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ANALYSIS OF GENE EXPRESSION BY EST FROM SSH LIBRARY IN DWARF OIL PALM

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ABSTRACT

Six subtracted cDNA libraries were constructed from PS1 and AG1 oil palm breeding lines using Suppression Subtractive Hybridisation (SSH) method. Six putative clones identified to be associated in dwarfism encodes for brassinosteroid biosynthesis-like protein (DWF1), brassinosteroid insensitive 1-associated receptor kinase 1 precursor (BRI1), late elongated hypocotyl protein (LHY), gibberellin receptor (GID1), sterol 24-methyltransferase 1 (SMT1) and E3 ubiquitin-protein ligase (E3Ub) were analysed. These candidate dwarfing genes are involved in various stages of brassinosteroids (BR), gibberellins (GA) biosynthesis and signaling pathways for plants growth and development. The expression levels of candidate genes were validated by quantitative Real-time PCR (qRT-PCR) by two reference genes, manganese superoxide dismutase (PD569) and predicted protein IFH-1 like (EA1332). Higher expression level of BRI1, LHY and SMT1 genes were observed in dwarf palms compared to standard palms with normalised fold-difference of 2.3285, 1.5620 and 4.9044, respectively. Statistical analysis of all potential transcripts showed further evidence of SMT1 as a potential molecular marker for the screening of dwarf palm planting materials with highly significant expression levels in dwarf palms compared to standard palms; however, the expression levels of BRI and LHY genes were statistically insignificant.

Keywords: dwarfism, Suppression Subtractive Hybridisation (SSH), brassinosteroids (BR), gibberellins (GA), quantitative Real-time PCR (qRT-PCR), expression level.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is currently the most versatile and important crop providing an alternative for cheaper and environment-friendly daily needs. In 1958, oil palm was ranked in the 10th placed among major oils and fats producing crops such as soyabean, cottonseed, groundnut, sunflower, rapeseed, corn, coconut, safflower, olive, castor, sesame and linseed seeds (Ramli, 2011). Later, in 2009, oil palm has preceded other oil-bearing crops becoming the world major producers in oils and fats (Ramli, 2011). It is estimated that 80% of palm oil is used in foods (Shahriza *et al*., 2010), while the non-edible oil palm by-products include soaps, candles, detergent, lubricant, biodiesel, paper, fertilisers, activated carbon, inks, furniture, polishing liquids and cosmetics. Therefore, maintaining the sustainability of the oil palm has to be a key factor to support the rapid growth of world's population of the future. In order to achieve this, many strategic plans have been developed to improve the oil palm productivity such as developing new varieties of dwarf palms with novel traits. One of the major

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concerns is to develop the molecular marker for detection of oil palm dwarfism. Identification of dwarfing genes in oil palm by molecular approach could be the starting point for screening of dwarf varieties. Dwarf plants have many advantages in agriculture particularly the ease of harvesting, produce denser growth, reduce loss during harvesting, reduce overall production and harvesting costs, and increase resistance to rain and storm damage (Qin *et al*., 2008; Kovi *et al*., 2011; Zhang *et al*., 2011a). Besides, the quality of fruit clusters is much well preserved. As such, the interest in breeding dwarf palms has great significance towards the sustainability of oil palm and further elevates the oil palm industries in Malaysia.

Previous studies have successfully elucidated the role of brassinosteroids (BR) that regulates the morphogenesis, such as cell division and elongation. The genes were present in vegetative tissues such as leaves, shoots and stems which have undergone the early developmental stage. Therefore, the use of spear leaves is appropriate to develop the dwarfing gene libraries. There are several other publications that have successfully identified potential markers for dwarfism extracted from leaves such as *Musa acuminata* (Chen *et al*., 2016), *Arabidopsis thaliana* (Chung *et al*., 2010), and chestnut (Xu *et al*., 2006). To date, there is limited information on dwarfing genes in oil palm. The efforts towards dwarf palms production were only restricted to the conventional method of palm breeding, *i.e*. by introgression of dwarf genes from 'Dumpy' palm into other oil palm population to generate dwarf palms with desirable traits. Various studies have successfully developed molecular markers for dwarf genes in other crops such as rice high-tillering, dwarf 1 and 2 (*htd-1* and *htd-2*) genes (Zou *et al*., 2005; Liu *et al*., 2009), rice dwarf mutant [*d162*(t)] gene (Zhang *et al*., 2011b), rice plant height 1 (ph1) which encodes a chitin-inducible gibberellin-responsive protein (CIGR) (Kovi *et al*., 2011), spring wheat reduced height (*Rht-B1b* and *Rht-D1b*) genes (Chapman *et al*., 2007), Chinese wheat reduced height (*Rht-B1b, Rht-D1b* and *Rht8*) genes (Zhang *et al*., 2006), barley semi-dwarfing (*sdw1* and *ari-e*.GP) genes (Chloupek *et al*., 2006), barley *HvDWARF* and *HvBAK1*/*SERK3* (Gruszka, 2011), barley *sdw1* and dwarf-denso genes (Jia *et al*., 2009; Ren *et al*., 2010) and *Brassica napus* dwarf mutant (*bnaC.dwf*) gene (Zeng *et al*., 2011). The molecular markers developed from these studies have provided significant advantage for selection of dwarf varieties in various plant species via marker assisted selection and genetic engineering.

Dwarf genes have been reported to be associated with plant growth hormones such as BR and gibberellins (GA). BR deficient dwarf mutants include *A. thaliana dwarf1*/*diminuto1* (dwf1; also called *dim* or *cbb1*) (Hossain *et al*., 2012), constitutive photomorphogenesis and dwarfism (*cpd*/*dwf3*), *dwf4*, *dwf5*, de-etiolated2 (*det2*/*dwf6*) (Chung *et al*., 2010), *ste1*/*dwf7*, pea *lka* and *lkb*, tomato *dwarf* (Bishop *et al*., 1999), *brassinosteroid-insensitive1* (*BRI1*; also called *cbb2*), and *brassinosteroid light and sugar1* (*bls1*) had short hypocotyls and concomitantly reduced cell elongation. Molecular genetic analyses have revealed a number of GA biosynthesis mutants such as *ga1, ga2, ga3, ga4* and *ga5* (Thomas *et al*., 2005). A novel dominant mutant, GA-insensitive dwarf1-1D (*gsd1-1D*) from *A. thaliana*, which regulates the bioactive GA level in *A. thaliana* has been screened and isolated (Soh, 2006). This mutant exhibited the characteristics phenotypes of GA-deficient mutants such as semi-dwarfism, dark-green leaves, late-flowering, and reduced fertility.

In this study, total ribonucleic acid (RNA) was extracted from leaf tissues to isolate the differentially expressed genes related to dwarfism using Suppression Subtractive Hydridisation (SSH) approach. The SSH procedure has provided an alternative strategy which is able to generate higher success rate compared to other methods such as Northern blotting and DNA fingerprinting. Identified genes associated with height will be cloned and sequenced to establish their expression profiles, and further validated using the quantitative Real-time PCR (qRT-PCR). In future, the molecular markers for height trait isolated from this study can be utilised to screen out the oil palm seedlings to predict dwarf population at the early stage. These hybrid progenies that possess slower height increment trait will subsequently reduce the overall production costs, increase crops productivity, simplify harvesting, and improve the quality and productivity of oil palm.

MATERIALS AND METHODS

Plant Materials

The spear leaves were obtained from the commercial *Dura* x *Pisifera* (DxP) MPOB Planting Series 1 (PS1) at the MPOB Research Station, Bangi Lama, Selangor, Malaysia and FELDA AGI at FELDA Plant Breeding Section (PPPTR), Jerantut, Pahang, Malaysia in December 2010. PS1 spear leaves were collected from standard palms, 409-1206 with a height increment of approximately 40 cm yr^{-1}, and dwarf palms (303-209, 303-210, 303-211) with height increment of 20 cm yr^{-1}; with the palm age of 12 years old. AGI spear leaves were collected from standard palms (AG1-9, AG1-13, AG1-44) with a height increment of approximately 68 cm yr^{-1}, and dwarf palms (AG1-4, AG1-12, AG1-22) with height increment of 31 cm yr^{-1}; with the palm age of 10 years old. The leaf tissues were cleaned with distilled water, cut into smaller fragments, immediately frozen in liquid nitrogen, and stored at -80°C.

Total RNA Extraction

Total RNA extraction was carried out using modified method of Prescott and Martin (1987). This method took three days to complete total RNA recovery. Ten grams of leaf tissues were pulverised into finely-ground powder in liquid nitrogen using pre-chilled mortar and pestle. The frozen powder was transferred into 20 ml cold extraction buffer containing 50 mM of Tris hydrochloride (Tris-HCl; pH 9), 150 mM of lithium chloride (LiCl) (Sigma-Aldrich®, Germany), 5 mM ethylenediaminetetra acetic acid (EDTA; pH 8) (Gibco BRL, USA), 5% (w/v) sodium dodecyl sulphate (SDS) (Merck, Germany), 2 mM aurin tricarboxylic acid (Merck, Germany) and sterile RNA water, followed by the addition of 2-Mercaptethanol (β-ME) (Sigma-Aldrich®, Germany) mixture. Next, $^1/_2$ volume of cold phenol (AppliChem, Germany) and $^1/_2$ volume of chloroform (Systerm®, Malaysia) were added and immediately homogenised using POLYTRON® PT 3100 homogeniser (Kinematica, INC., USA). Other extraction solution used in this method include chloroform:isoamylalcohol (24:1). The total RNA was washed and precipitated with cold 2 M LiCl for the next two days. On the final day, the RNA solution was pelleted by centrifugation at 10 000 g for 30 min at 4°C, and the pellet was washed with 2 M LiCl. Finally, the pellet was resuspended in 1 ml of ice-cold diethyl pyrocarbonate (DEPC)-treated water (Fermentas, USA) until fully dissolved. The extracted total RNA were further treated using RNase-free DNase Set (QIAGEN) according to the manufacturer's instructions to remove genomic DNA contaminants.

Quantification and Qualification of Total RNA

The extracted total RNA were further separated by running the 1% (w/v) agarose gel electrophoresis at 80 V for 1 hr 15 min. The gel was visualised under ultraviolet (UV) light and analysed using the GeneSnap Tool software (Syngene International Ltd, USA).

NanoDrop® ND-1000 (Thermo Scientific, Wilmington DE) was further used to estimate the quantity and quality of the total RNA that have been isolated. For valid expression study using qRT-PCR, RIN value of each total RNA were determined using Agilent's 2100 Bioanalyser (Agilent Technologies, Germany). The analysis was carried out using Agilent RNA 6000 Nano Kit (Agilent Technologies, Germany) according to manufacturer's instructions.

Suppression Subtractive Hybridisation (SSH)

SSH was carried out using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA) in accordance to the manufacturer's instructions. In this protocol, both purified total RNA populations were converted into cDNA. The cDNA from the dwarf palms was represented as 'tester', while cDNA from the standard palms as 'driver'. The control poly A+ RNA (from human skeletal muscle) provided in the kit was performed alongside with the 'tester' and 'driver' to examine the efficiency of SSH. The PCR products from primary and secondary PCR were analysed by 2% (w/v) agarose gel electrophoresis.

Construction of cDNA Libraries

The subtracted cDNA (secondary PCR products) were cloned directly into the yT&A cloning vector (Yeastern Biotech, Co., Ltd, Taiwan). The described cloning protocol was based on the manufacturer's instructions. The cloning procedure was carried out in 10 μl reaction mixture containing 3 μl of subtracted PCR products, 1X of ligation buffer A, 1X of ligation buffer B, 5 ng of yT&A cloning vector, 0.6 U of YEA T4 DNA ligase and 2 μl of deionised water.

The ECOS™ 101 competent cells (Yeastern Biotech Co., Ltd, Taiwan) containing DNA were plated on Luria Bertani (LB) (GIBCO-BRL, USA) media containing 50 μg ml^{-1} of ampicillin (Sigma®, Missouri), 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (MBI Fermentas, USA) and 50 μg ml^{-1} of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (Fermentas, USA). This procedures was carried out in the dark because X-Gal is light sensitive. The plates at 37°C in the dark for 12-16 hr. Single colony was inoculated into 7 ml of LB broth (GIBCO-BRL, USA) supplemented with 50 μg ml^{-1} of ampicillin and grown overnight at 37°C with agitation in shaker incubator.

Plasmids DNA were isolated and purified using the QIAprep Spin Miniprep Kit (QIAGEN, USA) according to the manufacturer's instructions. Plasmids were then digested with *Hin*dIII (Thermo Scientific, USA) restriction enzyme to screen for the presence of inserts. Digestion of plasmid DNA was carried out in 20 μl of reaction mixture containing 4 μl of plasmid DNA, 13 μl of nuclease-free water, 0.5 U of *Hin*dIII enzymes and 2 μl of buffer R. The mixture was incubated at 37°C overnight, and analysed by 1% agarose gel electrophoresis.

DNA Sequence Analysis

Plasmids harbouring putative transcripts were sent for DNA sequencing at the First BASE Laboratories Sdn Bhd, Malaysia. Quality control of sequence data was processed using Phred program (Ewing *et al*., 1998) to call bases with cut-off value of ≥ 20. The output sequences were manually edited to remove the poly A and poly T tracks, linearised vector and adaptor sequences. The clean sequences were collected and assembled by CAP3 in BioEdit

program. The consensus sequences were then compared to the non-redundant (nr) database in the GenBank databases using the BLASTX algorithms. Sequences with a BLASTX cut-off E-value ≤ 1e-05 were considered as significant. The Gene Ontology terms for functional, process and cellular categorisation were assigned by Blast2GO program (Conesa and Götz, 2008).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Primer designing. Primer pairs were designed to specifically amplify the region of interest, namely, the selected putative dwarfing genes. Primers were designed manually based on several important criteria for qRT-PCR based on Fraga *et al*. (2008). All primer sequences were subjected to BLASTX search to confirm specificity of the target genes prior to synthesis. The primer sequences used for qRT-PCR are summarised in *Table 1*.

Selection of candidate reference genes. Five reference genes (Bustin *et al*., 2009) were selected to normalise the qRT-PCR data: actin (ACTIN), polyubiquitin (UBIQUITIN), predicted 40S ribosomal protein S27-2 (PD380), predicted protein IFH-1 like (EA1332) and manganese superoxide dismutase (PD569). The reference genes' primers are summarised in *Table 2*.

cDNA synthesis. The cDNA was synthesised from 2 μg of total RNA in a 20 μl of reaction volume using the High Capacity Reverse Transcription Kit (Applied Biosystem, USA) according to the manufacturer's instructions. Three reaction mixtures for reverse transcription (RT), non-reverse transcription (NRT) and non-template control (NTC) were performed. The thermal cycler conditions were programmed as follows: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C for 10 min. The cDNA samples were stored at -20°C before further use.

TABLE 1. LIST OF PRIMER SEQUENCES FOR THE POTENTIAL CANDIDATE DWARFING GENES

Gene ID	Primer ID	Sequence (5′ --- 3′)	Annealing temperature (°C)	Amplicon length (bp)	Guanine-Cytosine (GC) content (%)
Gibberellin receptor GID1	GID1-F	GCTGCCGAGGTGTCTGGTG	65	141	68.4
	GID1-R	AAGAGCTCGATACCGTGGTG			55.0
Brassinosteroid insensitive 1-associated receptor kinase 1 precursor	BRI1-F	CCTCACCCTTTAAAAGCCTGAC	63	144	50.0
	BRI1-R	CAACTTGCTGACCCTTCTCTTG			50.0
Late elongated hypocotyl protein	LHY-F	CCATACTGGATAGGCATGAG	62	130	50.0
	LHY-R	CCTCCTCAGAGACTGCTTTC			55.0
Sterol 24-methyltransferase 1	SMT1-F	CCGAGCAAGGAAGAAGTAC	65	125	52.6
	SMT1-R	TTGCTCCGGCAGGCAGTTC			63.2
E3 ubiquitin-protein ligase	E3Ub-F	TCCCTCGCCTCCCAATTAC	63	144	57.9
	E3Ub-R	GATAACAGAACGTGTGCTCTC			47.6
Brassinosteroid biosynthesis-like protein	DWF1-F	GGGACCAGTGGTGGTTCAG	63	134	63.2
	DWF1-R	CAAGAGCATCCCCAACTTTG			50.0

TABLE 2. LIST OF REFERENCE GENES' PRIMER SEQUENCES FOR qRT-PCR

Gene ID	Primer ID	GenBank accession number	Sequence (5′ --- 3′)	Annealing temperature (°C)	Primer length (bp)	Primer GC (%)	Product size (bp)
Actin	ACTIN-F	AY550991	TGCTGATCGTATGAGCAAGGAAA	62.0	23	43.48	147
	ACTIN-R		GAAATCCACATCTGCTGGAAGGT		23	47.83	
Polyubiquitin	UBIQUITIN-F	EL689143	CCAGGCCAATCTCTCAGGATG	63.0	21	57.14	130
	UBIQUITIN-R		GGGGGATGCCCTCTTTATCC		20	60.00	
Predicted 40S Ribosomal protein S27-2	PD380-F	EY397675	GATGGTTCTTCCGAACGATATTGA	63.0	24	41.67	113
	PD380-R		TCACATCCATGAAGAATGAGTTCG		24	41.67	
Predicted protein IFH-1 like	EA1332-F	EY406625	AAACGAAGGTACGGCAAGTACAAG	60.0	24	45.83	111
	EA1332-R		CTTAGCACATGCAGAGCAGATGTT		24	45.83	
Manganese Superoxide dismutase	PD569-F	EL682210	CACCACCAGACGTACATCACAAA	60.0	23	47.83	129
	PD569-R		GATATGACCTCCGCCATTGAACT		23	47.83	

Note: qRT-PCR – quantitative Real-time polymerase chain reaction. GC– Guanine-Cytosine.

Two microlitres of RT-cDNA from each tested sample were pooled in a fresh 1.5 ml microcentrifuge tube (Eppendorf, Germany) to prepare for the stock of pooled cDNA. Stock of pooled NRT-cDNA and NTC-cDNA were also prepared. The stock of pooled RT-cDNA, NRT-cDNA and NTC-cDNA were each diluted to 1 ng and 4 ng. Then, the diluted RT-cDNA stock were further diluted to five data points by two-fold serial dilutions (1 ng, 2 ng, 4 ng, 8 ng and 16 ng) using nuclease-free water (Fermentas, USA). As for NRT and NTC, the stock cDNA from each reaction were diluted to 16 ng. These diluted cDNA samples were then subjected to primer efficiency test.

Primer efficiency test. The five point dilutions of pooled RT-cDNA were mixed with KAPA SYBR® Fast qPCR Kit Master Mix (2X) Universal (KAPA Biosystems, USA) according to manufacturer's instructions. As KAPA Master Mix (2X) is light-sensitive, this procedure was performed under a very dim light, and the reaction plate was covered with the aluminum foil throughout this experiment. The same procedure was carried out for the diluted pooled NRT-cDNA and NTC-cDNA.

A total of 20 μl reaction mixture containing 5.2 μl of nuclease-free water, 0.2 μM of forward and reverse gene specific primers: 1) gene of interest (GOI) and 2) reference (RF) genes, 1X KAPA SYBR® Fast qPCR Kit Master Mix (2X) Universal and 4 μl of cDNA template was performed in triplicates in MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, USA). The surface of the plate was sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems, USA), and briefly centrifuged to collect the content and eliminate any air bubbles. The qRT-PCR for primer efficiency test was carried out using Eppendorf Mastercycler® ep *realplex* (Eppendorf, Germany), and analysed by Realplex software version 2.2. The mastercycler conditions were programmed as follows: 95°C for 3 min, 40 cycles of 95°C for 3 s and 60°C (may vary depending on primer's annealing temperature) for 20 s, and a melting curve program at 95°C for 15 s, 60°C at 15 s and 95°C for 15 s, with a heating rate of 0.4°C per 1 s. The output data were imported to Microsoft Excel for further analysis.

The qRT-PCR amplification efficiencies of all tested primers were calculated using Pfaffl efficiency (E) formula (Pfaffl, 2001). The percentage of PCR amplification efficiency for each gene was calculated using the following formula:

$$E = 10^{[-1/\text{slope}]} - 1$$ (Bustin *et al*., 2009)

Relative quantitation of gene expression. A new set of cDNA samples were prepared for validation of putative GOI. First, each cDNA sample of RT, NRT and NTC reactions were diluted to a 2.5 ng μl^{-1}. The same procedure was carried out using KAPA SYBR® Fast qPCR Kit (KAPA Biosystems, USA) in accordance to manufacturer's instructions. Following the qRT-PCR, the average C_t values of each sample at 10 ng per reaction for GOI and RF genes were selected for analysis. Then, the average C_t values were converted to relative quantities for geNorm (version 3.4) input (Vandesompele *et al*., 2002). The relative quantities for each gene were calculated based on the comparative Ct method based on following equation:

$$\text{Relative quantities for each gene} = E^{(\text{minimum } C_t - \text{sample } C_t)}$$

The *E* value of two is equivalent to 100% efficiency. The highest relative quantities for each gene were set to 1. Then, the most stable reference genes were determined using geNORM (Vandesompele *et al*., 2002). The analysis was performed according to geNORM user manual available in geNORM website: http:// http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf.

Normalised GOI expression levels. The normalised GOI expression levels were calculated based on the geNORM manual according to the method described by Vandesompele *et al*. (2002). The normalised GOI expression levels for each tested sample from SSH hybrid were compared to verify the expression levels of dwarfing genes. Standard bar charts which represent each dwarfing gene were constructed to illustrate the expression levels in dwarf and standard palms. The GOI that is highly expressed in standard palm compared to dwarf palm signifies the false positive.

Fold-difference of the normalised expression levels between two compared samples was determined using the following formula:

$$\text{Normalised fold-difference} = GOI_{norm} \text{ of dwarf palm} / GOI_{norm} \text{ of standard palm}$$

Statistical Analysis of the Differentially Expressed Genes

Statistical analysis using SPSS 14.0 software was carried out to validate the efficacy of qRT-PCR output. The paired t-test was used to examine the difference in the expression levels of GOI in dwarf and standard palms. As this test does not require the exploratory data analysis for normality testing before the t-test, therefore, any outliers in the data were deselect before proceeding to the paired samples t-test analysis. The hypothesis for each GOI was formulated as follows:

i) Null hypothesis, H_0 = there is no difference in GOI expression levels in dwarf and standard palms.

ii) Alternative hypothesis, H_1 = GOI is differentially expressed in dwarf palms than in standard palms.

Once the statistical analysis has proven that the GOI expression levels in dwarf and standard palms were significantly different, the normalised GOI expression levels in all tested samples were computed using a calibrator (dwarf palm that showed significant GOI expression) as the control baseline (1.0000 expression level). The GOI expression level below 1.0000 indicates as down-regulation; and above 1.0000 is up-regulation.

RESULTS AND DISCUSSION

Qualification and Quantification of Total RNA

A clean and intact RNA is crucial for functional genomic studies. However, it is made difficult due to the presence of large quantities of polysaccharides and phenolic compounds (MacRae, 2007; Li *et al*., 2011). In this study, RNA extraction method established by Prescott and Martin (1987), with slight modification was carried out. Total RNA samples showed distinct 28S, 18S and ribosomal RNA (rRNA) bands, indicating effective isolation of total RNA (Intan Nur Ainni *et al*., 2014). The two intense 28S and 18S bands at approximately 2.1 and 1.9 kb respectively were observed (*Figure 1*). The RNA absorbance of each sample was determined at multiple wavelengths at 230, 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE). The $A_{260/280}$ ratio of all RNA samples obtained was in the range of 2.0 - 2.1; indicating that the RNA samples were of high quality and free from protein contaminants. The $A_{260/230}$ ratio of all samples ranged from 1.8 to 2.2, indicating low polysaccharide and phenol contaminations. Total RNA assessment using Agilent 2100 Bioanalyser showed RNA integrity (RIN) value ranged between 6.7 to 7.1 (*Table 3*). Generally, in order to establish a good expression data, a good quality RNA of $A_{260/280}$ ratio ranging from 1.8 to 2.0; and RIN value of at least 5 is acceptable for further downstream applications (Fleige and Pfaffl, 2006).

Construction and Analysis of SSH cDNA Libraries

The construction of cDNA library was established by SSH method using a small amount of good quality total RNA samples isolated from PS1 and AG1 spear leaf tissues. A total of six cDNA libraries using PS1 and AG1 populations were constructed using SSH. The cDNA which carry the specific and unique transcripts (*i.e.* the cDNA from dwarf palms: 303-209, 303-210, 303-211, AG1-4, AG1-12 and AG1-22) were represented as 'tester', and reference cDNA (*i.e.* the cDNA from standard palms: 409-1206, AG1-9, AG1-13 and AG1-44) were represented as 'driver'. The control skeletal muscle was used as PCR control sample, and performed alongside with the experimental cDNA to determine the efficacy of the subtraction. The *Rsa* I digestion has generated fragments ranging from 500 to 1500 bp. The subtracted and unsubtracted cDNA from the experimental samples showed significant difference in banding patterns based on the analysis of PCR products on 2% (w/v)

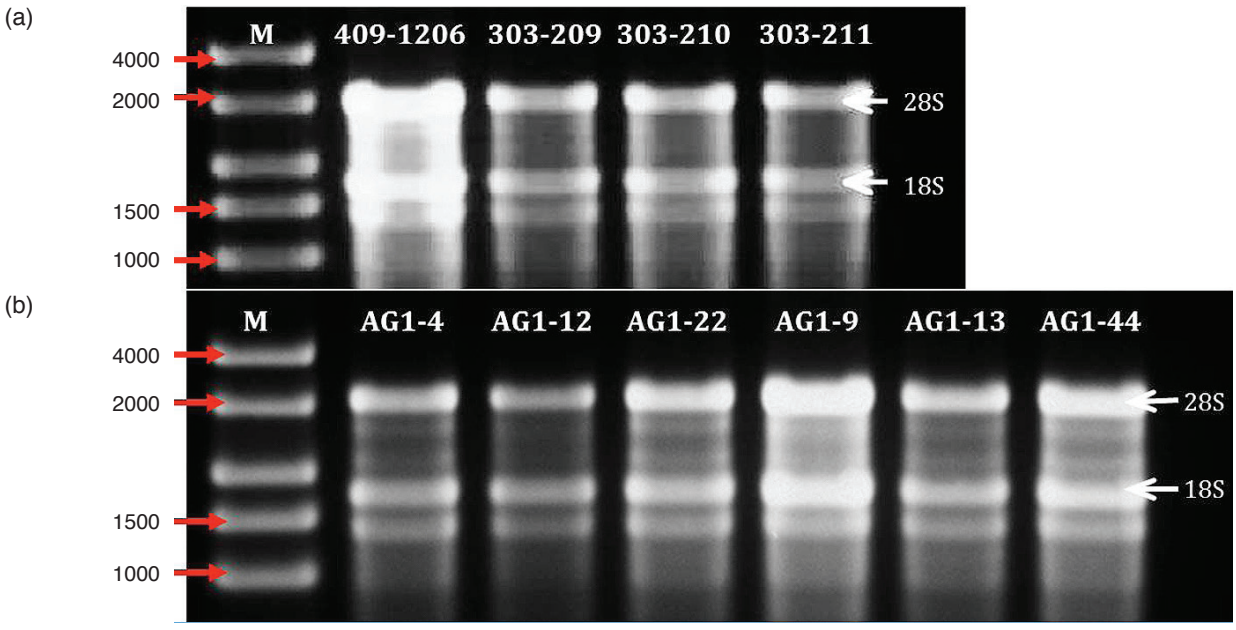

Figure 1. Gel electrophoresis of total ribonucleic acid (RNA) from oil palm spear leaf tissues. (a) MPOB PS1 and (b) FELDA AG1 total RNA samples were analysed on 1% (w/v) agarose gel. M: GeneRuler RNA ladder high range (Fermentas, USA).

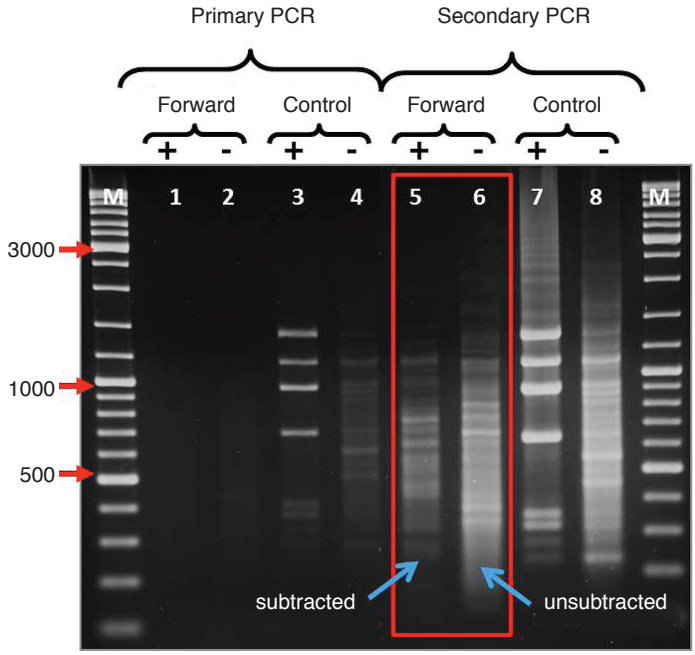

Figure 2. Analysis of polymerase chain reaction (PCR) products from suppression subtractive hybridisation (SSH). Primary (lanes 1-4) and secondary PCR (lanes 5-8) reactions were performed and analysed on a 2% (w/v) agarose gel. Amplification of subtracted and unsubtracted cDNA was revealed. Lanes labelled '+' are subtracted and '-' are unsubtracted. Lanes 1 and 5: subtracted experimental cDNA. Lanes 2 and 6: unsubtracted experimental cDNA. Lanes 3 and 7: subtracted control skeletal muscle cDNA. Lanes 4 and 8: unsubtracted control skeletal muscle cDNA. M: GeneRuler™ DNA Ladder Mix (100-10 000 bp), ready-to-use (Fermentas, USA).

TABLE 3. TOTAL RNA ASSESSMENT USING NanoDrop® ND-1000 and AGILENT 2100 BIOANALYSER

Palm population	Palm ID	RNA concentration (ng μl^{-1})	RNA purity		RNA integrity number (RIN)
			($A_{260/280}$)	($A2_{60/230}$)	
MPOB (PS1)	409-1206	382.0	2.11	2.22	7.0
	303-209	433.0	2.13	2.17	6.8
	303-210	415.0	2.13	2.19	6.7
	303-211	430.0	2.10	2.07	6.7
FELDA BACKCROSS (AG1)	AG1-4	515.0	2.07	1.84	6.8
	AG1-12	406.0	2.10	1.85	6.8
	AG1-22	379.0	2.12	2.15	6.9
	AG1-9	376.0	2.12	2.20	7.1
	AG1-13	361.0	2.12	2.04	6.8
	AG1-44	376.0	2.12	1.90	7.1

Note: RNA - ribonucleic acid.
The data presents the concentration, absorbance ratios and RNA integrity number (RIN) of the purified total RNA from MPOB (PS1) and FELDA BACKCROSS (AG1).

agarose gel (*Figure 2*). Based on the secondary PCR analysis, the unsubtracted experimental samples for all SSH libraries showed a long smear from 200-3000 bp with few distinct bands. As for the subtracted cDNA samples, few distinct bands indicating high abundant gene transcripts were observed at 180-2500 bp. The control PCR showed the reduction of cDNA transcripts in secondary PCR in comparison to the primary PCR. The experimental primary PCR products appeared as a smear from 200 to 2000 bp, with or without appearance of distinct bands. The experimental secondary PCR products showed a smear bands greater than 300 bp in addition to fewer distinct bands. The difference in banding patterns and the reduction of few bands were visibly observed, indicating the effectiveness of SSH to eliminate uniform cDNA present in both 'tester' and 'driver'. The similar banding patterns of the PCR control-subtracted cDNA and skeletal muscle control subtraction from the secondary PCR indicated successful subtraction of the two closely-related cDNA populations being compared.

Screening of the Putative Transformants

A total of 963 putative transformants were picked from the six cDNA libraries for screening of the positive clones containing inserts. Screening

results showed 860 clones harbouring inserts ranging from 200 to 1200 bp. Positive transformants were subjected to DNA sequencing at 1st BASE, Malaysia.

Sequence Data Analysis

The quality score for each putative sequence was determined using Phred program where the sequences with Phred value of ≤ 20 were eliminated due to low-quality of traces. Further elimination of undesired sequences including linearised vector, poly A and T, and adaptors were manually removed to isolate specific gene fragments without overlapping sequences. Phred program has yielded 973 high quality sequences with Phred value ≤ 20 for further sequence analysis. Assembly results by CAP3 algorithm have generated 279 unigenes, which consist of 227 contigs and 52 singletons. BLASTX analysis against the NCBI non-redundant databases has found 213 sequence matches to protein identified in various classes of organisms. The highest top-hit with highest percentage identity, score and E-value was identified in *Vitis vinifera* species with 35 hits, followed by *Elaeis guineensis* with 25 hits. The input sequence hits mostly to the *Oryza sativa* species because of the comprehensive genome database available, which encompasses highly well-characterised genes with known functions. This has proven that the input sequence was closely-related to oil palm species.

Gene Annotation and Selection of Candidate Dwarfing Genes

Gene ontology (GO) of entire differentially expressed cDNA was extracted from Protein Knowledgebase UniProtKB and InterPro database via Blast2GO. Sequence annotations generated by Blast2GO program were classified into molecular processes, biological processes and cellular components, based on their putative functions. Among 279 candidate genes, Blast2GO has assigned 224 sequence annotations. The remaining 55 sequences showed no available data due to non-matching sequences identified. A total of 154 gene transcripts were classified into 12 molecular processes including kinase activity (5%), transferase activity (3%), hydrolase activity (7%), other enzyme activity (23%), DNA/RNA binding (17%), protein binding (4%), nucleic acid binding (6%), nucleotide binding (16%), structural molecule activity (2%), transporter activity (3%), other molecular functions (3%) and other binding (10%). The remaining 70 sequences were classified into cellular components and biological processes (*Table 4*). Six selected putative dwarfing genes were chosen based on their molecular function in suppressing the active enzymes that regulate growth and development of plants.

The six selected potential candidates of dwarfing genes are: 1) brassinosteroid biosynthesis-like protein [AG1.2.17: DWF1], 2) BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative [Contig98: BRI1], 3) late elongated hypocotyl protein [Contig134: LHY protein], 4) gibberellin receptor GID1 [AG1.2.45F: GID1], 5) sterol 24-C-methyltransferase 1 [Contig210: SMT1], and 6) E3 ubiquitin-protein ligase MARCH6 [Contig218: E3Ub]. Functional annotation using Blast2GO has revealed that BRI1 was identified to be associated in kinase activity; SMT1 in transferase activity; GID1 in hydrolase activity; DWF1 and E3Ub in other enzyme activity; and LHY responsible in DNA or RNA binding. Differentially expressed genes were assigned in GenBank EST database with accession numbers from JZ905514 to JZ905764.

Analysis of Primer Efficiency

Eleven primer pairs were designed specific to the target genes as indicated by formation of single peak at one specific temperature in the melting curve analysis. Standard curve was constructed on each tested genes using diluted pooled cDNA from all tested samples to obtain a valid qRT-PCR results. These standard curves were made to calculate the gene-specific qRT-PCR amplification efficiency (E) based on slope and correlation coefficient (R^2) value generated from the standard curve. Primer efficiency test in 10 tested samples generated slopes between -3.1459 to -3.5976; and percent efficiency of 90% to 108%. The R^2 obtained were > 9.0 which provides an indication of efficient primers. Melting curve analysis showed specific primer amplification of all samples with the presence of single peak at melting temperature ranging from 80.4°C to 86.2°C. In this study, the percentage efficiency of the GOI and RF genes in all tested samples ranged from 94% to 107% and 90% to 108%, respectively (*Table 5*).

Five candidate RF genes were selected based on the preliminary test conducted by Low (2009) and Ooi *et al.* (2012) which have indicated the uniformity of the expression stability in various developmental stages in oil palm tissue culture material. In addition, the available microarray expression data (Low, 2009) and expressed sequence tags (EST) (Ho *et al*., 2007; Low *et al*., 2008) generated from various oil palm tissues have provided a selection of candidate RF genes. Chan *et al*. (2014), in her study has utilised the same RF genes with an additional of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), NADH dehydrogenase subunit 5-like gene (*NAD5*), and alpha-tubulin 1 (*TUBULIN*). This study has revealed that EA1332, PD380 and PD569 showed higher expression stability compared to the classical RF gene via geNORM analysis; where

TABLE 4. GENE ONTOLOGY (GO) ANNOTATIONS OF CANDIDATE GENES CLASSIFIED BY THEIR MOLECULAR FUNCTIONS ASSIGNED BY Blast2GO

Contig ID	GO ID[a]	Gene name	Annotation[b]	Sequence length (AA)	E-value[c]	Identity[d] (%)
Kinase activity (7)						
Contig1	GO:0019200	AT4G30310	FGGY family of carbohydrate kinase (*Arabidopsis thaliana*)	489	3e-93	83
Contig98[e]	GO:0004672	RCOM_0020950	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative (*Ricinus communis*)	491	3e-28	54
Contig143	GO:0016301	RCOM_0571360	Hypothetical protein MTR_029s0005 (*Medicago truncatula)*	321	6e-64	91
Contig205		RCOM_0811160	Methylthioribose kinase, putative (*Ricinus communis*)	442	1e-27	74
Contig147	GO:0004674	MTR_5g045190	Serine/threonine protein kinase (*Medicago truncatula*)	311	3e-15	68
Contig160		RCOM_0437810	Serine/threonine-protein kinase PBS1, putative (*Ricinus communis*)	421	1e-54	70
Contig177		STK	Serine-threonine kinase (*Persea americana*)	584	5e-114	85
Transferase activity (5)						
Contig27	GO:0016157		RecName: Full=sucrose synthase 2 (*Tulipa gesneriana*)	645	2e-137	93
Contig149		sucS1	Sucrose synthase, partial (*Poincianella yucatanensis*)	337	4e-66	92
Contig43	GO:0008171		Putative O-methyltransferase (*Elaeis guineensis*)	734	7e-102	86
Contig210[e]	GO:0003838	SMT1	Sterol 24-C-methyltransferase 1 (*Dioscorea zingiberensis*)	419	6e-63	71
Contig172	GO:0016740	RCOM_1207090	Bromodomain-containing protein, putative (*Picinus communis*)	438	5e-17	74
Structural molecule activity (3)						
Contig31		EgUbi	Ubiquitin (*Eustoma grandiflorum*)	307	5e-54	100
Contig80	GO:0003735		Unknown (*Populus trichocarpa*)	269	2e-15	100
Contig209		POPTRDRAFT_73793	Predicted protein (*Populus trichocarpa*)	633	2e-45	83
Transporter activity (6)						
Contig46			Hypothetical protein VITISV_023524 (*Vitis vinifera*)	644	1e-60	76
Contig72	GO:0012599	Rsdv1	Soyabean dwarf virus resistance protein (*Glycine max)*	366	2e-35	87
Contig67	GO:0008308	LOC100250071	PREDICTED: nitrate transporter 1.7 (*Vitis vinifera*)	463	5e-53	62
Contig89	GO:0005215	AQP	Aquaporin (*Manihot esculenta*)	499	5e-98	87
Contig99		AQP	Aquaporin (*Manihot esculenta*)	411	2e-83	90
AG1.3.38R		MTR_3g082050	Zinc transporter (*Medicago truncatula*)	336	4e-32	71
Hydrolase activity (11)						
Contig79		LOC100778704	Uncharacterised protein LOC100778704 (*Glycine max*)	592	9e-84	78
Contig104		XTH3	Xyloglucan endotransglucosylase/hydrolase (*Diospyros kaki*)	584	2e-93	74
AG1.2.45F[e]	GO:0004553	RCOM_1612620	Gibberellin receptor GID1, putative (*Ricinus communis*)	440	2e-12	52
AG1.2.201R		AT4G37550	Formamidase (*Arabidopsis thaliana*)	283	3e-46	80
Contig29	GO:0004519	MTR_5g051060	RRNA intron-encoded homing endonuclease (*Medicago truncatula*)	624	6e-23	88
Contig163			Uncharacterised protein LOC100280151 (*Zea mays*)	874	2e-112	73
Contig30	GO:0004013	SH6.2	RecName: Full=adenosylhomocysteinase (*Medicago sativa*)	765	2e-154	94

TABLE 4. GENE ONTOLOGY (GO) ANNOTATIONS OF CANDIDATE GENES CLASSIFIED BY THEIR MOLECULAR FUNCTIONS ASSIGNED BY Blast2GO (continued)

Contig ID	GO ID[a]	Gene name	Annotation[b]	Sequence length (AA)	E-value[c]	Identity[d] (%)
Contig78		LOC101027131	Aspartic proteinase nepenthesin-1 precursor (*Zea mays*)	725	2e-55	60
AG1.1.146F	GO:0004190		Aspartic proteinase (*Sonneratia alba*)	515	2e-51	70
AG1.1.146R			Aspartic proteinase (*Sonneratia alba*)	513	2e-51	70
Contig136	GO:0003993	umc2754	Stem 28 kDa glycoprotein precursor, putative (*Ricinus communis*)	551	2e-40	65
Other enzyme activity (35)						
Contig26			Pyruvate decarboxylase (*Lycoris aurea*)	822	1e-168	88
Contig167		MDH	NAD-dependent malate dehydrogenase (*Prunus armeniaca*)	602	2e-121	94
Contig105		csu928	Unknown (*Zea mays*)	367	5e-40	79
Contig119	GO:0003824	FBA	Fructose 1,6 bisphosphate aldolase class 1, partial (*Moringa oleifera*)	400	6e-69	95
Contig180			Malate dehydrogenase (*Zea mays*)	462	6e-84	91
Contig213		L2	ADP-glucose pyrophosphorylase leaves small subunit (*Zea mays sub. Mays*)	503	2e-106	92
AG1.2.145F		MTR_029s0030	Beta-glucosidase (*Medicago truncatula*)	303	2e-10	52
Contig17	GO:0016491	LOC100779153	PREDICTED: 3-oxoacyl-(acyl-carrier-protein) reductase FabG-like (*Glycine max*)	464	2e-53	66
Contig19			Peroxiredoxin (*Elaeis guineensis*)	608	1e-109	100
Contig215		Chyb	Beta-carotene hydroxylase (*Elaeis oleifera*)	311	5e-44	91
Contig94			Short-chain dehydrogenase Tic32 (*Elaeis guineensis*)	318	7e-22	100
Contig207	GO:0016491		Alcohol dehydrogenase (*Elaeis guineensis*)	439	1e-52	100
AG1.2.17[e]		DWF1	Brassinosteroid biosynthesis-like protein (*Zea mays*)	237	8e-45	96
AG1.3.77F		ndhD	NADH-plastoquinone oxidoreductase subunit 4 (*Elaeis oleifera*)	656	4e-142	99
Contig47	GO:0004601		Putative alpha-dioxygenase (*Cicer arietinum*)	539	2e-74	76
Contig23	GO:0004324	LOC100798467	PREDICTED: ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like isoform 1 (*Glycine max*)	453	2e-98	95
Contig57	GO:0003755		Cyclophilin (*Ziziphus jujube*)	701	5e-98	88
Contig90	GO:0004816	RCOM_1048270	Aspartyl-tRNA synthetase, putative (*Ricinus communis*)	391	7e-72	89
Contig91	GO:0004252	RCOM_0909450	Acylamino-acid-releasing enzyme, putative (*Ricinus communis*)	346	4e-57	80
Contig93	GO:0006537	RCOM_1579610	Glutamate synthase, putative (*Ricinus communis*)	405	3e-52	89
Contig113	GO:0016984	RBCS1	RecName: Full=ribulose bisphosphate carboxylase small chain, chloroplastic (*Musa acuminate*)	348	6e-50	84
Contig116	GO:0008233	CP-1	Cysteine protease (*Dimocarpus longan*)	368	5e-48	75
Contig121	GO:0008705	MS5	Vitamin-b12 independent methionine synthase (*Populus trichocarpa*)	402	2e-42	91
Contig113	GO:0016984	RBCS1	RecName: Full=ribulose bisphosphate carboxylase small chain, chloroplastic (*Musa acuminate*)	348	6e-50	84
Contig123		rbcS	Ribulose 1,5 bisphosphate carboxylase small subunit (*Fritillaria agrestis*)	316	1e-31	79

TABLE 4. GENE ONTOLOGY (GO) ANNOTATIONS OF CANDIDATE GENES CLASSIFIED BY THEIR MOLECULAR FUNCTIONS ASSIGNED BY Blast2GO (continued)

Contig ID	GO ID[a]	Gene Name	Annotation[b]	Sequence length (AA)	E-value[c]	Identity[d] (%)
Contig208	GO:0004222	LOC100822930	PREDICTED: putative zinc metalloprotease slr1821-like (*Brachypodium distachyon*)	492	2e-28	87
Contig223	GO:0016161	BMY	Chloroplast beta-amylase (*Musa acuminata* AAA Group)	441	1e-54	62
AG1.1.8R	GO:0016853	TPI	Putative triosephosphate isomerase (*Elaeis guineensis*)	320	1e-20	100
Contig59	GO:0004455	POPTRDRAFT_82783	Predicted protein (*Populus trichocarpa*)	971	8e-172	95
Contig60	GO:0004630	Os01g0172400	Os01g0172400 (*Oryza sativa* Japonica Group)	444	3e-88	99
Contig184		PLD	Phospholipase D (*Lolium temulentum*)	594	2e-119	94
Contig76	GO:0003994	RCOM_0782740	Cytoplasmic aconitate hydratase (*Arabidopsis thaliana*)	606	2e-57	95
Contig69	GO:0016630		RecName: Full=protochlorophyllide reductase, chloroplastic (*Cucumis sativus*)	1038	1e-170	84
Contig150	GO:0016874	MTR_4g068500	Annotation was added to scaffolds in November 2011~long-chain-fatty-acid-CoA ligase (*Medicago truncatula*)	566	5e-103	75
Contig218[e]		MTR_4g085550	E3 ubiquitin-protein ligase MARCH6 (*Medicago truncatula*)	663	2e-97	87
DNA or RNA binding (26)						
Contig45		LOC100839138	PREDICTED: cohesin subunit SA-1-like (*Brachypodium distachyon*)	748	1e-129	87
Contig71		Os09g0470500	RecName: Full=homeobox-leucine zipper protein HOX4 (*Oryza sativa* Indica Group)	479	1e-38	83
Contig87	GO:0003677	Os03g0743400	Putative histone H2 protein (*Oryza sativa* Japonica Group)	499	2e-40	100
Contig134[e]		LOC100192868	LHY protein (*Zea mays*)	618	6e-34	66
Contig202		HTA915	Histone 2 (*Populus trichocarpa*)	380	9e-32	100
AG1.3.134R		HTR8	Histone H3.3 (*Arabidopsis thaliana*)	423	2e-69	100
AG1.3.125R		ERF	Ethylene response factor (*Ophiopogon japonicas*)	446	3e-30	63
Contig28	GO:0016149		Unknown (*Populus trichocarpa*)	404	4e-80	91
Contig211		LOC100250061	PREDICTED: eukaryotic translation initiation factor 2 subunit alpha (*Vitis vinifera*)	259	6e-53	95
Contig32		LOC100244384	PREDICTED: 60S ribosomal protein L12 (*Vitis vinifera*)	561	4e-81	89
Contig34			Ribosomal protein L27a-like protein (*Solanum tuberosum*)	503	3e-35	77
Contig39		LOC100244384	60S ribosomal protein L12 (*Capsicum annuum*)	361	6e-72	93
Contig41			30S ribosomal protein S13-1 (*Arachis diogoi*)	306	2e-47	82
Contig52	GO:0003723		Ribosomal protein L10 (*Elaeis guineensis*)	386	8e-72	100
Contig73			Rribosomal protein L32 (*Elaeis guineensis*)	567	4e-50	100
Contig132		RCOM_0029690	50S ribosomal protein L15, putative (*Ricinus communis*)	601	4e-73	87
Contig197			60s ribosomal protein L21 (*Lycoris radiate*)	420	4e-46	94
Contig199		SORBIDRAFT_03g04	Hypothetical protein SORBIDRAFT_03g047210 (*Sorghum bicolor*)	327	2e-69	92
Contig179		LOC_Os02g10770	RecName: Full=DEAD-box ATP-dependent RNA helicase 41 (*Oryza sativa* Japonica Group)	672	1e-111	73

TABLE 4. GENE ONTOLOGY (GO) ANNOTATIONS OF CANDIDATE GENES CLASSIFIED BY THEIR MOLECULAR FUNCTIONS ASSIGNED BY Blast2GO (continued)

Contig ID	GO ID[a]	Gene Name	Annotation[b]	Sequence length (AA)	E-value[c]	Identity[d] (%)
Contig212	GO:0003723		Ribosomal protein L27a (*Petunia x hybrida*)	809	3e-10	64
Contig216			Ribosomal protein L10a (*Elaeis guineensis*)	294	2e-63	99
PS1.2.120R		AT3G44260	CCR4-associated factor 1-like protein (*Arabidopsis thaliana*)	284	3e-24	91
AG1.3.124F		rpl22	50S ribosomal protein L22 (chloroplast) (*Elaeis guineensis*)	537	6e-88	97
AG1.3.18F		rps3	30S ribosomal protein S3 (*Phoenix canariensis*)	394	4e-32	98
Contig124	GO:0003746		Translation elongation factor EF-1 beta chain (*Elaeis guineensis*)	575	2e-70	100
Contig225			elongation factor 1-alpha (*Dendrobium nobile*)	873	1e-136	97
Nucleotide binding (25)						
Contig3			Unknown (*Medicago truncatula*)	533	1e-96	95
Contig13		HSP70	PREDICTED: heat shock cognate 70 kDa protein-like (*Glycine max*)	610	2e-107	93
Contig35		LOC100259987	PREDICTED: 14-3-3 protein 4 (*Vitis vinifera*)	317	1e-12	92
Contig38		Os04g0397100	Os04g0397100 (*Oryza sativa* Japonica Group)	825	0.0	95
Contig20		RCOM_0710470	Groes chaperonin, putative (*Ricinus communis*)	495	2e-92	82
Contig42		SORBIDRAFT_04g03	Hypothetical protein SORBIDRAFT_04g032970 (*Sorghum bicolor*)	496	2e-99	92
Contig110	GO:0005524	RCA03	Rubisco activase (*Glycine max*)	346	5e-28	89
Contig114		RCA2	RecName: Full=ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic (*Larrea tridentata*)	438	9e-88	85
Contig146		RCOM_0464250	Heat shock protein, putative (*Ricinus communis*)	554	6e-109	97
Contig151			Chaperonin 60 beta subunit (*Capsicum annuum*)	280	7e-45	94
Contig169			Actin (*Ipomoea batatas*)	498	2e-70	100
PS1.1.33F		Gma.20674	PREDICTED: 6-phosphofructokinase 3-like (*Glycine max*)	610	6e-115	85
PS1.1.9R		VITISV_030204	Hypothetical protein VITISV_030204 (*Vitis vinifera*)	424	4e-24	86
AG1.3.94R			ATP sulfurylase (*Zea mays*)	269	2e-52	92
Contig68	GO:0005525		Putative ADP-ribosylation factor (*Hertia cheirifolia*)	302	2e-64	98
Contig109		TubA3	Alpha-tubulin (*Gossypium hirsutum*)	534	2e-77	100
Contig158	GO:0005525	ATub2	Alpha-tubulin 2 (*Gossypium hirsutum*)	420	8e-94	100
Contig157		QG8140	Putative ADP-ribosylation factor (*Hertia cheirifolia*)	357	4e-80	98
Contig174		OsI_01991	Hypothetical protein OsI_01991 (*Oryza sativa* Indica Group)	437	1e-28	72
Contig175		ATub2	Alpha-tubulin 2 (*Gossypium hirsutum*)	428	3e-91	98
Contig182			Ras-like GTP-binding protein 2, partial (*Dimocarpus longan*)	301	2e-69	99
AG1.3.8F		LOC100259150	PREDICTED: ras-related protein RABA2a (*Vitis vinifera*)	474	6e-85	84
Contig138	GO:0000166	RALY	Unnamed protein product (*Homo sapiens*)	272	3e-18	100
Contig194		Sb08g019140	Hypothetical protein SORBIDRAFT_08g019140 (*Sorghum bicolor*)	373	5e-13	63
AG1.3.85R		LOC_Os03g58810	Os03g0802700 (*Oryza sativa* Japonica Group)	401	4e-55	72
Nucleic acid binding (10)						
Contig103	GO:0003676		polyprotein (*Oryza australiensis*)	697	5e-32	60
Contig106			Polyprotein (*Oryza australiensis*)	664	2e-71	72
Contig127			Polyprotein (*Oryza australiensis*)	424	7e-20	70

TABLE 4. GENE ONTOLOGY (GO) ANNOTATIONS OF CANDIDATE GENES CLASSIFIED BY THEIR MOLECULAR FUNCTIONS ASSIGNED BY Blast2GO (continued)

Contig ID	GO ID[a]	Gene Name	Annotation[b]	Sequence length (AA)	E-value[c] (%)	Identity[d] (%)
Contig170		RCOM_0536310	r3h domain containing protein, putative (*Ricinus communis*)	368	1e-26	59
Contig186			Polyprotein (*Oryza australiensis*)	544	2e-09	44
Contig221			Polyprotein (*Oryza australiensis*)	458	1e-56	66
Contig222		VITISV_001808	Hypothetical protein VITISV_001808 (*Vitis vinifera*)	818	2e-39	71
PS1.3.205F			Polyprotein (*Oryza australiensis*)	657	3e-71	57
AG1.2.10F		LOC100246501	PREDICTED: 31 kDa ribonucleoprotein, chloroplastic isoform 1 (*Vitis vinifera*)	657	1e-98	75
AG1.3.95R		VITISV_011091	Hypothetical protein VITISV_011091 (*Vitis vinifera*)	671	5e-110	72
Protein binding (6)						
Contig224			Unknown (*Picea sitchensis*)	247	4e-26	91
PS1.1.28F	GO:0051082	dnaJ	Hypothetical protein OsI_08334 (*Oryza sativa* Indica Group)	496	2e-98	85
PS1.1.38F		dnaJ	Unknown (*Picea sitchensis*)	479	3e-60	85
Contig206	GO:0003779	ADF4	Actin depolymerizing factor 4 (*Gossypium hirsutum*)	436	2e-76	93
Contig201	GO:0046982	AUX/IAA	Auxin induced family protein (*Elaeis guineensis*)	669	1e-71	98
AG1.2.148F		RCOM_1051590	Auxin-responsive protein IAA27, putative (*Ricinus communis*)	554	6e-56	92
Other binding (15)						
Contig61	GO:0005509	RCOM_0603960	Oxygen-evolving enhancer protein 1, chloroplast precursor, putative (*Ricinus communis*)	342	5e-26	82
Contig125			Oxygen evolving enhancer protein 3 (*Bruguiera gymnorhiza*)	323	2e-32	83
Contig129		LOC100781848	PREDICTED: oxygen-evolving enhancer protein 2, chloroplastic-like (*Glycine max*)	581	2e-93	88
Contig12		LOC100259651	PREDICTED: protein OBERON 2 (*Vitis vinifera*)	489	4e-79	78
Contig53			Fibre protein Fb2 (*Elaeis guineensis*)	702	5e-87	99
Contig62	GO:0008270	LOC543182	RecName: Full=zinc finger protein 1 (*Triticum aestivum*)	323	2e-8	60
Contig75			Predicted protein (*Hordeum vulgare subsp. Vulgare*)	308	3e-16	54
Contig81			Hypothetical protein VITISV_030567 (*Vitis vinifera*)	297	7e-30	82
Contig83			gag/pol protein (*Bryonia dioica*)	269	2e-08	35
Contig97		VITISV_030567	Hypothetical protein VITISV_030567 (*Vitis vinifera*)	495	9e-53	66
Contig84	GO:0046923	RCOM_1417710	ER lumen protein retaining receptor, putative (*Ricinus communis*)	347	2e-13	66
Contig51	GO:0005543	SORBIDRAFT_01g01	Hypothetical protein SORBIDRAFT_01g014000 (*Sorghum bicolor*)	324	3e-21	80
Contig195	GO:0030246	LOC100252010	PREDICTED: uncharacterized protein LOC100252010 (*Vitis vinifera*)	313	6e-40	72
Contig178	GO:0030145	GLP1	Germin-like protein (*Camellia sinensis*)	385	5e-13	90
Contig133	GO:0046872		Metallothionein-like protein (*Typha latifolia*)	424	6e-20	87
Other molecular functions (5)						
Contig49		RCOM_0210880	Root phototropism protein, putative (*Ricinus communis*)	499	1e-25	67
Contig50	GO:0004871	FKF1	Circadian clock-associated FKF1 (*Glycine max*)	468	0.0	80
Contig51		LOC100264249	PREDICTED: root phototropism protein 3 (*Vitis vinifera*)	324	1e-109	81
PS1.3.209R	GO:0000156	Os11g0157600	Os11g0157600 (*Oryza sativa* Japonica Group)	299	2e-24	78
Contig166	GO:0004857		Protein E (*Enterobacteria* phage phiX174)	310	4e-34	98

Note: [a] Gene Annotation ID assigned by the Blast2GO program.
[b] Annotation of each contigs and singletons developed by Blast2GO program.
[c] The expect value (E-value) is the number of hits one can 'expect' to observe by chance when searching in the GenBank with cut-off value 10^{-5}. The lower the E-value, the closer it is to zero, the more 'significant' the match is.
[d] Percentage identity is the percent similarity between the query and subject sequences that falls within the length of coverage area.
[e] Differentially expressed genes that regulate dwarfism in oil palm.

TABLE 5. PRIMER EFFICIENCY TEST RESULTS OF ALL GENE OF INTEREST (GOI) AND REFERENCE (RF) GENES

Primer ID	Slope	Correlation coefficient (R^2)	Primer efficiency (%)
Gibberellin receptor (GID1)	-3.2522	0.9830	103
Brassinosteroid insensitive 1-associated receptor kinase 1 precursor (BRI1)	-3.3219	0.9901	100
Late elongated hypocotyl protein (LHY)	-3.3186	0.9854	100
Sterol 24-methyltransferase 1 (SMT1)	-3.2821	0.9956	102
E3 ubiquitin-protein ligase *MARCH6* (E3Ub)	-3.4847	0.9644	94
Brassinosteroid biosynthesis-like protein (DWF1)	-3.1558	0.9886	107
Actin (ACTIN)	-3.3684	0.9966	98
Polyubiquitin (UBIQUITIN)	-3.4349	0.9890	95
Predicted 40S ribosomal protein S27-2 (PD380)	-3.5976	0.9769	90
Predicted protein IFH-1 like (EA1332))	-3.5844	0.9970	90
Manganese superoxide dismutase (PD569)	-3.1459	0.9922	108

Chan *et al*. (2014) have revealed that PD380 and PD569 being the most stable expression in MA2 and MA8 tissue culture lines confirmed by geNORM, NormFinder and BestKeeper statistical algorithms.

Determination of the Most Stable RF Genes for Normalisation

Analysis of the most stable RF genes for normalisation by geNORM (version 3.4) showed that manganese superoxide dismutase (PD569) and predicted protein IFH-1 like (EA1332) genes were determined as the most stable RF genes for normalisation of gene expression indicated by the lowest expression stability (*M*) value of 0.3408. Polyubiquitin (UBIQUITIN), actin (ACTIN) and predicted 40S ribosomal protein S27-2 (PD380) were eliminated due to higher *M* values (*Figure 3a*). Pairwise variations with cut-off value of 0.15 showed the inclusion of the third RF gene, *i.e*., PD380 has no significant effect for all tested samples as indicated by lower $V_{2/3}$ value of 0.1154 (*Figure 3b*). Therefore, the geometric means of the most stable RF genes, PD569 and EA1332 were calculated to obtain the normalisation factor. Normalised GOI expression levels were determined in accordance to geNORM manual available in geNORM website: http://medgen.ugent.be/~jvdesopm/genorm/.

However, it should be pointed out that different sets of samples had their own best RF genes, because different tissues or plant species exhibit differential expression patterns. For instance, UBQ2C gene showed highly stable expression in banana, peach and *Arabidopsis* (Chen *et al*., 2011). Hence, the gene stability measure is crucial in order to obtain accurate and reliable normalisation prior to validation of GOI expression levels.

Validation of Selected GOI in the Dwarf Palms by qRT-PCR

The qRT-PCR has revealed six potential gene candidates related to dwarfism in oil palm have been identified. The gene functions were based on previous findings in other plant species. GOI expression analysis was carried out in 10 samples, normalised by two most stable RF genes, *i.e.* PD569 and EA1332. The expression levels of GOI were compared in dwarf and standard palm as shown in *Figures 4a* to *4f*. A total of three GOI showed higher expression in dwarf palms: 1) BRI1 in 303-211 with 1.0185 ± 0.0241 expression levels, and 2.3285 fold-differences to 409-1206; 2) LHY in AG1-12 with the expression value of 1.0000 ± 0.1351, and 1.5620 fold-differences to AG1-13; and 3) SMT1 in AG1-22 with 1.000 ± 0.0263 expression levels, and 4.9044 fold-differences to AG1-44. However, based on quantitative analyses of normalised qRT-PCR data, three GOI were deemed as false positive, *i.e.* DWF1, GID1 and E3Ub showing down-regulation in dwarf palms.

Sterol 24-C-methyltransferase 1 (SMT1). SMT1 acts as a catalyst involved in the initial methylation step in the plant sterol biosynthesis pathway, where the conversion of the phytosterol intermediate cycloartenol to the 24-alkyl sterols (Shi *et al*., 1996; Clouse and Sasse, 1998; Neelakandan *et al*., 2010). The occurrence of SMT1 resulted in the alteration of sterol contents in plant where it accumulates the cholesterols and alkylated sterols, therefore restrict further sterol transformation (Diener *et al*., 2000). SMT1 plants defective in sterol biosynthesis are characterised by short malformed roots, calcium sensitivity to roots, poor growth, greatly reduced fertility, and abnormal embryogenesis (Diener *et al*., 2000; Choe, 2010). Three SMT genes were identified in *A. thaliana* genome, *i.e.* SMT1 (At5g13170), SMT2 (At1g20330) and SMT3 (At1g76090) (Clouse and Sasse, 1998; Diener *et al*., 2000; Kwon and Choe, 2005).

SMT1 was also isolated from soyabean using etiolated hypocotyl (Shi *et al*., 1996). In soyabean, SMT transcript was highly expressed in flowers and growing vegetative tissues, where lower expression levels were identified in young pods and immature

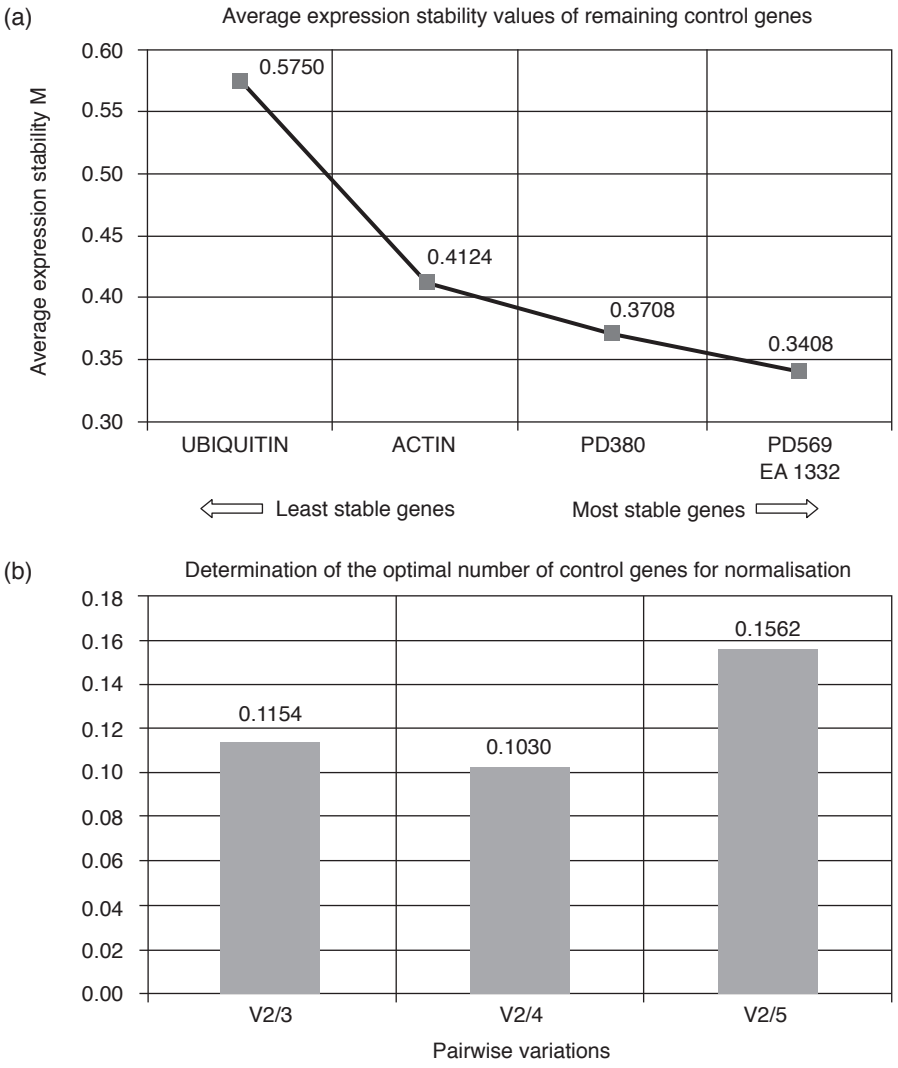

Figure 3. Determination of the optimal number of reference genes for accurate normalisation. a) The average expression stability values (M) *of the candidate reference genes. A lower* M *value indicates the more stable expression, as shown in PD569 and EA1332 genes. b) Pairwise variations (V) analysis.*

seeds (Shi *et al*., 1996). In addition, Diener *et al*. (2000) revealed that the expression of SMT1 was strongest in the growing regions especially at the shoot apex. In comparison with other BR dwarf mutant such as dwf1, dwf5 and dwf7, SMT1 showed the least reduction in the overall growth. This is probably due to the deficiency of specific sterols that are required at certain levels in particular tissues or cells (Choe, 2010). In contrast, the morphological phenotypes of dwf1 and dwf7 are rescued by brassinosteroids (Diener *et al*., 2000).

Brassinosteroid biosynthesis-like protein (DWF1). *DWF1* or also called *DIMINUTO1* (*DIM1*) was selected as the candidate dwarfing gene because it has been isolated in various plant species such as *A. thaliana* (Kwon and Choe, 2005; Hossain *et al*., 2012), pea (Fujioka and Yakota, 2003) and tomato (Bishop *et al*., 1999; Ahsan *et al*., 2007) to study plant dwarfism. For example, in *A. thaliana*, the *DWF1* (At3g19820) was identified to be defective in the early steps of BR biosynthesis pathway, during the conversion of 24-methylenecholesterol to campesterol (Choe, 2010; Hossain *et al*., 2012). The accumulation of 24-methylenecholesterol and lower level of campesterol observed in *DWF1* mutants suggested that the activation of *DWF1* was blocked during this conversion step (Choe, 2010). The seedlings of *DWF1* mutants were characterised by short hypocotyls, petioles and roots, whereas the adult plants showed extremely short inflorescences and flowers with shorts hypocotyls (Hossain *et al*., 2012).

BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative (BRI1). BRI1 or also known as *cbb2* was first identified and characterised in *Arabidopsis* (Clouse *et al*., 1996; Pereira-Netto, 2007) which possess dwarf characteristics due to disruption in BR signaling. It was also identified in pea (*Pisum sativum*) and tomato (*Solanum lycopersicum*) (Hossain *et al*., 2012). BRI1

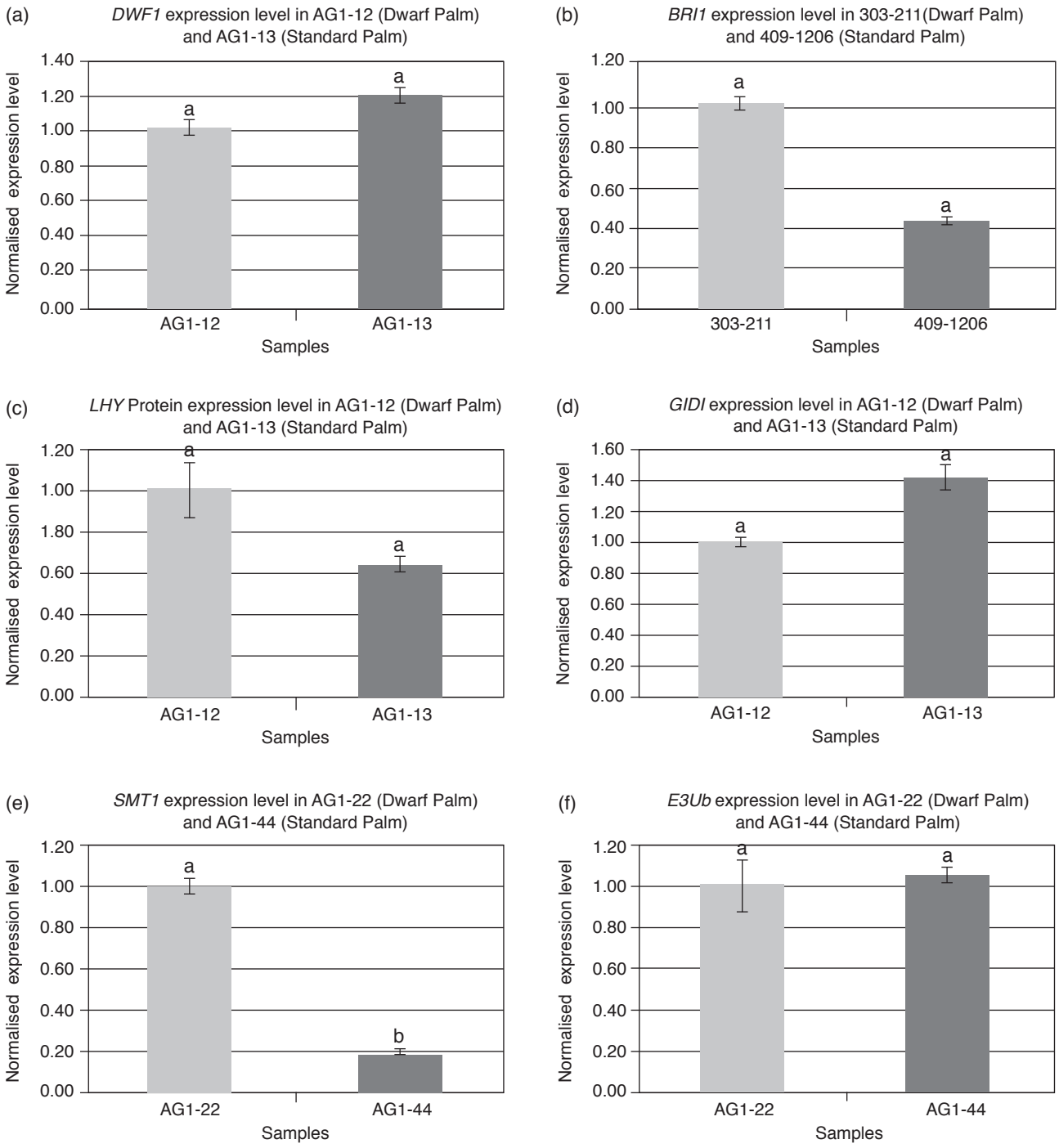

Figure 4. The normalised expression levels of gene of interest (GOI). Normalised expression levels of (a) brassinosteroid biosynthesis-like protein (DWF1), (b) brassinosteroid insensitive 1 (BRI1), (c) late elongated hypocotyl protein (LHY), (d) gibberellin receptor (GID1), (e) sterol 24-methyltransferase 1 (SMT1) and (f) E3 ubiquitin-protein ligase MARCH6 (E3Ub) in standard and dwarf palms. Different letters indicate significant differences at $p < 0.05$.

mutant deficient in BR response and biosynthesis was recognised by having short hypocotyls with reduced cell elongation (Hossain *et al*., 2012), the leaves are thickened, dark green and curled (Clouse *et al*., 1996), delayed development, reduced fertility (Clouse *et al*., 1996), and altered vascular structure (Clouse, 2002). The BRI1 occurs in BR signal transduction pathway, where BR are perceived through binding of BR with extracellular domain of the BRI1 receptor kinase, a leucine rich repeat (LRR) receptor-like kinase at plasma membrane, leading to the increase of kinase activity and further induce the dimerisation and phosphorylation with other co-receptor kinase-like protein, BRI1-associated receptor kinase called BAK1 (Tang *et al*., 2008). The BRI1-BAK1 complex initiates the BR signal transduction process (Pereira-Netto, 2007). The interaction of these receptors (BIN1 and BAK1) with several regulators via several phosphorylation processes has led to

the expression of BR-repressed genes which may affect the regulation of several BR responses such as cell elongation, cell division, leaf development, root growth, xylem development, flowering, male fertility, stress responses and senescence (Tang *et al*., 2008).

Late elongated hypocotyl protein. Late elongated hypocotyl protein or simply known as LHY protein is selected as candidate dwarfing gene due to its high interrelation with circadian clocks endogenous mechanisms, which controls numerous physiological and molecular processes in organisms (Lu *et al*., 2009). In plants, the circadian clocks take part in leaf movements, stomata opening, hypocotyl elongation, and transcription of a number of genes (De Grauwe *et al*., 2006; Lu *et al*., 2009). Hypocotyl elongation is influenced by various signals interaction including phytohormones, temperature and light (De Grauwe *et al*., 2006). For example, in *Arabidopsis*, LHY mutants exhibited short hypocotyls and petioles under continuous light exposure (Miyata *et al*., 2011). A dominant LHY mutant in *Arabidopsis* defects in circadian rhythm displayed elongated hypocotyls, reduced levels of endogenous LHY transcripts, and disrupts the rhythmic expression of genes at dawn (Schaffer *et al*., 1998; De Grauwe *et al*., 2006; Lu *et al*., 2009). Schaffer *et al*. (1998) also reported that LHY transcripts were 10 times more abundant in LHY mutants than in wild type plants.

Gibberellin-receptor GID1 (GID1). Gibberellin-insensitive Dwarf 1 or also known as GID1 acted as a gibberellin repressor. It is classified into DELLA protein family which suppresses the GA responses in various stages of plant development including seed germination, stem elongation, fruit/flower development (Ariizumi and Steber, 2006; Soh, 2006). The interaction of bioactive GA with GID1 will promote stronger and more stable DELLA protein, leading to degradation in GA signaling for plants development (Ariizumi and Steber, 2006). The disruption in GA signaling response moderated by DELLA proteins caused the alteration of plant growth and development. Loss of function in GA signaling will result in less requirement of GA hormone for stem elongation, therefore producing GA-insensitive dwarf phenotype (Dill *et al*., 2001). GID1 has been identified in rice (Ueguchi-Tanaka *et al*., 2005) and *A. thaliana* (Soh, 2006). GID1 mutants deficient in GA displayed several characteristics such as shortened petiole, reduced internode length, small dark-green leaves, delayed flowering, altered GA gene expression, and reduced fertility (Schomburg *et al*., 2003; Magome *et al*., 2004; Fleet and Sun, 2005; Soh, 2006). Magome *et al*. (2004) has identified that dwarfed and delayed flowering (DDF1) gene was similar to GA-insensitive mutants by means of GA deficiency.

E3 ubiquitin-protein ligase MARCH6 (E3Ub). The E3 ubiquitin ligase gene was actively found regulated during plant development and hormone signaling processes in a number of plant tissues such as *A. thaliana* (Mazzucotelli *et al*., 2006). Based on the *A. thaliana* genomic studies, a total of 1415 genes were classified into E3 ubiquitin ligase group which include HECT domain, RING, PUB, ASK, SCF (SKP1, CULLIN, F-box), APC and BTB (Ariizumi and Steber, 2006; Mazzucotelli *et al*., 2006). Mazzucotelli *et al*. (2006) have also reported that the *A. thaliana* genomic studies aiming to investigate the expression levels of E3 ubiquitin ligase gene showed up-regulation during the hormone treatments with cytokinin, salicylic acid, jasmonate, auxin, gibberellin 3, ethylene, brassinosteroids, 1-aminocyclopropane-1-carboxylic acid (ACC) and abscisic acid (ABA). However, the gene was down-regulated with hormone inhibitor treatments such as inhibitors of auxin, gibberellin, ethylene, and brassinosteroids.

In addition, E3 ubiquitin ligase is responsible in catalising the transfer of ubiquitin to the target protein, *i.e.* DELLA protein. *A. thaliana*, the DELLA proteins were negatively requlated by E3 sub group: F-box and SLEEPY1 (SCFSLY1) genes resulted in loss-of-function of SLY1 mutants (Ariizumi and Steber, 2006; Mazzucotelli *et al*., 2006). The SCF^{SLY1} mutants showed severe dwarfism, block of seedling development, death of young seedlings, reduced fertility, and delayed flowering (McGinnis *et al*., 2003; Fleet and Sun, 2005; Ariizumi and Steber, 2006; Mazzucotelli *et al*., 2006). Ariizumi and Steber (2006) explained the evidence indicating that SCFSLY1 acts as E3 ubiquitin ligase in GA signaling based on several facts: 1) high accumulation levels of DELLA proteins were identified in SCF^{SLY1} mutant plants, 2) the GA application to SCF^{SLY1} dwarf mutants failed to eliminate DELLA proteins, and 3) direct interaction of SCFSLY1 with DELLA proteins.

Statistical Analysis

Statistical analysis using Paired Samples T-test has proven that among all GOI, only SMT1 gene showed significant different of expression in dwarf and standard palms (*Table 6*). The results showed that the expression levels of SMT1 are highly significant in dwarf palms, being on average higher expression compared to standard palms. Based on the test results, the critical value for t at $\alpha = 0.05$, two-tailed test, degrees of freedom (df) = 2 is ± 4.303. Therefore, the t value of 4.447 falls within the critical region defined by the critical value of 4.303, and the p-value of 0.047 is lesser than α of 0.05. The results is written as follows: $t\ (2) = 4.447$; $p < 0.05$.

The expression profile of SMT1 using AG1-22 as a calibrator with control baseline of 1.0000 expression level in all tested samples has indicated

TABLE 6. STATISTICAL ANALYSIS OF GENE OF INTEREST (GOI) BASED ON THE EXPRESSION LEVELS IN DWARF AND STANDARD PALMS USING PAIRED-SAMPLES T-TEST

Gene ID	Sig. (2-tailed)	Statistical results	Expression levels in dwarf and standard palms
Gibberellin receptor (GID1)	0.265	t (2) = -1.534; p > 0.05	No significant difference
Brassinosteroid insensitive 1-associated receptor kinase 1 precursor (BRI1)	0.159	t (2) = 2.197; p > 0.05	No significant difference
Late elongated hypocotyl protein (LHY)	0.108	t (2) = 2.797; p > 0.05	No significant difference
Sterol 24-methyltransferase 1 (SMT1)	0.047	t (2) = 4.447; p < 0.05	Significant difference
E3 ubiquitin-protein ligase (E3Ub)	0.203	t (2) = -1.865; p > 0.05	No significant difference
Brassinosteroid Biosynthesis-Like Protein (DWF1)	0.353	t (2) = -1.199; p > 0.05	No significant difference

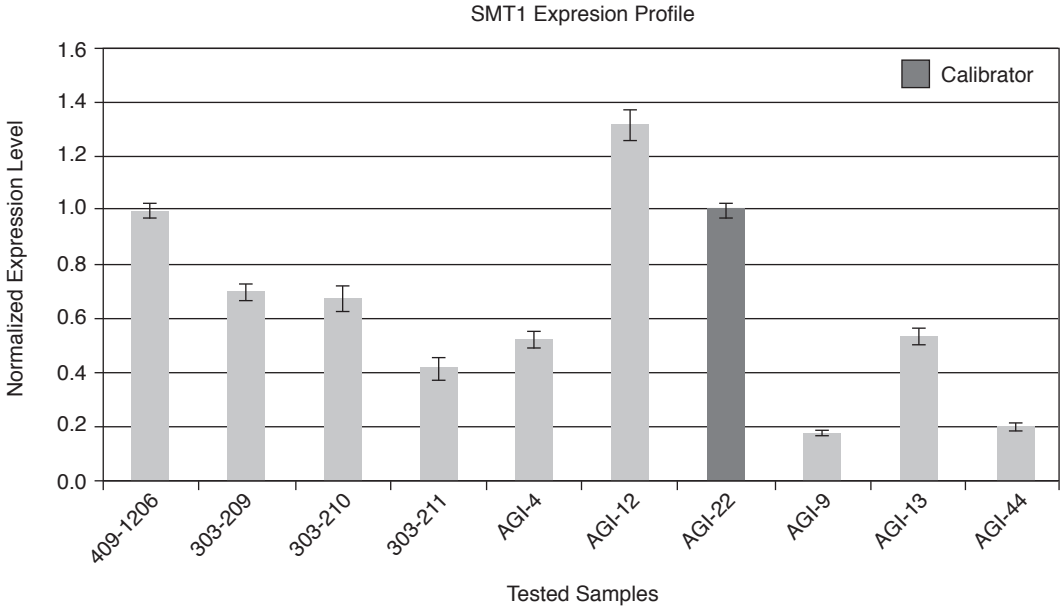

Figure 5. Representation of sterol 24-methyltransferase (SMT1) expression levels in 10 tested samples from standard and dwarf palms. Calibrator sample AG1-22 with expression value of 1.0000 represents as a control baseline. Each value signifies the means of three replicates, and vertical bars indicate the standard deviation.

that SMT1 was highly up-regulated in dwarf palm, AG1-12 with expression value of 1.3161 compared to the calibrator. Lower expression values of less than 1.0000 presented in 409-1206, 303-209, 303-210, 303-211, AG1-4, AG1-9, AG1-13 and AG1-44 showed down-regulation of SMT1 gene in the tested samples (*Figure 5*).

CONCLUSION

In this study, six potential candidate genes (DWF1, BRI1, LHY, GID1, SMT1 and E3Ub) associated with dwarfism were identified based on their putative functions. All GOI were identified to be associated with the alteration of BR and GA pathways, more likely to be in the dwarf phenotype. The expression levels of all GOI relative to RF genes, PD569 and EA1332 in all tested samples via qRT-PCR revealed that BRI1, LHY and SMT1 were highly expressed in dwarf palms. SMT1 has showed significant different of expression in dwarf palms based on T-test analysis. This study has provided complete information to conduct research on identifying the differentially expressed genes via SSH approach, and gene expression analysis via qRT-PCR particularly in oil palm study.

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