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Molecular cloning and characterization of *Ipomoea nil* metallothioneins

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Abstract: Metallothioneins are low-molecular-weight, cysteine-rich proteins able to bind a variety of heavy metal ions. They are involved in the maintenance of micronutrient homeostasis and detoxification of nonessential heavy metals. Plant metallothioneins are classified into four main types based on the number and arrangement of cysteine residues. Here we report on the isolation of novel complementary and genomic DNA from a model for a short-day plant, *Ipomoea nil* (Japanese morning glory), that encodes 66- or 76-amino acid residue proteins. In silico and phylogenetic analysis of putative amino acid sequences confirmed that these 66- and 76-amino acid residue proteins belong, respectively, to type 1 and type 2 plant metallothioneins. In genomic sequences of the analyzed metallothioneins, two introns were recognized, one of them located at an evolutionary conserved position. Furthermore, examination of the expression patterns of cloned metallothioneins in *I. nil* organs and in cotyledons in response to copper and zinc exposure suggested that their physiological functions could be diversified. Moreover, the observed changes of analyzed *I. nil* metallothioneins in response to heavy metal treatment indicate that InMT1 may be involved in copper homeostasis in *I. nil* cotyledons. The role of InMT2 in micronutrient homeostasis remains questionable.

Key words: Japanese morning glory, *Ipomoea nil*, metallothionein, heavy metal, micronutrient, gene expression

1. Introduction

Heavy metals are an ill-defined group of naturally occurring metals and metalloids with a density greater than 5 g dm^{-3}. They are widely distributed in the earth's crust, although higher concentrations of metals in soil, water, and sediments are observed in industrial areas and agricultural lands. Heavy metals include microelements essential to all living organisms, such as iron, zinc, or copper, as well as highly toxic elements without known physiological functions, e.g., cadmium and lead (Tchounwou et al., 2012).

Copper and zinc are essential micronutrients for plants involved in numerous physiological processes and their deficiency leads to severe impairment of plant growth and development (Broadley et al., 2007; Burkhead et al., 2009). Although zinc and copper are indispensable elements, their surplus may also be toxic for plants. For example, heavy metal excess may cause oxidative stress, disturbances in the regulation of several physiological processes, or the displacement of proper metals in metalloproteins. Therefore, plants have evolved multiple mechanisms ensuring tight control of metal ion concentrations in the cytosol (Hall, 2002). The significance of heavy metal homeostasis is illustrated by the fact that the maximum number of free metal ions is only a few per cell (Blindauer and Schmid, 2010). Metal homeostatic mechanisms constitute a tightly regulated network of proteins involved in the uptake, distribution, chelation, and sequestration of metals within the cell. Although during recent years several metal transporters, chelators, chaperones, and other small metal binding molecules have been identified and described in many plant species, the mechanisms underlying the regulation of metal concentrations in plant cells need to be further evaluated (Clemens, 2006).

Among the key agents in heavy metal homeostasis are the metallothioneins (MTs), which are low-molecular-weight (<10 kDa), cysteine-rich proteins able to bind a variety of heavy metal ions via multiple thiol groups. They are widely distributed in eukaryotes but also found in some prokaryotes including *Synechococcus*, *Pseudomonas*, and *Mycobacterium* (Mierek-Adamska et al., 2017). The first plant metallothionein, E_c (standing for "early cysteine-labeled"), was isolated from mature wheat embryos. The E_c protein, similarly to animal MTs, contains multiple cysteine residues grouped in characteristic Cys-X-Cys motifs, but in contrast to them it contains aromatic amino acids and shares no significant sequence similarity to archetypal MT (Lane et al., 1987; Kawashima et al., 1992).

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Therefore, MTs with high homology to the first isolated metallothionein were grouped into Class I, while the rest of the metallothioneins, including plant MTs (pMTs), were grouped into Class II (Robinson et al., 1993). However, plant MTs are much more diversified in terms of sequence length, number, and arrangement of cysteine residues than MT isogenes from other organisms, requiring a further level of classification, and so they were classified on the basis of number and arrangement of Cys residues into four main types. The number of Cys residues varies from 10 in type 3 pMTs and 12 and 14 in type 1 and type 2 MTs, respectively, to 17 in MTs belonging to type 4. Type 1–3 pMTs contain two Cys-rich domains separated by a Cys-free stretch of variable length, whereas in type 4 MTs three Cys-rich regions are separated by two short Cys-free stretches (Leszczyszyn et al., 2013).

Several lines of evidence have confirmed that MTs play a crucial role in heavy metal ion homeostasis in plant cells. First, a comparison of several *A. thaliana* ecotypes revealed a positive correlation between *AtMT2* expression level and seedling copper tolerance (Murphy and Taiz, 1995). Second, it has been shown that ectopic expression of pMTs in metal-sensitive yeast strains restores metal tolerance (Guo et al., 2008; Yang et al., 2011). Additionally, bacteria cells expressing pMTs also show enhanced tolerance to heavy metals (Jin et al., 2006; Zhigang et al., 2006; Kim et al., 2011). Finally, transgenic plants overexpressing metallothioneins show increased metal tolerance and/or metal accumulation, whereas a reduction in pMTs leads to reduced metal content and decreased metal tolerance (Zhigang et al., 2006; Guo et al., 2008; Ren et al., 2012).

Beyond functions related to reactions to metals, it has also been shown that the expression of pMTs is affected by multiple environmental stimuli (Hassinen et al., 2011; Hrynkiewicz et al., 2012; Dąbrowska et al., 2012, 2014). In silico analysis of MT promoter sequences in two model plants, thale cress and rice, revealed the presence of multiple putative regulatory elements (Dąbrowska et al., 2012). Therefore, reconciling all the available data on plant MTs and the diverse family of pMTs, it has been concluded that the functions of pMTs are not limited to metal ions and it seems that there is no single unifying function for pMTs, and a single pMT may fulfill more than one function (Leszczyszyn et al., 2013).

The aim of this study was to clone metallothioneins from *Ipomoea nil* (L.) Roth. (synonym: *Pharbitis nil* Choisy) (Japanese morning glory), a plant species that is widely used as a model plant for the study of photoperiodic flowering and flower coloration (e.g., Sage-Ono et al., 1998; Dąbrowska et al., 2002; Jaworski et al., 2012). The genus *Ipomoea* is the largest genus in the family Convolvulaceae. This family includes commercially important species such as *I. batatas* (sweet potato), *I. nil*, *I. purpurea*, and *I. tricolor*. Three full-length cDNAs encoding MT-like proteins from the leaves of sweet potato have been previously isolated and characterized (Huang et al., 2001; Chen et al., 2003). In addition, Huang et al. (2014) demonstrated that MT-I and MT-II in *I. batatas* tuberous root may have significant antioxidant activities. Our preliminary results indicated the involvement of *I. nil* MTs in photoperiodic flower induction (Dąbrowska, unpublished data). Moreover, there are over 1500 *I. nil* mutant lines and its draft genome has been recently published (Hoshino et al., 2016), which makes *I. nil* an attractive model plant.

We cloned *I. nil* MTs belonging to pMT types 1 and 2 and analyzed their nucleotide and putative amino acid sequences. Furthermore, we determined the expression pattern of cloned genes in *I. nil* organs. In order to gain some insight into the possible function of *I. nil* MTs in the maintenance of micronutrient homeostasis, the expression patterns of cloned genes were examined in cotyledons of young developing seedlings treated with copper and zinc.

2. Materials and methods

2.1. Plant material and heavy metal ions treatment

The seeds of *Ipomoea nil* 'Violet' (Marutane Seed Co., Japan) were soaked in 95% sulfuric acid for 30 min, rinsed several times with water, and soaked in water with stirring overnight. The imbibed seeds were sown in pots (8–10 seeds per pot) filled with vermiculite and sand (1:1, v:v) and cultivated in a grow chamber with 12-h photoperiods for 7 days at 26 °C. To induce flowering, 6-day-old seedlings were placed in undisturbed darkness for 16 h and then transferred back to the growth chamber with a 12-h photoperiod. Plants started to flower after a few weeks. Transcript levels of *InMT1* and *InMT2* were examined in dry seeds, 24-h imbibed seeds, cotyledons, the hypocotyls and roots of 6-day-old seedlings, and first emerging leaves and flowers.

In order to evaluate the effect of heavy metals on *InMT1* and *InMT2* mRNA levels, 6-day-old seedlings were treated with 200 μM $CuSO_4$ or 500 μM $ZnSO_4$, and cotyledons were collected 0.5, 2, 4, 10, and 24 h after application of metal ions.

Plant material was collected, frozen in liquid nitrogen, and stored at –80 °C until nucleic acid isolation was performed.

2.2. Identification of *InMT1* and *InMT2* sequences

In order to identify the cDNA sequences of *I. nil* metallothioneins, the cDNA sequences of *Ipomoea batatas* metallothioneins deposited in the NCBI GenBank (*I. batatas* type 1 metallothionein – accession number AF116845.1 and *I. batatas* type 2 metallothionein – accession number AB193157.1) were used for searching the *I. nil* EST database using BLASTN. From the numerous ESTs found, the following sequences were

chosen for further analysis: CJ743627.1 (type 1 MT) and BJ561016.1 (type 2 MT). Based on the sequences, specific primers were manually designed: InMT1_for 5'-GGCACGAGGGTTTGGGAGAG-3' and InMT1_rev 5'-ACACAACATACATTATTGAT-3', and InMT2_for 5'-CACTGCAAACCCCAATCTTT-3' and InMT2_rev 5'-ATTCCAAGAATTGCCTGCTG-3'.

2.3. Genomic DNA and total RNA isolation and reverse transcription reaction

Genomic DNA (gDNA) was isolated from *I. nil* cotyledons using the Gene MATRIX Plant and Fungi DNA Purification Kit (EURx, Poland) according to the manufacturer's protocol. Total RNA isolation was performed using TRI-Reagent (Sigma-Aldrich, Poland) according to the manufacturer's protocol. The integrity of the nucleic acids was checked on 1% agarose gel stained with ethidium bromide (EtBr) in TAE buffer.

To remove any DNA contamination, 3 µg of total RNA was treated with 200 U of DNase I (Thermo Scientific Fermentas, USA) for 30 min at 37 °C; the reaction was stopped by addition of 0.025 mM EDTA and incubation at 65 °C for 10 min. For reverse transcription (RT) reaction, the following mixture of a total volume of 14.5 µL was prepared: 3 µg of DNA-free total RNA, 0.5 µg of oligo$(dT)_{18}$ primer, and 1 µL of 10 mM dNTPs. The mixture was incubated at 65 °C for 5 min and then at 0 °C for 2 min. Subsequently, 40 U of RNase inhibitor, 4 µL of 5X RT buffer, and 200 U of RevertAid Premium Reverse Transcriptase (Thermo Scientific Fermentas, USA) were added. The reaction was performed at 50 °C for 30 min and then the enzyme was inactivated at 85 °C for 5 min. The obtained cDNA was stored at –20 °C until RT-PCR was performed.

2.4. Cloning of cDNA and genomic sequences of *InMT1* and *InMT2*

The PCR reaction mixture included cDNA or gDNA as a template, 0.5 µL of 10 µM forward and reverse primer, 0.4 µL of 10 mM dNTPs, 2 µL of 10X buffer, and 2.5 U of *Pfu*Plus! DNA polymerase (EURx, Poland) in a total volume of 20 µL. The thermal cycling conditions were as follows: 95 °C for 30 s (cDNA) or 2 min (gDNA), 51 °C (*InMT1*) or 55 °C (*InMT2*) for 40 s, and 72 °C for 40 s, for 30 cycles. The PCR products were ligated into the pJET1.2 vector (Thermo Fisher Scientific, USA) and sequenced at the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics, Polish Academy of Science (IBB PAS, Poland).

2.5. Semiquantitative RT-PCR (sqRT-PCR)

Semiquantitative RT-PCR (sqRT-PCR) was used to evaluate the mRNA levels of *InMT1* and *InMT2* in *I. Nil* organs and cotyledons of seedlings treated with heavy metal ions. *I. nil* actin (*InAct4*, AB054978.1) amplified transcripts (primers: 5'-CCCGGTATTGCGGATAGAA-3' and 5'-TTCCTGTGCACAATTGACGG-3') were used as the internal standard. The primers for *InMT1* and *InMT2*, designed using Primer3, were as follows: InMT1sq_for 5'-ATTAAGGGATGGGGCTTTTG-3' and InMT1sq_rev 5'-AGTCCAAACACCCAAGCAAC-3', and InMT2sq_for 5'-TGTGGGATGTACGCTGATGT-3' and InMT2sq_rev 5'-ATTCCAAGAATTGCCTGCTG-3'. For each primer set, PCR reaction parameters were optimized according to Marone et al. (2001). The PCR reaction mixture contained single-stranded cDNA as a template, 0.4 µL of each 10 µM primer, 0.4 µL of 10 mM dNTPs, 2 µL of 10X buffer, and 1.25 U of OptiTaq DNA polymerase (EURx, Poland) in a total volume of 20 µL. The amplification reaction was conducted as follows: 95 °C for 30 s, 53 °C (*InMT1*, *InAct4*) or 52 °C (*InMT2*) for 40 s, and 72 °C for 30 s, for 25 cycles (*InMT1*) or 28 cycles (*InMT2*, *InAct4*). PCR products were sequenced to confirm their specificity. PCR products were separated in 2% agarose gel in TAE buffer with EtBr and their quantity was analyzed by densitometric measurements using ImageGauge 3.46 software (Fujifilm, Japan). Statistical analyses performed using ANOVA analysis of variance, followed by Tukey's significant difference test (PAST3.10 software; Hammer et al., 2001), were used to determine the significant differences ($P < 0.05$) between means.

3. Results and discussion

3.1. Identification and in silico analysis of *Ipomoea nil* metallothioneins

Metallothioneins are some of the most important metal chelators in plants and their significant roles in Cu and Zn homeostasis have been widely reported and discussed (Guo et al., 2003; Yang et al., 2009; Ren et al., 2012; Benatti et al., 2014). However, other physiological functions of MT, including scavenging of reactive oxygen species, have also been proposed (Hassinen et al., 2011). In plants, genes encoding MTs form small families. Representatives of all four types of pMTs have been found in all sequenced angiosperm genomes. Usually, multiple genes for MTs are present; e.g., *Arabidopsis thaliana* has seven, *Oryza sativa* has eleven, *Glycine max* has nine, *Sorghum bicolor* has six, and *Brassica napus* has seventeen identified genes (information from NCBI; Leszczyszyn et al., 2013).

In Silico analysis of a type 1 metallothionein sequence (AF116845.1) obtained with InMT1_for and InMT1_rev primers has revealed the presence of an open reading frame of 201 nucleotides, flanked by a 63-bp 5'-UTR and 353-bp 3'-UTR, encoding a putative protein of 66 amino acids with a predicted molecular mass of 6.5 kDa. The sequence shares 91% identity with MT type 1 of *I. batatas* (BAD95644.1) and contains 12 cysteine residues grouped into 6 Cys-X-Cys motifs, 3 each at the N- and C-termini of the protein (Table; Figure 1). The cloned cDNA named

Table. Characteristics of cloned *I. nil* metallothionein sequences.

Gene NCBI number	gDNA (intron length) [bp]	cDNA [bp]	ORF [bp] / protein [aa]	Cysteine residue number	Cysteine sequence patterns
InMT1 MF061303	1491 (217 and 657)	617	201 / 66	12 (6 + 6)	Cys-X-Cys
InMT2 MF061304	861 (362 and 162)	337	231 / 76	14 (8 + 6)	Cys-Cys, Cys-X-Cys, Cys-X-X-Cys

Type 1

```
InMT1       M-SSGCKCGSDCKCGSNCGCEEV---------TTTVTIIKGAAPVKLSLEGSSEKATEGGHGCKCGSNCTCDP-CNC-
IbMT1       M-SSGCKCGSDCKCGSDCACEEV---------TTTVTIIEGVAPVKLTLEGSSEKATEGGHACKCGSNCTCDP-CNC-
PsMT1       M--SGCGCGSSCNCGDSCKCNKRSSGLSYSEMETTETVILGVGPAKIQFDGAEMSVAAEDGGCKCGDSCTCDP-CNCK
OsMT1       M--S-CSCGSSCGCGSNCTCGKMYPDLEEKSSSAQATVVLGVAPEKAHFEAAAES-GETAHGCGCGSSCKCNP-CNC-
AtMT1A      MADSNCGCGSSCKCGDSCSCEKNYNKECD-------------------------------NCSCGSNCSCGSNCNC-

Consensus         CxCxxxCxCxxxCxC                                              CxCxxxCxCxx-CxC
```

Type 2

```
InMT2       MSCCNGKCGCGSSCSCGSSCNGCGMYADVE---KASSVSSLIQGVAPMKN---SFEGGAEKATEGGHACKCGDNCKCNPCNC--
IbMT2       MSCCGGNCGCGSGCKCGSGCNGCGMYADVE---KPTTVSSLIQGVAPVKN---SFEGGAEKATEGGHACKCGDNCQCNPCNC--
OsMT2       MSCCGGNCGCGSGCQCGSGCGGCKMYPEMA--EEVTTTQTVIMGVAPSKGHAEGLEAGAAAGAGAENGCKCGDNCTCNPCNCGK
AtMT2A      MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKN--QYEASGESNNAENDACKCGSDCKCDPCTCK-
GmMT2       MSCCGGNCGCGSSCKCGNGCGGCKMYPDLSYTES-TTTETLVMGVAPV-KA--QFESAEMGVPAENDGCKCGANCTCNPCTCK-

Consensus     CCxxxCxCxxxCxCxxxCxxC                                          CxCxxxCxCxxCxC
```

Figure 1. Amino acid sequence alignment of InMT1 or InMT2 and representative members of type 1 or type 2 plant metallothioneins and consensus sequences of cysteine-rich domains (x denotes an amino acid other than Cys). Conserved cysteine residues are marked in bold. The following amino acid sequences deposited in GenBank were used: At – *Arabidopsis thaliana* (MT1A - AEE28147.1, MT2A - AEE74760.1), Gm – *Glycine max* (NP_001235506.1), Ib – *Ipomoea batatas* (MT1 - AAD10220.1, MT2 - BAD95645.1), Os – *Oryza sativa* (MT1 - NP_001049782.1, MT2 - NP_001042028.1), Ps – *Pisum sativum* (BAD18382.1).

InMT1 is likely full-length or nearly full-length, as its size is consistent with the sizes of full-length cDNA encoding MTs in other plant species.

In silico analysis of a 337-bp-long cDNA sequence obtained with InMT2_for and InMT2_rev primers, flanked by a 30-bp 5'-UTR and 76-bp 3'-UTR, has revealed the presence of a 231-bp-long open reading frame encoding a putative polypeptide of 76 amino acids with a predicted molecular mass of 7.5 kDa. The relatively short UTRs suggest that the cloned cDNA is only a partial-length sequence. A high similarity (89% of identical amino acids) was observed between the amino acid sequence predicted for *InMT2* and that of MT type 2 of *I. batatas* (BAD95645.1). This protein contains 14 cysteine residues grouped in plant-specific motifs: Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys (Table; Figure 1).

Both *I. nil* MTs contain two Cys-rich domains separated by a stretch of approximately 30–40 amino acids. Although the role of this region remains unclear (it may be involved in targeting the protein within the cell or in the formation of metal-binding clusters), it is the main feature that distinguishes pMTs from animal MTs (Freisinger, 2008). The low molecular mass is a characteristic feature of all proteins belonging to metallothioneins. Similar masses to those predicted for InMT1 and InMT2 were also calculated for MTs found in the closely related species *I. batatas* (Chen et al., 2003). However, in more evolutionarily distant species, including poplar (Kohler et al., 2004) and rice (Zhou et al., 2006), the length of MT1 peptides is greater than that observed for InMT1 (Figure 1), and hence the molecular mass of InMT1 is lower than the average masses of pMTs belonging to type 1 (approximately 7.6 kDa). The largest plant metallothioneins belong to type 2 (approximately 7.9 kDa), and the mass of InMT2 is slightly lower than average. The observed mass differences mainly originate from the different length of the cysteine-free region, while the cysteine-rich domains are highly evolutionarily conserved (Freisinger, 2011). Multiple sequence alignment (Figure 1) and phylogenetic analysis of these sequences (Figure 2) have revealed that InMT1 and InMT2 share high homology

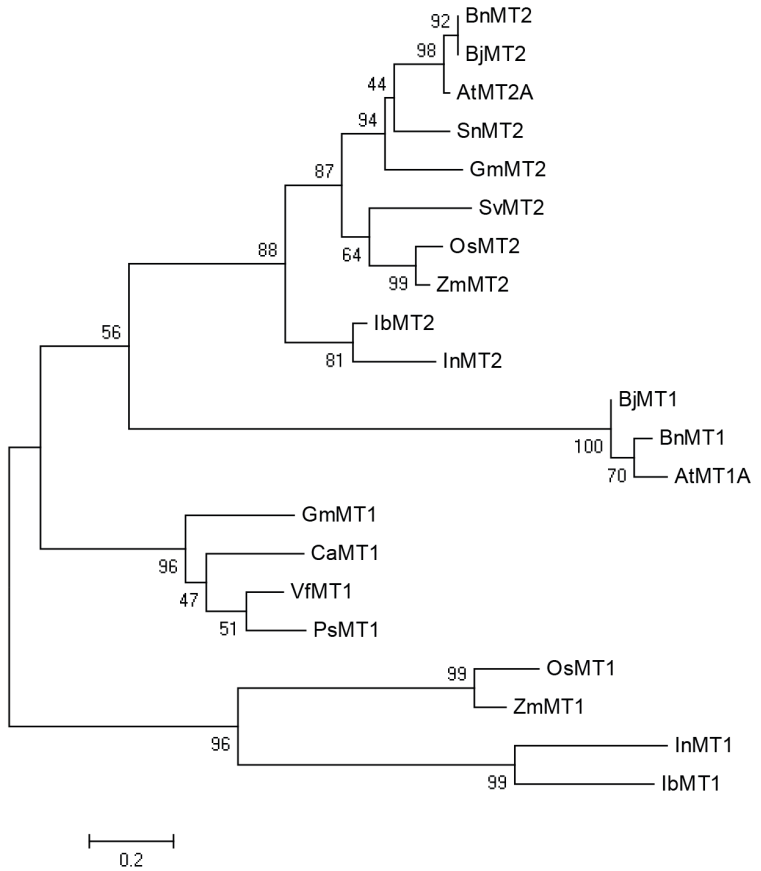

Figure 2. Phylogenetic analysis of pMT proteins obtained using MEGA6 (Tamura et al., 2013). Above or below nodes, the bootstrap values (1000 trials) are given. The following amino acid sequences deposited in GenBank were used: Bj – *Brassica juncea* (MT1 - ABO71662.1, MT2 - CAA71803.1), Bn – *Brassica napus* (MT1 - JX035784.1, MT2 - JX103200.1), Ca – *Cicer arientinum* (CAA65008.1), Gm – *Glycine max* (BAD18376.1), Sn – *Solanum nigrum* (ACF10395.1), Sv – *Silene vulgaris* (AAC72984.1), Vf – *Vicia faba* (CAA62551.1), Zm – *Zea mays* (MT1 - NP_001150528.1, MT2 - ACG42263.1). The rest of the sequences are listed in the caption of Figure 1.

with pMTs of types 1 and 2, respectively. Moreover, the C-terminal domains of InMT1 and InMT2 contain a highly conserved CxCxxxCxCxxCxC motif, while the N-terminal domain is specific to each type. Therefore, it was confirmed that the cloned *I. nil* cDNAs encode metallothioneins representing plant MT types 1 and 2, and this justified naming them InMT1 and InMT2, respectively.

To characterize the intron/exon structure of *InMT1* and *InMT2*, genomic fragments spanning the coding region were amplified by PCR. In both analyzed sequences, two introns were recognized: 217 bp and 657 bp in *InMT1*, and 362 bp and 162 bp in *InMT2* (Table; Figure 3). The first introns are located just before the end of the N-terminal cysteine-rich domains, while the second ones are found within Cys-free regions (Figure 3). All MT genes analyzed to date are characterized by a discontinuous structure. Two introns have been reported in the genes of MT1 in sweet potato (Chen et al., 2003) and rice (Zhou et al., 2006), as well as in MT genes of type 2 in *Helianthus tuberous* (Chang et al., 2004) and rice (Zhou et al., 2006). In contrast, in MTs of type 1 and 2, single introns have been described in *A. thaliana* (Guo et al., 2003), rapeseed (Dąbrowska et al., 2013), soybean (Pagani et al., 2012), and *Prosopis juliflora* (Usha et al., 2009). The number and position of introns is preserved in closely related species, e.g., *A. thaliana* and *B. napus* (Dąbrowska et al., 2013). Interestingly, the first intron is always located close to the end of the N-terminal cysteine-rich domain (Cobbett and Goldsbrough, 2002).

A. InMT1

```
GGCACGAGGGTTTGGGAGAGATATAATATTAGTTTGTTGTGTTTGAAGGGATCGGAGTAAGAGATGTCT  69
                                                                M  S  2
TCCGGTTGCAAGTGTGGCTCCGACTGCAAGTGCGGCAGTAACTGTGGGTAAGCTTCCCGGCCGGCGGCC 138
 S  G  C  K  C  G  S  D  C  K  C  G  S  N  C  G                      18
GGCCCGATCAACCACCATCATCTTATTTTTTCATACCAGATGAGAACAATAAGATTTGATCGAAACTAA 207
TTCTATTATTTGGATATATATATATATATATATAGAGAGAGAAAGAAAGAGAGGTGTAACTTACTATTA 276
AGTTGTATAGCTCAATTATGGATTACAATTTTGATTCTTTTTTTTTTTTCTTGGTAGATGTGAAGAGGT 345
                                                      C  E  E   21
GACCACCACCGTTACCATCATCAAGGGGGCTGCACCGGTGAAGTTGTAAGTGAACTTGATCTATTTATC 414
V  T  T  T  V  T  I  I  K  G  A  A  P  V  K  L                      37
CTTTTTAACATTATTTAAAGGATTAAGGTTTCGATTTATGCCTTTTGTATACGATAAAATTATACATGA 483
TATGTCTAGTTGTATTGGATACTATGGAGATTTGTATTTGGTTCAATCTAAATAGCATATATGGTTGTA 552
CTCGGTATAAAAGATATACATACATCTGCACCAGTGAAGTTGTATGTGAATTTCCATGATCTATTTATC 621
CTTTTTAACTTTCTTTAAGGGATTAAGGTGTCAATTTGTGTCTTTTGTATAGGATTCAACTACACATGA 690
TATACCTCATTGTATTGGATATTATGGAGATCGATTTGTATTTGATACAATCTAAATACCATATGGTTA 759
TATTCGGTACACTATGTACATACATCGACACTAATGAAGTTGTATGTGAATTTCTATGATCTATTTATC 828
CTTTTTAACATTCTTAAGGGATTAATTAAGGTGTCGATTTGTATCTTCTGTATACGATATAATTATACA 897
TGATATGTCTAGTTGTATTGGATACTATATATGGATATCAATATTTGGTACAATATATGGCTATTCTCG 966
GAACAAAAGATCTTAAAATATCTAATTTTTATGAGTGGAAAAAATATTTTATTTGTTTTTAATGTTGGG 1035
ATTATATTGCAGGAGCTTAGAGGGATCTTCTGAGAAGGCTACAGAGGGGGGACATGGCTGCAAATGTGG 1104
              S  L  E  G  S  S  E  K  A  T  E  G  G  H  G  C  K  C   55

ATCAAACTGCACCTGTGACCCTTGCAACTGTTAGGGCTAAAATAGTGCAAATATAATAATCACCCCTTC 1173
G  S  N  C  T  C  D  P  C  N  C  *                                  66
AAGCTATGTATGGATAGATCGGAGCATGTCTTATTAGGGTTTGTCTAAATATATATATATATACATATC 1242
TGTGTATGTACTGATGATAATTAAGGGATGGGGCTTTTGCAGTGATGATATATGATGAGTGTAATAATA 1311
AGCAGATTGCAGATGATGAGTTGTGCAGATCTTTGTTGAAGTGTCACTTTAGATTTGTGTGATTCATTT 1380
ATGTTTGGAATGTGTGGTTGCTTGGGTGTTTGGACTTTATCCTTAATGTATATTGAAAGAGGTTGACTG 1449
TACTGTATTGAACTAAAATGGTATCAATAATGTATGTTGTGT                           1491
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B. InMT2

```
CACTGCAAACCCCAATCTTTCATACAAAACATGTCTTGCTGCAACGGGAAATGTGGCTGCGGCTCTAGC   69
                                  M  S  C  C  N  G  K  C  G  C  G  S  S 13
TGCTCGTGCGGCAGCAGCTGTAATGGGTAAGTTCGCTATTTTTAGCGTACAAGTTTAAACTACTGAAAA  138
  C  S  C  G  S  S  C  N  G                                          22
TTATGAAATTTCTGAAAAAACCTTTGTTACTATTTCCGATCACCTTTTGCTTTCAACTTGATTCAAGAT  207
CATTTTTAGGTGGTCAGAAATTTCATAATTTCCAGCAGTGTTTCAACGAGCTTGAGAGATTCTATTTAT  276
TAGAATGACCTATTGGTCTTACGCTAATAAGTTATGAACAAATTAGACTAGTTTATCTCTTTTGTAACT  345
CTTTGCCTCCTTGAGTTACTCAGTACACACTACTGGTTGTGGCTGCGATTTTCCTAATCACCAAAAAAA  414
AAAAAAATTACTATGTTTAAGGATGTTTTTTTTATGATAACAGATGTGGGATGTACGCTGATGTTGAGA  483
                                              C  G  M  Y  A  D  V  E  30
AGGCCAGCAGTGTGAGCAGCCTCATCCAGGGCGTTGCACCAATGAAGAAGTAAGAAGTCAATTTCTCTT  552
K  A  S  S  V  S  S  L  I  Q  G  V  A  P  M  K  N                   47
TAACTGCTGTTATGTTTTGGCATAAGATCAGAAGTGTGAAATCTGGGAAGAATTGAAGAATAGGAAACT  621
AAAACAGGCAATTAAGTTTATTGATGAAGAAATTATGATTTATTGTTTATTTATACAAAATTTAATTTT  690
GCAGCAGCTTTGAGGGAGGAGCTGAGAAGGCAACAGAGGGAGGACATGCATGCAAGTGCGGAGATAACT  759
      S  F  E  G  G  A  E  K  A  T  E  G  G  H  A  C  K  C  G  D  N  68
GCAAATGCAACCCTTGCAACTGTTGACCAAACAAACTAATAACTATGTTGTCGTGTGTGAGTTTAATAA  828
R  K  C  N  P  C  N  C  *                                           76
TATAATAAGGTGGCAGCAGGCAATTCTTGGAAT                                    861
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Figure 3. Nucleotide and deduced amino acid sequences of *InMT1* (A) and *InMT2* (B) genes and their peptides. Exons are underlined and at the end of each line the number of the last nucleotide/amino acid is marked.

3.2. Analysis of *InMT1* and *InMT2* expression patterns in plant organs

The expression of pMTs is regulated by multiple environmental stimuli and endogenous factors, and it varies depending on the phase of plant development as well as being organ- and tissue-specific (Freisinger, 2008; Leszczyszyn et al., 2013).

In this study, sqRT-PCR was performed to establish the expression patterns of *InMT1* and *InMT2* in vegetative organs, flowers, and seeds. Differentiated expression of these genes in *I. nil* organs was revealed (Figure 4). The highest level of expression of both *I. nil* metallothioneins was observed in vegetative organs (roots, hypocotyls, cotyledons, and first emerging leaf). The differences between the expression of *InMT1* and *InMT2* in these organs were not statistically significant. In dry seeds both genes were expressed at a similarly low level, while in imbibed seeds the expression of only *InMT2* increased significantly, with a 3-fold difference being apparent. In contrast, *InMT1* was expressed at a significantly higher level, approximately 3-fold that of *InMT2* in *I. nil* flowers. The expression level of both genes was significantly lower in dry seeds, imbibed seeds, and flowers than in vegetative organs (Figure 4). A 6-fold difference was observed between the *InMT1* expression level in cotyledons and imbibed seeds, and between the expression level of *InMT2* in hypocotyls and dry seeds.

Based on the available data it is suggested that type 1 MTs are expressed mainly in roots, but also in leaves and shoots, whereas type 2 MTs are equally expressed in leaves and roots, but their transcripts are detected also in shoots, developing seeds, and fruits. Transcripts of *MT3* are present mainly in fruits and leaves, and the expression of type 4 *MTs* is embryo-specific (both types not studied in this paper) (Leszczyszyn et al., 2013). However, only in type 4 MTs in plant species analyzed to date is the expression restricted to developing and mature seeds and reduced at the early stages of imbibition (Kawashima et al., 1992; Ren et al., 2012; Dąbrowska et al., 2013). For types 1–3 pMTs, mRNA levels in different organs vary across plant species. Similar to *InMT1* and *InMT2* (Figure 4), comparable expression levels in roots, stems, and leaves were observed for *A. thaliana* (Ren et al., 2012) and *I. batatas* (Huang et al., 2001) MT1s, whereas the expression level of *AtMT2* was higher in shoots than in roots (Ren et al., 2012) and that of *IbMT2* was higher in leaves than in roots and stems (Chen et al., 2003). In poplar, it was shown that the expression of *MT2* is much higher than that of *MT1* in leaves, while the opposite is true for *MT1* in roots (Kohler et al., 2004). In the flowers of *A. thaliana,* transcripts of *MT1* and *MT2* are present, and similarly to *I. nil* MTs, the expression of *MT1* is significantly higher than that of *MT2* (Ren et al., 2012). Interestingly, in *B. napus* flowers no expression of type 1 MT was observed (Dąbrowska et al., 2013). Not much is known about the expression of metallothioneins during seed imbibition. Expression of *MT2* in *Nelumbo nucifera* increased throughout the 24-h period of imbibition and decreased during the subsequent 48 h (Zhou et al., 2012). In dry seeds and 24-h imbibed seeds of *B. napus*, the mRNA level of *MT2* was significantly higher than that of *MT1* (Dąbrowska et al., 2013). Differences in spatial expression patterns of metallothioneins observed across plant species may at least in part be a result of the analysis being of plants grown under distinct conditions and at different stages of development. Moreover, it was shown that spatial expression differs between MTs belonging to the same type in one plant species (Ren et al., 2012).

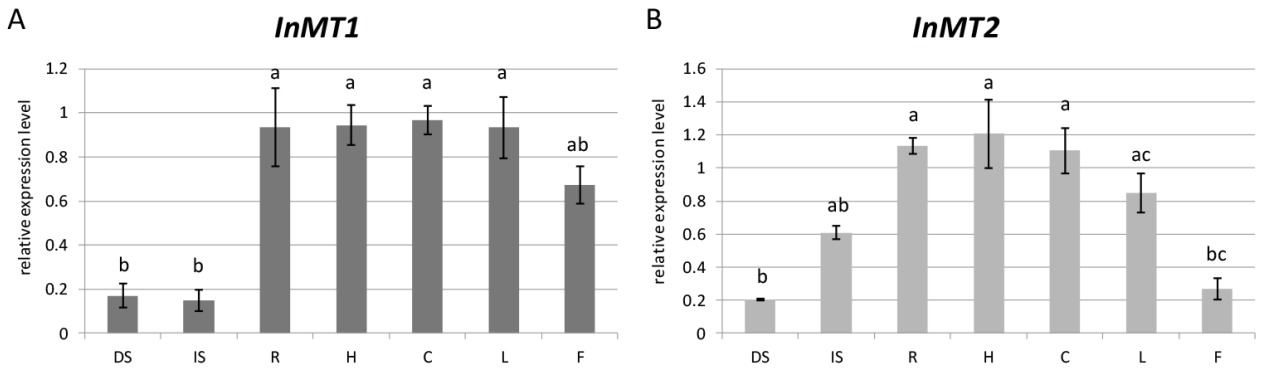

Figure 4. Analysis of *InMT1* (A) and *InMT2* (B) expression patterns in *I. nil* organs. Total RNAs were isolated from dry seeds (DS) and seeds imbibed for 24 h (IS), roots (R), hypocotyls (H), and cotyledons (C) of 6-days-old seedlings, first emerging leaves (L), and flowers (F). *β-actin* was amplified to confirm that a constant amount of total RNA was used. The graph shows relative expression level expressed as the ratio of the amount of RT-PCR product for *InMT1* or *InMT2* gene to the amount of RT-PCR product for the housekeeping gene, *InAct4* (data are means of three independent replicates ± SE). Different letters indicate a statistically significant difference ($P < 0.05$) between organs for each gene.

3.3. Analysis of *InMT1* and *InMT2* expression patterns in response to heavy metal ions

To address the hypothesis that *I. nil* MTs are involved in micronutrient homeostasis and/or detoxification of excess metals, 6-day-old *I. nil* seedlings were treated with copper or zinc and the expression of *InMT1* and *InMT2* was evaluated (Figure 5). Even though metallothioneins are able to bind multiple metal ions, zinc and copper seem to be the most biologically relevant (Krężel and Maret, 2017).

In cotyledons of water-treated seedlings (control), the basal expression of *InMT1* and *InMT2* was at a similar and constant level throughout the 24-h experimental period (data not shown). In Cu-treated seedlings the expression of *InMT1* was increased significantly after 2 and 4 h, with a 1.5-fold difference being apparent, and it remained slightly elevated until after 24 h. In contrast, the expression of *InMT2* remained generally unchanged throughout the whole experimental period; however, at 4 h and 10 h it was significantly decreased (Figure 5).

InMT1 expression was significantly (~1.4-fold) downregulated upon zinc treatment, just after metal application. At 2 h and 4 h, *InMT1* expression level was the same as in control samples, but during subsequent hours a slight reduction of *InMT1* expression was observed. The mRNA level of *InMT2* was permanently downregulated by zinc and remained significantly (~1.3-fold) lower than in the control throughout the 24-h experimental period (Figure 5).

Several lines of evidence have shown that the expression of plant MTs is affected by heavy metal ions in multiple plant species; however, the changes are dependent on the type of metal used for plant treatment, as well as on the type of pMT and the plant species (e.g., Guo et al., 2003; Usha et al., 2009; Ahn et al., 2012; Hrynkiewicz et al., 2012; Schiller et al., 2014). Our results demonstrated that *InMT1* and *InMT2* are differentially regulated by Cu and Zn and thus may fulfill diverse physiological functions in the maintenance of those micronutrients' homeostasis. The upregulation of *I. nil MT1* (Figure 5) in response to copper suggests that InMT1 may be involved in detoxification of Cu, at least in cotyledons of young developing seedlings. However, the observed increase of the expression is relatively small, and thus the contribution of InMT1 to Cu

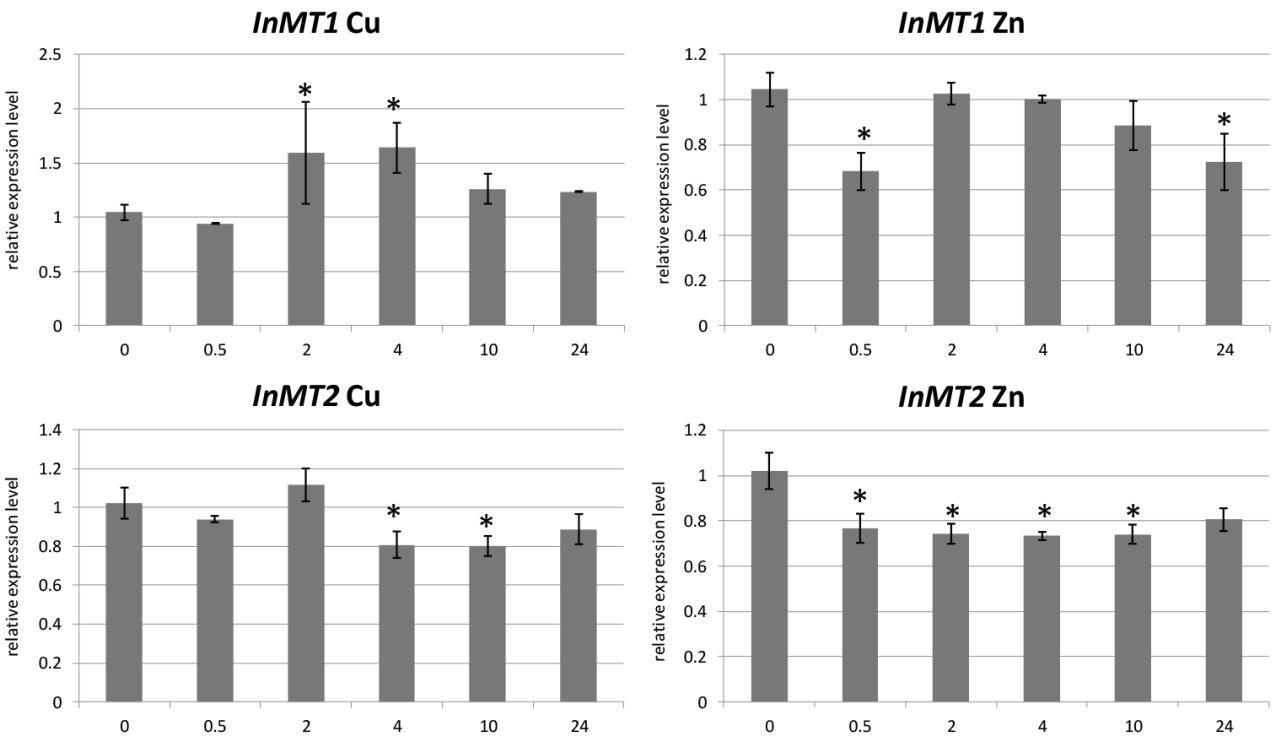

Figure 5. Analysis of *InMT1* and *InMT2* expression patterns in cotyledons of 6-day-old seedlings in response to heavy metal ion treatment. Total RNAs were isolated from cotyledons of 6-day-old seedlings treated with 200 µM $CuSO_4$ or 500 µM $ZnSO_4$. *β-actin* was amplified to confirm that a constant amount of total RNA was used. The graphs show a relative expression level expressed as the ratio of the amount of RT-PCR product for *InMT1* or *InMT2* gene to the amount of RT-PCR product for the housekeeping gene, *InAct4* (data are means of three independent replicates ± SE). The results obtained for each time point were compared with the expression level in untreated seedlings and an asterisk indicates significant differences ($P < 0.05$) between them.

homeostasis needs to be further evaluated. By contrast, the expression of *InMT2* was unaffected by copper (Figure 5), which may indicate that this metallothionein may be involved in the maintenance of Cu homeostasis when physiological amounts of this micronutrient are present, rather than in Cu detoxification, or that InMT2 is relevant to other micronutrients. The expression of type 2 MT was also not changed by Cu in *B. rapa* seedlings (Ahn et al., 2012). Zinc led to repression of *InMT1* and *InMT2* transcription (Figure 5), which suggests that those MTs are probably not involved in detoxification of excessive Zn in cotyledons of young seedlings. By contrast, increased expression of pMTs in response to zinc exposure was observed in several species, including hybrid poplar (Kohler et al., 2004), *P. juliflora* (Usha et al., 2009), and rice (Yang et al., 2009). The observed differences may, at least partially, emerge from the plant species having been examined at numerous stages of development. Therefore, it seems reasonable to speculate that the roles of pMTs in heavy metal metabolisms may be tissue- or organ-specific and may change during plant growth and development.

Taken together, the presented results show that *MT* genes encoding metallothioneins with pMT-typical numbers and arrangements of cysteine residues are present in the *I. nil* genome. Our data indicate that the physiological roles of InMT1 and InMT2 in the maintenance of zinc and copper homeostasis and/or detoxification of those micronutrients are diversified. However, the observed downregulation and relatively little upregulation of *InMT1* and *InMT2* expression levels in response to heavy metal treatment leave an unanswered question about the role of those MTs in micronutrient homeostasis. Therefore, further research is needed to pin down the physiological roles of InMT1 and InMT2.

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