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Gene Expression Analysis of Somatic Embryogenesis in Oil Palm

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ABSTRACT

Multiplication of oil palm through somatic embryogenesis is hampered by low callogenesis and embryogenesis rates. Molecular marker based on RNA (transcriptomes analysis) is considered as one of the most effective techniques for detection and differentiation of embryogenic and non-embryogenic callus. A previous research using microarray technique had shown some potential candidate genes related to oil palm somatic embryogenesis, such as: IAA-amino acid hydrolase ILR1-like1 (*ilr1*), late embryogenesis abundant (*lea2*), 26S proteasome non-ATPase regulatory subunit 13 homolog B (*26sp*), and alpha trehalose phosphate synthase [UDP-forming] 6-like (*tps6*). The objective of this study was to analyze the transcription level of *ilr1, lea2, 26sp*, and *tps6* using quantitative reverse transcription PCR (RT-qPCR). Non-embryogenic nodular callus and somatic embryo (coleoptile stage) of *Elaeis guineensis* var. Tenera (Deli Dura x AVROS Pisifera) leaf explants were collected from three palms for RNA extraction. The first strand cDNA was synthesized from RNA and used for gene expression analysis. The expressions of four embryogenesis-related genes were analyzed using Relative Quantification Standart Curve method. RQ value was analyzed with One-way ANOVA and Dunnett's test using Statistical Product and Service Solution (SPSS) 20.0 for windows. In RT-qPCR analysis, non-embryogenic nodular callus was used as calibrator sample and 40S ribosomal protein S27-2 (*40s*) was used as reference gene. The result shows that *ilr1* and *lea2* genes were significantly transcribed higher on coleoptile stage of the somatic embryo compared to callus, in other hand *26sp*, and *tps6* shows no expression difference on both samples. *ilr1* genes gave the highest expression in somatic embryo compared to callus in most tested palms. Thus, it indicated that *ilr1* may potentially involve in oil palm somatic embryogenesis and can be used as a candidate to develop the marker for embryogenesis in oil palm.

Keywords: *Elaeis guineensis*, IAA-amino acid hydrolase, RT-qPCR, tissue culture, transcript level analysis

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INTRODUCTION

Oil palm (*Elaeis guineensis*) is one of the most productive oil-bearing plants in comparison to other oil crops, such as rapeseed, sunflower, and soybean (Sumathi *et al.* 2008). Palm oil is used in many foods, household products, and biodiesel production. Due to the very high demand for vegetable oils, the increment in palm oil production is needed. It can be achieved by planting elite palms.

Tissue culture is one of the best techniques to multiple elite palms, as it could improve oil yield by 12-15% through cloning the top 5% of the palms and 30% by recloning the top clones after a clonal test (Soh 2012). Nevertheless, this technique is still hampered by low embryogenesis rate. Callogenesis and embryogenesis rates were very low, only about 19% (Corley & Tinker 2003) and 3%, respectively (Kushairi 2010).

Some factors that influence the success of somatic embryogenesis in oil palm are genotype (Sanputawong & Te-chato 2008), culture media, plant growth regulator, carbon sources (Kramut & Te-chato 2010), cellular competences, and microculture environment (light & temperature). In oil palm tissue culture, callogenesis and embryogenesis rate is influenced by different progeny (Nugroho *et al.* 2014). Genotypic variation of (three dura palms) in response to four auxins (2,4-D, Picloram, 2,4-D + Picloram and NAA) was observed both in callus induction and embryogenesis (Jayanthi *et al.* 2015). Mostly researchers had only focused to improve the success of embryogenesis through modification of culture media and culture condition.

Understanding of oil palm tissue culture's molecular basis could provide the necessary information needed to improve the efficiency of oil palm propagation, such as identification of genes related to embryogenesis in oil palm. It can be used in developing molecular marker to differentiate the embryogenic and non-embryogenic cultures. Many approaches had been taken to understand the complexities of gene expressions and interactions in oil palm embryogenesis, such as analysis with DNA microarray (Low *et al.* 2006 and Budinarta *et al.* 2012), analysis of Expressed Sequence Tags (ESTs) (Lin *et al.* 2009), and cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) (Pattarapimol *et al.* 2015).

Some embryogenesis-related genes in oil palm, such as lipid transfer protein homolog (*wbp1a*), somatic embryogenesis receptor kinase 1 (*serk1*), and defensin (*egad1*) (Shariff *et al.* 2008), have been reported. Other genes are also reported in carrot and Arabidopsis such as late embryogenesis abundant (*lea*), somatic embryogenesis receptor kinase (*serk*), agamous-like 15 (*agl15*), baby boom (*bbm*), leafy cotyledon 1 (*lec1*), fusca3 (*fus3*) and leafy cotyledon 2 (*lec2*) (Ikeda *et al.* 2006).

Previous research using microarray technique compared non-embryogenic nodular callus with the somatic embryo (Budinarta *et al.* 2012). This study was used many stages of somatic embryo (globular, coleoptilar, and scutellar) in bulk. The result showed that IAA-amino acid hydrolase ILR1-like 1 (*ilr1*) gene has the highest transcript levels (fold change is 142.62 up-regulated) in somatic embryo compared to non-embryogenic nodular callus.

Late embryogenesis abundant (*lea2*) gene have transcript levels 1.93 down-regulated in the somatic embryo (Budinartha *et al.* 2012). This result is in contrast with Lara-Chaves *et al.* (2012) result which the transcript levels of LEA gene was accumulated in developing Pinustaeda (pine) embryos and the highest transcript levels were in late cotyledonary phase.

26S proteasome non-ATPase regulatory subunit 13 homolog B (*26sp*) was the same with LEA genes. *26sp* had fold change 15.13 down-regulated in somatic embryo compared to callus (Budinartha *et al.* 2012), but this detected gene had the highest transcript levels in round/globular (embryo) stage in pine.

Trehalose phosphate synthase [UDP-forming] 6-like (*tps6*), also had the transcript levels about 11.44 down-regulated in somatic embryo compared to callus (Budinarta *et al.* 2012). This gene was one of three genes that had the highest interaction with others genes (embryogenesis-related genes) based on gene interaction analysis with Bioinformatic software Cytoscape_v2.8 and BioGRID 3.2 database based on Budinarta *et al.* 2012 result.

Thereby, the aim of this study was to analyze the transcripts level of *ilr1*, *lea2, 26sp,* and *tps6* genes in callus and embryo stage of oil palm using RT-qPCR.

MATERIALS AND METHODS

Plant Materials

The samples were obtained from the Tissue Culture Research Laboratory of PT SMART Tbk, West Java Indonesia. Callus (non-embryogenic nodular callus) and somatic embryo (coleoptilar stage) of *E. guineensis* var. Tenera (Deli Dura x AVROS Pisifera) were generated from leaf explants through indirect somatic embryogenesis.

The fresh samples of callus and somatic embryo from three palms of three different progenies (Table 1) were collected prior to RNA extraction. Callus and embryoids were induced from leaf explant with Murashige and Skoog (MS) basal medium incorporated with 2,4-Dichlorophenoxyacetic acid (2,4-D) and Naphthaleneacetic acid (NAA) (Wong *et al.* 1997).

RNA Extraction and cDNA Synthesis

Total RNA was isolated from callus and somatic embryos samples using a mortar and liquid nitrogen according to NucleoSpin® RNA Plant Protocol (Macherey-Nagel, Germany) followed by DNAse treatment. Isolated RNA was stored at-80 °C.

The quality and quantity of RNA were determined using NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, USA). Ten microliters of RNA samples was verified on denaturing Formaldehyde gels in 1X MOPS buffer (Mansour & Pestov 2013), ran at 100 volts for 30 min followed by EtBr staining, and documented using the UV trans-illuminator Gel-Doc™ XR (BIORAD, USA). Two hundred nanogram of total RNA was assessed for its integrity on capillary electrophoresis using QIAxcel RNA QC Kit v2.0 protocol (Qiagen, Germany) followed by RNA quantification by RNA Integrity Score (RIS) using QIAxcel ScreenGel Software 1.2 (Qiagen, Germany).

The first strand of cDNA was synthesized from 3 µg of RNA (1 µg for each reaction) following the QuantiTect® Reverse Transcription Kit protocol (Qiagen, Germany). To assess the purity of cDNA from DNA contamination, amplification using forward primer *40s*-1 5'GCTCCTTC-CCTTCCTCTCAA'3 and reverse primer 40s-1 5'CCTGGACTAACAAAATCAC-CGAT'3 was performed. This primer is intron-spanning primers that will generate the product size of about 456 bp for cDNA and ± 1000 bp for DNA. The cDNA samples were stored at -40 °C.

Construction of Standard Curve

The cDNA of callus was amplified with RT-qPCR primers (Table 2). Reaction mix was as followed: 1 µL of cDNA (1.5 µg μL^{-1}); 14 µL of primer mix (10 µM); 10.5 µL of 10X DreamTaq Buffer; 10.5 µL of

Table 1 Origin information and embryogenesis rate of samples

Palm No.	Progeny	Female	Male	(%) Embryo
1	9810093N	Deli dura 5005.126	AVROS pisifera 742.316	5.6
2	9010190E	Deli dura 711.614	AVROS pisifera 742.316	6.7
3	9102109E	Deli dura 703.802	AVROS pisifera 742.316	3.2

Table 2 Primer sets used for RT-qPCR

Gene abbreviation	Primer pair 5' → 3'	Product size (bp)	Ta (°C)*	Description	E-value	Ident (%)
ilr1	F CTCCTCAATTCGTTGCTTCAGTT R CGGAAAGGGAGGTCATGGT	250	64	PREDICTED: *E. guineensis* IAA-amino acid hydrolase ILR1-like 1 (LOC105047633)	8.0E-82	99
lea2	F ACAAACCCCAATCCCATCC R AATCGGCACATCAACAATGAG	243	64	PREDICTED: *E. guineensis* uncharacterized (LOC105049817)	7.0E-93	100
26sp	F AGCCTCTCGGTTCATCTTATTG R GTTGTTTTGGTCTCCTTCTGC	250	62	PREDICTED: *E. guineensis* 26S proteasome non-ATPase regulatory subunit 13 homolog B (LOC105060648)	4.0E-90	100
tps6	F CAGGAGAGGGGCTTATCACC R GCTTCTTACTGCTTGTTCTGAG	240	64	PREDICTED: *E. guineensis* alpha-trehalose-phosphate synthase [UDP-forming] 6-like (LOC105048485)	4.0E-85	100
40s	F GCTCCTTCCCTTCCTCTCAA R TACCACACACAACAACAGTCT	246	62	PREDICTED: *E. guineensis* 40S ribosomal protein S27-2 (LOC105035175)	2.0E-98	100

*Ta (°C) is annealing temperature of primers which were optimized with enzyme DreamTaq DNA Polymerase (Thermo Scientific, USA).

dNTP Mix, 2 mM each; 4.2 µL of $MgCl_2$; and 0.84 µL of DreamTaq DNA Polymerase (Thermo Scientific, USA), and nuclease-free water was added to reach the total volume of 100 µL.

The PCR program was at 94 °C for 5 min; 35 cycles of 94 °C for 1 min (denaturation), 62 or 64 °C for 30 s (Table 2) (annealing), 72 °C for 1 min (extension), and 72 °C for 7 min (final synthesize). The amplified products were separated on 2% agarose gel, 100V for 90 min. The PCR products were extracted from the gel and purified using QiAquick® Gel Extraction Kit (Qiagen, Germany).

The standard curve was conducted by two replications with ten fold dilutions of the purified PCR product (cDNA), ranging from 10-1ng μL^{-1}-10^{-7}ng μL^{-1}. All of the RT-qPCR assays showed a linear relationship between C_t value and the log of cDNA concentration (R^2>0.95) in all cases, which allowed concentration determination of the unknown samples based on their C_t value.

Relative Quantification

The expressions of four embryogenesis-related genes were analyzed using Relative Quantification Standart Curve method (Larionov *et al.* 2005). In this study, was compared the transcription level of two samples: non-embryogenic nodular callus with the somatic embryo, while the non-embryogenic nodular callus used as samples calibrator. This study also analyzes transcription level of four embryogenesis-related genes such as *ilr1, lea, 26sp*, and *tps6*.

In this study, RT-qPCR analysis used 40S ribosomal protein S27-2 (*40s*) as reference gene (Chan *et al.* 2014). *40s* selected used after analyze five reference genes: *act3, ef2, 40s, sodm,* and *tubβ1*. Melting curve analysis showed that three genes: *act3, 40s,* and *sodm* was specific (no dimer) (Figure 1). *40s* selected as *β*reference genes for RT-qPCR analysis because these reference genes have the most stable of C_t value on both tested samples (Table 3).

The RT-qPCR primers were designed using the Primer-Blast software (Ye *et al.* 2012) that generated amplicon with the size of 240-250 bp (Table 2). Primers were designed according to Wang and Seed (2006). PCR product was sent for sequencing to the 1st BASE (IDT, Singapore). Sequences analysis was carried out using Basic Local Alignment Search Tool Nucleotide (BLASTN) and BLASTX from the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nih.gov) (Altschul *et al.* 1997).

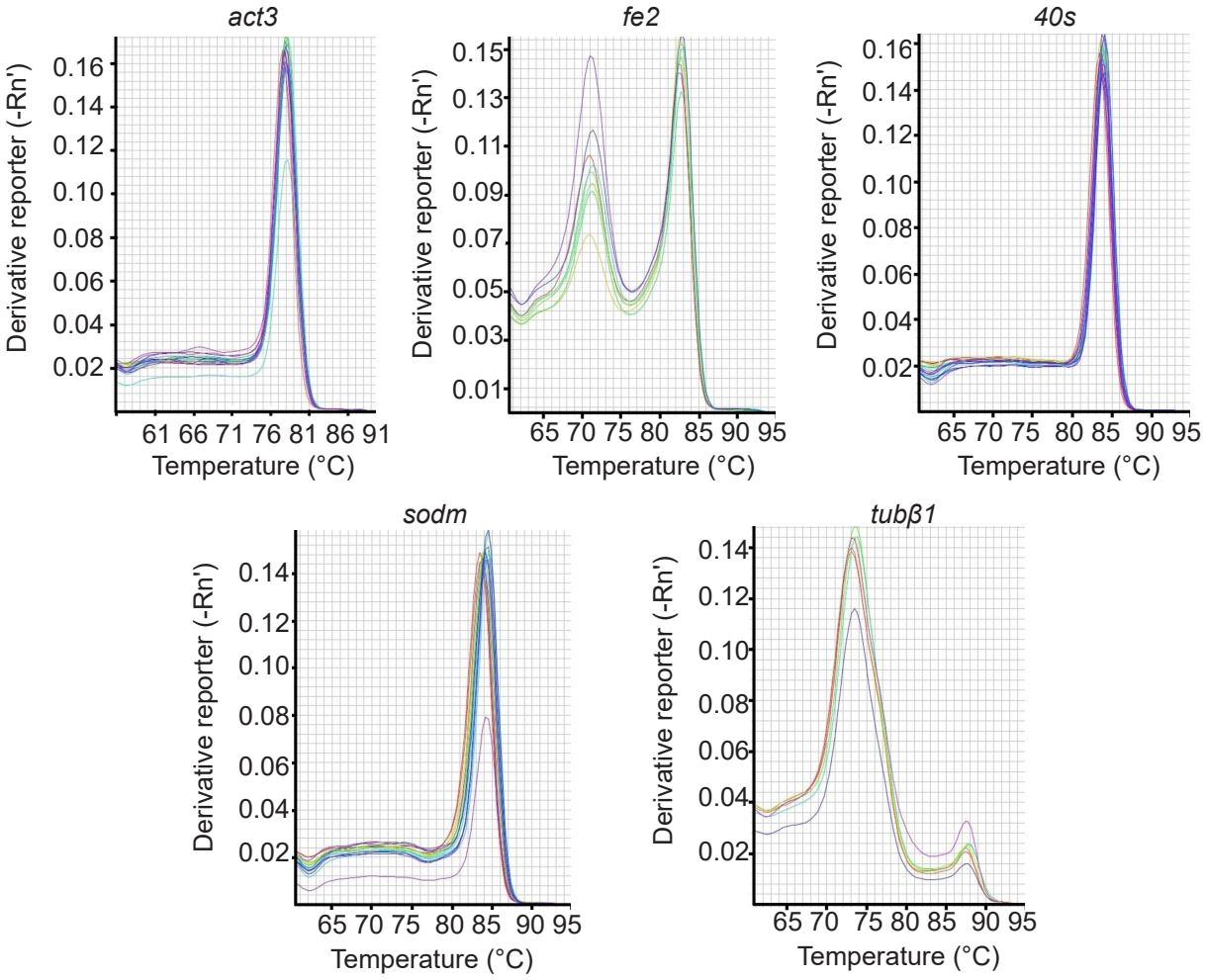

Figure 1 Melting curve analysis on five reference gene used oil palm embryo

Table 3 Coefficient of variation of three reference gene on callus and embryo

Gene	Samples	C_t Range*	SD of C_t	Mean of C_t	CV (%)
act3	Callus	26.65 – 29.84	1.160	27.999	4.145
	Embryo	25.05 – 27.64	0.994	26.514	3.748
40s	Callus	27.35 – 29.28	0.850	28.240	3.010
	Embryo	26.79 – 29.70	1.084	28.215	3.840
Sodm	Callus	30.90 – 34.99	1.660	32.244	5.148
	Embryo	29.96 – 33.28	1.378	31.555	4.366

*RT-qPCR analysis used five replicate for each samples.

A triplicate RT-qPCR reaction was performed on MicroAmp® Fast Optical 96-well reaction plate 0.1 mL (Applied Biosystems, USA) using QuantiFast® SYBR® Green PCR Kit (Qiagen, USA) and Applied Biosystems™ 7500 Fast Real-Time PCR System. RT-qPCR reactions were as followed: 200 ng μL^{-1} cDNA samples, 0.2 µM primer mix, 5 µL QuantiFast SYBR Green PCR Master Mix (2x), and RNase-free water to reach the total volume of 10 µL with PCR condition: pre-cycling 95 ºC for 5 min, cycling stage 95 ºC for 10 s (denaturation) and 62 ºC for 30 s (annealing and extension). The amplification was done for 40 cycles and Melting curve analysis was conducted at 95 ºC for 15 s, 60 ºC for 1 min, 95 ºC for 15 s, and 60 ºC for 15 s.

The expressions of four target genes using RT-qPCR by manual reaction setup were assessed with three biological replications (three palms) and triplicate technical replication (each palm three times RNA sample extraction). Standard curve and C_t were used for normalization, while quantity value was calculated automatically with 7500 Software v2.0.6 (Applied Biosystems, USA). Relative Quantification (RQ) value was calculated according to Real-Time Quantitative PCR guide from Applied Biosystems (2008). RQ value was analyzed with One-way ANOVA and Dunnett's test using Statistical Product and Service Solution (SPSS) 20.0 for windows. RQ value was calculated following this equation:

$$\text{Normalized target (test sample)} = \frac{\text{(quantity means of target)}}{\text{(quantity means of endogenous control)}}$$

$$\text{Normalized target (calibrator sample)} = \frac{\text{(quantity means of target)}}{\text{(quantity means of endogenous control)}}$$

$$\text{Target fold difference} = \frac{\text{(Normalized target (test sample))}}{\text{(Normalized target (calibrator sample))}}$$

RESULTS AND DISCUSSION

RNA was isolated from callus (nodular callus) and somatic embryos (coleoptilar stage) of *E. guineensis* var. Tenera (Deli Dura x AVROS Pisifera) from three different palm trees. A High quality of RNA has an $OD_{260/280}$ value with the ratio ranging from 1.8 to 2.0 (Sambrook *et al.* 1989), and had higher than five RNA Integrity Number (RIN/RQI) (Fleige & Pfaffl 2006). Concentration, purity and integrity score of RNA samples are shown on Table 4. The first strand of cDNA was synthesized from RNA using QuantiTect® Reverse Transcription Kit (Qiagen, Germany). Figure 2 shows that cDNA with primer *40s-1* amplification is free from DNA contamination and generated PCR product with the size of ±456 bp, while the DNA produce band with the size of ±1000 bp.

The four embryogenesis-related genes were analyzed on callus and somatic embryo using Relative Quantification Standart Curve method (Larionov *et al.* 2005). This method remains a reliable and simple alternative to the PCR-efficiency based

Table 4 Quantity and quality of RNA isolated from callus and somatic embryo of oil palm culture

Samples	Callus				Somatic embryo			
	Code*	[RNA] ng μL^{-1}	$A_{260/280}$	RIS	Code*	[RNA] ng μL^{-1}	$A_{260/280}$	RIS
Palm 1	C1	1225.2	2.03	4.8	E1	1002.3	2.15	5.3
	C2	1593.6	2.14	4.5	E2	1458.0	2.16	5.4
	C3	1455.8	2.12	5.5	E3	1438.6	2.15	5.8
Palm 2	C1	685.1	2.10	4.7	E1	391.6	2.14	5.8
	C2	1714.6	2.15	5.1	E2	971.9	2.15	5.4
	C3	1109.2	2.15	4.5	E3	1201.2	2.16	5.3
Palm 3	C1	878.6	2.16	6.5	E1	1308.8	2.15	5.7
	C2	1738.0	2.14	5.2	E2	649.4	2.14	5.4
	C3	1041.1	2.08	5.1	E3	608.1	2.15	5.0

*C is callus and E is somatic embryo.
C1 Palm 1, C2 Palm 2, and C2 Palm 3 selected as calibrator samples and used to construct the standard curve.
All somatic embryo used as target samples in RT-qPCR analysis.

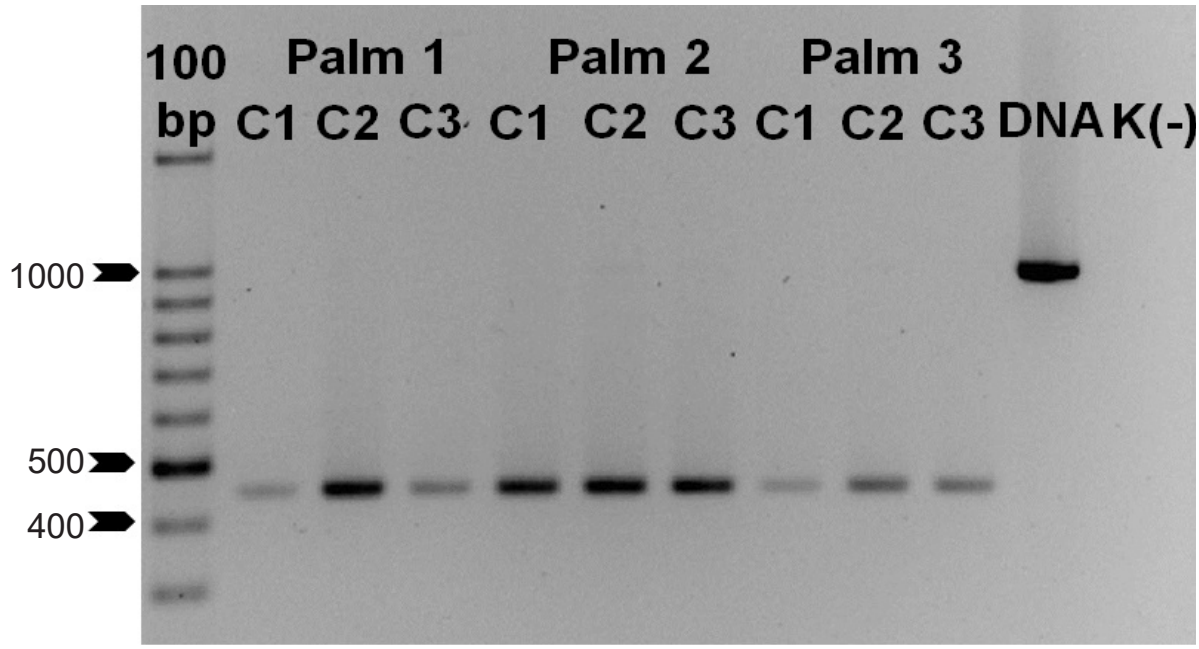

Figure 2 Amplification of *40s-1* gene on cDNA and DNA callus.

on calculations in relative Real-Time PCR (Larionov *et al.* 2005). Amplification of all primers had generated single band, which was sent for sequencing. Alignment with BLAST (BLASTN) showed high identity with *E. guineensis* genes (Table 2) and a single peak was observed after Melting curve analysis (Figure 3).

The result from RT-qPCR analysis showed that transcription level of *ilr1* was mostly higher in somatic embryo (coleoptile stage) than non-embryogenic nodular callus (calibrator samples) in every palm (Figure 4). Embryo Palm 1 and 2 was significant difference at $p<0.05$ (base on One-way Anova and Dunnett t-tests).

Embryo from Palm 2 gave the highest expression than embryo from two other palms. It indicated that every palm have different endogenous auxin levels. The high expression of *ilr1* gene was also reported in the previous research using

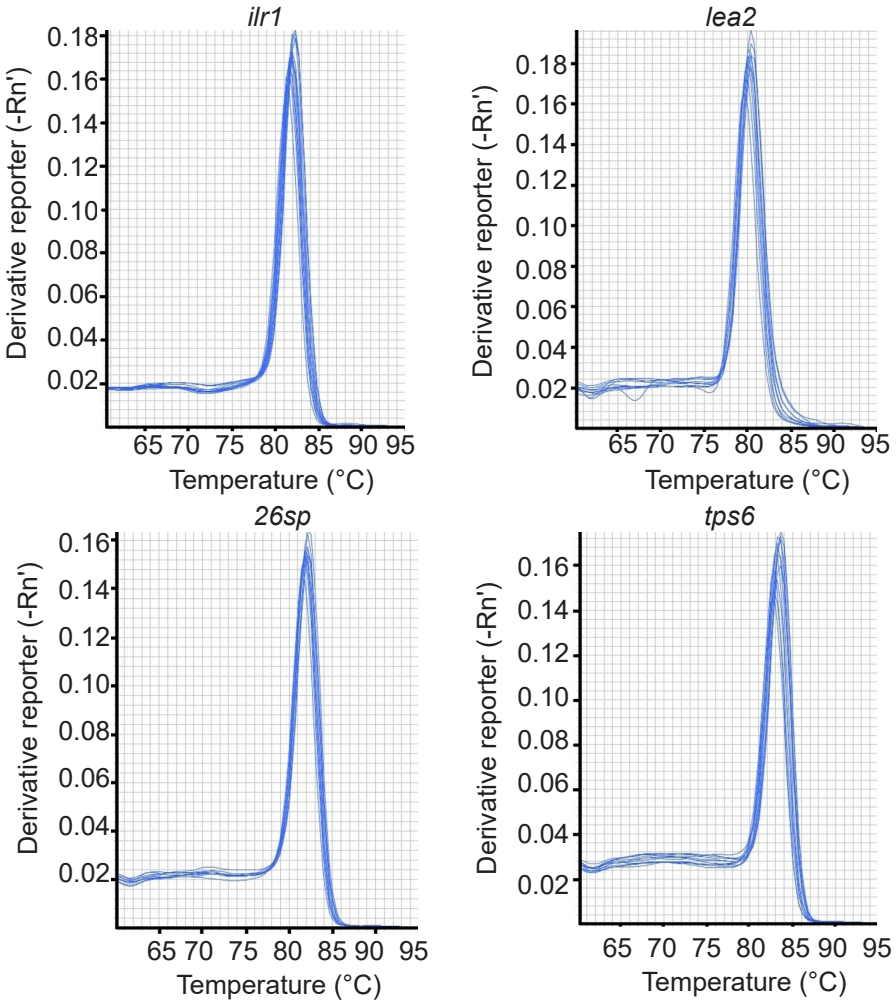

Figure 3 Melting curve analysis on four target genes (*ilr1, lea2, 26sp,* and *tps6*).

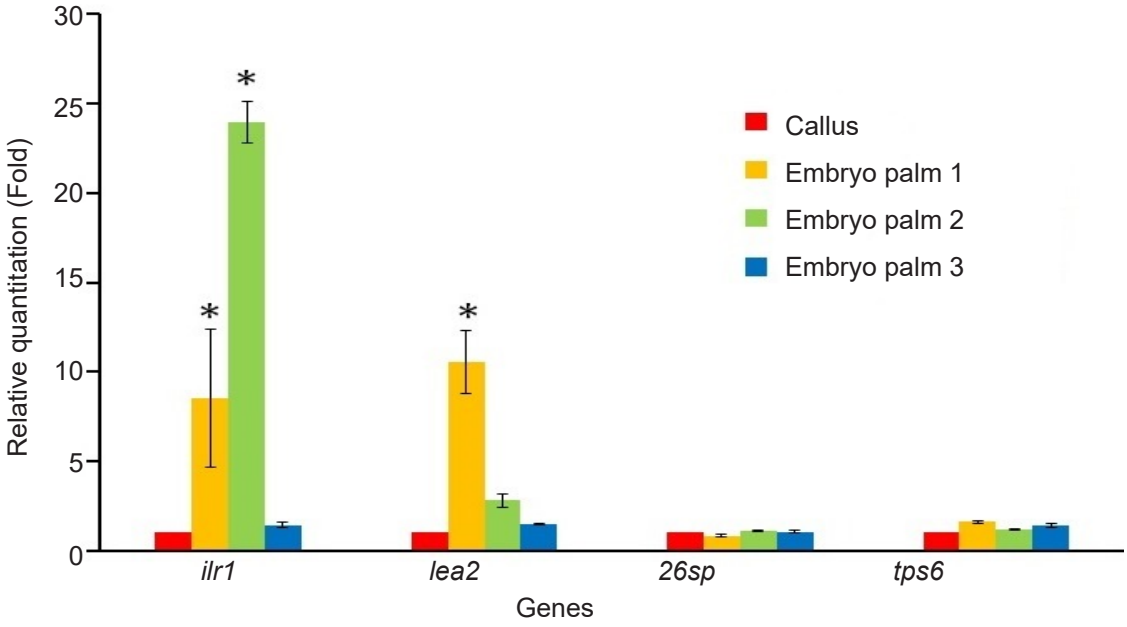

Figure 4 Transcription level analysis result (RQ value) of *ilr1, lea2, 26sp*, and *tps6* on callus and somatic embryo.

microarray analysis by Budinarta *et al.* (2012) which in that study, *ilr1* 142.63 fold up-regulated in somatic embryos compared to callus (Table 5).

IAA-amino acid hydrolase ILR1-like1 (*ilr1*) include in IAA-conjugates. This type of auxin hydrolase converted IAA conjugates to active IAA through tryptophan-independent pathways of IAA biosynthesis in plants (Bartel 1997). Auxin had emerged as one of the potent initiators of somatic embryogenesis (Raghavan 1997). Common synthetic auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) was used for inducing somatic embryogenesis in this study. The hormone generates DNA hypermethylation, which maintains a highly active mitotic stage, and induces pro-embryonic phase in the cells (Endress 1994).

Transcription level analysis showed that late embryogenesis abundant (*lea2*) gene was expressed higher in somatic embryo than callus, especially in the embryo from Palm 1 (Figure 4). In pine, lea was expressed either in somatic embryo or zygotic embryo and the expression increased simultaneously within embryo development (Lara-Chavez *et al.* 2012). LEA, a group of hydrophilic proteins, has been linked to plants and animals survival in periods of stress, putatively through the safeguarding of enzymatic function and prevention of aggregation in times of dehydration/heat (Amara *et al.* 2014). Late embryogenesis abundant proteins (LEA proteins) were first found in cotton (*Gossypium hirsutum*) seeds, accumulating in late embryogenesis (Dure *et al.* 1981).

The transcription level of *26sp* result showed that no significant expression difference on callus nor somatic embryoid stage. Moreover, some of the embryoids (embryo from Palm 1) were down-regulated (Figure 4), which was also reported in the previous research (*26sp* have fold change 15.13 down-regulated in embryo compared to callus) using microarray analysis (Budinarta *et al.* 2012). 26S proteasome non-ATPase regulatory subunit 13 homolog B (*26sp*) protein, involves in a wide range of processes, including embryogenesis, hormone signaling, and senescence (Moon *et al.* 2004). In pine, this gene was also expressed in somatic and zygotic embryos, with the higher expression in globular stage compared to others, such as early and late cotyledonary (Lara-Chavez *et al.* 2012).

The transcription level of *tps6* result also showed no significant expression difference in both callus and somatic embryo (Figure 4). This gene is one of the genes that involve in synthesis trehalose in the plant. Trehalose (a-D-glucopyranosyl-[1,1]-a-D-glucopyranoside) is a stable non-reducing sugar, widely found in living cells of bacteria, archaea, fungi, invertebrates, and plants (Avonce *et al.* 2006). In the plant, trehalose synthesis takes in two-steps: 1. Synthesis the trehalose-6-phos-

Table 5 Expression of four embryogenesis-related genes through microarray analysis

No		Genes	Fold change*	References
1	*ilr1*	iaa-amino acid hydrolase *ilr1*-like 1	142.63 Up	Chugh and Khurana 2002
2	*lea2*	late embryogenesis abundant protein	1.93 Down	Lara-Chavez *et al.* 2012
3	*26sp*	26s proteasome non-atpase regulatory subunit 13	15.13 Down	Lara-Chavez *et al.* 2012
4	*tps6*	Trehalose-6-phosphate synthase	11.44 Down	Eastmond & Graham 2002

*Fold change values based on microarray result by Budinarta *et al.* 2012.

phate (T6P) from sucrose through catalyzed by trehalose-6-phosphate synthase (TPS) and 2. T6P was consecutive dephosphorylation through catalyzed by trehalose-6-phosphate phosphatase (TPP) to produce trehalose (Ponnu *et al.* 2011).

Trehalose-6-phosphate synthase is essential for the embryo development and maturation of *A. thaliana* (Eastmond & Graham 2003). Trehalose is an osmoprotectant, which counteracts the desiccation effect of drought, salt, or low-temperature stresses in the basal lineages of life (Crowe *et al.* 1992). T6P plays an important role in orchestrating cell cycle activity and cell wall biosynthesis with cellular metabolism during embryo development (Gomez *et al.* 2006).

CONCLUSION

This study analyzed four embryo-genesis related genes: IAA-amino acid hydrolase ILR1-like1 (*ilr1*), late embryogenesis abundant (*lea2*), 26S proteasome non-ATPase regulatory subunit 13 homolog B (*26sp*), and alpha trehalose phosphate synthase [UDP-forming] 6-like (*tps6*). The transcription level result showed that *ilr1* and *lea2* genes were expressed higher on coleoptile stage of the somatic embryo compared to (non-embryogenic nodular) callus. The result also showed that *26sp* and *tps6* had no expression difference in both tested samples. *ilr1* genes gave the highest expression in somatic embryo compared callus in most tested palms and these genes may potentially be involved in oil palm somatic embryogenesis and can be used as a candidate to develop the marker for embryogenesis in oil palm.

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