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Genomic structure and characterization of a lipase class 3 gene and promoter from oil palm

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Abstract

Lipase class 3 is part of the triacylglycerol lipase family involved in lipid degradation, esterification, and transesterification processes in plants. In this study, a lipase class 3 gene and promoter from oil palm (*Elaeis guineensis* Jacq.) were isolated and characterized by Northern blot, Southern blot, oil palm genome sequence, and transient expression GUS assay. The full-length lipase class 3 (FLL1) deduced polypeptide encoded 483 amino acids and was identical to that deduced from lipase (EgLip1) cDNA (GI: 409994625). It contained the lipase consensus sequence, GxSxG motif, and a putative catalytic triad and had a 3-dimensional protein model similar to that of a lipase from *Giberella zeae* with a 50 % identity. The Northern blot and reverse transcription polymerase chain reaction (RT-PCR) show that *FLL1* was predominantly expressed in the mesocarp and the expression increased as fruits reached maturity. A lower expression was detected in germinated seedlings and especially in roots. The expression of *FLL1* was also enhanced in the mesocarp of cold treated fruits. A high oil accumulation in the mesocarp during fruit development makes this tissue a suitable target for a genetic modification, hence the isolation of the *FLL1* promoter. The transient expression of the β-glucuronidase (*GUS*) gene driven by the *FLL1* promoter detected the *GUS* expression in mesocarp slices, especially in vascular bundles. This suggests the potential role of using the promoter as tool to direct the expression of a transgene to the mesocarp of transgenic oil palm.

Additional key words: fatty acids, *GUS* gene, mesocarp, RACE, transient assay, triacylglycerides.

Introduction

Oil palm, *Elaeis guineensis* Jacq. is the most productive oil crop in the world. The most treasured component of the oil palm is the fruit which produces two different types of oil: the palm oil derived from the mesocarp, and the palm kernel oil from the kernel. Both oils differ in their fatty acid composition, hence are used in different applications. Palm oil contains 50 % saturated, 39 % unsaturated and 11 % polyunsaturated fatty acids (Tang *et al.* 2000), and the oil is mainly used as food (Miskandar *et al.* 2011). The palm kernel oil contains mostly medium-chain fatty acids and it is normally used for industrial purposes (Hazimah *et al.* 2011).

Triacylglycerides (TAGs) make up the main component of palm oil and are found in abundance in mesocarp tissues especially in mature fruits. TAGs, however, are prone to hydrolysis, which causes the

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Abbreviations: CARE - *cis*-acting regulatory elements; FFA - free fatty acids; FLL1 - full-length lipase class 3; GUS - β-glucuronidase; LD-PCR - long distance polymerase chain reaction; LRE - light responsive elements; LTRE - low temperature responsive elements; ORF - open reading frame; RACE - rapid amplification of cDNA ends; REG - regulatory element groups; RT-PCR - reverse transcription polymerase chain reaction; SDS - sodium dodecyl sulphate; SSC - sodium chloride-sodium citrate; TAG - triacylglycerides; TSS - transcription start site; UTR - untranslated region; WAA - weeks after anthesis; Y-patch - pyrimidine patch.
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release of free fatty acids (FFA) from the glycerol backbone. FFA content and its oxidation products cause an undesirable flavour and odour of oil (Chong 2011), which affects oil quality. The increase in FFA content in palm oil is also induced by an endogenous lipase that is present in the mesocarp (Sambanthamurthi *et al.* 1995). Thus, one of the ways to minimize the losses of oil quality is to use oil palms with lower lipase activities. Those could be obtained from screening the oil palm germplasm (Wong *et al.* 2005, Ngando Ebongue *et al.* 2008).

The lipase activity assay in the mesocarp has been reported by several researchers. Henderson and Osborne (1991) and Sambanthamurthi *et al*. (1991) demonstrated that the oil palm mesocarp contains an active endogenous lipase. Ngando Ebongue *et al.* (2006) demonstrated that the highest activity was detected at 35 °C and at pH 9. On the other hand, Sambanthamurthi *et al.* (1991) and Cadena *et al.* (2013) showed that by inducing the lipase activity at a cold temperature of 5 °C, a maximum FFA content is detected. Sambanthamurthi *et al.* (1995) also observed that the lipase activity is induced by ripening oil palm fruits.

Two lipase genes coding for a putative lipase homolog (P87EST) and a lipase class 3 family protein (O65EST) were discovered from a 17-week-old mesocarp cDNA library (Nurniwalis 2006, Nurniwalis *et al.* 2008). Both genes show differential expression patterns: The *P87EST* expression is constitutively low but in all tested tissues, whereas the expression of *O65EST* is high in mesocarp tissues. The increase in the expression of *O65EST* follows the ripening pattern of the oil palm fruit which coincides with the oil synthesis period. In this paper, we report the isolation of full-length cDNA encoding lipase class 3 (*FLL1*), the prediction of protein structure, the characterization of *FLL1* expression, the isolation of its promoter, and the transient promoter assay.

Materials and methods

Plants and treatments: Various tissues from commercial oil plant (*Elaeis guineensis* Jacq.) fruits were used. Mesocarp and kernel tissues were harvested from fresh fruit bunches at various weeks after anthesis (WAA) for RNA extraction. Spear leaves were obtained from unopened leaf fronds. Roots from 2-year-old and 1-week-old seedlings were also used. For a cold induced treatment, oil palm fruits were incubated at 7 °C for 5 h prior to RNA extraction.

Total RNA and DNA extraction: Total RNA from different oil palm tissues was extracted by a modified method of Prescott and Martin (1987). Frozen tissues (5 g) were ground and transferred to 15 cm^3 of an extraction buffer [50 mM Tris-HCl, pH 9.0, 150 mM LiCl, 5 % (m/v) SDS, 5 mM Na_2EDTA, 2 mM aurin tricarboxylic acid, and 0.4 % (v/v) β-mercaptoethanol] and followed by phenol:chloroform (50:50, v/v) and chloroform:isoamyl alcohol (24:1, v/v) extraction. The precipitation of RNA with 2 M LiCl was carried out at 4 ^oC overnight. The pellet obtained was washed with 2 M LiCl, dissolved in ice-cold sterile water, and used for Northern analyses and first strand cDNA synthesis. Genomic DNA from oil palm leaves was isolated as described previously (Nurniwalis 2006).

Rapid amplification of cDNA ends (RACE) and identification of transcription start site (TSS): The 5` region of the lipase class 3 gene (*FLL1*) was amplified and TSS identified using a *GeneRacer*™ kit (*Invitrogen*, Carlsbad, USA) which specifically targets the 5` capped mRNA structure to produce full-length cDNA. First strand cDNA was synthesized from the 17 WAA oil palm mesocarp total RNA. Touchdown PCR amplification was carried out in a 0.05 cm^3 reaction mixture containing a *RACE-Ready* cDNA template, a 1× *Expand HF* buffer with 15 mM $MgCl_2$ (*Roche*, Mannheim, Germany), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP (*Applied Biosystems*, Foster City, USA), a *GeneRacer* 5` primer, a 0.2 μM LAS2 primer, and a 0.1 U *Expand HF* PCR enzyme (*Roche*). The PCR was performed at 94 ºC (2 min), 94 °C (30 s), and 72 ºC (3 min) for 5 cycles; 94 °C (30 s) and 70 °C (3 min) for 5 cycles; 94 °C (30 s), 65 °C (30 s), and 68 °C (3 min) for 25 cycles, and a final extension at 68 °C for 10 min. The PCR products were purified using a *Qiaquick* gel extraction kit and cloned into a *pCR®II-TOPO®* vector (a *TOPO TA* cloning kit, *Invitrogen*). The plasmids were isolated using a *Qiagen* plasmid mini kit, digested with EcoRI, and sent for automated sequencing using *M13* reverse and forward universal primers.

Long distance (LD)-PCR: Full-length cDNA of the lipase class 3 gene was generated *via* LD-PCR using a *GeneRacer*™ kit (*Invitrogen*). Touchdown PCR amplification was carried out in 0.05 cm^3 of a reaction mixture containing 0.1 μg of a *RACE-Ready* cDNA template, a 1× *Advantage 2* PCR buffer (*Clontech*, Palo Alto, USA), a 1× *Advantage 2* polymerase mix, a 0.2 mM dNTP mix, 0.2 μM each of gene-specific primers LF6 and LAS10 with the following conditions: 94 °C (5 s) and 72 °C (3 min) for 5 cycles; 94 °C (5 s), 70 °C (10 s), and 72 °C (3 min) for 5 cycles; 94 °C (5 s), 68 °C (10 s), and 72 °C (3 min) for 22 cycles, and a final extension at 72 °C for 7 min.

Sequence analyses: Nucleotide and protein sequences were analysed using *Biology Workbench*, *v. 3.2* (http://workbench.sdsc.edu) and *Bioedit* tools (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence similarity searches were performed *via NCBI BLAST* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene structure was analyzed using *SPIDEY-NCBI*

(http://www.ncbi.nlm.nih.gov/SPIDEY). Protein motifs/ domains were analyzed using *CDD Search* (Marchler-Bauer *et al.* 2013), *Inter-Pro Scan* (http://www.ebi. ac.uk/Tools/pfa/iprscan/), and *MEME* (http://meme.sdsc. edu/meme/meme.html). Regulatory motifs in the promoter sequences were identified using *PlantCARE* (http:// bioinformatics.psb.ugent.be/webtools/plantcare/html/). The prediction of subcellular localization was performed using the *Target P program v. 1.1* (http://www.cbs. dtu.dk/services/TargetP). Hydropathy profiles were analyzed using *Kyte-Doolittle Plot*. The protein secondary structure prediction was carried out using the *PsiPred* program (http://bioinf.cs.ucl. ac.uk/psipred). The protein 3D model was predicted using *3D-JIGSAW* (http://bmm.cancerresearchuk.org/ ~3djigsaw/) and *I-Tasser* (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and viewed using the *RasMol* latest version (http://www. umass.edu/microbio/rasmol/getras.htm). Additional analyses and predictions not stated above were also performed using various programs in *Biology Workbench*, *Bioedit*, and *Expasy toolkit* (http://web. expasy.org/tools).

Expression analyses *via* Northern blot and RT-PCR: For Northern blot, 7 µg of total RNA was denatured at 55 ^{o}C for 15 min in a solution containing 78 % (v/v) deionized formamide, 16 % (v/v) deionized glyoxal and a 10 mM sodium phosphate buffer. The denatured RNA was separated on a 1.2 % (m/v) agarose gel for 3 h at 100 V in a 40 mM Tris-acetate buffer, pH 7.6, and transferred to a nylon charged membrane (*Hybond-N*$^{+}$, *Amersham*, Buckinghamshire, UK) overnight by capillary blotting (Sambrook and Russell 2001). The 3` untrans-lated region (UTR) was generated *via* PCR using gene-specific primers LF3 and LR1 and was used as probe for Northern hybridization. The probe was labelled with α^{-32}P dCTP by a random primer reaction using a *Megaprime* DNA labelling system kit (*Amersham*) following the manufacturer's instructions. The membrane was hybridized in 5× saline-sodium citrate (SSC), 5× *Denhardt*, 0.5 % (m/v) sodium dodecylsulphate (SDS) with the probe at 65 °C overnight, and the next day washed with 2× SSC and 0.1 % SDS at 65 °C for 10 min , 1× SSC and 0.1 % SDS at 65 °C for 15 min, and 0.5× SSC and 0.1 % SDS at 65 °C for 20 min. Finally, the membrane was exposed to an X-ray film at -70 °C overnight. For RT-PCR, a single stranded cDNA was synthesized from 2 µg of DNAse-treated total RNA using a high-capacity cDNA reverse transcription kit (*Applied Biosystems*). PCR amplification was carried out in 0.025 cm^3 of a reaction mixture containing 0.1 µg of cDNA, a 1× *Advantage 2* PCR buffer, a 1× *Advantage 2* polymerase mix, a 1× dNTP mix, 0.2 µM LF1, and 0.2 µM LR1 primers with the following conditions: denaturation at 95 ^{o}C (1 min); 95 °C (30 s) and 60 °C (1 min) for 30 cycles; and a final extension at 60 °C for 1 min. The amplification of the actin gene as internal control was performed in 0.025 cm^3 of a reaction mixture containing 0.1 µg of cDNA, a 1× *Advantage 2* PCR buffer, a 1× *Advantage 2* polymerase mix, a 1× dNTP mix, and 0.2 µM actin F and 0.2 µM actin R primers with the following conditions: denaturation at 95 °C (1 min); 95 °C (15 s), 55 °C (30 s), and 72 °C (2 min) for 30 cycles; and a final extension at 72 °C for 7 min.

Genomic DNA amplification, Southern analyses and gene family search: The amplification of the FLL1 genomic region was carried out in 0.05 cm^3 of a reaction mixture containing 0.1 µg of genomic DNA, a 1× *Advantage 2* PCR buffer, a 0.7 U *Expand HF* PCR system enzyme, a 1× dNTP mix, and 0.2 µM LF6 and 0.2 µM LAS10 primers with the following two-step PCR conditions: an initial denaturation at 95 °C (1 min); denaturation at 95 °C (30 s), simultaneous annealing and extension at 65 °C (1 min) for 30 cycles, and final annealing and extension at 65 ^{o}C for 3 min. A total of 15 µg of genomic DNA was digested individually with BamHI, EcoRI, SpeI, and XbaI (*Fermentas*, Germany), respectively. Southern analyses were carried out following a standard procedure described by Sambrook and Russell (2001). The membrane was subjected to hybridization with a 3' UTR cDNA probe in high stringency conditions (5× SSC, 5× Denhardt, 0.5 % SDS at 65 °C overnight) and then washed with 2× SSC and 0.1 % SDS at 65 °C for 10 min, 1× SSC and 0.1 % SDS at 65 °C for 15 min, and 0.5× SSC and 0.1 % SDS at 65 °C for 20 min. To search for other putative members of the lipase class 3 family, a *Hidden Markov* model (HMM) profile was build using the amino acid sequence of the conserved lipase class 3 domain (*Pfam ID*: PF10764) downloaded from *Pfam* (http://pfam. xfam.org) and searched against the translated transcriptome and gene model of oil palm (Singh *et al.* 2013) using the *HMMER3* (Eddy 2011) hmm search programme.

Promoter isolation: The isolation of the *FLL1* promoter was carried out using universal *Genome Walker* and *Advantage 2* PCR kits (*Clontech*). Four *Genome Walker* libraries were constructed *via* the digestion of genomic DNA with DraI, EcoRV, and PvuII dan StuI prior to ligation with the *GenomeWalker* adaptor. Primary PCR amplifications were performed using all four *Genome Walker* libraries as the DNA template. Each reaction mixture contained 100 ng of *Genome Walker* library, a 1× *Advantage 2* PCR buffer, a 0.2 mM dNTP mix, 0.2 µM each of primers AP1 and LAS11, a 1× *Advantage 2* polymerase mix, in a two-step cycle parameters: 94 °C (25 s) and 72 °C (3 min) for 7 cycles; 94 °C (25 s) and 67 °C (3 min) for 32 cycles, and a final extension at 67 °C for 7 min. The primary PCR product was diluted 50× and used as DNA template for secondary PCR. The secondary PCR mixture was similar to that of the primary PCR except the primers used were primers AP2 and LAS14. The PCR reaction was performed in 5 cycles of 94 °C (25 s) and 72 °C (3 min), followed by 20 cycles of 94 °C (25 s) and 67 °C (3 min), and a final extension at 67 °C for 7 min.

Promoter-vector construct and transient assay analysis: Plasmid *FLL1/GUS* was constructed by replacing the CaMV 35S promoter contained in pBI221 with the HindIII-Xba1 flanked *FLL1* promoter sequence from the position -664 to 83 bp. The amplification of the HindIII-Xba1 flanked *FLL1* promoter region was performed in 0.05 cm^3 of a reaction mixture containing 25 ng of plasmid DNA, a 1× *Expand HF* buffer with 15 mM $MgCl_2$ (*Roche*), a 0.2 mM dNTP mix (*Clontech*), 0.1 µM each of gene-specific primers PLF2 and PLR1, and a 0.1 U *HF Enzyme* mix (*Roche*) under the following conditions: 94 °C (3 min), 94 °C (1 min), 57 °C (1 min), and 72 °C (90 s) for 20 cycles, and a final extension at 72 °C for 10 min. The cloning procedure was confirmed *via* restriction enzyme analysis and the insert was verified by sequencing. The preparation of target materials for transformation, the bombardment parameters, and the GUS histochemical assay were carried out as described by Zubaidah and Siti Nor Akmar (2003).

Results and discussion

The primers used for PCR amplifications are listed in Table 1 Suppl. A 1448 bp cDNA fragment designated as 5`4LAS2 was amplified to represent the 5` region of the FLL1 cDNA *via* 5` RACE. The complete sequence of the *GeneRacer*™ RNA oligo primer was detected at the 5` region confirming that the full-length message from the 5` end was successfully obtained and that the PCR product was very specific to the respective gene (Invitrogen Instruction Manual of *GenerRacer*™ kit, 2004). A translation start codon (ATG) was detected at the 5` end and the sequence adjoining the start codon is adequate to the Kozak (1999) initiation motif (ANNATGG). The O65EST cDNA isolated from the 17-WAA mesocarp cDNA library contains a 350 bp cDNA fragment including a 22 bp poly $(A)^+$ tail (Nurniwalis 2006, Nurniwalis *et al.* 2008). By assembling the 5` RACE and O65EST cDNA sequences, an overlap of 112 nucleotides or 37 amino acid residues with a 100 % identity at the nucleotide and protein levels were observed (Fig. 1 Suppl.). The reconstitution of both the sequences resulted in the amplification of the full-length FLL1 cDNA *via* LD-PCR. The full-length cDNA was 1 721 bp long and contained an open reading frame (ORF) of 1 452 bp (Fig. 1 Suppl.). The ORF was flanked by 106 bp of 5` UTR followed by 141 bp of 3` UTR and a 22 bp poly $(A)^+$ tail. At the 3` UTR, two putative polyadenylation sites were found to differ slightly from the plant consensus sequence, *i.e.*, AATAAA (Joshi 1987a). Nevertheless, it contained four to five out of six base matches of the conserved sequence found in most plants (Hunt 1994). One polyadenylation site was located 20 bp upstream the poly $(A)^+$ tail. This matched an expected regulatory region (11 - 24 bp) for 3` end pre-mRNA cleavage and polyadenylation, but often, an accurate position and a detailed mechanism of 3` mRNA processing remains unclear (Jin and Bian 2004).

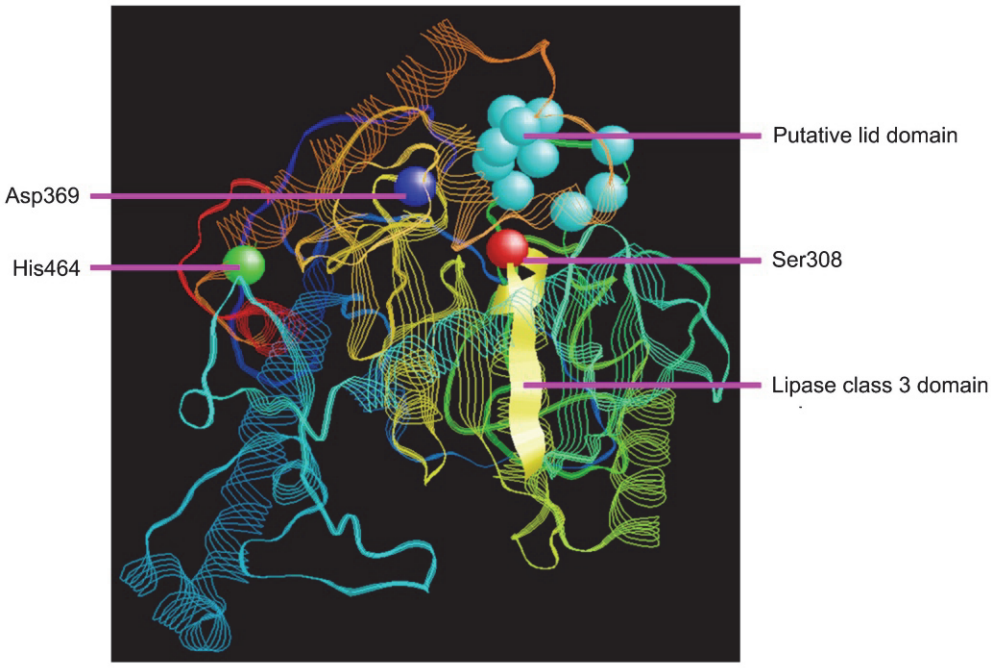

Fig. 1. A three-dimensional best model for FLL1 predicted protein selected based on a C-score using *I-Tasser*. The putative lipase catalytic triad Ser308, Asp369, and Hid464 (represented as the *balls*), the lipase class 3 conserved region (represented as the *ribbon*) and the putative lid domain (represented as the *balls*) are indicated. The model is presented using *RasMol v. 2.6*.

The nucleotide sequence alignment between *FLL1* and the oil palm transcriptome data from 15 WAA mesocarp tissues (Singh *et al.* 2013) showed a 100 % identity with an e-value of 0.0. The database searches for homologies against the nucleotide and protein sequences showed that *FLL1* is 99 and 100 %, respectively, identical to *EgLIP1*, a lipase class 3 cDNA (accession No. AFV50601.1) from oil palm at the nucleotide and protein level (97 % query coverage). The next closest and the following matches to the FLL1 deduced amino acid sequence share a much lower homology with a putative lipase (identity of 51 % and an e-value of $6e^{-144}$) from rice (acc. No. NP_001054678.2) followed by lipase (44 % identity and an e-value of $3e^{-128}$) and triacylglycerol lipase (43 % identity and an e-value of $2e^{-124}$) from castor bean (acc. Nos. AAV66577.1 and XP_002533321.1). The conserved domain and homology search showed that FLL1 contained the lipase consensus sequence [LIV]X[LIVAFY][LIAMVST]G[HYWV]SXG[GSTAC] that encoded the highly conserved lipase class 3 domain (Pfam ID: PF10764). The region of sequence similarities detected all three putative amino acid residues that form the lipase catalytic triad. The serine (S) residue was detected at position 308 and the aspartic acid (D) residue at position 368. Histidine (H), the third residue to complete the catalytic triad, had more diverse flanking sequences, but the exact H position was predicted at location 464 based on consensus sequence alignments (Fig. 2 Suppl.). In addition, putative active site lids at positions 223, 224, 226, and 229-236 as well as nucleophilic elbow at positions 306 - 310 were detected in FLL1 (Fig. 1 Suppl.).

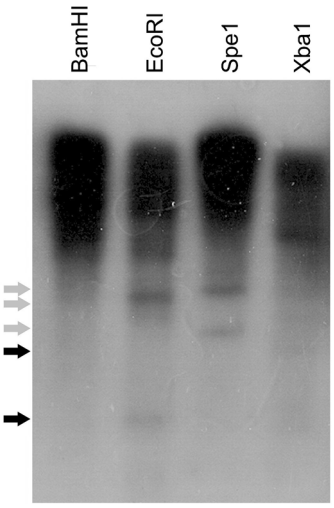

Fig. 2. The southern analysis of oil palm genomic DNA. Blots were hybridized with a gene-specific sequence based on the 3`UTR of the *FLL1* gene. Strong and faint signals are shown by the *grey* and *black arrows*.

The deduced amino acid sequence of FLLI was predicted to have a secondary structure that consists of 12 β-sheets, 14 α-helixes, and 25 coils. FLL1 had more hydrophobic residues (47.4 %), and a Kyte-Doolittle hydropathy profile showed the predicted protein was predominantly hydrophobic. The three dimensional protein prediction using *3D-JIGSAW* detected three family domains (pb073089, pf01764, and pb108722) in FLL1 but only pf01764 (the lipase class 3 domain) was homologous to FLLI. FLL1 had an identity of 50 % and an e-value of $1e^{-55}$ to 3ngmA, a crystal structure of lipase from *Gibberella zeae* from location 64 to 221. The structural protein prediction of FLL1 using *I-Tasser* generated five 3-D output models, ranking model 1 as best predicted (Zhang 2008) with a C-score more than -1.5 to indicate correct protein folding (Roy *et al.* 2010). Fig. 1 represents the best predicted model 1 with a C-score value of 2.83 based on the highest structural alignment to a lipase protein from *Gibberella zeae* (PDB hit:3ngmA). The predicted EC number for FLL1 is 3.1.1.3, which represents a triacylglycerol lipase. Based on a gene ontology (GO), the *FLL1* gene has a molecular function that is involved in triglyceride lipase activity (GO:0004806). No signal peptide or transmembrane domains were identified in FLL1 using various subcellular localization prediction searches, which would suggest that FLL1 is cytoplasmic.

Southern blot was used to determine the presence of lipase gene(s) in the oil palm genome. The oil palm genomic DNA was digested with BamHI, EcoRI, XbaI, and SpeI, respectively, followed by hybridization using the 3` UTR *FLL1* cDNA as probe. At high stringency conditions, more than two bands were detected in all the restriction enzyme-digested oil palm genomic DNAs (Fig. 2). This result indicates that *FLL1* was not a single copy gene and that it belonged to a multi gene family. Thus, the search of other putative members of this family was carried out using the *BLAST* program and a Pfam ID: PF10764 as query sequence on the oil palm transcriptome and genome data (Singh *et al.* 2013). To date, we have identified 27 putative genes encoding the lipase class 3 domains (data not shown) with protein identities ranging from 26 to 83 % to *FLL1*.

Northern blot and semi-quantitative RT-PCR were carried out using RNA from various oil palm tissues, such as the mesocarp at different developmental stages, namely 8, 10, 12, 15, 17, and 20 WAA, the kernel, spear leaves, roots, and germinated seedlings. Northern blot was performed using a pair of gene-specific primers that correspond to the 3` UTR of the *FLL1* sequence where it hybridized to a transcript with the size of about ~1.7 kb (Fig. 3). The size of the hybridized transcript was similar to that of the full-length *FLL1* cDNA which is approximately 1 721 bp long (including the poly $(A)^+$ tail). The expression of the mRNA transcript in the mesocarp tissue was found to gradually increase from the early fruit developmental stages up to when the fruit was fully ripened. This pattern of expression could possibly correlate closely with the pattern of oil synthesis in the mesocarp, and this result is in agreement to that found from the dot blot analysis (Nurniwalis 2006, Nurniwalis *et al.* 2008). The expression of the 3` UTR probe in the other tested tissues, however, appeared to be indistinct.

Hence, RT-PCR was conducted to examine the expression of *FLL1* in various oil palm tissues. For RT-PCR, two primers specific to the corresponding sequence of the lipase class 3 EST sequence, O65EST cDNA (ORF + 3` UTR), and *actin* as internal control were used. PCR products with the size ~400 bp were amplified and the size corresponded well to O65EST cDNA. The PCR products were amplified from all the mesocarp tissues from young fruits until the ripening period and the band intensities appeared to be strong in these tissues in comparison to the *actin* gene (Fig. 3). As RT-PCR is a more sensitive technique for mRNA detection and quantitation, trace amounts of PCR products were also detected in the germinated seedlings and even lower in the roots. No PCR product was detected in the rest of the tested tissues (the kernel, spear leaves, male and female flowers). RT-PCR was also performed to determine the expression pattern of *FLL1* in

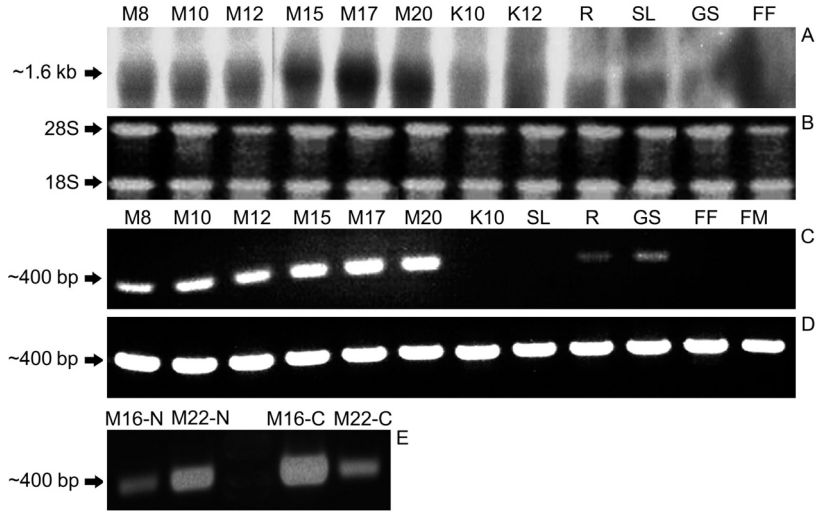

Fig. 3. Expression patterns of *FLL1* gene in various oil palm tissues as shown by the Northern analysis and RT-PCR. The blot was probed with a gene-specific sequence based on the 3`UTR (*A*); an ethidium bromide stained gel that shows approximate equal loading total RNA (*B*); the RT-PCR amplification of the 3`region of *FLL1* using gene-specific sequences (*C*); the amplification of the *actin* control gene (*D*); and the comparison of the *FLL1* cDNA in normal and cold treated fruits (*E*). M - mesocarp, K - kernel, R - roots, SL - spear leaves, GS - germinated seedlings, FF - female inflorescence, FM - male inflorescence, N - normal, and C - cold treated fruits. Numbers in the mesocarp and kernel - developmental stages at WAA.

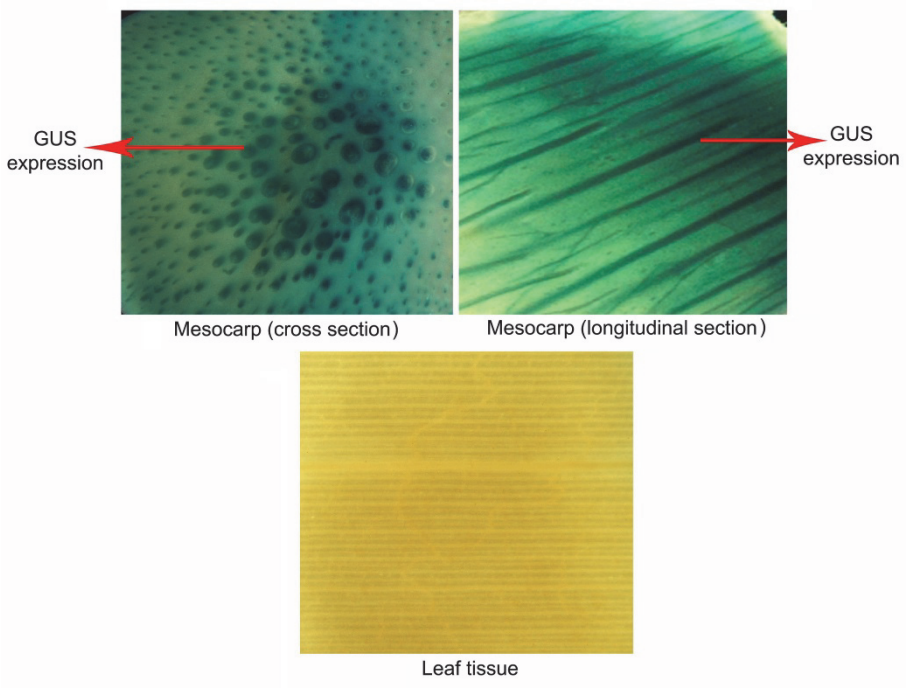

Fig. 4. An *FLL1pro:GUS* expression profile in the mesocarp and leaf tissues of oil palm.

the mesocarp of cold induced oil palm fruits. Comparison was carried out in normal and cold treated fruits at two stages of fruit development, *i.e*., 16 and 22 WAA. The results show that both the normal and cold treated fruits had a similar expression pattern. However, the expression of the *FLL1* gene was higher in the cold induced fruits than in the normal fruits (Fig. 3).

The amplification of the *FLL1* genomic sequence yielded a 5.5 kb fragment. The genomic organization and presence of *FLL1* in the oil palm genome were confirmed through a sequence alignment between the amplified *FLL1* genomic sequence and the scaffold p5-sc00064 of the fifth genome build [p5-build] (Singh *et al.* 2013). The result shows a 100 % identity at the nucleotide level with an e-value of 0.0. An exonerated search with a 60 % self-score threshold revealed *FLL1* at chromosome 3 in the EG5 build. The comparison of the *FLL1* genomic sequence and oil palm microsatellite genomic DNA by Morcillo *et al.* 2013 (acc. No. HE661587.1) revealed a 97 % identity suggesting that both regions are identical. The amplification of the *FLL1* genomic sequence and an exonerated search revealed the presence of four introns located in between five exons in *FLL1*. The size of the introns were 1 424, 555, 1 626, and 179 bp and the five exons 382, 224, 34, 620, and 251 bp, respectively. The intron-exon boundaries in *FLL1* conformed to the universal GT-AG rule for introns starting with a GT dinucleotide and ending with an AG dinucleotide (Breathnach and Chambon 1981).

The upstream genomic region of *FLL1* was further amplified to isolate its corresponding promoter using the Genome Walker approach. The PCR amplification resulted in the amplification of a 756 bp fragment. This genomic sequence contained 671 bp of promoter sequence and 85 bp of 5' UTR. An adenine residue located furthest at the 5' terminal of *FLL1* was selected as TSS. The adenine residue is most likely the TSS as the pyrimidine sequences flanking the adenine residue are often the preferred TSS motif in plants {C/TAC/T} (Joshi 1987b). The putative TSS motif also matches most of the TSS motifs in highly expressed genes in plants (Sawant *et al.* 1999).

The distribution of sequences in the FLL1 promoter is divided into three main groups which contain the TATA-box, pyrimidine patch (Y-patch), and regulatory element group (REG) (Yamamoto *et al.* 2007). The putative TATA-box, TATATATTA, was present 37 bp upstream of the TSS, consistent with the distance of 32 ± 7 bp (Joshi 1987b). The Y-patch motif was located within 100 bp upstream of the TSS and downstream of the TATA-box. The role of the Y-patch motif, a plant-specific core element, is unknown but the local distribution of short sequence analysis showed that it maked up one of the general/important components in the core promoters of dicots and monocots (Yamamoto *et al.* 2007). The REG group contains *cis*-acting regulatory elements (CARE) that correspond to known transcriptional regulatory sequences. In the *FLL1* putative promoter, a number of putative CAREs were identified, which included those that respond to environment signals as well as those required to direct specific expressions to specific tissues (Table 2 Suppl.).

A schematic diagram of the *FLL1* promoter cloned into the pBI221 transformation vector carrying *GUS* as reporter gene that replaced the 35S promoter is shown in Fig. 3 Suppl. The activity of the *FLL1* promoter in oil palm mesocarp slices was analysed using a transient *GUS* expression analysis. The transient *GUS* expression was driven by the *FLL1* promoter fused to the *GUS* reporter gene construct using optimized bombardment parameters (Zubaidah and Siti Nor Akmar 2003). The expression of the *GUS* gene was detected in the mesocarp slices at 12 WAA but not in the leaves (control tissue) (Fig. 4). These results support the expression analysis of *FLL1 via* Northern blot and RT-PCR. The lipase gene is expressed throughout the fruit developmental stages. Due to difficulty in handling the ripe fruit for a transient expression assay because of a high oil content, the 12 WAA stage was chosen as the target tissue to represent the expression of the promoter. Longitudinal and cross sections of the mesocarp tissues demonstrated that the *GUS* expression was targeted to vascular bundles. This result is similar to that of the *MT3A* promoter of metallothionein gene which directs a high expression in mesocarp tissues (Siti Nor Akmar and Zubaidah 2008, Zubaidah and Siti Nor Akmar 2010). Phloroglucinol-stained mesocarp sections for lignin detection also showed a similar result (Singh *et al.* 2013). Lignins are normally associated with the vascular bundle in plants. They are hydrophobic in nature which allows water transportation throughout the plant (Xu *et al.* 2009). In the mesocarp tissues of oil palm (Sambantamurthi *et al.* 1995) and olive (Panzanaro *et al.* 2010), lipase activities have been demonstrated to be associated with oil bodies and require a hydrophobic condition to function. Similar targeted expressions of the *GUS* gene and lignin to vascular bundles in mesocarp slices are unclear, but this raises the possibility that they could share a common trait or function that is still unknown.

We describe here the isolation and characterization of the full-length lipase class 3 gene (*FLL1*) and its corresponding promoter from oil palm. FLL1 contains the lipase consensus sequence ILVTGHSLGG which includes the active serine residue (S_{308}) that forms the esterase box GxSxG motif. Together with the aspartic acid (D_{368}) and histidine (H_{464}) residues, they form the putative Ser-Asp-His catalytic triad that is essential for esterase and lipase activities to hydrolyze/de-esterify fatty acids from complex lipids (Li *et al.* 2012). This feature is not only common in lipases from plants but in animal, fungi, and bacteria as well (Patil *et al.* 2011). FLL1 is hydrophobic and the hydrophobic residues in the putative lid domain is likely important for the catalytic activity of FLL1.

FLL1 was 100 % identical to the mesocarp trans-criptome and p5 build genome data at the nucleotide level. This result is in line to that of oil palm genome data where most of the oil palm genes involved in oil quality

were derived from the paternal pisifera (Singh *et al.* 2013). *FLL1* was also 99 % identical to *EgLIP1* (acc. No. AFV50601.1) at the nucleotide level and was 39 nucleotides longer at the 5` UTR possibly as result of a better cDNA amplification of only the full-length transcripts minus the truncated messages from the amplification process (Invitrogen Instruction Manual of GenerRacer™ Kit 2004). The sequence alignment of *EgLIP1* to the p5 build genome data shows a 99 % identity at the nucleotide level with an E-value of 0.0. It was also located on the same p5-sc00064 scaffold of the p5-build and chromosome 3, which strongly suggests that both the genes are identical.

The recent release of the oil palm genome sequence provides rapid means to identify genes of interest from oil palm (Singh *et al.* 2013). The preliminary search identified putative members of the lipase class 3 family in oil palm with identities ranging from 26 to 83 % to *FLL1*. This is possibly due to the nature of the lipase class 3 family where polymorphisms were observed within the gene members. Thirty-eight putative lipase class 3 proteins were identified in *Arabidopsis* containing the conserved lipase class 3 domain (PF01764) but there is quite a divergence in terms of their sequence homologies, gene structures, and expression patterns (Li *et al.* 2012). This result suggests that presence of other members of the lipase class 3 family in oil palm is possible and they may play specific or constitutive roles in plant growth and development especially with regards to TAGs.

The Northern and RT-PCR analyses indicated that *FLL1* was highly expressed in the mesocarp tissues of oil palm. The *FLL1* transcripts were detectable in all the tested fruit developmental stages and the expression was stronger as the fruits reached maturity. It was possibly induced in the cold treated fruits in agreement to what was reported by Sambanthamurthi *et al*. (1995). The activation of lipase at a low temperature in oil palm fruit bunches may provide an alternative to FFA production *via* fat splitting for use in the oleochemical industry (Sambanthamurthi and Kushairi 2002). FLL1 is also present in germinated seedlings as well as in roots but at very much lower amounts just as reported by Morcillo *et al.* (2013). Oo and Stumpf (1983) have detected a lipase activity in oil palm germinated seedlings. Because lipase is the first enzyme involved in TAG breakdown, the low expression of *FLL1* in the germinated seedlings is logical for early seedling growth although there is no evidence to prove that lipase class 3 genes are responsible for this. Apart from the well-studied role of lipase in germinated seedlings (Murphy 1993), the role of lipase in fruits is still unclear. It has been suggested that the lipase in the oil palm mesocarp serves to increase the palatability of fats (Morcillo *et al.* 2013). Another possible role of mesocarp lipase in trans-esterification during lipid synthesis has also been raised (Sambantahmurthi *et al*. 1995). The oil palm mesocarp tissue is also a suitable target for introducing novel characteristics for the production of high and value-added products and improving oil quality. Thus, from the molecular/genetic engineering aspect, the high expression of the *FLL1* gene encoding the lipase class 3 prompted the isolation of its corresponding promoter. This promoter has the potential to direct the expression of a desired gene carrying the trait of interest to the mesocarp for production of novel product(s) in oil palm.

The *in silico* analysis using *PlantCare* shows that the *FLL1* promoter contained several different *cis*-acting regulatory motifs, which suggest a complex regulatory system to regulate the *FLL1* expression. Majority of the CAREs present in the *FLL1* promoter are associated with light responsive elements (LREs). The promoter of a fruit-specific gene, *GalUR* from strawberry whose activity is specifically targeted to the fruit, is dependent on irradiance. It contains a G-box motif that is essential for a fruit specific expression (Agius *et al.* 2005). In addition, an I-box motif, another LRE, is also present in the *GalUR* promoter from strawberry as well as in other fruit and in tissue-specific promoters in melon (Yamagata *et al.* 2002) and oil palm (Siti Nor Akmar and Zubaidah 2008). To date, the role of the LRE in the *FLL1* promoter in association with irradiance is uncertain, but the detection of multiple LREs suggests that the promoter activity is most likely enhanced by irradiance.

Two low temperature responsive element (LTRE) sequences associated with a low temperature responsiveness were identified in the *FLL1* promoter in the forward and reverse orientations. In *Arabidopsis*, the LTRE elements in the promoter region of the *COR15A* gene demonstrated an involvement in cold, drought, and ABA-regulated gene expressions (Baker *et al.* 1994). Transgenic potato containing the LTRE element exhibited an extensive cold inducibility in a tuber-specific manner (Liu *et al.* 1994). Integration of LTRE and LRE signals in *Arabidopsis* is required for a full response towards a cold acclimation (Catalá *et al.* 2011). The LTR elements in the *FLL1* promoter suggests that plant lipases, especially *FLL1* encoding the lipase class 3, play a role in TAG hydrolysis possibly through the activation of its corresponding gene at a low temperature. This is somewhat consistent to that of the RT-PCR results as well as the findings reported by Sambanthamurthi *et al.* (1995) where lipases in oil palm are activated at a cold temperature.

The strength and activity of the promoter determines its effectiveness in controlling and regulating gene expression. The transient assay system using the *GUS* reporter gene is a common method in assessing the trait of a promoter. Testing the *FLL1* promoter activity *via* a transient *GUS* expression using biolistic methods on oil palm mesocarp slices is preferred in comparison to generating oil palm transgenics. The assay saves a lot of time and is also the preferred choice to test other oil palm promoters (Zubaidah and Siti Nor Akmar 2010, Masura *et al.* 2011). With the high expression and specificity of the *FLL1* promoter observed in the mesocarp tissues, it has a high potential to be used as biotechnology tool to genetically engineer oil palm for production of novel oils and products.

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