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ORIGINAL ARTICLE

Genome-wide identification and expression analysis of metal tolerance protein (MTP) gene family in soybean (*Glycine max*) under heavy metal stress

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Abstract

Aim Plant metal tolerance proteins (MTPs) are plant membrane divalent cation transporters that specifically contribute to heavy metal stress resistance and mineral uptake. However, little is known about this family's molecular behaviors and biological activities in soybean.

Methods and results A total of 20 potential MTP candidate genes were identified and studied in the soybean genome for phylogenetic relationships, chromosomal distributions, gene structures, gene ontology, cis-elements, and previous gene expression. Furthermore, the expression of MTPs has been investigated under different heavy metals treatments. All identified soybean MTPs (*GmaMTP*s) contain a cation efflux domain or a ZT dimer and are further divided into three primary cation diffusion facilitator (CDF) groups: Mn-CDFs, Zn-CDFs, and Fe/Zn-CDFs. The developmental analysis reveals that segmental duplication contributes to the *GmaMTP* family's expansion. Tissue-specific expression profiling revealed comparative expression profiling in similar groups, although gene expression differed between groups. *GmaMTP* genes displayed biased responses in either plant leaves or roots when treated with heavy metal. In the leaves and roots, nine and ten *GmaMTP*s responded to at least one metal ion treatment. Furthermore, in most heavy metal treatments, *GmaMTP1.1*, *GmaMTP1.2*, *GmaMTP3.1*, *GmaMTP3.2*, *GmaMTP4.1*, and *GmaMTP4.3* exhibited significant expression responses.

Conclusion Our findings provided insight into the evolution of MTPs in soybean. Overall, our findings shed light on the evolution of the *MTP* gene family in soybean and pave the path for further functional characterization of this gene family.

Keywords *Glycine max* · Heavy metals · Metal tolerance protein (MTP) · Gene expression

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Introduction

Metals function as cofactors, which is important in inactivating enzymes in plant cells to complete particular biological reactions [1]. The optimum concentration of numerous metal ions such as manganese (Mn^{2+}), iron ($Fe^{2+,3+}$), zinc (Zn^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}), vanadyl (VO^{2+}), and cobalt (Co^{2+}) is required for regulation of enzymatic activities in various biological processes. Still, the excess of these transition ions stimulates over-accumulation of reactive oxygen species (ROS), which seriously affects crop yield [2, 3]. For instance, two amidohydrolase compound families utilize Zn^{2+}, Ni^{2+}, and Fe^{2+} as cofactors during hydrolysis. Contrastively, minuscule amount of non-essential metals including mercury (Hg^{2+}), silver (Ag^{+}), cadmium (Cd^{2+}), chromium (Cr^{+2},Cr^{+3},Cr^{+6}), selenium (Se^{-2},Se^{+4},Se^{+6}), lead (Pb^{2+}) and arsenic (As^{-3}) is harmful to plant cells. [4, 5]. Plant roots assimilate metal ions from soil dissolved in water and store them in different tissues [6].

To deal with the harmful impacts of heavy metals stress, plants' biochemical and metabolic processes go through exact changes at a physiological and molecular level [7, 8]. Metal transporters are important for plant homeostasis in specific metal transport proteins, natural resistance-associated macrophage proteins, cation diffusion facilitators (CDFs), and heavy metal ATPase, which are initiated to prevent cell harm by metal stress, consistently [9, 10]. Physiological processes, for example, metal absorption, transport, accumulation, chelation, and efflux, are plant elements to keep up with metal homeostasis [11]. Some specific membrane-bound protein families serve as metal ions transport channels. Cation diffusion facilitators (CDFs) proteins serve as divalent cation transporters responsible for metal ions efflux from the cytoplasm in subcellular organelles or cell export [12]. In light of substrate diffusion, there are three classes of CDF families; Zn-CDF, Fe/Zn-CDF and Mn-CDF, which are responsible for Zn^{2+}, Fe^{2+} and Mn^{2+} moves alongside Cu^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} [11].

These CDFs transporters are also known as metal-tolerance proteins in plants, as explained in the accompanying seven gene subfamilies 1, 5, 6, 7, 8, 9, and 12 [12]. There are 12 MTP proteins in *Arabidopsis thaliana* [13], 11 in *Solanum Lycopersicum* [8], 11 in *Vitis vinifera* [14], 22 in *Populus trichocarpa* [15], 20 in *Triticum aestivum* [16], 12 in *Medicago truncatula* [17], 13 in *Camellia sinensis* [18], 26 in *Nicotiana tabacum*, 13 in *Nicotiana sylvestris*, and 12 in *Nicotiana tomentosiformis* [7], 18 in *Brassica rapa* [19], 12 in *Citrus sinensis* [20] and 7 in *Pyrus bretschneideri* [21]. Initially, a group of 8 MTPs was distinguished in *Stylosanthes hamata*, which increased resistance against Mn^{2+} toxicity on overexpression in Arabidopsis [22].

Numerous Zn-CDFs have been identified in Arabidopsis [13], such as *AtMTP1* and *AtMTP3* performs a vital role in $Zn^{2+}$and Co^{2+} disposition into vacuole [23], and *AtMTP5* and *AtMTP12* in the accumulation of Zn^{2+} into Golgi bodies [24]. Correspondingly, the elements of the Mn-CDF group, such as *AtMTP8,* play a significant function in Mn^{2+} homeostasis via their disposition as well as deposition of Fe^{2+} and Mn^{2+} in seeds of Arabidopsis during seed development and germination [22, 25, 26]. *OsMTP8.1*, *OsMTP8.2*, *HvMTP8.1,* and *HvMTP8.2* are localized in tonoplast and serve as Mn^{2+} deposition in Golgi apparatus in rice and barley [27–29]. Furthermore, cucumber *CsMTP8* confers Mn^{2+} tolerance when overexpressed in Arabidopsis and yeast [30].

Soybean is a leguminous crop rich in seed oil, proteins, vitamins, and isoflavones. Genetically, soybean is a legacy paleopolyploid and a pending model for elucidating the sequels of genome duplication in higher eukaryotes [31]. As of late, endeavors have been led to investigate model plants and common commercial species genomes [32]. Also, the accomplishment of great draft genomes in the soybean *MTP* gene family at the genome-wide level gave an event to play out an orderly examination. In this article, we recognized *GmaMTP* genes in soybean and investigated their structure, 3D protein structure, gene ontology, and cis-regulating elements. Expression outlines of *GmaMTP* genes in various soybean tissues under the five divalent heavy metals stresses were examined.

Our findings will provide a deep understanding of the MTP gene family involved in heavy metal stress response in a plant cell, as well as the founders and biological functions of GnaMTP proteins, which will open up new avenues of research in the area of molecular mechanisms of homeostasis and heavy metal transport, and will ultimately help to precisely engineer soybeans plants for heavy metal stress.

Materials and methods

Identification of soybean *GmaMTP* genes

The candidate soybean *GmaMTP* gene sequences were retrieved via blast analysis of open reading frames (ORFs) of *AtMTPs*, *MtMTPs,* and *PtMTPs* in an Integrating Genetics and Genomics to Advance Soybean Research (soybase.org/) database by adjusting the default parameters. Subsequently, BioEide 7.0 software was employed to construct a local database. Furthermore, candidate *GmaMTP* genes were analyzed for HMM profiling of two conserved *MTP* domains, such as PF16916 and PF01545, by searching the Pfam website (sanger.ac.uk/Software/Pfam). Every gene was assigned a specific name following the standard Mendel database for plant gene families listed in Commission on Plant Gene Nomenclature (CPGN) (mbclserver.rutgers.edu/

CPGN/), International Society of Plant Molecular Biology (ISPMB) [33].

Whole-genome blast analysis of putative MTP protein sequences of soybean was performed on the NCBI database (blast.ncbi.nlm.nih.gov/blast.cgi) and phytozome database (phytozome.jgi.doe.gov/). Finally, all retrieved protein sequences were confirmed by exploring CCD (www.ncbi.nlm.nih.gov/cdd/) and SMART (smart.embl-heidelberg.de/) programs. All retrieved GmaMTP protein sequences were anatomized at E-value $< 10^{-5}$ to identify the MTP domain by employing SMART (smart.embl-heidelberg.de/) tools [34]. All detailed inheritable information of the putative *GmaMTP* gene family, including chromosomal location and CDS, were acquired from the phytozome database (phytozome.jgi.doe.gov/). Likewise, MTP family proteins were analyzed for their molecular weight, number of atoms, number of amino acids, isoelectric point, and instability index using EXPASY PROTOPARAM (expasy.org/tools/protparam.html) [35]. Eventually, calculations of theoretical isoelectric point (pI) and molecular weight (MW) were attained using ExPASy ProtParam Tools (web.expasy.org/portparam) [18].

Phylogenetic analysis

In addition to protein sequences of *GmaMTPs* of *Glycine max*, AtMTP protein sequences of *Arabidopsis thaliana* (arabidopsis.org), CsaMTP sequences of *Cucumis sativus* (cucurbitgenomics.org/), PtrMTPs protein sequences of *Populus trichocarpa* (plantgdb.org/PtGDB/), OsMTPs protein sequences of *Oryza sativa* (rapdb.dna.affrc.go.jp/) and TaMTPs protein sequences of *Triticum aestivum* (wheat-genome.org/) were also recaptured and anatomized for genetic mapping. The ClustalX2.0 software with default parameters was used for multiple sequence alignments of all retrieved MTP protein sequences [36]. All alignments were uploaded in MEGA6.0 software with a Neighbor-Joining method to construct a phylogenetic tree [37]. Finally, bootstrap analysis was performed at iterations with a pair-wise gap deletion mode [38, 39].

Chromosomal localization and gene synteny analysis

Soybean genetic database (phytozome.jgi.doe.gov/) was searched to retrieve data about chromosomal localization of *GmaMTP* genes, and a high-fidelity genetic map was constructed by assuming MapChart (wur.nl/en/show/Mapchart.htm) software. The genes of single species placed in the same clade were declared asco-paralogs which were further explored for possible duplication events. The Phytozome (phytozome.jgi.doe.gov/) database was searched to explore segmental duplications among *GmaMTP* genes. The presence of paralogs was supposed to result from tandem duplication due to splicing two genes into five or further within a 100 kb stretch [40]. Also, co-paralogs located within duplicated chromosomal regions were supposed to be segmentally duplicated [41]. Smith-Waterman algorithm (ebi.ac.uk/Tools/psa/) was employed to calculate the local alignments of two protein sequences. The synteny analysis of the *GmaMTP* family members was performed using circos (circos.ca/) tools to localize different alleles distributed among chromosomes [42].

Gene structures, motif analyses, and cis-regulatory elements prediction

Each *GmaMTP* gene sequence was analyzed to investigate for number, size, and location of introns and exons in both gDNA and CDS sequences by deploying the Genes Structure Display Server program (GSDS) (http://gsds.gao-lab.org/) [43]. Furthermore, conserved gene family motifs were also identified by deploying a Multiple EM for motif elicitation (MEME) (meme.nbcr.net/meme3/meme.html) tool with the following parameters; a maximum of 20 motifs harboring 6–200 amino acids per motif [44]. To investigate regulatory mechanisms of *GmaMTP* genes, 2 kb upstream 3'UTR promoter region of each gene was downloaded from plant genomic resource (phytozome.jgi.doe.gov/). The cis-elements of *GmaMTPs* promoter were identified using the PLACE (dna.affrc.go.jp/PLACE/?action=newplace) and PlantCARE programs (bioinformatics.psb.ugent.be/webtools/plantcare/html/) [45, 46].

Protein modeling, protein–protein interaction, and Gene ontology (GO) analysis

The Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) website was searched to perform protein modeling, prediction of protein–protein interactions and detailed analysis of GmaMTP polypeptides [47]. The amino acid sequences of each GmaMTP family member were used to construct a protein–protein interaction network in the STRING database (string-db.org/). Finally, the following two softwares, Blast2GO v3.0.11 (www.blast2go.com) and OmicsBox were employed to predict GO enrichment of all identified GmaMTP protein sequences[48].

RNA-seq-based gene expression analysis

RNA-seq data of different organs of soybean plants were retrieved by integrating genetics and genomics to advance soybean research (soybase.org/soyseq/). The expression level of each *GmaMTP* gene was calculated in young leaves, one cm long pod, pod shell after 10 and 14 days of fertilization, seeds 10, 14, 21, 25, 28, 35, and 42 days after fertilization, roots, flowers, and nodule of soybean under normal

conditions by deploying cufflinks v.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 (heatmapper.ca/) [49].

Growth conditions and heavy metal treatments

The seeds of the soybean variety Zhonghuang-39 were sown during the Autumn of 2020 and placed in the experimental greenhouse of Yibin University (China). In order to perform surface sterilization, soybean seeds were first washed with 10% hypochlorous acid, followed by three washing items with distilled deionized water (ddH_2O). Subsequently, soybean seeds were spread on water-saturated filter papers and incubated in the dark for germination. After 10 days of germination, four uniform seedlings were shifted from filter paper to fertilized pit moss soil-filled plastic pots and placed under the following conditions; 16 h light (27ºC) and 8 hours dark (18ºC) with a relative humidity of 70%. For metal ions treatments, thirty-day-old soybean plantlets were dipped in 1/2 Hoagland solution (pH 6.0) supplemented with following different concentrations of heavy metals; 0.1 mM $CdCl_2$, 0.1 mM $CoCl_2$, 0.5 mM $FeSO_4$,1 mM $MnSO_4$, 0.5 mM $ZnSO_4$ and plane 1/2 Hoagland (CK)solution as a control [15]. After 24 h, the leaves and roots of soybean plantlets were detached and immediately preserved in liquid nitrogen for further experiments. The whole experiment was performed in a completely randomized design comprised of 6 treatments with three scientific repeats.

RNA extraction and qRT-PCR analysis

To extract RNA, each metal ion solution treated soybean leaf and root tissue was separately homogenized in liquid nitrogen in triplicate manners. Subsequently, the Trizol® reagent method (Invitrogen, USA) was used to extract RNA; integrity confirmation was performed by running 2 µl in 1% agarose gel prepared in RNase-free 0.5 × TAE buffer and reverse transcribed to cDNA by using SuperMix Kit (Transgen, Beijing) [50, 51]. The primers of all selected *GmaMTP* genes and *β-actin* as an internal control were designed using Primer Premier 5.0 software (Table S1). The real-time PCR was performed with the following ingredients in 20 µL reaction volume; 10 µl of 2 × SYBR premix Taq mixture, 1 µl of cDNA,0.5µL of each primer, and 8 µl of ddH_2O. The PCR conditions were adjusted as follows: initial denaturation at 95 °C for 10 min, extension at 95 °C for 15 s, total cycles 40, final extension at 60 °C for 1 min, and finally, storage at 4 °C for infinity [52]. The relative expression level of each *GmaMTP* gene was calculated using Livak $2^{-\Delta\Delta CT}$ values in triplicate manners for each sample [53].

Statistical analysis

The results of three biological replicates of expression analysis of each gene are presented in mean ± standard deviation (SD) at $p < 0.05$. The analysis of variance (ANOVA) among different values was compared at $p < 0.05$ using a student *t-test*.

Results

Identification of *GmaMTP* genes in *soybean*

After excluding reads with incomplete functional domains, only 20 candidate *GmaMTP* genes were obtained after blast analysis of 30 soybean *GmaMTP* gene sequences. Each gene's characteristics, such as gene locus, molecular weight, number of amino acids, grand average of hydropathicity, and isoelectric points, have been listed (Table 1). Except for the following seven chromosomes; 1,4,5,6,13,17, and 20, the remaining 13 chromosomes of soybean contain *GmaMTP* gene loci. The molecular weight of GmaMTP protein molecules differs from 38,221.62 to 53,254.09 Da (Table 1). We observed a variable number of inter and intra-protein ionic residues, such as the highest anionic residues observed in *GmaMTP1.2* and the lowest in *GmaMTP5.2*. Essentially, the most elevated cationic residues were seen in *GmaMTP10.2,* while the lowest was in *GmaMTP3.1* and *GmaMTP3.2.*).

Phylogenetic analysis of *GmaMTP* genes

An evolutionary relationship-based phylogenetic tree showed that all *GmaMTP* family genes and their orthologs *AtMTP*, *PtrMTP*, *OsMTP,* and *CsMTP* were divided into the following seven groups; 1, 5, 6, 7, 8, 9, and 12 (Fig. 1). The biggest number of *MTP* genes are exist in Group 9 including *GmaMTP9*, *GmaMTP10.1*, *GmaMTP10.2* and *GmaMTP11.1* along with *AtMTP9*, *AtMTP10* and *AtMTP11*; followed by Group 1 which is comprised of *GmaMTP1.1*, *GmaMTP1.2* and *GmaMTP3.1* along with *AtMTP1*, *AtMTP2*,*AtMTP3* and *AtMTP4*; followed by Group 8 which is comprised of *GmaMTP4.1* and *GmaMTP4.2* along with *OsMTP4*; followed by Group 5, 6 and 7 which are comprised of *GmaMTP5.1*, *GmaMTP5.2*, *GmaMTP2.1*, *GmaMTP2.2*, *GmaMTP7.1*and *GmaMTP7.2* along with *AtMTP5*, *AtMTP6* and *AtMTP7*. Notably, no *GmaMTP* secured a position in Group 12. Ionic bunching uncovered that 6 *GmaMTP*s were bunched in the Zn-CDFs group, 4 *GmaMTP*s were clustered in Fe/Zn-CDFs group, and 10 *GmaMTP*s were crowded in the Mn-CDFs group (Fig. 1).

Table 1 The characteristics of *GmaMTP* genes in soybean

MTPs	Gene NCBI symbol	Location	Strand	(−)	(+)	MW (Da)	aa	Instability	Aliphatic index	GRAVY	pI	Subcellular localization
GmaMTP1.1	LOC100790458	Chro. 7: 4171934..4176574	Reverse	47	34	45,248.03	408	33.30	103.95	0.059	6.23	Plasma memberane
GmaMTP1.2	LOC100794876	Chro 16: 1575487..1579996	Reverse	51	33	46,176.83	419	31.50	103.56	0.043	6.08	Plasma memberane
GmaMTP2.1	LOC100811927	Chro 2: 16208079..16216028	Forward	46	37	53,059.73	491	46.48	96.52	−0.009	6.46	Chloroplast
GmaMTP2.2	LOC100796505	Chro 10: 1758537..1771413	Reverse	45	42	53,254.09	490	39.78	95.94	−0.028	6.98	Chloroplast
GmaMTP3.1	LOC100789520	Chro11: 10283960..10286041	Reverse	41	24	43,782.19	395	33.86	107.32	0.127	6.02	Endoplasmic reticulum
GmaMTP3.2	LOC100777590	Chro12: 4256998..4259160	Reverse	40	24	42,650.26	388	32.80	112.04	0.248	5.85	Endoplasmic reticulum
GmaMTP4.1	LOC100787366	Chro 3: 37382032..37388757	Forward	48	36	44,139.07	395	43.32	108.33	0.145	5.38	Endoplasmic reticulum
GmaMTP4.2	LOC100802103	Chro 14: 48050141..48053661	Reverse	46	32	42,746.17	382	34.85	103.61	0.067	5.44	Plasma memberane
GmaMTP4.3	LOC100785952	Chro18: 4322227..4327055	Forward	49	39	45,795.62	409	5.54	102.98	−0.009	5.54	Plasma memberane
GmaMTP5.1	LOC100783643	Chro 3: 37241861..37246415	Forward	31	30	41,686.00	375	39.15	101.92	0.265	6.97	Plasma memberane
GmaMTP5.2	LOC100809496	Chro19: 41327188..41331874	Forward	29	31	40,477.67	363	36.76	103.14	0.263	8.40	Plasma memberane
GmaMTP7.1	LOC100812806	Chro 8: 18097949..18101526	Forward	41	45	47,356.41	427	33.41	92.95	−0.009	8.75	Plasma memberane
GmaMTP7.2	LOC100820261	Chro 7: 1573901..1578708	Reverse	41	46	47,493.51	429	31.44	95.03	−0.039	8.88	Plasma memberane
GmaMTP9	LOC100811575	Chro 9: 17483088..17489197	Forward	39	35	38,221.62	333	50.30	85.80	−0.320	6.52	Cytoplasm
GmaMTP10.1	LOC100797536	Chro 8: 13057785..13062016	Reverse	44	44	45,325.53	396	44.12	92.58	−0.098	7.28	Plasma memberane
GmaMTP10.2	LOC100802157	Chro 9: 30368289..30372262	Forward	48	47	46,006.26	400	43.21	94.32	−0.136	6.88	Plasma memberane
GmaMTP10.3	LOC100803531	Chro 15: 51477823..51482011	Forward	43	45	45,628.00	396	41.72	92.10	−0.096	8.29	Plasma memberane
GmaMTP10.4	LOC100805118	Chro 15: 51489192..51492878	Forward	43	44	45,560.77	397	43.71	96.50	−0.081	7.86	Plasma memberane
GmaMTP11.1	LOC100791229	Chro 2: 8518682..8522582	Forward	42	27	40,157.20	353	45.65	104.99	0.105	5.16	Plasma memberane
GmaMTP11.2	LOC100776339	Chro18: 56901488..56905136	Reverse	50	32	44,914.49	396	46.32	101.21	0.014	5.09	Plasma memberane

(−), (+), 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

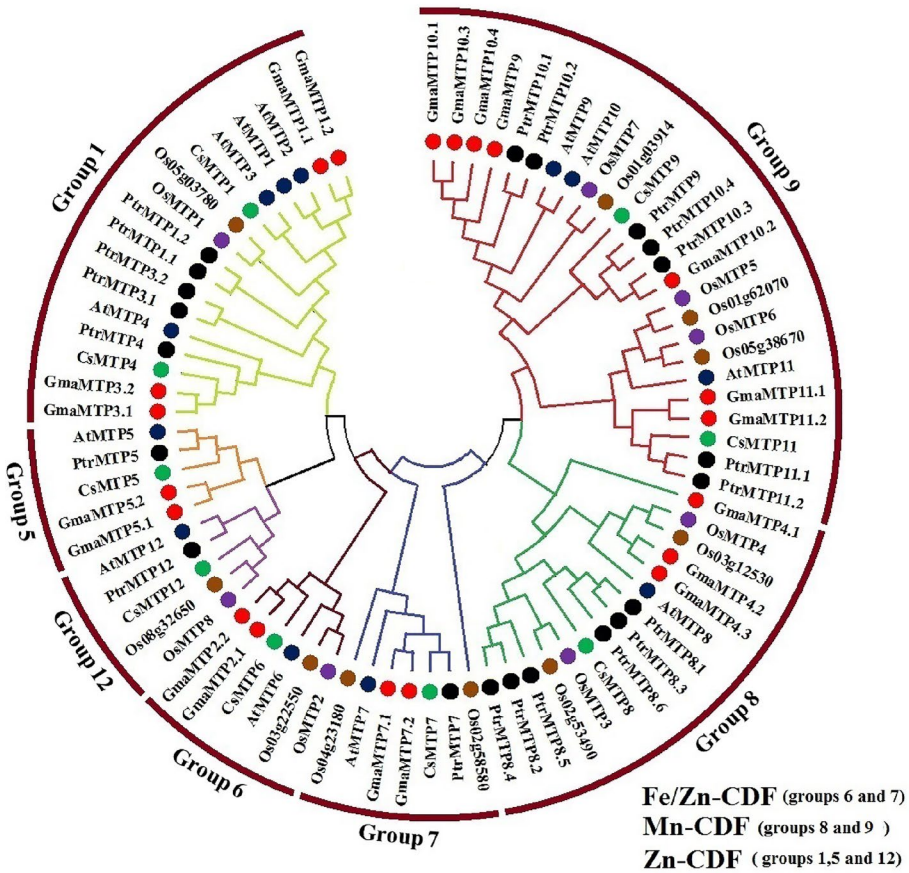


Fig. 1 Phylogenetic tree of 80 MTP proteins: 12 *Glycine max* (marked by a red circle),12 *Arabidopsis* (blue circle), 8 Wheat (purple circle), 10 Rice (brown circle), 9 Cucumber (green circle), and 21 Black Poplar (black circle). ClustalX1.83 was used for protein alignments and the phylogenetic tree's construction Neighbor-Joining (NJ) level with MEGA5.0 software at 1,000 replications bootstrap

Gene synteny analysis of *GmaMTPs*

Assessment of gene family increase and novel functions with the help of circos in the Plant Genome Duplication Database (PGDD) revealed segmental and tandem gene pair duplications (Fig. 2). We observed 70–100% identical collinearity after the ablation of segmental replication in numerous gene sets (Table S2). Segmental duplication resulted in a considerable level of homologies in *GmaMTP* gene pairs such as *GmaMTP1.1*/*GmaMTP1.2*, *GmaMTP1.2*/*GmaMTP3.2,* and *GmaMTP1.2*/*GmaMTP4.2* (Fig. 2). Similarly, two tandem replicated gene clusters were seen on chromosomes 3 and 15.

Gene structures and Construction of Conserved Motifs

Entire *GmaMTP* family genes were partitioned into six subfamilies; A, B, C, D, E, and F (Fig. 3a). Subfamily A has seven members, followed by subfamily F with four and then B with three. Finally, subfamilies C, D, and E each had only two genes. (Fig. 3a). All *GmaMTP* gene sequences harbor a variable number of introns except *GmaMTP1.1, GmaMTP1.2*, *GmaMTP3.1,* and *GmaMTP3.2*. Intron and exon analysis of all MTP genes revealed that each retrieved sequence of the *GmaMTP* gene family is a correct and true member of the six subfamilies (Fig. 3c).

All *GmaMTP* subfamilies harbor various introns except subfamily F, which does not harbor any intron. All *GmaMTP* genes have different introns and exons. Nonetheless, the similarity index in sub-families is very high, indicating a close evolutionary link among all retrieved *GmaMTP* genes. Conserved protein motifs in GmaMTP polypeptide are composed of various amino acids. The largest motif was 6, observed in 14 MTP members (Fig. 3b and Table 2). Noticeably, motifs' number, type, and order were more similar in the intrasubfamily than in the intersubfamily.

Cis-regulatory element in the upstream sequence of the MTP Family

Cis-elements associated with various stress reactions (ARE, WUN-motif, LTR, and MBS) were also discovered to support the majority of GmaMTPs (Fig. 4). Furthermore, cis-elements involved in plant advancement (MBS1, CAT-box, ERE, O2-site, and EBRE) were discovered in the promoter district of nearly all GmaMTPs. In the meantime, the cis-elements of the entire GmaMTPs

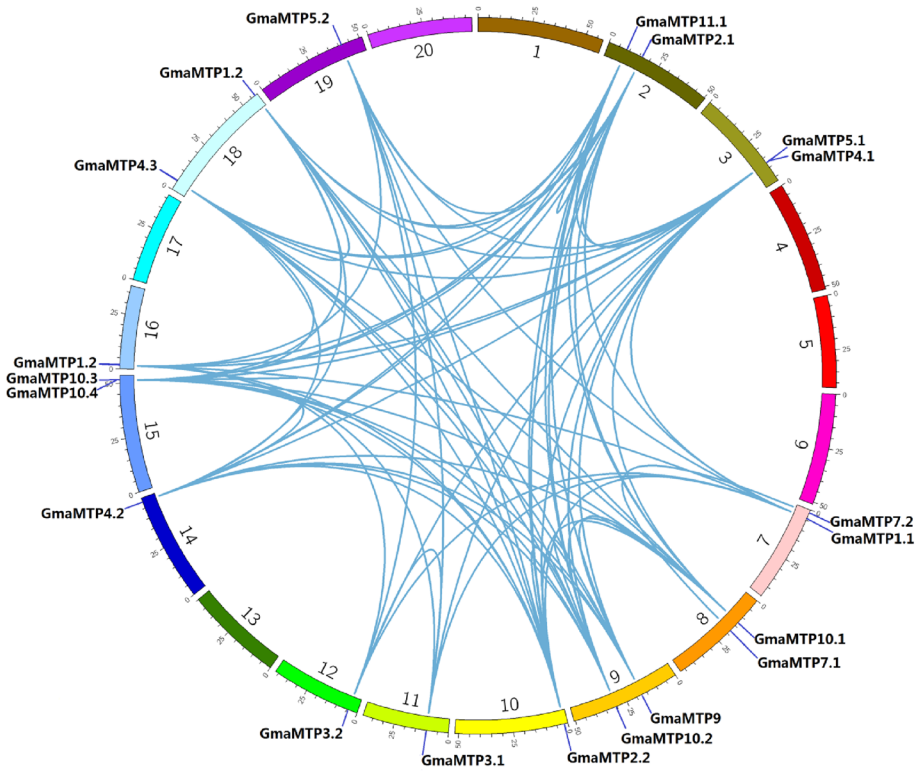


Fig. 2 Genome-wide synteny analysis of *MTP* gene family among 20 *Glycine max* chromosomes. The blue lines represented the syntenic orthologs and paralogs and displayed segmental duplication, while the tandem duplication was represented in the red box

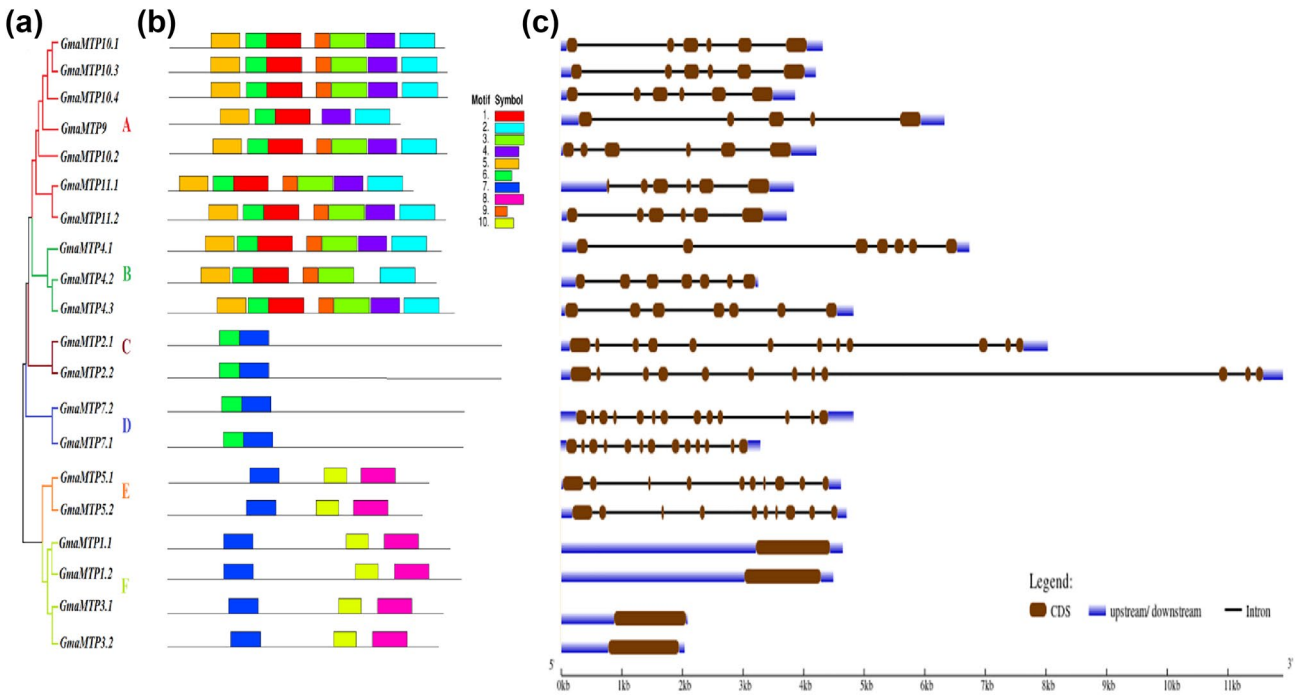
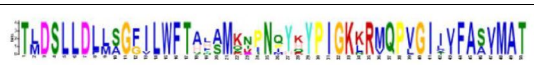
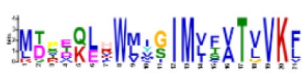


Fig. 3 Phylogenetic relationship, gene structure and conserved motif analysis of *GmaMTP* genes; **a** The neighbor-joining phylogenetic tree was constructed with MEGA7 using GmaMTP amino acid sequences with 1000 times replicate. **b** The motif composition of GmaMTP proteins using ten conserved motifs is represented by the unique colour mentioned in the box on the top lift. **c** The exon–intron structure of *Glycine max* MTP proteins where dark green boxes presented the exons and the black lines represented the introns. The blue boxes represented the untranslated regions (UTRs), with detailed size scales at the bottom

Table 2 Analysis of the 10 conserved motifs of *GmaMTP* genes in soybean

Motif	Logo	Best possible match	Width
1		TLDSLLDLLSGFILWFTAHAMKNPNQYKYPIGKKRMQPVGIIVFASVMAT	50
2		VRAYTFGAHYFVEVDIVLPEDMPLKZAHNIGESLQEKLEKLPEVERAFVH	50
3		LMVYCRSFKNEIVRAYAQDHFFDVITNSVGLAAAVLAVKFYWWIDPVGAI	50
4		ALYTINTWAKTVJENVWSLIGRTAPPEFLAKLTYLIWNHHE	41
5		QRKVAEYYKRQERLLEGFNEMDSMTESGGIPGSLTEDEMKQ	41
6		KVSNAANLVLFAAKVYASIASGSLAIIAS	29
7		AAHLVSDVALFAISLFSLWASRWEADPEHPYGYNRLEVLGAL	42
8		VLGTTLPLLRNIYGILMERTPPEIDTTKLEKGLLDIEGVEDVHELHIWA	49
9		MTPEQLHWMIGIMVFVTVVKF	21
10		TTKKNALNINLQGAYLHVLADSIQSIGVMIAG	32

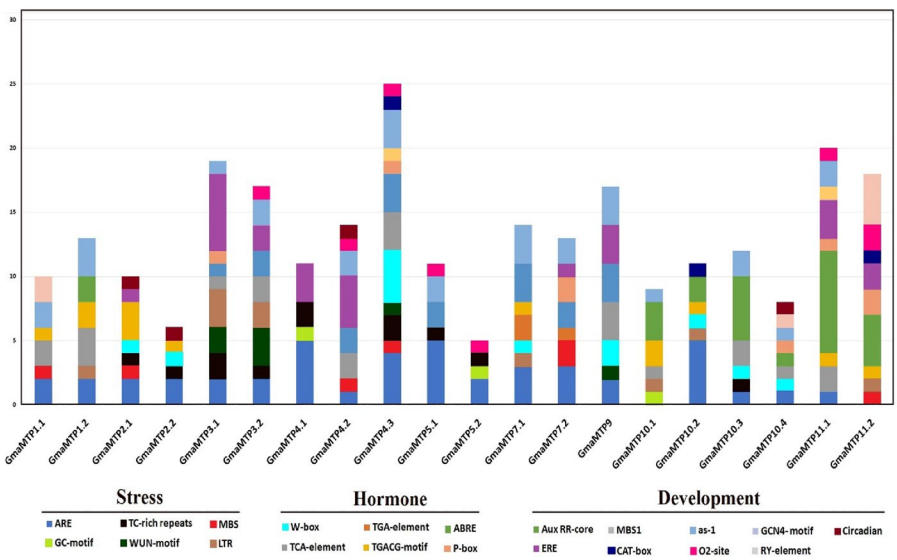


Fig. 4 Cis-regulatory elements in the promoter region of *GmaMTP* genes. Cis-regulatory elements were identified in the 2000 bp promoter sequence upstream of the start codon of *GmaMTP* genes using PlantCARE and PLACE. These elements are related to different functional diversity, represented by different colors

family were separated into various classifications given capacity expectations. The outcome showed that the most significant classification of cis-elements was hormone-related, trailed by stress-related and advancement-related cis-elements. In any case, numerous themes have not yet been practically described and whether these themes present remarkable utilitarian roles to GmaMTPs remains to be researched.

Protein modeling, protein–protein interactions

Phyre 2 web-based interface (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was utilized for protein displaying utilizing all MTP amino acid sequences (Figure S1 and Table S4). Each of the twelve anticipated models for MTP proteins was 100 percent in light of c6xpdB, c3j1zP, c2qfiB, and d2qfia2 layouts. On the contrary, the STRING examination of protein–protein interplay showed the physical (immediate) and the practical (circuitous) affiliations (Figure S2 and Table S4).

The outcome showed various associations inside the studied proteins, where the all-out number of hubs was 20, with an intermediate of 11. 4. The STRING information base investigation showed 114 edges and ten expected local network clusters, which were CL:48,378, 48,377, 48,387, 48,345, 48,298, 48,258, 48,504, 49,055, 48,255, and 48,260. Group 48,255 was the greatest, including 17 MTP proteins (Table S5). In addition, our protein investigation showed the presence of two ordinary spaces inside the checked member from the MTPs family, which were PF01545 (all proteins) and PF16916 (12 proteins) (Table S6).

GO enrichment analysis

The cellular component, molecular function, and biological processes were anticipated by GO improvement examination (Fig. 5 and Table S5). In subcellular localization investigation, the anticipated conveyance scores of MTP proteins were as follows; 20/71% in all layers 4/14% in the plasma membrane, vacuole, and Golgi apparatus. The GmaMTP1.1 and GmaMTP1.2 proteins were identified in 14 of the 17 sub-cellular compartments, highlighting their importance in metal stress resistance. The aggregate scores of MTP protein molecules during the biological process were as follows: trans-membrane transport of Zn + was 4/31%, Mn + ions were 6/46%, and transmembrane transport of protons was 3/23%. GmaMTP1.1, GmaMTP1.2, GmaMTP3.1, and GmaMTP3.2 performs a significant part in transmembrane transport of Zn^{+}, while GmaMTP4.1, GmaMTP4.2, GmaMTP4.3, GmaMTP9, GmaMTP11.1,and GmaMTP11.2 plays an essential part in transmembrane transport of Mn^{+}. The investigation of molecular function and biological processes uncovered huge roles in GmaMTP1.1, GmaMTP1.2, GmaMTP3.1, and GmaMTP3.2, which assume a critical part in the transmembrane transport of Zn^{+}. It affirmed the fundamental pieces of GmaMTP4.1, GmaMTP4.2, GmaMTP4.3, GmaMTP9, GmaMTP11.1, and GmaMTP11.2 in transmembrane transport of Mn^{+}.

Gene expression analysis by RNA-seq data

The tissue expression models of GmaMTPs were researched using transcriptome data in various soybean tissues (Fig. 6. A). As displayed in Fig. 6b and Table S6, all 20 *GmaMTP* genes were identified in the 14 tissues tested ($\log2(FPKM+1)>0$), except for *GmaMTP4.2* (expressed only in root tissue), *GmaMTP4.1* (expressed in young leaf, bloom, one cm unit, case shell, seed 21 DAF, root, and knob), and *GmaMTP10.2* (only expressed in flower, seed 42DAF, root, and nodule). Among these,13 genes (*GmaMTP1.1*, *GmaMTP1.2*, *GmaMTP2.1*, *GmaMTP2.2*, *GmaMTP3.1*, *GmaMTP4.3*, *GmaMTP5.1*, *GmaMTP5.2*, *GmaMTP7.1*, *GmaMTP7.2*, *GmaMTP9*, *Gma TP11.1 and Gma TP11.2*) expressed integral expression ($\log2(FPKM+1)>1$ in all tissues), and *GmaMTP11.1* had the most elevated expression levels contrasted and other GmaMTPs in completely identified tissues, besides in-unit shell 14 DAF, seed 14 DAF, seed 25 DAF, seed 42 DAF, root and knob, though *GmaMTP4.2* displayed the least expression levels in all tissues ($0<\log2(FPKM+1)<1$). Besides, a few genes displayed tissue-explicit expression. For example, four genes (*GmaMTP7.1, GmaMTP9, GmaMTP10.2,* and *GmaMTP11.1*) in the root, one gene (*GmaMTP7.1*) in the nodule, three genes (*GmaMTP1.2, Gma MP7.1,* and *Gma TP11.1*) in young leaf and two genes (*GmaMTP7.1* and *GmaMTP11.1*) in flower showed the highest transcript abundances.

Expression analysis of *GmaMTPs* in response to heavy metals treatment

Entire *GmaMTP* genes showed discrepant gene expression levels when treated with various heavy metals examined in the root and leaf tissues (Fig. 7). *GmaMTP1.1*, *GmaMTP1.2*, *GmaMTP3.1*, *GmaMTP4.1*, *GmaMTP4.3*, *GmaMTP10.4*, and *GmaMTP11.1* were upregulated in roots, while *GmaMTP5.1* and *GmaMTP7.2* were downregulated. Similarly, Co^{2+} increased the expression of *GmaMTP1.1*, *GmaMTP2.1*, *GmaMTP2.2*, *GmaMTP3.2*, *GmaMTP5.2*, *GmaMTP9*, *GmaMTP10.1*, *GmaMTP10.2*, *GmaMTP10.3*, *GmaMTP10.4*, and *GmaMTP11.2*. Under Fe^{2+} treatment, *GmaMTP2.2*, *GmaMTP4.1*, *GmaMTP4.3*, and *GmaMTP10.3* were upregulated, whereas *GmaMTP5.1* and *GmaMTP7.2* were downregulated. Under Mn^{2+} treatment, *GmaMTP1.1*, *GmaMTP1.2*, *GmaMTP2.1*, *GmaMTP3.2*, *GmaMTP4.1*, *GmaMTP4.2*, *GmaMTP4.3*, *GmaMTP5.2*, *GmaMTP9*, *GmaMTP10.1*, *GmaMTP10.4*, *GmaMTP11.1* and *GmaMTP11.2* were upregulated, while *GmaMTP2.2* was downregulated Under Zn^{2+} treatment, *GmaMTP10.4* and *GmaMTP11.1* were upregulated, whereas *GmaMTP2.1* and *GmaMTP7.1* were downregulated.

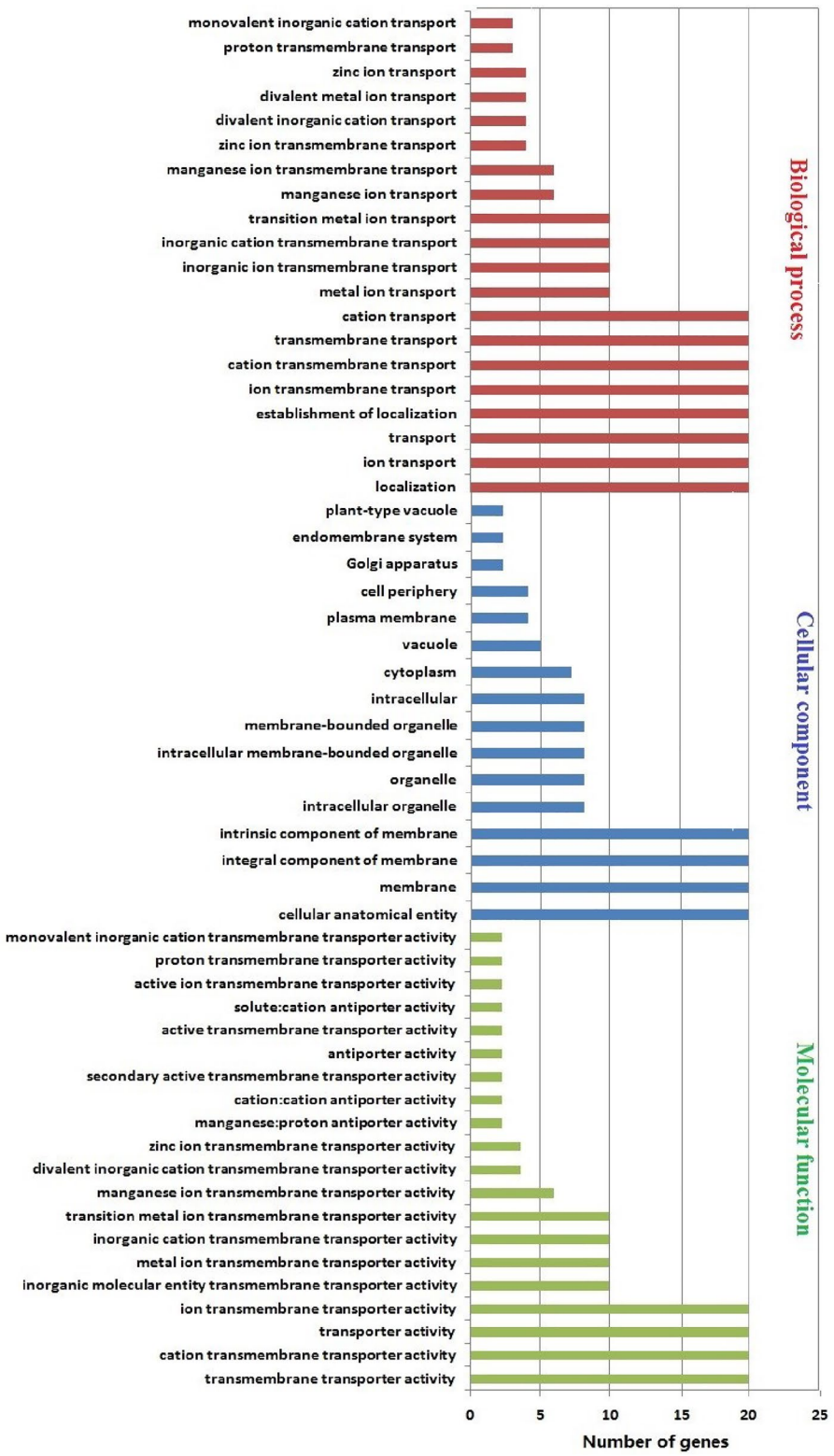


Fig. 5 Gene Ontology analysis of *Glycine max MTP* genes. Gene ontology showed the distribution of every *GmaMTP* gene in the plant, where a blue colour column mentioned the cellular component. In contrast, the MTP family participation's biological processes were mentioned in the red color column, and the molecular function was mentioned in the green colour

In leaf, Cd^{2+} treatment conclusion in enhancement expression of *GmaMTP1.1, GmaMTP1.2, GmaMTP3.1, GmaMTP3.2* and *GmaMTP4.1*, yet, a huge end in expression of *GmaMTP2.1* and *GmaMTP5.1*. Comparatively, Co^{2+} treatment fundamentally expanded the expression of *GmaMTP1.1*, *GmaMTP1.2, GmaMTP3.1, GmaMTP3.2, GmaMTP4.1, GmaMTP4.3* and *GmaMTP9* but decreased the expression of *GmaMTP5.1*. Fe^{2+} treatment decreased

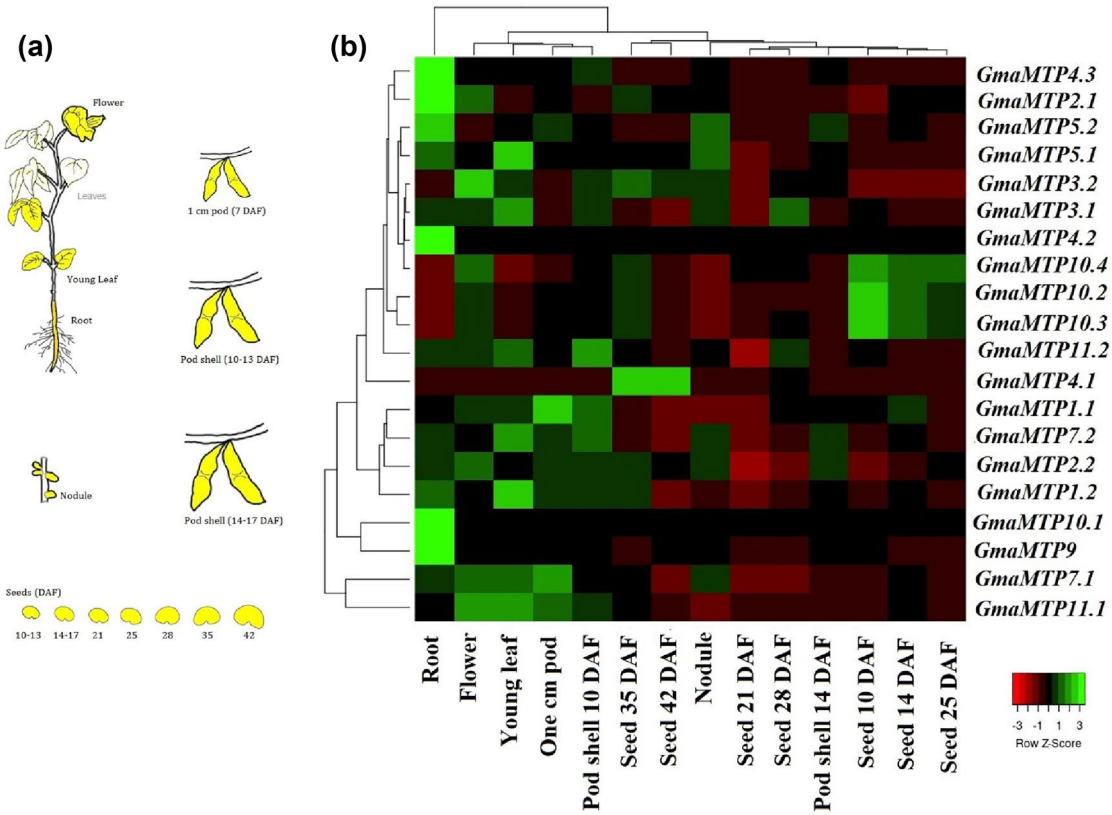


Fig. 6 The heat map of *Glycine max* 20 *GmaMTP* gene expression profiles based on RNA-seq data. The previous expression has been shown in root, leaf, flower, hypocotyl, seed coat, root tip, vegetative buds, stem, shoot and pod tissues

in expression of *GmaMTP2.1, GmaMTP7.2* and *GmaMTP10.1* but resulted in increased the expression of *GmaMTP1.1*, *GmaMTP1.2, GmaMTP3.1, GmaMTP3.2, GmaMTP4.1, GmaMTP4.3, GmaMTP5.2, GmaMTP9* and *GmaMTP10.3*. Mn^{2+} treatment brought about expanded expression of *GmaMTP1.1, GmaMTP1.2, GmaMTP3.1, GmaMTP3.2, GmaMTP4.1, GmaMTP4.3, GmaMTP9* and *GmaMTP11.1*, but decreased expression of *GmaMTP2.1*. Finally, Zn^{2+} treatment eventuated in improved expression of *GmaMTP1.1*, *GmaMTP1.2, GmaMTP3.1, GmaMTP3.2, GmaMTP4.1, GmaMTP9, GmaMTP10.4* and *GmaMTP11.1*, but decreased expression of *GmaMTP2.1* and *GmaMTP5.2*.

Discussion

Heavy metals majorly affect the environment and make them unsuitable for human utilization [54, 55]. Once delivered into the environment, they accumulate in plants and other living tissues utilizing the order of things and cause harmfulness even at lower concentrations [56]. *MTP* genes (membrane divalent cation transporters) are fundamental for transporting different heavy metals and upgrading plant tolerance against heavy metals stress [57]. They likewise play a normal part in plant mineral sustenance upkeep [7]. Additionally, these metal-binding proteins are currently being used as bio-natural markers for foreseeing heavy metal pollution in light of their expression levels [58]. They also function normally in plant mineral nourishment upkeep [7, 57]. The MTP family has formerly been examined in various plants, like Arabidopsis [13], tobacco [7], wheat [16], and Black poplar [15]. While this is the first genetic characterization research of the MTPs family in soybean. As a result, we identified 20 *MTP* genes in soybean, which were named based on the sequence similarities and orthologous relationships between them and *AtMTPs*. The MTP proteins' evolutionary links between soybeans and other relevant plant species were first determined in *A. thaliana,* which contained 12 *MTPs* in past research (*AtMTP1* to *12*) [16]. These findings would be the basis for determining the practical characteristics, especially the GmaMTP protein substrate-specificities. The CDS length, protein size, MW, pI, GRAVY, sub-cellular localization, and TMD quantity of the GmaMTPs were dissected and subsequently predicted. Following the investigation of Vatansever, Filiz and Eroglu [16], *NNtMTPs* may act as vacuole-localized cation transporters, as some MTP proteins are

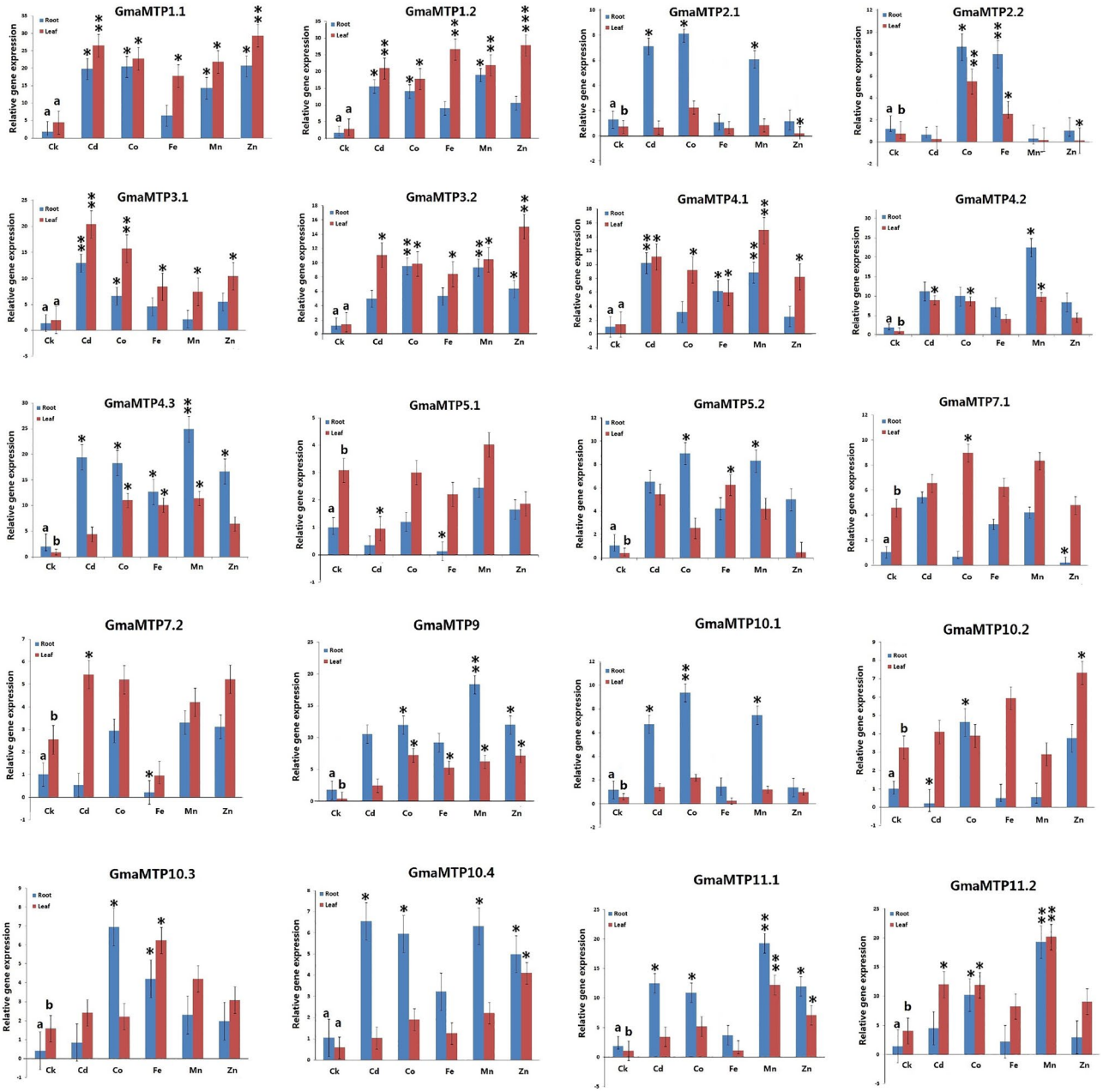


Fig. 7 The qRT-PCR expression of the *Glycine max MTP* genes from root and leaf samples. The reactions were normalized using the *β-actin* reference gene. The standard deviations have been represented by the error bars from three independent technical replicates. The mean expression levels of three replicates were analyzed with the five heavy metals treatments (Cd^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}) using *t*-tests ($p < 0.05$), while the CK represents control samples. Different letters (a, b, and c) indicate significant differences between the roots, and leaves under normal conditions. Asterisks indicate significant differences between the treatment samples and the corresponding control samples in the roots, and leaves. ($n = 9$, $p < 0.05$, Student's *t*-test)

anticipated to be found in the vacuole while others are found in the cellular membrane or nucleus. Unlike other plant MTP families, where the MTP12 has the largest molecular size [7], GmaMTP2.1 and GmaMTP2.2 were roughly one-third the mass of the other GmaMTPs. (Table 1). In our article, we explored the gene synteny and duplication examination for more information about the genome annotation and the development system of the MTP gene family in soybean (Fig. 2 and Table S2). The exit of at least two genes on a similar chromosome is frequently connected with tandem duplication, while segmental duplication regularly happens on various chromosomes [31].Our article explains two pair duplication sets while there were 28 segmental duplications. In-plant gene families, the gene duplication occasions in its

sorts are trailed by difference, think about standard highlights, and are connected with auxiliary plant metabolic gene [59]. Consequently, our outcomes about the gene replications affirmed their fundamental parts in the *MTP* gene family development. Practically all subfamilies contained similar quantities of introns and theme sequences, predictable with the past examinations where a comparative gene construction was found inside similar subfamilies [60, 61]. For instance, all of the gene individuals from subfamily A contain five introns. Be that as it may, subfamily F individuals contain no introns. These results showed that during the soybean advancement of *GmaMTPs*, some intron gain and misfortune occurred. A few genes have no intron except for one exon, which causes the lower capacity of exons in gain/loss rate because of higher selection pressure in the exons sequences [62]. In this way, with this multitude of perceptions, it is plausible that the situation divergences in intron numbers consider shared occasions connected with the gene family advancement [49]. In a comprehensive consideration of the MTP proteins, we anticipated their 3D design, which was considered a supportive tool for inspecting their function [63]. The four temples in soybean MTP proteins demonstrate that these proteins, with heavy metals where these carrier proteins are in the plant, are characterized as metal-take-up proteins that shift fundamental and poisonous heavy metals to the cytoplasm and metal-take-up proteins. Simultaneously, the other is metal-efflux proteins that assist the cell with eliminating any excess heavy metals [64]. Then again, the protein–protein connection investigation gave us more information about the plant developmental processes and their roles in response to environmental stresses [65]. Moreover, the cis-regulatory elements (CREs) examination investigated the potential administrative variables influencing the expression of GmaMTPs. At long last, we recognized 3440 CREs involved in numerous biological processes. Past examinations showed that a portion of these recognized CREs were more related to pressure reactions [7].On the other hand, gene ontology is a fundamental analysis to predict putative functional contributions across living organisms [66]. Additionally, gene ontology classes and ideas have been utilized to characterize the connections and gene functions existing between these concepts [67]. Our gene ontology examination uncovered the critical role of the soybean *GmaMTP* genes with heavy metals (Fig. 5). Besides, the GO showed the molecular functions, where most of them participate in metal-related processes, including cation transmembrane transporter activity, transporter activity, transmembrane transporter activity, and ion transmembrane transporter activity.

The previous transcriptomic data helps detect the presence, structure, and amount of RNA in any biological sample under certain conditions [68]. In this manner, we explored the expression profile of all individuals from the *MTP* gene family from recently distributed RNA-sequencing information, which showed the declaration of all gene individuals in thoroughly chosen soybean tissues (Fig. 6 and Table S5). Digital data analysis showed that the MTP gene's critical part could contribute to development and advancement. Considerable evidence about the fundamental part of soybean MTPs after tissue expression assessment has been obtained. For example, the exclusive expression of the three genes *GmaMTP1.2*, *GmaMTP7.1*, and *GmaMTP11.1* were in the young leaf, though *GmaMTP7.1* and *GmaMTP11.1* were most abundant in flowers, showing that they may be engaged with early leaf and flower enhancement. Besides the crucial expected part of *GmaMTP7.*1in nodule development and maturation, its appearance has expanded.

Nonetheless, just *GmaMTP4.2* was rarely expressed in totally analyzed tissues from all *GmaMTPs*. The archived down-regulation in some gene expressions is fundamental for keeping up with the gene duplicates and ancestral functions [69]. Subsequently, in our finding, the down-regulation of *GmaMTP4.2* expression is relied upon as essential for keeping their biological processes and keeping up with them from misfortune during cell evaluation.

The dependability of the transcriptome information was additionally approved by qRT-PCR; nevertheless, the minor imbalance between the two examinations might be because of various development conditions and soybean assortments, which at last impacted the spatial expression. We analyzed the expressional behavior of *MTP* genes under five divalent metals (Mn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, and Zn^{2+}). Various examinations in different plants showed the massive part of the *MTP* gene family to improve the plant tolerance against these metals [11, 15] as it was depicted as metal efflux transporters out of the cytoplasm, basically shipping Zn^{2+}, yet additionally moves Ni^{+2}, Co^{2+}, Cd^{2+}, Fe^{2+}, and Mn^{2+} [57]. The transcription of the transcript amassing a record of MTPs in light of different heavy metals fluctuated and confounded. Nonetheless, the gene's expressional reaction to various stresses usually is reflected in comparing gene functioning. In Arabidopsis, the tonoplast-limited Zn carrier *AtMTP1* expressed bit modifications in expression with an overabundance of Zn openness at transcription and translation levels [70, 71]. Furthermore, despite the fact that the high expression of *CsMTP1* encoded protein, gene expression in cucumber was consistent despite the high amount of Zn^{2+} [72].

As previously stated, *AtMTP12* up-regulation is independent of Zn concentration. Still, it can carry Zn via joining *AtMTP5* in a heterodimeric complex form [24], which is comparable to the findings of et al. [7] in tobacco. Furthermore, differing Mn^{2+} sources had little impact on Mn-CDF expression (*AtMTP8*, *9*, *10*, and *11*). [22]. Close Same findings have been reported in the tobacco industry [7]. Except for *GmaMTP5.1* and *GmaMTP5.2*, all Zn-CDF members

showed a considerable increase in expression when exposed to high Zn^{2+} in our investigation. Additionally, in various soybean tissues, the up-regulation of *GmaMTP7.2* of Zn/Fe-CDFs exceeds Zn^{2+}, but it is down-regulated by Fe^{2+}. Moreover, the deposition of Mn^{2+} significantly impacted all members of the Mn-CDF class, except for the tree genes *GmaMTP10.1* and *GmaMTP10.3*. As a result, our research will be critical in determining the molecular functions of MTP in soybeans during diverse heavy metal stresses. Generally, a total of 20 potential *GmaMTPs* in the soybean genome were successfully identified and analyzed for a phylogenetic relationship, chromosomal distributions, gene structures, gene ontology, cis-elements, and previous gene expression. Besides, the expression assay of *MTP*s has been examined under five divalent heavy metals (Cd^2, Co^2, Mn^2, Zn^2, and Fe^2) treatments.

Substantially, our discoveries will help to understand the functions of GmaMTP proteins in heavy metal resistance as well as the methodology of heavy metal transport regulated by GmaMTP proteins. These findings, taken collectively, would provide a practical and theoretical platform for future studies on the operational identification of *GmaMTP* genes. Consequently, depending on their expression levels, the most highly expressed *MTPs* (*GmaMTP1.1, GmaMTP1.2, GmaMTP3.1, GmaMTP3.2, GmaMTP4.1*, and *GmaMTP4.3*) can be used as bio-environmental markers for predicting the heavy metal accumulation.

Conclusions

In soybeans, genome-wide recognition identified twenty *MTP* genes, phylogenetically and extensively studied. The *GmaMTPs* were split into three substrate-specific clusters (Zn/Fe-CDFs, Zn-CDFs, and Mn-CDFs). Six groups seemed to have undergone expansion and gene loss after polyploidization via segmental duplication. The cation efflux region and/or ZT dimerization domain are projected to be present in all GmaMTPs, and each MTP within the same group has the same structural characteristics. Aside from predicting cis-elements, gene ontology provides valuable information regarding the critical functions of MTPs during plant growth and tolerance. The expression patterns of each *GmaMTPs* gene in retaliation to several heavy metals in diverse tissues revealed that these genes are essential for soybean growth and expansion. In addition, we discovered that *GmaMTP1.1, GmaMTP1.2, GmaMTP3.1, GmaMTP3.2, GmaMTP4.1*, and *GmaMTP4.3* play a significant role in heavy metal stress tolerance in plants.

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Author contributions Conceptualization: AHES, MA, HQ and JL. Formal analysis: AHES, JL, SHW and HQ. Writing an original draft: AHES. Methodology: AHES, ASE, ZN and NS. Writing, review and editing; AHES, MA, SAR, SHW, MB and JL. Investigation: AHES, HQ and RRM.

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Data availability Provided as supplementary material.

Declarations

Conflict of interest The authors have no conflict of interest and nothing to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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