DETERMINATION OF REFERENCE GENES FOR NORMALISATION OF GENE EXPRESSION STUDY OF *Ganoderma*-INFECTED OIL PALMS

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ABSTRACT

Basal stem rot (BSR) disease is a major threat to oil palm industry in Malaysia, caused by pathogenic fungus, Ganoderma boninense. *Quantitative real-time polymerase chain reaction (qPCR) has become a favourable method in quantification the levels of gene expression involved in disease development. In this study, 14 oil palm reference genes were tested for their suitability as reference genes for qPCR analyses using oil palm root taken from six-month old seedlings (nursery sample) and 15-year old mature palms (field sample). Only six reference genes were subjected to stability test via RefFinder. The GRAS and ACTIN genes were ranked as the best reference genes for nursery sample, whereas, GAPDH and GvHK genes for field samples. These reference genes were used in the qPCR analysis for accurate normalisation. Thus, results obtained in this study emphasise the importance of validating the stability of the reference gene and proving the credibility and reliability of RefFinder in determining the most stable reference genes in each specific experiment or biological setting used.*

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is currently one of the most economically important plantation crops in Malaysia with 5.74 million hectares of cultivation area (MPOB, 2017; Nadzri and Ahmad, 2016).

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However, Malaysian oil palm cultivation suffers from the most devastating fungal disease known as basal stem rot (BSR), which is caused by *Ganoderma boninense*. Oil palm from different geographical origins has been reported to have different level of susceptibility towards *G. boninense* infection, which may be resulting from the genetic resistance of each palm (Durand-Gasselin *et al.*, 2005; Idris *et al.*, 2004). Thereby, several studies have been conducted to identify key genes and elucidate biological pathways involved in the disease development of oil palm (Tan *et al.*, 2013; Tee *et al.*, 2013; Yeoh *et al.*, 2012; 2013).

Quantitative real-time polymerase chain reaction (qPCR) is an analytical technique that has the ability to detect and precisely quantify levels of gene expression by measuring relative small amount of messenger ribonucleic acid (mRNA) in

cells (Sanders *et al.*, 2014). The major advantage of qPCR is that the technique can analyse large number of samples rapidly with significantly high degree of sensitivity and specificity. Hence, this technique has become one of the most preferred methods to quantitatively determine the presence of the gene of interest in a cell population under specific conditions (Gadkar and Fillion, 2013). In qPCR assay, the expression level of target genes is determined by measuring the changes of expression across samples in different conditions (healthy and disease). However, gene expression assay is often prone to variations and errors associated with sampling methods, RNA preparations, reverse transcription and amplification efficiencies of the target genes across different samples in different conditions, which would cause variability and affecting the assay results (Vandesompele *et al.*, 2002; Bustin, 2002; Andersen *et al.*, 2004).

Normalisation of gene of interest with internal calibrator known as reference genes, which need to have constant expression levels across different sample experimental conditions or treatments, is essential to minimise variations and errors that may rise from the comparison of gene expression assay, as well as avoiding misrepresentation of the gene of interest expression fold. In oil palm, several stable reference genes have been reported, however, each of the reference gene has different stability in different species, varieties, tissues, developmental stages, organ, and under different experimental conditions (Chan *et al.*, 2014; Xia *et al.*, 2014; Yeap *et al.*, 2014). Therefore, selection and validation of appropriate reference genes are essential in obtaining accurate results during qPCR analysis.

In common practices, the expression stability of the reference genes is evaluated and ranked using four major softwares, such as geNorm (Vandesomple *et al.*, 2002), NormFinder (Andersen *et al.*, 2004), BestKeeper (Pfaffl *et al.*, 2004) and delta Ct method (Silver *et al.*, 2006). Each of the software has equal capability in determining the stability of the tested reference genes. However, every program uses different mathematical calculation methods, causing the stability ranking of the genes to vary, hence, making it difficult to achieve a consensus of the most stable reference genes under a particular condition from all these softwares (Zhang *et al.*, 2015).

A web-based integrated tool known as RefFinder (leonxie.esy.es/RefFinder/), which incorporates these four major program algorithms (geNorm, NormFinder, BestKeeper and delta Ct method), provides solution to overcome the different limitations of each computational algorithm by determining the stability of the reference genes based on the consensus ranking from all the programs. RefFinder enables the assessment of the most stable reference genes by balancing the reference genes

ranking across these algorithms via calculating the geometric mean of the genes and determining the overall comprehensive stability value for each reference gene (Xie *et al.*, 2012). RefFinder has been reported to successfully determine the most stable reference genes in different plants such as sugarcane (de Andrade *et al.*, 2017) and tree peony (Li *et al.*, 2016). Moreover, this program has been proven to generate reference gene stability ranks that are in agreement with geNorm, NormFinder, BestKeeper and delta CT method (De Spiegelaere *et al.*, 2015). Thus, the objective of this study was to assess the most stable reference genes for qPCR analysis of six-month old oil palm seedlings (nursery sample) and 15-year old mature palms (field sample) under *Ganoderma*-infected condition using RefFinder program.

MATERIALS AND METHODS

Selection of Reference Genes Candidate

Fourteen oil palm reference genes that have shown stable expression across various oil palm tissues as reported by Chan *et al.* (2014), Xia *et al.* (2014), and Yeap *et al.* (2014) were selected. The details of the primers are shown in *Table 1*.

Selection of Planting Materials

The uniformity of the reference genes were validated using RNA samples extracted from roots of six-month old oil palm seedlings grown in the nursery and 15-year old mature palms grown in the field. The root samples were taken based on two different conditions: healthy samples (uninfected/control) and samples infected with *G. boninense* (infected). The infected samples were selected based on visual external symptoms such as the wilting of leaves and the formation of fruiting body on the oil palm stem. For the nursery samples, both the healthy and infected root tissues were sampled from six-month old oil palm seedlings grown at MPOB-UKM nursery, Bangi, Selangor in Malaysia. The inoculated palms were artificially inoculated with *G. boninense* for three months before the root samples were harvested at 12 weeks post inoculation (wpi). As for the field samples, the primary root tissues were sampled from 15-year old oil palm at two different locations; the healthy tissues were taken from oil palm plantation at Keratong, Pahang in Malaysia while the infected tissues were taken from oil palm plantation at Seberang Perak, Perak. The root tissues were cleaned with tap water and wiped using 70% ethanol to remove external contaminants from the samples. The samples were immediately frozen in liquid nitrogen and stored in -80°C until further use.

TABLE 1. LIST OF SELECTED REFERENCE GENES

Gene name	Abbreviation	Description	Primer Sequence (5'→ 3')	Product length (bp)	References
Actin	ACTIN	Cell division	F-TGCTGATCGTATGAGCAAGGAAA R-GAAATCCACATCTGCTGGAAGGT	147	Chan et al. (2014)
Glyceraldehyde 3- phosphate dehydrogenase	GAPDH	Energy provider	F-GATCGAGAAATCAGCCACGTATG R-GTCACCAATAAAGTCGGTGGACA	124 109	Chan et al. (2014) Chan et al. (2014
Alpha-tubulin 1	TUBULIN	Cell growth and elongation	F-CATGGCTTGCTGCCTTATGTATC		Chan et al. (2014)
Polyubiquitin	UBIQUITIN	Abiotic stress	R-AGGACACCAGTCAACAAACTGGA F-CCAGGCCAATCTCTCAGGATG R-GGGGGATGCCCTCTTTATCC	130	Chan et al. (2014)
NADH dehydrogenase subunit 5-like gene	NAD5	Electrons transfe	F-CATTTCTGGTTCACACGACTTCAG R-AGAGAGTAAAACGACCCGAAATCC	112	Chan et al. (2014)
Cyclophilin	CYC	Abiotic stress tolerance	F-AGGGCTGTATGGGGATGATGT R-GCTTTTACCTCCAGTTCCATTTC	166	Xia et al. (2014)
Eukaryotic elongation factor	eEF	Elongation	F-CTAGCTTTCGAGTATTTGGGTGTG R-ATGCTCTTCTCGCCTTCACTCT	91	Xia et al. (2014)
Eukaryotic initiation factor 4A	eIF	mRNA binding	F-CCTCACCTATACTCTTCCCACCA R-GTCATCGCCCAGGCACAG	214	Xia et al. (2014)
C-repeat binding factor	E8CBF	Low temperature response	F-GATAAGTGGGTCTGCGAGGT R-AATCGGCAAAGTTGAGGCA	148	Xia et al. (2014)
Gibberellin-responsive protein 2	GRAS	Organ and tissues development	F-CGGAAGAGTCACATCAATCG R-GGAGCAAGAAGCCAACAC	103	Yeap et al. (2014)
Pre-mRNA splicing factor SLU7	SLU7	Protein-nucleic acid interactions	F-TAATCCACACATTCCTCAGT R-GCACCTCTGTCATACCAT	129	Yeap et al. (2014)
Superoxide dismutase [Mn]	GvHK	Oxidative stress control	F-TCAACCACTCAATCTTCTGG R-CTTCTGCGTTCATCTTTTGC	139	Yeap et al. (2014)
Protein Kinase domain containing protein	Kinase	Signal transduction.	F-ACAATCTACTCATACATTCA R-TTATCATCCTCCAACCTA	278	Yeap et al. (2014)
Triosephosphateisomerase	TrioP	Glycolysis and energy production	F-AAGAAGATTGTCACTACC R-ATAAGGAATGCCAAGATT	213	Yeap et al. (2014)

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Total RNA Extraction, RNA Quality Assessment and First Strand cDNA

RNA from oil palm roots were extracted using cetyl trimetylammonium bromide (CTAB) protocol according to Zeng and Yang (2002), with some modifications. The extracted RNA was treated with DNase 1 using the RQ1 RNase-Free DNase Kit (Promega, Madison, WI, USA). The concentration and the quality of the total RNA were determined using NanoDrop® ND-1000 Analysis (Thermo Scientific, Wilmington DE). The RNA integrity was assessed on 1.5% (w/v) agarose gel electrophoresis. First strand cDNA was synthesised from 2 µg of total RNA in a 20 µl of reaction volume using GoScript™ Reverse Transcription Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Specificity Test Using PCR

Fourteen candidate of reference genes were subjected to specificity evaluations. The PCR was carried out in 20 µl reaction mixture containing 10× PCR buffer; 25 mM of $MgCl_2$ 10 mM of dNTP mix; 5 U µl^{-1} of *Taq* polymerase; 0.4 µM of each forward and reverse gene specific primers; 1.0 µl of cDNA and 11.5 µl of nuclease-free water. The mixture was run in Bio-RAD thermo cycler (Bio-Rad, USA) according to the following programs: 2 min initial denaturation at 95°C; followed by 35 cycles of 1 min at 94°C, 30 s at 61°C, 1 min at 72°C; a 5 min final extension at 72°C and hold at 15°C. The amplicons were electrophorised in 1.5% agarose gel at 80 V constant for 60 min, followed by ethidium bromide (EtBr) staining for 10 min, destained in distilled water for 10 min, and visualised the gel under UV light using Bioimaging System (UVP, USA). The specificity of the amplification product for each primer pair was determined by the production of a single product within the expected amplicon length. The primers that generated specific product were chosen for amplification efficiency test using qPCR.

Efficiency Test of Primer Amplification via qPCR

The efficiency of the primers amplification towards the root tissues from oil palm nursery and field samples was tested by constructing the primers standard curves using cDNA as template in four-fold dilution series: 100 ng, 25 ng, 6.25 ng, 1.56 ng and 0.39 ng corresponded to 10^0, 10^{-1}, 10^{-3} and 10^{-4}, respectively, while the specificity of primers towards each of oil palm tissues was further confirmed from the melt curve analysis. The qPCR was carried out using SensiFast™ SYBR® No-ROX One-Step Master Mix Kit (2×) (Bioline, USA) according to manufacturer's protocol and performed in triplicates. The primer efficiency test was carried out using Bio-RAD CFX96 Real-time PCR thermo cycler (Bio-Rad, USA) with the cycling conditions as follows: 95°C of initial denaturation for 2 min, 40 cycles of 95°C for 5 s and 61°C for 30 s; followed by dissociation curve programmed at 65°C at 5 s and 95°C for 5 s with a heating rate increment of 0.1°C per 1 s. Two negative controls were included; no reverse transcriptase (NRT) and non-template control (NTC). The result of the primer efficiency was analysed by using the Bio-Rad CFX Manager V3.0 software and the PCR amplification efficiency (E) was calculated based on the slope data of the primer pair's standard curve with the following formula: $E = [10^{(-1/slope)}-1] \times 100\%$ (Pfaffl, 2001). The primers pair that amplified with efficiencies ranging from 90%-110%, had R^2 values greater than 0.98, slope value between -3.6 and -3.1, and produced single peak from melting curve analysis were selected for stability validation assay.

Stability Ranking of Reference Genes Using RefFinder

The expression stability of the reference genes were evaluated using cDNA of healthy and infected oil palm tissues as a template. The expression levels of the reference gene candidates were assessed based on the number of amplification cycles needed to reach fixed cycle threshold (Cq) during exponential phase. The raw Cq values of all reference genes in root tissues at different conditions (infected and control) were subjected to expression stability evaluations using the RefFinder program (leonxie.esy.es/RefFinder/) (Xie *et al*., 2012). Two reference genes ranked as the most stably expressed genes were subsequently used for normalisation in the expression profiling studies of the gene of interest.

RESULTS

Primers Specificity

Fourteen DNA primers pair designed for the candidate reference genes were tested onto root cDNA of healthy and *Ganoderma*-infected oil palm using PCR. The result showed that out of 14 reference genes, only eight of the genes, namely *ACTIN*, *eEF*, *TUBULIN*, *eLF*, *GRAS*, *GvHK*, *GADPH*, and *UBIQUITIN* were able to amplify an intact single band product (*Figure 1*). The formation of a single band indicates that these DNA primers were specific in amplifying target regions from the root cDNA. However, the result also revealed that primers for *EgCBF*, *NAD5*, *CYC* and *SLU7* reference genes produced multiple bands, while primers for *TrioP* and *Kinase* genes were unable to amplify desired product from the template. The DNA primers that failed to amplify a single product were subjected to optimisation by amplifying the genes at several annealing temperatures ranging from
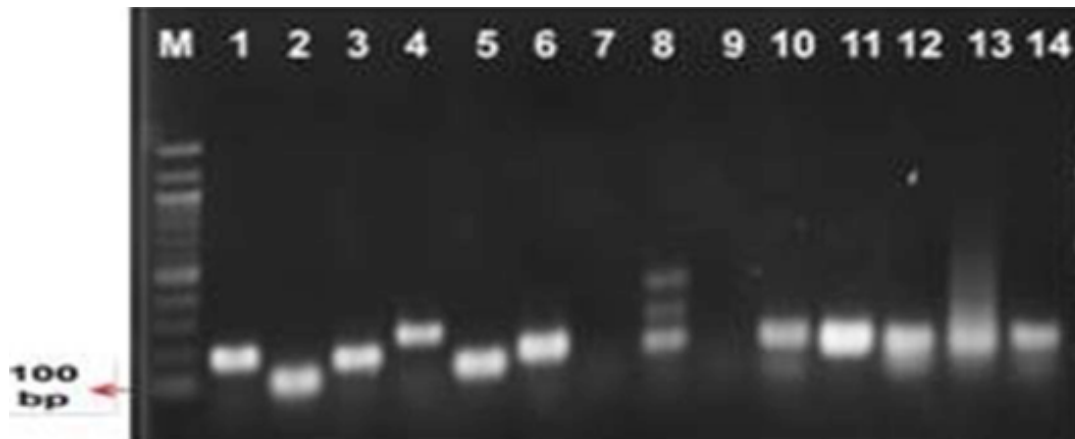
Figure 1. Polymerase chain reaction (PCR) amplification of the reference genes on oil palm root analysed on 1.5% (w/v) agarose gel. M: GeneRuler 100 bp (Fermentas, USA), Lane 1: ACTIN, Lane 2: eEF, Lane 3: TUBULIN, Lane 4: eLF, Lane 5: GRAS, Lane 6: GvHK, Lane 7: KINASE, Lane 8: SLU7, Lane 9: TrioP, Lane 10: CYC, Lane 11: GADPH, Lene 12: EgCBF, Lane 13: UBIQUITIN, Lane 14: NAD5.

55°C to 65°C to determine the optimal temperature (Fraga *et al.*, 2008). Nevertheless, the results obtained remained the same, indicating that the primers were unspecific toward the samples used in this study. Therefore, only the DNA primers that amplified intact single bands from the root samples were subjected to qPCR assay.

Amplification Efficiency Test

All eight DNA primer pairs of the reference genes (*ACTIN, eEF, TUBULIN, eLF, GRAS, GvHK, GADPH*, and *UBIQUITIN*) that could amplify a single PCR product were further evaluated via qPCR for the specificity and efficiency tests using melt curve and standard curve analyses before subjected to stability validation assay. The amplification efficiency (E) refers to the efficiency of the reaction in which *E*-value of 100% indicates that specific target product is amplified in each PCR cycle of the exponential phase (Fraga *et al.*, 2008). The correlation coefficient (R^2) value reflects the uniformity and a better fitting of the technical replicate for each cDNA serial dilution in generating experimental data in the standard curve, while the slope generated from the standard curve reflects the PCR amplification efficiency and a negative slope value indicates that the amplification efficiencies are comparable. According to the international MIQE guideline in conducting qPCR assay, the acceptable primer amplification efficiency is between 90% and 110% with good correlation coefficient value (R^2>0.98), slope value ranges from -3.6 to -3.1 and generate single peak in melt curve analysis (Bustin *et al.*, 2009).

Our result showed that six reference genes *ACTIN, GAPDH, TUBULIN, eEF, GvHK*, and *GRAS* generated single peak in melt curve analysis as depicted in *Figure 2*, confirming that the primer

pairs amplified specific products. Moreover, these genes also have amplification efficiency (*Table 2*) that ranges between 95.3% to 110.2% with R^2 value of 0.989 to 0.997 and slope value ranges -3.440 to -3.157, corresponded to the standard optimal result listed in MIQE guideline (Bustin *et al.*, 2009). However, as for the remaining two reference gene candidates, *eLF* and *UBIQUITIN* showed the production of non-specific products in both melt curve and standard curve analyses.

Standard curve of *eLF* reference gene showed the amplification efficiency was greater than 100%, which was thought to be influenced by the formation of two target products at temperature of 85°C and 89.5°C in the reaction (Fraga *et al.*, 2008). Meanwhile, for *UBIQUITIN* gene, a formation of primer dimer was observed at temperature between 75°C to 79°C, thus affecting the R^2 and forming positive slope, indicating that the amplification efficiency of *UBIQUITIN* is incomparable. Both primer sets for *eLF* and *UBIQUITIN* were optimised by adjusting the annealing temperature between 60°C to 70°C to increase the primer specificity and eliminate non-specific products. However, after optimisation step, the formation of non-specific products was still observed. The results indicated that the *eLF* and *UBIQUITIN* primer sets were unspecific in amplifying the targeted product from root tissues.

Reference Genes Stability Test via RefFinder

Six candidate reference genes (*ACTIN, GAPDH, TUBULIN, eEF, GvHK,* and *GRAS*) with efficient amplification were subjected to qPCR analysis using samples from the nursery and field at different treatment conditions (healthy and disease). The Cq values for each reference gene from all the root samples under different treatment conditions were calculated to determine the gene stability
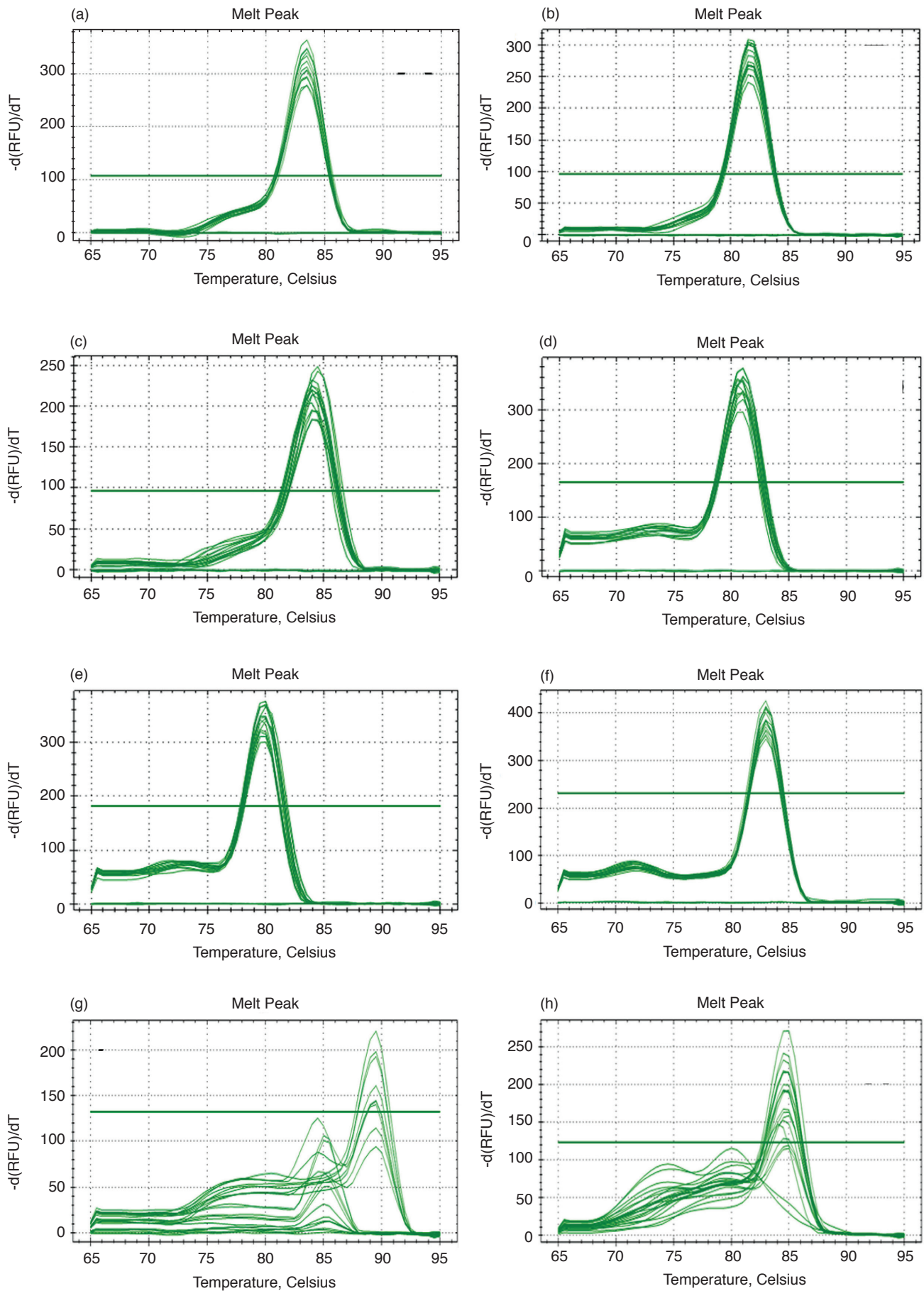
Figure 2. Melt curve analysis of the reference genes on oil palm root (a) ACTIN, *(b)* GAPDH, *(c)* TUBULIN, *(d)* eEF, *(e)* GRAS,
(f) GvHK, *(g)* eLF *and (h)* UBIQUITIN.

TABLE 2. SUMMARY OF PRIMER AMPLIFICATION EFFICIENCY TEST OF THE REFERENCE GENES FROM OIL PALM ROOT

Gene name	Efficiency (E, %)	Correlation coefficient (R²)	Slope	Melt temperature Tm (°C)
ACTIN	107.4	0.989	-3.157	83.5
GAPDH	99.1	0.996	-3.345	81.5
TUBULIN	110.2	0.997	-3.079	84.5
eEF	95.3	0.990	-3.440	81.0
GRAS	105.0	0.994	-3.209	80.0
GvHK	104.8	0.995	-3.212	83.0
eLF	144.0	0.888	-2.581	85.0/89.5
UBIQUITIN	97.2	0.320	0.641	77.0/84.5

using RefFinder. Based on the RefFinder result, the program ranked different reference genes for nursery and field samples as the most stable genes across different statistical algorithms as shown in *Table 3*.

For six-month old seedlings (nursery sample) *GRAS* was ranked as the most stable reference gene with stability value of 1.000 followed by *ACTIN* as the second most stable gene with stability value of 1.682 among the six reference gene candidates. The least stable reference gene was *eEF* with stability value of 6.000. The stability of the genes was arranged in a descending order as *GRAS > ACTIN > GvHK > GAPDH > TUBULIN > eEF*. Moreover, the stability ranking results generated from all four major algorithms (geNorm, NormFinder, BestKeeper and delta Ct method) revealed that all programs had

ranked *GRAS* as the most stable reference genes and *eEF* as the least stable reference genes.

As for the mature palms (15-year old field samples), RefFinder ranked *GAPDH* as the overall most stable gene with stability value of 1.414 followed by *GvHK* with stability value of 1.565. The least stable reference gene was *eEF* with stability value of 5.733. The stability of the genes was arranged in a descending order as *GAPDH >GvHK > GRAS > ACTIN > TUBULIN > eEF*. Slight differences in the ranking of the most stable genes were noticed among the four programs, where geNorm and NormFinder ranked *GAPDH* as the most stable reference gene followed by *GvHK*, while delta Ct method ranked *GvHK* as the most stable gene followed by *GAPDH*, and BestKeeper ranked *GAPDH* as the second stable reference gene whereas *GvHK* as the third stable reference gene. Meanwhile, geNorm, NormFinder, and delta Ct method ranked *eEF* as the least stable reference gene whereas BestKeeper ranked *eEF* as the second least stable reference gene and *TUBULIN* as the least stable reference gene.

DISCUSSION

BSR caused by *Ganoderma boninense* is the most economically disastrous disease to oil palm industry in Malaysia. In order to understand the molecular basis involved in plant response towards the fungal infection, studies with qPCR have been widely conducted for characterising gene expression patterns of oil palm defence mechanism towards the

TABLE 3. EXPRESSION STABILITY VALUE OF THE REFERENCE GENES FROM OIL PALM NURSERY ROOT IN ALL CONDITIONS AND THEIR STABILITY RANKING FROM DIFFERENT SOFTWARE ALGORITHMS INCORPORATED BY REFFINDER

Reference genes	RefFinder geomean ranking value		geNorm		NormFinder		BestKeeper		Delta CT method	
	Stability	Rank	Stability	Rank	Stability	Rank	Stability	Rank	Stability	Rank
Oil palm seedings root from nursery										
GRAS	1.000	1	0.133	1*	0.379	1	0.244	1	1.230	1
ACTIN	1.682	2	0.133	1*	0.418	2	0.267	2	1.263	2
GvHK	3.224	3	0. 340	2	0.485	3	0.472	3	1.316	3
GAPDH	3.722	4	0. 407	3	1.008	4	0.509	4	1.419	4
TUBULIN	5.000	5	1.208	4	2.126	5	1.753	5	2.495	5
eEF	6.000	6	1.769	5	2.677	6	1.991	6	2.892	6
Mature oil palm root from field										
GAPDH	1.414	1	0.706	1*	1.076	1	1.713	2	1.923	2
GvHK	1.565	2	0.706	1*	1.131	2	1.883	3	1.916	1
GRAS	3.224	3	0.963	2	1.217	3	2.156	4	2.034	3
ACTIN	3.344	4	1.948	4	2.066	5	0.404	1	2.630	5
TUBULIN	4.427	5	1.550	3	1.620	4	2.842	6	2.371	4
eEF	5.733	6	2.326	5	2.733	6	2.552	5	3.080	6

Note: *geNorm algorithm evaluates the expression stability value (M) of each reference gene based on mean pairwise variation between selected candidate reference genes. Therefore, the software will generate the average ratio of two best stable genes expression levels that are sufficient to be normalised in our study (Vandesompele *et al.*, 2002).

fungal disease (Tan *et al.*, 2013; Tee *et al.*, 2013; Yeoh *et al.*, 2012; 2013).

The accuracy of the relative quantification of gene expression levels using qPCR is highly dependent on the normalisation of the target genes with reference genes to minimise variation that arise during sample preparation and reaction. Theoretically, a good reference gene will express constantly with a minimal change of expression and its expression is independent regardless of any experimental condition and parameters. However, some studies revealed that oil palm reference genes expression are prone to undergo stability changes depending on the plant experimental conditions (Chan *et al.*, 2014; Xia *et al.*, 2014; Yeap *et al.*, 2014). Hence, preliminary evaluation and validation must be conducted in each experimental background to determine most stable reference genes to be used. In addition, it is recommended to use at least two reference genes for optimum normalisation of non-biological sample-to-sample variation introduced during gene expression analysis to avoid biases and misinterpretation of data that will yield inaccurate results (Nicot *et al.*, 2005).

Herein, the stability in reference gene expressions was evaluated on oil palm root seedlings and mature palms under two different conditions, healthy samples (uninfected/control) and samples infected with *G. boninense* (infected) via RefFinder. The RefFinder incorporates the

available major computational algorithms (geNorm, NormFinder, Bestkeeper and delta Ct method) that will allocate appropriate weight to each reference gene and calculates the geometric mean of the genes weights for determining the stability final ranking, named comprehensive ranking (Xie *et al.*, 2012). The selection of the most stable reference genes depends on the finding of reference genes with lowest Geomean value (de Andrade *et al.*, 2017).

In the present work, RefFinder analysis had ranked different set of most stable reference genes across different statistical algorithms for the nursery and field samples. *GRAS* and *ACTIN* genes are the best reference genes to be used in gene expression studies of oil palm seedlings (six-month old) whereas *GAPDH* and *GvHK* are the best reference genes for mature palms (*Figure 3*). Meanwhile, *TUBULIN* and *eEF* were ranked as the least stable reference genes by RefFinder across different statistical algorithms for both nursery and field samples. By comparing the stability ranking results generated from the four major algorithms, *i.e.* geNorm, NormFinder, BestKeeper and delta Ct method, all programs were in consensus to rank *GRAS* and *ACTIN* as the most stable reference genes for nursery samples, indicating high correlation among these comparative programs. However, in mature palm samples, ranking was slightly different among the four programs. These differences may have probably been affected by the sampling
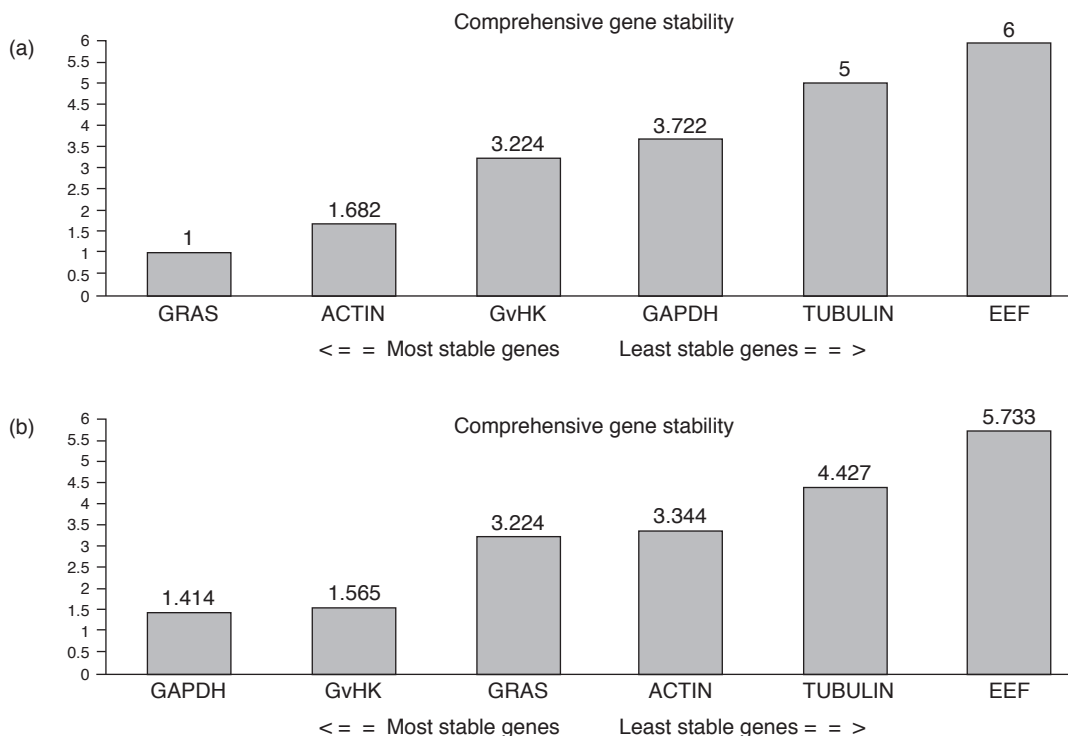

Figure 3. Expression stability of the reference genes in all conditions (healthy and disease palm) and their stability ranking generated by RefFinder. (a) Value of reference genes from nursery root with GRAS *and* ACTIN *ranked as the most stable reference genes and (b) value of reference genes from field root with* GAPDH *and* GvHK *ranked as the most stable reference genes.*

method; where each of the field sample collected for this study has different exposures toward variety of external abiotic stresses (*i.e.*, soil composition, water retention or rainfall condition, sunlight saturation, fertiliser, *etc*.), and biotic stresses (*i.e.,* insect, fungi, *etc*.) depending on their location in the plantation, in which these stresses also influence and contribute to the expression of the reference genes in oil palm (Yang *et al.*, 2011; Chan *et al.*, 2014; Ho *et al.*, 2018). The consensus ranking of reference genes calculated by RefFinder across all programs in both nursery and field samples showed the credibility and reliability of this program in determining the best reference genes to be used in normalisation of gene expression analysis.

Our results have shown that different reference genes were suggested for gene expression analysis at different stages of oil palm's development. The difference might be due to the age of oil palm used in this study where the root samples were taken from six-month old oil palm seedlings (nursery) and 15-year old mature palms (field). It has been reported that reference genes have different expression consistency across plant tissues under different developmental stages and different experimental or stress conditions (Chan *et al.*, 2014; Xia *et al.*, 2014; Yeap *et al.*, 2014). Several studies have assessed the stability of candidate reference genes in oil palm nursery samples, for example, Kwan *et al.* (2016) evaluated the stability of reference genes in three-month old oil palm seedling root tissues during *Ganoderma* infection, suggesting *NAD* (NADH dehydrogenase subunit 5-like), *Ubiquitin* and *MSD* (manganese superoxide dismutase) genes as the most stable. However, both *NAD5* and *UBIQUITIN* in our study exhibited unspecific amplification in root tissues during *Ganoderma* infection condition. Meanwhile, Yeap *et al.* (2014) reported the *GRAS* gene as the best reference gene in root vegetative tissues and reproductive tissues of six-month old oil palm seedling, in which consistent with our result of *GRAS* gene that was ranked as the most stable reference gene in the nursery samples. Hence, this explains *GRAS* gene has stable expression in six-month old oil palm seedlings (Yeap *et al.*, 2014). As for field samples, *GAPDH* that was ranked as the most stable reference gene was proven to be the most stable gene to be used for plantation samples that are exposed to variety of external stresses as it did not show any variation between different tissues under different experimental condition (Kozera and Rapacz, 2013).

Based from the RefFinder analysis, it was suggested to use two of the most stable reference genes (with lowest Geomean value) for qPCR assay normalisation in oil palm *Ganoderma*-infected root nursery and field samples as the accuracy of qPCR results will improve by the increasing number of reference genes used in a study (Nicot *et al.*, 2005;

Lim *et al.*, 2014). Moreover, the different set of stable reference genes generated by RefFinder for both oil palm nursery and field samples indicates that reference genes need to be validated before they can be used in normalisation of gene expression study, even though the samples are from the same plant tissues since the stability of each reference gene differs greatly depending on the plant ages, developmental stages or experimental conditions.

CONCLUSION

RefFinder is proven to be a suitable web-based program in determining the most stable reference genes for normalisation of qPCR analysis on oil palm samples of different ages and growth conditions. Consensus ranked generated by the RefFinder showed the most stable reference genes for both of the oil palm *Ganoderma*-infected root samples; *GRAS* and *GAPDH* were the best reference genes for nursery samples and field samples, respectively. Moreover, the stability ranking results obtained in this study highlighted the importance of validating the stability of the reference genes in each specific experiment or biological setting used; even the samples are of the same plant tissues.

ACKNOWLEDGEMENT

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