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

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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.

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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
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 Spieghelere et al., 2015; Chandna et al., 2012).

There are several genes including cyclophillin 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 (Elaeis guineensis Jacq.) (Clone 8A/PL233/5/9AP1/4/8/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)₁₄ 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
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 primer pairs was verified by melt curve analysis by increasing the temperature from 65°C to 95°C. Amplification efficiency was calculated as $E(\%) = \left[10^{-\frac{1}{\text{slope}}} - 1\right] \times 100$ 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 ($2^{-\Delta C_q}$) ($\Delta C_q = \text{corresponding} \ C_q \text{ value} - \text{minimum} \ C_q \text{ value}$). 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
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
statistical software, on the other hand, used pairwise 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

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.
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 R² > 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 R² value reflects the regression lines were fitted to different template concentrations from serial dilutions (Tellingshuizen 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.

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