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Comparative analysis of metallothionein proteins (MTs) from different grass species

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Plant metallothioneins (MTs) are small proteins, having rich cysteine residues and appear to play key roles in metal homeostasis. In current study, MTs of 9 grass species (*Brachypodium distachyon, Festuca rubra, Hordeum vulgare, Oryza sativa japonica, Oryza sativa indica, Saccharum officinarum, Sorghum bicolor, Triticum aestivum,* and *Zea mays*) were used for *in silico* comparative analysis. Physicochemical analyses showed that pI values and GRAVY index were found to be in the range of (4.44) - (7.36) and (-0.037) - (-0.376), respectively. All MTs investigated in this study exhibited hydrophilic character and 8 of 9 MTs had acidic nature. Also, there were no sequences containing tryptophan (Trp) residues. While the highest and lowest similar sequence identities were found between *S. bicolor* & *S. officinarum* (0.928), and *Z. mays* & *H. vulgare* (0.183), respectively. Phylogenetic analysis revealed that one main group observed including two subgroups. The highest bootstrap value was observed between *S. bicolor* and *S. officinarum* (92%). Interestingly, *H. vulgare* appears not to be within any grass species group.

Key words: Metallothioneins, grass speciess, comparative analysis, *Poaceae*.

Introduction

Metallothioneins (MTs) compromise a family of cysteine-rich, low molecular weight, metal-binding proteins found in various organisms including plants. Although MTs have been thought to play roles in metal ion metabolism, their functions are still unclear (Kagi, 1993). Metallothioneins are assumed to be connected with metal sequestration in plants and it may be associated with metal tolerance (Clemens *et al.*, 2002). Many plant MT genes have been isolated and characterized from different species including *Noccaea caerulescens* (Fernandez *et al.*, 2012), sugarcane (Sereno *et al.*, 2007), sweet potato (Chen *et al.*, 2003), *Vicia faba* (Foley and Singh, 1994), *Brassica napus* (Buchanan-Wollaston, 1994), barley (Heise *et al.*, 2007) and maize (White and Rivin, 1995).

Nine MT genes were identified in the *Arabidopsis* genome classified as two Type I genes (MT1a and MT1c); two Type II genes (MT2a and MT2b); one Type III gene (MT3) and three Type IV genes (MT4a, MT4b and MT4c) and a pseudogene (MT1b) (Zimeri *et al.*, 2005). The rice genome contains nine members of the MT gene family (Wong *et al.*, 2004) including three Type I genes (OsMT1a, OsMT1b, and OsMT1c), three Type II genes (OsMT2a,

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OsMT2b, OsMT2c), two Type III (OsMT3a and OsMT3b), and one Type IV (OsMT4). Some MT genes, namely HvMT-1a, HvMT -2b, HvMT -3a, HvMT-1b, HvMT -1c, HvMT -1d, and HvMT -2c were found to be in different tissues and organs in barley (Heise *et al.*, 2007). MT proteins were classified according to amino acid sequence into four types. Type 1 MTs contain a total of six Cys-Xaa-Cys motifs, type 2 and 4 MTs have two and three cysteine-rich domains, respectively. Type 3 MTs include four Cys residues in the N-terminal domain (Cobbett and Goldsbrough, 2002).

The grass family (*Poaceae*) comprises approximately 10,000 species, defined in 600 to 700 genera (Clayton and Renvoize, 1986). In general, *Poaceae* is divided into six or seven subfamilies, namely *Bambusoideae*, *Oryzoideae*, *Pooideae*, *Panicoideae*, *Arundinoideae*, *Chloridoideae*, and *Centothecoideae* (Renvoize and Clayton, 1992). This study was designed to analyse plant metallothionein protein sequences from different grass species (9) by using bioinformatics tools. Physiochemical properties of MTs proteins, and phylogenetic relationships between grass species based on MTs amino acid compositions were studied and the results were presented.

Materials and methods

Sequence data

The dataset of MTs protein sequences of grass species were obtained from NCBI (www.ncbi.nlm.nih.gov) database in FASTA format. The selected MTs were *Brachypodium distachyon* (XP_003577206.1), *Festuca rubra* (O24528.1), *Hordeum vulgare* (AFK12212.1), *Oryza sativa japonica* (Q5JM82.1), *Oryza sativa indica* (A2ZH20.1), *Saccharum officinarum* (ABP37784.1), *Sorghum bicolor* (EES00317.1), *Triticum aestivum* (P43400.1), and *Zea mays* (NP_001149669.1). Physiochemical data were generated from the Expasy's ProtParam server (Gasteiger, 2005) including sequence length, amino acid composition (Table 1), molecular weight, theoretical isoelectric point (pI), and grand average hydropathy (GRAVY) (Table 2). A multiple sequence alignment was done by using Clustal W (Larkin *et al.*, 2007) (Figure 1).

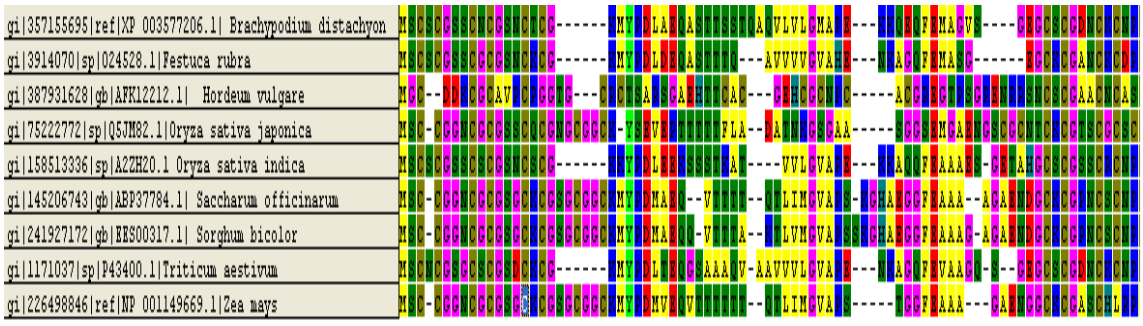

Figure 1. Multiple sequence alignment of MT protein sequences from amino acid residues 1–85.

The subcellular distribution and N-Glycosylation sites of the MT proteins were predicted by using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) and the NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) servers, respectively (Table 2).

Pfam (http://www.sanger.ac.uk/software/pfam/search.html) was used for domain analysis (Table 2). The Pfam database is a large collection of protein families, each represented by multiple sequence alignments and hidden Markov models (HMMs) (Punta *et al.*, 2012). The conserved

protein motifs were deduced by using MEME (Multiple Em for Motif Elicitation) software (Timothy *et al.*, 2009) (Figure 2 and Figure 3). Phylogenetic relationships among the MT protein sequences (Figure 4) and sequence identity matrix (Table 3) were generated using MEGA 5.1 **(**Molecular Evolutionary Genetics Analysis**)** software (Tamura *et al.*, 2011) by the bootstrap analyses with 1000 replicates (Felsenstein, 1985). The evolutionary history was inferred by using the Neighbor-Joining method based on the JTT (Jones-Taylor-Thomton) matrix-based model (Jones *et al.*, 1992). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965).

Table 1. Amino acid composition (in %) of MT proteins

	Plants								
Amino acids	Bd	Fr	Hv	Oj	Oi	So	Sb	Ta	Zm
Ala	6,67	8.57	11.69	6.25	9.46	9.88	10.71	12	8.42
Cys	16	17.14	22.08	21.25	16.22	17.28	16.67	16	11.58
Asp	2.67	4.29	2.60	1.25	1.35	2.47	2.38	4	5.26
Glu	6.67	5.71	5.19	5	8.11	4.94	4.76	5.33	4.21
Phe	1.33	1.43	0	1.25	1.35	1.23	1.19	1.33	1.05
Gly	10.67	12.86	16.88	21.25	9.46	19.75	20.24	14.67	18.95
His	0	1.43	2.60	0	1.35	1.23	1.19	0	3.16
Ile	0	0	0	0	0	1.23	0	0	1.05
Lys	5.33	7.14	1.3	3.75	8.11	6.17	7.14	5.33	3.16
Leu	4	1.43	0	1.25	2.7	1.23	1.19	2.67	3.16
Met	5.33	4.29	1.3	2.5	1.35	4.94	4.76	2.67	4.21
Asn	6.67	5.71	5.19	8.75	4.05	4.94	4.76	6.67	2.11
Pro	4	2.86	5.19	1.25	4.05	4.94	4.76	4	4.21
Gln	6.67	4.29	0	1.25	2.7	2.47	2.38	5.33	3.16
Arg	0	0	7.79	0	1.35	0	0	0	3.16
Ser	13.33	10	9.09	12.5	18.92	6.17	7.14	9.33	8.42
Thr	5.33	4.29	7.79	10	4.05	7.41	5.95	1.33	8.42
Val	4	7.14	1.3	1.25	4.05	2.47	3.57	8	4.21
Trp	0	0	0	0	0	0	0	0	0
Tyr	1.33	1.43	0	1.25	1.35	1.23	1.19	1.33	1.05

Notes: Bd: *B. distachyon*, Fr: *F. rubra,* Hv: *H. vulgare*, Oj: *O. japonica*, Oi: *O. indica*, So: *Sorghum bicolor*, Sb: *S. officinarum*, Ta: *T. aestivum*, and Zm: *Z. mays.*

Results and Discussion

A total of 9 plant MT proteins were analyzed by computational tools in grass species. GRAVY indices of MTs were ranging from -0.037 to -0.376. Physiochemical analysis revealed that all MTs proteins were in hydrophilic nature and in conjunction with this; this low range of GRAVY values supports the possibility of better interaction between MTs and

water. The computed pI value indicates that all MT proteins showed structure having basic character (pI<7) except one from *H. vulgare* (pI: 7.36). In general, MTs proteins were considered to be showing hydrophilic and acidic character (Table 2).

Table 2. Characteristics of MT proteins in grass species

Number	Accession number	Pfam family	Sequence length (aa)	M. wt. (Da)	pI	GRAVY	Predicted subcellular localization	N-glycosylation sites
1	XP_003577206.1	Metallothio 2	75	7705.6	4.49	-0.291	Chloroplast	14 NCTC
2	O24528.1	Metallothio 2	70	7096	5.05	-0.191	Unknown	No sites predicted
3	AFK12212.1	Metallothio PEC	77	7530.3	7.36	-0.375	Unknown	61 NCSC
4	Q5JM82.1	Metallothio 2	80	7601.3	4.58	-0.180	Unknown	58 NGSC
5	A2ZH20.1	Metallothio 2	74	7482.3	6.51	-0.376	Chloroplast	No sites predicted
6	ABP37784.1	Metallothio 2	81	7870.9	5.59	-0.130	Unknown	No sites predicted
7	EES00317.1	Metallothio 2	84	8099.1	6.49	-0.160	Unknown	No sites predicted
8	P43400.1	Metallothio 2	75	7376.2	4.44	-0.037	Unknown	No sites predicted
9	NP_001149669.1	Metallothio 2	95	9406.6	5.39	-0.271	Unknown	No sites predicted

Table 3. Sequence Identity Matrix for amino acid sequences of grass MTs.

	B. d.	***F. r.***	***H. v.***	***O. j.***	***O. i.***	***S. o.***	***S. b.***	***T. a.***	***Z. m.***
B. distachyon	1	0,693	0,207	0,305	0,641	0,471	0,454	0,684	0,352
F. rubra	0,693	1	0,262	0,349	0,605	0,482	0,500	0,720	0,393
H. vulgare	0,207	0,262	1	0,231	0,200	0,235	0,255	0,209	0,183
O. japonica	0,305	0,349	0,231	1	0,361	0,411	0,418	0,297	0,357
O. indica	0,641	0,605	0,200	0,361	1	0,458	0,453	0,623	0,360
S.officinarum	0,471	0,482	0,235	0,411	0,458	1	0,928	0,465	0,630
S. bicolor	0,454	0,500	0,255	0,418	0,453	0,928	1	0,482	0,588
T. aestivum	0,684	0,720	0,209	0,297	0,623	0,465	0,482	1	0,346
Z. mays	0,352	0,393	0,183	0,357	0,360	0,630	0,588	0,346	1

Based on amino acid composition, the most abundant three amino acids residues were found to be cysteine (Cys, in 5 sequences), glycine (Gly, in 4 sequences), and serine (Ser, in 1 sequences) respectively (Table 1). MTs were obtained with molecular weights and sequence lengths ranging from 7096 to 8099.1 Dalton and 70 to 95 amino acids, respectively. MTs are small ubiquitous cysteine-rich metal-binding proteins (≤10 kDa). A Cys-Gly-Gly-Cys motif is present at the end of the N-terminal cysteine-rich domain (Cobbett and Goldsbrough, 2002). Given data is consistent with our results showing that the largest molecular weights were found to be 8.0991 kDa (from *S. bicolor*) and cysteine (Cys) was predominant in 5 sequences,

respectively. MTs have rich cysteine residues and low aromatic amino acid contents with molecular weights between 6 and 8 kDa (Robinson *et al.*, 1993). In current study, MT proteins had low histidine (His), isoleucine (Ile), and arginine (Arg) residues and no any tryptophan (Trp) residue (an aromatic amino acid). This shows that there is an agreement between our data and previous data. When all MT sequences were subjected to MEME, a total of three motifs were observed (Fig. 2. and Fig. 3.). These motifs were KMYPDLAE QASTTTQTIILI (20 amino acids and 7repeats), AGAEHDGCKCGDNCRCNPC NC (21 amino acids and 8 repeats), and MNCGCGSSCKCGSNC (15 amino acids and 8 repeats) respectively. Type 2 MTs had Cys-Cys motif in amino acid positions 3 and 4 of these proteins and the C-terminal domain includes three Cys-Xaa-Cys motifs (Cobbett and Goldsbrough, 2002). Especially, motif II and motif III had rich cysteine residues presented in first five amino acid positions and included Cys-Xaa-Cys motifs (Figure 2). These findings support previous works (Cobbett and Goldsbrough, 2002; Kagi, 1993).

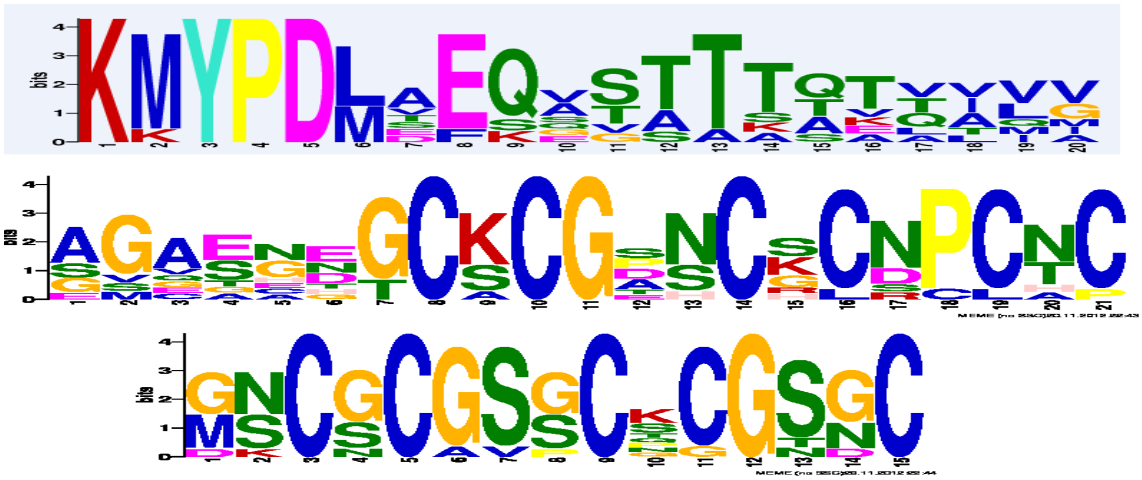

Figure 2. The conserved protein motifs in MTs (Motif I, motif II, and motif III, respectively)

Based on combine block diagram of MTs, 8 out of 9 MTs had motif III at first position and 7 out of 9 MTs contain motif I at second positions (Figure 3). Especially, motif III was present at first position in 8 sequences containing rich cystein residues except barley (*H. vulgare*). While 8 out of 9 grass species had metallothio 2 family members, a different protein family (Plant PEC family metallothionein) was detected in barley by using Pfam. In addition, to having a different protein family rather than metallothio 2 family, barley MT was the only having basic character (Table 2). It is known that there is a connection between protein structure divergence and sequence divergence (Lesk and Chothia, 1980). It is essential to understanding protein evolution and sequence differentiation for prediction of new functions (Bjorklund *et al.*, 2005). Despite the probability of radical substitutions, protein structure and function must be saved (DePristo *et al.*, 2005). These could show relation between MT proteins having different motif distribution arose during MTs evolution. In protein evolution, motifs are related with biological functions or protein structure and generally contain short amino acid residues (5-25 amino acids) (Saito *et al.*, 2007). Also, these motifs may be related with special physiological responses in plants.

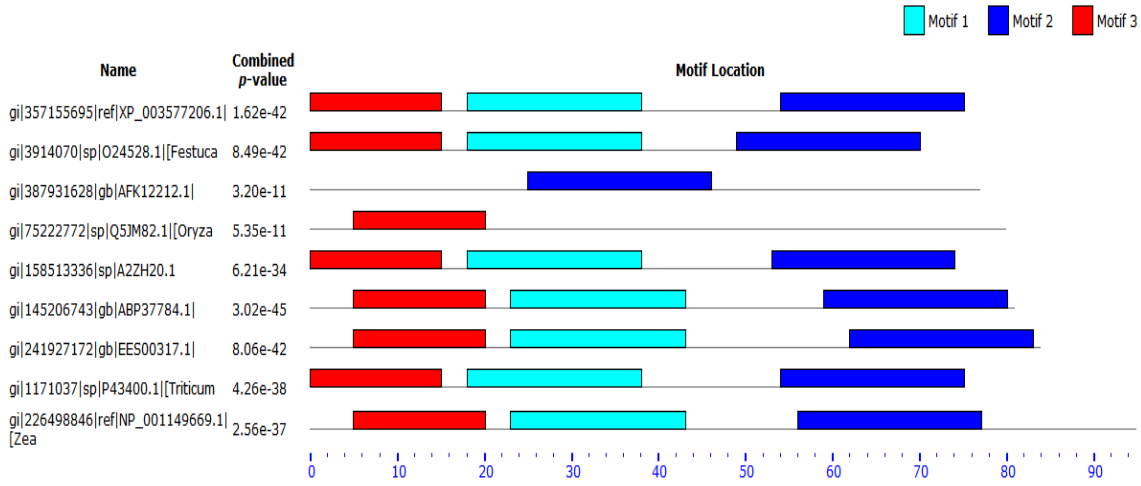

Figure 3. Combined block diagrams of the conserved protein motifs in grass MTs (Motif I, motif II, and motif III are KMYPDLAEQASTTTQTIILI, AGAEHDGCKCGDNCRCNPCNC, and MNCGCGSSCKCGSNC, respectively).

Predicted N-glycosylation sites were observed including 14 NCTC (Asparagine-cysteine-threonine-cycteine) for *B. distachyon,* 61 NCSC (Asparagine-cysteine-serine-cysteine) for *H. vulgare*, and 58 NGSC (Asparagine-glycine-serine-cysteine) for *O. japonica* (Table 2). N-linked glycosylation on protein sequences is a basic post-translational modification that causes covalent binding on asparagine residues owing to oligosaccharide attachment in polypeptide chains. The N-X-S/T consensus sequence was accepted as a general recognition element (Schwarz and Aebi, 2011). 3 out of 9 MTs are predicted to have N-glycosylation sites that may affect protein structure. It may lead to the emergence of resulted new catalytic functions in different plant tissues or organs. Subcellular localizations of 2 MTs (out of 9) were only predicted and this low prediction rate could be the result of poor-defined MTs. According to our sequence identity matrix data, the most closely related grass species were *S. bicolor* and *S. officinarum* (0.928) and those were followed by *T. aestivum* and *F. rubra* (0.720), and *B. distachyon* and *F. rubra* (0.693) respectively. The minimum identity value was observed between *Z. mays* and *H. vulgare* (0.183) (Table 3). The phylogenetic tree was established by using metallothionein protein sequences from nine grass species and *Vitis vinifera* was used as out group in this configuration (Figure 4).

The highest and lowest bootstrap values were found to be 92% (*S. bicolor* - *S. officinarum* cluster) and 51% (*Z. mays* - *H. vulgare* cluster), respectively. While barley (*H. vulgare*) was alone in group II, the other grasses were grouped together in group I. Group I consisted of three main subgroups (A, B, and C). Grasses have been classified into two major clades, BOP (*Bambusoideae*, *Oryzoideae*, *Pooideae*) and PACC (*Panicoideae*, *Arundinoideae*, *Chloridoideae*, *Centothecoideae*) (Grass Phylogeny Working Group, 2000). While subgroup A had three Pooideae's species (*T. aestivum*, *B. distachyon*, and *F. rubra*) and one *Oryzoideae*'s species (*O. indica*) with 94% bootstrap value, subgroup B had three *Panicoideae*'s species (*Z. mays*, *S. bicolor,* and *S. officinarum*) with 90% bootstrap value. *V. vinifera* as out-group species was grouped with *Panicoideae*'s species in subgroup B with 64% bootstrap value. The highest bootstrap value (92%)

and sequence similarity (0.928) were obtained between S. *bicolor* - *S. officinarum* clusters. The results of earlier studies show similarity with our findings. According to the results, *S. bicolor* and *S. officinarum* were found to be in *Panicoideae* subfamily (Watson and Dallwitz, 1992; Hilu, 2007). *O. indica* and *Poideae* species (*T. aestivum*, *B. distachyon*, and *F. rubra*) were grouped together in subgroup A (BOP clade). This is consistent with previous systematic data (Grass Phylogeny Working Group, 2000).

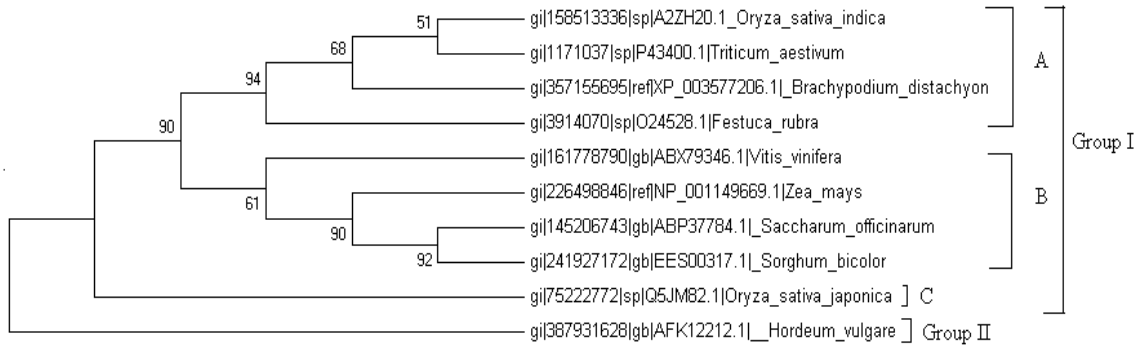

Figure 4. Phylogenetic tree of MTs representing grass species constructed by the NJ method. The bootstrap values are shown at the branch points.

Members of a gene family may contain tandem duplicates, dispersed duplications, and genome-wide duplications (Yuan *et al.*, 2002). Gene duplication may emerge through regional genomic events or genome-wide events (polyploidization) (Lawton-Rauh, 2003). It is well known that many land plant genomes harbor multiple copies of the whole genome occurred through polyploidy events (Soltis *et al.*, 2009). Gene duplications may lead to the formation of new functions of genes (Long and Langley, 1993). Also, gene duplication modes can alter expression divergence between duplicated genes (Wang *et al.*, 2012). Owing to duplication or speciation events, homologous proteins were diverged from related protein families by sequence substitutions (Orengo and Thornton, 2005). Also, insertions and deletions affected structural divergence efficiently (Flores *et al.*, 1993). Altough *H. vulgare* is known as a member of *Pooideae* subfamily, it was not grouped with the other grass species surprisingly. Also, *H. vulgare* had low sequence similarity values ranging from 0.183 to 0.262 with the other grasses. Its MT protein sequences may affect by evolutionary events such as sequence substitutions, mutations, and gene duplications in speciation. Consequently, our findings contribute to understand MT proteins in grasses. Also, new experimental and comprehensive analysis support discovering new putative MT proteins and understanding physiological roles of these proteins in annotated plant genomes.

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