Plant Molecular Biology **20**: 1019–1028, 1992.

Expression of the pea metallothionein-like gene $PsMT_A$ in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: Implications for $PsMT_A$ function

Katherine M. Evans, John A. Gatehouse, William P. Lindsay, Jianguo Shi, Andrew M. Tommey and Nigel J. Robinson *

Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, UK (author for correspondence)*

Received 12 May 1992; accepted in revised form 24 July 1992

Key words: copper detoxification, metallothionein, metal tolerance, pea, *Pisum sativum*, $PsMT_A$

Abstract

The $PsMT_A$ gene from pea (*Pisum sativum*) shares similarity with metallothionein (MT) genes and related sequences have also been isolated from a number of other higher-plant species. The proteins encoded by these genes have not yet been purified from plants and their functions remain unclear although, by analogy to MT, roles in the metabolism and detoxification of metal ions have been proposed. By contrast, correlation between transcript abundance and Fe availability has led to an alternative proposal that these genes are involved in mechanisms of Fe efficiency.

Phenotypic effects of constitutive $PsMT_A$ expression were examined in *Escherichia coli* and *Arabidopsis thaliana*. Copper accumulation by *E. coli* cells expressing recombinant $PsMT_A$ protein was approximately 8-fold greater than in control cells. No significant effects on the accumulation of Zn or Cd were detected. In segregating *A. thaliana* progeny, derived from a transgenic F_1 parent containing the $PsMT_A$ gene under the control of a CaMV 35S promoter, 75% of individuals accumulated more Cu (several-fold in some plants) than untransformed, control plants. These data suggest that $PsMT_A$ protein binds Cu *in planta* and that uncoupled (constitutive) expression of the $PsMT_A$ gene causes enhanced Cu accumulation.

Roots of *P. sativum* plants grown under conditions of low Fe availability showed elevated activity of root surface Fe(III) reductase and accumulated more Cu than roots of plants grown in an Fe-supplemented solution. Changes in the expression of MT-like genes, coincident with changes in Fe availability, are consistent with a role in Cu homoeostasis.

Introduction

Metallothioneins (class I and II) are low-molecular-weight, metal-binding polypeptides which are widespread throughout the animal kingdom [1–3] and have also been identified in cyanobacteria [4] and some fungi [5, 6]. Transcriptional activation of fungal MT genes by Cu [7] and animal and microbial MT genes by a range of trace metal ions [8, 9], linked with the metal-binding potential of their encoded proteins, indicates that MTs have a role in metal ion metabolism/detoxification.

However, induction of animal MTs by a number of other endogenous signals [2, 8] also suggests some as yet undefined, additional significance.

In higher plants, small metal-binding ligands with the general structure (γ-glutamylcysteinyl)$_n$ glycine, or (γEC)$_n$G, have been identified [10–12]. These polypeptides are synthesized from glutathione and/or γ-glutamylcysteine precursors and their synthesis increases in response to elevated levels of certain toxic trace metals [13, 14]. There is evidence that a function of (γEC)$_n$G, also termed class III MT [15], phytochelatin [16] or cadystin [17, 18], is to detoxify supra-optimal concentrations of certain metal ions (notably Cd and Cu) and it has been proposed that they may be functional analogues of MTs (class I and II) in plants. Recent evidence suggests, however, that higher plants also possess MT genes. The Zn-binding E_c protein, isolated from wheat germ [19], has been designated class II MT [2] and more recently a number of plant genes have been isolated which encode predicted proteins with sequence similarity to MTs. The open reading frame of the pea gene, *PsMT*$_A$, encodes a predicted protein which contains N- and C-terminal MT-like domains separated by a 'spacer' region [20]. Related sequences, all of which encode proteins with two MT-like domains, have been independently isolated from *Mimulus guttatus* [21], maize [22], soybean [23], barley [24] and *Arabidopsis* (M. Chino, personal communication). It would appear likely, therefore, that homologous MT-like genes are widespread throughout the plant kingdom although the proteins encoded by these genes have not yet been purified from plants and their functions remain to be determined. Specifically, it remains to be established whether or not the products of these genes bind metals *in planta*.

To examine *in vitro* metal-binding affinities of PsMT$_A$ protein, the *PsMT*$_A$ gene was expressed in *E. coli* as a carboxy-terminal extension of glutathione-*S*-transferase [25]. Metal ions (Cu, Zn and Cd) were associated with the purified recombinant GST-*PsMT*$_A$ fusion protein. The pH of half-dissociation of these metals ions from the GST-PsMT$_A$ fusion protein indicated high affinities, particularly for Cu, comparable to those of known MT. *In vitro* expression studies in *E. coli* have also indicated that metal ions bind to the MT-like terminal domains of the PsMT$_A$ protein rather than the 'spacer' region [26]. This observed metal-binding ability is consistent with the hypothesis that PsMT$_A$ is involved in the metabolism/detoxification of the above metal ions *in planta*, analagous to known MTs. By contrast, correlation between increased abundance of *ids-1* (a barley homologue of the *PsMT*$_A$ gene) transcripts and low Fe availability [24], has led to an alternative proposal that the products of these plant genes are involved in mechanisms of Fe efficiency, perhaps analogous to bacterial *fur* genes [27].

In order to investigate the function of MT-like plant genes, we have examined the phenotypic effects of constitutive *PsMT*$_A$ expression, in *E. coli* and *A. thaliana*, on the accumulation of metal ions. Levels of Cu in the roots of pea plants were additionally examined, following growth under low Fe, high Fe and high Fe/Cu conditions, to examine any correlation between Fe availability and Cu accumulation. The results of this study support a role for *PsMT*$_A$ in the metabolism/detoxification of Cu but no direct role in Fe accumulation. It is proposed that expression of such genes under conditions of Fe deficiency may be an indirect response attributable to concomitant increases in Cu.

Materials and methods

PCR amplification, cloning and sequence analysis of PsMT$_A$ *in pGEX3X*

Polymerase chain reaction (PCR) amplification products corresponding to the protein coding region of the pea (*P. sativum* L.) gene, *PsMT*$_A$, were generated and cloned (in-frame) into the glutathione-*S*-transferase (GST) fusion-protein expression vector, pGEX3X (Pharmacia), as previously described [25]. The sequence and reading frame of cloned *PsMT*$_A$ fragments in both positive (pGPMT3) and negative (pGPMT1) orientations were verified by sequencing in both directions using the PCR primers.

Expression of $PsMT_A$ *in* E. coli

Overnight cultures of *E. coli* JM101 containing either plasmid pGEX3X, pGPMT1 or pGPMT3, were diluted approximately 1:10 in fresh LB media supplemented with ampicillin (50 μg/ml) to an OD_{600} of 0.025. Cultures were grown at 37 °C to an OD_{600} of 0.3 and supplemented with high or low concentrations of either $ZnSO_4$ (1 mM or 500 μM), $CuSO_4$ (2 mM or 500 μM) or $CdSO_4$ (600 μM or 300 μM) before expression of GST or the recombinant fusion protein was induced by addition of isopropyl-*β-D*-thiogalactoside (IPTG) to a final concentration of 1 mM. Cultures were grown for a further 3 h at 37 °C and their OD_{600} monitored.

Analysis of metal accumulation in E. coli *cultures*

Standardized aliquots (equivalent absorption at OD_{600}) were removed from JM101 cultures and cells recovered by centrifugation at $5000 \times g$ for 10 min. Cells were washed twice in LB media and digested overnight in 70% v/v nitric acid/water. Metal content was determined by atomic absorption spectrophotometry and converted to amount of metal per 8×10^8 cells (assuming $OD_{600} = 1$ is equivalent to 8×10^8 cells ml^{-1}).

PCR amplification of $PsMT_A$ *and cloning into pROKII*

PCR primers 1 and 2, comprising 15 bp which corresponded exactly to regions within the 5′ and 3′ transcribed but untranslated $PsMT_A$ sequence respectively, were synthesized using an Applied Biosystems 381A DNA synthesizer. Primer 1 (5′-ATGGTACCAGCAACCAAGCAAT-3′) was designed to initiate extension from base −44 of the $PsMT_A$ gene [20] towards the 3′ terminus and primer 2 (5′-ATGGTACCAA-TATCTCTGCTTC-3′) from base 883 towards the 5′ terminus. The 5′ regions of each primer contained *Kpn* I and Asp718I (*Kpn* I isoschizomer) restriction endonuclease recognition sites to facilitate cloning into the vectors, pUC18 and pROKII.

PCR was performed essentially as described previously [28] using 1.5 μg of plasmid pλS2$PsMT_A$ (3.1 kb $PsMT_A$ genomic DNA fragment in pUC18) DNA as a template. Diagnostic-sized (969 bp) amplification products, which showed strong hybridization to [^{32}P]-labelled $PsMT_A$ probe, were electroeluted from 1% agarose gels, treated with T4 polymerase and ligated to *Hinc* II-digested pUC18. The sequence of the cloned fragment was verified by sequencing in both directions using fluorescent dye-linked universal M13 primers and analysed using an Applied Biosystems 370A DNA sequencer. Recombinant pUC18 was restricted with Asp718I, the $PsMT_A$ insert recovered from a 1% agarose gel by electroelution and ligated into Asp718I restricted pROKII following treatment with alkaline phosphatase. The orientation of the $PsMT_A$ sequence was determined by restriction mapping and plasmids containing the $PsMT_A$ sequence in the positive orientation designated p^+PsMT_A (Fig. 1). Plasmids containing the negatively orientated insert were designated p^-PsMT_A.

Transformation and propagation of A. thaliana

Plasmids, p^+PsMT_A or p^-PsMT_A, were transferred from donor *E. coli* DH5α cells into a disarmed *Agrobacterium tumefaciens* C58-derived strain (C58/3), following triparental matings [29] which also included the mobilizing *E. coli* strain pRK2013. Total DNA was extracted from recombinant *A. tumefaciens* cells [30], restricted with *Hind* III and Asp718I and analysed by Southern hybridization to [^{32}P]-labelled $PsMT_A$ probe prepared by random priming [31].

A. thaliana (cv. C24) root explants were transformed using acetosyringone-induced *A. tumefaciens* [32], containing either plasmid p^+PsMT_A or p^-PsMT_A. Transformed explants were plated onto SIM (Gamborgs B5 salts, 2% glucose, 5 mg/l 2ip, pH 5.7, 0.8% agar) containing 200 mg/l augmentin and 50 mg/l kanamycin and propagated until callus tissue developed and

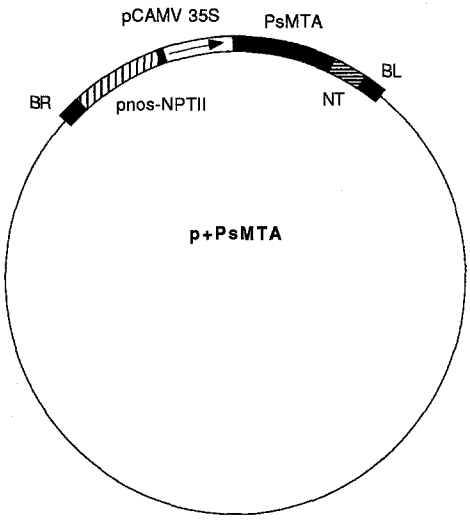

Fig. 1. The vector, $p^{+}PsMT_A$ (circular map shown), was constructed using the pBIN19-based vector pROKII. The $PsMT_A$ gene was inserted into a unique *Kpn*I site between the constitutive 35SCaMV promoter and *nos* terminator in each orientation to form $p^{+}PsMT_A$ (positive orientation) and $p^{-}PsMT_A$ (negative orientation). Symbols: p35SCaMV, 35S cauliflower mosaic virus promoter; pnos-NPTII, nopaline synthase promoter fused to the NPTII (kanamycin resistance) gene; $PsMT_A$, $PsMT_A$-coding region; NT, nos 3′ polyadenylation signal; BR, the right border of T-DNA; BL, the left border of T-DNA.

shoots were formed. Individual shoots were excised after approximately 5 weeks growth and transferred to GM (MS salts, 1% sucrose, 1 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, pH 5.7, 0.8% agar). Plants were selfed at flowering, siliques allowed to develop and the seeds collected. F_1 progeny plants were generated from these seeds, selfed at flowering and the seed collected.

Extraction of A. thaliana *genomic DNA and detection of* $PsMT_A$ *by PCR*

Single leaves were removed from F_1 seedlings, macerated in Eppendorf tubes for 15 s at room temperature and mixed with 400 μl of 200mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS. Cell debris was pelleted by centrifugation at 12000 × *g* for 1 min. DNA was recovered following isopropanol precipitation and resuspended in 100 μl TE. PCR was performed using primers 1 and 2 and either 2.5 or 10 μl of DNA as template. All reactions were UV-irradiated for 7 min prior to cycling. Amplification products were analysed by Southern hybridization to $PsMT_A$ probe.

Detection of $PsMT_A$ *mRNA expression in* A. thaliana

Small amounts of leaf tissue from F_1 seedlings were macerated in Eppendorf tubes, mixed with 20 μl of extraction buffer (0.5% NP-40, 10 mM Tris pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$) and incubated on ice for 5 min. Cell debris was removed by centrifugation (13000 × *g*, 2 min) and total cDNA synthesized from 10 μl of the resulting supernatant using reverse transcriptase (5 units) in a 20 μl reaction mixture which included 20–50 pmol oligo(dT) primer, 500 μM of each dNTP, 1 × *Taq* polymerase PCR buffer, 1 mM dithiothreitol and 2 units RNAse inhibitor. The reaction mixture was incubated at 37 °C for 1 h and PCR performed as described for plant genomic DNA using 10 μl of the reaction mixture as DNA template. PCR amplification products were analysed by Southern hybridization to $PsMT_A$ probe.

Propagation and metal analysis of F_2 A. thaliana *seedlings*

Seeds from untransformed plants and a selfed, transgenic ($PsMT_A$-expressing) F_1 parent were surface-sterilized in 20% sodium hypochlorite for 20 min with frequent agitation, rinsed three times in sterile water and germinated on GM. Four-week old seedlings were harvested, rinsed with sterile water, dry-blotted and weighed. Individual seedlings were quick-frozen in liquid nitrogen, ground in an Eppendorf tube with a homogenizer and digested overnight in 1 ml 70% v/v nitric acid/water. Extracts were centrifuged at 12000 × *g* for 5 min and the liquid fraction analysed for Cu content by atomic absorption spec-

trophotometry. Any remaining liquid from each sample was re-analysed first for Fe and then for Zn content but due to limited amounts of liquid, not all samples were analysed for these two metals. Consequently, more seedlings were analysed for Cu than for Zn and Fe.

Growth of pea seedlings

Pea (*P. sativum* L. cv. Feltham First) seeds were surface-sterilized in 20% sodium hypochlorite for 20 min, imbibed overnight in sterile, distilled water with constant aeration and germinated on moist filter paper. Seedlings were grown hydroponically in pots which contained 2.1 l of either unsupplemented or Fe-supplemented solution as described [33] and also in the Fe-supplemented solution with additional Cu (1 μM $CuSO_4$).

Copper accumulation and Fe-reductase activity in pea roots

Lateral roots were removed from 14-day old pea seedlings, rinsed in distilled water and dry blotted. Between 0.5 and 1 g of root material was placed in Eppendorf tubes and incubated with 1 ml 70% v/v nitric acid/water for 168 h. The supernatant was removed and analysed for Cu using atomic absorption spectrophotometry. Fe-reductase activity, a marker for the activation of Fe-efficiency mechanisms, was determined for intact root systems of 14-day old seedlings as previously described [33]. The volume of root material used in assays was determined by displacement of water [34].

Results

Expression of $PsMT_A$ *and metal accumulation in* E. coli

Following growth in media supplemented with 2 mM $CuSO_4$, cells transformed with plasmid pGPMT3 and expressing $PsMT_A$ protein as a carboxyterminal extension of GST, accumulated approximately 8 times more Cu than the equivalent cells containing either plasmid pGEX3X or pGPMT1 (Table 1). No significant effects of $PsMT_A$ expression on accumulation of Zn or Cd were detected in cultures grown in media supplemented with either high or low levels of these two metal ions.

Expression of $PsMT_A$ *in* A. thaliana

PCR followed by Southern analysis confirmed that F_1 progeny plants, derived from selfed parents transformed with construct p^+PsMT_A, contained the 969 bp $PsMT_A$ gene sequence and PCR using cDNA template verified that $PsMT_A$ mRNA of the predicted size (335 bases) was being transcribed (Fig. 2). The 969 bp $PsMT_A$ gene sequence was also detected in an F_1 progeny plant derived from a single, selfed parent transformed with construct p^-PsMT_A but $PsMT_A$ transcripts were not detected. Analysis of PCR products corresponding to the predicted size (335 bases) of $PsMT_A$ transcripts, therefore, confirmed that the

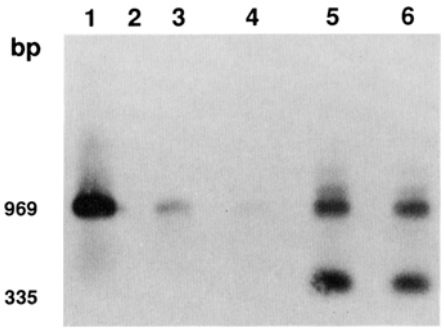

Fig. 2. Southern blot of PCR products probed with a 3.1 kb [^{32}P]-labelled DNA fragment containing the $PsMT_A$ gene sequence. PCR reactions were conducted as described using primers 1 and 2 and the following templates; lane 1, pλS32$PsMT_A$ positive control; lane 2, no template DNA negative control; lane 3, genomic and cDNA from an F_1 *A. thaliana* seedling derived from a selfed, transgenic parent transformed with p^-PsMT_A ($PsMT_A$ negative orientation); lane 4, genomic and cDNA from an untransformed *A. thaliana* seedling; lanes 5 and 6, genomic and cDNA from F_1 *A. thaliana* seedlings derived from selfed, transgenic parents transformed with p^+PsMT_A ($PsMT_A$ positive orientation).

Table 1. Accumulation of metal ions by *E. coli* JM101 cultures containing either plasmid pGEX3X, pGPMT3 or pGPMT1.

Replicate	Accumulated metal (nmol per 8×10^8 cells)					
	pGEX3X		pGPMT3		pGPMT1	
	H	L	H	L	H	L
Cadmium						
1	20.82	6.354	22.03	9.923	24.596	8.495
	(0.985)	(0.504)	(2.8)	(0.497)	(2.53)	(0.704)
2	22.478	4.724	29.11	10.149	17.747	6.083
	(1.99)	(0.348)	(0.512)	(0.162)	(3.73)	(0.208)
3	29.255	6.289	25.534	14.161	28.24	11.572
	(0.338)	(0.108)	(0.981)	(0.709)	(1.145)	(0.048)
Copper						
1	18.393	6.527	134.08	11.11	21.652	9.789
	(0.394)	(0.096)	(1.0)	(0.786)	(1.978)	(0.577)
2	24.175	5.642	121.06	8.746	32.03	7.538
	(3.358)	(0.410)	(7.01)	(0.450)	(1.51)	(0.943)
3	7.94	6.411	129.70	12.621	11.702	9.280
	(1.90)	(0.736)	(2.14)	(0.829)	(2.17)	(0.132)
Zinc						
1	12.052	2.137	11.662	3.244	12.793	7.308
	(0.196)	(0.179)	(0.505)	(0.038)	(0.770)	(0.189)
2	11.49	1.919	12.176	2.395	13.146	5.052
	(0.565)	(0.219)	(2.018)	(0.039)	(1.93)	(0.089)
3	10.41	1.939	12.036	3.213	11.96	5.725
	(1.08)	(0.212)	(0.383)	(0.325)	(1.32)	(0.129)

Standardized values for Cu, Zn or Cd concentrations (nmol per 8×10^8 cells) are presented as means of three cultures replicated in three separate experiments with standard deviations in parentheses. Cultures were grown in media supplemented with either high or low concentrations of respective metal ions. High: $ZnSO_4$ 1 mM, $CuSO_4$ 2 mM, $CdSO_4$ 600 μM; low: $ZnSO_4$ 500 μM, $CuSO_4$ 500 μM, $CdSO_4$, 300 μM.

introduced $PsMT_A$ gene sequence was only transcribed in progeny derived from selfed parent plants transformed with construct p^+PsMT_A. It is noted that a species of lower abundance with homology to the $PsMT_A$ gene sequence and of similar size was present amongst PCR products amplified from genomic and cDNA from an untransformed plant. It was considered that this low abundance product may have arisen due to amplification of a $PsMT_A$ homologue in *A. thaliana*. However, analysis of homologous *A. thaliana* sequences (M. Chino, personal communication: P.B. Goldsborough, personal communication) has revealed that primers 1 and 2, used in PCR analysis of putative transgenic and untransformed seedlings, were unlikely to bind to the targeted sites within these genes to yield products of the sizes observed. Furthermore, transcripts of the size predicted from the *A. thaliana* sequences were not detected by PCR using cDNA template from untransformed seedlings. This could suggest that the *A. thaliana* genes were either not being transcribed or that their coding regions shared insufficient homology with the [^{32}P]-labelled $PsMT_A$ probe for detection. An alternative explanation for the presence of the $PsMT_A$-hybridizing spe-

cies in untransformed seedlings is amplification from contaminating $PsMT_A$ genomic DNA sequences of similar size during PCR, although precautions were taken to minimize such contamination. $PsMT_A$ mRNA of the predicted size is, however, clearly only being transcribed in progeny seedlings derived from parent plants transformed with plasmid p^+PsMT_A.

Metal accumulation in A. thaliana

The Cu, Zn and Fe contents of untransformed seedlings and progeny seedlings derived from a single transgenic F_1 parent are presented in Fig. 3. Cu accumulation by different, untransformed seedlings was similar but there was marked variation in seedlings (Fig. 3: 1A and 1B) within the

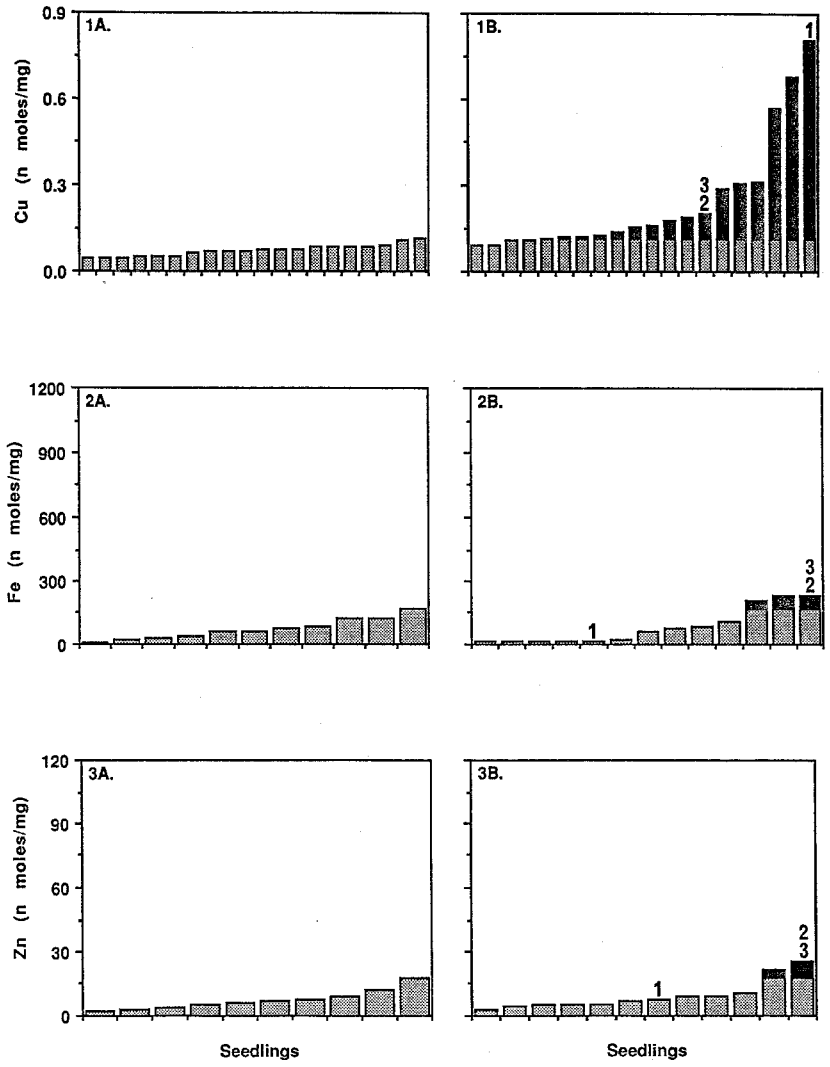

Fig. 3. Accumulation of metal ions by *A. thaliana*. Metal concentrations (nmol/mg fresh weight) in tissue from untransformed, control seedlings; 1A, Cu; 2A, Fe; 3A, Zn and in F_1 progeny seedlings derived from a selfed transgenic parent expressing $PsMT_A$; 1B, Cu; 2B, Fe; 3B, Zn. Highest metal-accumulating individuals from the total population are labelled above respective data blocks; 1, Cu; 2, Fe; 3, Zn. Seeds were germinated and seedlings grown for four weeks on non-supplemented GM (MS salts, 1% sucrose, 1 mg/ml thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, pH 5.7, 0.8% agar) before analysis.

F_1 progeny population, 75% of these seedlings accumulating more Cu than the highest accumulating control seedling. Furthermore, the amount of Cu accumulated by a number of these F_1 progeny seedlings was several-fold higher than that of the equivalent controls. Smaller increases in Zn and Fe accumulation, relative to the controls, were recorded for 2 and 3 F_1 progeny respectively (Fig. 3: 2A, 2B, 3A and 3C). The amount of Zn and Fe accumulated by individual seedlings did not appear to be related to Cu accumulation. Indeed, the Zn and Fe contents of the highest Cu-accumulating individual were similar to those of untransformed, control seedlings.

Copper accumulation and Fe-reductase activity in pea roots

The roots of pea plants grown hydroponically under low Fe conditions accumulated over twice as much Cu as roots of plants grown in an Fe-supplemented (2 μM Fe-EDDHA) solution (Table 2). The activity of a cell-surface Fe-reductase was also greater for these roots. Further increases in the Cu content of roots were recorded when 1 μM $CuSO_4$ was additionally added to the Fe-supplemented growth solution but root Fe-reductase activity was reduced.

Table 2. Copper accumulation (nmol/g root) and Fe-reductase activity (μmol Fe(II)$BPDS_3$ per ml root) for roots of 14-d-old pea (*Pisum sativum* L. cv. Feltham First) plants.

Treatment	[Cu]	Fe-reductase activity
Control	107 (9)	1.47 (0.34)
Fe-EDDHA	50 (2)	0.54 (0.01)
Fe-EDDHA/Cu	174 (21)	0.38 (0.03)

Seeds were germinated on moist filter paper and seedlings grown hydroponically in pots containing non-supplemented solution and Fe-supplemented solution with or without additional $CuSO_4$ (1 μM) as described. Data are presented as mean determination from nine plants, grown in three separate containers, with standard error values in parenthesis.

Discussion

The data reported here indicate that constitutive expression of the pea gene, $PsMT_A$, in *E. coli* cultures and *A. thaliana* seedlings enhanced the accumulation of Cu. This supports the proposal that $PsMT_A$, and homologous MT-like genes from other higher plant species, may have a role in the homeostasis of Cu. Analysis of Cu accumulation by the roots of pea plants grown under high and low Fe conditions also provides an alternative explanation for the observed induction of a $PsMT_A$ homologue from barley in response to low Fe availability [24], which is consistent with a proposed role in Cu detoxification.

Expression of the $PsMT_A$ gene in *E. coli* cultures grown in media supplemented with high levels of Cu, resulted in increased accumulation of this metal ion. No significant effects of $PsMT_A$ expression on the accumulation of Zn or Cd by similar cultures supplemented with either one of these metal ions were detected. In *A. thaliana* seedlings derived from a single, $PsMT_A$-expressing F_1 parent and segregating for $PsMT_A$, 75% of seedlings accumulated more Cu (up to 8-fold in the highest accumulating seedling) than untransformed, control plants. Conversely, only minor differences in Zn and Fe accumulation, relative to the controls, were detected in a limited number of these progeny. These data suggest that Cu accumulation by a significant number of the progeny within this segregating population is enhanced by constitutive expression of $PsMT_A$. The observed increases in Cu accumulation by *E. coli* and *A. thaliana*, following expression of the $PsMT_A$ gene, suggested that the encoded $PsMT_A$ protein bound Cu *in vivo* and support a function in the metabolism/detoxification of this metal ion *in-planta*.

It has been reported that the net uptake of Cu in wild-type and CUP1-deleted *Saccharomyces cerevisiae* cells is similar [35]. It is possible that the close coupling of CUP1 (which encodes yeast MT) induction to intracellular Cu levels avoids excess cellular accumulation of Cu. The metal tolerance of CUP1-deleted *S. cerevisiae* cells has additionally been examined following coupled

(Cu-induced) or uncoupled (constitutive) expression of the *Drosophila melanogaster* MT gene, MTn [36]. The correct coupling of MT expression in this system was more effective in achieving a Cu-resistant phenotype than uncoupled expression of the gene. In addition, a number of studies have shown that uncoupled expression of various MT genes in *E. coli* mediates enhanced metal accumulation [37, 38]. The observed increases in Cu accumulation by *A. thaliana* seedlings in the present study may, therefore, be attributable to uncoupled (constitutive) expression of $PsMT_A$ *in planta* whereas coupled (metallo-regulated) expression of $PsMT_A$ may not have affected Cu accumulation but could increase tolerance to this metal ion.

It was recently demonstrated that $PsMT_A$ protein, expressed in *E. coli* as a carboxyterminal extension of glutathione-*S*-transferase (GST), has strong affinities for a number of metal ions [25]. The pH of half-dissociation, a criterion used to distinguish MT from other metal-binding proteins, of Zn, Cd and Cu from the purified recombinant GST-$PsMT_A$ fusion protein was determined to be 5.35, 3.95 and 1.45 respectively. The apparent *in vitro* affinity of the GST-$PsMT_A$ fusion protein for Cu is higher than the equivalent value determined for equine renal MT under the same conditions. These data support the contention that $PsMT_A$ is a plant MT gene which encodes a protein with a high affinity for Cu ions. However, it has been suggested that the product of a $PsMT_A$ homologue from barley, *ids-1* [24], which again shares sequence similarity with animal and fungal MTs, does not function as an MT but may have a role in Fe uptake by regulating genes involved in mugineic acid synthesis or genes encoding proteins involved in Fe transport. While this is consistent with the increased abundance of *ids-1* transcripts in barley roots grown under low Fe conditions, there is an alternative hypothesis. Analysis of root material from pea plants grown hydroponically in solutions containing high and low concentrations of available Fe indicated that (i) the activity of a root surface Fe-reductase, a marker for the activation of Fe-efficiency mechanisms in *P. sativum*, was greater in plants grown under low Fe conditions and (ii) roots grown under low Fe conditions accumulated twice as much Cu as roots grown under conditions of high available Fe. A ten-fold increase in Cu accumulation, coincident with Fe deficiency, has also been recently observed in another study (L.V. Kochian, personal communication). Therefore, it is possible that increased transcription of MT-like genes under low Fe conditions may be a result of enhanced Cu uptake, following the activation of Fe efficiency mechanisms. Progressive increases in $PsMT_A$ transcript abundance have been observed in developing pea roots during the first 14 days after germination [39]. Stringent growth conditions, required to avoid activation of Fe efficiency mechanisms in *P. sativum* [33], were not employed in these studies. Increased expression of $PsMT_A$ may, therefore, coincide with the activation of Fe efficiency mechanisms following depletion of Fe stored in the seed.

Inverse correlation between Cu accumulation and Fe availability suggests that activation of transcription of MT-like plant genes, coincident with a reduction in available Fe, is also consistent with a role in Cu homoeostasis. Studies to investigate interactions between Cu and Fe concentrations and the regulation of $PsMT_A$ expression in pea plants are presently being conducted.

Acknowledgements

K.E. was supported by a SERC Quota Research Studentship. A.M.T. was supported by research grant PG12/519(PMB) from the AFRC. The authors thank J. Bryden for technical assistance. N.J.R. is a Royal Society University Research Fellow.

References

1. Karin M, Richards RI: Human metallothionein genes: molecular cloning and sequence analysis of mRNA. Nucl Acids Res 10: 3165–3173 (1982).
2. Hamer DH: Metallothionein. Annu Rev Biochem 55: 913–955 (1986).
3. Kägi JHR, Schäffer A: Biochemistry of metallothioneins. Biochemistry 27: 8509–8515 (1988).
4. Olafson RW, McCubbin WD, Kay CM: Primary- and secondary-structural analysis of a unique prokaryotic

metallothionein from *Synechococcus* sp. *cyanobacterium*: Biochem J 251: 691–699 (1988).
5. Munger K, Lerch K: Copper metallothionein from the fungus *Agaricus bisporus*: chemical and spectroscopic properties. Biochemistry 24: 6751–6756 (1983).
6. Lerch K, Beltramini M: *Neurospora* copper metallothionein: molecular structure and biological significance. Chem Scripta 21: 109–115 (1983).
7. Munger K, Germann UA, Lerch K: The *Neurospora crassa* metallothionein gene. J Biol Chem 262: 7363–7367 (1987).
8. Palmiter RD: Molecular biology of metallothionein gene expression. In: Kägi JHR, Kojima Y (eds) Metallothionein II, pp. 63–80. Birkhauser Verlag, Basel (1987).
9. Karin M, Najarian R, Haslinger A, Valenzuela P, Welch J, Fogel S: Primary structure and transcription of an amplified genetic locus: The CUP1 locus of yeast. Proc Natl Acad Sci USA 81: 337–341 (1984).
10. Robinson NJ, Ratliff RI, Anderson PJ, Delhaize E, Berger JM, Jackson PJ: Biosynthesis of poly(γ-glutamylcysteinyl)glycines in cadmium-tolerant *Datura innoxia* (Mill.) cells. Plant Sci 56: 197–204 (1988).
11. Robinson NJ, Jackson PJ: Metallothionein-like metal complexes in angiosperms; their structure and function. Physiol Plant 67: 499–506 (1986).
12. Jackson PJ, Unkefer CJ, Doolen JA, Watt K, Robinson NJ: Poly(gamma-glutamylcysteinyl)glycine: its role in cadmium resistance in plant cells. Proc Natl Acad Sci USA 84: 6619–6623 (1987).
13. Grill E, Winnacker EL, Zenk MH: Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells. FEBS Lett 197: 115–120 (1986).
14. Grill E, Winnacker EL, Zenk MH: Phytochelatins, a class of heavy metal-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci USA 84: 439–443 (1987).
15. Kägi JHR, Kojima Y: Chemistry and biochemistry of metallothionein. In: Kägi JHR, Kojima Y (eds) Metallothionein II, pp. 25–62. Birkhauser Verlag, Basel (1987).
16. Grill E, Winnacker EL, Zenk MH: Phytochelatins, the principal heavy-metal complexing peptides of higher plants. Science 230: 674–676 (1985).
17. Murasugi A, Wada C, Hayashi Y: Occurrence of acid-labile sulphide in cadmium-binding peptide I from fission yeast. J Biochem 93: 661–664 (1983).
18. Kondo N, Isobe M, Imai K, Goto T: Synthesis of metallothionein-like peptides cadystin A and B occurring in fission yeast, and their isomers. Agric Biol Chem 49: 71–83 (1985).
19. Lane B, Kajioka R, Kennedy T: The wheat germ Ec protein is a zinc-containing metallothionein. Biochem Cell Biol 65: 1001–1005 (1987).
20. Evans IM, Gatehouse LN, Gatehouse JA, Robinson NJ, Croy RRD: A gene from pea (*Pisum sativum* L.) with homology to metallothionein genes. FEBS Lett 262: 29–32 (1990).
21. De Miranda JR, Thomas MA, Thurman DA, Tomsett AB: Metallothionein genes from the flowering plant *Mimulus guttatus*. FEBS Lett 260: 277–280 (1990).
22. De Framond AJ: A metallothionein-like gene from maize (*Zea mays*): Cloning and characterization. FEBS Lett 290: 103–106 (1991).
23. Kawashima I, Inokuchi Y, Chino M, Kimura M, Shimizu N: Isolation of a gene for a metallothionein-like protein from soybean. Plant Cell Physiol 32: 913–916 (1991).
24. Okumura N, Nishizawa N-K, Umehara Y, Mori S: An iron deficiency-specific cDNA from barley roots having two homologous cysteine-rich MT domains. Plant Mol Biol 17: 531–533 (1991).
25. Tommey AM, Shi J, Lindsay WP, Urwin PE, Robinson NJ: Expression of the pea gene $PsMT_A$ in *E. coli*: Metal-binding properties of the expressed protein. FEBS Lett 292: 48–52 (1991).
26. Kille P, Winge DR, Harwood JL, Kay J: A plant metallothionein produced in *E. coli*. FEBS Lett 295: 171–175 (1991).
27. Bragg A, Neilands JB: Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbial Rev 51: 509–518 (1987).
28. Robinson NJ, Gupta A, Fordham-Skelton AP, Croy RRD, Whitton BA, Huckle JW: Prokaryotic metallothionein gene characterization and expresssion: chromosome crawling by ligation-mediated PCR. Proc R Soc Lond B 242: 241–247 (1990).
29. Ditta G, Stanfield S, Corbin D, Helsinki D: Broad-host-range DNA cloning system for gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77: 7347–7351 (1980).
30. Dhaese P, De Greve H, Decraemer H, Schell J, Van Montagu M: Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of *Agrobacterium tumefaciens*. Nucl Acids Res 7: 1837–1849 (1979).
31. Feinberg AP, Vogelstein B: A technique for radiolabeling restriction endonuclease fragments to a high specific activity. Anal Biochem 132: 6–13 (1983).
32. Sheikholeslam SN, Weeks DP: Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. Plant Mol Biol 8: 291–298 (1987).
33. Grusak MA, Welch RM, Kochian LV: Physiological characterization of a single-gene mutation of *Pisum sativum* exhibiting excess iron accumulation. Plant Physiol 93: 976–981 (1990).
34. Lang A, Thorpe MR: Xylem, phloem and transpiration flows in a grape: Application of a technique for measuring the volume of attached fruits to high resolution using Archimedes' principle. J Exp Bot 40: 1069–1078 (1989).
35. Lin C-M, Kosman DJ: Copper uptake in wildtype and copper metallothionein-deficient *Schizosaccharomyces pombe*. J Biol Chem 265: 9194–9200 (1990).
36. Silar P, Wegnez M: Expression of the *Drosophila melanogaster* metallothionein genes in yeast. FEBS Lett 269: 273–276 (1990).
37. Kille P, Stephens P, Cryer A, Kay J: The expression of a synthetic rainbow trout metallothionein gene in *E. coli*. Biochim Biophys Acta 1048: 178–186 (1990).
38. Jacobs FA, Romeyer FM, Beauchemin M, Brousseau R: Human metallothionein-II is synthesized as a stable membrane-localized fusion protein in *Escherichia coli*. Gene 83: 95–103 (1989).
39. Robinson NJ, Evans IM, Mulcrone J, Bryden J, Tommey AM: Genes with similarity to metallothionein genes and Cu, Zn ligands of *Pisum sativum* L. Plant Soil, In press.