REVIEW ARTICLE

Plant metallothioneins

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INTRODUCTION

The name metallothionein was first used to describe a protein isolated from equine renal cortex in 1957 that contained large amounts of sulphur and cadmium (Margoshes and Vallee, 1957; Kägi and Vallee, 1960). During the ensuing three decades, structurally related proteins were identified in diverse organisms and shown to associate with several metal ions, most commonly Zn^{2+} and Cu^{+} (or Cd^{2+} if Cd^{2+}-intoxicated) (Kägi and Kojima, 1987). Metallothioneins are thought to sequester excess amounts of certain metal ions although precise functions for most of these molecules remain the subject of debate. The specific metals sequestered by metallothioneins vary for the structurally distinct proteins/polypeptides occurring in different organisms (reviewed in Kägi, 1991). Roles in the regulation of gene expression have been proposed for some metallothioneins, in particular those in higher eukaryotes which co-ordinate Zn^{2+} and show programmed expression during development. Animal metallothionein genes respond to endogenous factors, which include a variety of hormones, second messengers, growth factors and cytokines, in addition to trace-metal levels. A proposed antioxidant role (Thornalley and Vasak, 1985) is supported by recent evidence that DNA strand-breakage, induced by oxidative stress, is reduced in the presence of elevated metallothionein levels and enhanced in Chinese hamster cells expressing a metallothionein antisense construct (Chubatsu and Meneghini, 1993). A wealth of information exists concerning the structure and regulation of expression of animal and fungal metallothioneins. These molecules are the subjects of a dedicated volume of Methods in Enzymology (Riordan and Vallee, 1991) and several articles thoroughly review the literature concerning animal and fungal metallothioneins and the genes encoding them (Karin, 1985; Hamer, 1986; Palmiter, 1987; Kägi and Schäffer, 1988; Thiele, 1992).

In 1985, it was reported that the major Cd^{2+} ligands in Cd^{2+}-intoxicated plant cells are composed of poly(γ-glutamyl-cysteinyl)glycine (Grill, 1985; Grill et al., 1985; Bernhard and Kägi, 1985; Robinson et al., 1985). These polypeptides, and other γ-glutamyl isopeptides in which Gly is either absent or substituted with β-alanine, are designated class III metallothioneins (Kojima, 1991). These compounds were first identified and characterized in the fission yeast *Schizosaccharomyces pombe* and termed cadystins (Murasugi et al., 1981; Kondo et al., 1984). Similar polypeptides were subsequently purified from plant cell cultures and termed phytochelatins (Grill et al., 1985). These class III metallothioneins have now been found in certain fungi and a broad spectrum of plant phyla (Grill, 1989). The structure, biosynthesis and proposed functions of these polypeptides have previously been reviewed (Tomsett and Thurman, 1988; Rauser, 1990; Steffens, 1990; Robinson, 1990; Jackson et al., 1990) and only work which postdates these reviews is described in any detail here. The principal focus of this article is a growing body of literature describing gene-encoded plant metallothioneins (see Figure 1). A brief section on metallothioneins in other organisms is included to provide a context for discussion of plant metallothioneins.

METALLOTHIONEINS IN OTHER KINGDOMS

Mammalian metallothioneins are composed of approx. 61 amino acids with M_rs of 6000–7000. They contain no aromatic amino acids and 20 Cys residues that co-ordinate seven divalent metal ions (or 12 monovalent ions such as Cu^{+}; Nielson and Winge, 1984) in two distinct metal clusters. The locations of the Cys residues in mammalian metallothioneins are invariant, and proteins from any phyla (for example *Neurospora crassa*, quoted in Münger et al., 1987; and *Agaricus bisporus*, quoted in Münger and Lerch, 1985) that have similar primary structures are designated class I metallothioneins. Class II metallothioneins are low-M_r Cys-rich metal-binding proteins, but the distribution of Cys residues does not correspond to that in mammalian metallothioneins. These proteins have been identified in cyanobacteria, yeast, the nematode *Caenorhabditis elegans* and a higher plant (wheat germ E_c protein) (cited in Kägi, 1991).

Synthesis of metallothionein increases following exposure to elevated concentrations of Cu^{+} and Ag^{+} in fungal cells (Karin et al., 1984; Fürst et al., 1988), Cd^{2+} and Zn^{2+} in cyanobacteria (Olafson et al., 1988) [although other metals also stimulate increases in the abundance of metallothionein transcripts in cyanobacteria (Huckle et al., 1993)], and a range of trace metals including the ionic species of cadmium, zinc, copper, mercury, gold, silver, cobalt, nickel and bismuth in animals (cited in Kägi, 1991). Induction is primarily regulated at the level of gene transcription. *Cis*-acting metal-regulatory elements of animal and fungal metallothionein genes are known (for reviews see Hamer, 1986; Palmiter, 1987; Theile, 1992) and the first reports describing the use of such elements to control the expression of foreign genes in transgenic animals (Palmiter et al., 1982, 1983) are widely cited. *Trans*-acting metal-responsive factors have been identified (Labbé et al., 1991, and citations therein), purified (Labbé et al., 1993) and cloned from animals (Radtke et al., 1993), cloned from cyanobacteria (Huckle et al., 1993; Morby et al., 1993) and cloned and structurally characterized in yeasts (Dameron et al., 1991, and citations therein). Accumulation of metallothionein in response to elevated metal ion concentrations, combined with its association with these ions, indicates a role in the sequestration of excess metal.

Hypersensitivity to elevated trace-metal concentrations has been observed in fungal (cited in Hamer, 1986) and prokaryotic (Turner et al., 1993) cells with deleted metallothionein genes,

Abbreviations used: EDDHA, *NN'*-ethylenebis-[2-(2-hydroxyphenyl)glycine]; ABA, abscisic acid; GST, glutathione S-transferase; BPDS, bathophenanthrolinedisulphonic acid; BCDS, bathocuproinedisulphonic acid.

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Type 1
.MSGCGCGSSCNCGDSCKCNKRSSGL.SYS.EMETTETVILGVGPA.KIQFEGAEMS.AASEDG.GCKCGDNCTCDPCNCK   Pea1
..MSCSCGSSCGCGSSCKCGKKYPDLEETSTAAQ..PTVVLGVAPEKKAAPEFVEAAAESGEAAHGCSCGSGCKCDPCNC  Maize2
..MSCSCGSSCGCGSNCNCGKMYPDLEEKSGATMQVTVIVLGVGSAKVQ....FEEAAEFGEAAHGCSCGANCKCNPCNC  Barley3
MSSGCSCGSGCKCGDNCSCSMYPDM......ETNTTVTMIEGVAP.LKMYSEGSEKSFGAEGGN.GCKCGSNCKCDPCNC  Mimulus4
    C C   C C   C C                                                 C C   C C  C C

Type 2
MSCCGGNCGCGSSCKCGNGCGGCKMYPDLSYT.ESTTTETLVMGVAPV.KAQFEGAEMG.VPAENDGCKCGPNCSCNPCTCK  Soyabean5
MSCCGGNCGCGSGCKCGNGCGGCKMYPDMSFS.EKTTTETLVLGVGAE.KAHFEGGEMGVVGAEEGGCKCGDNCTCNPCTCK  Castorbean6
MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESN.NAESDACKCGSDCKCDPCTCK  Arabidopsis7
  CC   C C   C C   C  C                                                C C   C C  C C

Barley B22E8
MSCCGGKCGCGAGCQCGTGCGGCKMFPDVEATAGAAAMVMPTASHKGSSGGFEMAGGETGGCDCATCKCGTRAAAPAAAASEPAPGRPAGRGEHEDERRTSNTNQAPSPSPSYHQ
  CC   C C   C C   C  C                                         C C  C C

Wheat Ec9
MGCDDKCGCAVPCPGGTGCRCTSARSGAAAGEHTTCGCGEHCGCNPCACGREGTPSGRANRRANCSCGAACNCASCGSATA
  C   C C   C     C C              C C   C C  C C                C C   C C  C
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Figure 1 Sequences of the predicted products of plant genes with similarity to metallothionein genes, and of the E_c protein, emphasizing the distribution of Cys residues (appended in red).

Sources: [1]Evans et al. (1990); [2]de Framond (1991); [3]Okumura et al. (1991); [4]de Miranda et al. (1990); [5]Kawashima et al. (1991); [6]Weig and Komor (1992); [7]Takahashi (1991); [8]Klemsdal et al. (1991); [9]Kawashima et al. (1992). The predicted proteins are separated into two groups (type 1 and type 2) based on the arrangement of Cys residues. The E_c protein and a second predicted protein from barley deviate from these two patterns.

while amplification of metallothionein genes has been observed in animal (Beach and Palmiter, 1981; Crawford et al., 1985) fungal (Fogel and Welch, 1982; Karin et al., 1984) and prokaryotic (Gupta et al., 1992, 1993) cells, selected for enhanced tolerance to certain trace metal ions. The phenotype of *Saccharomyces cerevisiae* cells lacking the metallothionein gene, *CUP1*, indicates that this gene performs no essential role(s) in cell growth, differentiation or 'normal' copper metabolism. These mutants grow with normal doubling times in standard low-copper media, and are capable of mating, diplophase growth, sporulation, germination, accumulation of copper and accumulation and activation of copper-dependent superoxide dismutase (cited in Hamer, 1986). *CUP1*-deficient cells are hypersensitive to elevated concentrations of copper. Sequestration of excess copper may be an exclusive role for metallothionein in *S. cerevisiae*. Similarly, cyanobacterial mutants deficient in the metallothionein locus, *smt*, are viable, but show reduced tolerance to Zn^{2+} and Cd^{2+} implying exclusive, but non-essential, roles in the homoeostasis/metabolism of Zn^{2+} and detoxification of Cd^{2+} (Turner et al., 1993).

The observations that animal metallothioneins show programmed expression during development and respond to certain endogenous signals suggest an undefined role in cellular regulation. This role may be mediated by the association of animal metallothionein with Zn^{2+}. More than 300 known enzymes, with representatives from all six recognized categories, and 200 known DNA-binding proteins require this divalent ion (cited in Vallee, 1991). While animal metallothionein has a high affinity for Zn^{2+}, the associated metal ions are also highly labile with rapid Zn^{2+} exchange between metallothionein molecules in solution (cited in Vallee, 1991). Animal metallothionein may thus serve as a Zn^{2+} store suited to the donation of metal ions when and where required.

Mammalian thionein (apo-metallothionein) can inactivate the Zn^{2+}-requiring transcription factor Sp1 (human) and can also acquire Zn^{2+} from *Xenopus laevis* transcription factor IIIA *in vitro* (Zeng et al., 1991a,b). In cultured adult rat hepatocytes, metallothionein accumulates in nuclei at early S-phase, but not at G_0 or G_1 when the protein is extranuclear (Tsujikawa et al., 1991). Modulation of animal thionein biosynthesis, or intracellular distribution (nuclear or extranuclear), could affect DNA binding by Zn^{2+}-requiring transcription factors and thereby regulate the activity of a large subset of genes (Zeng *et al.*, 1991a,b).

THE E_c PROTEIN FROM WHEAT GERM

At present, the wheat E_c protein is the only plant protein that can be unequivocally designated a metallothionein (Hofmann et al., 1984; Lane et al., 1987; Kawashima et al., 1992). While 'metallothionein-like' plant genes have been isolated from other species (Figure 1), and data indicate that these genes have roles in metal metabolism (see below), their translational products remain to be purified from plant material and sequenced.

The E_c protein is the dominant site of Cys incorporation during early germination, prior to degradation of mRNA species stored in the dry seed. A large proportion (20–25%) of radiolabelled [^{35}S]Cys is incorporated into a single protein following *in vitro* translation of bulk mRNA from dry wheat germ (Hanley-Bowdoin and Lane, 1983; Hofmann et al., 1984; Lane et al., 1987). The abundance of E_c mRNA declines rapidly post-imbibition and falls to imperceptible levels between 5 h and 10 h. In common with post-imbibition synthesis of other seed storage proteins, it is likely that synthesis of E_c is 'residual', resulting from a role during embryogenesis and subsequent survival of E_c transcripts in the desiccated seed (Hanley-Bowdoin and Lane, 1983).

The sequence of the E_c protein, purified from wheat germ, is shown in Figure 1. The location of the Cys residues, notably the presence of Cys-Xaa-Cys motifs, suggested that the protein may be capable of binding metal ions. Purified E_c protein was subsequently found to associate with Zn^{2+} at a stoichiometry (Zn^{2+}/protein) of approximately 5:1 (Lane et al., 1987). This led to its designation as a class II metallothionein (Kägi and Schäffer, 1988).

Recently, E_c genes have been isolated (Kawashima et al., 1992). They are located in single copies on the long arms of chromosomes 1A, 1B and 1D in hexaploid wheat, unlike animal metallothionein genes which are contained in multigene clusters (cited in Kawashima et al., 1992). An element with similarity to known abscisic acid (ABA)-responsive elements is located in the 5′ flanking region of wheat E_c genes but there are no clearly identifiable metal-responsive elements on the basis of sequence similarities (Figure 2). Northern blots confirm that E_c transcripts

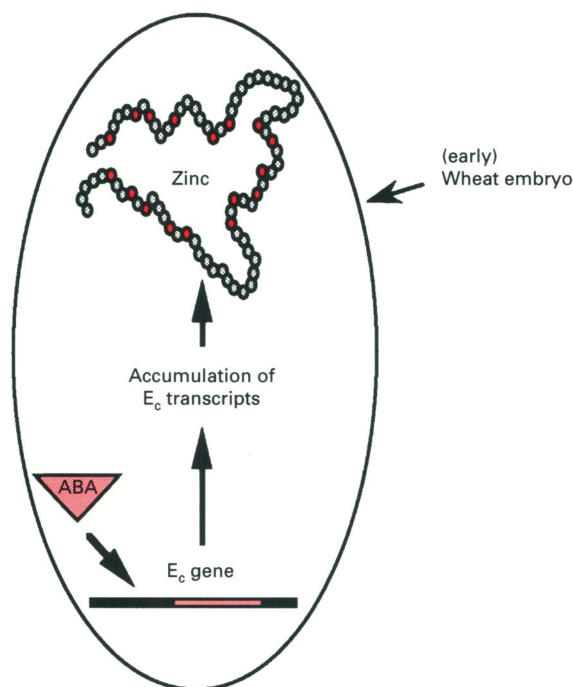

Figure 2 The E_c protein is expressed in early wheat embryos, binding Zn^{2+} and possibly modulating activity of Zn^{2+}-requiring proteins (after Kawashima et al., 1991)

E_c transcripts persist in the desiccated seed but rapidly decline at germination unless supplemented with ABA. The E_c gene and a metallothionein-like gene from barley (Klemsdal et al., 1991) contain consensus ABA-responsive elements, but in the latter exogenous ABA is known to repress expression during seed development. Cysteine residues in the protein are shown in red.

accumulate in immature embryos and the highest levels of E_c mRNA were detected at the earliest stages (15 days post-anthesis) of embryogenesis, shortly after the onset of rapid cell division and differentiation (Kawashima et al., 1992). Furthermore, the abundance of E_c mRNA increases following addition of ABA, but not Zn^{2+}, to germination media.

Kawashima et al. (1992) noted that wheat E_c genes are conspicuously expressed during embryogenesis under the control of endogenous factors, analogous to mammalian liver metallothionein genes. The hepatic concentration of metallothionein, primarily Zn^{2+}-thionein, is 20-fold greater in neonatal than in adult rats. Kern et al. (1981) observed that the deposition of animal metallothionein is generally allied with a shift between proliferative and differentiating stages of embryo development. This also applies to wheat E_c. A homoeostatic role that engages Zn^{2+}-E_c with Zn^{2+}-dependent DNA and RNA polymerases, as well as with Zn^{2+}-requiring *trans*-acting factors (Zn^{2+}-fingers; Zn^{2+}-twists; Zn^{2+}-clusters; refer to Vallee et al., 1991) has been proposed for E_c (Kawashima et al., 1992). Only a small proportion (approx. 5 %) of the total Zn^{2+} in mature wheat embryos is associated with E_c, suggesting that an alternative role in storage of Zn^{2+} for germination is less likely (Kawashima et al., 1992).

PLANT GENES WITH SIMILARITY TO METALLOTHIONEIN GENES

Genes that encode proteins with (some) sequence similarity to metallothioneins have been isolated from several plant species (Evans et al., 1990; de Miranda et al., 1990; Kawashima et al., 1991; Takahashi, 1991; Okumura et al., 1991; de Framond, 1991; Robinson et al., 1992). All of these genes encode predicted proteins with two Cys-rich domains containing Cys-Xaa-Cys motifs (where Xaa is an amino acid other than Cys). Computer-based searches select metallothioneins as the most similar known proteins. Comparison (DNASTAR software) of the predicted protein from *Mimulus guttatus* with sequences in the NBRF protein database identified 19 of the top 23 matches as metallothioneins (de Miranda et al., 1990). Similar analysis (Fast P software) of the predicted translational product of the pea gene, $PsMT_A$, also selected metallothioneins as the top 10 best matches (Evans et al., 1990). Matrix comparisons of the amino acid sequences of the predicted $PsMT_A$ protein and class I metallothionein from *Neurospora crassa* identify two regions of sequence similarity (amino acids 4–18 and 61–74 in the predicted pea protein), that correspond to the Cys-rich terminal domains. These two domains are linked by a central 'spacer' region of approx. 40 amino acids that is devoid of Cys residues. The Cys-rich terminal domains in the predicted sequences from different species are somewhat more conserved than the central 'spacer' regions (Figure 1).

Two categories of metallothionein-like proteins are proposed on the basis of the predicted locations of Cys residues and are designated types 1 and 2 (Figure 1). In type 1 there are exclusively Cys-Xaa-Cys motifs whereas in type 2 there is a Cys-Cys and a Cys-Xaa-Xaa-Cys pair within the N-terminal domain. Unlike the E_c protein, the translational products of these genes remain to be purified from plant tissue. Their discrimination from known Cys-rich plant proteins, such as leaf thioneins (Apel et al., 1990) and sulphur-rich prolamins (Shewry and Tatham, 1990), is based (primarily) upon the observed clustering of Cys residues to form these metallothionein-like domains although some other data support this distinction (see below). A second predicted protein sequence from barley (*Hordeum vulgare*) is also included in Figure 1.

Occurrence and isolation of plant genes with similarity to metallothionein genes

Several metallothionein-like plant genes have been isolated (by serendipity) via differential screening of cDNA libraries for root-abundant sequences (Evans et al., 1990; de Framond, 1991), sequences repressed by elevated copper (de Miranda et al., 1990) and sequences induced in response to depleted iron (Okumura et al., 1991). Sequences preferentially expressed in roots rather than other organs were isolated from cDNA libraries prepared from poly(A)$^+$ RNA from roots of garden pea (*Pisum sativum*) and maize (*Zea mays*) (Evans et al., 1990; de Framond, 1991). The corresponding genes, $PsMT_A$ and MT-L, were subsequently isolated from pea and maize genomic libraries respectively. These genes are members of small multi-gene families. Partial sequences of two further members of the pea gene family, $PsMT_B$ and $PsMT_C$, have been obtained following PCR-mediated cloning (Robinson et al., 1992). A related cDNA from barley, *ids-1*, was identified in a library prepared from root poly(A)$^+$ RNA isolated from plants grown under conditions of iron deficiency, following differential screening with probes prepared from poly(A)$^+$ RNA from iron-deficient and iron-sufficient roots (Okumura et al., 1991). A soyabean (*Glycine max*) sequence was isolated from a cDNA library following hybridization to a 21-mer oligonucleotide (5′ ATGGACCCCAACTGCTCCTGC 3′) that corresponds to a conserved region found at the N-terminus of mammalian metallothionein genes (Kawashima et al., 1991). Several of the above sequences have been used as probes to isolate clones containing homologues from other

species including tobacco (*Nicotiana tabacum*) (Robinson et al., 1992), alfalfa (*Medicago sativum*) (Robinson et al., 1992) and *Arabidopsis thaliana* (Takahashi, 1991) and to reveal cognates in several other higher plant species by Southern analysis.

The identification of metallothionein-like genes in representative monocotyledonous (e.g. barley, maize) and dicotyledonous (e.g. pea, *Mimulus*, soyabean) species suggests a broad species distribution, although an extensive survey of genera, similar to that conducted for class III metallothioneins (cited in Grill, 1989), remains to be reported.

Attempts to detect the translational products of metallothionein-like genes within plants

Low-M_r, metal-induced, metal ligands whose structures remain to be elucidated have been isolated from plants (see citations in Robinson and Jackson, 1986). Many partly characterized Cd^{2+} complexes have amino acid compositions consistent with class III metallothioneins, while several partly characterized copper complexes appear to be unlike these and are more similar to the products of metallothionein genes (Robinson and Jackson, 1986). It has been proposed that either (i) the presence of copper may lead to increased oxidation of thiol groups during purification, resulting in isolates of class III metallothionein containing large amounts of impurities, or (ii) plants produce metallothionein-like proteins, as well as small metal-binding polypeptides, but the proteins are more important in the metabolism of an essential metal such as copper than in Cd^{2+} detoxification (Robinson and Jackson, 1986).

In addition to characteristic class III metallothioneins, a low-M_r copper complex was also isolated from *Mimulus guttatus* which was unlike these polypeptides (Tomsett and Thurman, 1988; Salt et al., 1989; de Miranda et al., 1990). The amino acid composition and size of the purified polypeptide was similar to an average of the two terminal domains of the predicted product of the *Mimulus guttatus* metallothionein-like gene. It was proposed that the internal 'spacer' region of the predicted product of this gene may be removed to generate smaller metal-binding polypeptides composed of the Cys-rich terminal domains (de Miranda et al., 1990). Following expression of the pea gene $PsMT_A$ in *Escherichia coli*, proteolysis was observed within the equivalent region of recombinant $PsMT_A$protein (Kille et al., 1991; Tommey et al., 1991). Proteolytic cleavage within this region was further examined by exposure of purified $PsMT_A$ protein to proteinase K, followed by resolution of residual polypeptides by reversed phase f.p.l.c. (Kille et al., 1991). The amino acid composition of the residual polypeptides correlated with amino acids 2–21 and 56–75 from the Cys-rich N- and C-terminal domains of the full-length protein. Since cleavage within the $PsMT_A$ spacer region occurred in *E. coli*, it was proposed that this region may also act as a substrate for plant proteases (Kille et al., 1991). It is feasible that the spacer region could be important for folding the Cys-rich terminal domains into a conformation suitable for metal binding. Proteolysis of the spacer regions within plants may account for the failure of past attempts to isolate the native proteins.

The antigenic determinants for several vertebrate metallothioneins have been mapped close to the N-terminus within the region of residues 1–7 (cited in Katsuyuki et al., 1991). An epitope for many of these antibodies is a peptide region (amino acids 1–5) that includes the acetylated N-terminal Met. The epitope for antibody OAL-JI excludes the acetylated Met and is thought to include residues 3–7, i.e. Pro-Asn-Cys-Ser-Cys in vertebrate metallothionein. An equivalent heptapeptide occurs within the C-terminal region of the predicted product of the soyabean metallothionein-like gene, and a related tetrapeptide Asn-Cys-Thr-Cys is present within the C-terminal domain of the predicted product of the pea gene $PsMT_A$. OAL-JI showed strong cross-reactivity with antigens present in soyabean tissues and somewhat less reactivity with material from pea. OAL-JI is reported not to cross-react with wheat germ E_c protein or with protein extracted from *Mimulus guttatus* (Katsuyuki et al., 1991). However, full characterization of these antigens has not been reported and it thus remains to be established whether or not the antigens and the predicted products of the characterized metallothionein-like genes are synonymous.

Metal-binding characteristics of recombinant products of plant genes with similarity to metallothionein genes

The products of metallothionein genes from different organisms form conformations suitable for association with metal ions when expressed in *E. coli* and yeast (Romeyer et al., 1988, 1990; Jacobs et al., 1989; Kille et al., 1990; Silar and Wegnez, 1990). In the absence of purified native protein, the $PsMT_A$ gene was expressed in *E. coli* to facilitate examination of the metal-binding properties of its product (Tommey et al., 1991; Kille et al., 1991). Following growth in Cd^{2+}-supplemented media, cells expressing the $PsMT_A$ gene accumulated more Cd^{2+} than equivalent cells containing the vector (pPW1) alone (Kille et al., 1991). Increased accumulation of copper was also observed in *E. coli* cells expressing $PsMT_A$ as a C-terminal extension of glutathione S-transferase (GST) following growth in media supplemented with this metal (Evans et al., 1992). Such effects on metal accumulation have been used as a reliable indicator of intracellular production of metal–ligand complexes in *E. coli* (cited in Kille et al., 1991). The estimated $Cd^{2+}/PsMT_A$ stoichiometry ranged from 5.6 to 6.1 g-atoms of Cd^{2+} per mol of protein purified from *E. coli* (Kille et al., 1991). If all of these metal ions are associated with thiolate ligands (a total of 12 in the two Cys-rich domains) then the Cd^{2+}–Cys connectivities must differ from the pattern observed in other metallothioneins (Kille et al., 1991).

Estimations of the pH at which 50% of metal ions dissociate is a criterion used to distinguish metallothioneins from other metal-binding proteins (Vasák and Armitage, 1986). The pH of half-dissociation of Zn^{2+}, Cd^{2+} and copper ions from purified GST–$PsMT_A$ fusion protein was estimated to be 5.25, 3.95 and 1.45, respectively (Tommey et al., 1991). Comparison with equine renal metallothionein indicates that recombinant $PsMT_A$ protein has slightly lower affinities for Zn^{2+} and Cd^{2+}, but a slightly higher affinity for copper ions. Equivalent studies remain to be performed on the expressed products of the soyabean, castorbean and *Arabidopsis thaliana* sequences which contain Cys-Xaa-Xaa-Cys and Cys-Cys motifs that may modify metal binding. The metal-binding characteristics of the E_c protein and the product of a second related gene from barley also require further examination.

It is well documented that association with metal ions protects proteins from proteolytic degradation and this is consistent with the observation that residues 2–21 and 56–75 in the $PsMT_A$ protein are refractory to proteinase K (Kille et al., 1991). Despite cleavage within the central spacer region, the two Cys-rich domains appear to remain associated with each other indicating that the metal–protein bonds are capable of holding this portion of the cleaved molecule together. Based upon these observations, Kille et al. (1991) (tentatively) proposed a model (illustrated in Figure 3) for association of metal to $PsMT_A$, followed by proteolysis of the spacer region. Computer analysis of the encoded protein from *Mimulus gutattus* predicts extensive folding

Figure 3 A putative structure for $PsMT_A$ associated with metal ions

Adapted from Kille et al. (1991). Cysteine residues are shown in red.

of the Cys-rich terminal domains but an extended configuration for the 39-amino-acid intervening spacer region (de Miranda et al., 1990).

Evidence that the products of these metallothionein-like genes bind metal ions within plants has been obtained from studies with *Arabidopsis thaliana* transformed with a construct containing $PsMT_A$ under the control of the cauliflower mosaic virus 35 S (CaMV 35 S) promoter (Evans et al., 1992). In a segregating progeny, derived from a single transgenic F_1 parent expressing $PsMT_A$, 75% of individuals accumulated more copper than untransformed control plants.

Regulation of expression of plant genes with similarity to metallothionein genes

Information describing the expression of metallothionein-like plant genes is sparse (Table 1). Transcripts from type 1 genes are abundant in roots grown in nutrient solutions that have not been supplemented with supra-optimal concentrations of trace-metal ions (Evans et al., 1990; de Miranda et al., 1990; de Framond, 1991; Robinson et al., 1992). In pea plants *PsMT* transcripts were not detected in the embryonic radicle but increased in abundance during development of roots grown in hydroponic culture (Robinson et al., 1992). In soyabean and *Mimulus guttatus*, exposure to elevated concentrations of copper did not further increase transcript abundance in roots. Moreover, slight decreases in transcript abundance were observed in response to highly elevated copper concentrations (de Miranda et al., 1990; Kawashima et al., 1991). Transcripts encoded by the *ids-1* gene from barley were detected in roots grown under conditions of iron deficiency but not in roots supplemented with available iron (Okumura et al., 1991). The abundance of *PsMT* transcripts in pea roots also declines following supplementation with iron chelates (N. J. Robinson, unpublished work). The apparent constitutive expression of related genes observed in the roots of a number of species may correlate with iron deficiency since the stringent growth conditions required to maintain a supply of available iron, which depresses the activation of iron-efficiency mechanisms (Grusak et al., 1990), were not employed in these previous studies (Evans et al., 1990; de Miranda et al., 1990). Similarly, progressive increases in *PsMT* transcript abundance during root development will coincide with the activation of iron-efficiency mechanisms following depletion of iron stored (in plant ferritin; Laulhere and Briat, 1993) in the seed.

Table 1 Expression of genes with similarity to metallothionein genes in different plants

Refer to text for references.

	Transcript abundance											
	Localization			Elevated metal								
Plant	Root	Leaf	Seed	Zinc	Copper	Cadmium	Iron	Iron + copper	Hormone ABA	CXC only	ABA element	Metal binding
Pea	High	Low	Low	–	No	–	Down	Up	–	Yes	No	Cu > Cd > Zn (recombinant) (Cu, transgenic plants)
Mimulus	High	Low	–	Down	Down	Down	–	–	–	Yes	–	–
Maize	High	Low	Very low	–	–	–	–	–	–	Yes	No	–
Barley	High	–	–	–	–	–	Down	–	–	Yes	–	–
Arabidopsis	–	On	–	–	–	–	–	–	–	No	–	–
Castorbean	–	On	–	–	–	–	–	–	–	No	–	–
Soyabean	Low	High	–	–	Down	–	–	–	–	No	–	–
Barley2	–	–	High	–	–	–	–	–	Down	No	Yes	–
Wheat E_c	–	–	High	No	–	–	–	–	Up	No	Yes	Zn (native protein)

Table 2 Link between iron and copper metabolism in plants

There is an inverse correlation between iron availability, copper accumulation and copper and iron reduction by intact roots. Values in parentheses are S.D.s.

	Metal content of roots (nmol/g)		Metallo-reductase activity [μmol of Cu^{+}-BCDS (or Fe^{2+}-BPDS)/h per g]	
	Copper	Iron	Copper reduction	Iron reduction
Control	125(5)	89(5)	6.15(0.27)	3.29(0.3)
Fe-EDDHA	55(2)	160(11)	3.17(0.76)	1.36(0.11)

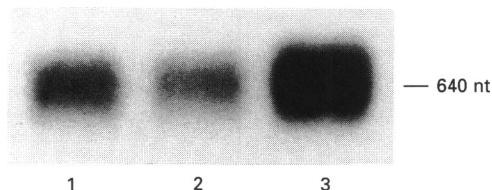

Figure 4 Northern blot showing that the abundance of *PsMT* transcripts in pea roots correlates with the concentration of copper in media supplemented with available iron chelate

Equivalent amounts of total RNA were resolved in each track and the resultant Northern blot was probed with $PsMT_A$. Total RNA was isolated from pea seedlings grown hydroponically in the presence of added Fe^{3+}-EDDHA (2 μM), plus: no added copper (lane 2), micronutrient copper (40 nM) only (lane 1), highly elevated copper (1000 nM) (lane 3) (A. M. Tommey, L. V. Kochian, R. M. Welch, J. E. Schaff, S. C. Schaeffer and N. J. Robinson, unpublished work).

A root surface ferric-chelate reductase (Fe^{3+} to Fe^{2+}) is expressed in pea plants grown under conditions of low available iron (Buckhout et al., 1989; Grusak et al., 1990). Inducible *trans*-plasma-membrane ferric reductases have been extensively characterized in yeast, where electrons are transferred from cytosolic NAD(P)H to extracellular ferric ions (Lesuisse et al., 1991; Dancis et al., 1992). Ferrous ions are then imported. In yeast, transcription of the *FRE1* gene, which encodes ferric reductase, increases under iron deficiency. The FRE1 reductase also reduces $Cu^{2+}$to Cu^{+} and appears to also be involved in the uptake of copper by *S. cerevisiae* (Lesuisse and Labbé, 1992). Correlative data indicating that pea root ferric-chelate reductase may similarly act as a cupric-chelate reductase and is implicated in copper acquisition includes the following: (1) increased cupric-chelate reductase activity coincides with increased ferric-chelate reductase activity under low-iron conditions (Table 2); (2) copper accumulation increases under low-iron conditions (Table 2; Evans et al., 1992); (3) cupric-chelate reductase activity increases in response to low copper (Welch et al., 1993); (4) ferric-chelate reductase activity increases coincident with the activation of cupric-chelate reductase activity under low-copper conditions (Welch et al., 1993); (5) iron accumulation increases under low-copper conditions (Welch et al., 1993). Expression of metallothionein-like genes under conditions of iron deficiency may be a direct response to concomitant increases in copper accumulation. Indeed, under conditions of high available iron, expression of *PsMT* transcripts correlates with the copper status of the growth media (Figure 4). Figure 5 summarizes these observations. According to this scheme, the products of type 1 metallothionein genes expressed under low-iron conditions chelate and thereby detoxify and store excess copper. However, if overaccumulation of copper in response to iron deficiency were also proposed to be the mediator of enhanced accumulation of *ids1* transcripts in barley, some other aspect of the iron-efficiency mechanism (other than inducible ferric-chelate reductase) would have to account for increased copper uptake (at least in graminaceous species such as barley). Graminaceous species have alternative iron-efficiency systems which do not include inducible ferric-chelate reductases (cited in Grusack et al., 1990).

Overaccumulation of copper in transgenic *Arabidopsis thaliana* containing $PsMT_A$ constructs (Evans et al., 1992) may be a reflection of constitutive expression of $PsMT_A$, whereas expression coupled to internal metal ion concentration may enhance tolerance without increasing metal accumulation. For example, net uptake of copper in wild type and *CUP1*-deleted *S. cerevisiae* is similar (Lin and Kosman, 1990). The metal tolerance of *CUP1*-deleted cells has additionally been examined following coupled (copper-induced) or uncoupled (constitutive) expression of the *Drosophila melanogaster* metallothionein gene, MTn (Silar and Wegnez, 1990). The correct coupling of metallothionein expression in this system is more effective in achieving a copper-resistant phenotype than uncoupled expression of the gene. In addition, a number of studies have shown that uncoupled expression of different metallothionein genes in *E. coli* mediates enhanced metal accumulation (Jacobs et al., 1989; Kille et al., 1990).

In soyabean, transcripts encoding the predicted metallothionein (type 2; Figure 1) are present in roots and leaves but are most abundant in leaves (Kawashima et al., 1991). Related cDNAs have been isolated from *Arabidopsis thaliana* and castor-bean libraries prepared from leaf poly(A)$^{+}$ RNA but the pattern of expression remains to be determined (Table 1). These observations raise the possibility that different members of a gene family, possibly encoding proteins with different metal-binding specificities, could be expressed in different organs or under different environmental conditions. Representatives of one group (type 1) appear to be expressed primarily in roots and are implicated in the metabolism or detoxification of copper, while others may be predominantly expressed in aerial tissues.

In maize, the abundance of MT-L transcripts in kernels is low (de Framond, 1991). In pea, *PsMT* transcripts are not detected in the embryonic radicle but transcripts of a slightly smaller size than those in roots are detectable in the embryonic cotyledon (Evans et al., 1990). Genes encoding analogues of the E_c protein may be preferentially expressed in seeds and are implicated in Zn^{2+} metabolism, possibly regulating the activity of Zn^{2+}-requiring factors as proposed by Kawashima et al. (1992), or serving as a storage form of Zn^{2+}. Related cDNAs from barley were isolated from libraries constructed from poly(A)$^{+}$ RNA from the aleurone/pericarp and embryo of developing grains and also from germinating scutella (Klemsdal et al., 1991). The sequence of the predicted B22E product is shown in Figure 1. B22E transcripts are repressed by ABA in developing seeds (Olsen et al., 1990) and the corresponding gene contains consensus abscisic acid response elements (Klemsdal et al., 1991). It

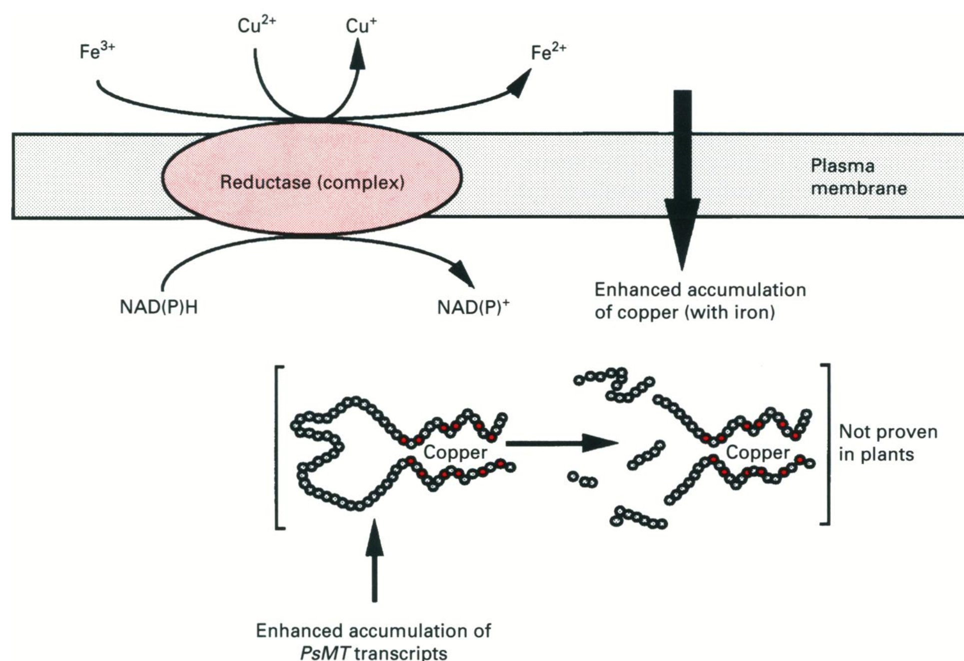

Figure 5 Hypothetical scheme linking iron availability, expression of root surface ferric-chelate reductase, copper accumulation and the expression of (type 1) plant genes with similarity to metallothionein genes

Under low-iron conditions there are coincident increases in root surface cupric-chelate reductase activity and copper accumulation. This scheme proposes that the ferric- and cupric-chelate reductases are synonymous. However, it is noted that the biochemical basis for enhanced copper accumulation under low-iron conditions remains to be established and that Cu^{2+} has previously been considered to be the transported form of copper in plants. Proposed proteolysis of the 'spacer' region of $PsMT_A$ within plants is also illustrated. Cysteine residues in metallothionein-like proteins are shown in red.

remains to be established whether or not the B22E product binds Zn^{2+} and is analogous to E_c in wheat.

In addition to an abscisic acid element, an element similar to that believed to be responsible for starchy-endosperm-specific expression of other cereal storage protein genes (Forde et al., 1985; Kreis et al., 1985) has also been detected in the B22EL8 gene, which encodes one class of B22E transcript (Klemsdal et al., 1991). Clearly, investigations are required to identify and characterize putative metallo-regulatory sequences in the flanking regions of type 1 genes, in addition to sequences responsive to endogenous signals (e.g. conferring spatial or temporal control of expression) such as the ABA elements in the E_c and B22EL8 genes (Klemsdal et al., 1991; Kawashima et al., 1992).

CLASS III METALLOTHIONEINS: γ-GLUTAMYL ISOPEPTIDES, CADYSTINS, PHYTOCHELATINS, POLY(γ-GLUTAMYLCYSTEINYL)GLYCINES

For more detailed discussions of these polypeptides, readers are referred to a number of reviews (Tomsett and Thurman, 1988; Rauser, 1990; Steffens, 1990; Robinson, 1990; Jackson et al., 1990). This section provides only a brief overview of these molecules and highlights some recent findings. Class III metallothioneins differ markedly from class I and II metallothioneins. They are enzymically derived and are most commonly composed of poly(γ-glutamylcysteinyl)glycine, (γ-Glu-Cys)$_n$Gly, where $n = 2$–11 depending on the organism, although the most common forms have $n = 2$–4 (Grill et al., 1986a). However, class III metallothioneins isolated from the Fabaceae contain β-alanine in the C-terminal position and these species produce predominantly homoglutathione (γ-glutamylcysteinyl-β-alanine) rather than glutathione (γ-glutamylcysteinylglycine) (Grill et al., 1986b). Des-glycine forms of class III metallothioneins have also been described (cited in Steffens, 1990).

Metal complexes containing these γ-glutamyl isopeptides have apparent native M_rs ranging from 2000 to 10000 depending upon the source and method of isolation, and include multiple polypeptides in a cluster (cited in Steffens, 1990; Rauser, 1990). Sulphide is sometimes present in Cd^{2+} complexes in varying amounts but has not been found in copper complexes (cited in Steffens, 1990; Rauser, 1990). High-M_r sulphide-containing complexes show enhanced affinity for metals (Reese and Winge, 1988). The structure of such complexes is of interest, being composed of a CdS quantum semiconductor crystallite core surrounded by polypeptides (Dameron et al., 1989). Sulphide-containing complexes have been described in *Candida glabrata* (Mehra et al., 1988), tomato (*Lycopersicon esculentum*) (Reese et al., 1992) and a selenium-tolerant wild mustard (*Brassica juncea*) (Speiser et al., 1992a). Metal-tolerant *Silene vulgaris* (Verkleij et al., 1990) and cell cultures of *Datura innoxia* (Robinson et al., 1990) incorporate greater amounts of S^{2-} into these complexes than their less tolerant counterparts. Most recently it has been observed that enzymes involved in purine biosynthesis are required for the introduction of S^{2-}into these complexes in *S. pombe* (Speiser et al., 1992b). The link between these two aspects of metabolism remains to be established.

Biosynthesis

Structural similarities between glutathione and class III metallothioneins suggest that the latter are synthesized from the former or its precursors. *In vivo* experiments demonstrate a significant

reduction of free glutathione upon exposure of plant cell cultures to Cd^{2+} (cited in Rauser, 1990; Steffens, 1990). Pulse–chase experiments, where the cellular glutathione pool is tagged with ^{35}S, show loss of radiolabel from glutathione with a concomitant increase in radiolabelled class III metallothioneins (Berger et al., 1989). Treatment of cell cultures with buthionine sulphoxamine, a potent inhibitor of γ-glutamylcysteine synthetase, results in the loss of metal tolerance and an inability to synthesize class III metallothioneins (Grill et al., 1987). In addition, mutants of the fission yeast deficient in enzymes of glutathione synthesis are unable to produce class III metallothioneins and are hypersensitive to Cd^{2+} (Mutoh and Hayashi, 1988).

There are several alternate pathways that might produce class III metallothioneins from glutathione or γ-glutamylcysteine. It has been reported that class III metallothioneins are synthesized from glutathione by the enzyme γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase) in *Silene cucubalus* cell suspension cultures (Grill et al., 1989). The M_r of the native protein was reported to be 95000; the protein is composed of four subunits, each with an M_r of approximately 25000. Enzyme activity is dependent upon the presence of metal ions, with addition of EDTA or apopeptides to reaction mixtures terminating synthesis (Löffler et al., 1989). The mechanism of biosynthesis requires two glutathione molecules or one glutathione plus a previously synthesized class III metallothionein molecule. The transfer of the γ-glutamylcysteine moiety of glutathione to another glutathione molecule or to a previously synthesized class III metallothionein does not require additional ATP.

In the fission yeast *S. pombe*, two pathways for the biosynthesis of class III metallothioneins have been detected in a cell-free system (Hayashi et al., 1991). The first is similar to that described (Grill et al., 1989) for *Silene cucubalus* cell cultures except that either glutathione or class III metallothioneins can act as donors for γ-glutamylcysteine. The second involves the polymerization of γ-glutamylcysteine by the transfer of γ-glutamylcysteine from glutathione to $(\gamma\text{-Glu-Cys})_n$ to produce $(\gamma\text{-Glu-Cys})_{n+1}$ plus Gly. This is followed by the addition of Gly to poly(γ-Glu-Cys). In maize, Cd^{2+}has also been shown to inhibit glutathione biosynthesis, and γ-glutamylcysteine accumulates (Meuwly and Rauser, 1992; Rüegsegger and Brunold, 1992; Ric De Vos et al., 1992). Accumulation of this dipeptide could drive the biosynthesis of class III metallothioneins in the presence of Cd^{2+}. Metal activation of an enzyme for γ-glutamylcysteine transfer from glutathione was not observed in crude enzyme preparations from *S. pombe*, and enzyme extracts from Cd^{2+}-induced and uninduced cells showed no difference in γ-glutamylcysteine transfer.

There are several reports of rapid synthesis of class III metallothioneins being insensitive to inhibitors of *de novo* protein synthesis in plant cell cultures exposed to Cd^{2+} (Scheller et al., 1987; Robinson et al., 1988) indicating that the enzymes responsible for the synthesis of class III metallothioneins and their precursors are constitutive in cells in the absence of excess metal ions. Furthermore, enzyme activity was detected in cell-free extracts from cultures not exposed to elevated metal ion concentrations (Grill et al., 1989).

Localization

In Cd^{2+}-exposed hydroponically grown *Nicotiana rustica* var. Pavonii, the main Cd^{2+}-binding components were $(\gamma\text{-Glu-Cys})_3$Gly and $(\gamma\text{-Glu-Cys})_4$Gly. The location of these polypeptides was determined following isolation of protoplasts and vacuoles from leaves of Cd^{2+}-exposed seedlings. Both class III metallothioneins and Cd^{2+} were found in the vacuoles (Vögeli-Lange and Wagner, 1990).

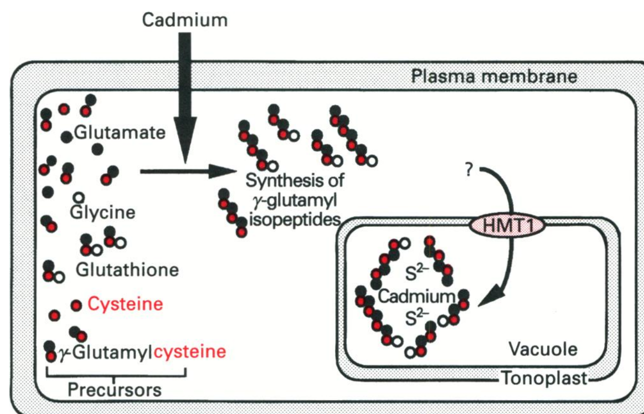

Figure 6 Plants and certain species of yeast accumulate γ-glutamyl isopeptides, designated class III metallothioneins, when exposed to Cd^{2+}

These thiol-rich compounds are also known as cadystins and phytochelatins. Cd^{2+} complexes contain multiple polypeptide molecules and some complexes also contain inorganic S^{2-} within the metal core. Vacuolar accumulation of such complexes has been observed in plants and in *S. pombe*. The product of the *hmt1* gene, localized to the vacuolar membrane, is required for the accumulation of these complexes in vacuoles of *S. pombe* (Ortiz et al., 1992). The predicted sequence of HMT1 is similar to those of ABC-type membrane transporters but it remains to be established which component(s) of the Cd^{2+} complexes (or their precursors) it transports. Red circles, cysteine residues; black circles, glutamate residues; white circles, glycine residues.

The recent observations of Ortiz et al. (1992) indicate that there is a specific transporter, designated HMT1, required for the accumulation of high-M_r CdS–(class III metallothionein) complexes in the vacuole of *S. pombe* cells. A Cd^{2+}-sensitive mutant of *S. pombe*, designated LK100, was isolated that accumulated less of these complexes than the wild type. LK100 cells transformed with *hmtF1* showed restored (increased) Cd^{2+} accumulation. The amino acid sequence deduced from *hmt1* cDNA suggests that its product is similar to ABC (ATP-binding cassette)-type membrane transport proteins (Ortiz et al., 1992). Subcellular fractionation of extracts from *S. pombe* containing an *hmt1–lacZ* fusion indicated that the encoded fusion protein is localized in the vacuolar membrane. At present it is not clear which of the components of the complex (Cd^{2+}, S^{2-}, polypeptides or their precursors) is transported into the vacuole via HMT1 (Figure 6). Delhaize et al. (1989) observed that Cd^{2+} tolerance in *Datura innoxia* cells correlated with the rapid assembly of class III metallothionein metal complexes, but not with modified rates of class III metallothionein synthesis. Modified activity of a plant analogue of HMT1 is one possible explanation for these observations.

CONCLUDING REMARKS: DIVERSITY OF PLANT METALLOTHIONEINS

Plants appear to contain a diversity of metal-binding metallothioneins with the potential to perform distinct roles in the metabolism of different metal ions. The E_c protein from wheat is implicated in the endogenous control of Zn^{2+} metabolism during embryogenesis (Figure 2) while the non-gene-encoded class III metallothioneins appear to play roles in the detoxification of Cd^{2+} (Figure 6) (and possibly of excesses of some essential metal ions). The putative type 1 products (Figure 1) of plant genes with similarity to metallothionein genes are implicated in the sequestration of copper in roots (Figure 5) while roles for type 2 products are even less certain. Clearly, there is an overwhelming necessity to isolate, from plants, the putative products shown in

Figure 1 and to establish whether or not species possess a complement of metallothioneins under the control of different exogenous and endogenous factors, fulfilling different biochemical requirements.

Support for work on plant metallothioneins from AFRC research grant PG12/519(PMB) is gratefully acknowledged. N. J. R. is a Royal Society University Research Fellow.

REFERENCES

Apel, K., Bohlmann, H. and Reimann-Philipp, U. (1990) Physiol. Plant. **80**, 315–321
Beach, L. R. and Palmiter, R. D. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 2110–2114
Berger, J. M., Jackson, P. J., Robinson, N. J., Lujan, L. D. and Delhaize, E. (1989) Plant Cell Rep. **7**, 632–635
Bernhard, W. R. and Kägi, J. H. R. (1985) in Abstracts of the Second International Meeting on Metallothionein and Other Low Molecular Weight Metal Binding Proteins, Zurich, p. 25
Buckhout, T. J., Bell., P. F., Luster, D. G. and Chaney, R.L (1989) Plant Physiol. **90**, 151–156
Chubatsu, L. S. and Meneghini, R. (1993) Biochem. J. **291**, 193–198
Crawford, B. D., Enger, D. M., Griffith, B. B., Griffith, J. K., Hanners, J. L., Longmire, J. L., Munk, A. C., Stallings, R. L., Tesmer, J. G., Walters, R. A. and Hildebrand, C. E. (1985) Mol. Cell. Biol. **5**, 320–329
Dameron, C. T., Reese, R. N., Mehra, R. K., Korfan, A. R., Carroll, P. J., Steigerwald, M. L., Brus, L. E. and Winge, D. R. (1989) Nature (London) **338**, 596–597
Dameron, C. T., Winge, D. R., George, G. N., Sansone, M., Hu, S. and Hamer, D. H. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6127–6131
Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G. and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3869–3873
de Framond, A. J. (1991) FEBS Lett. **290**, 103–106
Delhaize, E., Jackson, P. J., Lujan, L. D. and Robinson, N. J. (1989) Plant Physiol. **89**, 700–706
de Miranda, J. R., Thomas, M. A., Thurman, D. A. and Tomsett, A. B. (1990) FEBS Lett. **260**, 277–280
Evans, I. M., Gatehouse, L. N., Gatehouse, J. A., Robinson, N. J. and Croy, R. R. D. (1990) FEBS Lett. **262**, 29–32
Evans, K. M., Gatehouse, J. A., Lindsay, W. P., Shi, J., Tommey, A. M. and Robinson, N. J. (1992) Plant Mol. Biol. **20**, 1019–1028
Fogel, S. and Welch, J. W. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 5342–5346
Forde, B. G., Heyworth, A., Pywell, J. and Kreis, M. (1985) Nucleic Acids Res. **13**, 7327–7339
Fürst, P., Hu, S., Hackett, R. and Hamer D. H. (1988) Cell **55**, 705–717
Grill, E. (1985) in Abstracts of the Second International Meeting on Metallothionein and Other Low Molecular Weight Metal Binding Proteins, Zurich, p. 24
Grill, E. (1989) in Metal Ion Homeostasis: Molecular Biology and Chemistry (Hamer, D. H. and Winge, D. R., eds.), pp. 282–300, A. R. Liss Inc., New York
Grill, E., Winnacker, E.-L. and Zenk, M. H. (1985) Science **230**, 674–676
Grill, E., Winnacker, E.-L. and Zenk, M. H. (1986a) FEBS Lett. **197**, 115–120
Grill, E., Gekeler, W., Winnacker, E.-L. and Zenk, M. H. (1986b) FEBS Lett. **205**, 47–50
Grill, E., Winnacker, E.-L. and Zenk, M. H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 439–443
Grill, E., Löffler, S., Winnacker, E.-L. and Zenk, M. H. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6838–6842
Grusak, M. A., Welch, R. M. and Kochian, L. V. (1990) Plant Physiol. **93**, 976–981
Gupta, A., Whitton, B. A., Morby, A. P., Huckle, J. W. and Robinson, N. J. (1992) Proc. R. Soc. London B **248**, 273–281
Gupta, A., Morby, A. P., Turner, J. S., Whitton, B. A. and Robinson, N. J. (1993) Mol. Microbiol. **7**, 189–195
Hamer, D. H. (1986) Annu. Rev. Biochem. **55**, 913–951
Hanley-Bowdoin, L. and Lane, B. G. (1983) Eur. J. Biochem. **135**, 9–15
Hayashi, Y., Nakagawa, C. W., Mutoh, N., Isobe, M. and Goto, T. (1991) Biochem. Cell. Biol. **69**, 115–121
Hofmann, T., Kells, D. I. C. and Lane, B. G. (1984) Can. J. Biochem. Cell Biol. **62**, 908–913
Huckle, J. W., Morby, A. P., Turner, J. S. and Robinson, N. J. (1993) Mol. Microbiol. **7**, 177–187
Jackson, P. J., Unkefer, P. J., Delhaize, E. and Robinson, N. J. (1990) in Environmental Injury to Plants (F. Katterman, ed.), pp. 231–255, Academic Press, San Diego,
Jacobs, F. A., Romeyer, F. M., Beauchemin, M. and Brousseau, R. (1989) Gene **83**, 95–103
Kägi, J. H. R. (1991) Methods Enzymol. **205**, 613–626
Kägi, J. H. R. and Kojima, Y. (1987) Experientia Suppl. **52**, 25–61
Kägi, J. H. R. and Schäffer, A. (1988) Biochemistry **27**, 8509–8515
Kägi, J. H. R. and Vallee, B. L. (1960) J. Biol. Chem. **235**, 3460–3465
Karin, M. (1985) Cell **41**, 9–10
Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welsh, J. and Fogel, S. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 337–341
Katsuyuki, N., Suzuki, K., Otaki, N. and Kimura, M. (1991) Methods Enzymol. **205**, 174–189
Kawashima, I., Inokuchi, Y., Chino, M., Kimura, M. and Shimizu, N. (1991) Plant Cell Physiol. **32**, 913–916
Kawashima, I., Kennedy, T. D., Chino, M. and Lane, B. G. (1992) Eur. J. Biochem. **209**, 971–976
Kern, S. R., Smith, H. A., Fontaine, D. and Bryan, S. E. (1981) Toxicol. Appl. Pharmacol. **59**, 346–354
Kille, P., Stephens, P. E., Cryer, A. and Kay, J. (1990) Biochim. Biophys. Acta **1048**, 178–186
Kille, P., Winge, D. R., Harwood, J. L. and Kay, J. (1991) FEBS Lett. **295**, 171–175
Klemsdal, S. S., Hughes, W., Lönneborg, A., Aalen, R. B. and Olsen, O. A. (1991) Mol. Gen. Genet. **228**, 9–16
Kojima, Y. (1991) Methods Enzymol. **205**, 8–10
Kondo, N., Imai, K., Isobe, M., Goto, T., Murasugi, A., Wada-Nakagawa, C. and Hayashi, Y. (1984) Tetrahedron Lett. **25**, 3869–3872
Kreis, M., Shewry, P. R., Forde, B., Miflin, B. J. (1985) Oxford Surv. Plant Mol. Cell. Biol. **2**, 253–317
Labbé, S., Prevost, J., Remondelli, P., Leone, A. and Séguin, C. (1991) Nucleic Acids Res. **19**, 4225–4231
Labbé, S., Larouche, L., Mailhot, D. and Séguin, C. (1993) Nucleic Acids Res. **21**, 1549–1554
Lane, B., Kajioka, R. and Kennedy, T. (1987) Biochem. Cell Biol. **65**, 1001–1005
Laulhere, J.-P. and Briat, J.-F. (1993) Biochem. J. **290**, 693–699
Lesuisse, E. and Labbé, P. (1992) Plant Physiol. **100**, 769–777
Lesuisse, E., Horion, B., Labbe, P. and Hilger, F. (1991) Biochem. J. **280**, 545–548
Lin, C. M. and Kosman, D. J. (1990) J. Biol. Chem. **265**, 9194–9200
Löffler, S., Hochberger, A., Grill, E., Winnacker, E.-L. and Zenk, M. H. (1989) FEBS Lett. **258**, 42–46
Margoshes, M. and Vallee, B. L. (1957) J. Am. Chem. Soc. **79**, 4813–4814
Mehra, R. K., Tarbet, E. B., Gray, W. R. and Winge, D. R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8815–8819
Meuwly, P. and Rauser, W. E. (1992) Plant Physiol. **99**, 8–15
Morby, A. P., Turner, J. S., Huckle, J. W. and Robinson, N. J. (1993) Nucleic Acids Res. **21**, 921–925
Münger, K. and Lerch, K. (1985) Biochemistry **24**, 6751–6756
Münger, K., German, U. A. and Lerch, K. (1987) J. Biol. Chem. **262**, 7363–7367
Murasugi, A., Wada, C. and Hayashi, Y. (1981) Biochem. Biophys. Res. Commun. **103**, 1021–1028
Mutoh, N. and Hayashi, Y. (1988) Biochem. Biophys. Res. Commun. **151**, 32–39
Nielson, K. B. and Winge, D. R. (1984) J. Biol. Chem. **259**, 4941–4946
Okumura, N., Nishizawa, N.-K., Umehara, Y. and Mori, S. (1991) Plant Mol. Biol. **17**, 531–533
Olafson, R. W., McCubbin, W. D. and Kay, C. M. (1988) Biochem. J. **251**, 691–699
Olsen, O.-A., Jakobsen, K. S. and Schmelzer, E. (1990) Planta **181**, 462–466
Ortiz, D. F., Kreppel, L., Speiser, D. M., Scheel, G., McDonald, G. and Ow, D. W. (1992) EMBO J. **11**, 3491–3499
Palmiter, R. D. (1987) Experientia Suppl. **52**, 63–80
Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C. and Evans, R. M. (1982) Nature (London) **300**, 611–615
Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer R. E. and Brinster, R. L. (1983) Science **222**, 809–814
Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z. and Schaffner, W. (1993) EMBO J. **12**, 1355–1362
Rauser, W. E. (1990) Annu. Rev. Biochem. **59**, 61–86
Reese, R. N. and Winge, D. R. (1988) J. Biol. Chem. **263**, 12832–12835
Reese, R. N., White, C. A. and Winge, D. R. (1992) Plant Physiol. **98**, 225–229
Ric De Vos, C. H., Vonk, M. J., Vooijs, R. and Schat, H. (1992) Plant Physiol. **98**, 853–858
Riordan, J. F. and Vallee, B. L. (1991) Methods Enzymol. **205**
Robinson, N. J. (1990) in Heavy Metal Tolerance in Plants (Shaw, A. J., ed.), pp. 195–214, CRC Press, Boca Raton
Robinson, N. J. and Jackson, P. J. (1986) Physiol. Plant **67**, 499–506
Robinson, N. J., Barton, K., Naranjo, C. M., Sillerud, J. O., Trewhella, J., Watt, K. and Jackson, P. J. (1985) in Abstracts of the Second International Meeting on Metallothionein and Other Low Molecular Weight Metal Binding Proteins, Zurich, p. 25
Robinson, N. J., Ratliff, R. L., Anderson, P. J., Delhaize, E., Berger, J. M. and Jackson, P. J. (1988) Plant Sci. **56**, 197–204

Robinson, N. J., Delhaize, E., Lindsay, W. P., Berger, J. M. and Jackson, P. J. (1990) in Sulfur Nutrition and Sulfur Assimilation in Higher Plants (Rennenberg, H., Brunold, C. H., DeKok, L. J. and Stulen, I., eds.), pp. 235–240, SPB Academic Publishing, The Hague

Robinson, N. J., Evans, I. M., Mulcrone, J., Bryden, J. and Tommey, A. M. (1992) Plant Soil **146**, 291–298

Romeyer, F. M., Jacobs, F. A., Masson, L., Hanna, Z. and Brousseau, R. (1988) J. Biotechnol. **8**, 207–220

Romeyer, F. M., Jacobs, F. A. and Brousseau, R. (1990) Appl. Environ. Microbiol. **56**, 2748–2754

Rüegsegger, A. and Brunold, C. H. (1992) Plant Physiol. **99**, 428–433

Salt, D. E., Thurman, D. A., Tomsett, A. B. and Sewell, A. K. (1989) Proc. R. Soc. London B **236**, 79–89

Scheller, H. V., Huang, B., Hatch, E. and Goldsbrough, P. B. (1987) Plant Physiol. **85**, 1031–1035

Shewry, P. R. and Tatham, A. S. (1990) Biochem. J. **267**, 1–12

Silar, P. and Wegnez, M. (1990) FEBS Lett. **269**, 273–276

Speiser, D. M., Abrahamson, S. L., Banuelos, G. and Ow, D. W. (1992a) Plant Physiol. **99**, 817–821

Speiser, D. M., Ortiz, D. F., Kreppel, L., Scheel. G., McDonald, G. and Ow, D. (1992b) Mol. Cell. Biol. **12**, 5301–5310

Steffens, J. C. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**, 533–575

Takahashi, K. (1991) OWL; accession number X62818, EMBL, Heidelberg, Germany

Thiele, D. J. (1992) Nucleic Acids Res. **20**, 1183–1191

Thornalley, P. J. and Vasak, M. (1985) Biochim. Biophys. Acta **827**, 36–44

Tommey, A. M., Shi, J., Lindsay, W. P., Urwin, P. E. and Robinson, N. J. (1991) FEBS Lett. **292**, 48–52

Tomsett, A. B. and Thurman, D. A. (1988) Plant Cell Environ. **11**, 383–394

Tsujikawa, K., Imai, T., Katutani, M., Kayamori, Y., Mimura, T., Otaki, N., Kimura, M., Fukuyama, R. and Shimizu, N. (1991) FEBS Lett. **283**, 239–242

Turner, J. S., Morby, A. P., Whitton, B. A., Gupta, A. and Robinson, N. J. (1993) J. Biol Chem. **268**, 4494–4498

Vallee, B. L. (1991) Methods Enzymol. **205**, 3–7

Vallee, B. L., Coleman, J. E. and Auld, D. S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 999–1003

Vasák, M. and Armitage, I. (1986) Environ. Health Perspect. **65**, 215–216

Verkleij, J. A. C., Koevoets, P., Riet, J. V., Bank, R., Nijdam, Y. and Ernst, W. H. O. (1990) Plant Cell Environ. **13**, 913–921

Vögeli-Lange, R. and Wagner, G. J. (1990) Plant Physiol. **92**, 1086–1093

Weig, A. and Komor, E. (1992) OWL; accession number L02306, EMBL, Heidelberg, Germany

Welch, R. M., Norvell, W. A., Schaeffer, S. C., Shaff, J. E. and Kochian, L. V. (1993) Planta **190**, 555–561

Zeng, J., Heuchel, R., Schaffner, W. and Kägi, J. H. R. (1991a) FEBS Lett. **279**, 310–312

Zeng, J., Vallee, B. L. and Kägi, J. H. R. (1991b) Proc. Natl. Acad. Sci. U.S.A. **88**, 9984–9988