Full Length Article

Identification and transcriptional analysis of the metal tolerance protein (*MTP*) gene family in cassava under zinc deficiency

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Abstract

Available of zinc in cassava plants is essential for plant developmental stages as well as crop production. This paper was explored the molecular mechanism of zinc transport in cassava plants. Zinc deficient symptom was found in upper cassava leaves under zinc deficiency and identified the metal tolerance proteins (MTPs) family in cassava genome. MTPs are a metal cation efflux transporter that participated in zinc homeostasis. Computational analysis showed 12 *MeMTP* members in cassava genome with exons ranging from 1-12. Most of the MTP proteins were predicted to localize in plasma membrane and tonoplast except MeMTP4 and MeMTP8, which localized in ER membrane. Functional annotation verified that MeMTPs were cation efflux proteins/zinc transporters belonging to cation diffusion facilitator superfamily (CDF). Most of the cis-acting elements in *MeMTP* promoters were phytohormone responsive. TGA-element was identified in *MeMTP2*, *MeMTP5-7* and *MeMTP10* promoters, indicating its role in auxin regulation. MeMTP proteins were divided into three groups according to phylogenetic relationship. Moreover, RNA expression of 6 candidate *MeMTP* genes including *MeMTP1-5* and *MeMTP12* was evaluated under zinc deficiency. *MeMTP1* was up-regulated in roots and leaves under zinc deficiency whereas the expression of *MeMTP2*, *MeMTP4* and *MeMTP12* were tissue-specific. These findings will provide an important foundation of the MTPs in zinc homeostasis mechanism of cassava plants.

Keywords: zinc, zinc deficiency, cassava, metal tolerance proteins, gene expression

Introduction

Cassava (*Manihot esculenta*) is the second-most significant economic crops contributing to Thailand economy. Recently, it has been reported that production and harvesting area of cassava decreased continuously compared to planting area. Reduction of cassava yield results from viral diseases and inappropriate fertilizer management. Moreover, in Northeastern regions of the country, zinc deficiency also results in poor quality of cassava production. The main causes of zinc deficiency in the crops are an alkaline soil, calcareous soil and excess

macronutrient application (Alloway, 2009; Howeler, 2002; Noulas et al., 2018; Takrattanasaran et al., 2013).

Zinc is a micronutrient necessary for cell metabolisms such as cofactors in several enzymes, protein synthesis and precursors for auxin production (Broadley et al., 2012). Cassava plants appealing early symptoms of zinc deficiency may develop more fibrous roots rather than storage roots, which causes a reduction in yield. Normally, cassava plants accumulate zinc 50-100 µg/g in mature leaves but less than 20 µg/g in zinc-deficient leaves (Howeler, 2002). In cassava, zinc deficiency is characterized by chlorosis in interveinal regions of upper leaves. Growing of cassava plants in zinc-deficient soils for a long period will stop plant growth (Howeler, 2002).

To understand the zinc homeostasis in cassava plants whether mobilization, uptake, translocation or storage, the study of zinc uptake mechanisms in molecular level is not negligible. The knowledge also provides the baseline data for precision agriculture and farming management to improve crop production and quality. Zinc in a form of Zn^{2+} in soils is taken up across the membrane of root cells before transported to the upper part of the plants (Palmgren et al., 2008; Ricachenevsky et al., 2015; Scott Aleksander Sinclair & Krämer, 2012). These mechanisms require transporter proteins to sustain zinc homeostasis throughout plant organs.

Among major protein families responsible for zinc transport, the CDF (Cation Diffusion Facilitator) families, called MTP families in plants, function as a metal efflux transporter from cytoplasm and involve in sequestration of zinc into intracellular compartments such as vacuole and endoplasmic reticulum (ER) (Gupta et al., 2016). The MTPs transport divalent cations, mainly Zn^{2+} but also Mn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+} (Ricachenevsky et al., 2013). CDF members from several species, including bacteria, fungi, mammals and plants have been classified into three subgroups due to protein sequence analysis: Zn-CDF, Fe/Zn-CDF and Mn-CDF (Montanini et al., 2007). The members of MTP family are different upon plant species. For instant, rice, *Arabidopsis*, wheat, poplar and tobacco have 12, 12, 20, 22 and 26 MTP members, respectively (Gao et al., 2020; Kawachi et al., 2008; Liu et al., 2019; Vatansever et al., 2017).

Although MTPs are a crucial metal transporter linked to zinc homeostasis in many plants, the knowledge of MTPs in cassava is still limited. Here, we identified cassava *MTP* gene family (*MeMTPs*) and studied the transcription levels of 6 *MeMTP* genes under zinc deficiency. The coding regions and protein sequences of these MTP orthologs were analyzed with bioinformatics and online-based platforms.

Materials and Methods

Plant Materials

Cassava (*Manihot esculenta*) cultivar Kasetsart 50 (KU50) cuttings was kindly provided from the Thai Tapioca Development Institute in Nakhon Ratchasima Province, Thailand.

Plant Growth and Sample Collection

Cassava stems were cut into small sections with 2-3 nodes and nursed under hydroponics system for 3 weeks until leaves and roots were fully developed. The healthy plants were transferred to 3-liter pots containing Hoagland Solution (Hoagland & Arnon, 1950) with

2.5 µM $ZnSO_4$ (control) or without $ZnSO_4$ (Zn deficient condition) for 3 weeks. The daily average temperature in a greenhouse ranged from 28˚C in the morning to 38˚C in the afternoon. Youngest fully expanded leaves and fibrous roots were collected from 3 biological replicates at 3 weeks then frozen immediately in liquid nitrogen and kept at -80˚C until use for RNA extraction.

Identification of MeMTPs

To identify cassava MTP proteins. MTP protein sequences from *Arabidopsis* and rice databases were used as queries to search for homologous sequences in *Manihot esculenta* Phytozome database by BLASTP. We predicted putative transmembrane regions using TMHMM and predicted subcellular localizations of MeMTP proteins using PSORT (Peabody et al., 2016), CELLO (Yu et al., 2006) and Plant-mPLoc (Chou & Shen, 2010) servers.

Phylogenetic Analysis and Gene Structure

MeMTP protein sequences were aligned with MTP sequences from *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) using ClustalW. Phylogenetic tree was constructed by MEGAX (Kuma et al., 2018) via the neighbor-joining (NJ) method with 1000 bootstrap replicates. *MeMTP* gene structures were determined using the Gene Structure Display Server (GSDS) program (Hu et al., 2015).

Prediction of Cis-acting Regulatory Elements

DNA sequences located 2.0 kb upstream of *MeMTP* genes were acquired from Phytozome database for promoter analysis and the cis-acting regulatory elements on the promoter region were predicted using PlantCare (Lescot et al., 2002).

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from leaves and roots using the Plant RNA Purification Reagent, then treated with TURBO DNase™ Treatment (Thermo Fisher Scientific) to remove genomic DNA. Synthesis of cDNA was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Expression of 6 *MeMTP* genes was analyzed by RT-PCR using GoTaq® Green Master Mix (Promega) and normalized to *Me18S* and *MeGAPDH* genes. The transcript level of *MeZIP1* was used as a positive control under zinc deficiency. The primers used for RT-PCR are listed in Table 1.

Table 1. Primer characteristics

Gene name		Primer sequences	Nucleotide size (bp)	Tm (˚C)	Amplicon size (bp)	
					gDNA	cDNA
MeMTP1	Forward	5'-CCGTTGGGAGAAGTAGGATATG-3'	22	60	120	120
	Reverse	5'-GTGCTACTGCAATCAAGAGC-3'	20			
MeMTP2	Forward	5'-TGCTGCCCATTTGCTTTC-3'	18	58	124	124
	Reverse	5'-TGCACCTAGAATCTCAACCC-3'	20			
MeMTP3	Forward	5'-GCCATTGCTCGACTGATCTA-3'	20	60	118	118
	Reverse	5'-CATGCTGATGACCCAACAAG-3'	20			
MeMTP4	Forward	5'-CAGAACCTCGGGAAACAAG-3'	19	60	209	123
	Reverse	5'-GGGTCTATCCACCAGTAGAA-3'	20			
MeMTP5	Forward	5'-TGGGAATTGGGCTCTTCA-3'	18	60	282	131
	Reverse	5'-GTACACACGATCAGGCTTTC-3'	20			
MeMTP12	Forward	5'-ACCCAGTCTCACCATTCA-3'	18	58	181	124
	Reverse	5'-CAACTCCAACACTTCCCATC-3'	20			
MeZIP1	Forward	5'-TCACTTACGCAGGATTTGG-3'	19	58	209	124
	Reverse	5'-GACTCTGAGAAGCACCTAAAG-3'	21			
Me18S	Forward	5'-CGGAGAGGGAGCCTGAGAAA-3'	20	60	120	120
	Reverse	5'-CAGACTCGAAGAGCCCGGTATTA-3'	23			
MeGAPDH	Forward	5'-CGACTGTCCATGCAACTAC-3'	19	60	212	111
	Reverse	5'-CACCAGTGGAACTAGGAATG-3'	20			

Results and Discussion

Structural and functional annotation of cassava MTP gene family

Twelve putative *MTP* genes were identified in cassava genome on phytozome database as shown in Table 2. All their protein sequences were similar to the sequences in rice and *Arabidopsis*, which were used as queries. The *MTP* genes were classified into 3 groups (Figure 1). The exon-intron organization analysis of *MeMTP* genes showed number of exons ranging from 1-12. *MeMTP1-4* contained no intron in their gene structures whereas *MeMTP6* had highest number of introns and exons (Table 2, Figure 1). We found that *MeMTP9* and *MeMTP10* shared 90% homology according to the sequence alignment and contained the same number of exons. The notion that these genes was originated as a single gene before the divergent would be considered.

The predicted proteins coded from *MeMTP* genes consisted of 351-876 amino acids. Most of them were likely to localize in plasma membrane and tonoplast except MeMTP4 and MeMTP8, which had high potential to localize in ER membrane. Moreover, our hypothesis that MeMTPs are transmembrane proteins was supported by the presence of transmembrane helices (TMHs) in their structures (Table 2). Six TMHs are common in plant MTPs (Arrivault et al., 2006; Fujiwara et al., 2015; Gustin et al., 2011; Kawachi et al., 2008; Liu et al., 2019). In cassava, most of the MeMTPs contained 4-6 TMH domains, thus verifying the character of the MTP family. However, MeMTP6 did not hold any TMHs. In addition, the MeMTP6 was likely to be a mitochondrial protein, which was unique among other MeMTPs. Further in vivo experiments would be performed to unveil the cellular localization of MeMTP6 and its roles in plant metal homeostasis. Interestingly, MeMTP12 was a largest protein with highest TMH domains, indicating the distinctive biological function. These characters have also been reported in MTP12 from other plants (Fujiwara et al., 2015; Gao et al., 2020; Liu et al., 2019; Vatansever et al., 2017).

Functional annotation of the translated sequences from PANTHER, PFAM, KOG and KEGGORTH databases verified that cassava MTPs were cation efflux proteins/zinc transporter belonging to cation diffusion facilitator superfamily (CDF). Gene Ontology (GO) also classified MeMTPs as transmembrane proteins functioning in delivery of cations within or across a cell. However, the possibility to be an iron transporter would be considered in MeMTP6, MeMTP8, MeMTP9, MeMTP10 and MeMTP11 according to KOG annotation (Table 3).

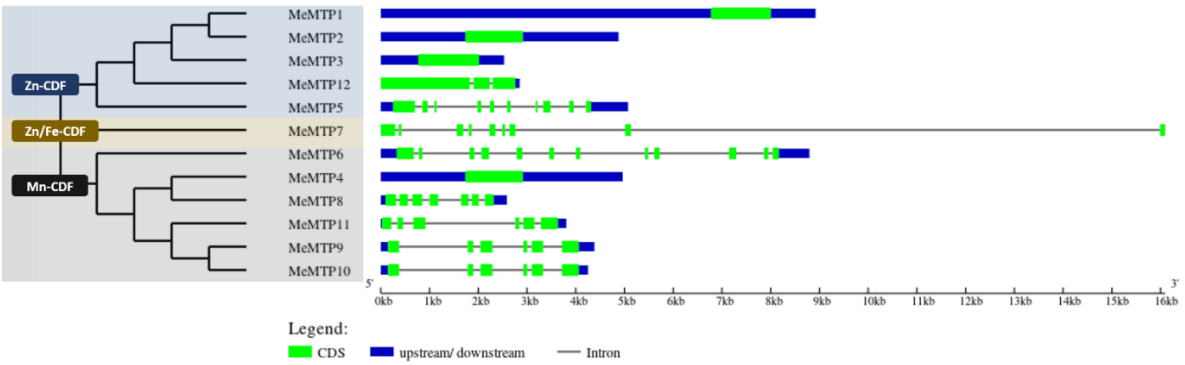

Figure 1. *MeMTP* gene structures. Blue boxes indicate untranslated 5′ and 3′ regions (UTR); green boxes indicate exons; black lines indicate introns.

Table 2. *MeMTP* genes encoding MTP proteins along with their general details in cassava

Gene name	Locus name	No. of Exons	Protein length	No. of TMHs	Subcellular localization
MeMTP1	Manes.05G016000.1	1	407	6	plasma membrane/tonoplast
MeMTP2	Manes.01G239300.3	1	390	6	plasma membrane/tonoplast
MeMTP3	Manes.02G000600.1	1	415	6	plasma membrane/tonoplast
MeMTP4	Manes.15G105000.1	1	404	5	ER membrane/tonoplast
MeMTP5	Manes.09G035800.1	10	404	6	plasma membrane/tonoplast
MeMTP6	Manes.05G065800.1	12	499	0	plasma membrane/mitochondria/tonoplast
MeMTP7	Manes.03G080500.1	9	351	4	plasma membrane/tonoplast
MeMTP8	Manes.08G042500.1	7	407	5	ER membrane/tonoplast
MeMTP9	Manes.S031300.1	6	413	4	plasma membrane/tonoplast
MeMTP10	Manes.16G046900.1	6	402	5	plasma membrane/tonoplast
MeMTP11	Manes.10G037600.1	6	394	4	plasma membrane/tonoplast
MeMTP12	Manes.S020800.1	3	876	13	plasma membrane/tonoplast

Analysis of cis-acting elements in the MeMTP promoters

Promoter regions are crucial for regulation of gene expression at the transcriptional level. To underly mechanisms in transcriptional regulation of *MeMTP* genes, cis-acting elements in *MeMTP* promoter regions were determined 2.0 kb upstream of a transcription start site. The results showed that most of the elements in *MeMTP* promoters were phytohormone responsive. Among them, the ethylene responsive element (ERE) was the most abundant one existed in all *MeMTP* genes, especially *MeMTP4* that contained up to 13 replicates. Therefore, the expression of *MeMTP4* may be regulated by ethylene signaling. The ERE was also found in *TaMTP1A* and *TaMTP4D* promoters of common wheat (Vatansever et al., 2017). ERE is a binding site of ethylene responsive factors (ERFs), which are transcription factors responding to ethylene signaling (Binder, 2020). Under metal stress, the production rates of ethylene are increased in plants (Keunen et al., 2016). Ethylene may stimulate the downstream signaling cascades including ERFs to trigger the expression of ERE responsive genes, including *MTPs*, resulting in the production of MTP efflux transporters to balance the concentration of metals in a cell.

TGA-element was identified in *MeMTP2*, *MeMTP5-7* and *MeMTP10* promoters, indicating its role in auxin regulation. Zinc is a precursor in a tryptophan synthesis for auxin production (Begum et al., 2016). Thus, auxin signaling may be promoted by zinc uptake via MTP transporters. Additional elements related to stress response, TC-rich repeats, were also found in *MeMTP1*, *MeMTP2* and *MeMTP3* promoters (Table 4). Therefore, *MeMTP* genes could be regulated by multiple stimuli.

Phylogenetic relationship of the MTP proteins

To further analyze the phylogenetic relationship of MeMTP proteins to their orthologs from *Arabidopsis* and rice, the tree was constructed using a total of 36 MTP proteins, comprising 12 from cassava, 12 from *Arabidopsis* and 12 from rice. As a result, the MeMTPs were divided into three major groups. Mn-CDF Group contained 6 MeMTP proteins (MeMTP4, 6, 8, 9, 10 and 11), while MeMTP7 was a single gene in Zn/Fe-CDF group. Zn-CDF Group contained 5 MeMTPs including MeMTP1, 2, 3, 5 and 12. Almost all of cassava MTP nomenclatures were categorized in the same manner of *Arabidopsis* MTPs, except MeMTP4 that shift to the same clade as OsMTP4 in rice. The result suggests that cassava *MTP* genes might be in the evolutionary history of the *MTP* gene family in *Arabidopsis* and rice (Figure 2).

Although the member of cassava MTP family is comparable to those reported in *Arabidopsis* and rice (Gustin et al., 2011; Kawachi et al., 2008; Montanini et al., 2007), different protein numbers are presented in various plants. The highest number has been reported in tobacco with 26 MTP proteins (Liu et al., 2019). It is possible that species containing high number of MTPs may be the result of gene duplication during evolution and each gene may start to develop its distinct functions.

In plants, metal transporters in the CDF family have been called the Metal Tolerance Proteins (MTPs) according to their functions in sequestration of metal (Me^{2+}) into organelles such as vacuole as well as the extracellular space to reduce cellular damage from high concentration of metals (Ricachenevsky et al., 2013). The plant MTPs have been clustered into 3 groups; the Zn-CDFs, Fe/Zn-CDFs, and Mn-CDFs, based on phylogenetic analysis and metal selectability (Ricachenevsky et al., 2013).

Table 3. Functional annotation of MeMTP proteins based on PANTHER, PFAM, KOG, GO and KEGGORTH databases

Database	Database ID	Functional annotation	Gene name											
			MeMTP1	***MeMTP2***	***MeMTP3***	***MeMTP4***	***MeMTP5***	***MeMTP6***	***MeMTP7***	***MeMTP8***	***MeMTP9***	***MeMTP10***	***MeMTP11***	***MeMTP12***
PANTHER	PTHR11562	CATION EFFLUX PROTEIN/ ZINC TRANSPORTER	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓
PANTHER	PTHR11562	METAL TOLERANCE PROTEIN				✓	✓	✓		✓	✓		✓	
PANTHER	PTHR11562:SF18	MITOCHONDRIAL METAL TRANSPORTER 1-RELATED						✓						
PANTHER	PTHR11562:SF13	ZINC TRANSPORTER 7												✓
PFAM	PF01545	Cation efflux family	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
PFAM	PF16916	Dimerization domain of Zinc Transporter				✓		✓			✓	✓		
KOG	KOG1482	Zn^{2+} transporter	✓	✓	✓									
KOG	KOG1484	Putative Zn^{2+} transporter MSC2 (cation diffusion facilitator superfamily)		✓		✓								✓

Database	Database ID	Functional annotation	Gene name											
			MeMTP1	***MeMTP2***	***MeMTP3***	***MeMTP4***	***MeMTP5***	***MeMTP6***	***MeMTP7***	***MeMTP8***	***MeMTP9***	***MeMTP10***	***MeMTP11***	***MeMTP12***
KOG	KOG1485	Mitochondrial Fe^{2+} transporter MMT1 and related transporters (cation diffusion facilitator superfamily)						✓		✓	✓	✓	✓	
GO	GO:0006812	The directed movement of cations, atoms or small molecules with a net positive charge, into, out of or within a cell, or between cell, by means of some agent such as a transporter or pore.	✓		✓	✓		✓	✓	✓	✓	✓	✓	✓
GO	GO:0016021	The component of a membrane consisting of the gene products and protein complexes having at least some part of their peptide sequence embedded in the hydrophobic region of the membrane.			✓	✓	✓	✓		✓	✓	✓	✓	✓
KEGGORTH	K14689	Solute carrier family 30 (zinc transporter), member 2		✓	✓									
KEGGORTH	K14692	Solute carrier family 30 (zinc transporter), member 5/7					✓							✓
KEGGORTH	K14696	Solute carrier family 30 (zinc transporter), member 9							✓					

Table 4. Predicted cis-elements in the promoter regions of *MeMTP* genes

Gene name	Stress responsive	Phytohormone responsive						
	TC- rich repeats	ABRE	ERE	CGTCA-motif	TGACG-motif	TGA-element	GARE-motif	TCA-element
MeMTP1	1	3	9					
MeMTP2	2	2	2	1	1	2		
MeMTP3	1	2	3					
MeMTP4			13					
MeMTP5		2	3	2	2	1	1	
MeMTP6		1	2	2	2	1		
MeMTP7			1	2	2	1	1	
MeMTP8			7	3	3			
MeMTP9		1	2	2	2			1
MeMTP10		3	3	2	2	2	1	2
MeMTP11		5	8	1	1			
MeMTP12		1	8					

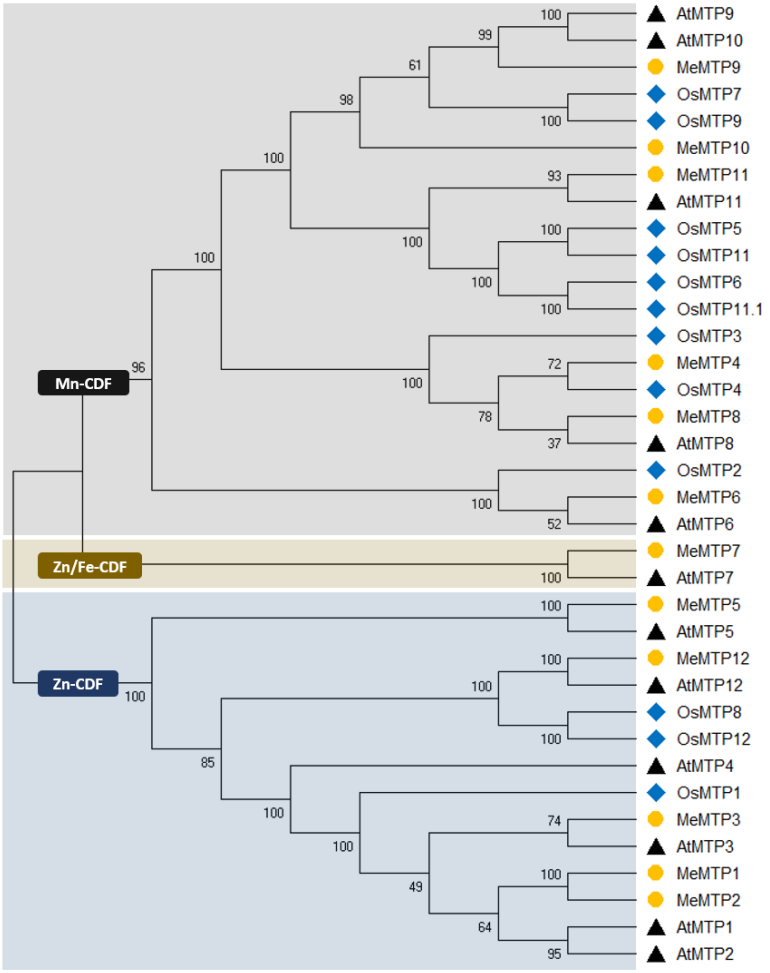

Figure 2. Phylogenetic analysis of MTP proteins in cassava, rice and *Arabidopsis*. The phylogenetic tree was constructed using neighbor-joining (NJ) method with 1000 bootstrap replicates.

In our case, the phylogenetic relationship also showed 3 main groups of MTPs similar to previous reports especially the *Arabidopsis* MTPs, except MeMTP4 that did not lie in the Zn-CDF groups (Figure 2) even the MeMTP4 was annotated as a zinc transporter (Table 3). MeMTP4 showed sequence similarity to rice MTP. In monocot such as rice and wheat, MTP4 (also called MTP8.1 in rice) has been characterized into Mn-CDFs (Chen et al., 2013; Vatansever et al., 2017).

However, a metal sensitivity assay in *Saccharomyces cerevisiae* has shown that a poplar MTP4 could not transport either zinc, iron or manganese (Gao et al., 2020). Therefore, roles of MTP4 might be different among plant species.

Morphological changes of KU50 cassava shoot under zinc deficiency

During the early growth stages, cassava plants were observed for 3 weeks for zinc deficiency symptoms. Initially, the plants exhibited interveinal chlorosis on young leaves then the leaves began developing small white, or light-yellow chlorotic spots between veins. The longer zinc deficient period, the smaller and more chlorotic appeared on the new emerging leaves. Moreover, foliar lobes became narrow, and curl upward compared to the control. These results provided evidence that zinc-deficient conditions were crucial for cassava development especially in the early growth stages (Figure 3). It has been reported that zinc deficiency is the most common symptom in cassava plants grown in high pH and calcareous soils due to the low availability of zinc to plant roots. Under severe conditions, plants may reduce growth and die at young age (Howeler, 1995; Watananonta et al., 2004).

Figure 3. Morphological changes of 3-week-old KU50 cassava shoot under zinc deficiency

Expression analysis of MeMTP genes in KU50 cassava leaf and root tissues under zinc deficiency

Gene identification using bioinformatic tools facilitates us to predict the existence of *MTP* gene family in cassava. However, verification of gene expression in vivo will provide further meaningful information to support the computational annotations. Here, we studied the expression of 6 candidate *MeMTP* genes including *MeMTP1-5* and *MeMTP12*, in cassava leaves and roots after transferring to zinc deficiency for 3 weeks (Figure 4).

RT-PCR data showed that the expression level of *MeMTPs* was expressed differently. Under zinc deficient conditions, the transcript level of *MeMTP1* was slightly increased in both leaves and roots, while *MeMTP5* was up-regulated in roots, suggesting that these genes play roles in cassava plants when zinc is scarce.

Previous studies have shown that overexpression of zinc transporters *AtZIP1* (*zinc-regulated transporter- like protein*) and *AtMTP1* in cassava plants triggers high zinc concentration in storage roots but reduces the tuber size. The overall yield also decreases due to zinc deficiency symptom in the aerial parts of the plants (Gaitán-Solís et al., 2015). These data imply that *MTP1* may correlate with zinc transportation in plants.

Recent study has shown that *Arabidopsis AtMTP2* transcript level in roots elevates under zinc deficiency condition. Analysis of *mtp2* mutant demonstrates the role of MTP2 in zinc translocation from roots to shoots when the zinc status in shoots is low (Scott A Sinclair et al., 2018). From our results, cassava *MeMTP2* expressed specifically in roots but did not response to zinc concentration. The MTP2 orthologs in different plants may have diverse function.

The expression levels of *MeMTP4* and *MeMTP12* were higher in leaves rather than roots under both +zinc and -zinc conditions. These results suggest that the expression of *MeMTP2*, *MeMTP4* and *MeMTP12* are tissue- specific. However, *MeMTP3* was rarely detectable in all conditions so that *MeMTP3* may not be a major gene for zinc transport compared to others in the MTP family.

The plant MTP protein family are well known for metal tolerance via sequestration of zinc into intracellular compartments. Some of MTP transcripts are up-regulated under zinc toxicity (Gupta et al., 2016). It is possible that the cassava MTP family may involve in both zinc deficiency and toxicity conditions, depending on the function of each individual protein. Study of *MeMTP* gene expression under zinc toxicity would provide further information of MTPs in cellular and molecular levels. Moreover, additional transcriptomic experiments in cassava under zinc deficiency would be developed for genome-wide identification of other zinc responsive genes.

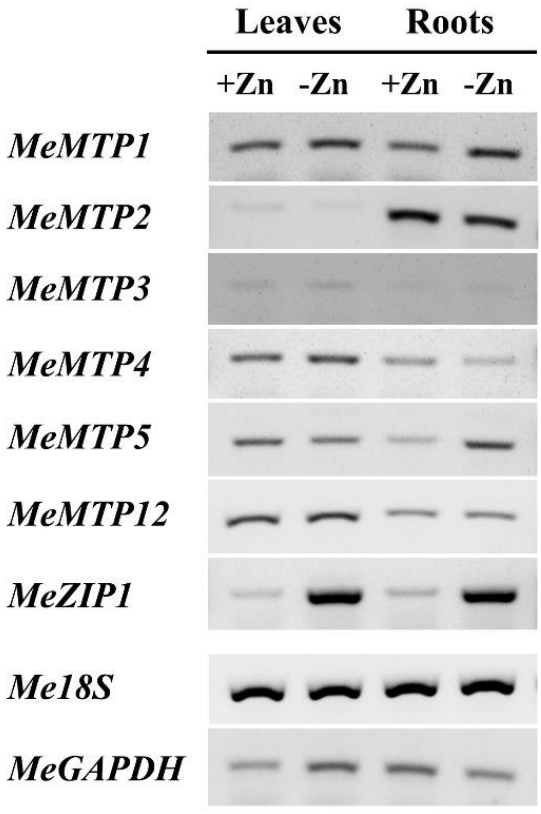

Figure 4. Expression of *MeMTP* genes in cassava leaves and roots under zinc deficiency

Conclusion

The MeMTP proteins have an influence on zinc transport in several plants. In this study, twelve *MeMTP* genes were discovered in cassava genome. *MeMTP1* was up-regulated in roots and leaves under zinc-deficient condition whereas the expression of *MeMTP2*, *MeMTP4* and *MeMTP12* were tissue-specific. These genes may be responsible for zinc balance in cassava plants.

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References

Alloway, B. (2009). Soil factors associated with zinc deficiency in crops and humans. *Environmental Geochemistry and Health, 31*: 537–548.

Arrivault, S., Senger, T., & Krämer, U. (2006). The *Arabidopsis* metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *The Plant Journal, 46*(5), 861-879.

Begum, M. C., Islam, M., Sarkar, M. R., Azad, M. A. S., Huda, A. N., & Kabir, A. H. (2016). Auxin signaling is closely associated with Zn-efficiency in rice (*Oryza sativa* L.). *Journal of Plant Interactions, 11*(1), 124-129.

Binder, B. M. (2020). Ethylene signaling in plants. *Journal of Biological Chemistry, 295*(22), 7710-7725.

Broadley, M., Brown, P., Cakmak, I., Rengel, Z., & Zhao, F. (2012). Function of nutrients: micronutrients. In *Marschner's mineral nutrition of higher plants* (pp. 191-248): Elsevier.

Chen, Z., Fujii, Y., Yamaji, N., Masuda, S., Takemoto, Y., Kamiya, T., Yusuyin, Y., Iwasaki, K., Kato, S., Maeshima, M., Ma, J. F., & Ueno, D. (2013). Mn tolerance in rice is mediated by MTP8.1, a member of the cation diffusion facilitator family. *Journal of Experimental Botany, 64*(14), 4375-4387.

Chou, K., & Shen, H. (2010). Plant-mPLoc: a top-down strategy to augment the power for predicting plant protein subcellular localization. *PLOS ONE*, 2010, 5: e11335.

Fujiwara, T., Kawachi, M., Sato, Y., Mori, H., Kutsuna, N., Hasezawa, S., & Maeshima, M. (2015). A high molecular mass zinc transporter MTP12 forms a functional heteromeric complex with MTP5 in the Golgi in *Arabidopsis thaliana*. *The FEBS Journal, 282*(10), 1965-1979.

Gaitán-Solís, E., Taylor, N. J., Siritunga, D., Stevens, W., & Schachtman, D. P. (2015). Overexpression of the transporters AtZIP1 and AtMTP1 in cassava changes zinc accumulation and partitioning. *Frontiers in Plant Science, 6*, 1-11.

Gao, Y., Yang, F., Liu, J., Xie, W., Zhang, L., Chen, Z., Peng, Z., Ou, Y., & Yao, Y. (2020). Genome-wide identification of metal tolerance protein genes in *Populus trichocarpa* and their roles in response to various heavy metal stresses. *International Journal of Molecular Sciences, 21*(5), 1-25.

Gupta, N., Ram, H., & Kumar, B. (2016). Mechanism of Zinc absorption in plants: uptake, transport, translocation and accumulation. *Reviews in Environmental Science and Bio/Technology, 15*(1), 89-109.

Gustin, J. L., Zanis, M. J., & Salt, D. E. (2011). Structure and evolution of the plant cation diffusion facilitator family of ion transporters. *BMC Evolutionary Biology, 11*(1), 1-13.

Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. *Circular. California agricultural experiment station, 347*(2nd edit).

Howeler, R. H. (1995). *Mineral nutrition of cassava.* Paper presented at the Mineral Nutrient Disorders of Root Crops in the Pacific. Proc. Workshop held in Nuku'alofa, Kingdom of Tonga.

Howeler, R. H. (2002). Cassava mineral nutrition and fertilization. *Cassava: biology, production and utilization*, 115-147.

Hu, B., Jin, J., Guo, A., Zhang, H., Luo, J & Gao, G. (2015). GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics, 31*(8):1296-1297.

Kawachi, M., Kobae, Y., Mimura, T., & Maeshima, M. (2008). Deletion of a histidine-rich loop of AtMTP1, a vacuolar Zn^{2+}/H^{+} antiporter of *Arabidopsis thaliana*, stimulates the transport activity. *Journal of Biological Chemistry, 283*(13), 8374-8383.

Keunen, E., Schellingen, K., Vangronsveld, J., & Cuypers, A. (2016). Ethylene and metal stress: small molecule, big impact. *Frontiers in Plant Science, 7*, 1-18.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35*:1547-1549.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., & Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic acids Research, 30*(1), 325–327.

Liu, J., Gao, Y., Tang, Y., Wang, D., Chen, X., Yao, Y., & Guo, Y. (2019). Genome-wide identification, comprehensive gene feature, evolution, and expression analysis of plant metal tolerance proteins in tobacco under heavy metal toxicity. *Frontiers in Genetics, 10*, 1-20.

Montanini, B., Blaudez, D., Jeandroz, S., Sanders, D., & Chalot, M. (2007). Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity. *BMC genomics, 8*(1), 1-16.

Noulas, C., Tziouvalekas, M., & Karyotis, T. (2018). Zinc in soils, water and food crops. *Journal of Trace Elements in Medicine and Biology, 49*, 252-260.

Palmgren, M. G., Clemens, S., Williams, L. E., Krämer, U., Borg, S., Schjørring, J. K., & Sanders, D. (2008). Zinc biofortification of cereals: problems and solutions. *Trends in Plant Science, 13*(9), 464-473.

Peabody, M. A., Laird, M. R., Vlasschaert, C., Lo, R. & Brinkman, F. S. (2016). PSORTdb: expanding the bacteria and archaea protein subcellular localization database to better reflect diversity in cell envelope structures. *Nucleic Acids Research, 44*(D1):D663-8.

Ricachenevsky, F. K., Menguer, P. K., Sperotto, R. A., & Fett, J. P. (2015). Got to hide your Zn away: molecular control of Zn accumulation and biotechnological applications. *Plant Science, 236*, 1-17.

Ricachenevsky, F. K., Menguer, P. K., Sperotto, R. A., Williams, L. E., & Fett, J. P. (2013). Roles of plant metal tolerance proteins (MTP) in metal storage and potential use in biofortification strategies. *Frontiers in Plant Science, 4*, 1-16.

Sinclair, S. A., & Krämer, U. (2012). The zinc homeostasis network of land plants. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1823*(9), 1553-1567.

Sinclair, S. A., Senger, T., Talke, I. N., Cobbett, C. S., Haydon, M. J., & Krämer, U. (2018). Systemic upregulation of MTP2-and HMA2-mediated Zn partitioning to the shoot supplements local Zn deficiency responses. *The Plant Cell, 30*(10), 2463-2479.

Takrattanasaran, N., Chanchareonsook, J., Johnson, P. G., Thongpae, S., & Sarobol, E. (2013). Amelioration of zinc deficiency of corn in calcareous soils of Thailand: zinc sources and application methods. *Journal of Plant Nutrition, 36*(8), 1275-1286.

Vatansever, R., Filiz, E., & Eroglu, S. (2017). Genome-wide exploration of metal tolerance protein (MTP) genes in common wheat (*Triticum aestivum*): insights into metal homeostasis and biofortification. *Biometals, 30*(2), 217-235.

Watananonta, W., Tungsakul, S., Phetprapi, P., & Howeler, R. H. (2004). *The response of micronutrients on root yield of 2 cassava varieties.* Paper presented at the Proceedings of the 42nd Kasetsart University Annual Conference, Kasetsart, Thailand, 3-6 February 2004.

Yu, C. S., Chen, Y. C., Lu, C. H., & Hwang, J. K. (2006). Prediction of protein subcellular localization. *Proteins: Structure, Function and Bioinformatics, 64*:643-651.