Journal of Oil Palm Research Vol. 31 (2) June 2019 p. 204-211
DOI: https://doi.org/10.21894/jopr.2019.0010

IDENTIFICATION OF RELIABLE REFERENCE GENES FOR GENE EXPRESSION STUDIES OF OIL PALM PLANTLETS USING NORMFINDER AND BESTKEEPER ALGORITHMS

MUHAMAD AFIQ, A H*; SHAHARUDDIN, N A and ZUBAIDAH, R***

ABSTRACT

Gene expression profiling analysis using real-time quantitative polymerase chain reaction (qRT-PCR) is commonly used due to its reliability and accuracy. Gene expression profiles of particular genes in different tissues and developmental stages aid in the understanding of their biological functions. However, the accuracy and reliability of qRT-PCR to study gene expression demand the use of species-specific reference genes for normalisation. Therefore, appropriate reference genes for qRT-PCR need to be determined for oil palm (Elaeis guineensis) *tissues. In this study, six candidate reference genes namely* actin, Cyp2, GRAS, EgEfa1, F-box, *and* GLU *were examined in leaf, node, internode, root, cabbage, shoot primordial and basal stem tissues of two-year old clonal oil palms. Two statistical algorithms, NormFinder and BestKeeper verified the stability of* GRAS *and* Cyp2 *reference genes in the oil palm tissues tested. The combination of* Cyp2 *and* GRAS *reference genes are recommended for gene expression normalisation profiles in nursery-stage oil palm plantlets.*

Keywords: oil palm, reference gene, quantitative real-time (qRT), normalisation.

Date received: 21 May 2018**; Sent for revision:** 22 May 2018**; Received in final form:** 24 September 2018**; Accepted:** 11 April 2019.

INTRODUCTION

Palm oil is the most traded oil in the world. The demand of palm oil to the world's oils and fats market has steadily increased over the years. In 2017, Malaysia produced 19.92 million tonnes of crude palm oil, 15% increased from previous year (Zulkifli *et al*., 2017), accounting for over one-third of total palm oil production in global trade exports. In comparison to annual oilseed crops, oil palm produces more oil per hectare and it is universally recognised that oil palm is the most efficient, effective and highest yielding crop for edible oil production (Murphy, 2014). Improvement of oil palm crop through genetic engineering has been identified as one of the key areas in oil palm research (Zubaidah *et al*., 2017; Rahman *et al*., 2016). The sequencing of oil palm genome was a key step towards this goal (Singh *et al*., 2013).

Gene expression profiling is used to determine gene expression patterns particularly at the transcription level thus provides understanding of complex regulatory networks of cellular function. Reverse-transcription-polymerase chain reaction (RT-PCR) is often used to semi-quantitatively determine levels of gene expression. On the other hand, quantitative real-time-PCR (qRT-PCR) is more preferable because it is highly accurate, specific and sensitive (Navarro *et al*., 2015). Nevertheless, the precision of qRT-PCR is determined by several

* Malaysian Palm Oil Board, 6 Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia. E-mail: bai@mpob.gov.my

** Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

factors such as amplification efficiencies of primers, sample genotype, type of tissues and treatments (Karuppaiya *et al*., 2017; Chao *et al*., 2012). Inaccuracy of qRT-PCR is also contributed by variations in sample volume, template quality including technical and systemic errors (De Ronde *et al*., 2017; Udvardi *et al*., 2008).

In order to minimise the effect of experimental induced variation, normalisation of gene expression using a set of reference genes is an approach to equilibrate the variability from transcript expression (Navarro *et al*., 2015). Thus, normalisation of target genes with the high stability reference genes can improve the accuracy of qRT-PCR. Optimum normalisation of the target gene expression requires more than one reference gene (De Spiegelaere *et al*., 2015; Chandna *et al*., 2012).

There are several genes including cyclophilin 2 (*Cyp2*), glutaredoxin (*GLU*) and gibberellin-responsive protein 2 (GRAS) that have been used as reference genes in oil palm studies (Lim *et al*., 2017; Kwan *et al*., 2016; Xia *et al*., 2014; Yeap *et al*., 2012). Normalisation in studies on basal cell metabolism, cytoskeletons, and intracellular functions previously used conventional housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *actin*, 18S and 28S ribosomal RNA for internal control (Li *et al*., 2017; Thuc *et al*., 2011; Zheng and Sun, 2011). The housekeeping genes were thought to be constitutively expressed in all tissues. However, increasing evidences have shown that the expression stability of housekeeping genes varies depending on experimental conditions, plant species and developmental stages (Li *et al*., 2017; Chandna *et al*., 2012). Significant bias and misinterpretation of data may result in inaccurate analysis due to inappropriate use of housekeeping genes for normalisation in relative quantification of gene expression. Thus, selection and validation of suitable reference genes using qRT-PCR for expression studies are necessary to minimise non-biological sample-to-sample variations.

Selection of the most stable reference genes requires evaluation of several candidate genes depending on different experimental or developmental conditions. Statistical algorithm software have been developed to determine and assess the expression stability of multiple reference genes that are required for gene expression profiling. There are a number of freely accessible and friendly user software available such as NormFinder (Andersen *et al*., 2004) and BestKeeper (Pfaffl *et al*., 2004).

This study reports on the selection of suitable reference genes for gene expression profiling using qRT-PCR in oil palm. In this study, six candidate reference genes obtained from previous studies (Yeap *et al*., 2013) and transcriptome results (Ooi *et al*., 2012), namely *actin*, *Cyp2*, *GRAS*, *EgEfa1*, *F-box*, and *GLU* were evaluated using two statistical software for selection of qRT-PCR reference genes in oil palm studies based on their stability.

MATERIAL AND METHODS

Plant Material

Tissue samples were obtained from oil palms planted in a greenhouse at the Malaysian Palm Oil Board (MPOB), Bangi, Selangor, Malaysia. Vegetative tissues (leaf, node, internode, root, cabbage, shoot primordial and basal stem) from two-year old clonal oil palms (*Elaies guineensis* Jacq.) (Clone 8A/PL233/5/9AP1/4/S/R) were excised and immediately frozen in liquid nitrogen and stored at -80°C prior to ribonucleic acid (RNA) extraction.

Total RNA Extraction, Purification and Quality Assessment

Total RNA extraction was carried out using RNeasy® Plant Mini Kit (Qiagen, Germany) as per the manufacturer's protocol. Total RNA was purified from traces of genomic DNA using RNeasy® MinElute Cleanup (Qiagen, Germany). The concentration and purity of the total RNA were quantified using a NanoDrop Spectrophotometer (ND-1000, Thermo Scientific, USA). The integrity of the total RNA was assessed by electrophoretic fractionation on Fragment Analyser (Advanced Analytic Technology Inc., USA).

First-strand cDNA Synthesis

Total RNA was used as the template to synthesise first-strand complementary DNA (cDNA). SuperScript™ III Reverse Transcriptase (Invitrogen, USA) was used to synthesise first-strand cDNA from 1 µg of DNase-treated RNA using 50 µM oligo $(dT)_{20}$ in a reaction volume of 20 µl according to the manufacturer's instruction.

Candidate Gene Selection and Primer Design

Six candidate reference genes, *Cyp2*, *GRAS*, *EgEfa1*, *F-box*, and *GLU* were selected from literature review (Yeap *et al*., 2013) for evaluation of expression stability. A conventional housekeeping gene, *actin* (Ooi *et al*., 2012), commonly used as internal control in gene expression studies was also evaluated. Primer sequences for the candidate reference genes are as listed in *Table 1*. RT-PCR was used to optimise and evaluate the amplification efficiency, specificity and annealing temperature with synthesised cDNA as template. The RT-PCR reactions were performed using ProFlex™ 3x32-Well PCR System (Applied

TABLE 1. LIST OF PRIMERS USED IN qRT-PCR ANALYSIS

Gene	Gene name	Function	Sequence (forward/reverse) (5′-3′)	Length (m)	Amplicon length (bp)	Reaction T_A (°C)
Cyp2	Cyclophilin 2	Peptidyl prolyl isomerase activity	CTCGTCTGATGTCGTCTA CTGCTGGTACTCTGGTAA	18 18	163	48
GRAS	Gibberellin-responsive protein 2	Transcription factor	CGGAAGAGTCACATCAATCG GGAGCAAGAAGCCAACAC	20 18	103	51
EgEfa1	Elongation factor	Protein synthesis on the ribosome	TAT CAA AGG ATG GGC AGA CC TCA TCA TAC CTT GCC TTG GA	20 20	127	50
F-box	F-box domain containing protein	Cell cycle signal transduction and regulation	TCT GGA CTC TAC CTA ACA ATG CTG ATG ACA ACT ACA	18 18	144	49
GLU	Glutaredoxin	Redox regulation of protein activity	CTC TTC TAA TCC CGT CAT CAC ATC CAA CTC AAT CAC	18 18	112	45
actin	Actin	Cytoskeletal structural protein	TGCTGATCGTATGAGCAAGG GAAATCCACATCTGCTGGAAG	20 21	146	60

Note: qRT-PCR – quantitative real-time polymerase chain reaction.

Biosystem, USA). The amplicons were further confirmed by sequencing. Primer pairs that were able to amplify single and specific amplicons were selected for qRT-PCR amplification efficiency test. Specificity of the primers pair was verified by melt curve analysis by increasing the temperature from 65°C to 95°C. Amplification efficiency was calculated as $E\ (\%) = [10^{(-1/\text{slope})} - 1] \times 100$ obtained from the efficiency and specificity tests using 10-fold serial dilution from the standard curve with satisfactory linear relationship ($R^2 \geq 0.99$) (Tellinghuisen and Spiess, 2014).

Quantitative Reverse Transcription Real-time PCR (qRT-PCR) Reaction

Synthesised first-strand cDNA were used as templates in the qRT-PCR reactions. The qRT-PCR reactions containing 10 µl 1× iTaq™ universal SYBR® Green Supermix (BioRad, USA), 500 nM of each primer and 5 ng of cDNA were prepared and amplifications were performed using CFX96™ Real-time PCR Detection System (BioRad, USA). Thermal cycling conditions were started with initial activation at 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 30 s. Melt curve was generated at dissociation stage of each run for evaluation of amplification specificity. Genomic DNA contamination was precluded with no-reverse transcriptase (NRT) control in each run. Negative control, a non-template control (NTC) was also included for each primer pair. All reactions were performed in triplicates. Quantification cycle (C_q) values generated by the qRT-PCR were used for gene expression stability analysis.

Data Analysis

Expression stability of the reference genes was analysed to determine the most stable reference genes using two different statistical algorithm softwares; BestKeeper, (http://www.gene-quantification.com/bestkeeper.html) and NormFinder (https://moma.dk/normfinder-software) (De Spiegelaere *et al*., 2015). The statistical algorithm softwares incorporated Microsoft Excel Spreadsheet to simplify calculations and rank the reference genes in terms of expression stability.

BestKeeper ranked expression stability of the reference gene based on Pearson correlation coefficient (*r*) and the probability BestKeeper index (*p*) using pair-wise correlation analysis. The stability of gene expression was determined by the *r* value. NormFinder determines the expression stability using comparative C_q method ($2^{-\Delta Cq}$) (ΔCq = corresponding Cq value – minimum Cq value). The results were analysed using default parameters.

RESULTS

RNA Quality

The extracted total RNA showed a purity level ranging from 1.8-2.0 and 2.00-2.40 for $A_{260/280}$ and $A_{260/230}$ ratios, respectively, with high concentration of 100-850 ng μl^{-1} indicating minimal protein and organic compound contamination. Total RNA from all tissues produced two intact and distinct 28S and 18S ribosomal RNA fragments with approximately 2:1 peak intensity ratio (*Figure 1*). RNA quality is expressed as the RNA Quality Number (RQN). The RNA samples were in good quality and highly intact, fulfilling the minimal sample requirement for qRT-PCR analysis (Martin *et al.*, 2016; Bustin *et al*., 2009).

Candidate Reference Genes for Oil Palm

The selection of most stable reference genes was determined based on the expression stability of six candidate reference genes. Primer pair specificity

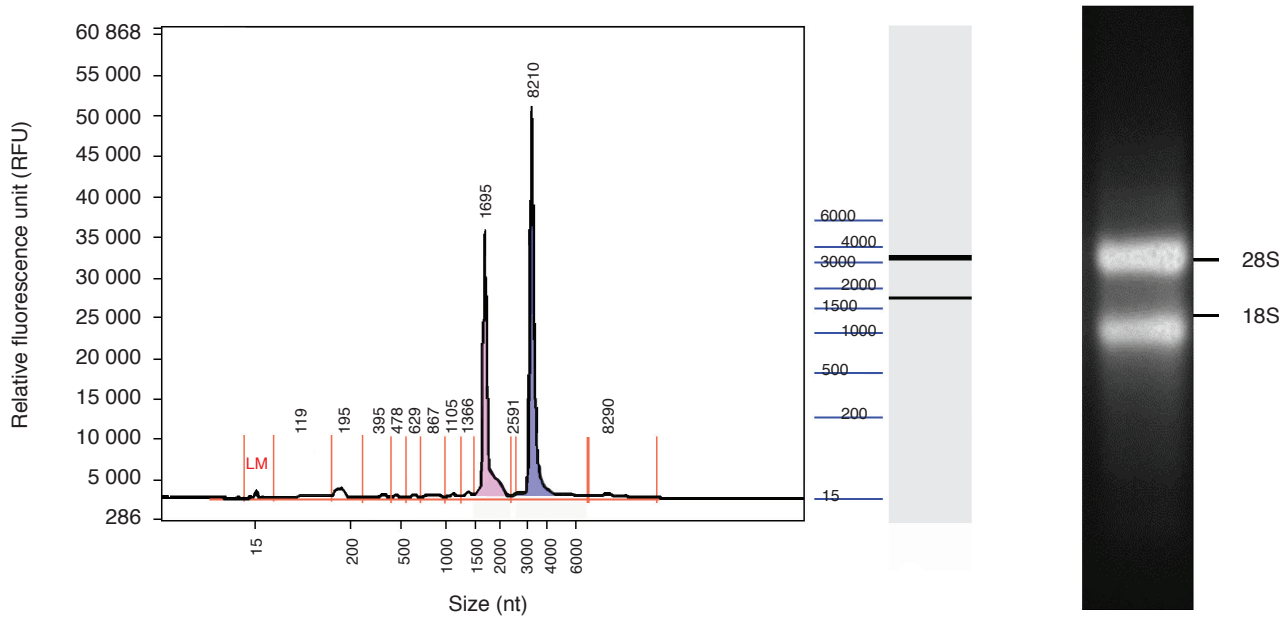

Figure 1. Total ribonucleic acid (RNA) integrity (representative). Intact 28S and 18S fragments was clearly separated as two distinct peaks. Total RNA integrity tested on 1% (w/v) agarose gel showing intact 28S and 18S fragments.

was evaluated using PCR and melt curve analysis. All reference genes produced single bands in agarose gel electrophoresis indicating high specificity of the primers (data not shown) when tested with leaf, node and internode tissues. However, further specificity evaluation using melt curve analysis in qRT-PCR revealed multiple peaks in *actin* gene analysis indicating multiple PCR products with approximately the same size. The *Cyp2*, *GRAS*, *EgEfa1*, *F-box* and *GLU* genes, however, generated single peaks in melt curve analyses (*Figure 2*). All the genes were further characterised by sequencing using the PCR products.

The amplification efficiency (*E*) and correlation coefficient (R^2) of the reference genes were calculated from standard curves generated from serial dilution (*Table 2*). The amplification efficiencies of the candidate reference genes ranged from 80.0%-99.5%, indicating that the primers were able to amplify the genes efficiently. Regression lines which fitted to the data points leading to the correlation coefficients of the candidate reference genes in the range of 0.98-0.99.

The gene expression levels of the reference genes were calculated based on C_q values generated from qRT-PCR. Low C_q indicated high level of expression. The candidate reference genes were expressed differently. The *Cyp2* exhibited the highest expression levels with an average C_q of 17.70±0.05 whereas *actin* exhibited the lowest expression level with average C_q of 26.06±0.08.

Expression Stability of Reference Genes

The *Cyp2* and *GRAS* were ranked as the most stable reference genes according to the NormFinder algorithm. This was determined by their lowest expression stability value (*Table 3*). The other reference genes were ranked from the most stable to less stable; *F-box*, *EgEfa1* and *GLU*. BestKeeper

TABLE 2. AMPLIFICATION EFFICIENCY (E), R^2 VALUES AND SLOPE VALUES OF CANDIDATE REFERENCE GENES

Candidate reference gene	Amplification efficiency (%)	R^2 value	Slope value
Cyp2	99.5	0.987	-3.333
GRAS	81.2	0.996	-3.875
EgEfa1	81.8	0.996	-3.852
F-box	91.4	0.985	-3.548
GLU	92.1	0.999	-3.527
actin	80.0	0.993	-3.197

TABLE 3. COMPARISON OF GENE EXPRESSION STABILITY CALCULATED BY NORMFINDER AND BESTKEEPER

Rank	NormFinder		BestKeeper		
	Gene	Stability	Gene	*r* value	*p* value
1	*Cyp2*	0.004	*GRAS*	0.93	0.001
2	*GRAS*	0.005	*Cyp2*	0.92	0.001
3	*F-box*	0.005	*EgEfa1*	0.92	0.001
4	*EgEfa1*	0.007	*F-box*	0.89	0.001
5	*GLU*	0.007	*GLU*	0.87	0.001
6	*actin*	-	*actin*	-	-

Note: *Candidate reference genes were ranked from the most stable to the least stable.

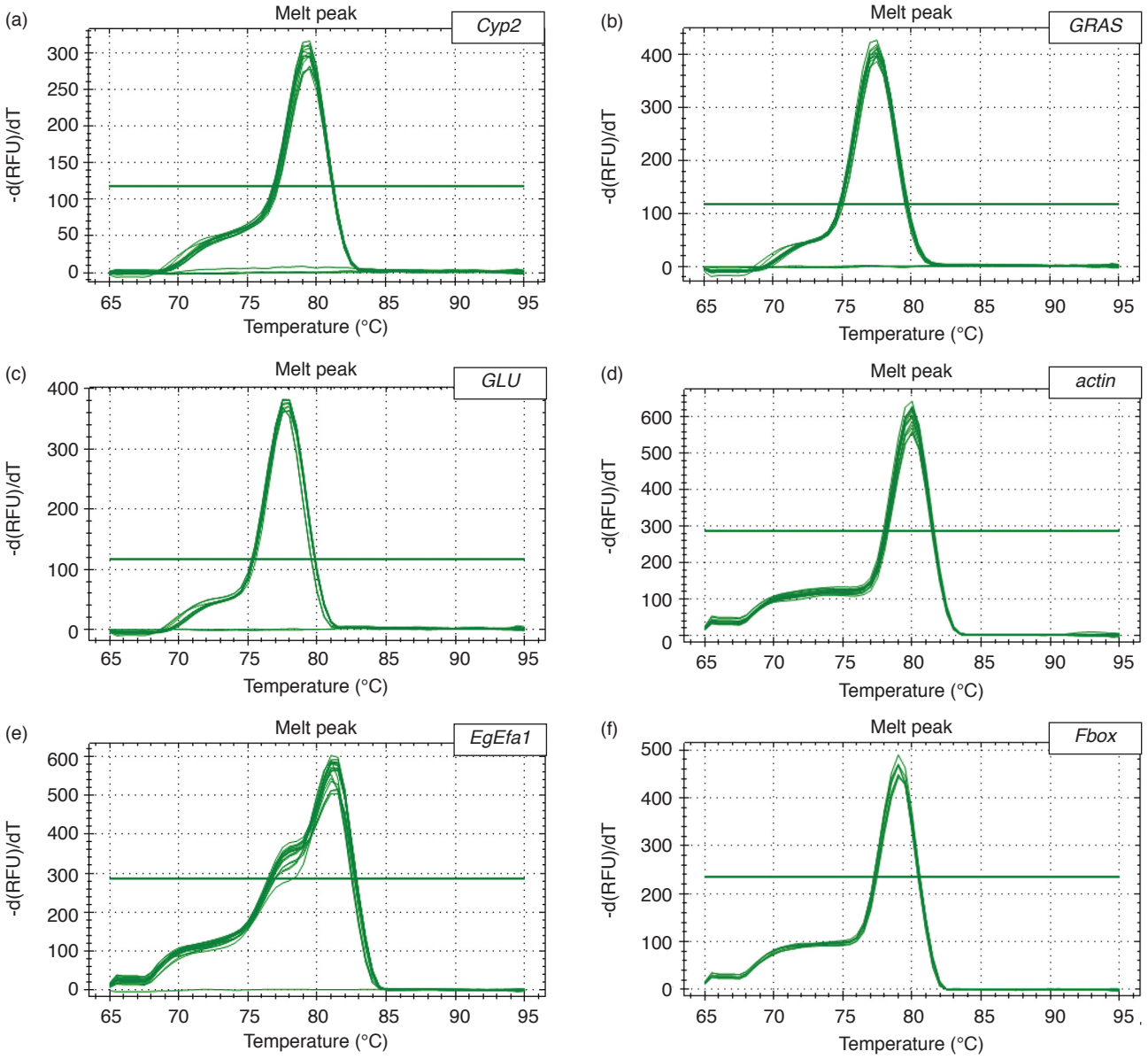

Figure 2. Amplicons melt curve analysis generated from leaf, node, internode, root, cabbage, shoot primordial and basal stem. (a) Cyp2, *(b)* GRAS, *(c)* GLU, *(d)* actin, *(e)* EgEfa1 *and (f)* F-box.

statistical software, on the other hand, used pair-wise correlation and regression analysis to rank the stability of the candidate reference genes. BestKeeper ranked the expression stability of the genes as follows; *GRAS*, *Cyp2*, *EgEfa1*, *F-box* and *GLU*. Two reference genes with the most stable expression, *Cyp2* and *GRAS* were determined based on correlation (*r* value) and BestKeeper index (*p* value) (*Table 3*). From this analysis, *Cyp2* and *GRAS* were concluded as suitable reference genes with high expression stability. Genes with lower expression stability were *EgEfa1*, *F-box* and *GLU*. Although two different softwares were used, similar results were obtained in terms of stability ranking. However, *actin* was excluded from the analysis due to the unspecific amplification of the primers.

DISCUSSION

The preferences of using qRT-PCR for gene expression profiling are prevalent especially for understanding gene function. The qRT-PCR is rapid, reliable and sensitive which is critical for gene expression studies. Nevertheless, the qRT-PCR accuracy and reliability are determined by many factors (Yeap *et al.*, 2013). A set of reference genes to normalise gene expression is essential to minimise the variability and achieve reliable and accurate results. However, the set of reference genes is species-specific and highly dependent on the type of tissue, treatment and experimental conditions. Thus, validation and selection of reference genes prior to gene expression profiling is important to

achieve high accuracy. Several statistical algorithm softwares have been developed to evaluate the stability of reference genes such as NormFinder, BestKeeper, geNorm, and qBase plus. Previous studies suggested that multiple reference genes are required for expression normalisation of target genes due to intrinsic variation (Sheshadri *et al*., 2018; Li *et al*., 2012; Xiao *et al*., 2012).

Gene expression profiling studies using qRT-PCR employed several reference genes to reduce intrinsic variation (Bustin *et al*., 2009; Li *et al*., 2012; Vandesompele *et al*., 2002; Expósito-Rodríguez *et al*., 2008). Basically, gene normalisation using optimal number of reference genes is evaluated by experimental conditions (Chandna *et al*., 2012). Normalisation using multiple reference genes would eliminate intrinsic variation and normalisation errors (Barsalobres-Cavallari *et al*., 2009; Xiao *et al*., 2012).

In the present study, several candidate reference genes for gene expression normalisation in oil palm tissues were validated and evaluated. The accepted amplification efficiency and coefficient correlation for qRT-PCR were 90.0% -110.0% and R^2 > 0.98 respectively. Amplification efficiency close to 100%, as determined by the slope value (desirable value of -3.3 to -3.8), indicates the reliability of the primer pairs to generate doubling increment of amplicons during each amplification cycle. The R^2 value reflects the regression lines were fitted to different template concentrations from serial dilutions (Tellinghuisen and Spiess, 2014; Pfaffl *et al*., 2004). The qRT-PCR melt curve analysis shows the amplification specificity of the candidate reference genes' primer pair. Single peak indicates that the amplification generated a single amplicon throughout the amplification reaction. This study demonstrated that the primer pair for *actin* gene was not specific and thus discarded from the analysis, although a single band was observed on agarose gel electrophoresis. This indicates that the sensitivity of qRT-PCR outperformed the conventional RT-PCR in studying gene expression.

NormFinder and BestKeeper, freely accessible softwares, were commonly used to select the most stable reference genes. The algorithm showed different ranking of the reference gene stability. The differences in stability ranking were due to differences in data interpretation by each software (Ransbotyn and Reusch, 2006). The genes were ranked by stability values from NormFinder and *r* value from BestKeeper. Nevertheless, each algorithm has its own advantages and limitations. Thus, to overcome the limitation of the algorithm, consensus ranking from both algorithm softwares was considered to determine the most stable reference genes (Yeap *et al*., 2013). Based on the consensus ranking from both algorithm softwares, the most stable reference genes for gene expression normalisation were *Cyp2* and *GRAS* whereas *GLU* was identified to be the least stable. The *Cyp2* and *GRAS* genes had the least variation in expression stability whereas *GLU* had the highest variation.

The reference gene *Cyp2* encoded an ubiquitous protein that was constitutively expressed in all subcellular compartments which is essential for many developmental processes and stress responsiveness involving signal transduction, protein transport, maturation, and mRNA processing (Romana *et al*., 2004). The reference gene *GRAS* encoded a protein involved in phytochrome A signal transduction and root/shoot development (Cenci and Rouard, 2017; Hirsch and Oldroyd, 2009). High expression in active tissues has been a major factor the preference of *GRAS* as a reference gene. Yeap *et al*. (2012) found that *Cyp2* and *GRAS* are highly stable and preferred as reference genes as they are not affected by abiotic stressed conditions including salinity, drought, submergence, cold and heat stresses in leaf discs.

In the present study, two years old nursery-stage clonal palm tissues were used to identify the most suitable reference genes for gene expression normalisation whereas Yeap *et al*. (2013) used 20 years old matured oil palm tissues that were selected based on seven years of yield records. The identification of the reference genes of *Cyp2* and *GRAS* were similar regardless the developmental stage of the oil palm.

CONCLUSION

Based on the comparison of statistical algorithm softwares, NormFinder and BestKeeper, *Cyp2* and *GRAS* are selected as the most suitable reference genes for gene expression normalisation in two-year old nursery-stage clonal palm due to their high expression stability. On the other hand, *GLU* gene displayed the highest variation and was the least stable among the reference genes tested and is thus not recommended to be used for normalisation in gene expression studies in oil palm vegetative tissues.

ACKNOWLEDGEMENT

The author would like to thank the Director-General of MPOB for permission to publish this article. We appreciate the help of Dr Ooi Siew Eng and Leslie Ooi Cheng Li of Breeding and Tissue Culture Unit, and Genomic Unit, Advanced Breeding and Biotechnology Centre (ABBC), MPOB, respectively, for critically reviewing this manuscript. We would also like to acknowledge all members of the Gene Functional Group of Biotechnology Functional Unit, ABBC, MPOB for their assistance.

REFERENCES

Andersen, C L; Jensen, J L and Orntoft, T F (2004). Normalization of realtime quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research, 64*: 5245-5250.

Barsalobres-Cavallari, C F; Severino, F E; Maluf, M P and Maia, I G (2009). Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions. *BMC Molecular Biology, 10*: 1.

Bustin, S A; Benes, V; Garson, J A; Hellemans, J; Huggett, J; Kubista, M; Mueller, R; Nolan, T; Pfaffl, M W; Shipley, G L; Vandesompele, J and Wittwer, C T (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry, 55*: 4.

Cenci, A and Rouard, M (2017). Evolutionary analyses of GRAS transcription factors in angiosperms. *Frontiers in Plant Science, 8*: 273.

Chandna, R; Augustine, R and Bisht, N C (2012). Evaluation of candidate reference genes for gene expression normalization in *Brassica juncea* using real-time quantitative RT-PCR. *PLoS ONE*, *7(5)*: e36918.

Chao, W S; Dogramaci, M; Foley, M E; Horvart, D P and Anderson, J V (2012). Selection and validation of endogenous reference genes for qRT-PCR analysis in leafy spurge (*Euphorbia esula*). *PLoS ONE, 7*: e42839.

De Spiegelaere, W; Dern-Wieloch, J; Weigel, R; Schumacher, V; Schorle, H; Nettersheim, D; Bergmann, M; Brehm, R; Kliesch, S; Vandekerckhove, L and Fink, C (2015). Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS ONE*, *10(3)*: e0122515.

De Ronde, M W; Ruijter, J M; Lanfear, D; Bayes-Genis, A; Kok, M G; Creemers, E E; Pinto, Y M and Pinto-Sietsma, S J (2017). Practical data handling pipeline improves performance of qPCR-based circulating miRNA measurements. *RNA, 23(5)*: 811-821.

Expósito-Rodríguez, M; Borges, A A; Borges-Pérez, A and Pérez, J A (2008). Selection of internal control genes for quantitative realtime RT-PCR studies during tomato development process. *BMC Plant Biology, 8*: 131.

Hirsch, S and Oldroyd, G E D (2009). Mini-review GRAS-domain transcription factors that regulate plant development. *Plant Signaling Behaviour, 4*: 698-700

Karuppaiya, P; Yan, X X; Liao, W; Wu, J; Chen, F and Tang, L (2017). Identification and validation of superior reference gene for gene expression normalization via RT-qPCR in staminate and pistillate flowers of *Jatropha curcas* - A biodiesel plant. *PLoS ONE*, *12(2)*: e0172460.

Kwan, Y M; Meon, S; Ho, C L and Wong, M Y (2016). Selection of reference genes for quantitative real-time PCR normalization in *Ganoderma*-infected oil palm (*Elaeis guineensis*) seedlings. *Australasian Plant Pathology, 45(3)*: 261-268.

Li, W; Zhang, L; Zhang, Y; Wang, G; Song, D and Zhang, Y (2017). Selection and validation of appropriate reference genes for quantitative real-time PCR normalization in staminate and perfect flowers of andromonoecious *Taihangia rupestris*. *Frontiers in Plant Science*, *8*: 729.

Li, Q; Fan, C-M; Zhang, X-M and Fu, Y-F (2012). Validation of reference genes for real-time quantitative PCR normalization in soybean developmental and germinating seeds. *Plant Cell Reports, 31*: 1789-1798.

Lim, F H; Fakhrana, I N; Rasid, O A; Idris, A S; Ho, C L; Shaharuddin, N A and PARVEEZ, G K A (2017). Molecular cloning and expression analysis of *Ganoderma boninense* cyclophilins at different growth and infection stages. *Physiological and Molecular Plant Pathology*, *99*: 31-40.

Martin, L B; Nicolas, P; Matas, A J; Shinozaki, Y; Catalá, C and Rose, J K (2016). Laser microdissection of tomato fruit cell and tissue types for transcriptome profiling. *Nature Protocols*, *11(12)*: 2376.

Murphy, D J (2014). The future of oil palm as a major global crop: Opportunities and challenges. *J. Oil Palm Res. Vol. 26(1)*: 1-24.

Navarro, E; Serrano-Heras, G; Castaño, M J and Solera, J (2015). Real-time PCR detection chemistry. *Clinica chimica acta*, *439*: 231-250.

Ooi, S E; Choo, C N; Ishak, Z and Ong-Abdullah, M (2012). A candidate auxin-responsive expression marker gene, *EgIAA9*, for somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.). *Plant Cell, Tissue and Organ Culture*, *110(2)*: 201-212.

Pfaffl, M; Tichopad, A; Prgomet, C and Neuvians, T (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnology Letters, 26*: 509-515.

Rahman, M N H A; Shaharuddin, N A; Nurniwalis, A W; Wahab, P E M; Abdullah, M O; Abdullah, N A P; Ghulam Kadir Ahmad Parveez; Jeremy, A R and Zubaidah, R (2016). Impact of paclobutrazol on the growth and development of nursery grown clonal oil palm (*Elaeis guineensis* Jacq.). *J. Oil Palm Res. Vol. 28(4)*: 404-414.

Ransbotyn, V and Reusch, T B (2006). Housekeeping gene selection for quantitative real-time PCR assays in the seagrass *Zostera marina* subjected to heat stress. *Limnology Oceanography Methods, 4*: 367-373.

Romana, P G N; Horton, P and Gray, J E (2004). The *Arabidopsis* cyclophilin gene family. *Plant Physiology, 134*: 1268-1282.

Sheshadri, S A; Nishanth, M J; Yamine, V and Simon, B (2018). Effect of melatonin on the stability and expression of reference genes in *Catharanthus roseus*. *Scientific Reports*, *8(1)*: 2222.

Singh, R; Ong-Abdullah, M; Low, E T L; Manaf, M A A; Rosli, R; Nookiah, R; Ooi, L C L; Ooi, S E; Chan, K L; Halim, M A and Azizi, N (2013). Oil palm genome sequence reveals divergence of interfertile species in Old and New worlds. *Nature*, *500(7462)*: 335.

Tellinghuisen, J and Spiess, A N (2014). Comparing real-time quantitative polymerase chain reaction analysis methods for precision, linearity, and accuracy of estimating amplification efficiency. *Analytical Biochemistry*, *449*: 76-82.

Thuc, L V; Sarpan, N; Ky, H; Ooi, S E; Napis, S; Ho, C L; Ong-Abdullah, M; Chin, C F and Namasivayam, P (2011). A novel transcript of oil palm (*Elaeis guineensis* Jacq.), Eg707, is specifically upregulated in tissues related to totipotency. *Molecular Biotechnology, 48*: 156-164.

Udvardi, M K; Czechowski, T and Scheible, W R (2008). Eleven golden rules of quantitative RT-PCR. *Plant Cell, 20*: 1736-1737.

Vandesompele, J; De Preter, K; Pattyn, F; Poppe, B; Van Roy, N; De Paepe, A and Speleman, F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3(7)*: 34.

Xia, W; Mason, A S; Xiao, Y; Liu, Z; Yang, Y; Lei, X; Wu, X; Ma, Z and Peng, M (2014). Analysis of multiple transcriptomes of the African oil palm (*Elaeis guineensis*) to identify reference genes for RT-qPCR. *J. Biotechnology*, *184*: 63-73.

Xiao, D; Zhang, N-W; Zhao, J-J; Bonnema, G and Hou, X-L (2012). Validation of reference genes for real-time quantitative PCR normalisation in non-heading Chinese cabbage. *Functional Plant Biology, 39*: 342-350.

Yeap, W C; Ooi, T E K; Parameswari, N; Harikrishna, K and Ho, C L (2012). *EgRBP42* encoding an hnRNP-like RNA binding protein from *Elaeis guineensis* Jacq. is responsive to abiotic stresses. *Plant Cell Reports, 31*: 1829-1843.

Yeap, W C; Loo, J M; Wong, Y C and Kulaveerasingam, H (2013). Evaluation of suitable reference genes for qRT-PCR gene expression normalization in reproductive, vegetative tissues and during fruit development in oil palm. *Plant Cell, Tissue and Organ Culture, 116(1)*: 55-66.

Zheng, W and Sun, L (2011). Evaluation of housekeeping genes as references for quantitative real time RT-PCR analysis of gene expression in Japanese flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunology, 30*: 638-645.

Zubaidah, R; Nurniwalis, A W; Siti Nor Akmar, A and Ghulam Kadir Ahmad Parveez (2017). The use of *Arabidopsis thaliana* model system for testing oil palm promoter: Case study on oil palm MT3-A promoter. *J. Oil Palm Res. Vol. 29(2)*: 189-196.

Zulkifli, Y; Norziha, A; Naqiuddin, M H; Fadila, A M; Nor Azwani, A B; Suzana, M; Samsul, K R; Ong-Abdullah, M; Singh, R; Ghulam Kadir Ahmad Parveez and Kushairi, A (2017). Designing the oil palm of the future. *J. Oil Palm Res. Vol. 29(4)*: 440-455.