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Characterisation of Type 2 and 3 Metallothioneins from Hevea brasiliensis *Bark*

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A small collection of ESTs was generated from the bark of the rubber tree clone RRIM 2025. Low molecular weight metal-binding proteins, metallothioneins (MTs), were found to be very abundant in the bark gene expression profile. Multiple sequence alignment and phylogenetic analysis between three Hevea *MTs and other plant MTs identified them as type 2 and type 3 proteins. Semi-quantitative PCR analysis indicated that each MT was differentially expressed between bark, leaves and latex, with the lowest transcript abundance in latex. In latex from ethephon-treated trees, all MT isoforms were induced at four hours after treatment while only one of them showed decreased expression after 24 hours. Our findings indicate the existence of a multiple gene family for* Hevea *MTs and varying roles played by isoforms derived from different rubber tree tissues.*

Keywords: *Hevea brasiliensis*; metallothionein; bark; EST; DNA sequencing; isoform; gene expression

Metallothionein (MT) was first discovered as a cadmium-binding protein from horse renal cortex tissue[1] and has since then, been found across plant and fungi kingdoms. In plants, MT was first reported in germinated wheat embryo as a zinc-containing protein[2]. MTs are present in the cytosol and display metal-binding property which is attributed to their cysteine-rich domains[3]. Heavy-metal inducible MT transcription has also been reported in plants such as *Brassica rapa*[4], *Oryza sativa*[5] and cotton[6]. In addition, MTs have been shown to act as scavengers of reactive oxygen species (ROS) during various physiological events such as senescence[7,8], abiotic stress[5,9–11] and wounding[12,13]. In the rubber tree, the MT

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type 2 protein was previously shown to be an effective scavenger of ROS[14].

MTs can be categorised based on their cysteine residue arrangements[3,15]. Class I MTs have been reported in vertebrate animals, whereas Class II MTs are abundant among plants, fungi and non-vertebrate animals. While animal MTs contain 20 highly conserved cysteine residues, plant MTs display far more variation in their cysteine-rich motifs[3]. In plants, cysteine distribution and number including length of linker regions between cysteine domains result in the further sub-division of plant MTs into types 1, 2, 3 and 4[3,16,17].

Expressed sequence tags (ESTs) are single-pass DNA sequences generated from randomly selected clones of a cDNA library. Information gathered from EST analysis can help in assessing the types and expression patterns of genes in a particular tissue. Since latex is the primary commercial product from the *Hevea brasiliensis* tree, the first rubber ESTs were generated from the laticifers[18–20]. Subsequently, RNA-Seq transcriptomes were generated from latex, leaf and bark tissues[21–23]. In this work, we have isolated and characterised three members of a gene family of MTs from an EST collection generated from *Hevea* bark tissue (clone RRIM 2025). We observed that MT transcripts were among the most highly expressed bark ESTs and show results of their expression patterns in different tissues and in latex following ethephon treatment.

MATERIALS AND METHODS

Plant Material

Bark shavings (approximately 1 cm from the trunk surface) from the tapping cut and young leaves were collected from three mature *Hevea* trees (clone RRIM 2025) grown in the Rubber Research Institute of Malaysia Research Station, Sungai Buloh, Selangor, Malaysia. Bark and leaf samples were transported in an ice box to the laboratory before storing at –80ºC. Latex (clone RRIM 2025) from three trees was collected directly into RNA extraction buffer[24] and flash frozen in liquid nitrogen before storing at –80ºC. To observe the effect of ethephon on MT gene expression, 2.5% (v/v) ethephon was applied along the tapping cuts of three RRIM 2025 trees. Latex was collected from ethephon-treated and control trees (in the same plot) at 2, 4 and 24 h after ethephon application.

RNA Isolation

Latex total RNA was isolated according to the method of Kush *et al*. (1990)[24]. Bark and leaf total RNA samples were isolated using RNeasy Plant Mini Kit (Qiagen). RNA concentration and quality were assessed by agarose gel electrophoresis and by using the NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies). An $OD_{260/280}$ ratio of 1.8-2.0 was accepted as indication of good quality RNA.

cDNA Library Construction and EST Sequencing

A bark cDNA library was constructed using the SMART cDNA Library Construction Kit (Clontech Laboratories). After conversion of recombinant λTriplEx2 clones to pTriplEx2 plasmids, individual plasmids were sequenced from the 5' end of the cDNA insert using the λTriplEx 5' sequencing primer (nucleotide 490-507). All sequencing reactions were set up with the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing products resolved with a 3730 DNA Analyser (Applied Biosystems).

EST Assembly and Annotation

Raw reads were processed and assembled using StackPack (SANBI, http://www.sanbi.ac.za/Dbases.html) according to pipeline and parameters reported previously[20,25]. Sequence annotation of unique transcripts (UTs) was performed using blastx[26]. Raw ESTs and UTs are available at www.genomemalaysia.gov.my/nrestdb/barkests/.

Metallothionein Sequence Analysis

Three metallothionein transcripts identified in this study were assembled from bark ESTs. MT DNA and protein sequences from plant species were obtained from GenBank (www.ncbi.nlm.nih.gov). Sequence alignments were done using ClustalW (www.ebi.ac.uk/ClustalW). Phylogenetic tree construction was done using MEGA5[27]. Initially, the protein sequences were aligned by MUSCLE with default gap penalties and then, a neighbour-joining phylogenetic tree was built with 1000 replicates of bootstrap analysis.

Primer Design

Primers for three MT transcripts (MTAfw 5'-GCC AGT GCG TGA AGA AGG-3'; MTArv 5'-CCA TAC CCA CAA GTA TTA CAA CAG-3'; MTBfw 5'-GCC ATT CGC ACT ATA ACC TTC-3'; MTBrv 5'-TCA ACA GCA AAC ACA TTA TTT AGC-3'; MTCfw 5'-CCT GAC ATC GTA GAG AAC ACC-3'; MTCrv 5'-ATG AAA CTG CTG GAA GGA AATC-3') and the 18S ribosomal RNA (18S rRNA; Accession No. AY496880) (18Sfw 5'-AAA GAC GAA CAA CTG CGA AAG-3'; 18Srv 5'-GCT CCA CCA ACT AAG AAC GG-3') were designed using the Beacon Designer version 4.0 (Premier Biosoft International) software. Primers were designed according to the following requirements: primer length between 18 – 21 mers, T_m range of 54 – 56ºC and amplicon length between 250 – 400 bp.

Semi-quantitative PCR

Reverse transcription of total RNA was carried out using the ImProm-II Reverse Transcription System (Promega). First-strand cDNA was synthesised from 1 µg total RNA (at 6mM $MgCl_2$ final concentration), primed by oligo $(dT)_{15}$ primer for MT transcripts and by random hexamers for 18S rRNA transcript.

PCR amplification was performed in a final volume of 50 µl, whereby each reaction contained 1X Green GoTaq® Flexi buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 1 µM each forward and reverse primers and 1.25 units GoTaq® Flexi DNA polymerase (reagents from Promega). PCR was performed in the PTC-200 DNA Engine (MJ Research) according to the following profile: initial denaturation step of 2 min at 94ºC followed by 35 times amplification cycles of 1 min at 94ºC (denaturation), 1 min at 56ºC (annealing), 2 min at 72ºC (elongation) and a final elongation of 2 min at 72ºC. Aliquots of PCR products were taken at cycles 15, 20, 25, 30 and 35, and were separated in 0.8% agarose gel. Ethidium bromide-stained agarose gels were viewed using AlphaImager®-HP Imaging System (Cell Biosciences).

RESULTS

EST Sequence Generation and Annotation

StackPack analysis of 490 cleaned bark ESTs showed that they consisted of 129 unique transcripts (UTs) (*Table 1*) where EST frequencies ranged from 1 – 240 (*Figure 1*). *Table 2* shows the identities of the top 20 bark UTs (representing 359 ESTs or 73.3% of 490 bark ESTs analysed) obtained by

TABLE 1. STATISTICS OF BARK EST ASSEMBLY USING STACKPACK. CONSENSUSES WERE GENERATED AFTER CLEANED EST SEQUENCES WERE ASSEMBLED INTO CLUSTERS, FOLLOWED BY CONTIGS. SINGLETONS ARE INDIVIDUAL ESTs WITH INSUFFICIENT DNA IDENTITY TO BE ASSEMBLED. UNIQUE TRANSCRIPTS (UTs) CONSIST OF CONSENSUSES AND SINGLETONS.

StackPack Analysis	Bark ESTs
Cleaned sequences entered into analysis pipeline	490
Consensuses	45
Singletons	84
Unique transcripts (UTs)	129

TABLE 2. BLASTX ANNOTATION OF BARK UTs WITH EST FREQUENCIES 3 – 240. UTs WITH KNOWN IDENTITIES ARE HIGHLIGHTED IN BOLD. METALLOTHIONEIN UTs ARE REFERENCED TO THEIR SEQUENCE CODES.

EST Frequency	Functional description based on blastx	E-value
240	Hypothetical protein NitaMp027 (*Nicotiana tabacum*), YP_173374.1	8e-23
18	Hypothetical protein (*Vitis vinifera*), CAN65763.1	4e-29
16	Hypothetical protein (*Vitis vinifera*), CAN65763.1	4e-29
11	**Metallothionein 3a (*Populus trichocarpa x Populus deltoides*), AAT02526.1[1]**	6e-20
8	No significant similarity found	-
7	Hypothetical protein (*Vitis vinifera*), CAN78405.1	5e-13
7	Hypothetical protein (*Vitis vinifera*), CAN65763.1	0.058
7	**ABRH15 (*Marsilea quadrifolia*), AAQ14305.1**	**0.004**
6	**Metallothionein 3a (*Populus trichocarpa x Populus deltoides*), AAT02526.1[2]**	**3e-17**
5	Hypothetical protein NitaMp027 (*Nicotiana tabacum*), YP_173374.1	3e-40
5	Unknown (*Picea sitchensis*), ABK21263.1	2.5
4	Hypothetical protein (*Gluconacetobacter diazotrophicus PA1 5*), ZP_02984755.1	8e-04
4	No significant similarity found	-
3	Hypothetical protein (*Vitis vinifera*), CAN62391.1, 1.1	1.1
3	**40S ribosomal protein S4, 081363**	**7e-27**
3	Predicted protein (*Nematostella vectensis*), XP_001624571.1	8e-16
3	Hypothetical protein (*Photorhabdus luminescens subsp. laumondii* TTO1), NP_928183.1	4.2
3	**Pectin methylesterase (*Citrus bergamia*), ABE67980.1**	**1e-81**
3	**Metallothionein 1a (*Populus trichocarpa x Populus deltoides*), AAT02522.1[3]**	**3e-07**
3	**Ribosomal protein S2 (*Lotus japonicas*), NP_084799.1**	**4e-35**

[1]MTB, [2]MTA, [3]MTC (UTs are cn13, cn12 and cn15 respectively)

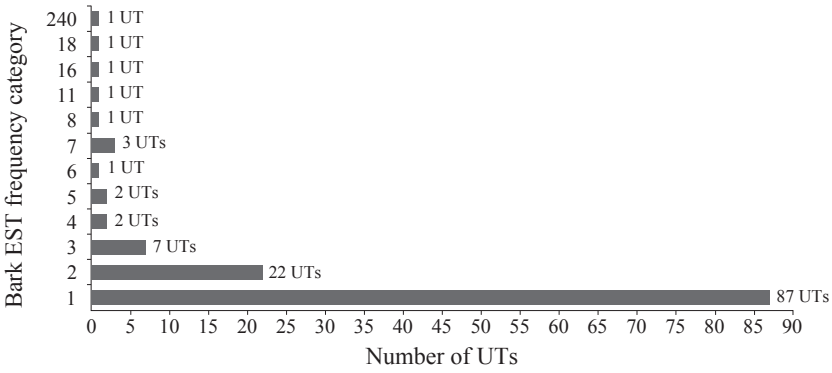


Figure 1. Distribution of unique transcripts (UTs) in relation to EST frequency categories. The number of UTs for each EST frequency category is indicated.

blastx comparison with sequences in the non-redundant GenBank databases. The top three UTs did not match with any sequence of well-characterised function in the public databases and only seven UTs had assigned known functions (*Table 2*).

MT transcripts were found to dominate UTs with known functions: two UTs (frequencies 11 and 6) and one UT (frequency 3) (*Table 2*). This is in contrast to latex EST profiling where the rubber particle proteins, the rubber elongation factor (REF) and the small rubber particle protein (SRPP), constituted 12% of latex ESTs analysed[20]. Among the 490 bark ESTs, only two singletons analysed were found to encode REF and SRPP respectively (data not shown). Therefore, the major difference observed between bark and latex EST profiles was REF and SRPP dominated latex ESTs with known gene function while bark ESTs were dominated by MT. Because of this distinctness, we decided to characterise MT isoforms in bark and their expression.

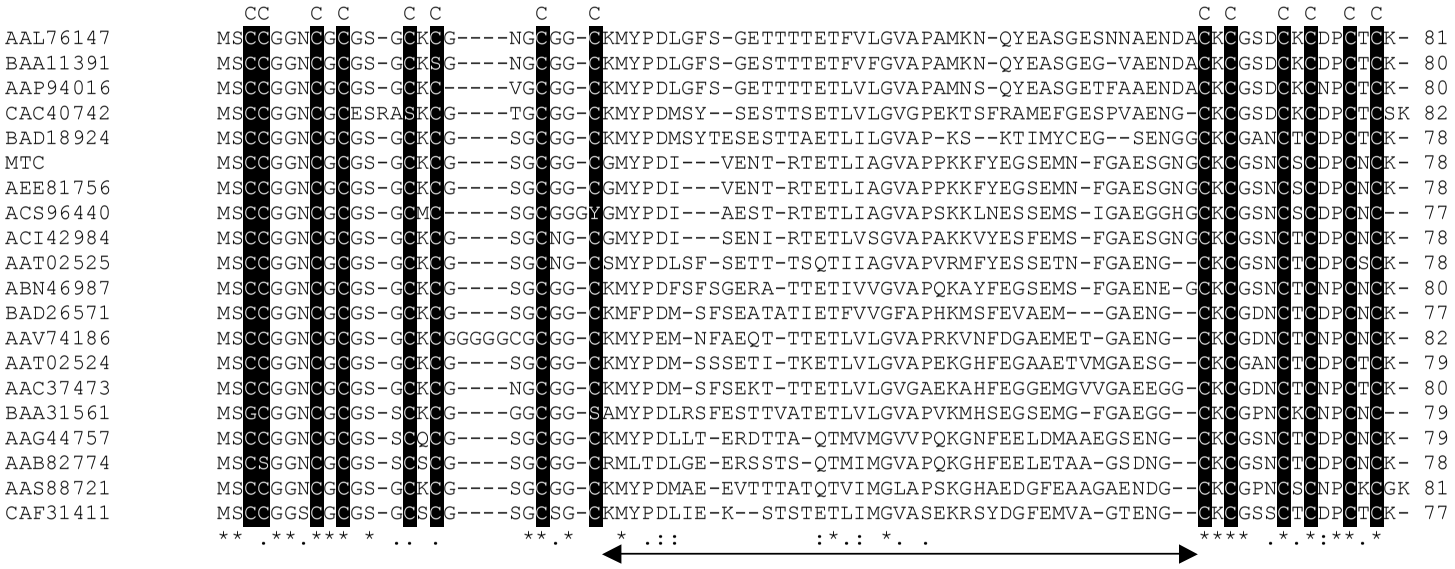
Characterisation of MT Sequences

As shown in *Table 2*, three consensuses from the *Hevea* bark EST assembly were found to encode MT (MTA, 535 bp; MTB, 482 bp and MTC, 563 bp). Only MTA and MTB contained poly (A) tails (data not shown). Sequence analysis showed that MTA, MTB and MTC contained complete open reading frames that translated into 66, 67 and 78 amino acids respectively. Alignments of MTA, MTB and MTC proteins with those from other plants are shown in *Figure 2A*. Based on the conservation and arrangement of cysteine rich domains, two type 3 (MTA and MTB) and one type 2 (MTC) MT were confirmed (*Figure 2A*). Subsequently, phylogenetic analysis of MTA, MTB and MTC further supported the classification of MTA and MTB as MT type 3 proteins and MTC as an MT type 2 protein (*Figure 3*).

With reference to *Figure 2B*, the three *Hevea* MT proteins used corresponded to GenBank nucleotide sequences, FJ229481, HM178927 and HQ687666. DNA alignment of these sequences with MTA, MTB and MTC revealed that only five were unique since MTA was identical to the HM178927 sequence with the exception of the former having longer 5' and 3' untranslated regions (data not shown). At the protein level, MTA and MTC were found to be the same as ADR30789 and AEE81756 proteins respectively (*Figure 2B*). Therefore,

A

(i)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BAA11391 MSCCGGNCGGGS-GCKSG----NGCGG-CKMYPDLGFS-GESTTTETFVFGVAPAMKN-QYEASGEG-VAENDACKCGSDCKCDPCTCK- 80
AAP94016 MSCCGGNCGGGS-GCKC-----VGCGG-CKMYPDLGFS-GETTTTETLVLGVAPAMNS-QYEASGETFAAENDACKCGSDCKCNPCTCK- 80
CAC40742 MSCCGGNCGCESRASKCG----TGCGG-CKMYPDMSY--SESTTSETLVLGVGPEKTSFRAMEFGESPVAENG-CKCGSDCKCDPCTCSK 82
BAD18924 MSCCGGNCGGGS-GCKCG----SGCGG-CKMYPDMSYTESESTTAETLILGVAP-KS--KTIMYCEG--SENGGCKCGANCTCDPCTCK- 78
MTC MSCCGGNCGGGS-GCKCG----SGCGG-CGMYPDI---VENT-RTETLIAGVAPPKKFYEGSEMN-FGAESGNGCKCGSNCSCDPCNCK- 78
AEE81756 MSCCGGNCGGGS-GCKCG----SGCGG-CGMYPDI---VENT-RTETLIAGVAPPKKFYEGSEMN-FGAESGNGCKCGSNCSCDPCNCK- 78
ACS96440 MSCCGGNCGGGS-GCMC-----SGCGGGYGMYPDI---AEST-RTETLIAGVAPSKKLNESSEMS-IGAEGGHGCKCGSNCSCDPCNC-- 77
ACI42984 MSCCGGNCGGGS-GCKCG----SGCNG-CGMYPDI---SENI-RTETLVSGVAPAKKVYESFEMS-FGAESGNGCKCGSNCTCDPCNCK- 78
AAT02525 MSCCGGNCGGGS-GCKCG----SGCNG-CSMYPDLSF-SETT-TSQTIIAGVAPVRMFYESSETN-FGAENG--CKCGSNCTCDPCSCK- 78
ABN46987 MSCCGGNCGGGS-GCKCG----SGCGG-CKMYPDFSFSGERA-TTETIVVGVAPQKAYFEGSEMS-FGAENE-GCKCGSNCTCNPCNCK- 80
BAD26571 MSCCGGNCGGGS-GCKCG----SGCGG-CKMFPDM-SFSEATATIETFVVGFAPHKMSFEVAEM---GAENG--CKCGDNCTCDPCNCK- 77
AAV74186 MSCCGGNCGGGS-GCKCGGGGGCGCGG-CKMYPEM-NFAEQT-TTETLVLGVAPRKVNFDGAEMET-GAENG--CKCGDNCTCNPCNCK- 82
AAT02524 MSCCGGNCGGGS-GCKCG----SGCGG-CKMYPDM-SSSETI-TKETLVLGVAPEKGHFEGAAETVMGAESG--CKCGANCTCDPCTCK- 79
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AAG44757 MSCCGGNCGGGS-SCQCG----SGCGG-CKMYPDLLT-ERDTTA-QTMVMGVVPQKGNFEELDMAAEGSENG--CKCGSNCTCDPCNCK- 79
AAB82774 MSCSGGNCGGGS-SCSCG----SGCGG-CRMLTDLGE-ERSSTS-QTMIMGVAPQKGHFEELETAA-GSDNG--CKCGSNCTCDPCNCK- 78
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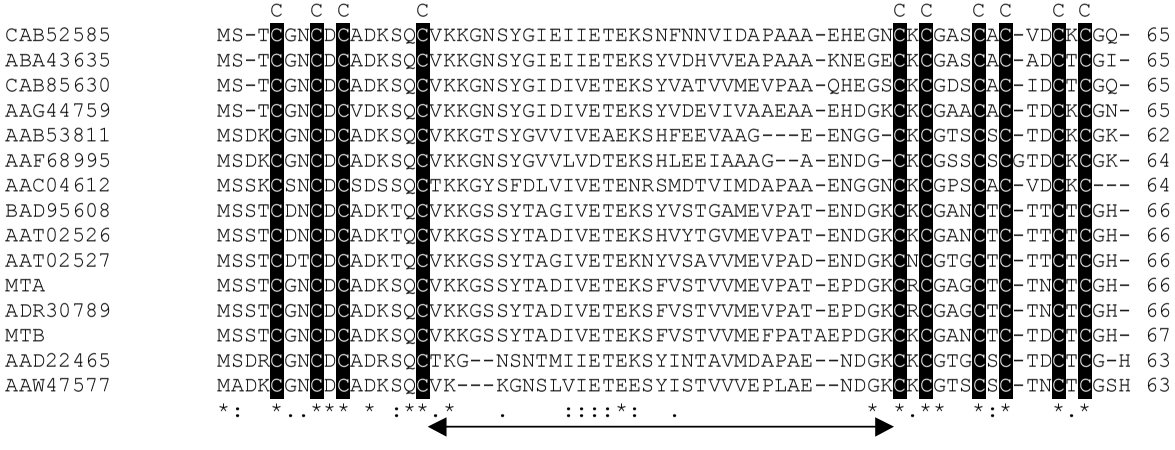
CAF31411 MSCCGGGSCGGGS-GCSCG----SGCSG-CKMYPDLIE-K--STSTETLIMGVASEKRSYDGFEMVA-GTENG--CKCGSSCTCDPCTCK- 77

(ii)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AAT02527 MSSTCDTCDCADKTQCVKKGSSYTAGIVETEKNYVSAVVMEVPAD-ENDGKCNCGTGCTC-TTCTCGH- 66
MTA MSSTCGNCDCADKSQCVKKGSSYTADIVETEKSFVSTVVMEVPAT-EPDGKCRCGAGCTC-TNCTCGH- 66
ADR30789 MSSTCGNCDCADKSQCVKKGSSYTADIVETEKSFVSTVVMEVPAT-EPDGKCRCGAGCTC-TNCTCGH- 66
MTB MSSTCGNCDCADKSQCVKKGSSYTADIVETEKSFVSTVVMEFPATAEPDGKCKCGANCTC-TDCTCGH- 67
AAD22465 MSDRCGNCDCADRSQCTKG--NSNTMIIETEKSYINTAVMDAPAE--NDGKCKCGTGCSC-TDCTCG-H 63
AAW47577 MADKCGNCDCADKSQCVK---KGNSLVIETEESYISTVVVEPLAE--NDGKCKCGTSCSC-TNCTCGSH 63

B

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MTA             MSSTCGNCDCADKSQCVK--KGSSYTADIVETEK--SFVSTVV--------MEVPAT-EPDGKCRCGAGCTCTNCTCGH 66        Type 3
ADR30789        MSSTCGNCDCADKSQCVK--KGSSYTADIVETEK--SFVSTVV--------MEVPAT-EPDGKCRCGAGCTCTNCTCGH 66        Type 3
MTB             MSSTCGNCDCADKSQCVK--KGSSYTADIVETEK--SFVSTVV--------MEFPATAEPDGKCKCGANCTCTDCTCGH 67        Type 3
MTC             MSCCGGNCGCGSGCKCGSGCGGCGMYPDIVENTRTETLIAGVAPPKKFYEGSEMNFGAESGNGCKCGSNCSCDPCNCK- 78        Type 2
AEE81756        MSCCGGNCGCGSGCKCGSGCGGCGMYPDIVENTRTETLIAGVAPPKKFYEGSEMNFGAESGNGCKCGSNCSCDPCNCK- 78        Type 2
ACI42984        MSCCGGNCGCGSGCKCGSGCNGCGMYPDISENIRTETLVSGVAPAKKVYESFEMSFGAESGNGCKCGSNCTCDPCNCK- 78        Type 2
                **.  ***.*.. .:* .   *..  .** *. :  :::: *.         *.    *... *:**:.*:*  *.*
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Note:
GenBank accession nos. represent MT proteins from *Arabidopsis thaliana* (AAL76147), *Atropa belladonna* (CAC40742), *Brassica rapa* (BAA11391), *Citrullus lanatus* (BAD26571), *Citrus unshiu* (BAA31561), *Codonopsis lanceolata* (BAD18924), *Cynodon dactylon* (AAS88721), *Elaeis guineensis* (CAB52585), *Gossypium hirsutum* (AAV74186, AAD22465 and AAW47577), *Hevea brasiliensis* (ACI42984, ADR30789 and AEE81756), *Jatropha curcas* (ACS96440), *Metroxylon sagu* (ABA43635), *Musa acuminata* (AAG44757, AAB82774 and AAG44759), *Nelumbo nucifera* (ABN46987), *Oryza sativa* (AAB53811), *Populus alba* x *Populus tremula* (BAD95608), *Populus trichocarpa* X *Populus deltoides* (AAT02524-7), *Porteresia coarctata* (AAF68995), *Posidonia oceanica* (CAF31411), *Pringlea antiscorbutica* (AAP94016), *Prunus avium* (AAC04612), *Ricinus communis* (AAC37473) and *Vitis vinifera* (CAB85630).

Figure 2. Alignment of MT protein sequences showing homology between (A, i) MTC and other plant MT type 2 proteins (A, ii) MTA, MTB and other plant MT type 3 proteins (B) MTA, MTB and MTC and three Hevea MT proteins in the public domain. Conserved cysteine residues are shaded. Double-headed arrows indicate interspace region that separate cysteine-rich domains in MT proteins.

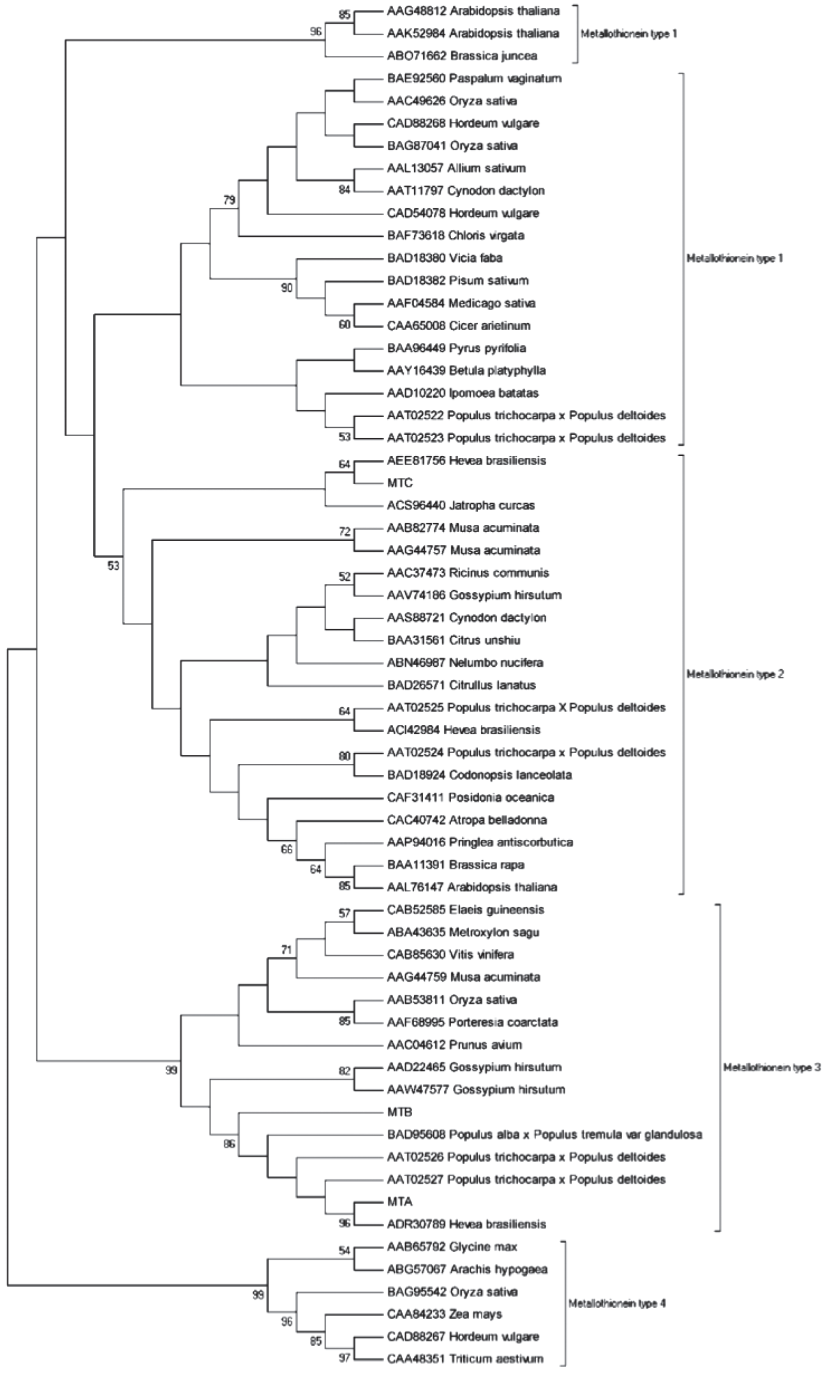

Figure 3. Phylogenetic tree of MT protein sequences from Hevea *and other plants. Two clades are shown for MT type 1 because members of the Brassicaceae family in one of the clades have a shorter sequence interspace between the N- and C-terminal cysteine domains.*

taken together, all six MT sequences from *Hevea* known to date represent five unique nucleotide sequences but only four unique proteins.

MT Gene Expression in Different Tissues

Semi quantitative RT-PCR was used to investigate the expression of *Hevea* MT type 3 (MTA and MTB) and type 2 (MTC) isoforms in latex, leaf and bark, with the 18S rRNA transcript as the housekeeping control gene. It was noted that the order of expression level of three MT isoforms in bark tissue (MTB>MTA>MTC; *Figure 4A*) was consistent with the order of their corresponding EST frequencies (*Table 2*). This lent support to the validity of the relative gene transcript frequencies based on 490 bark ESTs. MTA, MTB and MTC were expressed in all three tissues but each isoform displayed lowest expression in latex (*Figure 4A*). MTA showed very similar expression levels in leaf and bark. MTB was more abundant in bark compared to leaf while MTC was expressed relatively higher in leaf than in bark. In each tissue, MTC expression was lowest compared to MTA and MTB. By contrast, MTB showed strongest expression in each tissue except in leaf.

Effect of Ethephon on MT Gene Expression

Use of latex yield stimulants such as the ethylene releaser, ethephon (2-chloroethylphosphonic acid), increases latex production through prolonged latex flow[28]. To investigate the effect of ethephon application on MT transcription, MTA, MTB and MTC expression patterns were examined in latex from ethephon-treated trees at 2, 4 and 24 h after application. As shown by expression analysis in different tissues (*Figure 4A*), MTs are already lowly expressed in latex. Therefore, changes in latex MT transcript level would be more difficult to perceive by semi-quantitative RT-PCR. Nonetheless, we observed that compared to control untreated trees, all three MT isoforms were repressed at the 2 h time point and up-regulated 4 h after stimulation (*Figure 4B*). However, 24 h after ethephon treatment, MTC decreased in expression while MTA and MTB transcript levels showed little change.

DISCUSSION

Rubberwood is an important commercial product of the *Hevea* tree. Unlike latex, very few studies have been done on *Hevea* bark genes[29,30] and a second generation sequencing bark transcriptome was only recently reported for the development of EST-SSR markers[22]. Although we have generated far less transcripts in the form of Sanger ESTs, the salient observation from this collection was the abundance of MT in *Hevea* bark. Interestingly, the dominance of MT abundance in ESTs has also been reported in fruit tissues: pineapple[31] and sweet orange[32]. Metallothionein expression in fruit tissues may be related to plant developmental stages as reported in examples such as embryo development in oil palm[33], root formation in hybrid poplar[34] and seed maturation in sesame[35]. Past work on latex EST profiling revealed that latex gene transcripts were dominated by rubber particle membrane proteins which consist of numerous isoforms of REF and SRPP[18–20]. In contrast, REF and SRPP were distinctly low in expression among *Hevea* bark ESTs. It is possible that the low abundance of REF and SRPP bark may be related to the distinctly different physiology of bark which is not a primary latex-bearing tissue. Therefore, this formed the basis for characterising sequence and gene expression patterns of the three *Hevea* MT sequences generated from bark EST analysis, MTA, MTB and MTC.

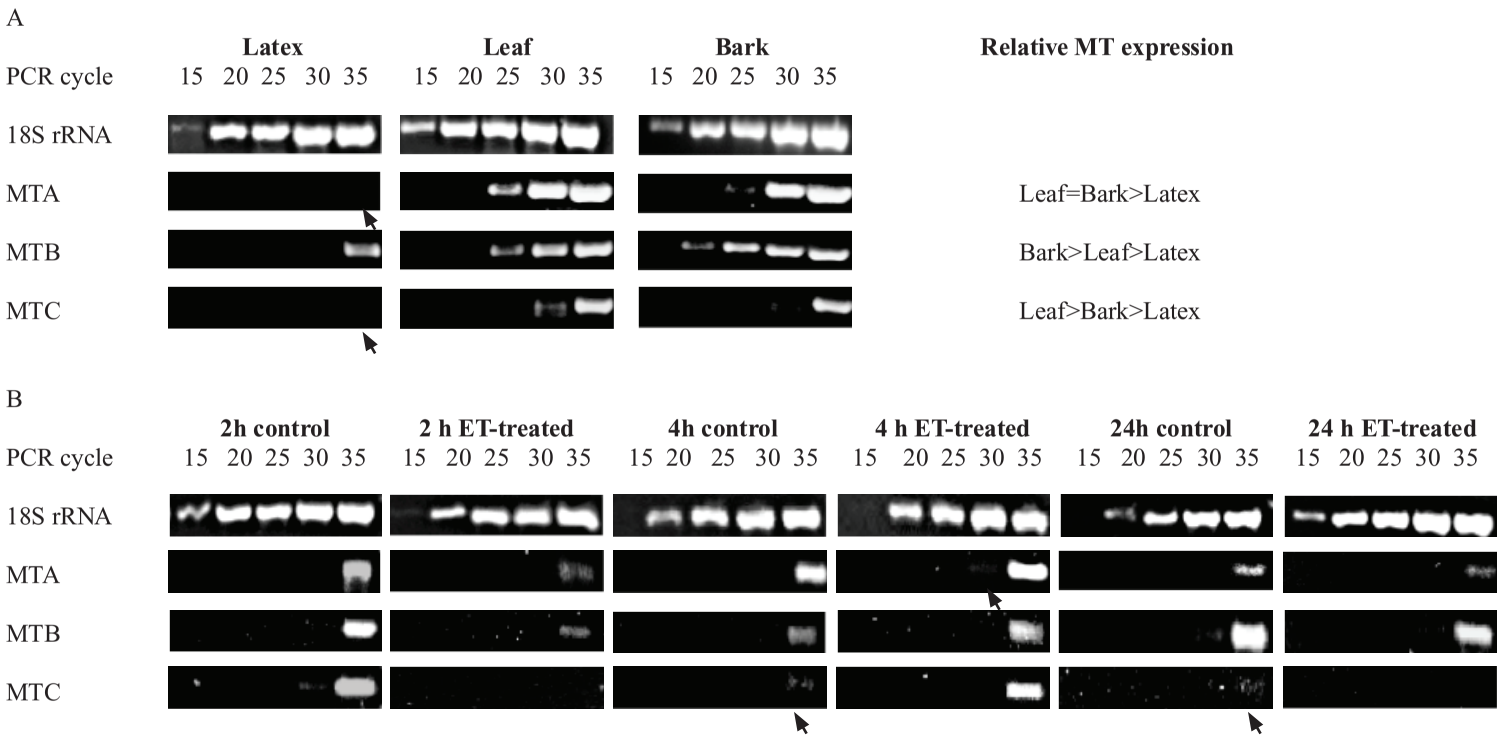

Figure 4. Analysis of MT gene expression by semi quantitative PCR in (A) latex, leaf and bark (B) latex from ethylene-treated and control untreated trees at 2, 4 and 24 h after ethephon (ET) treatment. Cycle numbers where PCR reaction aliquots were taken are indicated. Lanes with PCR products that were faintly stained by ethidium bromide are marked by arrowheads. Overall, the level of 18S rRNA transcripts was similar across different tissues and treatments (apparent variation in band intensities due to conditions of image capture).

Three *Hevea* MT sequences have been deposited in the GenBank database (accession nos. FJ229481, HM178927 and HQ687666). After considering sequence redundancy of these together with MTA, MTB and MTC, five DNA sequences representing four proteins were determined. All of these MTs were derived from different rubber tree clones (RRIM 600, RRIM 2025 and CATAS 7-33-97) and tissues (latex, leaf and bark), and consist of types 2 and 3 only. Collectively, they indicate the existence of a multiple gene family in *Hevea*. This is consistent with MT gene families found in other plant species, where there are eleven MT genes in rice[36], ten in barley[37], seven in *Arabidopsis*[7,38] and six in hybrid poplar[34]. We found only type 2 and type 3 MTs among bark ESTs and so far, no type 1 or 4 MT has been identified for *Hevea*. This is probably because MT type 1 is predominantly expressed in subterranean tissues[7] and MT type 4 in seeds[35,39], neither of which tissues were used for isolation of all *Hevea* MTs known to date.

Numerous studies have shown MT isoforms to be differentially expressed across many tissue types. In *Arabidopsis*, MT type 1 was highly expressed in roots, MT type 2 in leaves, MT type 3 in ripening fruits and MT type 4 in seeds[7,38,40]. However, abundance of an MT type in a particular tissue may differ between plant species. For example, unlike in *Arabidopsis*, rice MT type 1 was abundantly expressed in leaves, MT type 2 in roots and stems, and MT type 3 and type 4 in roots and seeds[36,39.] The same was also observed in *Hevea* MT gene expression whereby HbMT2 (accession no. FJ229481) transcription in RRIM 600 latex by Zhu *et al*.[14] was higher in contrast to the low expression of MTC (also a type 2 isoform) in RRIM 2025 latex in this study. Possibly, the differences in the type 2 MT expression may be attributed to isoforms that were derived from different tissues and rubber tree clones that were used in both studies.

Yield stimulants such as the ethylene releaser, ethephon (2-chloroethylphosphonic acid) increases latex production through prolonged latex flow but over-stimulation may result in oxidative stress in the laticifers[28,30]. MTs are known to play a role in scavenging reactive oxygen species[3] (ROS). The antioxidant ability of recombinant *Hevea* MT type 2 has been demonstrated and the same workers also reported that the transcript can be induced by ethephon within 1 h after treatment[14]. Additionally, we showed that MT type 3 isoforms can also be induced by ethephon, although in our study, both MT type 2 and 3 isoforms were up-regulated only 4 h after ethephon application. But since all expression experiments to date were based on latex from normal field-grown trees, further experiments on the expression MT in excessively stimulated trees would be necessary to deduce the role of MT under such a condition.

In conclusion, analysis of bark ESTs generated in this study has revealed previously unknown *Hevea* MT transcript sequences. Although PCR expression analysis were not fully quantitative, the results revealed preliminary observations of relative transcription patterns of type 2 and 3 MT isoforms from tree clone RRIM 2025. All four MT types are seldom found simultaneously in one study and therefore, future work could investigate whether type 1 and 4 MTs are really present in *Hevea*. Variation in transcript levels of MTA-C within bark, latex or leaf suggested differential roles played by these isoforms in various tissues. In bark, highest abundance of the MTB type 3 isoform has been shown from both EST and semi-quantitative PCR analyses). Since MTs are involved in diverse plant processes, further analysis of the MTB type 3 isoform in future would be useful to deduce its specific role in *Hevea* bark.

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