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Genetic transformation and molecular analysis of polyhydroxybutyrate biosynthetic gene expression in oil palm (*Elaeis guineensis* Jacq. var Tenera) tissues

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Abstract

Bioplastics are an alternative substitute for petrochemical synthetic plastics. *Polyhydroxybutyrate* (PHB) genes are involved in bioplastic synthesis. In this study, bioplastic synthesis genes were incorporated into the genome of oil palm because this plant has a high concentration of the PHB precursor acetyl-CoA. Immature embryos (IEs) of *Elaeis guineensis* var Tenera were infected with *Agrobacterium tumefaciens* LBA4404 that contained the binary vector pJLPHB3, which encoded the *phb* genes, β-ketothiolase (*bktB*), acetoacetyl-CoA reductase (*phaB*) and PHA synthase (*phaC*) flanked by a modified CaMV35S promoter, a plastid targeting sequence and the *nos* terminator. GUS assay revealed that about 78-100% transient transformation frequency was obtained for calluses and 55-65% for plantlets 1 month after transformation. However, GUS assays of leaf tissue from 12-month-old plantlets showed that only 10-33% transformation frequency was obtained. The presence of the *phb* genes in GUS positive plantlets was confirmed using PCR and multiplex PCR analyses. Southern blot analyses verified that the *phb* genes were integrated in transformed leaves and calluses using the *phaB* probe (0.805 kb) and *phaC* probe (1.730 kb). Quantitative transgene expression comparison in the transformed tissues measured using real-time PCR showed that the expression levels of the *phaB* and *phaC* transgenes were 6.06- and 6.02-fold higher compared to the non-transformed oil palm.

Keywords: *Agrobacterium tumefaciens*, binary vector, *Elaeis guineensis*, genetic transformation, immature embryo, polyhydroxybutyrate.

Abbreviations: IE_Immature embryo; mPCR_multiplex PCR; PHA_polyhydroxyalkanoate; PHB_polyhydroxybutyrate; PHBV_poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate; WAA_weeks after anthesis.

Introduction

Oil palm (*Elaeis guineensis*) is considered the golden crop in Malaysia for its high commercial value. Currently, palm oil is the second most important vegetable oil after soybean, accounting for almost 20% of the world's production (Abdullah, 2005). The rapid growth of the palm oil industry over the past 30-40 years has enabled Malaysia to become the second largest palm oil exporter after Indonesia; in 2008, Malaysia produced 4.4 billion tons of crude palm oil, compared to 4.17 and 3.66 billion tons in 2007 and 2004, respectively (Oil World Annual, 2007). Besides the exploitation of palm oil in the food, manufacturing and oleochemical industries, extensive research has been conducted to add additional value to oil palm via biotechnology.

Plant genetic transformation is a useful tool to transfer a foreign gene into a target plant genome. For the past three decades, genetic transformation experiments on oil palm have been carried out using various techniques, such as biolistic and *Agrobacterium*-mediated transformation, to produce insect- and disease-resistant oil palms. For instance, plants resistant to basal stem rot disease and the bagworm *Metisa plana* Walker have been produced. The production of a bioplastic was first attempted in *Arabidopsis thaliana* by Poirier et al. (1992). Researchers have since focused on bioplastic production in oil palm because it naturally possesses a high content of acetyl-CoA, which is a precursor for polyhydroxybutyrate (PHB) synthesis. The fatty acid metabolism pathway in oil palm can be modified by introducing the *phb* genes isolated from *Ralstonia eutropha* into the oil palm genome (Nawrath et al., 1994).

Polyhydroxybutyrate (PHB) is the simplest form of polyhydroxyalkanoates (PHAs), a class of biodegradeable polyoxoesters produced by a wide range of bacteria as a carbon and energy source (Willis et al., 2008). The production of PHB requires the sequential action of three enzymes. First, reversible condensation of two acetyl-CoA molecules by β-ketothiolase (*bktB*) results in acetoacetyl-CoA formation. Second, acetoacetyl-CoA reductase (*phaB*) reduces the acetoacetyl-CoA to R-(-)-3-hydroxybutyryl-CoA, which is further polymerized by the third enzyme, PHA synthase (*phaC*), into PHB (Jube and Borthakur, 2007; Poirier et al., 1992; Slater et al., 1988). However, PHB is a highly crystalline, stiff and brittle homopolymer. These poor physical properties limit its use in many commercial products. Thus, a co-polymer named poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate)(PHBV) was produced by incorporating three or five carbon monomers of 3-hydroxybutyrate. PHBV has a better commercial value because it is less stiff and stronger compared to PHB (de Koning, 1995; Poirier et al., 2002).

The traditional method of transforming a vector containing a single gene followed by cross hybridization between parent plants produced a low yield of PHB, only 0.1% from the fresh weight of *A. thaliana* leaves (Poirier et al., 1992). Subsequently, Nawrath et al. (1994) constructed three transformation vectors each carrying a *phb* gene with a plastid-targeting sequence. The PHB was expected to accumulate PHB in plastids because they have a double membrane, which would isolate the PHB, thereby preventing it from disrupting other organelles (Abdul Masani et al., 2008) and reducing the damage to plant growth. Using this approach, the production of PHB in *A. thaliana* was reported to be 14% of the leaf dry weight (Nawrath et al., 1994). Further modification of the method using binary vector increased the transformation efficiency, thus the yield of PHB to up to 40% of the *A. thaliana* leaf dry weight. However, many of the transformed plants displayed serious growth retardation, including chlorotic leaves and poor seed formation (Bohmert et al., 2000).

On the other hand, the production of PHBV in *Arabidopsis* has been reported by Valentin et al. (1999) and Slater et al. (1999). The PHBV only accumulated up to 1.5% and 1.6% of the *Arabidopsis* dry weight in studies done by Valentin et al. (1999) and Slater et al. (1999), respectively. However, these numbers were far below the target for commercialization, which was proposed to be 15% (Slater et al., 1999). The discovery of this technique had led to the expression of *phb* genes in many different plants, such as cotton (John and Keller, 1996), *Brassica napus* (Houmiel et al., 1999), alfalfa (Saruul et al., 2002), *Nicotiana tabaccum* (Bohmert et al., 2002), sugar beet (Menzel et al., 2003) and maize (Mitsky et al., 2000). Nevertheless, many studies are still in progress to develop the best transformation method that could produce the highest level of PHB without negatively affecting plant growth.

The objective of this study was to transform the pJLPHB3 vector, containing the three *phb* genes, into oil palm immature embryos (IEs) using *Agrobacterium tumefaciens*-mediated transformation. The transformation event was confirmed by GUS assay and multiplex PCR. The integration of the *phb* genes into the plant genome was determined by Southern blot analysis and gene expression levels were quantitated by real-time PCR.

Materials and methods

Plant material

Oil palm (*Elaeis guineensis* Jacq. var. Tenera) fruits 10-12 weeks after anthesis (WAA) were obtained from the Malaysia Palm Oil Board Research Centre (MPOB-UKM), Bangi, Malaysia.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain LBA 4404 (Hoekema et al., 1983), which harbored the plasmid pJLPHB3, was obtained from our laboratory and used to infect the target tissues. The plasmid backbone was pCAMBIA1301, which contained the *gusA* reporter and *hpt* genes for hygromycin selection.

Genetic transformation

Plasmid confirmation

The plasmid was isolated from *E. coli* using the modified alkaline lysis method (Birboin and Doly, 1979). pJLPHB3 (1 µg) was digested separately using 1 U of the *Hind*III, *Eco*RI, *Xba*I and *Bam*HI restriction enzymes. The digestion mixtures were incubated at 37ºC for 3 to 4 hours. The digestion products were checked by electrophoresis on a 1.0% agarose/EtBr gel.

Pre-culture condition for explants

Immature embryos (IE) were excised from the seeds, inoculated in $N_6$2.5 medium supplemented with 2.5 mg l^{-1} 2,4-D auxin (Chu et al., 1975) and incubated 5-7 days in the dark before transformation.

Preparation of Agrobacterium for transformation

A. tumefaciens was cultured in YM medium (0.04% yeast extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM $MgCl_2$ and 2.2 mM K_2HPO_4, pH 7.0) containing 50 µg ml^{-1} kanamycin and 100 µl ml^{-1} streptomycin. The bacteria culture was incubated at 28ºC with shaking at 200 rpm for 48 hours. Bacteria growth was measured with a spectrophotometer until the OD reached 0.4-0.55. The bacteria culture was further diluted in $N_6$6 medium and incubated until the bacteria reached log phase at OD 0.5 prior to transformation.

Co-cultivation of explants with A. tumefaciens

The pre-cultured IEs were cut in half using a scalpel and soaked in diluted *A. tumefaciens* culture for 30 min. After the incubation, the bacterial culture was discarded and the infected IEs were inoculated into N_6O medium and $N_62.5$ medium. The IEs were co-cultivated with *A. tumefaciens* for 3 days in the dark. The IEs were later transferred to N_6O and $N_62.5$ medium supplemented with 500 µg ml^{-1} carbenicillin or 250 µg ml^{-1} cefotaxime to prevent excessive bacteria growth.

Regeneration of putative transformants

The IEs cultured on N_6O medium were incubated at 25ºC under light conditions to regenerate the plantlets, whereas the IEs inoculated in $N_62.5$ medium were kept at 25ºC in the dark to induce callus formation. After 3 weeks, if over-growth of *A. tumefaciens* was not observed, the IEs were transferred to N_6O and $N_62.5$ medium without antibiotics. The IEs were sub-cultured every 3-4 weeks until they became plantlets and calluses.

Rooting and acclimatization

The plantlets were transferred to test tubes containing R68 rooting medium to induce root growth. The rooted plantlets were then planted in soil using poly bags and kept in the greenhouse.

Confirmation of transformation

GUS assay

GUS assay was performed on the putative transformants according to Jefferson et al. (1987). The transient expression of the *gus* gene was determined from calluses one month after infection by *A. tumefaciens,* whereas stable *gus* expression of the regenerated plantlet leaves was examined 3 months after co-cultivation. The putative transformants were immersed in the X-Gluc solution (0.2 M $NaPO_4$ buffer, pH 7.0; 0.1 M $K_3[Fe(CN)_6]$; 0.1 M $K_4[Fe(CN)_6]$; 0.5 M EDTA, pH 7.0; 0.1% Triton® X-100 and 1 mg ml^{-1} X-Gluc) and incubated at 37ºC overnight in the dark. After incubation, the samples were rinsed with distilled water and fixed in FAA solution [85% ethanol (v/v), 5% glacial acetic acid and 10% formalin].

Genomic DNA isolation and Multiplex Polymerase Chain Reaction

Genomic DNA from putatively transformed calluses and plantlet leaves were isolated according to the modified CTAB method (Doyle and Doyle, 1990). PCR was performed to verify the presence of the *phb* genes in the genomes of the putative transformants. An endogenous gene, *nad5,* was used as a control to determine the quality of the DNA isolated. Primers used for the *nad5* gene and *phb* genes are shown in Table 1.

Table 1. Primers sequences for PCR amplification.

Gene	Primer sequence (3'-5')	Amplicon size (bp)
bktB	F: GAATACGCTCAAGCTTCGAGGTCC R: CGGGACGATCTGGTCCTTGAAGTA	1146
phaB	F: TGACTCAGCGCATTGCGTATG R: GCTGGCTGCACCGCAATAC	805
phaC	F: GGCAAGTCCCAACCATTCAAGGTC R: CATGCCTTGGCTTTGACGTATCGC	1730
nad5	F: TAGCCCGACCGTAGTGATGTTAA R: ATCACCGAACCTGCACTCAGGAA	800

Table 2. Primers sequences for real-time PCR

Gene	Primer sequence (3'-5')	Amplicon size(bp)
bktB	F: AAG TGG TAG TGG TAA GCG GTG TC R: CAC AGG CGG TTC ACG GTC AG	259 pb
phaB	F: ACC AAC TAC TCC ACC GCC AAG R: GAC TCC TCC GAC GAC AAC CAG	236 pb
phaC	F: ATG TCG CCC GCC AAC TTC C R: CAC CGT ATG TCC CTG CTC CAC	354 pb
nad5	F: TCG GGT CGT TTT ACT CTC TTT C R: ATG CGA TCC TAT CTG TGC	174 pb

Multiplex PCR (mPCR) was performed to amplify all three *phb* genes in one tube. Optimization was carried out on the primer concentrations, DNA template concentrations and PCR parameters. A 25-µl or 12.5-µl PCR reaction contained 200 ng of DNA template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 µM of forward and reverse primers for each of the *phb* genes, 0.2 mM of each dNTP and 1 U of Taq DNA Polymerase (Biotools). The following profile for mPCR was used: 1 min pre-denaturation at 95°C; 30 cycles of 1 min denaturation at 95°C, 2 min annealing at 60°C and 3 min extension at 72°C and a final extension at 72°C for 10 min. The PCR products were separated by 1.0 % agarose/EtBr gel electrophoresis, examined under UV light and photographed.

Real-time PCR (qPCR) analysis

Total RNA was isolated from both putatively transformed calluses and plantlet leaves using the RNeasy Plant Mini kit (QIAGEN), digested with RNase-free DNase I (Fermentas) and converted into first strand cDNA by the RevertAid™ first strand cDNA synthesis kit (Fermentas), according to manufacturer's instructions. Primer sets for each *phb* genes and the *nad5* gene (Table 2) were designed using the Beacon Designer 4 (Premier Biosoft International, Palo Alto, CA) primer design software.

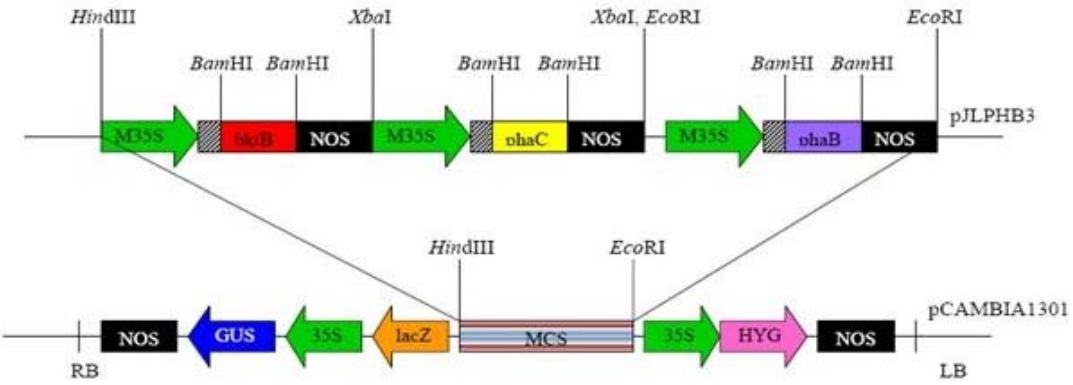

Fig 1. Restriction map for pJLPHB3 (18.04 kb) based on the digestion products of different restriction enzymes.

Plastid targeting sequence

Optimization was carried out for the template and primer concentrations. Template concentrations that showed amplification with Ct values between 20-30 were used for qPCR. In addition, primer concentrations that showed low Ct values, high end point fluorescence (dRn), no primer dimer formation and no signal in the non-template control (NTC) were chosen for qPCR amplification.

For each gene, a 20-µl PCR reaction was set up by mixing 1X RealMasterMix and the correct concentrations of primers and template. Relative quantitation and standard curve generation for each gene were performed by qPCR using the Mx3000P™ Real-Time PCR System (Stratagene, USA) instrument. The qPCR parameters used were as follows: 10 min of DNA Polymerase activation at 95ºC and 40 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s and extension at 68ºC for 30 s. Melting curves were performed according to the following parameters: 1 min denaturation at 95ºC, 30 s annealing at 55ºC and 30 s extension at 95ºC.

Results and discussion

Plasmid confirmation

The presence of the PHB cassette, which contained the *bktB*, *phaB* and *phaC* genes, flanked by modified 35 promoter, the plastid-targeting sequence and the *nos* terminator in pJLPHB3 were confirmed by plasmid digestion prior to genetic transformation (results not shown). Based on the single and double digestion results, a diagram of pJLPHB3 was constructed as shown in Figure 1. This plasmid was then transformed into oil palm immature embryos to test the feasibility of using immature embryos as target tissues and the transformation frequency and stability of the transgenes in calluses and regenerated plantlets.

Effect of pre-culture treatment and co-cultivation conditions

The IEs of *Elaeis guineensis* Jacq. var. Tenera used in this study were between 10-12 weeks after anthesis. IEs were chosen as the target tissue because they are able to regenerate into whole plants. In addition, IEs were also reported to have high levels of physiological uniformity (Texeira et al., 1993). The addition of the plant growth regulator 2,4-D auxin was used to enhance cell division because cells that are actively dividing should be more susceptible to *Agrobacterium* infection. Furthermore, we observed in our previous work that IEs pre-cultured on medium with 2,4-D auxin for 7 days will be 3-4 mm in length, which makes the IEs easy to cut into halves and allows *A. tumefaciens* to infect the wounded part more effectively. The probability of obtaining transgenic plants is higher if *A. tumefaciens* infection takes place at a wounded area, especially in the shoot and root meristem.

The "browning" phenomenon was observed at the wounded tissues as a consequence of the secretion of phenolic compound. This condition can be controlled by sub-culturing the explants in medium containing activated charcoal. Otherwise, the excess phenolic compound will cause IE fatality. The co-cultivation period is also an important factor that determines the success of a transformation event. Co-cultivation for too short of a period will cause insufficient infection, whereas an incubation time that is too long will cause the explants to die. We found that 30 min of co-cultivation was enough for *A. tumefaciens* to infect IEs. Excessive *A. tumefaciens* were eliminated with the cefotaxime and carbenicillin antibiotics. In this study, a total of 2160 IEs were transformed with pJLPHB3, of which 1080 were used to induce calluses and the other 1080 were regenerated into plantlets.

Confirmation of transformation
GUS assay

The histochemical GUS assay was used to determine the success of the transformation events in both calluses and plantlets. The transformed IE-derived calluses showed strong GUS activity at the lower part of the callus, which were produced radically, indicating that the T-DNA region from the pJLPHB3 construct had been transferred into the genomes of the IEs. However, there was a clear difference in the tube inten-

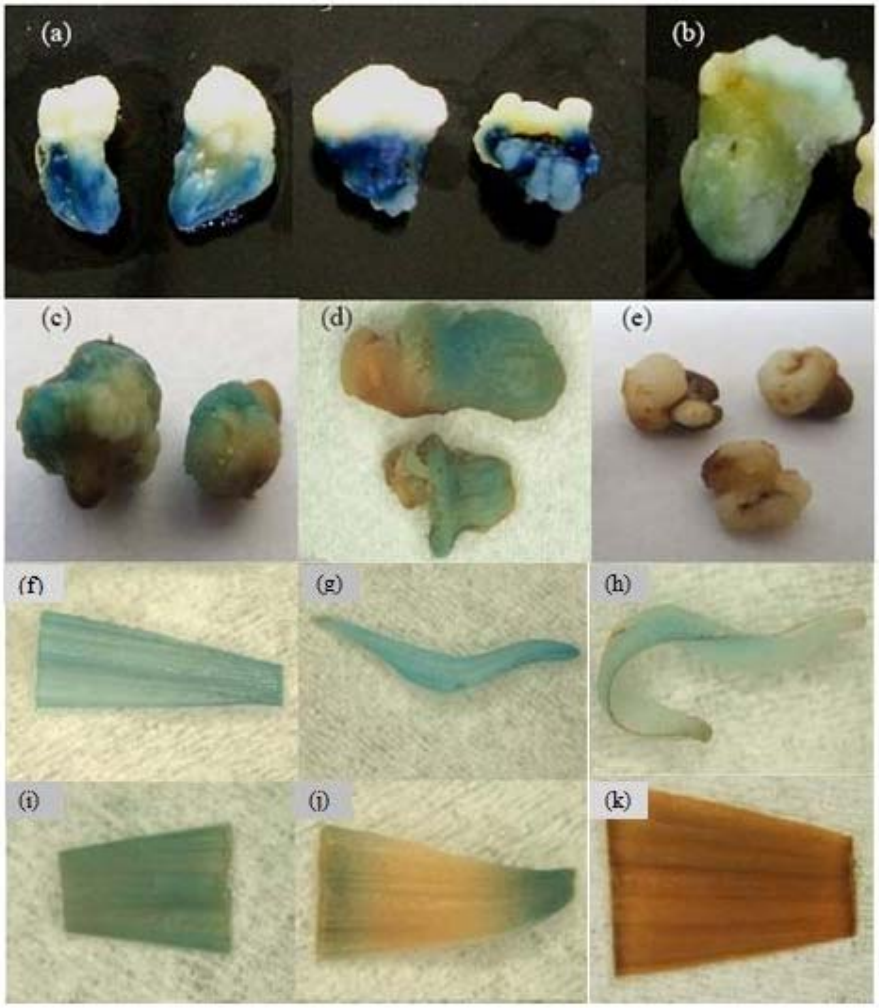

Fig 2. Different levels and expression patterns of the *gus* gene in the putatively transformed calluses and plantlets. Transient GUS expression in (a-d) an IE-derived callus and (e) an untransformed IE as a negative control. Stable GUS expression in (f-j) plantlet leaf tissue and (k) negative control oil palm leaves.

sities between different samples (Figure 2 a-d). We noticed that the surface of the callus that contacted the culture medium exhibited a stronger blue color. This result suggests that the presence of 2,4-D auxin in the $N_6$2.5 medium triggered cell division, and thus the IEs became more susceptible to *Agrobacterium* infection.

In addition, the leaf tissues also exhibited GUS activity, though the blue color was not as prominent as that observed in the callus. The color was distributed throughout the leaves, indicating that the *gus* gene was stably expressed in the regenerated plantlets (Figure 2 f-j). The differences between the expression levels of GUS may be caused by two factors. First, the transgenes may be integrated into the genome at different positions. Second, different copy numbers of the transgenes could have been inserted into the genome (Saini et al., 2003). For the transformed tissues that lacked GUS activity, instability of the transgenes, inefficient transgene integration or gene silencing could have resulted in no GUS activity. On the contrary, no blue color was observed as expected in both untransformed IE-derived calluses and leaves, which served as negative controls in the GUS assay (Figure 2 e & k).

The transformation frequency was then calculated according to the number of calluses and leaves that exhibited GUS activity. We observed that the IE-derived calluses showed a 78-100% transformation frequency, which is much higher compared to the IE-derived plantlets (10.26-32.79%) (Tables 3 & 4). The IE-derived plantlets demonstrated lower expression of the *gus* gene because the putative transformed tissues did not undergo hygromycin selection, which will kill not only untransformed tissues but also chimeric samples that carry the transgenes. It is also possible that dead untransformed tissues will inhibit nutrient uptake from the medium of the nearby transformed tissues (Ebinuma and Komamine, 2001) and thus only transgenic tissues would survive. The morphology of the transformed plantlets also showed symptoms of inhibition. Chlorosis was clearly seen on leaves, and the transformed plantlets grew slower than normal plants. Thus, the plantlet survival rates were also considerably lower (10.83-24.44%).

Table 3. Transformation frequency calculated based on transient *gus* gene expression in putative transformed IE-derived callus, one month after infected by *A. tumefaciens*

Treatment	Number of transformants	Number of plantlets used for GUS assay	Positive GUS	Transformation frequency
1st replication	360	18	14	77.78
2nd replication	360	18	18	100.00
3rd replication	360	18	15	83.33

Multiplex PCR amplification (mPCR)

To verify the presence of the *phb* genes in the oil palm genome, multiplex PCR was performed on the transformed IE-derived calluses. One of the advantages of using mPCR, which was first described by Chamberlain in 1988, is that two or more loci can be amplified at the same time in one PCR reaction (Chamberlain et al., 1988). Therefore analysis of transgenes using mPCR saves time and energy, and thus more samples can be analyzed compared to conventional PCR (Guillemaut and Marechal-Drouard, 1992).

Before mPCR was carried out, the optimum conditions for pJLPHB3 as the template were determined. The parameters tested were annealing temperature (58ºC and 60ºC) and extension time (2, 3 and 4 minutes) (data not shown). *nad5* gene was used as an internal control because it is an endogenous, multicopy gene present in the mitochondria (Mannerlöf and Tenning, 1997). In this study, the *phb* genes were successfully amplified from the transformed tissues by mPCR. The distinct bands visible by 1% agarose gel electrophoresis were *bktB* (~1146 bp) and *phaC* (~1730 bp). However, the band corresponding to *phaB* (~805 bp) was very faint and almost undetectable (Figure 3). As expected, no bands were detected in untransformed oil palm tissues.

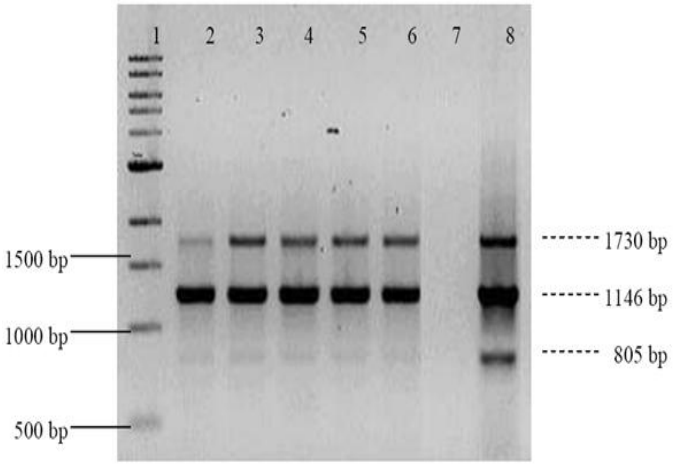

Fig 3. mPCR analysis of genomic DNA of transformed IE-derived calluses to detect the presence of the phb genes. Lane 1: 1 kb DNA ladder. Lanes 2-6: genomic DNA of IE-derived calluses. Lane 7: genomic DNA of an untransformed callus (negative control). Lane 8: pJLPHB3 DNA.

Real-time PCR analysis

Quantitative analysis of the transgenes was performed by real-time PCR to determine the expression levels of the *phb* genes. Real-time PCR was chosen because it is the most effective and sensitive method for determining expression levels and quantities of certain genes (Giulietti et al., 2001; Muller et al., 2002), even when the amount of the DNA template is very low (Pfaffl, 2001). In this case, only the *phaB* and *phaC* genes were tested in the transformed tissues in comparison with untransformed tissues. *nad5* was used as internal control as in the mPCR analysis. Before real-time PCR was performed, optimization was performed to determine the appropriate concentrations of the single stranded cDNA template and both forward and reverse primers. Six different concentrations of cDNA (500 ng, 100 ng, 20 ng, 4 ng, 0.8 ng and 0.16 ng) were tested, and the concentrations of primers used in these experiments were between 50 nM to 200 nM. The optimum concentration of cDNA was determined by analyzing the melting curves for the *nad5*, *phaB* and *phaC* genes, where the specificity of each amplicon can be observed. Based on the results obtained, the appropriate cDNA concentrations for *nad5*, *phaB* and *phaC* were 200 ng because they showed Ct values between 20 and 30. Single peaks were observed at different melting temperatures, which were 82.5ºC for *nad5*, 91.2ºC for *phaB* and 92.5ºC for *phaC,* implying that the amplifications were specific in the real-time PCR reactions (data not shown). In addition, the concentrations of the primers were determined by analyzing the Ct value for each gene. The optimum concentrations for the primers should not yield primer dimers, should show the lowest Ct value and highest fluorescent end point (dRn), and should not display signal in the non-template control (NTC). We determined that the optimum forward/reverse primer concentrations for *nad5* were 200 nM/200 nM. The primer concentrations for *phaB* and *phaC* amplification were 200 nM/50 nM and 100 nM/200 nM, respectively.

On the other hand, the standard curve served as the starting reaction for optimization of samples used to normalize (*nad5*) and calibrate (untransformed IE-derived callus) the transgenes in the relative quantification experiment. The starting quantity of each sample could also be determined by comparing the Ct values from samples with unknown concentrations and the Ct values from samples with known concentrations on the standard curve. In this experiment, relative quantification was done according to $2^{-\Delta\Delta Ct}$ formula.

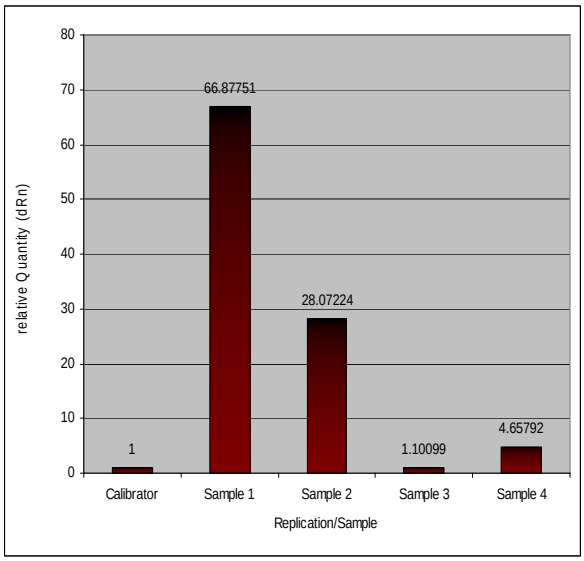

Fig 4. The *phbB* gene expression profile in different putatively transformed samples. Samples 1 and 2 are plantlet leaf tissues; Samples 3 and 4 are callus tissue.

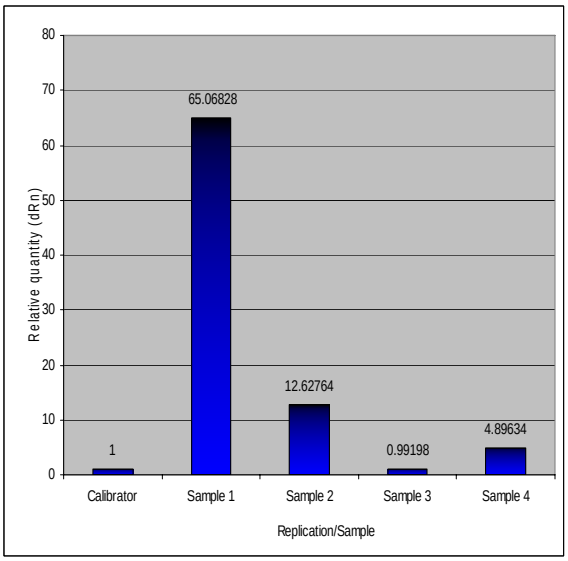

Fig 5. *phaC* gene expression profiles in different putatively transformed samples. Samples 1 and 2 are plantlet leaf tissues; Samples 3 and 4 are callus tissues.

The efficiency for the normalization gene and target genes must not be more than 5% different from each other and must be near 100% to compare the results (Bustin, 2000). In addition, normalization has to be carried out to identify the biological differences between samples. The normalization method that uses an endogenous gene is the most popular strategy (Kubista et al., 2006) because normalization towards total RNA or ribosomal RNA could mask the heterogeneity between samples. In this study, the endogenous gene *nad5* was used to normalize expression because it is naturally present in the mitochondrial respiration pathway. It has also been used previously for normalization in the multiplex RT-PCR analysis of apple virus (Menzel et al., 2002). The expression changes of the *phaB* and *phaC* transgenes were normalized to *nad5* for comparison. The results of this quantitative comparison are shown in the relative quantity chart (Figure 4 & 5).

According to Figure 4, the expression of *phaB* in leaf sample 1 was 66.88-fold greater than the untransformed oil palm leaves, followed by leaf sample 2, which was 28.07-fold greater than untransformed leaves. In contrast, *phaB* expression in callus samples was much lower than in leaves, which showed only 4.66-fold and 1.10-fold greater expression than untransformed calluses in callus sample 4 and callus sample 3, respectively. The expression of the *phaC* gene showed a similar pattern to *phaB* (Figure 5). The highest expression of *phaC* was found in leaf sample 1, which was 65.07-fold greater than untransformed control. This was followed by expression in leaf sample 2, which was 12.63-fold greater than untransformed leaves. On the contrary, the *phaC* gene showed much lower expression in callus samples, which were 4.9- and 0.99-fold greater than the untransformed control in callus samples 4 and 3, respectively.

The differences in the samples compared to the control were used to determine whether a gene was upregulated or downregulated. All of the samples tested in this study, especially transformed leaf tissues, showed upregulation of both *phaB* and *phaC* expression, except for transformed callus tissues (Sample 3), which showed downregulated gene expression. The differences between the *phb* gene expression levels may be due to differences in plant physiology and anatomy. Leaves may have higher expression of *phaB* and *phaC* because they consist of organized cells and mature organelles. In contrast, callus tissues consist of actively dividing cells and unorganized organelles. Besides, there may be more plastids in the leaves compared to calluses, which would contribute to the increase in transgene expression since they were targeted to plastids.

The promoter used may be one of the factors that influenced the transgene expression levels in both leaves and calluses. The CAMV35S promoter was reported to be active during S phase of the cell cycle (Nagata et al., 1987) because there is an increase in the number of meristem cells and almost no cell division in this phase (Harper and Steward, 2000). This fact indicates that the modified CAMV35S promoter used in this study functioned more efficiently in leaf tissues than in callus tissues.

The expression levels of *phaB* and *phaC* were different within the experimental samples, such as between leaf samples 1 and 2 and between callus samples 3 and 4. This situation might be caused by many factors, including gene silencing (Iyer et al., 2000), effects of somaclonal variation and regulatory sequences (Butaye et al., 2005). The copy number of the transgenes, the transgene integration position, the degree of homology with endogenous genes (Matzke and Matzke, 1995), with endogenous genes, epigenetics that result from *in vitro* culture and the

Table 4. Transformation frequency calculated based on by stable *gus* gene expression in putative transformed IE-derived plantlets, 3 months after infected by *A. tumefaciens*.

Treatment	Number of transformants	Number of plantlets used for GUS assay	Positive GUS	Transformation frequency	Percentage of plantlets survival
1st replication	360	39	4	10.26 %	10.83 %
2nd replication	360	88	18	20.45 %	24.44 %
3rd replication	360	61	20	32.79 %	16.94 %

transformation process (Zhang et al., 1996), and transgenic locus structure (Pawlowski and Somers, 1998) could also affect these results.

In this study, the three *phb* genes were flanked by the same sequences or repeated sequences, such as the CaMV35S modified promoter, plastid-targeting sequence and *nos* terminator. These repeated sequences and the integration of multiple copies of the transgenes have been reported as the main cause of gene silencing during transformation (Fagard and Vaucheret, 2000). Gene silencing is thought to be more prevalent in genetic transformation mediated by *Agrobacterium* compared to biolistic methods because biolistic methods transfer a limited number of copies of a linear expression vector, which only consists of a promoter, coding sequence and terminator signal. This method circumvents integration of the vector sequence into the plant genome (Fu et al., 2000).

Vector sequences that originate from the bacteria could trigger gene silencing because the foreign sequences are unable to bind eukaryotic nuclear proteins (Sandhu and Altpeter, 2008). Moreover, the sequences would induce spreading of dense methylation to the nearby transgenes (Jakowitsch et al., 1999). The vector backbone sequence would also cause recombination at a hotspot region, such as AT-rich sequences or origins of replication, which could induce rearrangement of the transgenes through plasmid-plasmid recombination (Kohli et al., 1999; Muller et al., 1999; Zhang et al., 2008). On the other hand, the position effect caused by the random genome integration of the transgenes (Daniell and Dhingra, 2002) would also contribute to the different levels of *phb* gene expression in this study. The position effect phenomenon usually does not occur in chloroplast genetic engineering applications because the transgene integration occurs in the target organelle.

In conclusion, multiplex PCR indicated that the *bktB*, *phaB* and *phaC* genes were successfully transferred into oil palm immature embryos. However, the expression levels of the transgenes in both calluses and plantlet leaves were influenced by gene silencing factors and position effects of transgene integration in oil palm DNA genome.

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