Genome-Wide Identification and Expression Analysis of Metal Tolerance Protein (MTP) Gene Family in *Medicago Truncatula* Under a Broad Range of Heavy Metals Stress

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Abstract

Metal tolerance proteins (MTP) encompass plant membrane divalent cation transporters to specifically participate in heavy metal stress resistance and minerals acquisition. However, the molecular behaviors and biological functions of this family in *M. truncatula* are scarcely known. We identified 12 potential MTP candidate genes and analyzed for a phylogenetic relationship, chromosomal distributions, gene structures, protein structures, gene ontology, and previous RNA-seq data. MtMTPs were classified into three major cation diffusion facilitator (CDFs) groups; Mn-CDFs, Zn-CDFs, and Fe/Zn-CDFs. Structural analysis of SlMTPs displayed high gene similarity within the same group where all of them have cation_efflux domain or ZT_dimer. RNA-seq and gene ontology analysis revealed a significant role of MTP genes during *M. truncatula* growth and development.MTP genes showed tissue-specific and variable expression levels under the stress of the following five divalent heavy metals (Cd^{2+}, Co^{2+}, Mn^{2+}, $Zn^{2+,}$ and Fe^{2+}). Expression levels of Fe^{2+}/*MtMTP11* and Mn^{2+}/*MtMTP4* were upregulated, while Mn^{2+}/*MtMTP5* was downregulated. In conclusion, *MtMTP1.1, MtMTP1.2*, and *MtMTP4* play a key role under heat and heavy metal stress in *M. truncatula*.

1. Introduction

M. truncatula is a diploid plant species ($2n = 2x = 16$) with a comparatively small and its genome has been sequenced, that is being recursively used as a model plant for legume genetic research (Benedito et al. 2008, Tang et al. 2014, Young et al. 2011). It is one of the best essential forage crops widely cultivated across the world, same as alfalfa (Young &Udvardi 2009). *M. truncatula*is the best species to explore functional genomics of metal tolerance, although it does not accumulate metals by itself (Zhou et al. 2012).

Generally, metals act as co-factor, which has essential implications inactivating enzymes in plant cells to perform the specific biological reaction. In contrast, the higher accumulation of these metals in the cytosol is toxic for plant cells and resulted in necrosis (Thomine &Vert 2013).

Plant response to heavy metal stress activates a complex signal transduction network. Heavy metal acts as external stimuli to activate *in-vivo* biosynthesis of stress-related proteins and signaling molecules which subsequently activate transcription of specific metal-responsive genes to counter metal stress (El-Sappah et al. 2012). The significant consequence of abiotic stress factors, i.e., heavy metals, is an accumulation of reactive oxygen species (ROS) (BERTAMINI 2001). The ROS include but not limited to superoxide radical (O^{-}), hydrogen peroxide (H_2O_2), hydroxyl (OH^{-}), which are extremely toxic and negatively regulate plant growth (El-Sappah et al. 2017, Maksymiec 2007), by damaging DNA, proteins, lipids and chlorophyll (Schutzendubel &Polle 2002). In response to counter ROS, plants harbor antioxidants i.e., superoxide dismutase, peroxidase, catalase, glutathione reductase, and low non-enzymatic molecule antioxidants i.e., proline, tocopheroles, carotenoids, glutathione, ascorbic acid (Apel &Hirt 2004).

In nature, many transporters are belonging to different gene families play an essential role in metal regulatory processes which the plant divalent cation transporters or the plant metal tolerance proteins (MTPs) are one of them (Migeon et al. 2010). The cation Diffusion Facilitator (CDF) family genes, known in the plant as MTP, are integral membrane divalent cation transporters that play essential roles in transportation the divalent metals ions between the cell and the vacuole or the extracellular space beside their role in homeostasis and provide tolerance of cells to divalent metal ions, such as Cd^{+2}, Zn^{+2}, and Co^{+2} (Montanini et al. 2007, Nies &Silver 1995). The plant MTP proteins are homodimers with six transmembrane domains (TMDs), in which hydrophobic C-terminal is embedded in cell membrane while hydrophilic N-terminal faces towards cytosol (Gao et al. 2020, Kolaj-Robin et al. 2015). Based on *Arabidopsis* MTP sequencing annotation, MTPs are further classified into the following seven groups; 1, 5, 6, 7, 8, 10, and 12. A total of 10 *MTPs* in the *Arabidopsis* genome and the first identified *MTP* gene was *AtMTP1* (van der Zaal et al. 1999),while wheat contain 20 *MTPs* (Vatansever et al. 2017). Many MTPs have been characterized in *Arabidopsis*, such as *AtMTP1* is significantly expressed, which plays key role in Zn^{2+} transport between cytosol and vacuoles (Kobae et al. 2004). Similarly, *AtMTP3* is vacuolar membrane-localized, which plays a fundamental role in Zn^{2+} transport and metal stress tolerance during Fe^{2+} deficiency (Eroglu et al. 2016, Vatansever et al. 2017). Unlike other members of the MTP gene family, *AtMTP12* harbors 14 TMDs and interact with *AtMTP5* for Zn^{2+} transport between cytosol and Golgi apparatus (Fujiwara et al. 2015). *AtMTP8* and *AtMTP11* play key role in plant protection from Mn^{2+} toxicity *via* the overproduction of endosomal vesicles, which engulf Mn^{2+} and dispose it out (Delhaize et al. 2007, Peiter et al. 2007).

In this study, we identified 12 MtMTPs in *M. truncatula* and characterized them for their structural, functional and evolutionary relationship. Furthermore, change in their expression level was evaluated under the stress of the following five heavy metals; Cd^{2+}, Co^{2+}, Mn^{2+}, $Zn^{2+,}$ and Fe^{2+}. Our findings will provide deep insight into the MTP gene family involved in heavy metal stress response in a plant cell, founders and biological functions of MtMTP proteins, that will open new avenues of research in the area of the molecular mechanism of homeostasis, heavy metal transport, and finally, help to precise engineer *M. truncatula* plants for heavy metal stress.

2. Materials And Methods

2.1. Identification of MTP genes in M. truncatula

The MTP gene sequences were retrieved from an online available *M. truncatula* genome database (http://www.medicagogenome.org/), and a local database was constructed with the help of BioEide 7.0 software. Candidate *MtMTP* genes were analyzed for HMM profiling of the following two *MTP* domainsPF16916 and PF01545 on the Pfam website (http://www.sanger.ac.uk/Software/Pfam). Whole-genome blast analysis of putative MTP protein sequences of *M. Truncatula* was performed on NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi), and phytozome (https://phytozome.jgi.doe.gov/).

All retrieved MTP protein sequences were analyzed at E-value < $10^{-5}$to identify the MTP domain *via* SMART (http://smart.embl-heidelberg.de/) tools (Letunic et al. 2004). All detailed genetic information of the putative *MTP* gene family, such as chromosomal location and CDS, was obtained from the phytozome database (https://phytozome.jgi.doe.gov/). Furthermore, MTP family proteins were analyzed for their molecular weight, a number of atoms, amino acids, isoelectric point, and instability index using EXPASY PROTOPARAM (http://www.expasy.org/tools/protparam.html) (Gasteiger et al. 2003). Finally, theoretical PI and molecular weight were obtained using ProtParam Tool (http://web.expasy.org/portparam).

2.2. Phylogenetic analysis

In addition to *M. truncatula*, MTP gene family members of *Arabidopsis thaliana* (http://arabidopsis.org), *Cucumis sativus* (http://cucurbitgenomics.org/), *Populus trichocarpa* (http://plantgdb.org/PtGDB/), *Oryza sativa* (https://rapdb.dna.affrc.go.jp/) and *Triticum aestivum* (https://www.wheatgenome.org/) were also retrieved and analyzed for genetic mapping. The CLUSTALX 2.0 software with default parameters was used for MTP proteins multiple sequence alignments. All alignments were uploaded in MEGA 6.0 software with a Neighbor-Joining method to construct a phylogenetic tree. Finally, bootstrap analysis was performed at 1,000 iterations with a pair-wise gap deletion mode (Tamura et al. 2011).

2.3. Chromosomal locations, and synteny analysis

M. truncatula genetic database (https://phytozome.jgi.doe.gov/) was analyzed to retrieve information about chromosomal localization of *MTP* genes, which were subsequently used to construct genetic map by MapChart software (https://www.wur.nl/en/show/Mapchart.htm). Two genes of the same species located in the same clade were defined as coparalogs to identify tandem or segmental duplication events. Simultaneously, the Phytozome database (https://phytozome.jgi.doe.gov/) was analyzed to identify segmental duplication in *M. truncatula* genes. The paralogs were identified, which were considered results of tandem duplication due to splicing of two genes into five or more genes within a 100 kb region (Tang et al. 2008). Similarly, coparalogs located within duplicated chromosomal regions were considered segmental duplications (Wei et al. 2007). Smith-Waterman algorithm (http://www.ebi.ac.uk/Tools/psa/) was employed for the calculation of local alignments of two protein sequences. The synteny analysis of the *MtMTP* gene family was performed using circos (http://circos.ca/) to localize different alleles distributed on different chromosomes (Krzywinski et al. 2009).

2.4. Gene structures and motif analyses

Structural analysis of all *MTP* gene family members was performed to explore the organization of intron/exon by using both gDNA and CDS sequences by deploying an online tool, namely Genes Structure Display Server program (GSDS)(http://gsds.cbi.pku.edu.cn/index.php) (Hu et al. 2015). The conserved gene family motifs were detected using a Multiple EM for motif elicitation (MEME) (http://meme.nbcr.net/meme3/meme.html) by adjusting the following parameters; maximum 20 motifs and 6-200 amino acids per motif (Bailey et al. 2006).

2.5. Protein modeling, prediction and the gene ontology annotation (GO).

The Phyre2 (sbg.bio.ic.ac.uk/phyre2/) website was employed for protein modeling, prediction and analysis of MtMTP proteins (Kelley et al. 2015). Blast2GO v3.0.11 (https://www.blast2go.com) and OmicsBox software were applied on all identified MTP protein sequences for GO annotation analysis (Conesa &Götz 2008).

2.6. Gene expression analysis based on RNA-seq data

RNA-seq data obtained from different organs of *M. truncatula* was downloaded from Gene Expression Atlas Project (MtGEA, https://mtgea.noble.org/v3/) (He et al. 2009). Furthermore, we analyze retrieved data for the actual expression level of MTP genes obtained from leaves, roots, seed coat, and flowers of *M. truncatula* under normal conditions. Subsequently, MTPs gene expression was also analyzed by deploying cufflinks (version: 2.2.1). Finally, absolute FPKM values were divided by their mean, transformed into a ratio of log2, and MeV 4.5 was employed to cluster expression data into a heat map (http://heatmapper.ca/) (Babicki et al. 2016, Saeed et al. 2006).

2.7. Growth conditions and heavy metal treatments

In this study, *M. truncatula* (cv. JemalongA17) line was cultivated during the Autom of 2020 at the experimental greenhouse of Yibin University (China). First, the seeds were washed with 10% hypochlorous acid and distilled water. The seeds have been germinated using water-saturated filter paper, and then transferred to fertilized pittmoss soil with germination conditions of 16 hours light (27ºC) and 8.0 dark (18ºC) with a relative humidity of 70%. Four seeds were planted in each plastic pot. After emergence, thinning was performed to maintain two uniform seedlings per pot.Thirty-day-old *M. truncatula* was placed in 1/2 Hoagland solutions (pH 6.0) with different heavy metal concentrations 0.1 mM $CdCl_2$, 0.1 mM $CoCl_2$, 0.5 mM $FeSO_4$,1 mM $MnSO_4$ and 0.5 mM $ZnSO_4$ respectively, while normal 1/2 Hoagland solutions as the control (CK) (Desoky et al. 2020, Gao et al. 2020). The experimental pots were positioned in a complete randomized block design. The experiment was composed of 6 treatments, as shown above, and each treatment was repeated with 3 pots. Then, 24 h later, the leaves and roots of tube plantlets were collected and used as RNA extraction materials. Three biological replicates of expression analyses have been performed for each treatment.

2.8. RNA extraction and qRT-PCR analysis

Trizol reagent (Invitrogen, USA) was used for RNA extraction from all plant samples (leaf, stem, and root), and subsequently reverse transcribed to cDNA using SuperMix Kit (Transgen, Beijing). Primer 5.0 tool was used to design specific primers of all selected genes, includingβ-actin as a housekeeping gene (Table S1). Total, 20μL reaction mixture was used to perform real-time PCR containing the following reagents; 10 μL 2×SYBR premix Taq, 1 μL cDNA,0.5μL of each primer, and 8 μL ddH_2O. Real-time PCR reaction conditions were adjusted as; 95°C for 10 min,95°C for 15 sec,60°C for 60 sec, and 40 cycles in total. The relative expression level was calculated by applying Livak Eq. $2^{-\Delta\Delta CT}$ with three replications for each sample (Livak &Schmittgen 2001).

2.9. Statistical analysis

The data is presented in the form of mean ± standard deviation (SD). After testing the homogeneity of the experimental errors by Bartlett's test (Steel 1997), the data was also subjected to one-way analysis of variance (ANOVA)and Duncan's Multiple Range Test to show significant variations among means that were compared at $p \leq 0.05$.COSTAT computer software (CoHort Software version 6.303, Berkeley, CA, USA) was used for the statistical analysis.

3. Results

3.1. Identification of *MTP* genes in *M. truncatula*

In total, 27 genes were identified via blast analysis; subsequently, genes with incomplete functional domain were excluded for the next study, and finally, 12 candidate genes were selected for further analysis. Every gene was assigned with a specific name i.e., *MtMTP1.1*, *MtMTP1.2*, *MtMTP2*, *MtMTP4*, *MtMTP5*, *MtMTP7*, *MtMTP8.1*, *MtMTP8.2*, *MtMTP9*, *MtMTP10.1*, *MtMTP10.2*and *MtMTP11*. Characteristics of all 12 genes such as gene locus, molecular weight, number of amino acids, grand average of hydropathicity, and isoelectric points (Table 1). Except chromosome 6, all other 7 chromosomes of *M. truncatula* were the locus of *MTP* genes. The molecular weight of all MTP protein molecules varied from 39491.59 to 53259.81 kDa. The total number of inter and intra protein ionic residues were variable i.e., the highest anionic residues were in MTP1.1, and lowest in MTP5. Similarly, the highest cationic residues were in MTP10.2 and lowest in MTP4 and MTP11. All MtMTP members harbor a variable number of introns, but *MtMTP1.1, MtMTP1.2* and *MtMTP4* were without any intron.

3.2. Phylogenetic analysis of *MTP* gene families

In order to unreveal the evolutionary footprints of the *MTP* gene family in *M. truncatuala*, comparison among *MTP* gene families in different species was performed. We retrieved 12 *AtMTP* genes of *Arabidopsis thaliana*, 9 *CsMTP* genes of *Cucumis sativus*, 21 *PtMTP* genes of *Populus trichocarpa*, 10 *OsMTP* genes of *Oryza sativa*, and 8 *TaMTP* genes of *Triticum aestivum* were aligned against 12 *MtMTP* genes of *M. truncatula*and phylogenetic tree was constructed for comparison of evolutionary relationship. All *MTP*gene families were divided into seven groups i.e., Group1, 5, 6, 7, 8, 10, and 12 (Fig. 1).

The highest number of *MTPs* were pooled in Group10 such as *MtMTP9*, *MtMTP10.1*, *MtMTP10.2*, and *MtMTP11* along with *AtMTP9*, *AtMTP10*, and *AtMTP11*; than in Group 1 such as *MtMTP1.1*, *MtMTP1.2*, and *MtMTP4*along with *AtMTP1*, *AtMTP2*,*AtMTP3*, and *AtMTP4*; than in Group 8 such as *MtMTP8.1*, and *MtMTP8.2* along with *AtMTP8*; than in Group 7 such as *MtMTP7*, along with *AtMTP7*, than in Group 6 such as *MtMTP2*, along with *AtMTP6*, and finally in Group 5 such as *MtMTP5*, along with *AtMTP5*, and no any *MtMTP* was placed in Group 12. Noticeably, ionic clustering revealed that 4 *MtMTP*s were clustered in Zn-CDFs group, 2 *MtMTP*s were clustered in Fe/Zn-CDFs group, and 6 *MtMTP*s were clustered in Mn-CDFs group (Fig. 1).

3.3 Chromosomal locations and synteny analysis of *MtMTP* gene family

Synteny analyses were performed to unreveal the distribution of genes on different chromosomes. We observed that *MtMTP* genes are distributed among all seven chromosomes. Furthermore, for evaluation of gene family expansion and novel functions, we also investigated gene duplication and divergence with the help of circos. We observed only segmental gene pair duplication from PGDD (Plant Genome Duplication Database). Collinearity due to excision of segmental duplication was observed in many gene pairs with 70-100% identity percentage (Table S2). Segment duplication resulted in many homologies of *MTP* genes between *M. truncatula* chromosome pairs, such as what occurs with the genes, *MtMTP1.1*/ *MtMTP1.2*,*MtMTP4*/ *MtMTP5*,and *MtMTP8.2*/ *MtMTP10.2* (Fig.2). Except for *MtMTP7*, all rest of *MtMTP* genes in *M. truncatula* displayed single and multiple genetic duplications. Noticeably, we did not observe any obvious tandem duplication among all *MtMTPs*.

3.4 Gene structures and motif analyses

All *MTP* family genes were further divided into six subfamilies (A, B, C, D, E, and F) (Fig. 3a). Subfamilies A and F were the largest among all subfamilies with six members in each, followed by subfamily B with 2 members, whereas subfamilies C, D, and Eeach contain only one gene (Fig.3a). Intron and exon analysis of all *MTP* genes revealed that each retrieved sequence of the *MtMTP* gene family is a correct and true member of six subfamilies (Fig.3c). Although there was variation in size and location of intron and exon of all *MtMTP* genes, the similarity index was higher among all subfamilies, which proved a close evolutionary relationship among *MtMTP* gene family members. All *MTP* family genes contain a variable number of introns, but all members of subfamily F were without any intron. Amino acid sequence-based conserved motifs of *MTP* were analyzed using MEME (Fig.3b and Table S3). All conserved motifs comprised 50 amino acids except motif 10which was comprised of only 41 amino acids. The largest motifs were 3 and 6, which were observed in all subfamilies, followed by motif 10, 83.3% of aforementioned. Noticeably, the number, type, and order of motifs were similar in intrasubfamily than inter subfamilies.

3.5 Protein modeling, sub-cellular localization, and GO enrichment analysis

Phyre 2 web portal (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was employed for protein modeling using all MTP aminoacid sequences (Fig.4, and Table S4). All twelve predicted models for MTP proteins were 100% based on c6xpdB, c3j1zP, c2qfiB, and d2qfia2 templates. Similarly, sub-cellular localization, molecular function, and biological process were predicted by GO enrichment analysis (Fig.5 and Table S5). In sub-cellular localization analysis, the predicted distribution scores of MTP proteins were as following;12/60% in all membranes, 3/15% in the plasma membrane and vacuole, and 1/5% in Golgi apparatus and root hair. Noticeably, the *MtMTP1.2* gene was localized in 12 sub-cellular compartments out of all 14, which underlined the significant role of *MtMTP1.2*in metal stress resistance. Collective scores of MTP protein molecules during biological processes were as following; trans-membrane transport of Zn^{+} and Mn^{+} ions was 3/43%, while trans-membrane transport of cations was 1/14%. More precisely, *MtMTP1.1*, *MtMTP1.2,* and *MtMTP4* play a key role in transmembrane transport of Zn^{+}, while *MtMTP8.1*, *MtMTP8.2*, and *MtMTP11* play a crucial role in transmembrane transport of Mn^{+}. Molecular function analysis revealed significant roles of *MtMTP8.2 and MtMTP11* in heavy metal processes.

3.6 Gene expression analysis by RNA-seq data

MtMTP family genes expression profiling was performed by analyzing previously sequenced RNA-seq data (https://mtgea.noble.org/v3/) of the following tissues of *M. truncatula*; leaf, bud, shoot, hypocotyl, stem, flower, seed coat, pod, root, and root tip (Table S6). A heatmap diagram was constructed to show the differential expression level of each *MtMTP* gene in all tissues (Fig. 6). Comparatively, *MtMTP1.1* displayed the highest expression level in a pod, while *MtMTP1.2* displayed the highest expression level in the root tip. Similarly, the highest expression level of *MtMTP2* was observed in plant shoots, while the expression level of *MtMTP5* was mild in hypocotyl, root, and root tip. The expression level of *MtMTP7* was also mild in buds while higher in the shoot. *MtMTP11* displayed the highest expression level in approximately all tissues, while *MtMTP10.1*only in hypocotyl and seed coat, *MtMTP8.1* only in the root, and *MtMTP9* only in flower tissue. Noticeably, *MtMTP8.2 and MtMTP10.2* displayed the lowest expression level in all tissues but the highest expression in root.

3.7 qRT-PCR analysis of *MtMTPs* under the effect of heavy metals

All *MtMTP*genes displayed differential gene expression levels under treatment of different types of heavy metals investigated in the following tissues; root, stem, and leaf (Fig. 7). In roots, *MtMTP1.2* and *MtMTP4* displayed the highest expression level, while *MtMTP5, MtMTP7,* and *MtMTP9* displayed the lowest expression level under the treatment Cd^{2+}. Similarly, *MtMTP1.1* and *MtMTP11* displayed the highest expression level, while *MtMTP7* displayed the lowest expression level under the treatment of Co^{2+}.*MtMTP1.1*, *MtMTP4*, *MtMTP5*, *MtMTP8.1*, *MtMTP8.2* and *MtMTP11* displayed highest expression level, while *MtMTP10.2* displayed lowest expression level under the treatment of Fe^{2+}. *MtMTP1.1* and *MtMTP4* displayed the highest expression level, while *MtMTP5* displayed the lowest expression level under the treatment of Mn^{2+}. *MtMTP1.1, MtMTP1.2* and *MtMTP4* displayed highest expression level, while *MtMTP2*, *MtMTP5*, *MtMTP7* and *MtMTP11* displayed lowest expression level under the treatment of Zn^{2+}.

In stem, Cd^{2+} treatment resulted in increased expression of *MtMTP1.2* and *MtMTP4*, but a significant halt in expression of *MtMTP2* and *MtMTP5*. Similarly, Co^{2+} treatment significantly increased the expression of *MtMTP11*, but decreased the expression of *MtMTP7*. Fe^{2+} treatment increased the expression of *MtMTP4*, *MtMTP5* and *MtMTP11* but resulted in decrease in expression of *MtMTP2* and *MtMTP10.2*. Mn^{2+} treatment resulted in increased expreesion of *MtMTP4* and *MtMTP10.1*, but decreased expression of *MtMTP5*, *MtMTP7* and *MtMTP10.2*. Finally, Zn^{2+} treatment resulted in enhanced expression of *MtMTP1.1*, *MtMTP1.2* and *MtMTP4*, but decresed expression of *MtMTP5, MtMTP7* and *MtMTP11*.

In leaf, Cd^{2+} treatment resulted in increased expression of *MtMTP4*, but decresed expression of *MtMTP5*. Similarly, Co^{2+} treatment resulted in increased expression of *MtMTP1.1*, *MtMTP4* and *MtMTP5*, but decreased expression of *MtMTP7*. Fe^{2+} treatment displayed increased expression of *MtMTP1.2*, but decreased expression of *MtMTP2*. Mn^{2+} treatment resulted in increased expreesion of *MtMTP1.1* and *MtMTP4*, while decreased expression of *MtMTP1.2*, *MtMTP5* and *MtMTP7*. Finally, Zn^{2+} treatment resulted in increased expression of *MtMTP1.1*, *MtMTP1.2* and *MtMTP4*, while decresed expression of *MtMTP2*, *MtMTP5* and *MtMTP7*.

4. Discussion

MTP proteins are divalent cation transport channels responsible for cross membrane movement of heavy metals and play a key role in the acquisition of mineral nutrition. Additionally, these proteins are also responsible for tolerating heavy metals in plants grown in saline soil (Liu et al. 2019, Ricachenevsky et al. 2013). The *MTP* gene family has rigorously investigated in many plant species, including; *Arabidopsis* (van der Zaal et al. 1999), tobacco (Liu et al. 2019), wheat (Vatansever et al. 2017), and black poplar (Gao et al. 2020). Herein, we identified 12 putative *MTP* genes in *M. truncatula* via genome-wide identification analysis, which were further divided into six groups (Group 1, 5, 6,7,8 and 10) and three clusters Zn-CDFs, Fe/Zn-CDFs, and Mn-CDFs based on phylogenetic analysis (Fig. 1), and our findings are in consistent with previous studies Liu et al. (2019).

Based on phylogenetic and functional domain analysis, MTP proteins in *M. truncatula* were further divided into the following six subfamilies (A, B, C, D, E, and F (Fig. 3a and b). *MtMTP* gene structure analysis revealed that introns, exons, and motif sequences share great similarities with previously explored *MTP* gene subfamilies (Liu et al. 2018). For example, all members of the A-subfamily harbor five introns. At the same time, F-subfamily did not have even a single intron, which shows structural evolutionary changes in the *MTP* gene family in *M. truncatula*. The absence of introns in the F-subfamily also revealed a lower selection ability of introns gain or loss rate due to higher selection pressure of exons sequences (Harrow et al. 2006). Noticeably, the number and placement divergences of introns depend upon history and evolutionary events (Babicki et al. 2016, Jeffares et al. 2006, Rogozin et al. 2012).

Annotation and expansion analysis of the *MTP* gene family in *M. truncatula* revealed the existence of segmental duplication (Fig. 2 and Table S2), similar to other species (Schlueter et al. 2007). In total, we observed 28 segmental duplications in following gene pairs; *MtMTP2/ MtMTP8.1*, *MtMTP2/ MtMTP10.2* ,and *MtMTP8.2/ MtMTP9*. The existence of genetic duplication confirms that *MtMTP* gene family members are predominantly involved in secondary metabolism because genes involved in secondary metabolism often go under different types of gene duplication (Ober 2005). Amino acid sequences of each member of the *MtMTP* gene family were analyzed to predict the 3D structure of proteins, which is responsible for protein function (Fig. 4 and Table S4) (Büyükköroğlu et al. 2018). The c3j1zP template was employed to model MtMTP8.1 and MtMTP8.2 proteins, and all rest of the MtMTP proteins were analyzed with c6xpdB template at 100% confidence level. Noticeably, c6xpdB template specifically used for transport proteins and in PDB database entitled as; cryo-em structure of human znt8 double mutant-d110n and d224n, 2 which determine outward-facing conformation. Similarly, the c3j1zP template is specific to metal transport and in the PDB database entitled as; the inward-facing conformation of the zinc transporter yip revealed by cryo-electron microscopy. Besides major templates c6xpdB (for all protein) and c3j1zP (SlMTP2 and SlMTP9), other templates used in protein modeling were c2qfiB_ (transport protein) and d2qfia2 (Cation efflux protein transmembrane domain-like). All four templates predicted the possible role of MtMTP proteins as heavy metal-uptake proteins, which provide a path for transport of essential metals towards cytoplasm and toxic heavy metals export out of the cytoplasm. Similarly, metal-efflux proteins play a key role in detoxifying cells by eliminating excessive toxic heavy metals (Mani &Sankaranarayanan 2018).

Gene ontology (GO) term enrichment analysis revealed vital roles of *MtMTPs* in heavy metal management such as; trans-membrane transporter activity, cation transmembrane transporter activity, transporter activity, and ion transmembrane transporter activity (Fig. 5 and Table S5). Similarly, RNA-seq is a robust technique that is used for the detection and quantification of mRNA profiling (Zambounis et al. 2020). RNA-seq analysis revealed a significant change in the expression pattern of *MTP* genes in different tissues and at different developmental stages of *M. trunactula* under various metal ions stress, which was similar to *P. trichocarpa*, and these findings endorsed GO enrichment analysis (Gao et al. 2020). Noticeably, the elevated expression level of the following six genes, *MtMTP1.2*, *MtMTP2*, *MtMTP4*, *MtMTP7*, *MtMTP9*, and *MtMTP11*, in plant root tips indicated a key role of them in metal ions uptake from the soil. Moreover, *MtMTP10.1* was abundantly expressed in the seed coat, which suggests this gene might have a role in the deposition of metal ions in seed coat, but further

studies are invited for authentication (Fig. 6 and Table S6). Similarly, a higher expression level of *MtMTP11* in buds shows its roles in shoots and leaves development similar to *OsMTP11* in rice (Tsunemitsu et al., 2018). Regulation of gene expression is a fundamental phenomenon to regulate metabolism associated with duplicate genes (Qian et al. 2010), so down regulation in the expression of *MtMTP8.2* and *MtMTP10.2* was in order to maintain equilibrium during the metabolic pathway. qRT-PCR was performed to authenticate RNA-seq data analysis (Fig. 7); however, the slight variations between the results of both techniques are due to non-uniform growth conditions and varietal response. We examined the behaviour of these cation family with five divalent metals where numerous studies in other plants indicated the major roles of them with plant cell to enhance their tolerance against divalent metals such as Mn^{+2}, Cd^{+2}, Co^{+2} and Fe^{+2} (Gao et al. 2020, Montanini et al. 2007). The variable transcriptional pattern of *MtMTPs* in response to various heavy metals was complicated, which reveal their key role in heavy metal stress management, just like a tonoplast-localized Zn^{+} transporter *AtMTP1* in *Arabidopsis* (Dräger et al. 2004, Kobae et al. 2004), and steady *CsMTP1* expression under high concentration of Zn^{2+} in cucumber (Migocka et al. 2015).

As mentioned before, upregulation of expression of *AtMTP12* was not due to Zn^{+}treatment, but it was due to the formation of a heterodimeric complex with *AtMTP5* for Zn^{+}transport (Fujiwara et al. 2015), same in tobacco (Liu et al. (2019). Moreover, variable concentrations of Mn^{2+} slightly affect expression of Mn-CDFs (*AtMTP8*, *AtMTP9*, *AtMTP10*, and *AtMTP11*) (Delhaize et al. 2007), same with tobacco (Liu et al. 2019). Contrarily, all Zn-CDF members displayed significant variation in their expression under treatment of Zn^{2+}, such as *MtMTP2* and *MtMTP7* of Zn/Fe-CDFs were down-regulated under treatment of higher concentration of Zn^{2+}. Furthermore, *MtMTP10.1* of Mn-CDF class was highly affected by the accumulation of Mn^{2+} only in stem. Our findings provide deep insights in molecular function of *MTP* genesin *M. truncatula* under various heavy metals stresses, which will invite researchesrs to precisely identify function of desired *MTP* genes in *M. truncatulavia* wet lab experiments.

5. Conclusion

Genome-wide identification analysis revealed 12 *MTP* genes in *M. truncatula*, which were further phylogenetically and comprehensively analyzed. The *MtMTPs* were divided into three major substrate-specific clusters (Zn/Fe-CDFs, Zn-CDFs, and Mn-CDFs), and six groups that were subjected to polyploidization under segmental duplication. All *MtMTPs* are predicted to harbor cation_efflux domain and/or ZT_dimerdomain, during each MTP share the same structural characteristics within the same group. The expression patterns of each *MtMTPs* gene in response to various heavy metals in different tissues indicated that these genes play a vital role in the growth and development of *M. truncatula*. Furthermore, our gene expression analysis revealed significant *MtMTP1.1*, *MtMTP1.2*, and *MtMTP4* in heavy metal stress tolerance in plants.

Declarations

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Authors Contributions :

Conceptualization, A.H.El-S (Ahmed H. El-Sappah); Formal analysis, A.H.El-S., J. L , Y. W.,Y. Z. and W. B; Methodology, A.H.El-S , M. A., X. Z. and R. G. E.; Writingoriginal draft, A.H.El-S.; writing, review and editing, A.H.El-S, K. Y., R. G. E. and Z. X; Correspondance, Z. X, and M. A.

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Tables

Table 1 The characteristics of MTP genes in *M. truncatula*.

No.	MTP	Gene NCBI symbol	Location	(-)	(+)	MW KDa	aa	Instability	Aliphatic index	GRAVY	PI
1	*MtMTP1.1*	LOC11443599	Chro2; 15887267..15891212	53	29	45054.52	407	31.92	106.81	0.053	5.89
2	*MtMTP1.2*	LOC25500609	Chro 8; (8867176..8870206	45	30	42458.02	385	31.15	112.7	0.201	6.02
3	*MtMTP2*	LOC25484765	Chro 1; 43633283..43640692	48	39	53259.81	491	40.20	89.06	-0.097	6.43
4	*MtMTP4*	LOC25492544	Chro 4; 32388504..32391373	36	26	43976.56	394	27.69	100.91	0.080	6.33
5	*MtMTP5*	LOC11428206	Chro 7; 43924325..43932959	33	34	43507.07	390	42.01	97.21	0.177	7.76
6	*MtMTP7*	LOC25491241	Chro 4; 1626723..1631936	42	40	48487.48	438	36.64	92.65	0.028	6.83
7	*MtMTP8.1*	LOC11413755	Chro 3; 31742366..31745903	50	38	45251.13	403	49	109.83	0.062	5.3
8	*MtMTP8.2*	LOC11425928	Chro 5; 33499438..33502150	46	30	44510.32	395	38.79	102.91	0.115	5.22
9	*MtMTP9*	LOC25501161	Chro 8; 18810864..18814971	45	41	44922.87	394	47.54	96.22	-0.091	6.53
10	*MtMTP10.1*	LOC25486917	Chro 2; 33136428..33141227	46	44	45006.21	393	43.79	95.98	-0.015	6.62
11	*MtMTP10.2*	LOC11432698	Chro 3; 39685592..39688357	47	47	46241.68	401	40.83	100.4	-0.109	7.16
12	*MtMTP11*	LOC11438849	Chro 7;7882546..7888430	41	26	39491.59	347	44.73	102.59	0.148	5.07

(-), (+), MW, aa, GRAVY and PI refer to, the total number of negatively charged residues (Asp + Glu), the total number of positively charged residues (Arg + Lys), molecular weight, amino acid number, Grand average of hydropathicity and isoelectric points, respectively

Figures

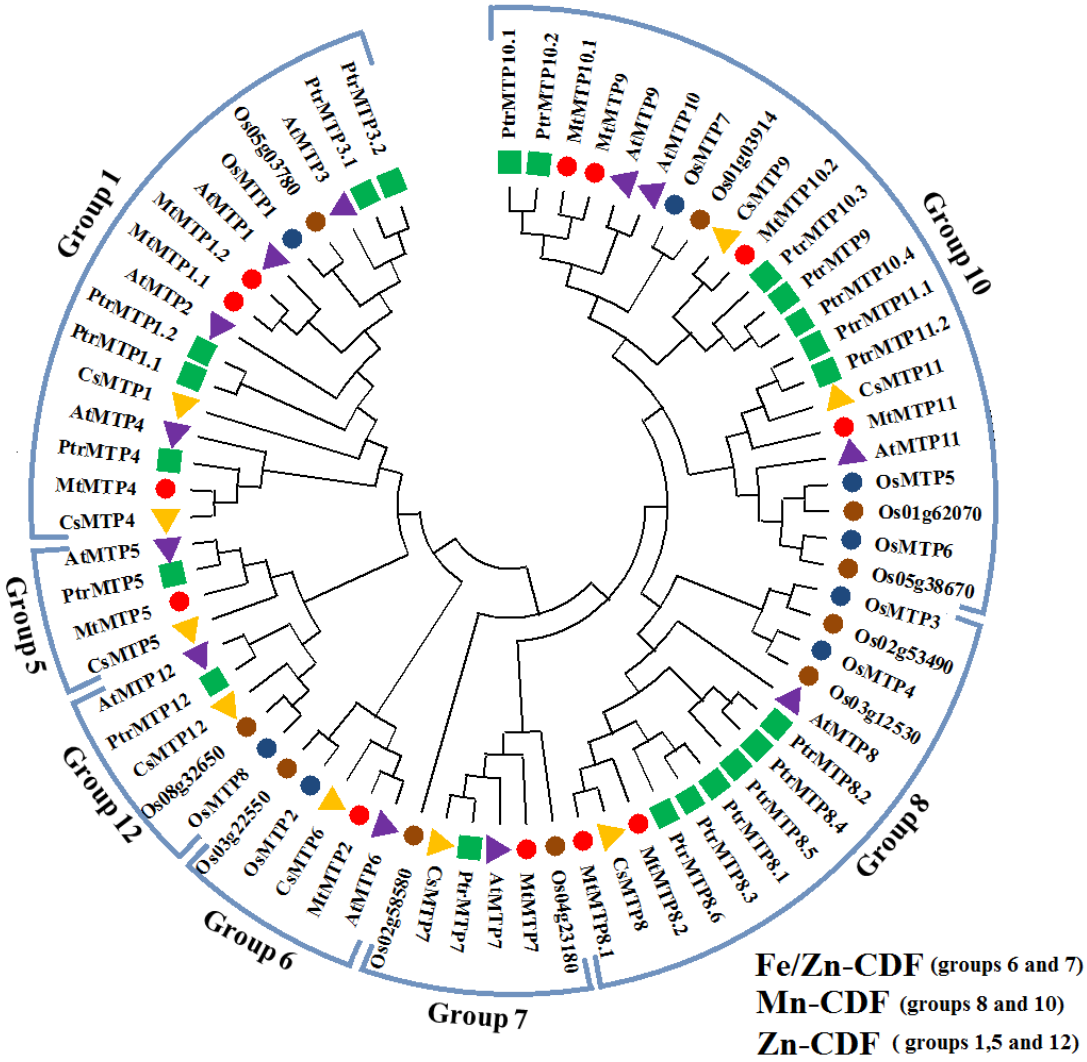

Figure 1

Phylogenetic tree of 72 MTP proteins: 12 M. truncatula (marked by red circle),12 Arabidopsis (purble triangle), 8 Wheat (blue circle), 10 Rice (brown circle), 9 Cucumber (yellow triangle), and 21 Black Poplar (blue square). ClustalX1.83 was used for protein alignments and the phylogenetic tree's construction Neighbor-Joining (NJ) level with MEGA5.0 software at 1,000 replications boot-strap.

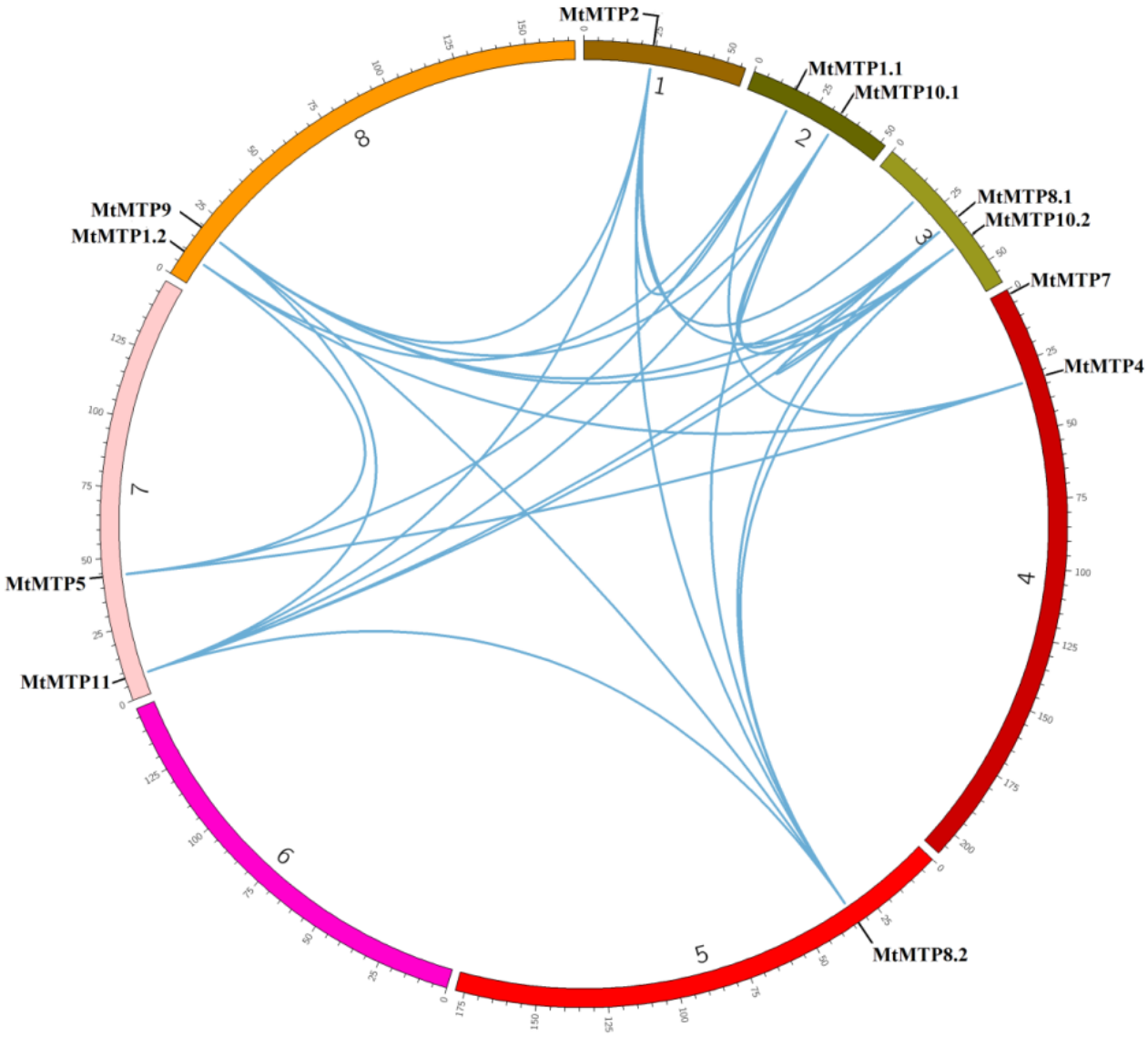

Figure 2

Genome-wide synteny analysis of MTP gene family among 8 M. truncatula chromosomes. The blue lines represented the syntenic orthologs and paralogs and displayed segmental duplication.

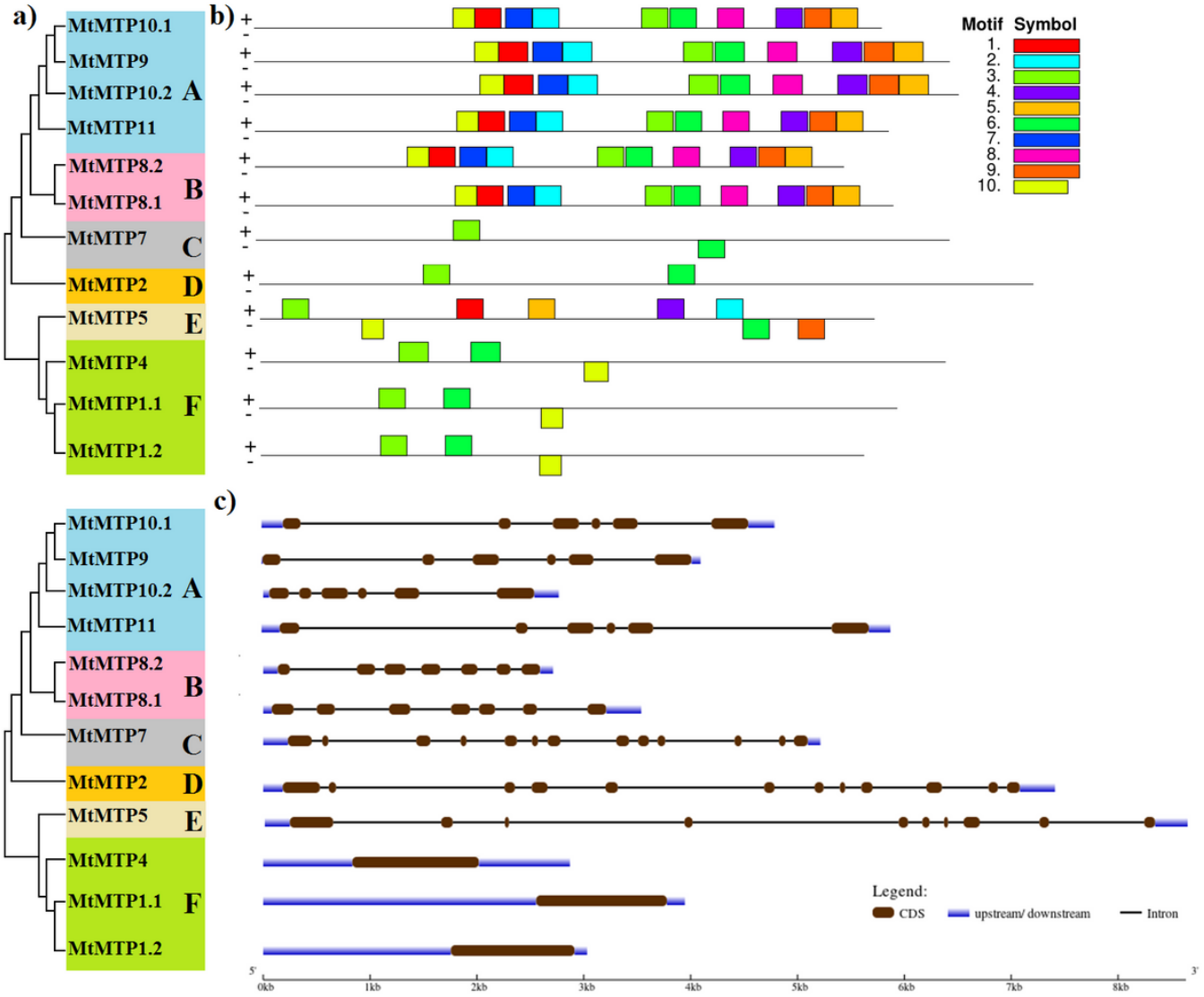

Figure 3

Phylogenetic relationship, gene structure and conserved motif analysis of MtMTP genes;(a) The neighbor-joining phylogenetic tree was constructed with MEGA7 using MtMTP amino acid sequences with 1000 times replicate. (b) The motif composition of MtMTP proteins using ten conserved motifs is represented by the unique colour mentioned in the box on the top lift. (c) The exon-intron structure of M. truncatula MTP proteins where dark green boxes presented the exons, and the black lines represent the introns. The blue boxes represented the untranslated regions (UTRs), with size scales detailed at the bottom.

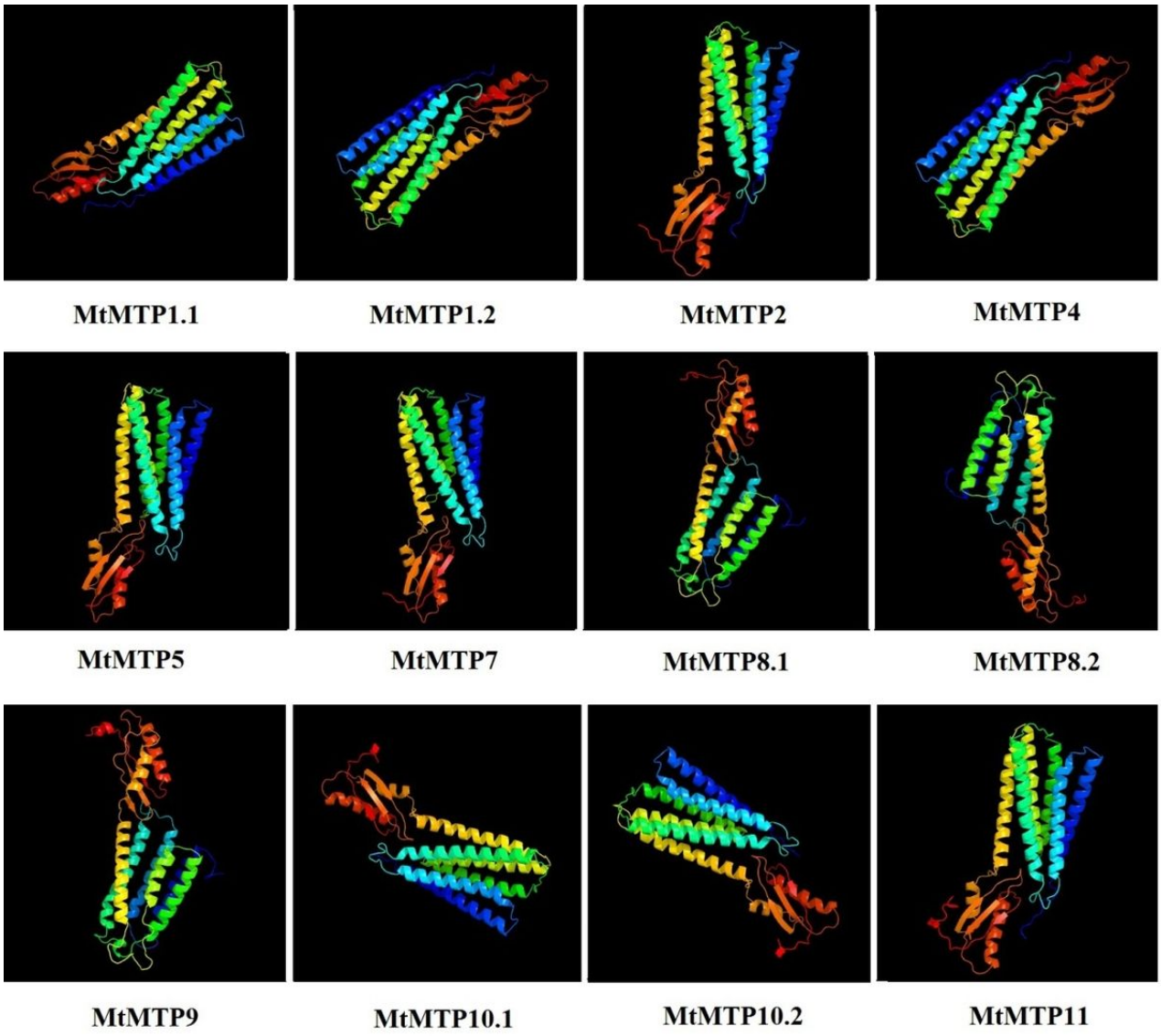

Figure 4

Predicted 3D models of M. truncatula MtMTP proteins. Models have been generated by using the Phyre 2 server in intensive mode. Models were visualized by rainbow colour from N to C terminus.

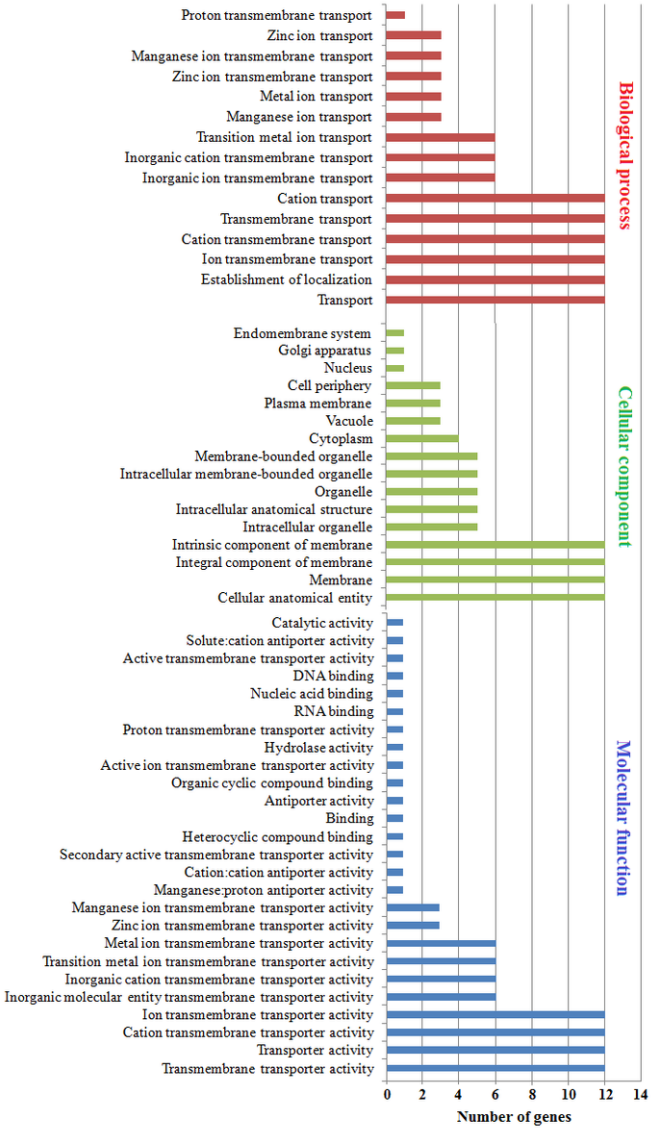

Figure 5

Gene Ontology analysis of M. truncatula MtMTP genes. Gene ontology showed the distribution of every MtMTP gene in the plant, where a red colour column mentioned the cellular component. In contrast, the biological processes in which the MTP family participate were mentioned by the blue colour column, and the molecular function was mentioned by move colour.

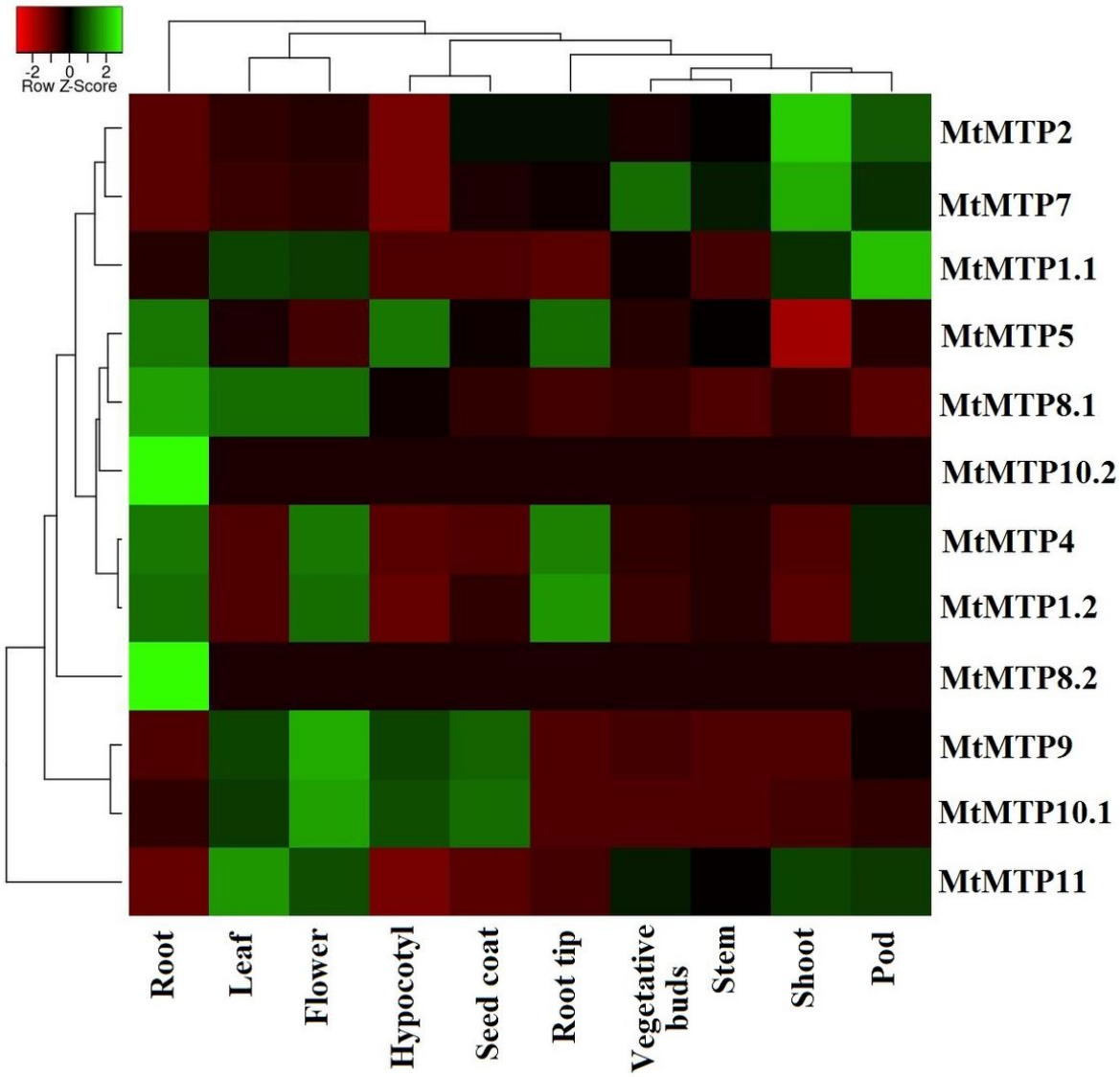

Figure 6

The heat map of M. truncatula 12 MtMTP genes expression profiles based on RNA-seq data. The previous expression has been shown in root, leaf, flower, hypocotyl, seed coat, root tip, vegetative buds, stem, shoot and pod tissues.

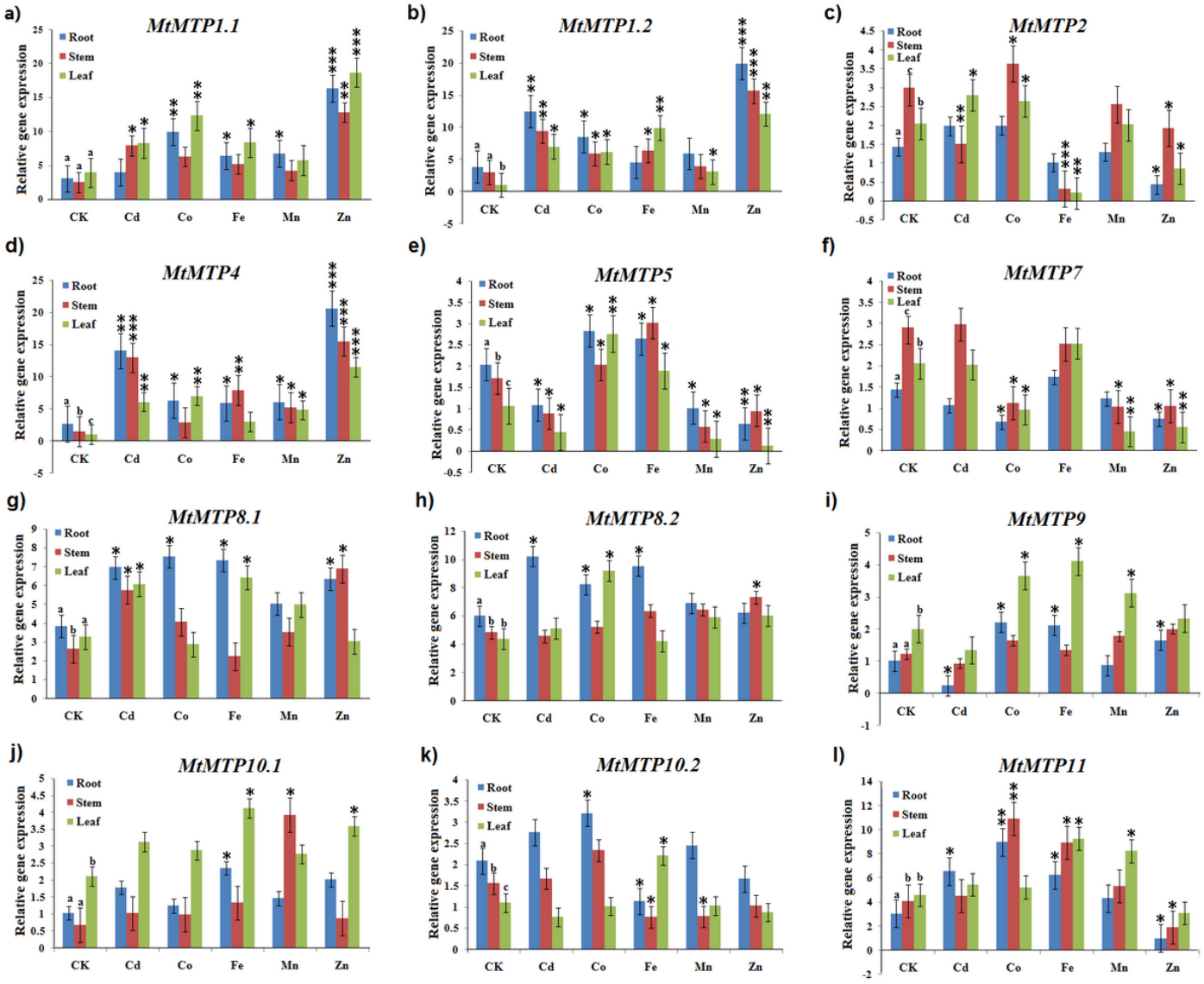

Figure 7

The qRT-PCR expression of the M. truncatula MtMTP genes from leaf samples. The reactions were normalized using the β-actin reference gene. The standard deviations have been represented by the error bars from three independent technical replicates. The mean expression levels of three replicates were analyzed with the five heavy metals treatments (Cd2+, Co2+, Fe2+, Mn2+, and Zn2+) using t-tests ($p< 0.05$) while the CK represents control samples. Asterisks indicate significant differences between the treatment samples and the corresponding control samples in roots, stems, and leaves. ($n = 9$, $p < 0.05$, Student's t-test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.primersforMTPfamilyexpression.docx
- TableS2Mt.MTP.synteny.xlsx.xlsx
- S3Motifsequances.docx
- tableS4.proteinmodlingsummary.xlsx
- TableS5geneontology.xlsx
- TableS6RNAseq.xlsx