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Original Article

Over expression and application of the β-carboxyltransferase *(EgaccD)* gene in oil palm (*Elaeis guineensis* Jacq)

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Abstract

Oil palm is an economic crop with its oil currently in high demand. In this work, the numbers of *EgaccD* copies, one of the key genes involved in fatty acid synthesis and oil production, were compared from two populations of oil palm, one a low and the other a high oil content productivity cultivar. Real time PCR was employed and the copy number of the *EgaccD* from the high oil yielding cultivar was much higher than from the low oil yielding cultivar. The *EgaccD* was then over expressed in oil palm calli under the control of its promoter to establish if its overexpression could enhance the oil content of transgenic calli. An increase in the lipid accumulation by the modified calli was detected by Sudan black B staining. Based on this finding, genetic manipulation of *EgaccD* seemed to be one promising method to try to increase the oil content. Also it seemed possible to use the level of this gene as a marker to assist selection of possible high oil yielding cultivars in breeding programs.

Keywords: copy number, *EgaccD*, marker assisted selection, palm oil content

1. Introduction

Oil from the fruit of the oil palm (*Elaeis guineensis* Jacq) is becoming one of the major sources for bio-diesel production. Its annual production per hectare of planted area can be as high as 7,250 liter/year, which is far higher than for coconut and soybean (Majid and Parveez, 2007). The preferred cultivation is tenera (Sh^+Sh^-) hybrid was derived exclusively from the dura (Sh^+S^+) X pisifera (Sh^-Sh^-) of *E. guineensis* Jacq. Tenera produces higher yields than its parents so its planting is recommended and widely distributed (Billotte *et al.*, 2010). At present, the worldwide demand for palm oil is continuously increasing. However, production of new and better elite genotypes from breeding programs has been extremely slow and has only increased yields slightly (Corley and Tinker 2003). However, the use of high yielding planting materials is one of the most important ways that could lead to increased oil palm productivity. This would decrease the time necessary to quickly disseminate these elite genotypes obtained from breeding programs (Lin *et al.*, 2009). Even though conventional breeding programs have made some contributions to the production of high yielding oil palm planting materials, progress is considered to be slow due to a limited genetic diversity and the length of the selection cycle for oil palm (Wong and Bernardo, 2008). Further improvements using molecular genetic strategies have now been given a priority to ensure successful manipulation of oil palm for years to come. Such molecular techniques have shortened the duration of the breeding programs from years to months, weeks, or even eliminated

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the need for them altogether (Mayes *et al.*, 2008; Stuber *et al.*, 1999).

One of the approaches that might overcome potential problems is to try metabolic engineering of some of the key genes required for oil biosynthesis and/or for use as a marker to select high yielding oil palm cultivars (Tester and Langridge 2010; Varshney *et al.*, 2007). Several publications have shown a correlation between the activities of acetyl-CoA carboxylase (ACCase) and the rate of fatty acid synthesis and oil accumulation (Francki *et al.*, 2002; Klaus *et al.*, 2004). In barley, maize and tobacco, ACCase was proven to be the key enzyme in controlling triacylglycerol biosynthesis (Page *et al.*, 1994; Shintani *et al.*, 1997). The ACCase enzyme is a multisubunit complex of four components, a biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and the α-subunit and the β-subunit of carboxyltransferase (CT). These four components are encoded by four subunit genes named *accB*, *accC*, *accA*, and *accD*, respectively, as reviewed by Li *et al.* (2010). In a previous study, there was a strong indication that the expression level of β-carboxyltransferase *(EgaccD*) mRNA was directly correlated with the oil palm's productivity (Nakkaew *et al.*, 2008). It was of interest, therefore, to use a plastid-encoded subunit of the *EgaccD* gene as a marker to assist in the selection of high oil producing variants. Also was it possible to determine if there was any correlation between the copy numbers of the *EgaccD* gene from the plastid genome and a high productivity plant. Furthermore, a transgenic approach was used to determine the importance of the *EgaccD* from a plastid genome for production and accumulation of oil. In this study, transgenic oil palm calli were produced and checked for their lipid accumulation. This work is the first attempt to enhance oil palm productivity by genetic modification of the *EgaccD*, b subunit of ACCase in oil palm.

2. Materials and Methods

2.1 Plant materials

Different *tenera* cultivars of *Elaeis guineensis* were used as the source of the embryos to produce callus. The sterilised fruits were halved and the embryos were extracted. These embryos were cultured on MS media supplemented with 2.5 mg/L 2, 4-D at 25°C and subcultured onto fresh media every month. After one month of culture, small calli were observed and they were used for transgenic studies. The leaves were harvested from fifteen-year old oil palm plants for the isolation of chromosomal DNA for the detection of the copy number of *EgaccD* in *E. guineensis* tenera varieties that had been produced from low and high oil yielding plants.

2.2 The copy number of *EgaccD* in chromosomal DNA

To measure the copy number of *EgaccD*, chromosomal DNA was isolated from 16 samples of *E. guineensis* leaves (tenera variety types) including 8 samples of low and 8 samples of high oil content oil palms and treated with the RNaseH (Invitrogen) according to the manufacturer's instructions. The real-time PCR was conducted by amplifying 0.1 µg of DNA with the iQ™ SYBR® Green Super Mix (BioRad) on the MX3000P™. The efficiency of the real-time PCR reactions was determined by performing the reaction using a DNA standard in the range of a 10^5- 10^9 copy numbers and the cycle-threshold (Ct) values were plotted against the log10 concentration of the template. To estimate the copy number of the target gene (*EgaccD*) in the oil palm genome, the real-time PCR technique was used to quantitate relative to a single-copy endogenous gene, (the defensin gene, *EgAD1*; GenBank accession no. AF322914) (Weng *et al.*, 2004). The genome of the 16 leaf samples was extracted and analyzed for the estimated number of copies of *EgaccD*. The PCR reaction was performed to amplify a 394-bp of *EgaccD* using the gene-specific primers set (EgaccD F and EgaccD R, Table 1) and *EgAD1* was used as the control by using the *EgAD1* gene-specific primer set (EgADF and EgADR, Table 1). The specificity of the amplification products was determined by the use of dissociation curves and then the genomic copy number of the samples was calculated from the Ct by interpolation from the standard curve by MaxPro QPCR Software Version 4.01 (Stratagene) (Nakkaew *et al.*, 2008; Weng *et al.*, 2004).

2.3 Cloning of EgaccD and an Eg rbcl-accD promoter for plant transformation

Total RNA was isolated from 100 mg of young *E. guineensis* leaves using the RNeasy extraction Kit (Qiagen). Fragments of *EgaccD* were amplified using an RT-PCR reaction, according to the manufacturer's instructions (Qiagen). The specific primers were designed from the *EgaccD* (GenBank accession no. DQ004687). The specific primers used were as follows; the EgaccD forward and EgaccD reverse primer were used to amplify the full length of *EgaccD*. The RT-PCR products with the expected sizes were purified and inserted into the pGEM-T Easy Vector (Promega). Subsequently, the recombinant DNA plasmid was sequenced and used as the template for construction of the *EgaccD* for plant transformation. The forward and reverse PCR primer corresponding to the beginning of the ORF with the addition of an upstream in-frame *Nco* I restriction site (*EgaccD Nco*I F and *EgaccD Nco*I R, Table 1) were used to amplify *EgaccD*. The PCR products were ligated to a similarly digested pCAMBIA 1303 (www.cambia.com.au), carrying the *gus* and *mgfp5* reporter gene controlled by a CaMV35S promoter. The recombinant plasmid DNA fragments were sequenced using the 3730 DNA sequencer to ensure the authenticity of the cloned nucleotide sequence and it was named p3EgD plasmid.

For amplification of the *Eg rbcl-accD,* a promoter sequence of the *E. guineensis* chloroplast genome was used to design the primer sets. The forward PCR primer cor-

Table 1. Sequences of the primers that were used in this study.

Primer name	Sequences (5' → 3')
EgaccD F	CT ATA GCA ATT GGA GTT ATG AAT T
EgaccD R	CY GCT TGT GAA CCT TCR GGY AC
EgADF	ATG GAG CAC TCT CGG CGA ATG CTT
EgADR	TTA ACA CTT GAT CTC CTT CAG CC
EgaccD forward	ATG GAA AAA TGG TGG TTC AAT TCG ATG TT
EgaccD reverse	CTC GAA TCA AAA CTC AAA AAA TTT ATA A
EgaccD Nco I F	CCA TGG AAA AAT GGT GGT TCA ATT CGA T
EgaccD Nco I R	CCA TGG ATA TTT TTG AGT TTT GAT T
rbcl-accD BamH I	GGA TCC GTG ATC TTG CTC GTG AAG GTA ATG AA
rbcl-accD Hind III	AAG CTT TTA TAA ATT TTT TGA GTT TTG ATT
CaMV35S promoter	ATG CCA TCA TTG CGA TAA AGG AAA GG
Egrbcl-accD F	GGA TCC GTG ATC TTG CTC GTG AAG GTA ATG AA
Egrbcl-accD R	ACT TAA ATG TAA CTG TCA CTG TAA TTG TC
GUSF	ATG GTA GAT CTG ACT AGT TTA CGT
GUSR	GCA TAC GCT GGC CTG CCC AAC CTT
18SF	CAA AGC AAG CCT ACG CTC TG
18SR	CGC TCC ACC AAC TAA GAA CG

responded to the chloroplast partial *rbcL* gene (AJ404830.1) with the addition of an upstream *BamH* I restriction site; the *rbcl-accD BamH*I primer and the reverse primer corresponding to the 3´ end of the *EgaccD* was flanked by a *Hind* III restriction site; the *rbcl-accD Hind*III primer, were used to amplify *Eg rbcl-accD*. The oil palm chromosomal DNA was isolated by DNeasy kit (Qiagen) and was amplified by using Platinum *Pfx* DNA polymerase according to the manufacturer's instructions (Invitrogen). The PCR product was purified and cloned into the pGEM-Teasy vector (Promega) followed by sequencing to ensure that the cloned nucleotide sequence was correct. The inserted plasmids were double digested with *BamH*I and *Hind*III before being ligated into the *p3EgD* to create the *prbcl-D3EgD*. Subsequently, the recombinant DNA plasmid, *prbcl-D3EgD,* was verified by sequencing and used for transformation of oil palm calli.

2.4 Plant transformation

The calli, approximately 3 mm in size were selected from the same callus line and bombarded using the PDS-1000/He particle delivery system (BioRad). The conditions for biolistic-mediated gene transfer for oil palm were optimised using callus and the pressure of 1100 psi that was found to be the best for bombardment and the frequency of transient transformants. Bombardments without DNA and with an empty vector were used as experimental controls. After bombardment, the calli were selected for transformants in media containing hygromycin (50 mg/mL). The expression of the reporter protein, *mgfp5* gene (green fluorescence protein; GFP) in one-month transgenic calli were visualized with a confocal laser scanning microscope (FV300, Olympus) fitted with a GFP filter set for excitation between 455 nm and 490 nm and emission above 543 nm (Nakkaew *et al.*, 2010).

2.5 DNA analysis of transgenic calli

To analyse for the integration of the *EgaccD* gene into the transgenic oil palm calli, genomic DNA was isolated from one-month old transformants and was used for PCR analysis. The PCR reaction was performed using the primer sets with the specific site, *CaMV35S* promoter and EgaccD R as the primer set were used for PCR amplification of a *CaMV35S-EgaccD* fragment. The *gus* primer set, GUSF forward and GUSR reverse primer, was used as a positive control to ensure a complete transgenic callus at a 298 bp of *gus* amplicon size. An *18S rDNA* primer set of 540 bp fragment size (18SF and 18SR) was used for an internal positive control in the PCR reactions.

2.6 Histological analysis

The control and bombarded calli at the same development stages (2 months after transformation) were fixed in a Navashin solution containing 90 mL of 70% ethyl alcohol, 5 mL of glacial acetic acid and 5 mL of formalin solution. These tissues were dehydrated through an ethanol-tertiary butanol series for 48 h and embedded in paraplast. The specimens were sectioned at 6 µm and stained in a saturated solution of Sudan black B in 70% ethanol for 1 h, washed in 70% ethanol, and rinsed in water (O2Brien and McCully, 1981). All the sections were mounted with gelatin permount and were viewed with bright-field illumination and an Olympus microscope.

3. Results and Discussion

3.1 Estimating the gene copy number of EgaccD by real time quantitative PCR assay

The quantitative PCR of the *EgaccD* copies was performed with specific oligonucleotide primers using the chromosomal DNA samples collected from the leaves of low and high oil content productivity oil palm cultivars (Table 2). The specificity of the amplified PCR products was determined by melting curve analysis, directly following the real-time quantitative PCR analysis. Sequencing of the amplified fragments confirmed the identity of the sequences to the previously assembled *EgaccD* (GenBank accession no. DQ004687) from oil palm. DNA standards for *EgaccD* and *EgAD1* in the range of 10^5- 10^9 copy numbers was used to calculate the gene copy number of both genes in individual samples. Each sample was performed in triplicate and all reactions were independently repeated twice to ensure the reproducibility of the results. The observed gene copy numbers of *EgaccD* by real-time PCR assay was estimated relative to the PCR product of the single-copy of the endogenous, *EgAD1* in the same sample. There was a significantly higher genomic copy number of *EgaccD* in the high oil content compared to the low oil content plants (Figure 1). The average oil content of the high and low oil content productivity plants over a 6 year period are shown in Table 2. Our previous study had shown a correlation between the expression of *EgaccD* from mature oil palm leaves and the yield of the plant (Nakkaew *et al.*, 2008) but we did not know if the

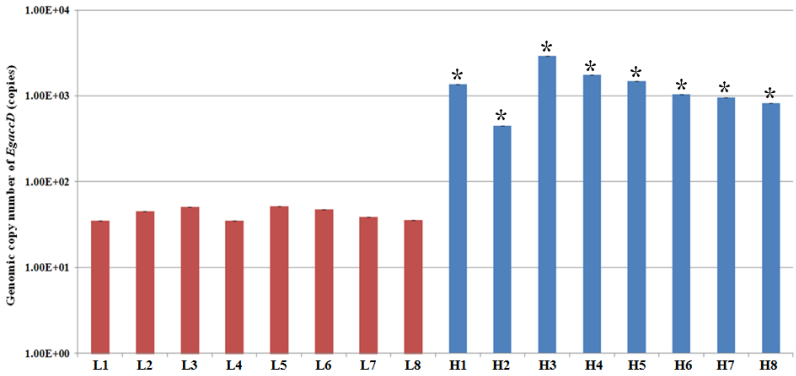

Figure 1. Quantitation of the *EgaccD* copy number in the chromosomal DNA samples collected from low and high productivity oil palm leaves (n=8) by using real time PCR. Each sample was analyzed in triplicate (p = 0.05).

Table 2. Yield of oil content from oil palm fruit that were determined by the value of fresh palm oil (kg/plant/year) that were collected from each tree over a period of 6 years

Sample	Oil yield content (kg/palm/year)						Sum	Average
	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Oil content	Oil content
L1	ND	7.26	24.71	4	ND	4.18	40.15	10.04
L2	2	20.36	11.75	1.48	0.52	5.31	41.42	6.90
L3	2.7	5.46	6.21	4.35	13.87	15.66	48.25	8.04
L4	1.95	10.28	9.14	6.33	10.27	4.52	42.49	7.08
L5	9.8	9.94	19.59	10.34	ND	ND	49.67	12.42
L6	ND	ND	ND	17.27	5.23	ND	22.5	11.25
L7	ND	5.62	ND	ND	ND	ND	5.62	5.62
L8	ND	1.48	ND	3.04	1.43	ND	5.95	1.98
H1	8.2	41.1	25.73	18.32	28.29	36.28	157.92	26.32
H2	18.64	47.5	40.39	28.31	23.73	34.7	193.27	32.21
H3	15.86	44.62	82.75	41.69	41.65	35.48	262.05	43.68
H4	7.8	31.82	39.31	24.36	24.15	37.06	164.5	27.42
H5	2.17	48.68	34.85	57.22	23.09	57.69	223.7	37.28
H6	22.69	48.48	28.54	25.47	42.21	50.75	218.14	36.36
H7	27.86	32.48	44.62	49.79	45.33	55.17	255.25	42.54
H8	25.63	27.68	13.8	40.04	40.02	34.28	181.45	30.24

ND: Not determined.

expression of *EgaccD* from leaves from younger plants would provide a similar correlation. We now know that the relative copy numbers of *EgaccD* obtained from the plastid genomes of leaves from either young or old plants reflected their potential for a low or high productivity phenotype. As *EgaccD* gene is the plastid gene so the copy number of the gene also reflexes the chloroplast number as wellas the productivity of plant. Therefore, determining the genomic copy number of *EgaccD* from the younger plant is perhaps a much better method to use to select for a high oil content yielding plant. This occurrence of a high *EaccD* genomic copy number was positively correlated to the high oil content and productivity of oil palm groups and supported the use of *EgaccD* for MAS selection of high oil yielding palm plants produced by breeding (Francki *et al.*, 2002; Madoka *et al.*, 2002). In a similar way, Gaines et al. (2010) have reported that the genomic copy number of *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) in resistant plants (*Amaranthus palmeri*) was higher than in sensitive plants by 5-160 fold.

3.2 Isolation and sequence analysis of the Eg rbcl-accD franking region

An *Eg rbcl-accD* (GenBank accession no. JQ616774) fragment of 2308 bp was cloned and subjected to DNA sequence analysis for determination of sequences with known binding motives. The 2308 bp fragment contained 829 bp of the putative 5' UTR region of *EgaccD* (called the *accD* promoter) and a 1479 bp including an open reading frame (ORF) that encodes a putative protein of 492 amino acid residues of the beta-carboxyltransferase from oil palm (*Elaeis guineensis* Jacq). The *accD* promoter region (Figure 2) analysis performed with the aid of the Proscan (Version 1.7) detected the putative transcription start site at position -484 and a putative TATA box position at -528/-532. Plantcare also predicted the presence of some cis-regulatory elements related to its metabolic expression and light induction or stress responses, such as a putative GATA-motif, Sp1 and a putative CAAT box at –741/-737, that was relatively close to the transcriptional start site (Figure 2). In addition, a promoter motif search of the *EgaccD* was carried out to define putative transcriptional elements using the MatInspector 7.7.3 software programs on the Genomatix server (Cartharius et al., 2005). A number of potential regulatory motifs corresponding to known cis-regulatory signals of plant genes were found to be present in the sequence. In addition, several core fragments of importance for transcription factors such as MYB proteins (orange line), MADS box proteins (blue line), DNA binding with one finger (DOF) (red line), WRKY domains (green line) and a motif conserved among the sucrose box (yellow bar) as shown in Figure 2.

3.3 Confirmation of transgenic calli by laser-scanning microscope and polymerase chain reaction analysis

The *Eg rbcl-accD* promoters were cloned to *3EgD* at the *BamH*I and *Hind*III sites to produce *prbcl-D3EgD*. Then

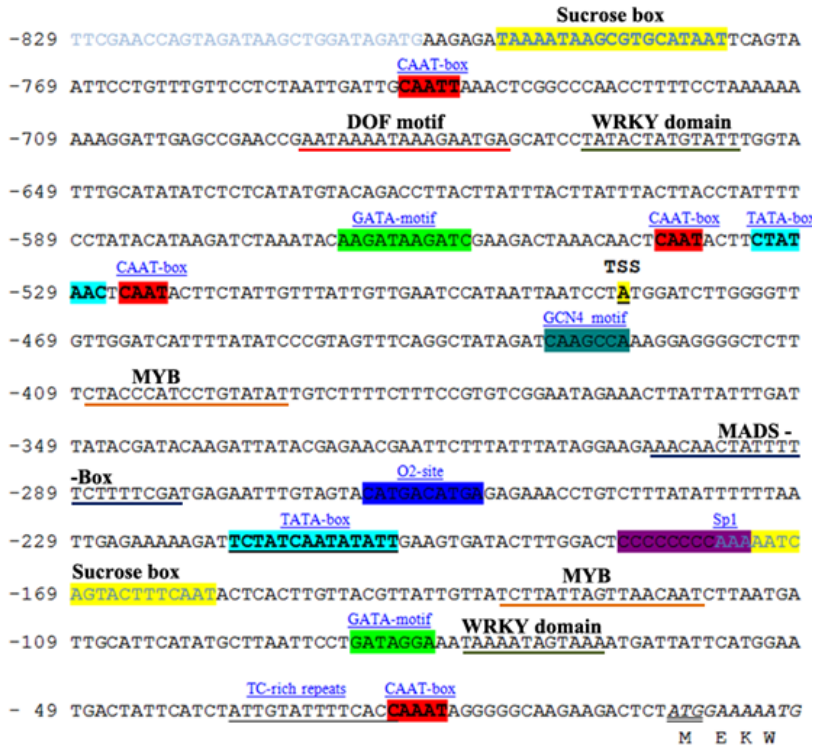

Figure 2. The nucleotide sequence of the 5’ prime region of *EgaccD* gene showing the 829 bp DNA fragment that represents the promoter region of *EgaccD* and has the start codon at position +1 (ATG) and the Transcription start site (TSS) at position -484.

the *prbcl-D3EgD* was transformed into oil palm calli and selected using a growth medium supplemented with 50 µg/L hygromycin. Tissue sections obtained from these transgenic calli were examined directly for GFP fluorescence with a confocal laser scanning microscope. Callus bombarded with an empty vector (Figure 3B) and recombinant *prbcl-D3EgD* (Figure 3C) showed many bright green spots from the GFP fluorescence spots inside the cells compared with no fluorescence after bombardment with only gold particles (Figure 3A). Surviving transformed calli were selected for checking the insertion of recombinant DNA (*prbcl-D3EgD*) into the plant genome, using specific primer sites: CaMVF and accDR to amplify a 1624 bp of the prbcl_D3EgD (Figure 4A). Confirmed transformed callus was also tested for *gus* expression, using the specific primers set for *gus* (298 bp) and for *18S rDNA* (540 bp) a housekeeping region, as internal positive controls in the PCR reactions (Figure 4A). The target sites of *prbcl-D3EgD* were identified in the callus to clearly demonstrate the presence of the recombinant DNA. Hence, GFP and PCR analysis demonstrated the successful transcription of *EgaccD* genes in the transformed oil palm calli and confirmed the efficiency of the biolistic delivery of the DNA. The mRNA expression level of *EgaccD* was measured by real time PCR and revealed a high copy number in the *EgaccD* transformed calli (prbcl-D3EgD 1-5) compared with the control calli as shown in Figure 4B, in which each sample

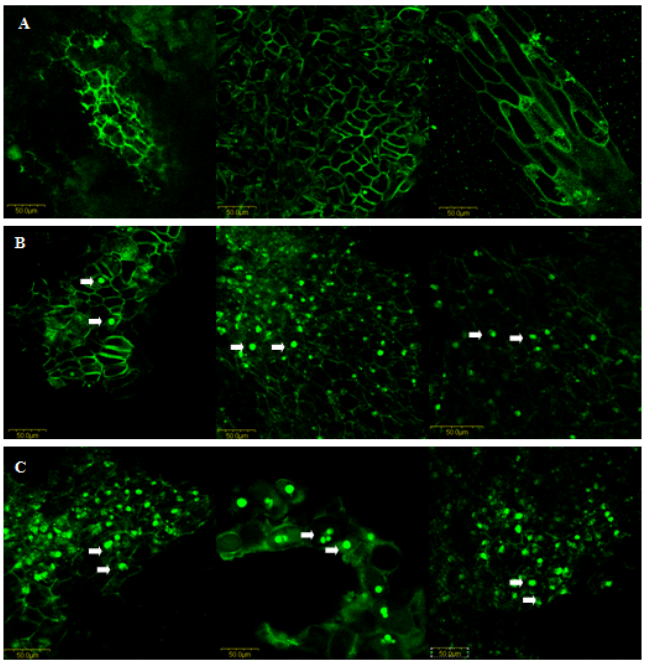

Figure 3. Expression of the GFP (bright green spots at white arrow) in the transformed oil palm calli; *prbcl-D3EgD* (C), compared with the control empty vector (B) and gold particle (A) transgenic calli, the *prbcl-D3EgD* transgenic calli shown many bright green spots using an Olympus confocal laser scanning microscope.

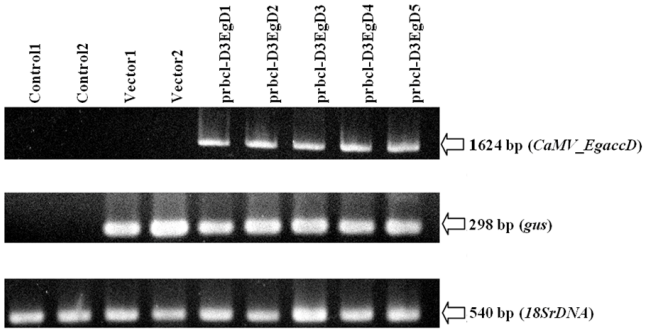

Figure 4A. PCR analysis of transgenic calli using specific primers to amplify the chromosomal DNA from bombarded calli and identify the *CaMV* promoter and *EgaccD* fragment; *CaMV-_EgaccD* (1624 bp), *gus* (298 bp) and *18S rDNA* (540 bp) on *prbcl-D3EgD* bombarded transgenic calli and compare with the gold particle (Control 1-2) and empty vector (Vector 1-2) transgenic calli i to confirm of integrated of T-DNA in transgenic.

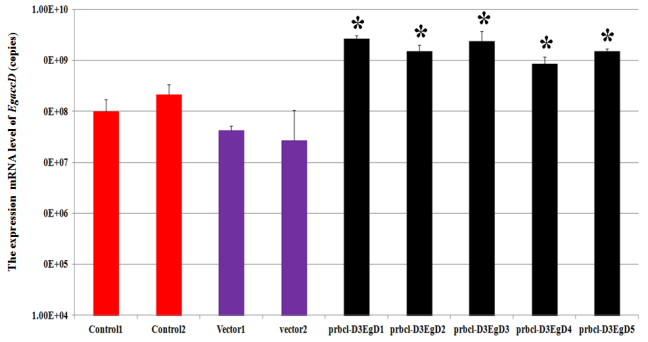

Figure 4B. Observed of the *EgaccD* gene expression level by real-time PCR in the samples of the EgaccD transgenic calli (*prbcl-D3EgD* 1-5) compared with the control.

was tested in triplicate and all reactions were independently repeated twice to ensure the reproducibility of the results.

3.4 Histological analysis

Light microscopy (LM) was conducted to visualize any lipid oil bodies that occurred in the three month transgenic oil palm calli (Figure 5C1-C2) and control (untransformed and empty vector transformed calli, Figure 5A and 5B, respectively). Intracellular oil droplets in callus tissue were observed after histological staining with Sudan black B as dark brown areas from several sections of tissue isolated from *prbcl-D3EgD* calli (Figure 5C1-C2) when compared with the transformed control calli. The section of transgenic oil palm showed oil droplets within cells and showed dark brown stained oil droplets on the periphery of the cell surfaces of the callus. In addition, Lipid staining is one of the screening methods used. However, determination of the amount of lipid is a further requirement for future work

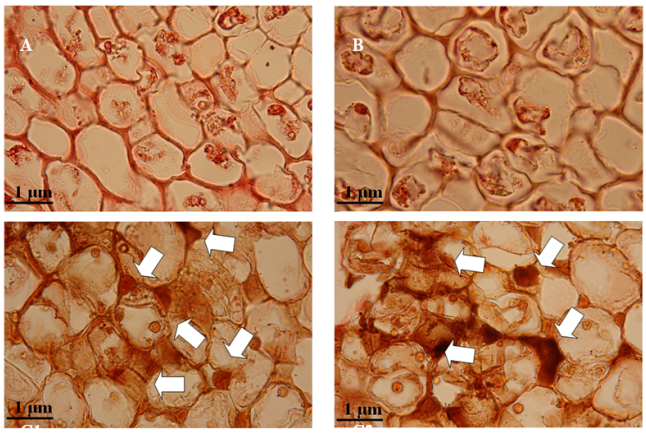

Figure 5. Histochemical analysis of lipid and oil droplets in sudan black B stained sections of *prbcl-D3EgD* transformed calli (C1-2), empty vector transformed calli (B) and non transformed calli (A), Bar = 1 ìm.

(Francki *et al.*, 2002). Over expression of *EgaccD* might increase fatty acid production and oil accumulation in calli. We expressed *EgaccD* with a 5'-untranslated region (UTR) because the 5' UTR acts as a translational enhancer or a stabilizer of the transcripts that require a specific nuclear-encoded RNA polymerase (NEP) (Hirata *et al.*, 2004) for expression. We found that the level of *EgaccD* expression correlated with the copy number and accumulation of lipid in the transformed callus cells. The over expression of the *accD* gene in tobacco showed a significant increase of the oil content in their leaves together with an increased leaf longevity compared to the wild type tobacco plant. It is also possible that the storage oil might enhance the growth of a transgenic plant because fatty acids are important regulators of plant growth and differentiation (Madoka *et al.*, 2002; Ohlrogge and Browse, 1995; Topfer *et al.*, 1995).

In conclusion, the results of this work provide evidence that the level of *EgaccD* supports the level of oil synthesis in transgenic oil palm calli and this could result in an increase in the overall level of oil produced by commercial oil crops. In addition, this study showed that a high copy number of *EgaccD* genes was correlated with a high oil content productivity of the oil palm in field trials. It will be easy and practical to determine the genotype number of this gene at an early stage for selection of varieties with the potential for improved oil yields and plantation. This could significantly reduce the time now required to detect and grow high yielding oil palm commercial cultivars and eventually could more efficiently increase further the productivity of oil palm plants to provide larger amounts of industrial oil.

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