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Identification of metal tolerance proteins (MTP) and their gene expression under drought stress in potato (*Solanum tuberosum* L.)

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Abstract: Metal tolerance proteins (MTPs) are as metal efflux transporters, existing extensively at all plant sections and play significant roles in regulation of the metal levels in biological processes. In the current study, phylogenetic relationships, gene structures, conserved motifs, and StMTP domains were analyzed. Here, 12 *MTP* genes in *S. tuberosum* were detected and categorized in three major clusters namely *Fe/Zn-MTP*, *Zn-MTP*, and *Mn-MTP* and seven groups (1, 5, 6, 7, 8, 9, and 11) according to phylogenetic relationships. Based on *in silico* and qPCR analysis, all of *StMTPs* included a cation diffusion facilitator (CDF) domains and the putative *Mn-MTP* harbored the ZT-dimer. An evolutionary analysis indicated that *StMTP* genes had undergone gene duplication leading to gene loss and gene expansion events. Analysis of transcription factor binding sites (TFBS) and microRNA in promoter region and coding sequence of *StMTP* genes revealed the presence of 5312 putative TFBS and 13 StmiRNAs. The analysis of promoter regions of *StMTP* genes possess various frequencies of TFBS, illustrating various responses in different growth and developmental stages as well as under abiotic stress. Expression profile analysis revealed that the *StMTP9* were up-regulated in leaves and stem, while, *StMTP8* up-regulated in leaves. Both genes down-regulated in tubers, roots as well as under drought stress. These results will provide a better insight for functional characterization of StMTP genes and can be helpful to elucidate the biological structure of their genes in potato.

Key words: biological processes, transporter, metal tolerance proteins, *S. tuberosum,* gene expression

Določanje na kovine tolerantnih beljakovin (MTP) in izražanje njihovih genov v razmerah sušnega stresa pri krompirju (*Solanum tuberosum* L.)

Izvleček: Na kovine tolerantni proteini (MTPs) so transporterji kovin iz celice, ki so prisotni v velikem številu pri vseh rastlinah in igrajo pomembno vlogo pri uravnavanju količine kovin v bioloških procesih. V raziskavi so bila analizirana filogenetska razmerja, zgradbe genov, ohranjena zaporedja in *StMTP* domene. V krompirju je bilo ugotovljeno 12 *MTP* genov, ki so bili razporejeni v tri glavne skupine in sicer *Fe/Zn-MTP*, *Zn-MTP* ter sedem skupin *Mn-MTP* genov (1, 5, 6, 7, 8, 9, in 11) glede na filogenetska razmerja. Na osnovi *in silico* in qPCR analize so se vsi *StMTPs* geni vključevali domene za olajšanje difuzije kationov (CDF) in gene z domnevno isto funkcijo (*Mn-MTP*), ki so vsebovali ZT-dimer. Evolucijska analiza je pokazala, da so *StMTP* geni prešli podvojevanje, kar je vodilo do izgube genov in njihovega povečevanja. Analiza mest vezave transkripcijskega faktorja (TFBS) in mikroRNK v promotorski regiji in kodirajočih sekvencah *StMTP* genov je odkrila prisotnost 5312 možnih TFBS in 13 StmiRNAs. Analiza promotorske regije *StMTP* genov je pokazala, da ti vsebujejo različne frekvence TFBS, kar kaže na različne odzive v različnih rastnih in razvojnih fazah kot tudi učinke abiotičnega stresa. Analiza izražanja profila je odkrila, da so geni *StMTP9* bolj aktivni v listih in steblu med tem, ko so geni *StMTP8* bolj aktivni v listih. Obe skupini genov sta manj aktivni v gomoljih in koreninah kot tudi v razmerah sušnega stresa. Ti rezultati prispevajo boljši vpogled v funkcionalno opredeljevanje *StMTP* genov in bi lahko bili koristni za razjasnitev biološke zgradbe genov v krompirju.

Ključne besede: biološki procesi, transporter, proteini tolerance na kovine, *S. tuberosum,* izražanje genov

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1 INTRODUCTION

Transition metals participate in many biological and physiological processes. Since they act as essential cofactors for many enzymes, they are components of transcription factors and other proteins and are important for both mitochondrial and chloroplast functions. However, high concentration together with non-essential metals can lead to extremely toxicity and can cause oxidative damages or compete with other essential ions. The physiological range of transition metals from deficiency to toxicity is extremely narrow and therefore a network to control the micronutrient fluctuations is required for all organisms. Since transmission metals are also essential components in reaction centers of enzymes, deficiency will also cause stress symptoms (Ducic and Polle, 2005). To regulate toxic effects of high and low concentration metals, it is necessary that plants maintain metal homeostasis at cellular levels (Hall and Williams, 2003). Special transporters were encoded by multigenic families which are responsible for the uptake and secretion of metal cations in different organelles (Montanini et al., 2007).

Transporters of the cation diffusion facilitator (CDF) family are namely Zn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, and Mn^{2+}, first detected by Nies and Silver (1995). CDFs are divided into three substrate-specific clades Zn-CDF, Fe/Zn-CDF, and Mn-CDF. These transporters are ubiquitous and spanning in all three kingdoms of organisms: Archaea, Eubacteria, and Eukaryotes. Expressed fundamentally in both root sand shoots, *AtMTP1* is over-expressed conferring Zn tolerance in rice (Zhang and Liu, 2017). *OsMTP1*, a cation transporter localized in tonoplast, possesses low affinity to Co, Fe, and Cd, controlling ion hemostasis in rice (Menguer et al., 2013). AtMTP5 and AtMTP12, other functional complex members of Zn-CDF proteins, were found to transport Zn into the Golgi apparatus (Fujiwara et al., 2015). In Mn-CDF groups, there were four AtMTP proteins (AtMTP8-11), which both AtMTP9 and AtMTP11 functioned as a Mn transporter. AtMTP11 was involved in maintaining Mn hemostasis and localized in pre-vacuolar compartment and/or trans-Golgi. Mutation of *atmtp11* display Mn sensitivity and higher levels of Mn in shoots and roots than the wild-type plants were accumulated. Five Mn-CDF members (OsMTP8.1/8.2/9/11/11.1) with known functions are classified into groups 8 and 9. ShMTP8, another member of Mn-CDF, is isolated from the Mn-tolerant legume *Stylosanthes hamata* (L.) Taub.. ShMTP8 is localized in tonoplast, exhibiting Mn-tolerance when expressed in *A.thaliana* (L.) Heynh. (Delhaize et al., 2003). In *Arabidopsis* and cucumber, AtMTP7, CsMTP7 act as Fe transporter member and are localized in plant mitochondrial (Migocka et al., 2018). CsMTP8 was found in vacuolar membrane and participated in the maintenance of Mn hemostasis (Migocka et al., 2014). CsMTP9 is involved in the efflux of Mn^{+2} and Cd^{+2} from cucumber root cells using H+-coupled with manganese and cadmium antiporter (Migocka et al., 2015).

Potato (*S. tuberosum* L.) is one of the largest non-cereal food crop worldwide and sequencing of its entire genome is completed (Zhang et al., 2017). Potato can be utilized for molecular plant biological research and to facilitate gene discovery and comparative genetics (Jaillon et al. 2007). There has been few relevant research on the *StMTP* genes in potato. The present genome-wide survey was conducted to identify the *MTP* gene family in *S. tuberosum* and systematically analyzed their sequence and structural characteristics as well as evolutionary relationships. Besides, the transcription factor binding sites distributions, and the potential microRNA target sites in *StMTP* genes were predicted. In addition, the expression profiles of *StMTP* genes in different potato tissues and in response to abiotic and biotic stresses were analyzed using a microarray data approach. Results in this study could provide a better insight into the biological functions of StMTP proteins and the molecular mechanisms underlying these metal transporters and the homeostasis maintained by them in potato.

2 MATERIAL AND METHODS

2.1 IDENTIFICATION OF *MTP* GENES IN *S. tuberosum*

The *MTP* genes of *A.thaliana* and *O.sativa* L. were taken from TAIR and RAP-DB databases, respectively.

To detect the potential *StMTP* genes in potato, the HMM file of the MTP domain (PF01545) was taken from the Pfam database and utilized to perform the HMMER search. Then, the resulting MTP sequences were adopted for tBLASTn. Finally, following the removal of redundant predicted sequences, the sequences all putative MTPs were further confirmed using InterProScan.

2.2 SEQUENCE ALIGNMENT AND PHYLOGENETIC

Sequence similarity analysis of MTPs proteins between *S. tuberosum* and *A. thaliana* were performed in blastp at NCBI. Each protein sequence of MTPs in *Arabidopsis* and *O.sativa* was used at the query, and all 12 StMTP protein sequences were used as the subject sequence.

For phylogenetic analysis, multiple sequence align-

ments at protein levels were performed by ClustalX, and MEGA 6.0. Phylogenetic tree construction was established by the Maximum likelihood method (Tamura et al., 2013). The MTPs sequence from *S. tuberosum, A. thaliana*, and *O. sativa* were downloaded from the above databases, as described by Liu et al (2019).

2.3 AMINO ACID PROPERTIES AND STRUCTURE CHARACTERISTICS OF MTP PROTEINS

The molecular weight, Pi, and peptide length were evaluated using the ProParam software and prediction of protein transmembrane helices was examined using protter. Sub-cellular localization was predicated using Plant-Mploc server (Hall 2002). MEME program was utilized to detect the conserved motifs (Bailey et al., 2009; Finn et al., 2016). Motifs functions were determined using the hmmscan tool. Then, detected MTP sequences were aligned using Muscle, and identity residue was calculated. The exon-intron structures of StMTP genes were characterized using GSDS program.

2.4 TFBS ANALYSIS AND MIRNA TARGET SITES PREDICTION

The promoter regions (up-stream 1000 bp) of *StMTP* genes were extracted to predict the TFBS using PlantPAN. The miRNA target sites of *StMTPs* were examined using small RNA target analysis server.

2.5 PLANT GROWTH AND qRT-PCR OF *StMTP* GENES

To analyze specific expression in root, stem and leaf tissue, samples were taken from two-week-old seedlings. Three tubers were planted in the pot. To analyze the expression under drought stress, two treatments of drought and irrigation were used. Each treatment was in a completely randomized block design with three blocks. In the first six weeks, all the plants in each two treatments were watered equally. After 2 weeks of stress, leaf and tuber samples were taken under the mentioned conditions. Then the leaves and tubers were immersed in liquid nitrogen and kept at -80 temperature until RNA extraction. RNA extraction was performed using the Synaclone kit. Then cDNA synthesis was performed as follows. Potato *EF-1α* gene was used as internal control. All primers used in gene expression analysis are listed in Table S1. Real time was done using SYBR Green Supermix. Relative expression was determined via $2^{-\Delta\Delta Ct}$.

3 RESULT

3.1 IDENTIFICATION AND CLASSIFICATION OF *MTP* GENES IN POTATO

Using 12 and 10 AtMTP and OsMTP protein sequences as the queries, a total of 12 *MTP* genes were detected in *S. tuberosum*. Subsequently, HMM verification was performed in 12 MTP sequences, including the cation efflux domain in the potato genome. According to sequence identity, cover value, and orthologous relationship, the 12 StMTP proteins were designated as StMTP1 to StMTP11. For each AtMTP protein, there was at least one MTP homolog in *S.tuberosum* except for AtMTP2, AtMTP10, and AtMTP12, where no corresponding StMTP was found. To understand the evolutionary relationships of MTP gene family members among potato, *Arabidopsis*, and rice 35 MTP protein sequences from three species were comprehensively analyzed and a phylogenetic tress was constructed. According to the classification of previous surveys (Montanini et al., 2007; Shirazi et al., 2019), 35 MTP proteins were divided into three substrate-specific groups (Zn-CDFs, Zn/Fe-CDFs, and Mn-CDFs) and seven primary groups (1, 5, 6, 7, 8, 9, and 11) that were similar to the AtMTPs and OsMTPs. Of the seven groups, group 1 had the maximum StMTP with 12 members, whereas groups 5, 6, and 7 contained the minimum StMTP with three members each. There are four, five, and five StMTP members in groups 11, 8, and 9, respectively. StMTP1, StMTP3, StMTP4, and StMTP5 belonged to Zn-CDF family; StMTP6 and StMTP7 to Fe/Zn-CDF family and StMTP8, StMTP8.1, StMTP9, StMTP9.1, StMTP9.2 and StMTP11 to Mn-CDF family (Fig 1).

3.2 STRUCTURE AND CHARACTERISTIC ANALYSIS OF *STMTP* GENES

The characteristics of the *StMTP* genes were analyzed in detail. The length of protein sequences of *StMTP* genes ranged from 86 to 503 amino acids. The molecular weights and pIs of these potato proteins ranged from 9781.63 to 55006.92 KDa and 4.98-10.45, respectively (Table 1). Most of the StMTP proteins included five to six

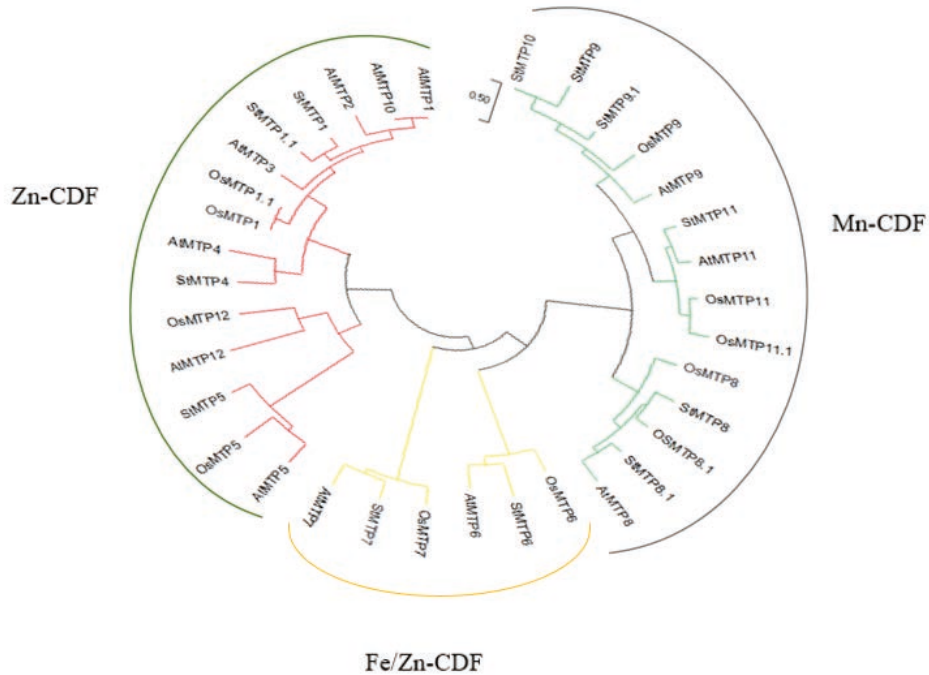

Figure 1: Phylogenetic relationship of MTP proteins in three main plants of *Arabidopsis*, rice, and potato. The tree was constructed using the MEGA 6.0 software by the Maximum likelihood method. The identical proteins were categorized into three sub-families (Mn-MTPs, Zn-MTPs, and Zn/Fe-MTPs). The Zn-MTP sub-family (red line) contains 1: (MTP1:4), 5: MTP5 (red); Zn/Fe-MTPs (yellow line) includes (MTP6-7); and Mn-MTP (green line) contains (MTP8-11)

putative transmembrane domains (TMDs), StMTP8 and StMTP11 had only four TMDs, StMTP7 contained three TMDs, and StMTP6 carried twelve TMDs. Particularly, StMTP5 and StMTP8.1 proteins lacked any of the TMDs.

To examine differences in *StMTP* genes, the exon and intron structures of 12 potato MTP genes were compared. As illustrated in Figure 2, the number of introns in the *StMTP* genes ranged from 1 to 12. Further, the results showed that most of the *StMTP* in same groups exhibited similar exon-intron compositions. Most members of Mn-CDF had six exons, all of StMTP in Fe/Zn included two exons, and more members in ZN-CDF possessed variable number of exons (Fig 2a).

To obtain more insight into the structure characteristics of the StMTP proteins and conserved motif analysis, their amino acid sequences were submitted to MEME program. As shown in Figure 3 and Table 2, ten motifs were in total identified in StMTP family members, while only four of them were explored to encode functional domains when subjected to Pfam. Motif 1, 4, and 8 were annotated as cation_efflux (PF01545), motif 3 as ZT-dimer (PF16916) while motifs 2, 5, 6, 7, 9, and 10 were not assigned by the Pfam. Highly similarity motifs are expected to have similar functions. StMTPs belonged to Mn-CDF group included three motifs sequences namely, motif 1 and 4. StMTPs relevant to Zn-CDF contained three motif (4, 8, and 9). StMTPs (1/3/4/5) included both motifs 4 and 8 cation-efflux. Whereas, StMTP 6 and StMTP7 had only one of 4 motif which belonged to Zn/Fe-CDF groups (Fig 2b). As explained earlier, the cation efflux domain is a typical feature of the MTP transporters. Hence, the domain architectures in StMTP proteins were analyzed. Results showed that all the StMTP proteins included the cation efflux domain. However, the members of groups 8, 9, and 11 (except to 9.2) possessed a ZT.dimer which is a significant zinc transporter dimerization domain.

3.3 MULTIPLE SEQUENCE ALIGNMENT, CONSERVED MOTIFS, AND DOMAIN ARCHITECTURES IN STMTPS PROTEINS

To evaluate the sequence of the StMTP proteins, the amino acid sequences of the AtMTPs, OsMTPs, and StMTPs from the three substrate-specific groups were multiple aligned by ClustalX, respectively. Results revealed that total of the AtMTPs, OsMTPs, and StMTPs proteins has one and two conserved HxxxD residues in Zn/Fe-

Table 1: MTP proteins information for potato

Gene	Accession number	Peptide lenght	MW (kDa)	pI	No. of TMDs N to C	Subcellular localization
StMTP9	PGSC0003DMG400004287	317	36293.33	6.41	5/into in	Cell memebrane. Vaculoe.
StMTP9.1	PGSC0003DMG400009656	86	9781.63	10.45	5/into in	Cell membrane. Vacuole.
StMTP9.2	PGSC0003DMG400011247	413	47080.09	6.22	5/into in	Vacuole.
StMTP8.1	PGSC0003DMG400001111	373	42328.02	4.98	0	Cell membrane. Vacuole.
StMTP8	PGSC0003DMG400032189	405	45429.20	5.07	4/into out	Vacuole.
StMTP7	PGSC0003DMG400026506	463	50752.05	6.36	3/into out	Vacuole.
StMTP6	PGSC0003DMG402011364	503	55006.92	6.19	12/out to out	Vacuole.
StMTP5	PGSC0003DMG400014975	387	43337.68	6.67	0	Vacuole.
StMTP4	PGSC0003DMG400030333	380	42495.88	5.85	6/into in	Vacuole.
StMTP3	PGSC0003DMG400030740	385	42861.52	5.86	6/into in	Vacuole.
StMTP1	PGSC0003DMG400030701	415	45972.68	6.05	6/into in	Vacuole.
StMTP11	PGSC0003DMG400023516	401	45240.14	5.03	4/ into out	Vacuole.

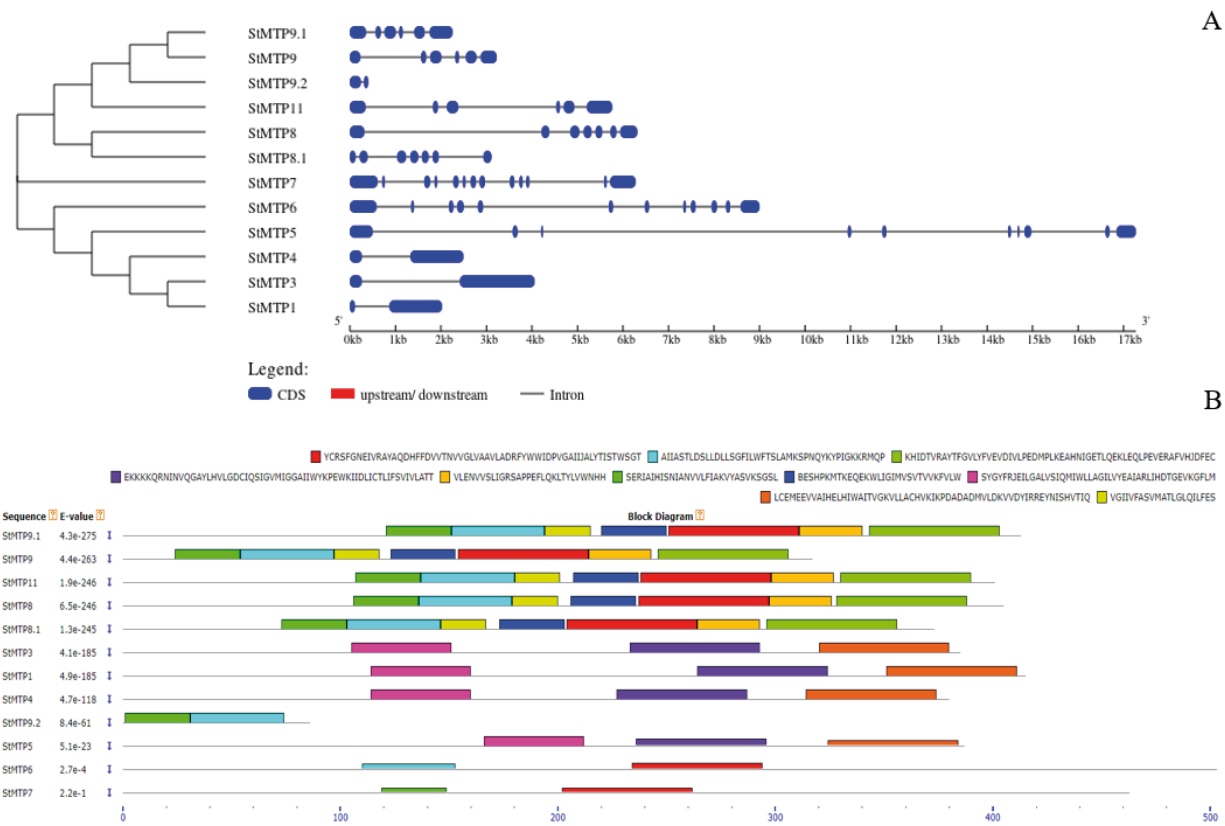

Figure 2: A) Distributions of the conserved domains in StMTP proteins. B) Conserved motifs detected by MEME and displayed in different colored boxes

Table 2: The sequences and the Pfam annotations of conserved motifs in StMTP proteins

Motif ID	Motif sequence	Length	Pfam
Motif 1	YCRSFGNEIVRAYAQDHFFDVVTNVVGLVAAVLADRFYWWIDPVGAIIJALYTISTWSGT	60	Cation-efflux, Pfam, PF01545
Motif 2	AIIASTLDSLLDLLSGFILWFTSLAMKSPNQYKYPIGKKRMQP	43	No motif was found in Pfam
Motif 3	KHIDTVRAYTFGVLYFVEVDIVLPEDMPLKEAHNIGETLQEKLEQLPEVERAFVHJDFEC	60	ZT_dimer, PF16916
Motif 4	EKKKKQRNINVQGAYLHVLGDCIQSIGVMIGGAIIWYKPEWKIIDLICTLIFSVIVLATT	60	Cation-efflux, Pfam, PF01545
Motif 5	VLENVVSLIGRSAPPEFLQKLTYLVWNHH	29	No motif was found in Pfam
Motif 6	SERIAIHISNIANVVLFIAKVYASVKSGSL	30	No motif was found in Pfam
Motif 7	BESHPKMTKEQEKWLIGIMVSVTVVKFVLW	30	No motif was found in Pfam
Motif 8	SYGYFRJEILGALVSIQMIWLLAGILVYEAIARLIHDTGEVKGFLM	46	Cation-efflux, Pfam, PF01545
Motif 9	LCEMEEVVAIHELHIWAITVGKVLLACHVKIKPDADADMVLDKVVDYIRREYNISHVTIQ	60	No motif was found in Pfam
Motif 10	VGIIVFASVMATLGLQILFES	21	No motif was found in Pfam

CDFs and Zn-CDFs, respectively, and two DxxxD residues were explored in the Mn-CDF subgroups (Fig 3).

3.4 POTENTIAL MicroRNA TARGET SITES IN *StMTP* GENES

MicroRNA (miRNAs) are small non-coding RNA molecules that can play key roles in gene expression (Zhang and Chen, 2013). With the expectation score lower than 3.0, a total of 13 StmiRNAs comprising target sites in three *StMTP* genes were detected (Table 3). Two members of group 1 can be targeted by stu-miR7992-3p. Moreover, StMTP5 was targeted by stu-miR5303g, stu-miR5303i, stu-miR5303h, stu-miR5303j, stu-miR156e, stu-miR156f-5p/g-5p/h-5p/i-5p/j-5p/k-5p, and stu-miR5303f. All identified miRNAs-targeted *StMTP* genes were predicted to be silenced by cleavage inhibition. Given that miRNA regulate a large section of mRNA transcripts, resulting nearly all biological events are affected by miRNAs (Bartel, 2009). The findings showed that the UPE ranged from 18.379 (stu-miR7992-3p/ StMTP3) to 23.914 (stu-miR5303f/ StMTP5) (Table 3).

3.5 ANALYSIS OF THE TFBS IN THE PROMOTER REGIONS OF *STMTP* GENES

TF binding sites (TFBS), regions of DNA binding sites in promoter, are important in transcription initiation of its target genes (Yu et al., 2016). As shown in Table 4, 7 TFBS groups, containing elements associated to biotic and abiotic stresses, light response, developmental response, cell cycle, basic transcription, phytohormonal response, and other binding sites were annotated. Among the more common TFBS, MYB and bZIP appeared to be the most frequent elements (with 1046 and 606 numbers, respectively), and were commonly established by all *StMTP* genes. Notably, elements involved in light control was distributed in the promoter regions of all *StMTP* genes. While, elements involved in hormone responsiveness were less abundant than the others (Table 4), it appears that the presence of these elements are an indication that *StMTP* genes could be transcriptionally regulated by different hormones (Table 4).

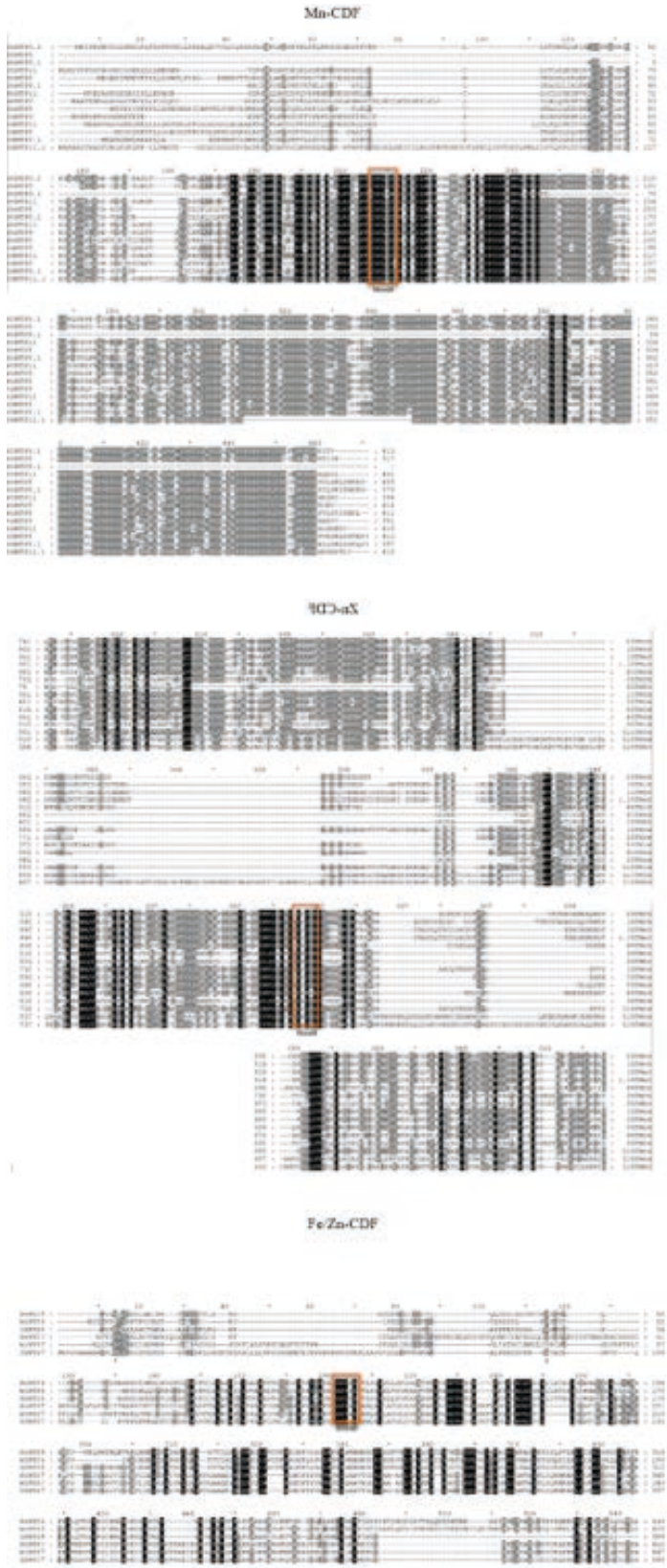

Figure 3: Multiple sequence alignment of StMTP, AtMTP, and OsMTP proteins. The signature sequences and the consensus sequence HXXXD or DXXXD (X = any amino acid) are indicated with black line and open boxes, respectively

Table 3: The potential miRNA target sites in *StMTP* genes

	miRNA_Acc.	Target_Acc.	Expectation	UPE	miRNA-length	Target Start-End	miRNA aligned fragment	Target aligned fragment	Inhibition
StMTP3	stu-miR7992-3p	StMTP3	3	18.379	22	1342-1364	UGUCUAGAUGUGCAUUUCAAAGU	UCCAUGAAUUGCACAUUUGGGCG	Cleavage
StMTP1	stu-miR7992-3p	StMTP1	3	20.777	22	1101-1123	UGUCUAGAUGUGCAUUUCAAAGU	UCCAUGAAUUGCACAUUUGGGCG	Cleavage
StMTP5	stu-miR5303g	StMTP5	1	20.855	23	13278-13301	AUAUUUUUGAAGAGUCUGAG-CAAC	GUUGCUCGGACUCUUCAAAAAU-GU	Cleavage
StMTP5	stu-miR5303i	StMTP5	1	20.855	23	13278-13301	AUAUUUUUGAAGAGUCUGAG-CAAC	GUUGCUCGGACUCUUCAAAAAU-GU	Cleavage
StMTP5	stu-miR5303h	StMTP5	1.5	19.962	23	13279-13302	AACAUUUUUGAAGAGUCUGAG-CAA	UUGCUCGGACUCUUCAAAAAU-GUC	Cleavage
StMTP5	stu-miR5303j	StMTP5	2	19.962	23	13279-13302	AAUAUUUUUGAAGAGUCUGAG-CAA	UUGCUCGGACUCUUCAAAAAU-GUC	Cleavage
StMTP5	stu-miR156e	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156f-5p	StMTP5	3	18.572	19	16072-16091	CUGACAGAAGAGAGUGAGCA	AGCCUACUCUUUUCUGUCAC	Cleavage
StMTP5	stu-miR156g-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156h-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156i-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156j-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR156k-5p	StMTP5	3	19.533	19	16071-16090	UGACAGAAGAGAGUGAGCAC	AAGCCUACUCUUUUCUGUCA	Cleavage
StMTP5	stu-miR5303f	StMTP5	3	23.914	23	13308-13331	AUUUUUGGAGAAUCU-GACACGGGU	GUGCAUGGCGAAUUCUCCAAAA-GU	Cleavage

Table 4: Summary of the transcription factor binding sites (TFBS) detected in the promoter regions of *StMTP* genes

TFBS related to hormone/ tissue-specific/ stress response/binding site	Name of TFBS	StMTP5	StMTP3	StMTP1	StMTP6	StMTP8	StMTP8.1	StMTP9.1	StMTP9.2	StMTP9	StMTP11	StMTP4	StMTP7	Expected function
Tfs related to hormone response	AP2	87	92	78	51	53	14	5	19	15	39	28	37	Ethylene -responsive element
Tfs related to hormone response	BBR-BPC	6	2	0	0	0	0	0	0	0	0	0	0	Cytokinin -responsive element
Tfs related to hormone response	BES1	4	3	0	2	0	0	0	1	2	0	0	2	Strigolactone and Brassinosteroids -responsive element
Tfs related to hormone response	ARF	2	1	0	0	0	0	0	0	0	0	0	0	Auxin -responsive element
Tfs related to hormone response	EIN3 ; EIL	8	3	7	5	4	2	0	0	0	0	3	0	Involved in ethylene and JA signaling
Tfs related to hormone response	VOZ	9	3	0	0	0	0	0	0	0	0	1	0	Gibberellin -responsive element
TFs related to light response	bHLH	28	17	8	36	36	14	5	37	38	7	34	34	Light-responsive element
TFs related to light response	Dof	36	54	51	16	11	12	9	13	15	13	11	15	Light-responsive element
TFs related to light response	GATA	30	26	26	17	18	13	4	7	15	13	13	11	Light-responsive element
TFs related to tissue-specific localisation	AT-Hook	30	18	18	29	19	21	3	13	27	23	11	26	Vasculature-specific expression
TFs related to tissue-specific localisation	SBP	26	5	5	21	19	2	2	18	13	16	3	17	Involved in flower and fruit development
TFs related to tissue-specific localisation	LOB	1	0	0	0	0	0	0	0	0	0	0	0	Involved in lateral organ development
TFs related to tissue-specific localisation	MADS box	8	3	0	0	0	0	0	0	3	0	0	8	Involved in flowering development
TFs related to tissue-specific localisation	MADF	16	0	0	0	0	0	0	3	1	0	1	1	Involved in flower and fruit development

Continued on the next page

TFs related to tissue-specific localisation	TCR	14	0	6	4	3	4	0	4	0	0	4	0	Involved in development male and female reproductive tissues/ tissue-specific expressions
TFs related to tissue-specific localisation	WOX	9	0	2	0	0	1	0	1	0	0	1	1	Tissue-specific expressions
TFs related to cell cycle	E2F/DP	3	1	0	1	0	0	0	0	0	2	0	0	Involved in cell polification
TFs related to stress response	MYB	259	214	165	70	58	37	9	47	46	49	32	60	responsive to environmental stress
TFs related to stress response	WRKY	106	55	3	33	27	34	2	13	20	23	33	27	involved in developmental and physiological processes
TFs related to stress response	HSF	20	2	2	2	0	3	0	0	0	0	0	20	Involved in cell differentiation, and proliferation
TFs related to stress response	C2H2	50	37	37	14	11	9	4	9	7	9	11	9	responsive to stress and the hormone signal transduction
Tfs related to basic transcription	NF-Y	7	2	3	2	1	1	4	1	2	2	3	3	Involved in transcription by recognizing and binding to a CCAAT motif in promoters
other tfs binding sites	WRC;GRF	4	0	0	0	0	1	1	1	1	0	0	0	Involved in stem and leaf development
other tfs binding sites	Sox	7	2	4	4	0	2	0	2	3	3	0	3	Involved in cell cycle regulation
other tfs binding sites	FAR1	1	0	0	0	1	0	0	1	0	0	0	0	Light-responsive element
other tfs binding sites	SRS	4	0	2	4	0	0	0	0	0	2	2	2	Involved in style and stigma development
other tfs binding sites	NAC	175	75	16	7	18	6	0	6	6	12	2	11	Involved in developmental process and stress responses
other tfs binding sites	bZIP	121	87	66	51	53	26	5	34	48	56	22	37	Developmental and physiological processes

Continued on the next page

other tfs binding sites	Homeodomain	69	42	56	30	30	31	1	27	26	36	29	27	Involved in cell fate and differentiation
other tfs binding sites	Storekeeper	4	0	0	1	0	0	0	0	0	2	0	1	Involved in sucrose inducible expression of patatin gene
other tfs binding sites	B3	37	28	13	21	18	12	9	13	13	21	7	20	Involved in developmental process
other tfs binding sites	Trihelix	24	6	0	0	0	0	0	0	0	0	3	1	Tissue-specific expressions
other tfs binding sites	TCP	22	5	0	14	8	2	6	9	4	23	9	5	Involved in plant morphology
other tfs binding sites	ZF-HD	13	13	12	0	0	0	1	0	0	0	0	0	Involved in spike development

3.6 EXPRESSION PATTERNS OF *STMTP* GENES UNDER DROUGHT STRESS AND TISSUE-SPECIFIC ANALYSIS

3.6.1 The expression patterns of *StMTP* under drought stress

To better understand the expression of *StMTP* genes under the influence of drought stress, two *StMTP* genes were selected and their expression levels were checked by qPCR in leaves and tubers under stress. The expression levels of drought and normal treatments are given in Figure 1. The analysis results showed that *StMTP8* and *StMTP9* showed the highest level of expression in leaf and tuber (natural) under normal treatment, while both genes decreased under drought conditions. In leaves and tubers, the expression level of *StMTP9* was higher than *StMTP8* in both leaves and tubers (normal) (Fig 4a).

3.6.2 The expression patterns of *StMTP* in tissue-specific

The tissue expression patterns of *StMTPs* were investigated based on the qPCR data. As shown in Fig 1B and C, both genes (*StMTP8* and *StMTP9*) were expressed in the four determined tissues. The results of qPCR analysis revealed that the *StMTP9* gene significantly had higher expression levels as compared with *StMTP8* in all tissues such as root, stem, leaf, and tuber. *StMTP9* and *StMTP8* genes exhibited maximum levels of gene expression in leaf whereas, the minimum levels had in tuber. Moreover, the high expression of *StMTP9* gene was observed in the stem (Fig 4b,c).

4 DISCUSSION

In the present study, a total of 12 *StMTPs* were detected in potato. The *MTPs* were named based on the sequence similarities and orthologous relationships between them and the *AtMTPs*. First, the phylogenetic relationships of the MTP proteins between *S.tuberosum, A. thaliana,* and *O.sativa* were assessed. Based on previous studies, *A.thaliana* included 12 MTPs (AtMTP1-12). Contrasted with *Arabidopsis*, *S.tuberosum* genome carried multiple MTP homologs for each AtMTP, but the homologs for AtMTP2 and AtMTP3 were absent. There were two, four, and six *StMTP* genes belonging to Fe/Zn-CDFs, Zn-CDFs, and Mn-CDFs, respectively. It is established that phylogenetic relationships can be utilized to infer structure and functional roles among species (Vatansever et al. 2017). This finding could provide clues to

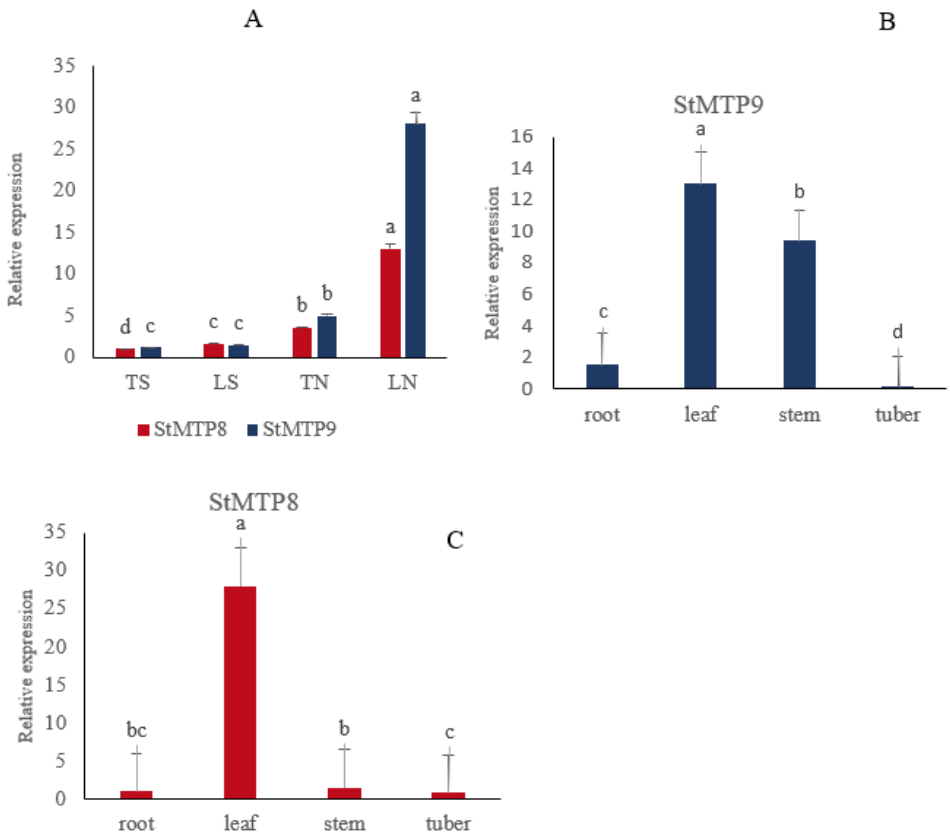

Figure 4: A) The qPCR expression of the potato *StMTP8* and *StMTP9* genes from tuber and leaf samples under drought stress. B, C) The expression of the potato *StMTP8* and *StMTP9* genes among different tissues in organs such as root, leaf, stem, tuber under control condition. Tuber normal, (TN), Tuber stress (TS), Leaf normal, (LN), Leaf stress (LS)

discover the functional characteristics, particularly the substrate-specificities of StMTP proteins. Montanini et al. (2007) identified a modified signature available in the trans-membrane regions of the metal tolerance proteins, and proposed a functional role the conserved group-residues in metal selectivity (Montanini et al., 2007). Further, the signature sequences HxxxD (x = any amino acid) and DxxxD were detected to illustrate the sequence characteristics of the both Zn-CDFs and Fe/Zn-CDFs, and Mn-CDFs, respectivey.

Features of the *StMTP* genes including peptide length, MW, Pi, sub-cellular, and TMD localization were analyzed. Our results agree with previous studies in wheat and tobacco (Vatansever et al., 2017; Liu et al., 2019), StMTP proteins were mainly predicted to be localized to vacuole, whereas some others are localized in cellular membrane and nucleus. It is suggested that StMTPs could function as the vacuole-localized cation transporters. Other studies in *Arabidopsis* revealed that AtMTP1 and AtMTP3 are involved in the transport of excess Zn into vacuoles, regulating cellular Zn hemostasis (Kobae et al. 2004; Arrivault et al. 2006). Although all of the StMTPs were identified the cation efflux domain and the modified features, however, some other motifs were not present in some StMTP members. StMTP6 and StMTP8.1 do not possess any TMD, a common signature of membrane proteins, which may have distinct biological functions and novel roles except other transporters.

Besides the transmembrane region, the modified signature sequence between TMDs I and II, and the characteristics C-terminal cation-efflux domain are two structural features of MTP proteins. Our findings revealed that all the StMTP proteins included two typical structural characteristics. Further, the signature sequences HxxxD and DxxxD were also detected in associated members of three main substrate-specific groups, which were in accordance with consensus residues. Also, these results provided a precious support for our phylogeny tree. Moreover, ZT-dimer was as molecule of zinc transporter that formed a homodimer during activity (Lu and Fu, 2007). The existence of ZT-dimer in specific StMTPs suggested that these proteins could require to organize heterodimers and homodimers when ministering as metal ion transporters. In this study, the ZT-dimer was iden-

tified in members of groups 8, 9 (except for StMTP9.2), and 11. Overall, these structure features of StMTP proteins were consistant with the structure characteristics of MTP transporters. These results revealed that there are structural similarity of StMTPs within the same groups.

The regulatory mechanisms controlling *StMTP* gene expression were evaluated at two levels, transcriptional and post-transcriptional using TFBS and the microRNA target sites in the promoter regions and the coding sequences of *StMTP* genes, respectively. A total of 5312 putative TFBS involved in multiple biological processes and thirteen StmiRNAs were detected. Former studies have revealed that some of these detected miRNAs were implicated in abiotic and biotic stress response. For example, the expression of stu-miR156e, stu-miR5303f, stu-miR5303g, stu-miR5303h, and stu-miR5303j would be up-regulated under late blight infection in potato (Kumar et al., 2018). In addition, mir156 possesses various functional roles in response to heat, cold, drought, and hypoxia (Stief et al., 2014). Stu-miR7992-3p was up-regulated in defense-related miRNAs to virus (Kondhare et al., 2018). Stu-miR5303g might also respond to Li^+ stress through regulating their target genes (Kwenda et al., 2016). Thus, it would be of interest to discover the functions of *StMTP* genes in this biological and physiological processes in latter studies.

The importance of *StMTP* function in potato growth and developmental stages could be identified through tissue expression profile analysis. For example, StMTP8 is highly expressed in leaf, an indication that it might be vital for potato leaf development. On the other hand, the expression levels of StMTP8 was most abundant in all three types of leaf structures during leaf formation, indicating it might be involved in regulating leaf development. StMTP9 was exclusively expressed in leaf and stem, indicating they have important roles in leaf and stem growth and development stages in potato. StMTP9, was slightly expressed in root and tuber, demonstrated a non-significant role in root growth and development. Also, StMTP8 was not or rarely induced in the evaluated root and tubers. In *Populus trichocarpa,* PtrMTP9 is expressed in roots and is sharply up-regulated by excess Fe (Gao et al., 2020). Earlier study has shown that OsMTP9 knockout significantly decreased Mn uptake and root-to-shoot translocation (Sasaki et al., 2016).

Drought treatment expression analysis showed that the *StMTP8* and *StMTP9* genes were down-regulated. Previous studies suggested that both AtMTP9 and VvMTP9 share two identical orthologues in potato, which StMTP9 is expected to be down-regulated in response to drought stress (Shirazi et al., 2019). However, both genes (AtMTP9 and VvMTP9) were upregulated in response to drought, salinity, osmotic shock, and hormonal stresses. AtMTP8 is expressed in root while it is orthologues with StMTP8 in potato. *StMTP8* gene is expressed in leaves whereas, it is slightly expressed in roots, stem, and tubers. *AtMTP 1* and *AtMTP3* were up-regulated in response to ABA, but were down-regulated in response to biotic and abiotic stresses. Additionally, *AtMTP2* was down-regulated in response to all stresses. Other *AtMTPs* varied with respect to expression, up and down regulation. This result showed that *MTP* genes have diverse roles in adaptation of plants under various stresses.

Using analysis of each StMTP genes promoter regions, different elements were identified that may regulate gene expression in developmental stages and drought stress in potato. In cucumber, MTP8 is a Mn transporter which maintain Mn homeostasis in root. CsMTP7 is constitutively induced in all cucumber tissues during plant development, a putative Fe/Zn transporter (Migocka et al., 2014). OsMTP1 was widely expressed in mature leaves and stems. Analysis of expression profiles revealed that StMTP might be involved in several aspects of potato development, and also be significant in leaf and shoot development. Further, *StMTP* genes may play significant roles drought and abiotic stress.

The expression profiles of the *MTP* genes under drought stress could reflect differences in the type and number of TFBS in the promoter region of the genes. As a result, different genes can respond to various stresses (Vatansever et al. 2017; Saidi et al., 2020a, b). The MYB and bZIP were two common TFBS found in the upstream regions of *StMTP* genes at a high frequency.

MYB plays a key role in plants under metal stress. In *Arabidopsis*, MYB4 is induced following exposure to Cd and Zn while MYB43, MYB48, and MYB124, member of MYB family were found to be particularly expressed in roots in response to Cd stress. MYB28 is as another member of MYB family which is induced after Cd-stress. Moreover, MYB, bZIP, AP2 play vital role in regulating the specific response of plants under Cd stress through modulating the particular responsive genes (Wu et al., 2012; Hajibarat and Saidi, 2022 a, Hajibarat et al., 2022b). BZIP has been identified as one of the most TFBS in *Arabdiopsis*, bean, sesame, and wheat, involved in adapting to zinc deficiency through inducing the expression of members of membrane transporters (ZIPs) (Wang et al., 2018; Saidi et al., 2020a, b).

In the current study, diverse MTPs revealed drought responses to stress conditions, the lowest level expression was observed for StMTP9 in response to drought stress. These results did not agree with our findings in *Arabiopsis*. Previous studies showed that some genes were up/down-regulated with similar stress with corresponding genes to same group (Li et al., 2018; Shirazi et al., 2019; Saidi and Hajibarat, 2019). AtMTP9, 10, 11 were up-reg-

ulated in plants exposed to drought, cold and salt stresses, regulating gene expression and functional proteins to enhance stress tolerance. In addition, drought stress lead to changes in plant metals concentration in *Brachypodium* (Chen et al., 2018). In general, our findings could provide significant evidence for highlighting the metal transport mechanism mediated by StMTP proteins in growth and developmental stages and drought stress.

5 CONCLUSION

Twelve *MTP*s in *S. tuberosum* were identified in the current study. Using bioinformatics tools, comprehensive analysis of *StMTP* genes were performed including protein properties, analysis of TFBS and structure, MicroRNA analysis, and analysis of gene expression in developmental and growth and drought stress. Based on phylogenic study, StMTPs were clustered into three subfamilies and seven groups (1, 5, 6, 7, 8, 9, and 11), similar to the MTP genes in *Arabidopsis*, rice, and tobacco. The MTP genes may have apparently been underwent gene loss and expansion via tandem duplication after polyploidization. All StMTPs contained cation-efflux and signature sequence, while, few of them also possess the ZT-dimer. The expression profiles of *StMTP* genes in tissue-specific and in response to drought stress predicted that *StMTP* genes had the necessary roles in potato developmental stage, particularly in drought stress. This study provides valuable resources for better insight into the biological roles of *StMTP* genes in potato.

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