Extent of Cd Complexation

Gel-filtration analyses of alkaline extracts from roots, cultured plant cells, and algae exposed to a variety of Cd concentrations for different times show that usually in excess of 90% of the buffer-soluble Cd occurs as Cd-binding complex and that free Cd^{2+} is absent. It may be concluded that the Cd in the cells is essentially totally complexed by γ-Glu-Cys peptides. To model the speciation of Cd in tissues or cells, however, it is necessary to quantitate the Cd-binding complex in relation to the total Cd in the tissue. In the initial extract of *Silene vulgaris* roots and cultured cells of *Rauvolfia*, 90 and 97%, respectively, of the Cd occurred as Cd-binding complex during gel filtration (Verkleij et al., 1990; Kneer and Zenk, 1992), and yet when based on the total tissue Cd the proportion was 52%. It is possible that this value is an underestimate because the initial extract comprised an unstated part of the total Cd. In maize roots the initial extract contained 63 to 74% of the

root Cd, and yet after six sequential extractions 92 to 94% of the tissue Cd was buffer soluble. In these combined extracts 19% of the root Cd was found as HMW complex 1 d after exposure to Cd and increased to 59% by d 7 (Rauser and Meuwly, 1995). Since the procedure used did not account for the putative LMW complex, the above values are probably also underestimates. Better estimates of the quantities of Cd in tissues or cells sequestered in LMW and HMW complexes would reveal whether additional mechanisms participate in Cd retention. These are fundamental aspects to modeling Cd speciation within plants.

Metal Tolerance

The term metal tolerance is used in different ways by various workers, leading to some difficulties in acceptance of interpretations. Figure 3 shows ideal dose-response curves. Curve B-D is the reaction of a normal, wild-type plant to an essential metal (e.g. Cu, Zn) and shows deficient, adequate, and toxic zones. Curve A-D is the reaction of a normal plant to a nonessential metal (e.g. Cd) and exhibits only adequate and toxic zones. The adequate zone may be specified by the low and high concentrations that cause growth reductions of 5 to 10% from maximum. This is analogous to plant mineral nutrition for which yield is plotted against metal concentration in tissue and the adequate zone lies between the concentrations for critical deficiency and critical toxicity (Bouma, 1983). Adequate zones are windows of concentration (e.g. w1 through w5 in Fig. 3) that vary as the toxicity thresholds change for the organism and nutrient concerned. Response curves B-D and B-E apply to Cu and Zn in different ecotypes of *S. vulgaris* and other species (Macnair, 1993). Response curves A-D and A-E apply to Cd in different ecotypes of *S. vulgaris* and *Holcus lanatus* and between nonselected and selected cultured cell lines of *Datura innoxia* and tomato (Macnair, 1993; Chen and Goldsbrough, 1994; De Knecht et al., 1994). The ecotypes with enlarged adequate zones (e.g. w3 in curve B-E and w4 in curve A-E) are mostly from metal-contaminated sites, those with smaller zones (e.g. w1 and w2) from normal sites. Increases in toxicity thresholds of Cd, Cu, and Zn are attributed to tolerance mechanisms

Figure 3. Ideal plots of growth as a function of the concentration of essential and nonessential metals. The deficiency zone (def.) applies only to essential metals. The concentrations encompassing maximal growth are the variable adequate zones w1 to w5; at higher concentrations toxicity effects arise.

rather than through avoidance by metal exclusion from the plant. Since the responses to varying applications of metal are continuous rather than discrete, there are degrees of tolerance, reflected as variable adequate zones or tolerance zones w1 through w5. Those plants with enlarged tolerance zones (e.g. w3 and w4) are traditionally called metal-tolerant ecotypes. By comparison the normal, wild-type plants are sensitive or nontolerant ecotypes. The sensitive designation is consistent with the accepted term hypersensitive. The *cad1* and *hmt1* mutants of Arabidopsis and *S. pombe*, respectively (Ortiz et al., 1992; Howden et al., 1995b), are Cd-hypersensitive strains with response curve A-C where the tolerance zone w5 is smaller than that of the wild type (w2, curve A-D). Mutants hypersensitive of an essential metal are unlikely to survive. The *S. pombe* strains harboring an *hmt1*-expressing multicopy plasmid making them more tolerant of Cd (Ortiz et al., 1992, 1995) would have a growth curve approaching A-E with a tolerance zone larger than w2 for the wild-type strains. Difficulty with the term metal tolerance arises from one's view of the normal, wild-type response. From recent work with organisms where the size of the tolerance zone can be manipulated by insertion or deletion of genetic material, the normal, wild-type organism can be viewed as tolerant compared to hypersensitive variants (e.g. w2 > w5), making those with enlarged tolerance zones (e.g. w4) hypertolerant. It is proposed that the terms hypersensitive, sensitive, and tolerant be used to describe the organisms responding with curves A/B-C, A/B-D, and A/B-E, respectively. Hypersensitive and sensitive organisms are still nontolerant. The term differential metal tolerance (Macnair, 1993) specifies comparisons between any pair of response curves in Figure 3.

Identification of differential metal tolerance in plants depends on assays of root growth, itself governed by an unspecified number of characters. Genetic analyses have consequently focused on genes with large individual effects. In *H. lanatus*, *Agrostis capillaris*, *Chlamydomonas reinhardtii*, *M. guttatus*, and *S. vulgaris* one or two major genes give differential tolerance to arsenate, Cd, or Cu with other genes as modifiers (Macnair, 1993). No locus of differential tolerance has been isolated and cloned.

Explanations for differential metal tolerance among naturally occurring selected ecotypes have been sought on the basis of γ-Glu-Cys peptides because of their propensity to bind metals. Extensive and critical comparisons of phytochelatin concentrations in complete dose-response curves were made for populations of *S. vulgaris* with differential tolerance of Cu, Zn, or Cd (De Knecht et al., 1994, and refs. therein). Concentrations of nonprotein thiols or of phytochelatins were the same in populations with small and large tolerance zones or even higher in the populations with a small tolerance zone, the so-called sensitive ecotypes. In the case of Cu and Cd the analyses were for the apical portion of roots, the regions actually exhibiting differential growth. It was concluded that differential synthesis of phytochelatins (i.e. elevated production) was not instrumental in producing differential metal tolerance. The
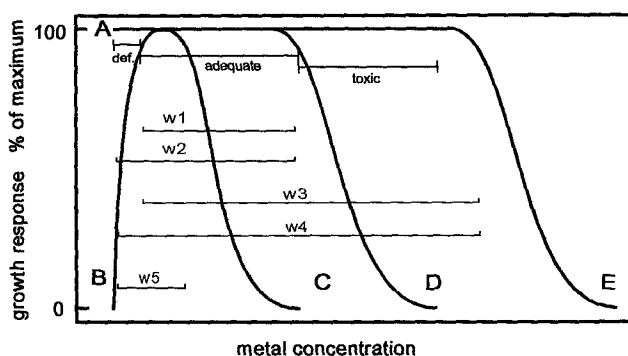
Cd-binding complex in entire roots of *Silene* was seen as a sink for excess Cd rather than the cause of differential tolerance.

The Cu- and Cd-binding complexes characterized for whole roots originate from predominantly mature tissue. In the absence of direct evidence, it is assumed that metal complexation is the same in the apical portion of roots as in the mature parts. Quantitative measurements of metal-binding complexes in root apices are not available for ecotypes exhibiting differential metal tolerance. Vacuoles occupy a lesser volume in root apical cells than in mature cells. In poorly vacuolated cells the HMW Cd-complex and putative storage form (Fig. 2) would be of lesser import than the LMW complex. Evaluating the extent of Cd chelation through LMW and HMW complexes in apices of ecotypes exhibiting differential Cd tolerance would address the functional aspect of γ-Glu-Cys peptides, aspects not probed by the available measurements of concentrations of thiol-rich peptides in apices.

The necessity of Cd complexation through the HMW complex to growth is undisputed for Cd-exposed Arabidopsis, cultured plant cells, and the fission yeast *S. pombe*. Absence of the HMW complex, either through mutation or inhibition of γ-Glu-Cys peptide synthesis by buthionine sulfoximine in otherwise Cd-resistant cell lines, markedly decreases the tolerance zone for Cd (Mutoh and Hayashi, 1988; Ortiz et al., 1992; Chen and Goldsbrough, 1994; Howden et al., 1995b). The γ-Glu-Cys peptides function as a sink for chelation of excess metal.

FUTURE DIRECTIONS

The diverse knowledge of metal-binding γ-Glu-Cys peptides has fostered some understanding of their role in plants and certain yeasts. The models devised for the nonessential element Cd may have to be tested directly for different metals and organisms. Further development of techniques, such as miniaturization and quantitation of specific complexes in various plant tissues, will enhance progress toward evaluating the actual functional import of the γ-Glu-Cys peptides in cellular metal sequestration. Elucidating the nature of the gene for differential metal tolerance and the connection with the gene for dipeptidyl transpeptidase may offer additional tools for amelioration of metal impacts in food production and bioremediation of contaminated soils.

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