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The response of the different soybean metallothionein isoforms to cadmium intoxication

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ABSTRACT

Cadmium is a highly toxic heavy metal for both plants and animals. The presence of Cd in agricultural soils is of major concern regarding its entry into the food chain, since Cd compounds are readily taken up by plants, and accumulated in edible parts due to their high solubility. In this study, we first demonstrate the high capacity for Cd concentration of soybean grains. Consequently, we considered the study and characterization of the molecular determinants of Cd accumulation –such as metallothioneins (MT)– to be of major practical importance. We report here the first characterization of the soybean MT system, with the identification of nine genes (one of which is a truncated pseudogene), belonging to the four plant MT types. The most highly expressed of each type was chosen for further function analysis. All of them are expressed at high levels in soybean tissues: GmMT1, GmMT2 and GmMT3 in roots, shoots and seeds, and GmMT4 only in seeds. The corresponding recombinant soybean MTs, synthesized in *Escherichia coli* cells cultured in metal supplemented media, exhibit greater cadmium than zinc binding capacity. These results suggest a definite role of GmMTs in Cd(II) accumulation as one of the main responses of soybean to an overload of this metal.

1. Introduction

Of all the heavy metals, cadmium is considered to have one of the highest toxicities for humans and all other living organisms, without performing any known biological function [1]. Although its levels in the lithosphere are low, a major increase in the agricultural soil environment has been registered over the last 200 years, mainly associated to anthropogenic activities. The most widely known sources of Cd contamination are atmospheric deposition associated to mining, smelting industries and fossil fuel combustion, organic amendments derived from sewage sludge, manure and wastewater, and the application of phosphate fertilizers [2]. Phosphate fertilizers contain variable amounts of Cd, depending on the origin of the rocks from which they were obtained, those from the north of Africa being richer [3]. The presence of cadmium in agricultural soils is of major concern regarding the entry of the metal in the food chain. Cadmium compounds are more soluble than other heavy metals, so they are more available and readily absorbed by plants, which accumulate them in different edible parts. Although Cd-induced phytotoxicity is rarely of concern –because of the high basal tolerance of vegetables and the low contamination levels usually found in most agricultural soils– the amount of metal accumulated in plant tissues has to be considered a real threat for human health [4], considering that vegetables contribute to more than 70% of daily Cd intake [5]. The joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) Committee on Food Additives and Contaminants proposes a limit of 0.1 mg/kg of Cd for cereals and grains [6], while the European Community has a limit of 0.2 mg/kg for wheat grain [7], and the FAO/WHO have proposed a maximum tolerable daily intake of 70 μg of Cd for humans [6], due to the risk associated with the long term consumption of Cd contaminated crops.

Different vegetable species vary widely in terms of Cd uptake and accumulation capacity. Some plants, including corn, pea and oat, accumulate low amounts of heavy metals, while leafy vegetables such as lettuce and spinach concentrate metals in their leaves [8]. Soybean, the worldwide main source of oil and high protein feeds for the livestock sector [9], has a high Cd accumulation capacity in the grain [10]. Differences in the ability to accumulate Cd can also be found between cultivars of the same plant [11,12]. If the limits proposed by the FAO/WHO, European Community and the Codex Alimentrius are established, it will be of outmost importance to identify soybean genotypes that translocate low levels of Cd to the seed.

Therefore, the global characterization of the molecular mechanisms involved in cadmium accumulation in edible plants, such as soybean, is of major interest in agriculture and food exploitation. Plants produce different types of peptidic defenses against heavy metals. On one hand, phytochelatins are enzymatically-synthesized peptides that capture heavy metals by coordination and polinuclear cluster formation [for a recent review, see 13]. They were identified

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as the major agents in charge of the defense against Cd in plants [14], playing a role of direct chelators for metal immobilization, and nowadays they have been also characterized as transporters through the vegetal organisms to cope with cadmium root overload [15]. On the other hand, metallothioneins (MTs) remain the main gene-encoded peptides acting as a response to an inadequate type/dose of heavy metals, also operating by chelation and immobilization. MTs are small cysteine-rich proteins with the ability to coordinate heavy metal atoms through metal–thiolate bonds, which are widely distributed among the animal and plant kingdoms [cf. recent review in 16]. MTs are currently clustered in fifteen families following taxonomical criteria, since they show extremely heterogeneous amino acid sequences. Plant MTs, placed in family 15, generally contain two small cysteine-rich domains (4 to 8 cysteines each) and a large spacer region (30–50 residues) devoid of this amino acid. As opposed to the large amount of knowledge of animal MTs, the structural and functional properties of plant MTs are little known (for recent plant MT reviews cf. [17,18]). The distribution of cysteine residues, as well as the length of the spacer region, served to further classify plant MTs into four types [19,20]. Only a small number of plant MTs differ from the described canonical primary structure: type 4 MTs, characterized by three cysteine-rich regions, and the *Brassicaceae* subtype of Type 1 MTs, bearing a short spacer of less than 10 residues. In *Arabidopsis*, seven actively expressed MT genes have been identified that include representatives of the four types [19,21]. The further isolation of representatives of the four types in the distant model *Oryza sativa* (rice) [22] indicated that the multiplicity of MT forms precedes the dicot/monocot split in angiosperms, and suggests that it is a common feature among this entire group of plants. Single MT forms have been also isolated in gymnosperms [23] and algae (*Fucus vesiculosus*) [24]. In general, plant MT genes respond to a wide variety of stresses (metal ions, abscisic acid, salt and oxidative stress, temperature, wounds and pathogen invasions), with a generally ubiquitous expression [25]. Specifically, it has been shown that Cd triggers plant MT gene response as a means of conferring protection against this metal [26–28]. Some trends in spatial distribution have been assigned to plant MT genes. Hence, there is an increased type 1 expression in subterranean tissues, while type 2 preferential synthesis seems to be located in aerial organs, type 3 MT mRNAs have been mainly purified from ripening fruits or leaves, and type 4 is practically restricted to seeds [21,25,29–31].

To undertake a full characterization of the response of soybean to cadmium overload, we have started by determining the main features of the accumulation of this heavy metal in the plant, as well as by defining the composition of its MT system and determining the behavior of one member of each isoform subfamily under Cd stress. Overall, our results fully support the need to further deepen the knowledge of the MT system to fully understand cadmium accumulation in soybean tissues.

2. Experimental

2.1. Plant culture conditions

Soybean seeds (*Glycine max* cv. Williams 82), surface sterilized by a 2-min treatment with 10% (v/v) household bleach, were germinated for 3 days in Petri dishes in darkness and then 3 plants per biological replicate were transferred to individual 12-L pots with humus soil. Previously, pots had been moistured and spiked with the exact amount of 0.1 M $CdCl_2$ solution to reach 1 ppm or 1.8 ppm of total cadmium (medium pollution), while no $CdCl_2$ was supplemented for control conditions. Hence, during the entire experiment only water was added at reposition (100% field capacity). These values represented an available cadmium concentration of 0.04 μM (control), 5.62 μM (1 ppm) and 12.2 μM (1.8 ppm) in the soil solution. Plants were grown in a greenhouse with 14 h light/10 h darkness for the whole lifecycle until maturity, spanning 135 days for the used cultivar, after which the leaves, roots and seeds were separately collected, frozen in liquid nitrogen and stored at −80 °C (for quantitative real time PCR (qPCR) experiments) or dried at 80 °C (for Cd content measurements). Short-time or acute cadmium exposure treatments were performed on 21 day-old plants, grown on perlite with Hoagland nutrient solution, supplemented or not with 200 μM $CdCl_2$ for 16 h or 40 h, after which the leaves and roots were collected separately, frozen in liquid nitrogen and stored at −80 °C until use. All experiments were performed in triplicate.

2.2. Metal content determination in soil and vegetal tissues

Soil samples were dried in an oven at 80 °C for 48 h and then ground and 1-mm sieved. The amounts of available Cd were extracted with Mehlich 1 solution (0.05 M HCl + 0.0125 M H_2SO_4; pH 1.2) in a soil:solution ratio of 1:10, shaken for 1 h on a reciprocating shaker at 120 oscillations/min and left to stand overnight. After 16 h, cadmium in the extracts was determined by graphite furnace atomic absorption spectrophotometry (GFAAS) with a GBC 906AA system.

Harvested soybean leaves, roots and seeds were dried at 80 °C for 72 h until reaching constant weight. All leaves, roots and about 10 seeds were ground, and 0.5 g of each sample was completely digested at 100 °C in a dry bath, with 50% v/v HNO_3, at reflux. Determination of cadmium concentration was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES) in a Polyscan 61E (Thermo Jarrell Ash) spectrometer, measuring Cd at 228.802 nm. To measure the Cd content of the commercial soybean seeds used to grow the plants of all the experiments, seeds were ground and then dried at 80 °C for 48 h, and 0.1 g of the ground powder was completely digested at 80 °C with 1 mL of HNO_3. Determination of Cd was carried out by means of ICP-MS, measuring Cd^{111} in an Elan 6000 Perkin Elmer spectrometer. All samples were extracted in triplicate in three different biological samples, totaling to nine replicates for determination.

2.3. In silico identification of soybean MTs. Availability of cDNA clones

The sequences of the four metallothioneins under study here were obtained by searching with the NCBI Basic Local Alignment Search Tool (BLAST), specifically the nucleotide BLAST program using the blastn algorithm, in the EST library database, limiting the results to the *G. max* organism. *Arabidopsis thaliana* metallothionein mRNA sequences NM_100633.2 (MT1a), NM_111773.3 (MT2a), NM_112401.1 (MT3) and NM_127888.1 (MT4a) were introduced as queries. Of the retrieved sequences, those showing a higher number of ESTs – indicative of a higher expression level– for each type of plant MTs, were selected. The EST clones BQ742738.1 (GmMT1), BQ629803.1 (GmMT2) and CA819971.1 (GmMT3) were acquired from Biogenetic Services (USA). Since there was no available EST clone for a GmMT4 isoform, the corresponding cDNA was synthesized as described in Section 2.5. With the release of the complete soybean genome assembly 1.01 during the course of this work, the search for MT genes was performed with the Phytozome BLAT alignment tool using the four previously selected cDNA GmMT sequences as queries.

2.4. Quantitative real time PCR (qPCR)

Three biological replicates, consisting each sample of leaves, roots or seeds pooled from three plants, were analyzed. Total RNA was isolated with TRIZOL® reagent (Invitrogen) following the manufacturer's protocol and treated with DNase I (Fermentas). The cDNA was synthesized using an oligo(dT_{18}) primer from 1 μg of total RNA with the reverse transcriptase RevertAid™ (Fermentas) and RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen). Quantitative real-time PCR reactions were performed in a 20-μL reaction volume with 0.5 μM gene-specific primers (listed in Table 1), 2 μL of 1/100 diluted cDNA as a template,

and SYBR Green I (Invitrogen) as detection reagent. Soybean actin 11 (Glyma02g10170) was used as a reference gene. The amplicon lengths were 158 bp for GmMT1, 182 bp for GmMT2, 59 bp for GmMT3, 105 bp for GmMT4 and 94 bp for actin 11. The reactions were performed in an MX3000P QPCR System (Stratagene) in triplicate (technical replicates). PCR conditions were: 94 °C for 5 min, followed by 45 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 15 s. After final annealing (72 °C, 5 min), a melt curve analysis was made by increasing the temperature from 65 °C to 95 °C at 0.5 °C intervals to check on the specificity of the assays, all real time PCR reactions passed this quality control. The SYBR® Green I fluorescent signal was determined for each cycle at the end of the extension step. The fold-change in gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) [32]. The correlation coefficient of amplification, determined from serial dilutions, was 0.996 for GmMT1, 0.998 for GmMT2, 0.995 for GmMT3 and 0.999 for GmMT4 and actin 11.

2.5. Recombinant synthesis of soybean MTs

Each of the four soybean MT cDNAs indicated in Section 2.3. (GmMT1 to GmMT4) was amplified by PCR, using the primers shown in Table 1. The templates for GmMT1 to GmMT3 were the cDNAs recovered from the EST clones acquired. cDNA for GmMT4 was obtained from reverse transcription using total RNA from developing seeds, performed at the same conditions described in the preceding section. The 35-cycle PCR amplification was performed with the thermo resistant Taq DNA polymerase (Invitrogen) under the following conditions: 30 s at 94 °C (denaturation), 30 s at 50 °C (annealing) and 20 s at 72 °C (elongation). This reaction added a 5′ *Bam*HI and a 3′ *Xho*I restriction site to the GmMT coding regions, for cloning into the pGEX-4T1 (General Electric HC) *Escherichia coli* expression plasmid downstream from the glutathione-S-transferase (GST) open reading frame. The correct construction of the recombinant plasmids, pGEX–GmMT1 to pGEX–GmMT4, was confirmed by DNA sequencing (through Macrogen-Korea) and thereafter they were individually transferred into the protease defective strain *E. coli* BL21 (DE3).

GmMT-GST fusion polypeptides were biosynthesized in 5 L-cultures of transformed *E. coli* cells (BL21 strain). Expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) and cultures were supplemented with final concentrations of 300 μM $ZnCl_2$ or 300 μM $CdCl_2$, and were allowed to grow for a further 3 h. Total protein extract was prepared from these cells as previously described in [33]. Metal complexes were recovered from the fusion constructs by thrombin cleavage and batch-affinity chromatography using Glutathione-Sepharose 4B (General Electric HC). After concentration using Centriprep Microcon 3 (Amicon), the metal complexes were finally purified through FPLC in a Superdex75 column (General Electric HC) equilibrated with 50 mM Tris–HCl, pH 7.0. Selected fractions were confirmed by 15% SDS-PAGE and kept at −80 °C until further use. All procedures were performed using Ar (pure grade 5.6) saturated buffers. Further details on the purification procedure specifically for a recombinant plant MT (*Quercus suber*) can be found in [34,35]. As a consequence of the cloning procedure, the dipeptide Gly-Ser is added to the N-terminus of the corresponding GmMT polypeptides, in relation to the sequences shown in Fig 2. This minor modification of the native form was previously shown not to alter any of the MT metal-binding capacities [36].

2.6. Characterization of the recombinant metal MT complexes

The S, Zn, Cd and Cu contents of the Zn–, Cd– and Cu–GmMT preparations were analyzed by means of ICP-AES in a Polyscan 61E (Thermo Jarrell Ash) spectrometer, measuring S at 182.040 nm, Zn at 213.856 nm, Cd at 228.802 and Cu at 324.803 nm. Samples were treated as in [37], but were alternatively incubated in 1 M HCl at 65 °C for 15 min prior to measurement in order to eliminate possible traces of labile sulfide ions, as otherwise described in [38]. Protein concentrations were calculated from the acid ICP-AES sulfur measure, assuming that the GmMT peptides contributed to all S atoms.

Molecular weight determinations were performed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) on a Micro Tof-Q Bruker instrument interfaced with a Series 1200 HPLC Agilent pump, equipped with an autosampler, all of which were controlled by the Compass Software. Calibration was attained with 0.2 g of NaI dissolved in 100 mL of a 1:1 H_2O:isopropanol mixture. Samples containing Zn– or Cd–GmMT complexes were analyzed under the following conditions: 20 μL of protein solution injected through a PEEK (polyether heteroketone) column (1.5 m × 0.18 mm i.d.), at 40 μL min^{-1}; 5 kV capillary counter-electrode voltage; 90–110 °C desolvation temperature; 6 Lmin^{-1} dry gas; and spectra collection range of 800–2000 m/z. The carrier buffer was a 5:95 mixture of acetonitrile:ammonium acetate/ammonia (15 mM, pH 7.0). Alternatively, for analysis of the GmMT apoforms, preparations at an acidic pH of 20 μL of the corresponding sample were injected at 40 μL min^{-1}; 3.5 kV capillary counter-electrode voltage; 4 kV lens counter-electrode voltage; 80 °C dry temperature; and 6 Lmin^{-1} dry gas. Here, the carrier was a 5:95 mixture of acetonitrile:formic acid at pH 2.4, which caused the complete demetalation of the Zn(II)- or Cd(II)-loaded peptides.

3. Results and discussion

3.1. Cadmium content in soil and vegetal tissues

Cadmium is extremely rare in the earth's crust with a mean concentration of 0.06 ppm but anthropogenic activities are raising its levels in the lithosphere [39]. The cadmium content of non-polluted soil is usually in the range of 0.1–2 ppm and mostly below 1 ppm [40]. The humus soil used in the experiment was naturally low in cadmium content. To simulate the conditions of medium contamination,

Table 1
Sequence of the oligonucleotides used for rtPCR assays (named GmMT) and for PCR synthesis of the cDNA for cloning into pGEX plasmids (named MT-*Bam*HI and MT-*Xho*I). The introduced restriction sites are underlined.

Direct oligonucleotide		Reverse oligonucleotide	
Name	Sequence	Name	Sequence
GmMT1 F	5′-TTTGACTTGAGTTACGTTGAGAAG	*GmMT1 R*	5′-CTTGCAGTTGCATGGGTCAC
GmMT2 F	5′-TGTACCCAGACTTGAGCTACAC	*GmMT2 R*	5′-TCAGCGGGAACACCCATTTC
GmMT3 F	5′-ACATCGAGACTGTTGTCATGGA	*GmMT3 R*	5′-CACACTTGCACTTCCCATCA
GmMT4 F	5′-TGGAGGAGATGCAGTGAGACC	*GmMT4 R*	5′-TGTCATGCCGACACTTGTGC
GmACT F	5′-GCACCCAGCAGCATGAAGA	*GmACT R*	5′-AGGTGCTAAGAGATGCCAAGA
MT1-BamHI F	5′-ATCGGATCCATGTCTAGCTGTGG	*MT1-XhoI R*	5′-ATCCTCGAGTTACTTGCAGTTGC
MT2-BamHI F	5′-ATAGGATCCATGTCTTGCTGCGGTG	*MT2-XhoI R*	5′-TCCCTCGAGAACACCTCACTTGC
MT3-BamHI F	5′-AAAGGATCCATGTCGAACACATGC	*MT3-XhoI R*	5′-ACACTCGAGAACACATGGCAATTAC
MT4-BamHI F	5′-GAGGGATCCATGGCTGATACAAGTG	*MT4-XhoI R*	5′-GGTCTCGAGTTAAGTGCGGCAAGAG

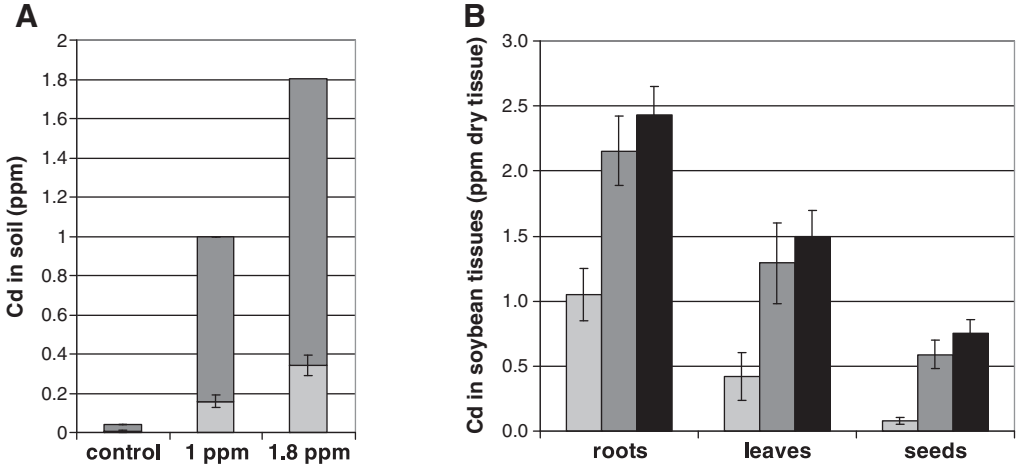
$CdCl_2$ in solution was added up to a total level of 1 and 1.8 ppm of cadmium in the soil. After 1 month of stabilization, available Cd was extracted with Mehlich 1 solution and measured by GFAAS. Fig. 1A shows that the amount of available cadmium is approximately 17% of the quantity of added cadmium, confirming the fact that its compounds are more soluble than other heavy metals with much lower solubilities. The total cadmium concentration shown in Fig. 1A for the control soil has been estimated as 0.04 ppm –well below the considered hazardous concentrations for agricultural soils– taking into account the calculated Cd availability.

Although only a fraction of cadmium compounds are accessible to be taken up by soybean roots, in all three cadmium levels tested the concentration of the metal accumulated in all the vegetal tissues analyzed was higher than the available cadmium in the corresponding soil (Fig. 1B). For instance, the highest concentration index is observed for the roots in the control experiment where the accumulated cadmium is 150 times the amount of the available metal in the soil. In seeds, the commercially valuable and edible part of the plant, this concentration index ranges from 2 to 11. All these values point towards an active transport of the metal from the roots. Table S1 summarizes all the results for cadmium accumulation in different parts of the plants in relation to the available Cd in the soil.

The mean Cd concentration of the commercial seeds from which the plants used in this study were grown was 0.074 μg Cd ± 0.009 per g of dried seed. Therefore it has to be assumed that this content does interfere with none of the results presented in this work. When comparing plants grown in moderately cadmium polluted soils to the control situation, the metal content in the roots is doubled, tripled in the leaves, and increased more than seven times in the seeds. It seems that when the buffering capacity of the roots is exceeded, the aboveground tissues accumulate much higher amounts of cadmium. Nevertheless, more importantly, the seeds from metal treated plants had concentrations of cadmium that are 6–7 times above the 0.1 ppm limit proposed by the Codex Alimentarius Commission [6], in agreement with the results reported by other authors [41]. While the level of cadmium accumulated by the plants used in this study might be higher than the levels occurring under agricultural field conditions, these results reinforce the need to monitor the concentrations of cadmium in food crops and the maximum allowable amounts of cadmium in agricultural fields.

3.2. In silico analysis of the soybean MT system

As mentioned before, MT is the most important gene system devoted to metal defense in all types of organism. At the beginning of this study, no information was known for the soybean MT family members, either at the protein or gene level, so we took advantage of the release of the soybean genome sequence to analyze the composition of the *G. max* MT system using in silico genomic screening. In total, nine genes, easily assignable to the four plant MT types, were identified. The corresponding translated protein sequences are shown in Fig. 2. For type 1, characterized by two 6-Cys-containing domains separated by a commonly 40-amino acid long spacer, three genes were identified in chromosomes 03, 14 and 17. The three GmMT1 isoforms exhibit high sequence similarity, with only a few amino acid substitutions and a 2-residue deletion in Gm03 MT1. Two coordinating domains of 8 and 6 Cys, also separated by an approximately 40-residue long stretch, characterize type 2 MTs. In the soybean genome, we retrieved two genes for type 2 MTs, but only one (situated in chromosome 07) appeared as a functional copy, since the other (chromosome 18) is a truncated copy, lacking the full first exon (Fig. 2). Also, two genes were retrieved for the MT3 type, situated in chromosomes 06 and 12, containing the canonical 4- and 6 Cys-domains separated by a 38-spacer region. The most atypical MT4 type –also known as *pec* or Ec proteins– is constituted by peptides with two coordinating domains, but the second is, in turn, formed by two Cys-containing regions. Therefore, three Cys-rich boxes are found in Ec MTs, encompassing 6, 6 and 5 Cys



	Bioavailable Cd
Control soil	0.007 ± 0.005
1 ppm Cd soil	0.158 ± 0.032
1.8 ppm Cd soil	0.343 ± 0.051

	Control soil	1 ppm Cd soil	1.8 ppm Cd soil
roots	1.05 ± 0.20	2.15 ± 0.27	2.43 ± 0.22
leaves	0.42 ± 0.18	1.29 ± 0.31	1.50 ± 0.20
seeds	0.08 ± 0.03	0.59 ± 0.11	0.75 ± 0.11

Fig. 1. Cadmium determinations in soils by GFAAS (A) and in soybean tissues by ICP-OES (B). In soils, light gray bars indicate the amount of Mehlich 1 extractable Cd (bioavailable), dark gray bars show the unextractable Cd, and the whole bar accounts for the total cadmium concentration. In soybean tissues, light gray bars represent the cadmium accumulated in plants from the control soil experiment whereas dark gray and black bars correspond to the cadmium concentration in plants from 1 and 1.8 ppm cadmium soils, respectively. For both graphs, tables with the measured values are provided to help follow some of the observations included in the text.

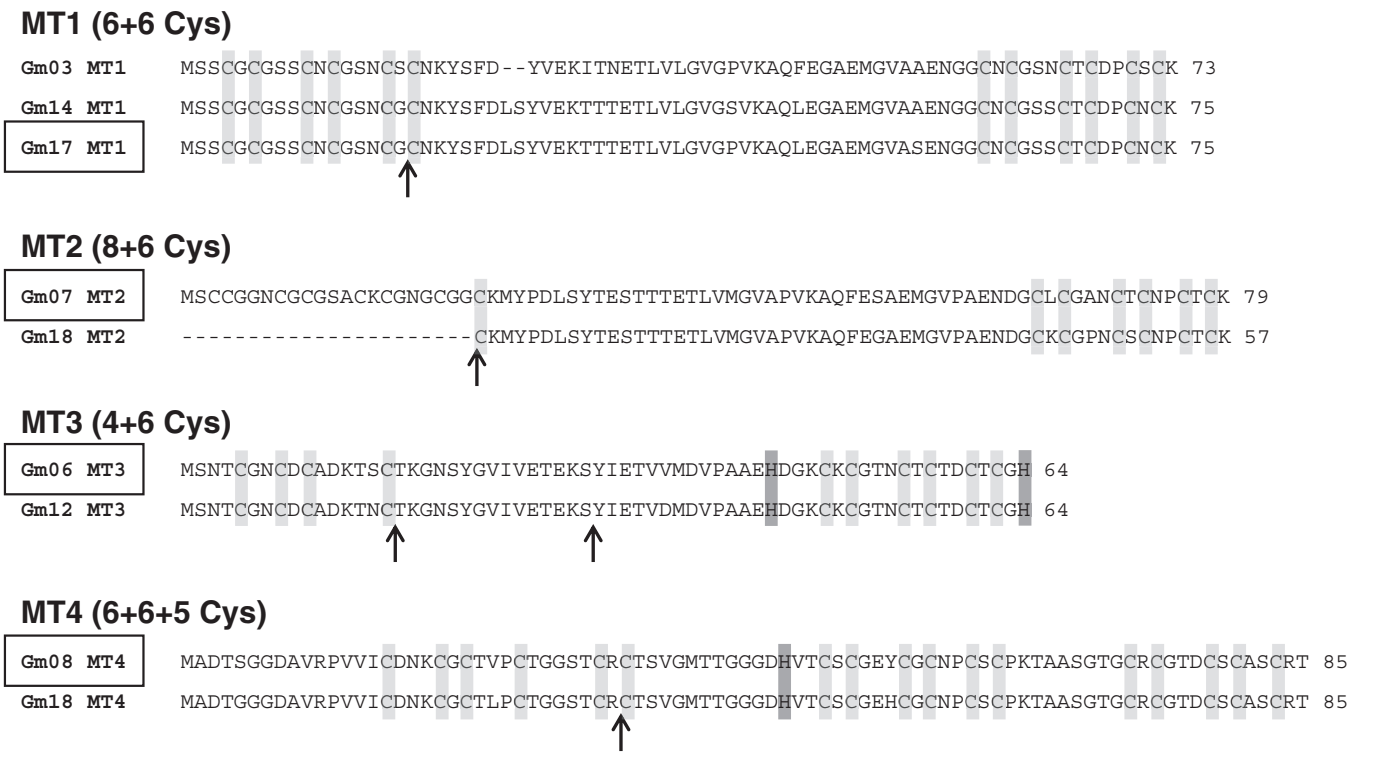


Fig. 2. Sequences for all the members in the soybean metallothionein family. Each metallothionein is named with the chromosome number where it is encoded. Light gray and dark gray show the conserved cysteines and histidines, respectively, within each family type. Arrows indicate the exon–exon junctions. Boxes indicate the metallothioneins in this study.

residues. Two GmMT4 soybean proteins, exhibiting this basic structure, are encoded by the respective genes identified in the 08 and 18 chromosomes. The two GmMT4 isoforms differ in three amino acid changes, two of which are conservative, and, strikingly, the third substitutes the second His in the polypeptide with a Tyr, which implies that Gm08 MT4 is the first member of this subfamily to lack its coordinating His, conversely including an aromatic residue in this sequence position. Supplementary Fig. S1 shows the sequence alignment of all GmMTs against metallothioneins from the model plants *A. thaliana* and *O. sativa*, as well as MTs from other plant

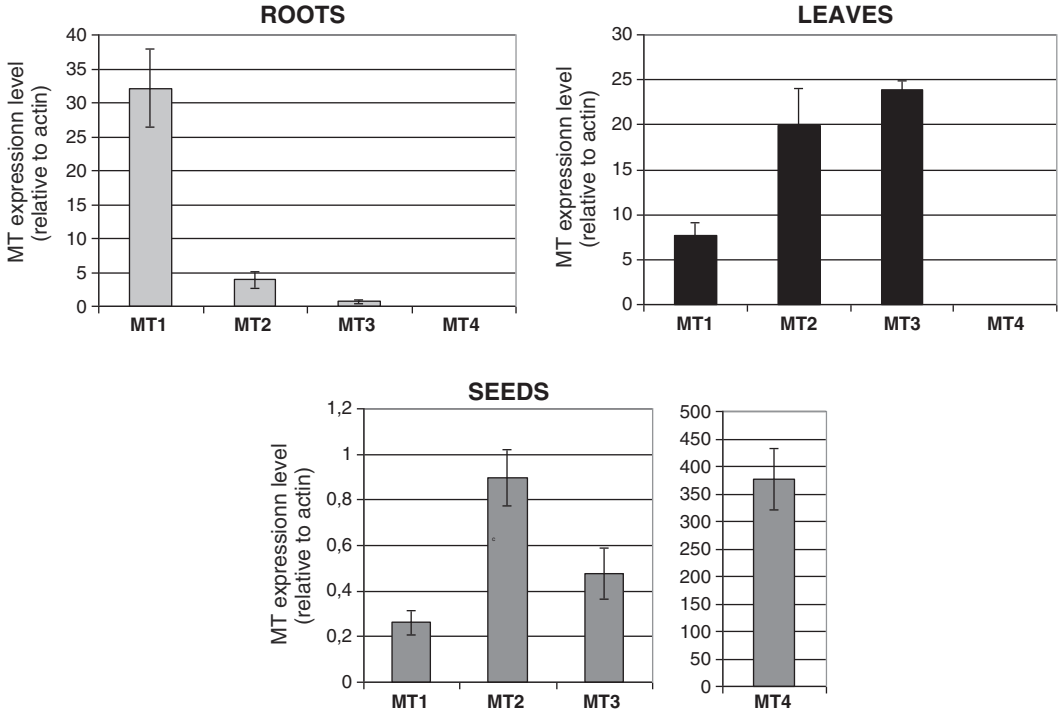


Fig. 3. Real time PCR analysis of soybean metallothionein basal expression in roots (light gray), leaves (black) and seeds (dark gray) of mature plants. The means were generated from three independent measurements, and the bars indicate standard deviations.

species discussed in Section 3.4, and Supplementary Tables S2–S5 present the sequence identity matrices for GmMTs compared to those MTs. As expected, a perfect conservation is observed for canonical cysteines; there is also a great conservation for some other residues within cysteine rich domains, and strikingly, some residues from the linker region of type 1, 2, and 3 MTs are almost fully conserved as well.

3.3. Basal and cadmium induced metallothionein expression

Expression of each *GmMT* gene was evaluated by quantitative real time PCR with SYBR Green I detection in roots, leaves and seeds of control and cadmium treated plants at harvest. In control plants, the expression patterns of all four MTs coincide with those described in the literature for each plant MT type [19]. *GmMT1*, *GmMT2* and *GmMT3* are expressed in all tissues studied, whereas *GmMT4* expression is restricted only to seeds (Fig. 3). *GmMT1* mRNA levels are the highest in roots, while the main isoforms in leaves are GmMT2 and GmMT3. Type 3 MTs are highly expressed in fleshy fruits, but in plants that do not produce them, such as soybean, they are instead expressed at high levels in leaves. In seeds, GmMT4 is by far the most expressed isoform. These results also confirm that *MT* genes are expressed at very high basal levels in plant tissues, at least in terms of transcript abundance.

A short-time, acute Cd exposure experiment was performed on 21-day old soybean plants treated with 200 μM $CdCl_2$ (aprox. 22.5 ppm Cd) for 16 h or 40 h and then harvested. Fig. 4 shows the expression level of all GmMTs present in the roots and leaves of the control and cadmium exposed plants, GmMT4 not being expressed either in roots or in leaves at any treatment condition. At 16 h treatment only *GmMT2* in leaves is significantly induced but a clear induction of *GmMT1*, *GmMT2* and *GmMT3* can be observed in leaves at 40 h treatment, with an increase in the transcription level of 3.25, 4.59 and 3.03 for each gene respectively. On the contrary, we observed a strong repression of *GmMT1* in roots at 16 h treatment –almost recovering its expression level at 40 h treatment– but no significant changes in the expression level of *GmMT2* and *GmMT3* at 16 h or 40 h treatments. Concordant results have been observed before for other plant species; *MT3* is induced in the leaves of buckwheat at 5 and 24 h cadmium exposure [42] and in the leaves of *Prosopis juliflora* at 24, 48 and 72 h cadmium treatments [43], there is a strong correlation between foliar *MT2b* expression in hybrid aspen and Cd concentration [44], whereas *MT1* and *MT2* are inhibited in sugarcane roots, in this case at long time cadmium exposure [45]. While the inducibility of *GmMT1*, *GmMT2* and *GmMT3* indicates a role in cadmium defense/detoxification for these proteins, it is striking the inhibition of *GmMT1* in roots at 16 h treatment. We are currently performing a set of different stress experiments to see if the last is a common stress response or if it is specific for cadmium toxicity. It is our hypothesis that GmMT1 –the main isoform in roots– is repressed in this tissue –the site of first contact with the stressing agent– to allow these cells to send signals in order to prevent or reduce the damage in other tissues. Systemic ROS signaling is one of those signals [46] and it is likely that activation of ROS scavengers, such as MTs, would diminish ROS accumulation being thus detrimental for the induction of the adaptative stress response, as it has been demonstrated for rice OsMT2b during biotic stress [47].

Quantitative real time PCR was also performed in order to determine the expression of all *GmMT* genes in roots, leaves and seeds of soybean plants grown in medium polluted soil (1 ppm and 1.8 ppm Cd) until maturity. No significant differences were observed in this case (data not shown), nevertheless it is important to mention that the high expression levels of all *GmMT* genes shown in Fig. 3 were maintained.

3.4. Metal binding capacities of the four soybean MT isoforms

Recombinant expression of the pGEX–GmMT constructs yielded GmMT polypeptides whose identity, purity and integrity were confirmed by ESI-MS of the respective apoforms obtained by acidification at pH 2.4 of the corresponding Zn–GmMT complexes. For the four cases, a single polypeptide of the expected molecular mass was detected: 7696.58 Da for GmMT1, 8085.20 Da for GmMT2, 6878.65 Da for GmMT3, and 8452.50 Da for GmMT4 (cf. sequences in Fig. 2). Commonly, the Zn– and Cd–GmMT complexes were recovered at a concentration range of about 1×10^{-4} M (cf. Table 2). Exceptions were the synthesis of GmMT1, which invariably yielded a very low amount of protein both when synthesized in zinc- and cadmium-enriched cultures, and, notably, the production of the GmMT3 isoform as Cd-complex, which rendered a much lower yield than when produced as Zn-complex (Table 2).

When GmMT1 was synthesized as Zn-complex, the major species recovered was Zn_4–GmMT1 (Table 2, Fig. 5). Conversely, recombinant synthesis of GmMT1 yielded a major Cd_6S_1–GmMT1 species, together with minor Cd_5S_6–, Cd_7S_1– and Cd_5–GmMT1. (Table 2, Fig. 6). The presence of sulfide ligands in a subpopulation of the cadmium complexes was fully corroborated by the clear differences between the normal and acid ICP sulfur measurements [38]. For GmMT2, the results followed a similar trend but with a lower sulfide content and a slight increase in the metal stoichiometries of the recovered Zn- and Cd-species, probably due to the two additional Cys residues of GmMT2 in relation to GmMT1. The globally diminished Zn(II)- and

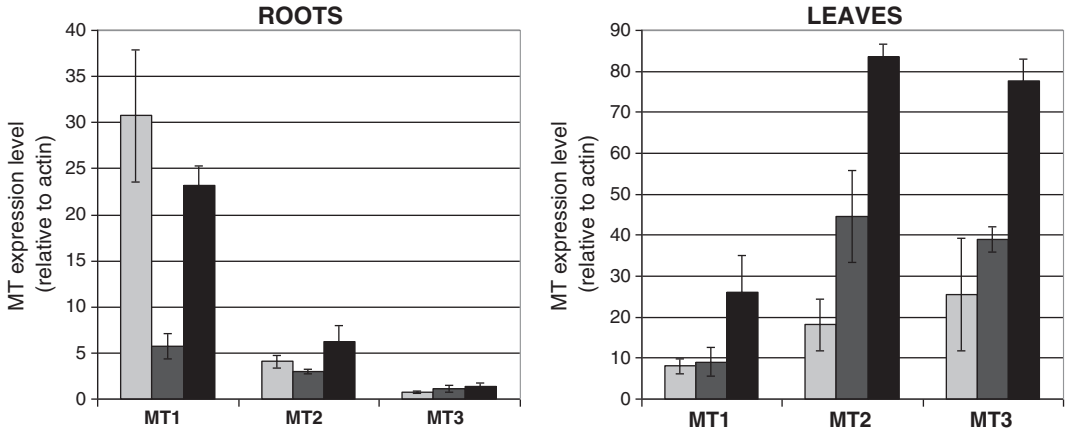
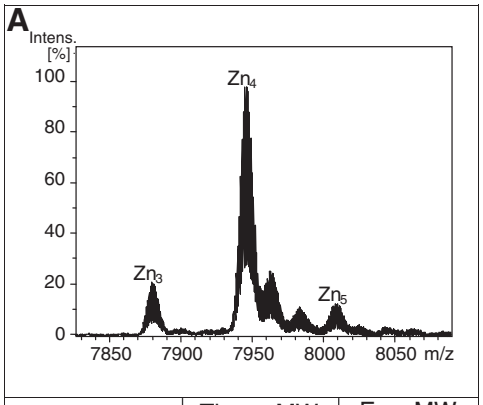
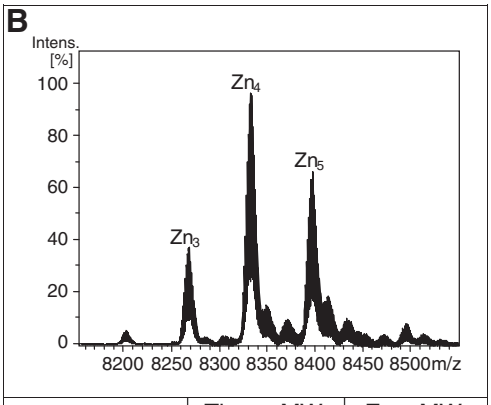


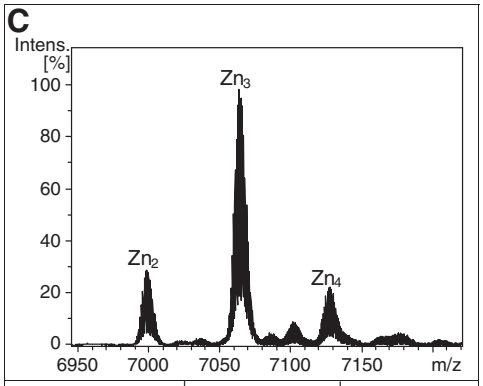
Fig. 4. Real time PCR analysis of soybean metallothionein expression in roots and leaves of plants treated with 200 μM $CdCl_2$ for 16 h (dark gray) and 40 h (black) vs. untreated plants (light gray). GmMT4 expression was measured in both tissues and both treatments and it was not detected (data not shown). The means were generated from three independent measurements, and the bars indicate standard deviations.



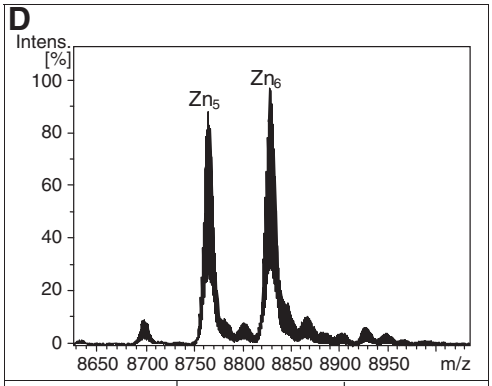
	Theor. MW	Exp. MW
Zn_3-GmMT1	7886.7	7883.0
Zn_4-GmMT1	7950.1	7949.0
Zn_5-GmMT1	8013.5	8013.0



	Theor. MW	Exp. MW
Zn_3-GmMT2	8275.3	8273.0
Zn_4-GmMT2	8338.7	8337.9
Zn_5-GmMT2	8402.1	8401.8



	Theor. MW	Exp. MW
Zn_2-GmMT3	7005.4	7002.7
Zn_3-GmMT3	7068.8	7067.6
Zn_4-GmMT3	7132.2	7131.5



	Theor. MW	Exp. MW
Zn_5-GmMT4	8769.4	8768.0
Zn_6-GmMT4	8832.8	8832.9

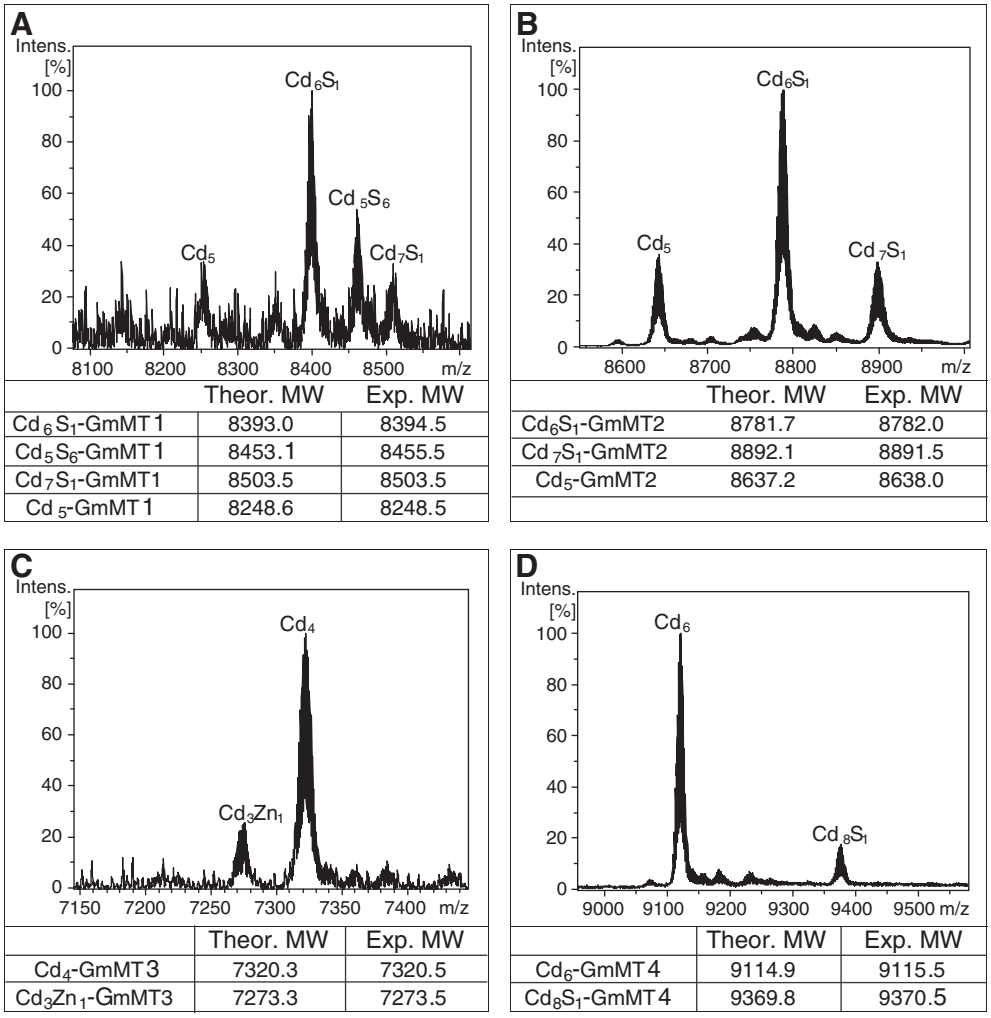
Fig. 5. Deconvoluted ESI-MS spectra of the recombinant preparations obtained from Zn-supplemented *E. coli* cultures of (A) GmMT1, (B) GmMT2, (C) GmMT3, and (D) GmMT4, at pH 7.0. The error associated with the experimental MW values was always lower than 1%, which allows a perfect correlation with the theoretical MW.

Cd(II) binding capacity of the GmMT3 isoform, rendering Zn_3–GmMT3 and Cd_4–GmMT3 respectively as major species, matches well with its lower Cys content (Table 2, Figs. 5 and 6). But significantly, for GmMT3, the major Cd-species were sulfide-devoid complexes rather than sulfide-containing species as for the previous cases. Finally, GmMT4 was the isoform that exhibited the highest Zn(II) and Cd(II) binding capacities, with Zn_6–GmMT4 and Zn_5–GmMT4 as major Zn(II) species and Cd_6–GmMT4 as the major product of synthesis in Cd(II) enriched media, accompanied by only very minor sulfide-containing (Cd_8S_1–GmMT4) complexes (Table 2, Figs. 5 and 6). It is worth noting that the Zn(II) and Cd(II) ion contents here reported for the four GmMT isoforms are in good concordance with those for other plant MT isoforms found in the literature. Native metal–MT complexes have only been isolated and characterized for type 4 MTs, due to the purification impairments presented by 1, 2 and 3 plant MT types. Therefore, the few stoichiometry data available for these isoforms are from recombinant proteins synthesized in *E. coli*, usually as GST-fusion proteins, thus ensuring their comparability beyond small differences due to protein sequence variability. Hence, the available divalent-metal-ion-to-protein ratios for type 1

Table 2
Analytical characterization of the recombinant Zn- and Cd-complexes yielded by GmMT1, GmMT2, GmMT3 and GmMT4.

	Cys/His content	Protein concentration of Zn-complexes[a] ($\times 10^{-4}$ M)	Zn/GmMT content[b]	Zn–GmMT species[c]	Protein concentration of Cd-complexes[a] ($\times 10^{-4}$ M)	Cd/GmMT content[b]	Cd–GmMT species[c]
GmMT1	12 Cys 0 His	0.25/0.27	5.0/3.8	$Zn_4 \gg Zn_3 > Zn_5$	0.13/0.06	3.9/8.1	$Cd_6S_1 > Cd_5S_6 > Cd_7S_1 \sim Cd_5$
GmMT2	14 Cys 0 His	0.8/0.87	4.4/4.3	$Zn_4 > Zn_5 > Zn_3$	1.28/0.90	5.6/6.7	$Cd_6S_1 > Cd_7S_1 \sim Cd_5$
GmMT3	10 Cys 2 His	1.10/1.00	3.2/3.2	$Zn_3 \gg Zn_4 \sim Zn_2$	0.09/0.10	4.2/4.3	$Cd_4 \gg Cd_3Zn_1$
GmMT4	17 Cys 1 His	1.10/1.24	5.8/5.6	$Zn_6 \sim Zn_5 \ggg Zn_4$	0.95/1.00	6.0/7.4	$Cd_6 \gg Cd_8S_1$

[a] Protein concentration calculated from the sulfur content calculated in normal/acid ICP-AES measurements, respectively.
[b] Metal per GmMT molar ratio calculated from the zinc or cadmium and sulfur content measured by normal or acid ICP-AES, respectively.
[c] Metal per GmMT molar ratio calculated from the difference between holo- and apoprotein molecular masses, obtained from ESI-MS.



	Theor. MW	Exp. MW
Cd_6S_1-GmMT1	8393.0	8394.5
Cd_5S_6-GmMT1	8453.1	8455.5
Cd_7S_1-GmMT1	8503.5	8503.5
Cd_5-GmMT1	8248.6	8248.5

	Theor. MW	Exp. MW
Cd_6S_1-GmMT2	8781.7	8782.0
Cd_7S_1-GmMT2	8892.1	8891.5
Cd_5-GmMT2	8637.2	8638.0

	Theor. MW	Exp. MW
Cd_4-GmMT3	7320.3	7320.5
Cd_3Zn_1-GmMT3	7273.3	7273.5

	Theor. MW	Exp. MW
Cd_6-GmMT4	9114.9	9115.5
Cd_8S_1-GmMT4	9369.8	9370.5

Fig. 6. Deconvoluted ESI-MS spectra of the recombinant preparations obtained from Cd-supplemented *E. coli* cultures of (A) GmMT1, (B) GmMT2, (C) GmMT3, and (D) GmMT4, at pH 7.0. The error associated with the experimental MW values was always lower than 1%, which allows a perfect correlation with the theoretical MW.

MTs are 5.6 Zn(II) for *Pisum sativum* MT1 [48,49], 4–5 Zn(II) or Cd(II) for *Cicer arietinum* MT1 [50] and 4 Cd(II) for *Triticum durum* MT1 [51], which are in full concordance with the here reported major Zn_4–GmMT1, as well as the only identified S^{2-}-devoid Cd(II) species (Cd_5–GmMT1). Type 2 MTs of *Q. suber* [35] and *C. arietinum* [52] data are also fully coincident with those of Gm-MT2: 4 and 5 Zn(II), respectively (cf. major Zn_4– and minor Zn_5–GmMT2); and again Cd_5–MT2 as the only S^{2-}-devoid Cd (II) species for the three cases, while for *Q. suber* MT2, Cd_6S_x–MT2 sulfide-containing complexes were the major constituent of the preparation, as for GmMT2 (Table 2). For GmMT3, the most comparable data, from a methodological point of view, were those of the banana *Musa acuminata* MT3, yielding Zn_3– and Cd_4–MT3 complexes [53], exactly as the soybean type 3 isoform. Finally, the similarity between the recombinant complexes rendered by Gm-MT4 and those for the wheat Ec-1 (type 4) protein are fully commented at the end of this section.

A comprehensive consideration of these results leads us to conclude that the divalent metal ion binding capacities of the four GmMT isoforms would be on the basis of their differential role as putative cadmium detoxification/accumulation agents. A common trait observed for all cases is that the mean metal content of the cadmium preparations was always higher than that of the corresponding zinc-supplemented synthesis, so that cadmium stoichiometries invariably showed higher values than their cognate zinc-stoichiometries (Table 2). Globally, these stoichiometric values correlate well with the Cys content of the respective peptides. GmMT1 and GmMT2 share a similar Zn(II) and Cd(II) binding behavior, with sulfide-containing Cd-complexes prevalent over their sulfide-devoid counterparts (cf. Table 2, Figs. 5 and 6). This is the typical behavior of Cu-thioneins, and is therefore indicative of a poor intrinsic ability to coordinate divalent metal ions [54,55]. The remarkable instability of the GmMT1 recombinant protein, which leads to a minimal recovery yield from the corresponding preparations, suggests native functions that are barely related with cell Zn(II) or Cd(II) handling. GmMT3 exhibits a metal binding behavior compatible with a clear Zn-thionein character [54], although with a relatively low capacity due to the limited number of coordinating residues in its polypeptide sequence. In this scenario, it is clear that our results for GmMT4 reflect the best ability to act as a divalent metal ion chelator in soybean (Table 2, Figs. 5 and 6). It is worth remembering that the wheat Ec-1 metallothionein, the paradigm of plant MT4 isoforms, is natively isolated from seeds as Zn-complexes [56], so that it has been hypothesized as a Zn(II) reservoir for plant embryo development. Wheat Ec-1 coordinates 6 Zn(II) ions in two independent domains: two in the N-terminal (γ) domain, comprising 6 Cys, and four in the β_E C-terminal end, with 11 Cys and 2 His contributing to the typical Zn(II)–Cys2His2 cluster formation [57]. In our case, GmMT4 renders major equimolar Zn_6– and Zn_5–GmMT4 species, but this decrease in metal binding capacity could be attributed to the lack of one His residue, precisely that included in the first

Cys box of the β_E domain in wheat Ec-1 (Fig. 2). However, the major $M(II)_6$ stoichiometry is recovered in Cd(II)-complex syntheses, since they render an almost unique Cd_6–GmMT4 species. The fact that this major product lacks sulfide anions is in total agreement with a clear Zn-thionein character of GmMT4. It is worth considering how the behavior of this soybean GmMT4 isoform, holding only one His residue, completely matches the results obtained for the Zn(II) and Cd(II) coordination with the H40A site-directed-mutant of wheat Ec MT [58]. Thus, as in that case, the decrease in Zn(II) binding capacity and the increased stability of the Cd(II) complexes would be explained by the loss of the Cys2His2 site for optimal Zn(II) coordination and the preference of Cys binding for Cd(II). Further characterization of GmMT metal binding features, and behavior in relation to other stresses, is underway.

4. Conclusions

Analysis of the *G. max* (soybean) accumulation response to cadmium overload is essential for toxicological/nutritional purposes. Our results invariably show that, upon root uptake, the concentration of Cd(II) accumulated in all the analyzed soybean plant tissues was higher than the available cadmium in the corresponding soil. Precisely in seeds, the commercially valuable part of the plant, the concentration capacity ranges from a 2 to 11 factor, which means that in our experimental conditions, the seeds from metal treated plants accumulated Cd(II) levels clearly above the 0.1 ppm limit proposed by the Codex Alimentarius Commission. Since it was likely that MTs, the most relevant proteins devoted to metal defense in all types of organism, were responsible for at least part of the detected Cd(II) accumulation, we undertook the characterization of the unreported soybean MT system. The *G. max* genome includes nine MT genes, eight of which are identified as fully active by detection of the corresponding ESTs in databanks. The other corresponds to a truncated copy generated by the loss of an exon. The predicted GmMT polypeptides match well with the motives defining the four plant MT types, so that soybean representatives for each of them were identified. One *G. max* MT gene/protein of each type (GmMT1 to GmMT4) was selected for further studies. The described *GmMT* expression patterns were fully coincident with data on the literature for other angiosperm plants, with *GmMT1*, *GmMT2* and *GmMT3* ubiquitously expressed, and GmMT4 synthesis restricted to seeds. GmMT1 is the main isoform in roots, while in leaves GmMT2 and GmMT3 are predominant. GmMT1, GmMT2 and GmMT3 were highly responsive to cadmium intoxication, which indicates their significant role in cadmium defense/detoxification mechanisms. The determination of the Zn(II) and Cd(II) binding abilities of the four GmMT peptides, recombinantly synthesized in metal supplemented *E. coli* cultures, suggested that GmMT3 and GmMT4 are the most optimal isoforms for divalent metal chelation, both exhibiting a significant Zn-thionein character. These results are fully consistent, on one hand, with the high inducibility of *GmMT3* by cadmium, and on the other hand, with the previously reported isolation of type 4 plant MTs as zinc complexes from seeds. Although neither Cd–MT complexes nor native MT protein has been isolated, and thus quantified, in this work, our data on increased MT mRNA synthesis on one hand, and increased Cd accumulation on the other, fully suggest the easy link between these two phenomena, particularly for GmMT3 in leaves. In fact, the relation between cadmium binding by MTs and cadmium tolerance and accumulation has been directly stated before in other plants, such as *A. thaliana* [27] and *Vicia faba* [28].

Abbreviations

ESI-MS	electrospray ionization mass spectrometry
ESI-TOF MS	electrospray ionization time-of-flight mass spectrometry
GFAAS	graphite furnace atomic absorption spectrophotometry
GmMT	soybean (*Glycine max*) metallothionein
ICP-AES	inductively coupled plasma atomic emission spectroscopy
MT	metallothionein
qPCR	quantitative real time PCR

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2012.08.020.

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