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Effects of Over-Expressing Ethylene Responsive Transcription Factor on Expression of Selected Fruit Ripening-Related Genes in Oil Palm (*Elaeis guineensis* Jacq.) Mesocarp

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ABSTRACT

Gene expression is regulated mainly at the transcriptional level through binding of transcription factors to specific promoter regulatory elements. The expression profile of *EgDREB*, a transcription factor belonging to the AP2 family, was determined by reverse transcriptase (RT)-PCR and it was found that it was expressed at different stages of oil palm mesocarp development as well as in vegetative tissues (roots and leaves) but not in the mesocarp at the early ripening stage, which is 12 WAA (weeks after anthesis). Thus, the effects of over-expressing *EgDREB* on the transcriptional regulation of genes from five functional groups related to ripening were investigated in 12 WAA mesocarp of oil palm. Co-bombardment of 12 WAA mesocarp tissues with recombinant vector construct harbouring *EgDREB* and plasmid containing the GFP reporter gene was carried out. Fluorescent detection of GFP and verification via RT-PCR using GFP-specific primers enabled selection of successfully transformed tissues. Using transient expression assay, it was demonstrated that over-expression of *EgDREB* results in up-regulation of translationally controlled tumor protein (*TCTP*) and type 2 metallothionein-like genes (*MET2a* and *MET2b*). These proteins are categorised under biogenesis of the cellular component and proteins with binding functions or cofactor requirements. More specifically, the roles of metallothioneins are in homeostasis of essential metal ions and oxidative stress response. This may suggest that *EgDREB*

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is involved in regulating cellular processes related to the roles of these three proteins.

Keywords: Elaeis guineensis Jacq., fruit ripening, microprojectile bombardment, transcription factor, transient expression assay

INTRODUCTION

The oil palm (*Elaeis guineensis* Jacq.) is the most important industrial crop in Malaysia. It is an economically important plant species that provides the world's number one source of edible vegetable oil (Tranbarger *et al*., 2012). It is a monocotyledonous perennial tree which can produce fruit bunches after two and a half years with good agronomic practices while 20-22 weeks are required from anthesis to fruit maturity. Palm oil and the palm kernel oil are extracted from the fruit mesocarp and nut, respectively (Hartley, 1988).

Generally, fruit development, maturation and ripening are important biological processes unique to plants. The molecular basis of oil palm fruit development, maturation and ripening has, however, received less attention. A series of physical and chemical changes occurs in ripening fruits of oil palm (Prada *et al*., 2011) where colour development is one of the most significant criteria related to fruit maturity (Alfatni *et al*., 2008). Differential expression of various genes that are tissue-specific and developmentally regulated genes lead to the occurrence of several physiological and biochemical changes during the fruit ripening stage. Various changes take place in the oil palm mesocarp during the late period of fruit ripening, including cell wall expansion and oil accumulation as well as carotenoids, vitamin E and ethylene production (Bapat *et al.*, 2010).

Understanding the biological and physiological mechanisms at the molecular levels during the ripening stage of the oil palm fruit will help enhance specific characteristics to improve production and quality of the oil. Al-Shanfari *et al.,* (2012) took an important step in unravelling these mechanisms by characterising the structure and function of genes expressed during the late ripening period of the oil palm fruits. Suppression subtractive hybridisation technique successfully identified twenty unigenes encoding abundant transcripts expressed in the mesocarp at the late fruit ripening stage. Based on gene ontology classifications, the genes were grouped under cellular component biogenesis; cell rescue, defence and virulence; protein with binding function or cofactor requirement; metabolism; cell cycle and DNA processing.

Various approaches have been carried out to unveil the complexity of gene expression and interaction in plants (Low *et al.,* 2008). Understanding the mechanisms of transcriptional regulation underlying gene expression in its entirety is essential. One of the most important mechanisms of gene expression regulation occurs at the transcriptional level through binding of transcription factors to specific promoter motif of genes whose expression they regulate (Laurila & Lahdesmaki, 2009). Transcription appears to be controlled by various transcription factors that mediate

the effects of intracellular and extracellular signals. The transcription factors are responsible for enhancing or suppressing the transcription of specific genes through a complex regulatory network (Phillips & Hoopes, 2008).

The AP2/ERF transcription factors are widely found in both monocot and dicot plant species and are reported to be important in regulating plant development and responses to biotic and abiotic stresses (Nakano *et al.*, 2006). They can be divided into subfamilies with diverse roles (Saleh & Montserrat, 2003). The AP2 family members of transcription factors are distinguished by the presence of the AP2-DNA binding domain specific to plants (Licausi *et al.*, 2010). The AP2 domain sequence comprises approximately 70 amino acids that have a high level of amino acid conservation among its members. DREB is a sub-family of the AP2/ERF superfamily of transcription factors. These genes are involved in regulation of abiotic stress responses, including response to low temperature, dehydration and salt stress through induction of expression of stress response genes (Wang *et al.*, 2011).

From a recent study, a transcription factor belonging to the AP2 family designated as *EgDREB* (Genbank accession no. ABF59742), which is highly expressed in ripening oil palm fruit mesocarp, was isolated and sequenced. In the present study, further characterisation of this transcription factor was conducted in order to identify genes whose expression is regulated by this transcription factor in the mesocarp tissue. This was achieved through co-bombardment of the mesocarp tissue at an early stage of the ripening period with the transcription factor and GFP reporter gene followed by analysis of the effects on the expression of selected groups of the oil palm ripening-related genes (Al-Shanfari *et al.*, 2012) in GFP positive-bombarded tissues.

MATERIALS AND METHODS

Plant Materials

Oil palm (*Elaeis guineensis* Jacq.) variety Tenera (*Dura* × *Pisifera*) fruits at different stages of development (7, 10, 12, 15, 17 and 19 WAA), roots and young leaves from 5-month-old polybag-grown seedlings were used in the present study. The oil palm fruits were obtained from the Malaysian Palm Oil Board Research Station, Bangi, Selangor, while the 5-month-old seedlings grown in polybags were obtained from Sime Darby, Banting, Selangor. The samples were sterilised in 20% (v/v) Clorox and followed by thorough rinsing with distilled water. The exocarp was removed and the mesocarp section of the fruits was excised and immediately frozen in liquid nitrogen and stored at −80°C.

Plasmid

pMDC32 plasmid carrying the 35S promoter of Cauliflower Mosaic Virus (CaMV35S) (ABRC) and pMDC32-EgDREB plasmid carrying oil palm ethylene responsive transcription factor gene (*EgDREB*) fused to CaMV35S promoter, constructed by Azzreena Mohamad Azzeme (Institute

of Tropical Agriculture, Universiti Putra Malaysia) were used. [Fig.1 (a)] and 35SpEGFP plasmid carrying GFP reporter gene, fused to CaMV35S promoter (Clontech) [Fig.1 (b)]. The 35SpEGFP plasmid was constructed by inserting an 800 bp *Hin*d III – *Sma*I fragment containing the CaMV35S promoter into the multiple cloning site of pEGFP-1 (Clontech). The plasmids were purified using the QIAprep® Spin Miniprep Kit (Qiagen).

Preparation of Target Tissue for Bombardment

Preparation of the target tissue for bombardment was carried out based on the procedure described by Agius *et al.* (2005). The oil palm fruit bunches at 12 WAA were obtained from University Agriculture Park, Universiti Putra Malaysia. The fruits were surface sterilised in Clorox (20%), then rinsed in sterile distilled water. The mesocarp was excised to produce 1 cm × 1 cm slices. The mesocarp tissue slices were subsequently placed in the middle within a diameter of about 3 cm in petri dishes filled with solid media (Murashige and Skoog 1962). They were kept at 28° 1 day before being used for bombardment.

Preparation of DNA-microcarrier

Gold microcarriers were prepared according to the manufacturer's (Bio-Rad) instructions. Gold microcarriers (60 mg of 1 µm diameter) were re-suspended in 100% ethanol (1 ml) by vigorous vortexing for 2 minutes followed by centrifugation at 10,000 rpm for 1 minute. The supernatant was removed and the pellet was washed with sterile distilled water and centrifuged at 10,000 rpm for 1 minute and the supernatant discarded. The recovered pellet was washed again with sterile distilled water and centrifuged at 10,000 rpm for 1 minute and the supernatant discarded. The microcarriers were re-suspended in 1 ml of sterile distilled water and kept at 4°C.

The oil palm mesocarp tissue slices were co-bombarded with pMDC32-EgDREB and 35SpEGFP. Into an aliquot of 50 µl of microcarriers, 2.5 µl of each plasmid DNA (1 µg/µl) was added followed by 50 µl of 2.5 M $CaCl_2$ and then 20 µl of 0.1 M spermidine, with continuous vortexing. An additional 3 minutes of vortexing was performed followed by recovery of the DNA microcarriers by centrifugation at 10,000 rpm for 10 seconds. After discarding

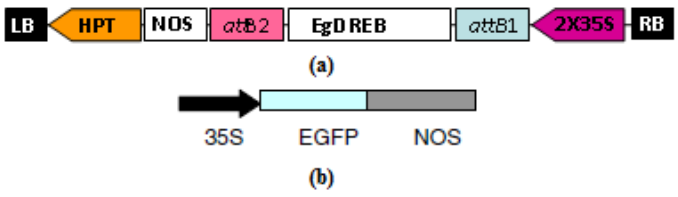

Fig.1: The schematic representation of pMDC32-EgDREB (a), and 35SpEGFP (b) used in this study

the supernatant, the pellet was washed 2X with 250 µl of 100% ethanol and finally re-suspended in 60 µl of 100% ethanol.

Biolistic Bombardment

Biolistic transformation was carried out using PDS-1000/He™ System (Bio-Rad) by co-bombardment of pMDC32-EgDREB and 35SpEGFP plasmids into the mesocarp tissue. For particle bombardment experiments, samples were arranged in a completely randomised design with three independent bombardments (replicates) which were 5 samples per replicate. The controls included unbombarded tissues and tissues bombarded with empty vector (pMDC32), which was plasmid without the *EgDREB* gene.

Each co-bombardment involved 10 µl of DNA-coated microcarriers loaded onto the centre of macrocarrier. The optimised parameters for transient transformation of oil palm tissues as described by Ramli and Abdullah (2003) were followed. The distances between the rupture disk and macrocarrier, macrocarrier and stopping screen, and stopping screen to target were 6 mm, 11 mm and 6 cm, respectively. The rupture disks of 1,550 psi and vacuum pressure of 27 mmHg pressure were used for the bombardment. The rupture disk, macrocarrier and stopping screen were soaked in 70% ethanol for 30 minutes prior to bombardment. The bombarded tissues were kept at 28°C for 48 hours prior to reporter gene assay.

Green Fluorescent Protein Assay

GFP analysis of the mesocarp tissue slices was carried out at 12 hours and 2 days after co-bombardment. The GFP spots were detected 2 days after co-bombardment, using Nikon SMZ1000 microscope equipped with UV source and GFP filter (excitation at 360 to 480 nm and emission at 480 to 500 nm). The transiently transformed tissues expressing GFP were used for RT-PCR analysis. The total RNA from the unbombarded and bombarded mesocarp tissue slices was extracted according to the modified method by Prescott and Martin (1987).

Semi-quantitative RT-PCR

A semi-quantitative RT-PCR was performed to measure the effects of over-expressing *EgDREB* in 12 WAA mesocarp tissues on the expression of fruit ripening-related cDNAs, which had been divided into 5 groups based on gene ontology classification (Table 1). For greater reliability of the results, the RT-PCR was performed in triplicate. The representative gel electrophoresis results of the RT-PCR products were used for product quantification.

The gene-specific primers used were from Al-Shanfari *et al.* (2012). As an internal standard, the GAPDH housekeeping gene was used. Initial standardisation of the PCR using different cycles ranging from 23 to 38 revealed that the 28 cycles provided the optimal result within the exponential range for product quantification for all expressed cDNAs. Hence, 28 cycles were

subsequently used in all of the quantification assays. RT-PCR analysis of *EgDREB* and GFP reporter genes was also carried out in order to provide internal and positive controls, respectively.

QIAGEN®OneStep RT-PCR Kit (Qiagen) was used for RT-PCR. The reactions were set up separately for each gene in a 50 µl reaction mixture, containing 10 µl of 5X QIAGEN OneStep RT-PCR Buffer, 2 µl of 25 mM dNTP mix, 3 µl each 0.6 µM gene-specific forward and reverse primers, 2 µl of QIAGEN OneStep RT-PCR Enzyme Mix, 1 µl of total RNA (1 µg/µl) and 29 µl of RNase-free water. The RT-PCR cycling was as follows: 30 minutes at 50°C for reverse transcription, initial PCR activation step for 15 minutes at 95°C, then 28 cycles of denaturation, annealing and extension steps for 1 minute at 94°C, 1 minute at 51 to 64.2°C (based on T_m values of the primers) and 1 minute at 72°C, respectively. This was followed by a final extension step for 10 minutes at 72°C. Electrophoresis analysis was performed using 1.2% agarose gel at 80 V for 1 hour and 20 minutes. RT-PCR analysis was also carried out for the *EgDREB* gene in fruit (mesocarp) at different stages of development and young leaves and roots from 5-month-old polybag-grown oil palm seedlings in order to check for the expression profile of the gene.

Quantification of Gene Expression

The AlphaEaseFCTM software (version 4.0.0; Alpha Inotech Corporation, San Leandro, CA, USA) was used to quantify the RT-PCR bands. The relative expression level of each target cDNA was represented by the band intensity ratio of the target cDNA/GAPDH. The fold expression was represented by cDNA relative expression level ratio of bombarded mesocarp tissue with pMDC32-EgDREB/unbombarded mesocarp tissue and bombarded mesocarp tissue with pMDC32/unbombarded mesocarp tissue. Only the band intensity that was ≥1.5-fold was considered significant.

Sequence Analysis

Sequence analysis was carried out in order to confirm the identity of the RT-PCR products. The RT-PCR bands were purified using QIAquick Gel Extraction Kit (Qiagen) and sent for sequencing. The obtained sequences were analysed using the workbench software (http://workbench.sdsc.edu).

RESULTS

RT-PCR Analysis of EgDREB Gene in Various Oil Palm Tissues

As shown in Fig.2 (a), RT-PCR analysis of the *EgDREB* gene at different stages of fruit (mesocarp) development (7, 10, 12, 15, 17 and 19 WAA) showed the expression of the *EgDREB* gene at all stages of fruit (mesocarp) development except for the 12 WAA fruit mesocarp tissues. The expected size of the *EgDREB* cDNA based on the primers was 700 bp. However, no expression of *EgDREB* gene was observed in the mesocarp of fruits at 12 WAA, suggesting that there is no endogenous DNA binding activity of the *EgDREB* gene

in unbombarded 12 WAA mesocarp tissues. As shown in Fig.2 (b), RT-PCR analysis of the *EgDREB* gene in the leaf and root tissues showed expression of the *EgDREB* gene in the vegetative tissues.

Green Fluorescent Protein Assay

Fig.3 (a) illustrates the transient expression of the GFP reporter gene in the mesocarp tissue slices 2 days after co-bombardment with pMDC32 and 35SpEGFP plasmids, whereas Fig.3 (b) illustrates the transient expression of the GFP reporter gene in the mesocarp tissue slices 2 days after co-bombardment with pMDC32-EgDREB and 35SpEGFP plasmids. However, no transient expression of the GFP reporter gene was detected in the unbombarded mesocarp tissue slices [Fig.3 (c)].

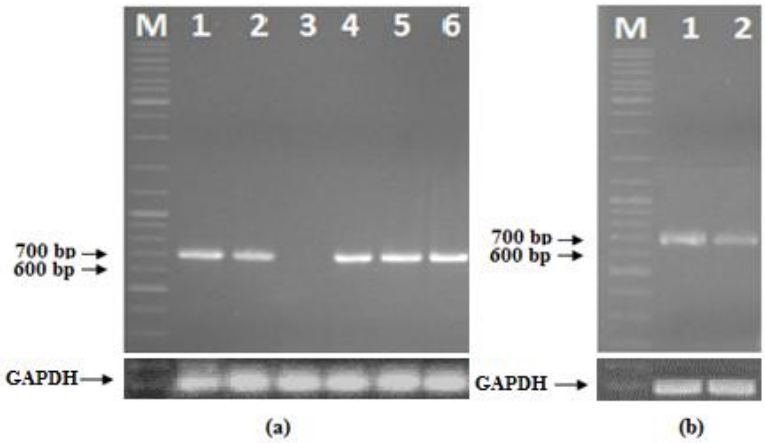

Fig.2: RT-PCR analysis of *EgDREB* gene in different stages of fruit (mesocarp) development. M: DNA Ladder Mix. Lanes 1, 2, 3, 4, 5 and 6 representing mesocarp at 7 WAA, 10 WAA, 12 WAA, 15 WAA, 17 WAA and 19 WAA, respectively(a), and vegetative tissues. M: DNA Ladder Mix. Lane 1 and 2 represents leaves and roots, respectively (b). GAPDH was used as an internal control

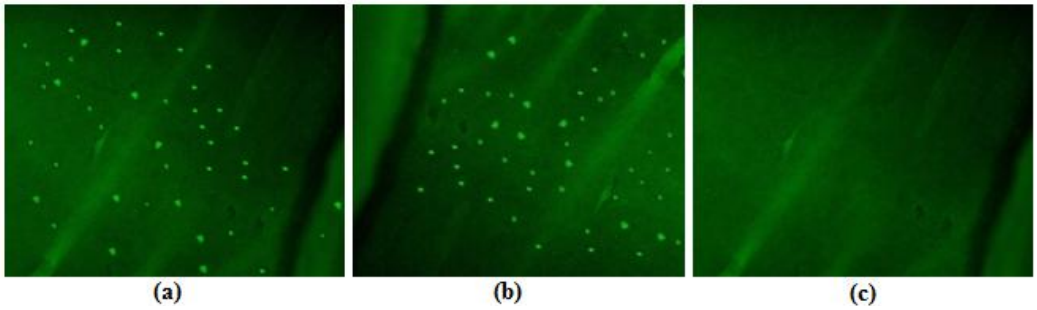

Fig.3: GFP-expressing mesocarp tissue slices co-bombarded with pMDC32 and 35SpEGFP (a), GFP-expressing mesocarp tissue slices co-bombarded with pMDC32-EgDREB and 35SpEGFP(b), and GFP expression not detected in unbombarded mesocarp tissue slices (c)

Co-bombardment of pMDC32 and pMDC32-EgDREB with GFP reporter gene provides a very useful indicator to measure the efficiency of DNA delivery and allows quick and easy selection of transiently transformed tissues.

RT-PCR Analysis of Controls

As shown in Fig.4 (a), RT-PCR analysis of the *EgDREB* gene showed that the expected size band of 700 bp was observed in the 17 WAA mesocarp tissue and the 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid. No expression of *EgDREB* gene was detected in the unbombarded 12 WAA mesocarp tissue and 12 WAA mesocarp tissue bombarded with pMDC32 plasmid. It was shown that *EgDREB* gene that was highly expressed in ripening oil palm fruit mesocarp (17 WAA) was only present in the 17 WAA mesocarp tissue and the 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB. As shown in Fig.4 (b), RT-PCR analysis of the GFP reporter gene showed that the expected size band of 200 bp was observed in the 12 WAA mesocarp tissue co-bombarded with pMDC32 and 35SpEGFP. It was also detected in the 12 WAA mesocarp tissue co-bombarded with pMDC32-EgDREB and 35SpEGFP. No expression level of GFP reporter gene was detected in the unbombarded 12 WAA mesocarp tissues. Thus the GFP reporter gene was only present in the bombarded mesocarp tissues.

Successful transcription of the *EgDREB* and GFP reporter gene in the bombarded mesocarp tissues showed the efficiency of transformation by biolistics as well as the accuracy of the RT-PCR approach. The RT-PCR was performed in triplicate for more reliable results and interestingly, the same results were observed for all the replicates which showed that the expression of introduced genes was found to be stable and most probably, did not interact with other metabolic pathways that result in suppression of gene expression.

RT-PCR Analysis of Fruit Ripening Related Genes

For Group 1, as shown in Fig.5(a), RT-PCR analysis of the *TCTP* gene showed expression of the *TCTP* gene based on the presence of the expected size of PCR product of 197 bp. The expected size bands were observed in the unbombarded 12 WAA mesocarp tissue, 12 WAA mesocarp tissue bombarded with pMDC32 plasmid, 17 WAA mesocarp tissue and 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid, with differences in intensity. The RT-PCR analysis showed that over-expression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded mesocarp tissues had resulted in up-regulation of the *TCTP* gene, based on the presence of bigger and enhanced intensity of the expected size band. From the representative gel electrophoresis result, it can be suggested that the *EgDREB* transcription factor gene regulates the expression of the*TCTP* gene.

For Group 2, RT-PCR analysis of the *Pp* [Fig.6 (a)], *ERP* [Fig.6 (b)], *USP-L* [Fig.6 (c)] and *USP* [Fig.6 (d)] genes showed

that no expression level was detected in the unbombarded mesocarp tissue, 12 WAA mesocarp tissue bombarded with pMDC32 plasmid and 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid. RT-PCR analysis showed that over-expression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded 12 WAA mesocarp tissue had not induced expression of *Pp*, *ERP*, *USP-L* and *USP* genes. This may suggest that the *EgDREB* transcription factor is not involved in regulating the expression of genes involved in cell rescue, defence and virulence.

For Group 3, RT-PCR analysis of *Met2a* [Fig.5 (b)] and *Met2b* [Fig.5 (c)] genes showed expression of these genes

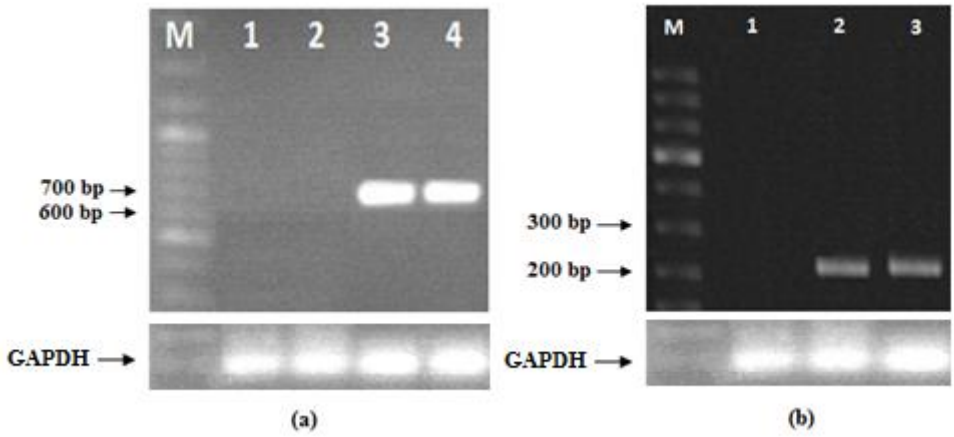

Fig.4: RT-PCR analysis for *EgDREB* gene in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: Bombarded 12 WAA mesocarp tissue with pMDC32. Lane 3: 17 WAA mesocarp tissue. Lane 4: Bombarded 12 WAA mesocarp tissue with pMDC32-EgDREB, and RT-PCR analysis for GFP reporter gene in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: Bombarded 12 WAA mesocarp tissue with pMDC32. Lane 3: Bombarded 12 WAA mesocarp tissue with pMDC32-EgDREB (b). GAPDH was used as an internal control

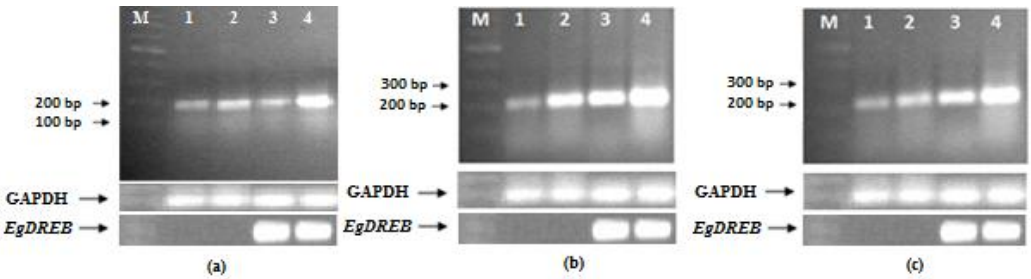

Fig.5: RT-PCR analysis for*TCTP* (a), *Met2a*(b), and*Met2b*(c) genes in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: 12 WAA mesocarp tissue bombarded with pMDC32. Lane 3: 17 WAA mesocarp tissue. Lane 4: 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB. GAPDH and *EgDREB* were used as an internal control

based on the presence of the expected band sizes. The expected sizes of the *Met2a* and *Met2b* genes, based on the primers used, were 209 and 239 bp, respectively. The expected band sizes were observed in the unbombarded mesocarp tissue, bombarded mesocarp tissue with pMDC32 plasmid, 17 WAA mesocarp tissue and bombarded mesocarp tissue with pMDC32-EgDREB plasmid, with differences in intensity. RT-PCR analysis showed that overexpression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded 12 WAA mesocarp tissue had resulted in up-regulation of the *Met2a* and *Met2b* genes based on the presence of the bigger expected sized bands in the bombarded as compared to the unbombarded 12 WAA mesocarp tissues. From the representative gel electrophoresis results, it can be suggested that the *EgDREB* transcription factor gene regulates the expression of *Met2a* and *Met2b* genes where its expression will lead to an increase in the expression level of both the *Met2a* and *Met2b* genes.

The results of Group 4 and 5 were identical to those of Group 2. For Group 4, as shown in Fig.6 (e), RT-PCR analysis of the *His3p* gene showed no detectable expression in the unbombarded mesocarp tissue, bombarded mesocarp tissue with pMDC32 plasmid and bombarded mesocarp tissue with pMDC32-EgDREB plasmid. As for Group 5, as shown in Fig.6 (f), RT-PCR analysis of the *FtsL* gene showed no detectable expression in the unbombarded mesocarp tissue, bombarded mesocarp tissue with pMDC32 plasmid and bombarded mesocarp tissue with pMDC32-EgDREB plasmid. Thus, it can be suggested that the *EgDREB* transcription factor gene does not regulate the expression of the *His3p* and the *FtsL* gene.

RT-PCR analysis of the 9 fruit ripening related genes (*TCTP*, *Pp*, *ERP*, *USP-L*, *USP*, *MET2a*, *MET2b, His3p* and *FtsL*) was conducted in triplicate for more reliable results. Interestingly, the same results were observed in all the replicates, which showed that the expression of the introduced *EgDREB* gene was found to be stable and most probably does not interact with other metabolic pathways. The representative gel electrophoresis results of the RT-PCR analysis showed that over-expression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded 12 WAA mesocarp tissues had resulted in increased expression of *TCTP*, *MET2a* and *MET2b* genes. No detectable expression of *Pp*, *ERP*, *USP-L*, *USP*, *His3p* and *FtsL* genes was observed in the bombarded mesocarp tissues as well as before bombardment of the 12 WAA mesocarp tissues.

Quantification of Gene Expression

The relative expression level of each target cDNA was represented by the band intensity ratio of the target cDNA/GAPDH. According to Barber *et al.* (2005), usually, quantitative expression levels of genes are normalised to the expression levels of the control, which is the housekeeping gene. RT-PCR analysis of the GAPDH gene showed expression in all of the tissues analysed. The expected size of the GAPDH

gene, based on the primers, was 100 bp. The expected size band was observed in the 12 WAA unbombarded mesocarp tissues, 12 WAA mesocarp tissue bombarded with pMDC32 plasmid, 17 WAA mesocarp tissue and 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid. RT-PCR analysis of the GAPDH housekeeping gene showed that the gene was expressed in all the mesocarp tissues being analysed.

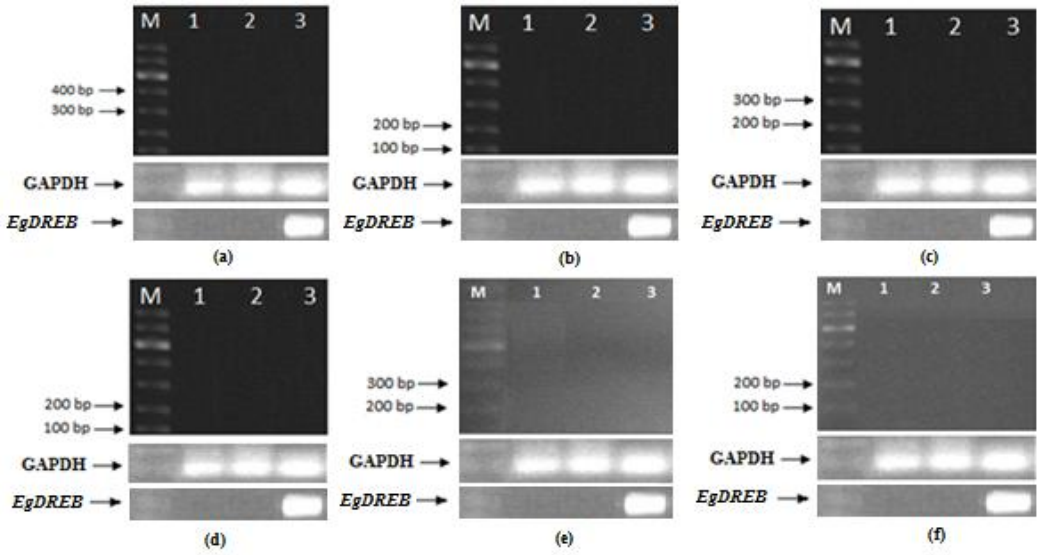

Fig.6: RT-PCR analysis for*Pp* (a), *ERP* (b), *USP-L* (c), *USP* (d) *His3p* (e), and*FtsL*(f) genes in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: 12 WAA mesocarp tissue bombarded with pMDC32. Lane 3: 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB. GAPDH and *EgDREB* were used as an internal control

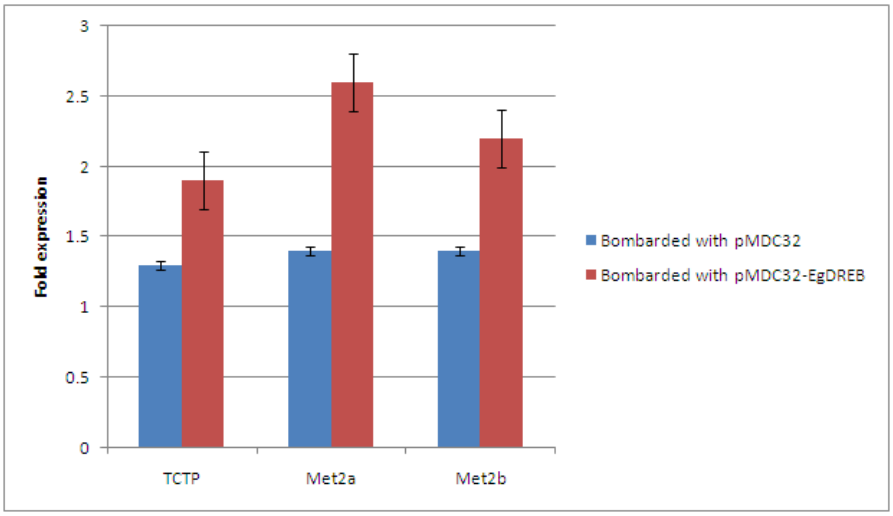

Fig.7: The fold expression of each target cDNA relative expression level in oil palm mesocarp tissues

The fold expression was represented by each target cDNA relative expression level ratio of bombarded mesocarp tissue with pMDC32-EgDREB/unbombarded mesocarp tissue and bombarded mesocarp tissue with pMDC32/unbombarded mesocarp tissue (Fig. 7). The band intensity that was ≥1.5-fold was considered significant (up-regulated). All 3 genes (*TCTP*, *MET2a* and *MET2b*) in the bombarded 12 WAA mesocarp tissue with pMDC32-EgDREB were found to be up-regulated with various expression values ranging between 1.9- to 2.6-fold. The *TCTP* gene showed 1.9-fold up-regulated expression, the *Met2a* gene showed 2.6-fold-up-regulated expression and the *Met2b* gene showed 2.2-fold up-regulated expression in the oil palm mesocarp tissue at 12 WAA. These genes are involved in biogenesis of the cellular component and protein with binding function or cofactor requirement, and were classified in Groups 1 and 3, respectively.

Bombarded mesocarp tissue with pMDC32 (empty vector without the gene of interest, *EgDREB* gene) showed no effects in the expression levels of *TCTP*, *MET2a* and *MET2b* genes, which indicates that the introduction of the pMDC32 plasmid in the bombarded mesocarp tissues did not affect the expression of these 3 genes and in fact the expression of the *TCTP*, *MET2a* and *MET2b* genes after bombardment with pMDC32-EgDREB plasmid is the absolute result of the over-expression of the *EgDREB* introduced via the pMDC32-EgDREB expression vector construct.

Sequence Analysis

Sequence analysis of *TCTP*, *Met2a* and *Met2b* RT-PCR products was conducted to confirm their identity. Significant sequence identities with 100% similarity with the oil palm genes reported by Al-Shanfari *et al.*, 2012 were observed for all 3 genes (*TCTP*, *Met2a* and *Met2b*).

DISCUSSION

This is the first report on applying transient expression assay as a model system to investigate the effects of a transcription factor in oil palm tissues in order to identify target genes whose expression is regulated. Successful transient gene expression assay was demonstrated by Omidvar *et al.* (2008) using a microprojectile bombardment-based approach in oil palm tissues. In both studies, the GFP gene was selected as a reporter for non-destructive monitoring and verification of successful transformed tissue in a manner similar to that reported by Kanchanapoom *et al.* (2008).GFP has gained widespread attention as a reporter gene system for plants as it need no external substrate for detection (El-Shemy *et al.*, 2008), and there have been no reports of detectable detrimental effects on the fitness of plants that express it (Sheahan *et al.*, 2004). The green fluorescence can be directly, easily and inexpensively assessed with fluorescence microscope as reported by Wurster *et al.* (2012). Even though GFP can provide non-destructive monitoring and be used to confirm the success of the transformation process (Hraska *et al.*, 2006), the transient expression of the GFP reporter

gene in the present study does not promise the successful expression of the *EgDREB* gene.

The *EgDREB* was shown to be constitutively expressed in different oil palm tissues including the roots, leaves and the mesocarp at different developmental stages except for the mesocarp at 12 WAA. Oil palm fruit development can be divided into different phases, including the cell division and expansion starting at 4 WAA, differentiation phase from 9 WAA followed by the ripening or maturation phase, with an active period of oil synthesis at 15-16 WAA until fruit maturity at about 20 WAA (Tranbarger *et al.*, 2011). This may suggest that the *EgDREB* may play a role in cell growth and development in young mesocarp tissues and also during the ripening period when oil accumulation occurs. Similar findings were observed for the fatty acid biosynthetic gene, stearoyl-ACP desaturase, which was found to be highly expressed in actively dividing young mesocarp tissues and its expression picked up again during the active oil synthesis period. It was suggested that it played a role in provision of fatty acids for membrane lipids in young tissues and for production of storage oil during the ripening stage (Siti Nor Akmar *et al.*, 1999). For *EgDREB*, the negligible expression provided an opportunity to look at genes whose expression is induced by this transcription factor by its introduction into mesocarp slices at 12 WAA.

The translationally controlled tumour protein (*TCTP*; also called p21, p23, histamine releasing factor and fortilin) is ubiquitously expressed and widely spread in eukaryotes (Berkowitz *et al.*, 2008; Susini *et al.*, 2008; Bommer & Thiele 2004). *TCTP* is an evolutionally highly conserved protein (Nagano-Ito & Ichikawa, 2012), and its expression is regulated at the levels of transcription and translation and also by a wide range of extracellular signals. *TCTP* plays a role in important cellular processes such as cell growth, cell cycle progression and malignant transformation and in the defence of cells against several stress conditions and apoptosis (Chen *et al.*, 2007; Bommer & Thiele, 2004). A study conducted by Al-Shanfari *et al.* (2012) reported that *TCTP* was found to be up-regulated at the late fruit ripening stage (17 WAA) of oil palm but is expressed at much lower levels at the earlier stage, at 12 WAA, the stage where the mesocarp tissues were used in the current study. In the present study, *TCTP* was found to be up-regulated with 1.9-fold expression value in response to over-expression of the *EgDREB* gene at the early ripening stage of fruit mesocarp development. The result suggests that the *EgDREB* gene may regulate the expression of genes related to growth and development based on the most widely reported role of *TCTP*. However, in recent years, microarray and proteomic analysis suggest the involvement of plant *TCTP* in abiotic stress signaling such as aluminum, salt and water deficit. Furthermore, over-expression of *Arabidopsis thaliana TCTP* was found to enhance drought tolerance in transgenic plants (Kim *et al.*, 2012). Hence, in oil palm the *TCTP* gene may also play a

role in abiotic stress signaling and the oil palm *EgDREB* is involved in regulating its expression at the transcriptional level.

Metallothioneins (MT) is defined as low molecular weight, cysteine rich metal binding proteins (Kumar *et al.*, 2010; Alizadeh *et al.*, 2011), whose expressions are induced by various factors (Yin *et al.*, 2005). These intracellular proteins are characterised by their unique high cysteine content (30%) and the absence of aromatic amino acids. MTs bind to a few trace metals like cadmium, mercury, platinum and silver, due to the high thiol content, (Sigel *et al.*, 2009) and also defend cells and tissues against toxicity of heavy metals (Thirumoorthy *et al.*, 2007). Research by Al-Shanfari and Abdullah (2014) reported that two different unigenes encoding type 2 MT-like proteins, namely *Met2a* and *Met2b*, were up-regulated in 17 WAA oil palm fruit mesocarp. This result suggests its role as a metal ion binding gene in the stress response to enhance the process of fruit ripening or to defend against oxidative damage. In the present study, *Met2a* and *Met2b* were found to be up-regulated with 2.6 and 2.2-fold expression value, respectively, in response to over-expression of the *EgDREB* gene. Their expression at the early stages of ripening is significantly lower. The result suggests that the *EgDREB* gene may regulate expression of genes that have transition metal ion binding functions or genes involved in oxidative stress response, consistent with the reported roles of metallothioneins. Metallothioneins have been reported to be involved in scavenging reactive oxygen species during oxidative stress (Ning *et al.*, 2010). Hence if the *EgDREB* is involved in regulating expression of *Met2a* and *Met2b*, its ultimate effect would be directly related to controlling the oxidative stress response. A recent study conducted by Hwang *et al.* (2012) which showed that the oxidative stress tolerance of DREB2C-overexpressing transgenic plants was significantly greater than that of wild-type plants supports our findings.

A study conducted by Al-Shanfari *et al.* (2012) on transcripts which encode the predicted protein (Pp), ethylene-responsive protein (ERP), USP-like protein (USP-L) and universal stress protein (USP) that were related to cellular stress, under cell rescue, defence and virulence, indicates that the oil palm fruit possesses a notably elevated level of stress genes in response to its environment. Genes encoding proteins that consist of the conserved 140-160 residues USP domain have the capability to respond to environmental stresses in bacteria, archaea, fungi, protozoa and plants. Genes containing the USP domain are induced by nutrient starvation, drought, high salinity, extreme temperatures and exposure to toxic chemicals (Isokpehi *et al.*, 2011). ERP proteins are essential in plant responses to stress (Zhang *et al.*, 2009; Wu *et al.*, 2008). In the present study, no detectable expression for Pp, ERP, USP-L and USP was found in response to over-expression of the *EgDREB* gene at the early ripening stage of fruit mesocarp development. The results suggest that the *EgDREB* gene is not involved in inducing expression of these

genes even though these genes have been reported to be involved in abiotic and biotic stress response. Hence, it can be suggested that while not all stress responsive genes are regulated by EgDREB there are certain genes whose expression is influenced by EgDREB.

CONCLUSION

This represents a basic study on screening the effects of *EgDREB* on different genes that have been grouped based on gene ontology classification. The results thus far seem to indicate that *EgDREB* may be involved in enhancing the expression of genes involved in growth, abiotic and oxidative stress response based on its ability to increase the expression of the *TCTP*, *Met2a* and *Met2b* genes. However, it appears that not all abiotic stress responsive genes are regulated by EgDREB as there may be certain signalling pathways or molecular mechanisms of stress response that are affected by EgDREB.

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