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Isolation and expression analysis of transcripts encoding metallothioneins in oil palm

A.B. AL-SHANFARI[1]* and S.N.A. ABDULLAH[1,2]

Department of Agriculture Technology, Faculty of Agriculture[1], and Laboratory of Plantation Crops, Institute of Tropical Agriculture[2], University of Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Abstract

Two of the abundant transcripts encoding type 2 metallothionein (MT) proteins designated as *MET2a* and *MET2b* were selected in our previous study due to their high abundance (16.05 %) in the suppression subtractive hybridization library and their involvement in fruit development and maturation. The present study involves the isolation of the full-length cDNA encoding *MET2a* and *MET2b* from the ripening oil palm fruit mesocarp, examining their expression pattern compared to the other two previously reported type-3 MT members (MT3-A and MT3-B) in various oil palm organs including different vegetative and reproductive tissues. The full-length cDNA sequences of *MET2a* and *MET2b* were 571 and 553 bp and they were designated as *EgMT2a* and *EgMT2b*, respectively. The sequences of the *EgMT2a* and *EgMT2b* were then compared for sequence similarities in the database using both *BLASTN* and *BLASTX* programs. Their sequences were homologous (67 - 77 %) with several type-2 MTs in plants. All four MT encoding genes were differentially expressed in the ripening oil palm mesocarp tissues, but undetectable in the vegetative tissues examined. All *MT* genes examined were significantly up-regulated in the mature developmental stages of oil palm fruit mesocarp, except for *EgMT2b* which was expressed only at 17 weeks after anthesis. The type 2 MT proteins are related to a greater degree to the late fruit-ripening stage than the type 3 MT proteins consistent with their reported functions in homeostasis or detoxification. The findings in the present study contribute to better understanding the molecular mechanisms involved in fruit ripening in oil palm.

Additional key words: cysteine, *Elaeis guineensis*, fruit ripening, gene expression, mesocarp, phylogenetic tree, RACE.

Introduction

Metallothioneins (MTs), which are widely distributed in eukaryotic and prokaryotic organisms, are a family of cysteine-rich (20 - 30 %) heavy metal-binding proteins of low molecular mass (2 - 8 kDa; Jia *et al*. 2012). The first plant MT was discovered in the roots of soybean (Casterline and Barnett 1982). Different MT types have been reported in plants such as rice, hybrid poplar, oil palm, and lichens (Abdullah *et al*. 2002, Kohler *et al*. 2004, Backor and Loppi 2009, Usha *et al*. 2011). Plant MTs are divided into four types based on the arrangement of Cys residues. Generally, type 1 MTs are predominantly expressed in roots, type 2 in leaves, type 3 in ripening fruits, and type 4 in seeds (Cobbett and Goldsbrough 2002). However, the expression characteristics of each type of plant *MT* gene differ from species to species. The MT function differs among organisms and isoforms. They play a role in metal homeostasis and detoxification of heavy metals due to their metal-binding activity and inducibility by heavy-metal ions (Jia *et al*. 2012).

Fruit ripening in different fruit types is a complex process that involves drastic changes in various physiological and biochemical events, including fruit softening (Kojima *et al*. 1994), senescence (Qian *et al*. 2012), increased conversion of starch to sugars (Hill and Ap Rees 1995), and increased accumulation of storage oil (Shaarani *et al*. 2010). Several ripening-related genes,

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Abbreviations: BLAST - basic local alignment search tool; cDNA - complementary DNA; CDS - coding regions; EgMT2 - *Elaeis guineensis* type two metallothionein; NCBI - National Center for Biotechnology Information; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription-polymerase chain reaction; UTR - untranslated regions.

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* Corresponding author: fax: (+603) 8946 4146, e-mail: ahmed.b.alshanfari@gmail.com

including those encoding MT proteins, have been identified previously. The *MT* genes have been shown to express temporally and differentially during fruit development (Liu *et al*. 2002) and ripening (Moyle *et al*. 2005).

In oil palm, MT cDNAs have been isolated previously (Abdullah *et al*. 2002, Ho *et al*. 2007, Low *et al*. 2008, Nurniwalis *et al*. 2008, Rival *et al*. 2008, Alizadeh *et al*. 2011), but the role of these MTs and their regulation during fruit development and ripening is still not clearly understood. Gene expression studies are the major source of data aiming at elucidating the function of plant MTs (Freisinger 2008, Xue *et al*. 2009, Huang and Wang 2010). Also, several studies have characterized *in vivo* and/or *in vitro* metal binding of MTs (Abdullah *et al*. 2002, Mir *et al*. 2004, Bilecen *et al*. 2005). In the present study, the expression profiles of four *MT* genes whose transcripts have been found in oil palm fruits were compared. The genes were *MT3-A* and *MT3-B*, previously reported by Abdullah *et al*. 2002, and *MET2a* and *MET2b* which were reported in our previous study (Al-Shanfari *et al*. 2012). The objectives of this study were to obtain and characterize the full-length cDNA clones of *MET2a* and *MET2b* from oil palm and to compare the expression profiles of the four different *MT* genes at different stages of development in the fruit mesocarp and in vegetative tissues.

Materials and methods

Elaeis guineensis Jacq. cv. Tenera roots, leaves, and fruits were collected and immediately washed with distilled water, frozen in liquid nitrogen, and stored at -80 °C for future RNA extraction. Roots and leaves were cut from adult tree and 7-month-old seedlings in a nursery of the Agricultural Technology Department, University of Putra Malaysia, Serdang, Selangor. The fresh fruit bunches at 7, 10, 12, 15, 17, and 19 weeks after anthesis (w.a.a.) were obtained from the Malaysian Palm Oil Board Research Station, Bangi, Selangor.

RNA was extracted from leaf, root, or mesocarp tissues (3 g; 10 repeats of each tissue) using the method described by Prescott and Martin (1987). The RNA integrity was checked by electrophoresis on a 1.2 % (m/v) agarose gel. A 1/6 volume of 6× loading dye (*Fermentas*, Maryland, USA) was added to the tested RNA and the mixtures were loaded into the wells of the submerged gel. Gel electrophoresis was carried out at 80 V with 1× TAE buffer for 80 min. After migration of the dye at 2/3 of the distance, the gel was removed from the running buffer and stained with ethidium bromide (0.5 μg cm^{-3}) for 20 min at room temperature and viewed using a UV transilluminator (*ChemiDoc™ XRS*, *Bio-Rad*, Hercules, USA). The image was captured using the *Syngene* (Cambridge, UK) gel imaging system.

The purity and quantification of RNA samples were measured in quartz cuvettes by a UV spectrophotometer (*BioQuest CE2502*, Cambridge, UK) and absorbance was read at 260 and 280 nm (A_{260}/A_{280} ratio provides an estimate of the purity of RNA).

The PCR-RACE technique was used to isolate the full-length cDNAs of the two previously reported genes *MET2a* and *MET2b*. RACE of 5′ and 3′ was carried out using *SMARTer*™ RACE cDNA amplification kit (*Clontech*, Mountain View, USA). For preparation of 5′-RACE-ready cDNA, 1.6 μg of 17 w.a.a. mesocarp RNA was mixed with 1 mm^3 of 5′-RACE CDS primer A, which was provided as a kit, with sterile H_2O to a final volume of 3.75 mm^3. At the same time, the same amount of RNA was mixed in another micro centrifuge tube with 1 mm^3 of 3′-RACE CDS primer A with sterile H_2O to a final volume of 4.75 mm^3 for 3′-RACE-ready cDNA. The reaction mixtures were incubated at 72 ºC for 3 min and cooled to 42 ºC for 2 min. To just the 5′-RACE cDNA synthesis, 1 mm^3 of the *SMARTer IIA* oligo was added to the reaction mixture. To each reaction tube, the following components were added: 2 mm^3 of a 5× first strand buffer, 1 mm^3 of 20 mM DTT, 1 mm^3 of 10 mM dNTP mix, 0.25 mm^3 of an RNase inhibitor provided in the kit (40 U dm^{-3}) and 1 mm^3 of *SMARTScribe*™ reverse transcriptase (100 U). The reaction mixtures were incubated at 42 ºC for 90 min in a hot-lid thermal cycler. After incubation, the tubes were heat inactivated at 70 ºC for 10 min. The first strand reaction product of each RACE reaction was diluted with 100 mm^3 of Tricine-EDTA and stored at -20 ºC.

The amplification was carried out using two primers designed on the sequences from both ends of the partial cDNA fragments of both *MET2a* and *MET2b*. Primer pairs used for amplification of all the genes studied are presented in Table 1. For both 5′- and 3′-RACE cDNA, the PCR amplification was carried out in 0.05 cm^3 of a reaction mixture containing 34.5 mm^3 of PCR-grade water, 5 mm^3 of a 10× *Advantage 2* PCR buffer, 1 mm^3 of a 10 mM dNTP mix, 1 mm^3 of a 50× *Advantage 2* polymerase mix, and a 5 mm^3 of 10× universal primer A mix (UPM). For 5′-RACE, 1 mm^3 of 10 μM pMET2a-F and pMET2b-F primers were added separately in addition to 2.5 mm^3 of 5′-RACE-ready cDNA for each reaction. For 3′-RACE, 1 mm^3 of 10 μM pMET2a-R and pMET2b-R primers were added separately in addition to 2.5 mm^3 of 3′-RACE-ready cDNA for each reaction. The PCR program was as follows: 35 cycles of denaturation at 94 ºC for 30 s, annealing for 30 s at 64 ºC for *MET2a* and at 57.2 ºC for *MET2b*, followed by extension at 72 ºC for 3 min. Cloning resulting PCR products of 5′ RACE and 3′ RACE of *MET2a* and *MET2b* was performed using the *yT&A*® cloning vector kit (*Yeastern Biotech*, Taipei,

Taiwan). Plasmid DNA was used as a template for sequencing, and all sequencing reactions were carried out from the 5'-end of the cDNA using universal primer M13 forward and from the 3'-end using M13 reverse (provided in the *yT&A*® cloning vector kit). To exclude sequencing errors, the clones were sequenced in triplicate. The DNA sequences were made available in *FASTA* form files. Vector sequences at both 5'-ends of the sense strand and 3'-ends of the antisense strand were removed.

The end-to-end PCR method was performed to amplify the complete cDNA sequence of the two MT selected genes *MET2a* and *MET2b*. Three end-to-end PCRs of each gene were carried out by combining the forward and reverse gene-specific primers based on the extended consensus sequence of 5′ RACE and 3′ RACE of *MET2a* and *MET2b*. The end-to-end gene-specific primer pairs were designed as EgMT2a-F and EgMT2a-R for *MET2a* full-length, whereas EgMT2b-F and EgMT2b-R were designed for *MET2b* full-length (Table 1). The end-to-end PCR reaction was carried out in 25 mm^3 of a reaction mixture containing 5 mm^3 of a 10× PCR buffer (minus Mg), 1.5 mm^3 of $MgCl_2$ (50 mM), 1 mm^3 of a dNTP mix (10 mM), 1.0 mm^3 of each end-to-end gene specific forward and reverse primers (10 μM), 1.0 mm^3 of 2.5 U Taq DNA polymerase (*Perkin Elmer*, Norwalk, USA), and 14.5 mm^3 of sterile water. The PCR program was started with an initial temperature at 94 ºC for 2 min followed by 36 cycles of denaturation at 94 ºC for 30 s, annealing for 30 s at 64 ºC for *MET2a* and 57.2 ºC for *MET2b* (depending on the T_m values of the primer pairs), and extension at 72 ºC for 3 s. The program was then ended with the final extension at 72 ºC for 10 min. The PCR reactions were conducted in a personal thermocycler (*BioMTra*, Goettingen, Germany). The PCR products were separated on a 1.2 % agarose gel. The amplified products were sequenced as purified PCR products using an outsourced commercial sequencing service provider (*First Base Laboratories Sdn Bhd*, Selangor, Malaysia). The full lengths of the cDNA sequences of the *MET2a* and *MET2b* were then compared for sequence similarities with other sequences in the database using the *BLAST* program (basic local alignment search tool) at *NCBI* (National Center for Biotechnology Information). The *BLAST* program used both the *BLASTN* and *BLASTX*. The obtained full lengths of *MET2a* and *MET2b* cDNAs were designated as *EgMT2a* and *EgMT2b*, respectively.

Gene expression analyses of the obtained *EgMT2a* and *EgMT2b*, in addition to *MT3-A* and *MT3-B* (GenBank accession number AJ236913 and AJ236914, respectively) obtained from oil palm by Abdullah *et al*. (2002), were performed by using semi-quantitative RT-PCR. RT-PCR was carried out in triplicates on independent RNA extractions for greater reliability of the results using a *SuperScript*™ III reverse transcriptase kit (*Invitrogen*, New York, USA). Samples of total RNA (5 μg) from each tissue were used to synthesize first strand cDNA in 20 mm^3 of a reaction mixture containing 5 mm^3 of a 10× PCR buffer (minus Mg), 1.5 mm^3 of $MgCl_2$ (50 mM), 1 mm^3 of a dNTP mix (10 mM), 1.0 mm^3 of each gene specific forward and reverse primers (10 μM), 1.0 mm^3 of 2.5 U Taq DNA polymerase (*Perkin Elmer*) and 9.5 mm^3 of sterile water. The primer pairs for *EgMT2a* and *EgMT2b* transcript amplifications were those used to amplify the full-length cDNAs (Table 1). The gene-specific primers used by Abdullah *et al.* (2002) were used to amplify the transcripts of *MT3-A* and *MT3-B* (Table 1). The oil palm housekeeping gene, *actin*

Table 1. Primer pairs used for PCR amplification of MT proteins in oil palm. T_m stands for melting temperature.

Protein	Primer name	Primer sequence	T_m [°C]	Amplicon size [bp]
pMET2a5	pMET2a-F	5′-TGGAGGATGCAAGATGTACCCTGACAT-3′	73.7	400
	UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′	76.3	
pMET2a3	pMET2a-R	5′-AGTTGGATCCGCACTTGCAGCCGCCGT-3′	79.1	500
	UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′	76.3	
pMET2b5	pMET2b-F	5′-TTCACACACATGGTAGAGGAGAGGAGC-3′	66.5	380
	UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′	76.3	
pMET2b3	pMET2b-R	5′-CACACAGACACTAGAAATTAACAACAC-3′	65.8	430
	UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′	76.3	
EgMT2a	EgMT2a-F	5′-AGTACATGGGGGCCGTGTTTGAGAGAAAGA-3′	61.6	571
	EgMT2a-R	5′-GAGAGATAGAAAGCCGGATAAGCTCTATTT-3′	61.6	
EgMT2b	EgMT2b-F	5′-ATGGGGGTGTTTGAGAGAAAGAGAGAGAGT-3′	67.8	553
	EgMT2b-R	5′-ACCACCCCCCATTGGGGGCTGGGGCGGGGG-3′	67.8	
MT3-A	MT3-A-F	5'-CACGAGTATCAGAGTTACAAAC-3'	59.8	446
	MT3-A-R	5'-GTGCGAAACTGGATAAGGAAG-3'	59.8	
MT3-B	MT3-B-F	5'-CTTCTCTCGAGTCACCTG-3'	59.0	429
	MT3-B-R	5'-GTCCGTTAAACAGGATAGG-3'	59.0	
ACTIN	ACTIN-F	5'- AGGCCGACCTCGTCACACGG -3'	68.6	204
	ACTIN-R	5'- ACAGGGTGCTCCTCAGGGGC-3'	68.6	

(GenBank accession number AAT45848), was used as a constitutive and internal positive control in the transcription analysis of MTs. The used primer sequences of the *actin* gene are also listed in Table 1. In a noncompetitive PCR, the targets and *actin* were amplified in different PCR tubes using a different primer set for each. The PCR conditions were started with an initial temperature at 94 ºC for 2 min followed by 28 cycles of denaturation at 94 ºC for 30 s, annealing 30 s at 58 ºC for *actin*, 52.5 ºC for *EgMT2a*, 57.2 ºC for *EgMT2b*, and 51 ºC for both *MT3-A* and *MT3-B* (depending on the T_m values of the primer pairs), and extension at 72 ºC for 3 s. The program was then ended with the final extension at 72 ºC for 10 min. The PCR reactions were conducted in a personal thermocycler (*BioMTra*). In order to amplify genes in the exponential phase, 28 amplification cycles were used to ensure reliability of measurements of transcript levels before they reached saturation (Franklin *et al.* 2008). The PCR products of each MT were mixed with the PCR products of the internal control in the same PCR tube and separated on a 1.2 % agarose gel. The amplified products were sequenced as purified PCR products using an outsourced commercial sequencing service provider (*First Base Laboratories Sdn Bhd*). The expression of the examined *MT* genes was quantified and calculated using the *AlphaEaseFCTM* software (*version 4.0.0*; *Alpha Inotech Corporation*, San Leandro, CA) according to the manufacturer's instructions. The relative expression level of each target cDNA was represented by the band intensity ratio of target gene/actin. Reported values correspond to the mean of five measurements for the fold expression. Only band intensities that were ≥ 1.5-fold higher were considered as significant.

The amino acid sequences of *EgMT2a* and *EgMT2b* analogs were compared with other known monocotyledon MTs using the *CLUSTALW* program (Thompson *et al.* 1994) available at *EBI* (http://www.ebi.ac.uk/Tools/clustalw/). Phylogenetic analysis of the aligned MT protein sequences was done by constructing a phylogenetic tree using the *ClustalW2* program (http://www.ebi.ac.uk/Tools/phylogeny/).

Results

Using a gene-specific primer pMET2a-F designed from the 5′ end of partial *MET2a* cDNA, a single PCR product of about 0.4 kb was successfully amplified from the 5′ RACE-ready cDNA pool and designated as *pMET2a5* (Fig. 1). Using the sense gene-specific primer pMET2a-R designed based on the 3′ end of the partial *MET2a* cDNA, a single PCR product of about 0.5 kb was successfully amplified and designated as *pMET2a3* (Fig. 1). The two partial cDNAs comprised of *pMET2a5* and *pMET2a3* were sequenced and assembled to generate full-length cDNA of *MET2a via* long distance PCR. The end-to-end primers derived from both extreme 5′ and 3′ ends of the *pMET2a5* and *pMET2a3* cDNAs were designated as EgMT2a-F and EgMT2a-R, respectively (Table 1). The full length PCR amplification was successful in amplifying a single PCR product of about 0.57 kb of *EgMT2a* (Fig. 1).

The upstream region of the *MET2b* gene was obtained using gene-specific primer pMET2b-F designed from the 5′ end of partial *MET2b* cDNA. A single PCR product of about 0.38 kb was successfully amplified from the 5′ RACE-ready cDNA pool and designated as *pMET2b5* (Fig. 1). Using the sense gene-specific primer pMET2b-R designed based on the 3′ end of partial *MET2b* cDNA, a single PCR product of about 0.43 kb was successfully amplified and designated as *pMET2b3* (Fig. 1). The two partial cDNAs comprised of *pMET2b5* and *pMET2b3* were sequenced and assembled to generate full-length cDNA of *MET2b via* long distance PCR. The primers derived from both 5′- and 3′-untranslated regions (UTR) of the *pMET2b5* and *pMET2b3* cDNAs were designated as EgMT2b-F and EgMT2b-R, respectively (Table 1). The full length PCR amplification was successful in amplifying a single PCR product of about 0.55 kb of *EgMT2b* (Fig. 1).

Sequence analysis revealed that the full-length

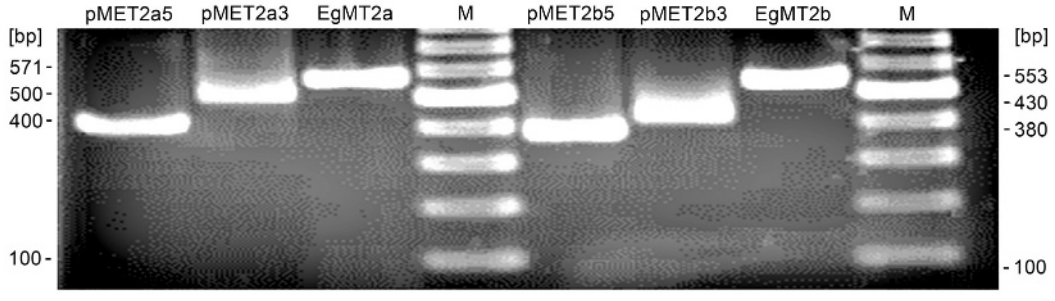

Fig. 1. Amplification products of RACE-PCR and full-length of *MET2a* and *MET2b* cDNAs. *Lane pMET2a5* - 5′ RACE (400 bp), *lane pMET2a3* - 3′ RACE (500 bp), *lane EgMT2a* - end-to-end fragment (571 bp), *lane pMET2b5* - 5′ RACE (380 bp), *lane pMET2b3* - 3′ RACE (430 bp), *lane EgMT2b* - end-to-end fragment (553 bp), *lane M* - DNA ladder mix. Samples were electrophoresed on a 1.2 % agarose/EtBr gel.

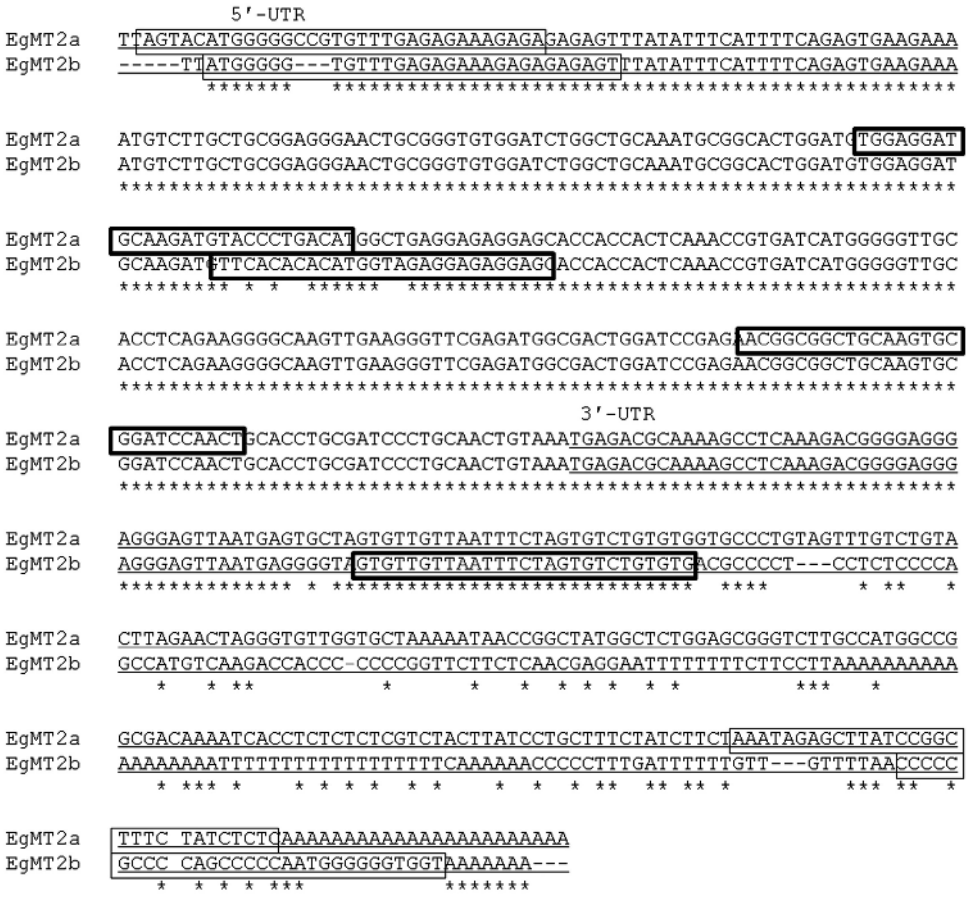

Fig. 2. Alignment between the full-length nucleotide sequences of oil palm *EgMT2a* and *EgMT2b*. The *underlined* DNA sequences indicate both 5′ and 3′-UTR. The sequences in *thick line boxes* are primer sequences for 5′- and 3′-ends amplification. The sequences in *thin line boxes* are end-to-end primer sequences for full-length gene amplification.

Table 2. Characteristics of *EgMT2a* and *EgMT2b* genes in oil palm fruit.

Characteristics	*EgMT2a*	*EgMT2b*	Identity [%]
Genbank acc. No.	JQ684027	JQ684028	
Insert size [bp]	571	553	
Coding-nucleotides			97.46
5'UTR			95.00
3'UTR			95.18
Amino acids in ORF	79	79	94.94
Number of Cys in ORF	14	14	
Molecular mass [kDa]	7.91	7.95	
Isoelectric point	5.14	6.57	

EgMT2a cDNA (GenBank accession No. JQ684027) is 548 bp [excluding the poly $(A)^+$ tail] and contains a 237 bp open reading frame (ORF). The ORF is flanked by 67 bp of 5′ UTR followed by 244 bp of 3′ UTR and a 23 bp poly $(A)^+$ tail (Fig. 2). The ORF encodes for 79 amino acids (aa) with a predicted molecular mass of 7.91 kDa and an isoelectric point (pI) of 5.14. It contains 14 Cys residues. Sequence analysis also revealed that the *EgMT2b* (GenBank acc. No. JQ684028) is 546 bp [excluding the poly $(A)^+$ tail] and contains a 237 bp ORF. The ORF is flanked by 59 bp of 5′ UTR followed by 250 bp of 3′ UTR and a 7 bp poly $(A)^+$ tail (Fig. 2). The ORF encodes for 79 aa with a predicted molecular mass of 7.95 kDa and pI of 6.57. It contains 14 Cys residues. The calculations of the molecular mass and isoelectric point was carried out using *Biology Workbench v. 3.2*. The presence of the start codon (ATG), stop codon (TAG), and total poly$(A)^+$ tail confirms that the isolation of the full length *EgMT2a* and *EgMT2b* containing the complete ORF, 5′ UTR, and 3′ UTR from the oil palm was successful.

Searches for homologies using the *BLASTP* program available in the GenBank public database showed that the oil palm *EgMT2a* and *EgMT2b* have a high sequence similarities to the MT protein type 2 from a wide range of plants species. The polypeptide encoded by the *EgMT2a* gene shares 77, 76, and 75 % of amino acid sequence identity with clones isolated from *Zea mays*, *Narcissus pseudonarcissus*, and *Typha angustifolia*, respectively. For *EgMT2b*, the highest similarity was found with sequences of *Musa acuminata* and *Narcissus*

pseudonarcissus with an amino acid sequence identity of 71 %, followed by *Typha angustifolia* and *Zea mays* with a 67 % sequence identity.

The comparison of the *EgMT2a* and *EgMT2b* nucleotide sequences show 97.5, 95.0, and 95.2 % sequence identities in the coding 5′ and 3′-UTR, respectively (Fig. 2, Table 2). The homology search using the *BlastX* database retrieved the conserved domains of the two MT candidates and shows them as type 2 MT super family. The deduced amino acid sequences of both *EgMT2a* and *EgMT2b* show 95 % identity (Fig. 3*A*). Both of the MTs were characterized by the presence of Cys-rich domains in both N- and C-termini. The amount of Cys residues in *EgMT2a* and *EgMT2b* was 18 % of the total number of amino acids in each MT. The sequences of coding regions of type 2 MT of oil palm were aligned with other eight plant species using the *ClustalW* program (Fig. 3*B*). The alignment shows that the encoded proteins of both *EgMT2a* and *EgMT2b* have the typical structure of plant type 2 MT proteins with two cysteine rich terminal domains (N- and C-terminus) separated by a cysteine-free central domain. The presence of the same distribution of cysteine residues of oil palm in the two domains (N- and C-terminus) as in the other type 2 MT genes from different plant species confirmed that the obtained sequences (*EgMT2a* and *EgMT2b*) belong to this group of proteins. *ClustalW2* software from EMBL-EBI was used to search for similarities to eight other plants (Fig. 4) and the resulted dendrogram indicates that oil palm *EgMT2a* and *EgMT2b* were closer to the *Zea mays* type 2 MT proteins.

Transcript accumulation in relation to fruit development was investigated by semi-quantitative RT-PCR for four MT genes (*EgMT2a*, *EgMT2b*, *MT3-A*, and *MT3-B*). The expression of the four members of MT genes could not be detected in vegetative tissues (roots and leaves), but it was strongly detectable in the fruit mesocarp tissues (Fig. 5). Furthermore, differential

Fig. 3. *A* - Alignment between the deduced amino acid coding region sequences of oil palm *EgMT2a* and *EgMT2b*. *B* - Alignment of CDS deduced amino acid sequences encoded by *EgMT2a* and *EgMT2b* of oil palm metallothionein with other selected plants encoding type 2 metallothioneins. The distribution of cysteine residues is shown in both N-terminus and C-terminus. GenBank accession numbers are indicated in brackets. Sequences were aligned by the *CLUSTALW* program. Consensus key: "*" - single, fully conserved residue, ":" - conservation of strong groups, "." - conservation of weak groups, "-" - no consensus.

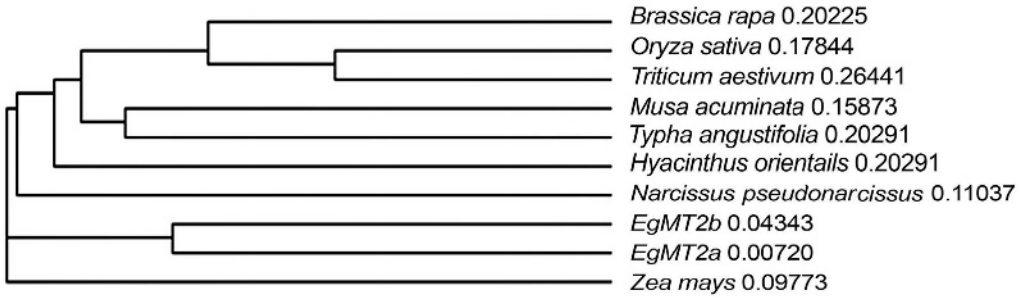

Fig. 4. The phylogenetic tree of oil palm *EgMT2a* and *EgMT2b* and eight plant type 2 M proteins based on deduced amino acid sequences. The alignment involved use of the *clustalW2* program. The dendogram indicates that oil palm *EgMT2a* and *EgMT2b* genes are closely related to the *Zea mays* type 2 MT proteins.

Table 3. Fold expression of metallothionein genes in oil palm that showed up-regulated expression in reproductive tissues at different stages. The fold expression of each gene is calculated using AlphaEaseFCTM. Means ± SE. Fold expressions that were ≥ 1.5-fold were considered as significant (*). Metallothionein genes were not expressed in vegetative tissues.

w.a.a.	*EgMT2a*	*EgMT2b*	*MT3-A*	*MT3-B*
7	0.00	0.00	0.00	0.00
10	1.73 ± 0.15*	0.00	1.65 ± 0.05*	0.87 ± 0.07
12	2.55 ± 0.29*	0.00	1.72 ± 0.04*	1.40 ± 0.00
15	1.63 ± 0.09*	0.00	2.57 ± 0.22*	2.20 ± 0.11*
17	2.67 ± 0.08*	0.54 ± 0.07	1.88 ± 0.06*	1.73 ± 0.03*
19	0.70 ± 0.05	0.00	1.46 ± 0.15	0.60 ± 0.06

expression of the four genes was also observed among different developmental stages of the fruit mesocarp. The RT-PCR analysis showed that the accumulation of MT transcripts was associated with stages of fruit development, with a different expression pattern for each MT member. The expression of the MT genes increased gradually during fruit development and was the highest in mature fruit. The expression of *EgMT2a*, *MT3-A*, and *MT3-B* started to increase at 10 w.a.a., reaching maximum before or at 17 w.a.a., and the expression dropped thereafter. *EgMT2a* transcripts were most abundant at 12 and 17 w.a.a., whereas transcripts of *MT3-A* and *MT3-B* were most highly expressed at 15 w.a.a. With respect to *EgMT2b*, it was expressed only at 17 w.a.a.

All of the MT gene members showed significant expression in mature oil palm fruit (Table 3). The expression of *EgMT2a* was significantly up-regulated at 10, 12, 15, and 17 w.a.a. with fold expression values of 1.73, 2.55, 1.63, and 2.67, respectively, relative to the internal housekeeping control gene *actin* (Table 3). Although *EgMT2b* was not significantly up-regulated (0.54-fold) (Table 3), the induced expression of this gene at 17 w.a.a. indicates its importance during the late ripening process of oil palm fruit. The expression pattern of *MT3-A* was generally similar to that of *MT3-B*, except that the relative expression of the former in 15 w.a.a. (2.57-fold) was even higher than that of the latter (2.20-fold) (Table 3). In comparison, among MT genes expressed in oil palm fruit mesocarp, *EgMT2a* was the highest, followed by *MT3-A*, *MT3-B*, and *EgMT2b* with a maximum fold difference of 2.67, 2.57, 2.20, and 0.54, at 17, 15, 15, and 17 w.a.a., respectively.

Discussion

Several authors suggested that MTs may be associated with fruit development and ripening, judging from the relatively high frequency of MT cDNAs being isolated from the library that was constructed from the fruit tissues. For example, different scales of the EST sequencing project identifying 10, 50, 37.6, and 6 % as sequences encoding MT from the banana, pineapple, oil palm, and citrus fruit cDNA library, respectively (Liu *et al*. 2002, Moyle *et al*. 2005, Low *et al*. 2008, Ye *et al*. 2010). In our previous study, also two different partial cDNA sequences encoding type 2 MT proteins designated as *MET2a* and *MET2b* occurred at a relatively high abundance (16.05 %) at the late ripening stage of the oil palm fruit (Al-Shanfari *et al*. 2012). Sequence analysis revealed that these cDNAs encode small polypeptides with Cys-rich N- and C-terminal domains which are the common features of plant MT and MT-like proteins (Robinson *et al*. 1993). The *EgMT2a* and *EgMT2b* were classified as type-2 MT according to Robinson *et al*. (1993) and in view of the presence of Cys-Cys, Cys-X-Cys, Cys-X-Cys, and Cys-X-X-Cys motifs in the N-terminal domain and Cys-X-Cys, Cys-X-Cys, and Cys-X-Cys motifs in the C-terminal, where X is any amino acid other than Cys. The amount of Cys residues in the *EgMT2a* and *EgMT2b* coding regions was 18 % of the total number of amino acids in each MT. The same amount was reported in other plants such as banana (Liu *et al*. 2004) and cork oak (Mir *et al*. 2004). Although

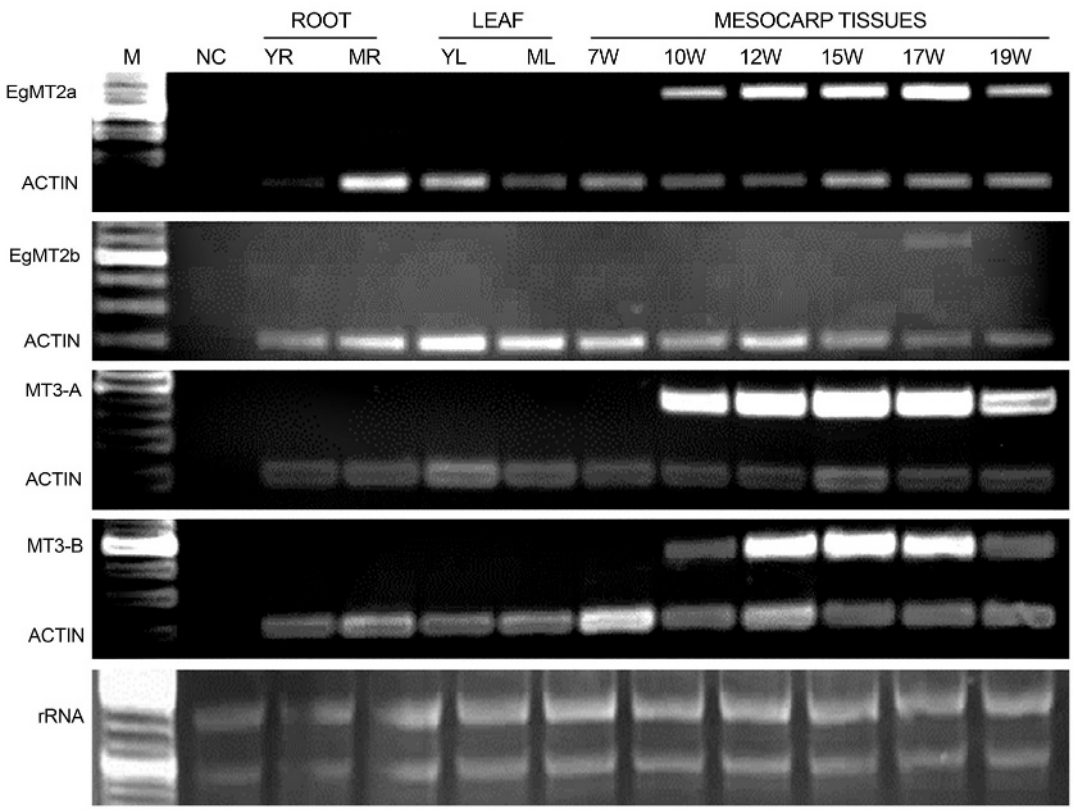

Fig. 5. Expression of *EgMT2a*, *EgMT2b*, *MT3-A*, and *MT3-B* in various oil palm tissues. *M* - molecular mass marker, NC - negative control, YR - young root, MR - mature root, YL - young leaf, ML - mature leaf, 7W to 19W - fruit mesocarp tissues at different stages (7 - 19 w.a.a.). *ACTIN* gene (size 200 bp) was used as internal control. rRNA represents the extracted total RNA from each sample. Each lane was loaded with 10 mm^3 of PCR products. Samples were electrophoresed on a 1.5 % agarose/EtBr gel.

the pattern of Cys distribution between the two candidates was identical in both N- and C-termini, they were strongly different in four amino acids in the central domain that was devoid of Cys (Fig. 3*A*). Nevertheless, these sequence characteristics were unique to MTs and MT-like proteins in plants (Robinson *et al*. 1993). Chen *et al*. (2003) have reported the isolation of a type 2 MT from sweet potato which shows an additional amino acid in the central spacer region.

The full-length sequences of the two partial cDNAs (*MET2a* and *MET2b*) were successfully isolated using the *SMARTer*™ RACE cDNA amplification kit and in generating complete cDNA sequences from the 5′ and 3′-ends (Fig. 2). To the best of our knowledge, the full-length sequences of the two types of type 2 MT proteins are the first ones reported for oil palm. The high degree of homology of the *EgMT2a* amino acid sequence with corresponding sequences of *Zea mays* (77 %) and *EgMT2b* with *Musa acuminata* and *Narcissus pseudonarcissus* (71 %) suggests that they belong to particularly closely related proteins. The deduced amino acids sequences of the two candidates have the characteristic arrangement of Cys residues just as other plant type 2 MT proteins (Fig. 3*B*). The sequences of *EgMT2a* and *EgMT2b* contain a 237 bp ORF coding for 79 aa with a predicted molecular mass of 7.9 kDa which is similar to the type 2 MT protein isolated from a cDNA library of *Helianthus tuberosus* (Chang *et al*. 2004) and *Cicer microphyllum* (Singh *et al*. 2011). The differences in the 3′-UTR and the coding regions between *EgMT2a* and *EgMT2b* indicate their characteristic differences at the nucleotide and amino acid levels, although the coding regions show high (95 %) sequence identity. In addition, their differential expression pattern during ripening further confirmed that *EgMT2a* is different from *EgMT2b.* The phylogenetic tree shows the high homology of the oil palm type 2 MT proteins with the corresponding sequence of *Zea mays* (Fig. 4). This could indicate the same role of the *MT2* gene in the fruit development. The yield and the quality of palm oil were highly influenced by genes expressed during the final weeks of ripening (Shaarani *et al*. 2010). From the point view of metal ion-binding, the yield and quality of fruit oil are affected by deficiency or excess of macro- and micro-elements (Franco *et al*. 2012). Therefore, it can be assumed that the high homology of the type 2 MT proteins between the two crops, oil palm and maize, may indicate that the MTs have a biosynthesis function as metal chelators, thereby enhancing the oil production process. However, further investigation needs to be

carried out to ascertain the assumption.

In many species, MTs were reported to express differentially in different organs. For example, the expression of *PaMT* genes in pineapple is confined to specific stages of fruit development (Moyle *et al*. 2005), and the rice *OsMT2b* gene is highly expressed in roots and seed embryos (Yuan *et al*. 2008). In the buckwheat, *FeMT3* transcripts are in mesophyll and guard cells of leaves, vascular tissue of roots, and throughout the whole embryo (Samardzic *et al*. 2010). In the present study, all four MT proteins, *EgMT2a*, *EgMT2b*, *MT3-A*, and *MT3-B*, show different patterns of expression in oil palm organs. The transcripts of all members are generally more abundant in ripening fruit tissues and are undetectable in vegetative tissues (Fig. 5) similarly as MT genes expression in Cavendish banana (Liu *et al*. 2002). However, it is somewhat different from the result of a previous study, in which transcripts encoding for type 2 MT proteins (GenBank Acc. No. ABQ14530.1) were highly expressed in 3-month-old oil palm seedling roots (Ho *et al*. 2007). In other studies of oil palm MT genes, it was found that *MT3-A* was slightly expressed in roots of 3- to 4-month-old seedlings and in senescing leaves, whereas *MT3-B* was strongly expressed in roots but no expression was detected in leaves or senescing leaves (Abdullah *et al*. 2002, Alizadeh *et al*. 2011). The variability of MT gene expression patterns in the vegetative tissues could be related to age differences between the plant tissues. The same variability has been reported for the *methyltransferase I* gene (*EgMET1*) during floral development of oil palm inflorescence tissue (Rival *et al*. 2008). Although it is not sufficiently clear whether gene expression is affected by age, the findings of the present study indicate that the transcript abundances of MT genes are associated to oil palm fruit ripening. This is evidenced by the accumulation of transcripts in the late ripening stage, starting with a low amount at 10 w.a.a. and reaching high expression at 15 - 17 w.a.a. The expression reaches its maximum a few weeks before maximal ripeness of the fruit with a significant reduction thereafter. Similar results have also been reported in banana (Liu *et al*. 2002) and oil palm (Abdullah *et al*. 2002).

The present study also shows differential expressions of different members of the MT gene familt during fruit ripening. All MT members were significantly up-regulated in mature development stages of oil palm fruit mesocarp (Table 3). Even though *EgMT2b* was not significantly up-regulated, its faint expression showing a marked detection of *EgMT2b* transcript at 17 w.a.a. was in response to the fruit ripening process. This indicates its unique functional importance in oil palm fruit ripening. Therefore, the unique gene expression at this interval of fruit ripening could give priority to *EgMT2b* as a candidate gene marker for the oil palm fruit ripening process. The comparison among the remaining three MT members also show how the gene expression was related to the stage of fruit ripening. The fluctuation of the expression of *EgMT2a* was in contrast with *MT3-A* and *MT3-B* at 15 w.a.a., whereas the relative expression of the former gene declined at 15 w.a.a. The latter two genes reached their peak of expression at the same stage of fruit ripening. This suggests that type 2 MT proteins are more related to the late fruit ripening process than the type 3 MT proteins which is consistent with the reported functions of metallothioniens. The comparison between the two classes of type 3 MTs shows that the expression of both *MT3-A* and *MT3-B* gradually increased until 15 w.a.a. and further declined. Thus, both members have the same expression pattern, although *MT3-A* shows a higher expression at 15 w.a.a. than the *MT3-B*. This is in agreement with Abdullah *et al*. (2002), and may indicate the sensitivity of this ripening stage to the gene expression. Therefore, up-regulation of the *MT* genes in the late fruit ripening stage of oil palm in the current and previous studies (Abdullah *et al*. 2002, Ho *et al*. 2007, Nurniwalis *et al*. 2008, Rival *et al*. 2008, Ramli and Abdullah 2010) and in other fruits, such as Japanese pear (Itai *et al*. 2000), Cavendish banana (Liu *et al*. 2002), and pineapple (Moyle *et al*. 2005) suggest that the possible role of these proteins in fruit ripening is to enhance the metal ion binding and so guarantee homeostasis or a pool of available micronutrients, or to provide a protection against oxidative damage (Mir *et al*. 2004). However, the mechanisms that regulates the expression of MT genes in ripening fruits has yet to be elucidated.

Based on the sequence alignment and expression pattern, the two members (*EgMT2a* and *EgMT2b*) described here have not been previously reported in oil palm fruit. More importantly, the present study provides unequivocal evidence that type 2 MT proteins are closely associated with the late period of fruit ripening to a greater degree than type 3 MT proteins in oil palm fruit. In view of the high abundance of MT transcripts during fruit ripening and previous reports on metal-MT coordination, these proteins may play an important role in metal homeostasis and detoxification during fruit development.

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