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Super Bimary Vector System Enhanced Transformation Frequency and Expression Level of Polyhydroxyvalerate Gene in Oil Palm Immature Embryo

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Abstract: The aim of this study was to compare the efficiency of two transformation vectors: binary and super binary vector in the process of gene transfer and expression in oil palm tissues. Two transformation vectors, namely pFA2 (binary vector, ~24.4 kb) and pFA3 (super binary vector, ~39.4 kb) that contains four essential genes (*bkt*B, *pha*B, *pha*C and *tdc*B) to produce polyhydroxyvalerate have been successfully constructed. The two transformation vectors and pMR505, which used as a control were later transformed into oil palm tissues mediated by *Agrobacterium tumefaciens*. Oil palm immature embryos were cocultivated with *A grobacterium. tumefaciens* that carry pFA2, pFA3 and pMR505 separately and also contain the higromycin phosphotransferase (hpt) and α-glucuronidase (gus) genes. GUS assay was carried out on putatively transformed immature embryos as an indicator of successful gene transfer. From the analysis, high percentage of transient GUS expression ranging from 52.6-85.7, 70.5-96.7 and 80.9-93.8% for pFA2, pFA3 and pMR505, respectively were obtained from putative transformed immature embryos. The successful integration of *bkt*B (1.2 kb), *pha*B (0.8 kb), *pha*C (1.0 kb) and *tdc*B (0.9 kb) genes into the immature embryos genome were confirmed by PCR analysis. Relative quantification through real-time PCR analysis indicates that the expression of PHBV gene in oil palm immature embryos transformed with pMR505 is 7 times higher than non transformed immature embryo, followed by transformed with pFA2 (2 times) and pFA3 (0.7 times), respectively. Thus, this data suggest the efficiency of super binary vector compared to binary vector in transferring foreign genes and enhancing its expression in oil palm tissues.

Key words: α-glucuronidase, immature embryo, oil palm, super binary vector, *Agrobacterium tumefaciens*

INTRODUCTION

Bioplastic is a pure biopolymer that is synthesized naturally in numerous organisms (Akar *et al*., 2006). Following the successful gene transfer and the manipulation of specific pathway in plants, biodegradable thermoplastics, poly-3-hydroxybutyrate (PHB) has been synthesized in a number of crops, such as cotton (John and Keller, 1996) and rape seeds (Slater *et al*., 1999). In microbes, bioplastics that have been abundantly produced are polyhydroxyalcanoates (PHA) and its derivatives (polyhyroxybutyrate/polyhydroxyvalerate). PHB synthesis requires the expression of two bacterial genes, which are acetoacetyl-CoA reductase and PHB synthase, as well as the involvement of plant endogenous gene, 3-kethotiolase. In contrast, the biosynthesis of PHBV, which is a copolymer of PHB with a slightly longer chain, requires another gene, *tdc*B or *ilv*A as well as the three genes that involves in the production of PHB. PHBV has additional improved quality with lower melting point, better flexibility and also less brittle compared to PHB (Kragelund *et al*., 2005). The production of PHA and its derivatives in plants are economically cheaper than in bacterias and other microbes. Unlike bacterial cells, plant cells possess subcellular compartments, which make it handy for PHA storage. Acetyl-CoA is the precursor for PHB synthesis in microbs. In plants, the thio-ester presents in the cytosol, plastid, mitochondria and peroxisome. Thus, PHB synthesis can be targeted to those subcellular compartments (Hanley *et al*., 2000; Moire *et al*., 2003). Nawrath *et al*. (1994) and Saruul *et al*. (2002) have shown that bioplastic synthesis in plants can be enhanced by targeting the expression of genes into the plastid. Constructs with the three genes in one vector

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have been introduced to numerous plants. The genes were attached to a particular DNA segment that encodes the chloroplast transit peptides, CaMV35S promoter and terminator sequence.

Over the years, numerous studies have shown that PHB and PHBV can be synthesized in dicot and monocot plants. Recently, the advantage of oil palm as a highly efficient oil yielding crop with its abundant pool of acetyl-CoA has been manipulated for the synthesis of PHB/PHBV. Genes that are involved in the conversion of acetyl-CoA to bioplastics have been transformed to oil palm tissues (Abdullah *et al*., 2005). Genetic transformation of oil palm via *Agrobacterium* and biolistic method has been carried out to transfer *PHB/PHBV* gene cassettes driven by constitutive and tissue-specific promoters into the oil palm tissues (Abdullah *et al*., 2005). Parveez (2003) has proposed the role of transit peptides from oil palm ACP gene and Matrix Attachment Region (MAR) for the purpose of plastid targeting and transgene expression stability. In order to produce PHBV, threonine dehydratase (*tdc*B) will be transformed together with the other three PHB genes.

In the process of gene transfer via *Agrobacterium* into the plant cells, transformation vector plays an important role in ensuring the success of gene integration into the plant genome. Since the last three decades, the existence of mega virulent plasmid has been recognized in *Agrobacterium* strains (Zaenen *et al*., 1974). Genetic experiments have showed that certain class of plasmids, which is known as Ti (Tumor-inducing) or Ri plasmid (Root-inducing) are responsible in causing tumorigenesis (Gelvin, 2003). The T-DNA region, which is a part of this plasmid, can be transferred into the plant cells and furthermore incorporate into the plant genome. Thus, Ti plasmid has been manipulated as a vector to introduce foreign genes into plant tissues.

Researchers have developed a strategy which is called the cointegrate vector system, in order to introduce foreign genes into the T-region. This is due to the fact that Ti plasmid is too big and there are no endonuclease restriction sites to clone foreign genes in this region. The strategy involved indirect cloning into the Ti plasmid, where the foreign genes were located in *cis* orientation with the *virulence* genes on the same plasmid. However, the complexity of this method in introducing foreign genes into the T-region has forced researchers to develope an alternative strategy to transfer the transgenes into plant tissues. Hoekema *et al.* (1983) have shown that the T-region and *vir* genes can be placed at two different replicons. Thus, when the two replicons present in the same *Agrobacterium* cell, *vir* genes product will act in *trans* in the T-region to affect the process of T-DNA transfer into the plant cells. This method is known as the binary vector system, where T-region replicon contains binary vector, whereas the *vir* gene replicon is known as the helper vir (Gelvin, 2003).

A significant modification to the binary vector has been developed by researchers. Hiei *et al.* (1994) has constructed the super binary vector pTOK233 by cloning *vir* B, *vir* G and *vir* C genes from pTiBo542 into pGA472. This vector has shown successful gene transfer into various rice cultivar (Kumria *et al*., 1998; Dong *et al*., 1996), sorghum (Zhao *et al*., 2000) and *Allium cepa* (Zheng *et al*., 2001). New copies of *vir* genes and its combination have been placed in *Agrobacterium* cells using alternative Ti plasmid, helper plasmid or additional *vir* genes in binary vector. Different *vir* genes have shown the increment of tumor formation (Jin *et al*., 1987), transient gene expression (Ke *et al*., 2001) and cells or tissues transformation stability (Tang *et al*., 2003). As a result, genetic engineering in the species that was previously recalcitrant to transformation, such as monocot plants, can be achieved by the combination of super virulent *Agrobacterium* and super binary vector (Hiei *et al*., 1994). The purpose of this study was to compare the efficiency of binary and super binary vector systems in the aspect of gene transfer, integration and expression in the oil palm tissues. The results have been compared qualitatively and quantitatively using molecular biology techniques, such as PCR and real-time PCR.

MATERIALS AND METHODS

Plasmids and bacterial strains: The experiments were carried out at the Molecular Biology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, National University of Malaysia. In this study, binary vector (pFA2) and super binary vector (pFA3) have been successfully constructed by cloning *bkt*B and *pha*C genes cassettes from pFA1 into pMR503 and pMR504, respectively. pFA1 (~10.9 kb) acted as the intermediate plasmid and each gene was individually flanked by ubiquitin promoter, plastid targeting sequence and poly A signal. Later, the two genes cassettes were cloned sequentially into pMR503 and pMR504, which already contained the other two genes cassettes, *pha*B and *tdc*B. In contrast to *bkt*B and *pha*C, these two genes were under the control of CaMV35S promoter and individually flanked by plastid targeting sequence and NOS terminator. pFA3 (~39.4 kb) was larger than pFA2 (~24.4 kb), due to the additional of the virulent gene in pMR504. Apart from these two plasmids, a stable super binary plasmids, namely pMR505 also used for transformation.

The steps required for cloning and transformation process were plasmid DNA isolation, digestion with specific restriction enzymes and Calf Intestine Alkaline Phosphatase (CIAP), plasmid DNA purification, ligation, competent cells preparation and also plasmid transformation. The Lysis Alkaline method (Birnboim and Doly, 1979) and GFX Microplasmid Prep kit (Amersham Biosciences, UK) was used to isolate the plasmid DNAs, whereas Geneclean ® (BIO 101 Inc, USA) kit was used to purify the DNA fragments. The *E. coli* competent cells were prepared using the Rubidium chloride method, in contrast to the preparation of *Agrobacterium* cells, which was prepared according to calcium chloride method. The medium for bacterial culture consists of LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH7), LB-G with 5 mM glucose and YM (0.04% yeast extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM $MgSO_4$, 2.2 mM K_2HPO_4, pH 7.0). All the plasmids were in *E. coli* strain DH5α and later transferred separately into *A. tumefaciens* strain LBA4404 via direct transformation.

Target tissue preparation and plant transformation: Oil palm *Elaeis guineensis* fruits were cut into two sections. The resulting immature embryos were then pre-cultured on N_6OPS medium and incubated for 7-12 days. This medium contained 2,4-D, which is a hormone that regulates callus formation. These target tissues were later used for the infection of *Agrobacterium*.

The plasmids, which were in the bacterial glycerol stocks, were then inoculated in LB-G and YM mediums that contained streptomycin and kanamycin antibiotics. The culture was incubated in the orbital shaker at 28°C until it reached OD 0.4-0.6. In the meantime, the pre-cultured immature embryos were cut into two sections to provide wounds for bacterial infection. The infection process took place when the immature embryos were added into N_6 medium and incubated on the belly shaker. The infected immature embryos were later placed on cefotaxime-containing N_6OPS medium before the tissues were ready for analysis.

GUS assay and transformation frequency: GUS histochemical analysis was carried out using modified 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc) substrate (Kosugi *et al*., 1990). GUS assay buffer (0.062 M $Na_2HPO_4{\cdot}_2H_2O$, 0.038 M $NaH_2PO_4 \cdot H_2O$, 0.001 M $K_3[Fe(CN_6)]$, 0.001 M $K_4[Fe(CN_6)]$, 0.01 M EDTA, 0.1% Triton X-100, 20% (v/v) methanol, 100 mg X-gluc) was prepared and stored in the dark. Putative transformants were stained in the buffer and incubated at 37°C overnight. FAA solution 42.5% (v/v) ethanol, 42.5% (v/v) glacial acetic acid, 8.5% formalin) was later used to get rid of pigments such as chlorophyll and carotenoid.

DNA isolation and polymerase chain reaction: Genomic DNA isolation was carried out according to CTAB method (Murray and Thompson, 1980) and Ultra Clean™ Plant DNA Isolation kit (MO Bio Laboratories, USA). PCR analysis has been carried out to prove successful gene transfer. Each PCR reaction mixture contained 100 ng DNA templates, 100 ng reverse and forward primers, 0.2 mM dNTP (BioTools, Spain), 1.5 mM $MgCl_2$ (BioTools, spain), 1X reaction buffer (BioTools, Spain) and 2.5 U Taq DNA polymerase (BioTools, Spain) in the total volume of 25 μL. The following primers were used: *bkt*B (F: 5,-TGACGCGTGAAGTGGTAGTGG-3' ; R: 5'-GTTCTTCGTCAGCGAAGCAAGG-3'), *pha*B (F: 5'-TGACTCAGCGCATTGCGTATG-3' ; R; 5'-GCTGGCTGCACCGCAATAC-3'), *pha*C (F: 5'-CGCCGTGCATCAACAAGTACT-3' ; R: 5'-CGCCGTGCATCAACAAGTACT-3') and *tdc*B (F: 5'-CGAATTTATAAAACAGGCATGCCTC-3' ; R: 5'-CGAAACCGGTGATTTGAGA-3'). Each primers hybridized to the specific genes sequences to produce a 1.176 kb (*bkt*B), 0.765 kb (*pha*B), 0.999 kb (*pha*C) and 0.900 kb (*tdc*B) PCR products. For all primers, the following PCR conditions were used: 1 min denaturing at 95°C, 1 min annealing at 55.5°C and 2 min elongating at 72°C. The reaction was repeated 30 times and the products were later analyzed using 0.8% agarose gel electrophoresis at 85V for 1 h and 30 min and later stained with EtBr under UV light.

RNA preparation and real-time RT-PCR: For real-time RT-PCR analysis, total RNA isolation was performed using Qiagen RNeasy Plant Mini (Qiagen, Germany) according to the standard protocol. The RNAs were later converted to cDNAs using Quantitect® Reverse Trancription (Qiagen, Germany) according to the protocol that has been suggested by Qiagen, Germany.

Real-time RT-PCR primers were designed using the primer design program of Beacon Designer 4 (Premier Biosoft International, Palo Alto, CA) Two sets of primers were designed for *nad*5 (F: 5'-TCGGGTCGTTTTACTCTCTTTC-3' ; R: 5'-AGTATGCGATCCTATCTGTGC) and *tdc*B (F: 5'-TGGCGGTTTAATTGCTGGTATTGC : R: 5'-CGGGCGGGAGACATCACAAC-3') genes. The PCR product's size for *nad*5 gene amplification is 172 bp, whereas the size for *tdc*B is 174 bp.

The single-stranded cDNAs (which contained pFA3, pFA2 and pMR505) have been used as templates for real-time RT-PCR reaction. 1/10 of the PCR mix total volume was taken from the reverse transcription product and added to the PCR mixture that contained 2.5X Real Master Mix (Eppendorf, Germany), 150 nM forward primer, 100 nM reverse primer and PCR graded distilled water. PCR conditions for amplifying DNA from *nad*5 and *tdc*B genes was 95°C for 5 min to activate DNA polymerase, 95°C for 1 min to denature, 60°C for 30 sec to anneal, 68°C for 30 sec to elongate, which were repeated 40 times. Each DNA sample was analyzed two times in separate reactions and the average of the two replicates was used for the analysis.

RESULTS AND DISCUSSION

We determined the efficiency of two transformation vector, which are super binary vector (pMR505 and pFA3) (Fig. 1, 2) and binary vector (pFA2) (Fig. 3) in transferring foreign genes into oil palm tissues and how they affect the expression level of the transgenes. Initially, pFA2 (~24.4 kb) and pFA3 (~39.4 kb) were successfully cloned and transformed into *Agrobacterium* LBA4404 strain. Together with these two plasmids, pMR505 (~37.3 kb), a stable super binary vector were simultaneously transformed into oil palm immature embryos.

Prior to transformation event, the immature embryos were pre-cultured for 5-12 days on N_6OPS medium that contained specific nutrients, vitamins, charcoal and 2,4-D. The effect of explants pre-culture towards transformation process was still not very clear. However, Abdullah *et al.* (2005) reported that oil palm immature embryos pre-culture prior to *Agrobacterium* infection exhibited high percentage of plant regeneration. On the contrary, pre-culture period that was less than 7 days resulted in 'browning' phenomena which eventually led to cell death after co-cultivation. The presence of charcoal and 2,4-D assisted the immature embryos to divide actively and eased the *Agrobacterium* infection process. In similar studies, 2,4-D hormone made the target tissues more susceptible to *Agrobacterium* infection. After 7-12 days, the target tissues started to bud. In this study, immature embryos with 3-4 mm in size was used for

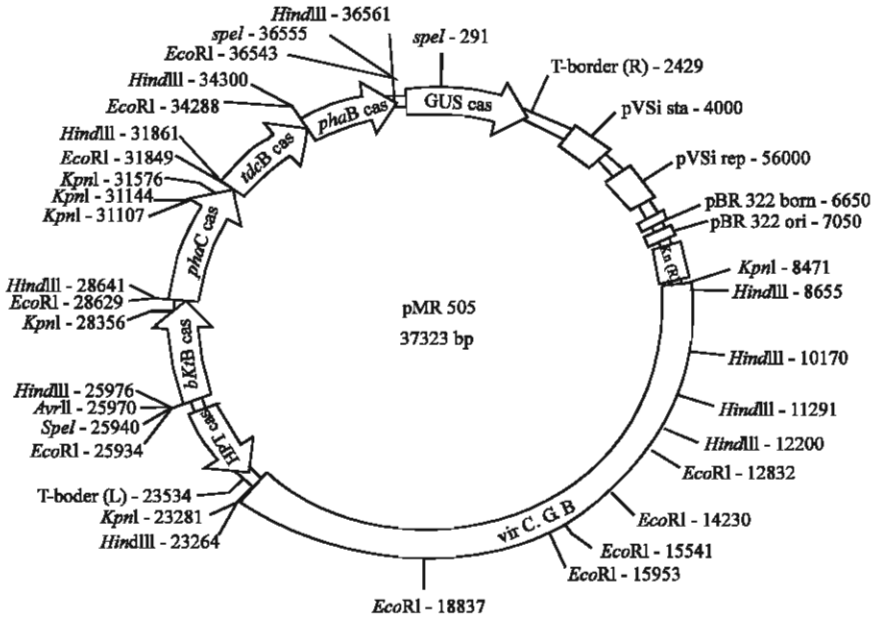

Fig. 1: The diagram of super binary vector pMR505

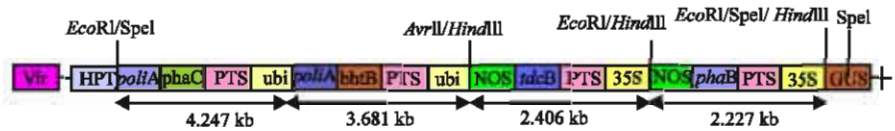

Fig. 2 : The T-DNA region of super binary vector, pFA3

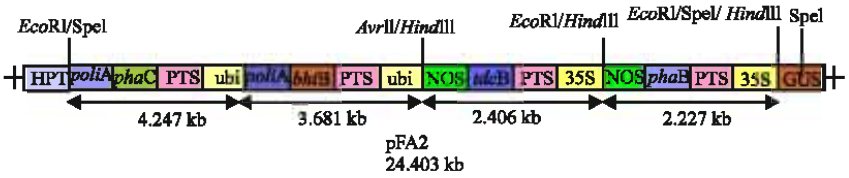

Fig. 3: The T-DNA region of binary vector, pFA2

Fig. 4: GUS assay after 20 days transformation event (A) pFA2 transformed immature embryos, (B) pFA3 transformed immature embryos, (C) pMR505 transformed immature embryos and (D) Non-transformed immature embryos

transformation. The 3-4 mm size immature embryos were easier to generate callus after *Agrobacterium* infection, compared to smaller or bigger sized immature embryos. The selected immature embryos were then cut into two sections to produce wound for bacterial infection. It has been reported that the wound will make the plant cells released chemical substances which attracted hormone which are essential to induce callus formation. *Agrobacterium* to the wounded cells. *Agrobacterium* would bind to the plant cell surface and started to synthesize cellulose, which eventually resulted in stronger binding between the two symbiotic cells (Tzfira *et al*., 2006). Apart from that, immature embryos amputation yielded more tissues for the bacteria infection.

Within 20 days after the transformation event, transient analysis through GUS assay was carried out to confirm successful gene transfer and transformation frequency. In this study, GUS assay on pFA3, pMR505 and pFA2 freshly co-cultivated target tissues exhibited uniform and well spread GUS activity, resulting in blue colored immature embryos (Fig. 4A-C). In spite of that, the blue intensity seemed to differ between each individual cells, although the promoter that drives the expression is the same, which is CaMV35S promoter. However, it is noteworthy to note that pMR505 co-cultivated target tissues exhibited stronger *gus* expression, followed by pFA3 and pFA2, respectively. This result suggest possible influence of plasmids used in delivering transgenes into target tissues and also other extrinsic factors, such as size, wound sites or pre-treatments prior to transformation. This result was compared to non-transformed immature embryos, which acted as the negative control (Fig. 4D). As expected, the negative control did not show any GUS activity.

Table 1: Transformation frequency in putative pFA2, pFA3 and pMR505 transformed immature embryos (IEs)

Experiment	Gene construct	No. of IEs	No. of assayed IEs	GUS positive IEs	Transformation frequency (%)
1	pFA2	72	19	10	52.6
	pFA3	76	17	12	70.5
	pMR505	80	21	17	80.9
2	pFA2	76	27	19	70.3
	pFA3	101	22	17	77.0
	pMR505	99	21	18	85.7
3	pFA2	110	28	24	85.7
	pFA3	177	31	30	96.7
	pMR505	137	34	33	93.8

In this study, pMR505 and pFA3 co-cultivated target tissues, which were super binary vectors, recorded the highest transformation frequency between 70.9-98.3 (pMR505) and 70.5-96.7% (pFA3), respectively. In contrast, pFA2 showed lower percentage which ranges between 52.6-85.7% (Table 1). The difference was significant to Abdullah *et al.* (2005) report that showed higher GUS activity and transformation frequency from those using super binary vectors. In another similar studies, Zhao *et al.* (1998) also reported that the use of super binary vector system has led to 33-51% transformation efficiency in transformed maize embryo compared to only 5.5% when binary vector was used (Frame *et al*., 2002). These studies may have correlation to Hamilton *et al.* (1996) studies, which proposed that additional copies of *vir*G and *vir*E genes were able to enhance high-molecular weight T-DNA (approximately 150 kb) transfer to tobacco genome. Vain *et al.* (2004) in his more recent studies also indicated that the addition of *vir* gene to the background vector may influence plant transformation efficiency and integration of background vector sequences.

In determining GUS activity in oil palm tissues, we also has reported the role of a number of constitutive and specific tissue promoters in driving *gus* expressions in oil palm mesocarp, leaves and immature embryos. This study showed that CaMV35S, which is a constitutive promoter, exhibited high GUS activity in immature embryos and mesocarps, whereas rubisco (tissue-specific promoter) drove high level of *gus* expression in leaves. Rashdan (2001), on the other hand, reported the role of vectors in influencing *gus* expression patterns and magnitudes in oil palm tissues. He reported that super binary vector system showed higher GUS activity compared to those using binary vector, as well as the role of infection sites in determining GUS activity, which usually converge at actively divided parts, such as embryos and specific tissues.

Based on a number of studies that were conducted on other monocot plants such as maize and rice, Hiei *et al.* (1994) has concluded 5 important factors that involves in influencing the success of genetic transformation via *Agrobacterium*. This includes *vir* gene induction, actively dividing cells in target tissues, medium composition, vectors and *Agrobacterium* strain and genotypes. Winnie (2004) in her more recent work demonstrated a number of factors that involves in determining high *Agrobacterium*-mediated transformation efficiency, which were bacterial density, co-culture periods and the effect of acetosyringone towards high GUS activity in oil palm tissues. These reports suggested a variety of factors that may contribute to the enhancement of *Agrobacterium*-mediated transformation, which will eventually lead to high expression of genes of interest. To determine the presence of PHBV genes in oil palm tissues, PCR analysis was performed on the transformed immature embryos genomic DNA. PCR amplification has verified the existence of *bkt*B (~1.176 kb), *pha*B (~0.765 kb), *pha*C (~0.999 kb) and *tdcB* (~0.909) genes in the transformed tissues. The distinct DNA bands were shown in Fig. 5A-D. The resulting PCR amplifications were compared to those from non-transformed immature embryos that acted as the negative control.

Real-time PCR analysis was performed to compare the efficiency of both vectors in the aspect of gene expression level. In this case, only *tdc*B was analyzed as the target gene of interest. *Tdc*B encodes threonine dehydratase, which controls the conversion of threonine to 2-ketobutyrate in isoleusine pathway. Thus, to divert 2-ketobutyrate towards PHBV biosynthetic pathway, the quantity of the substrate has to be increased. Besides, all three *tdc*B gene cassettes in pFA3, pFA2 and pMR505 were driven by CaMV35S promoter, which makes the analysis standardized towards the role of transformation vectors in effecting gene expression.

In real-time PCR analysis, primer design is the most critical step in ensuring the success of the experiment. In this study, a set of primers rtTdcBF/rtTdcBR has been designed to amplify *tdc*B (172 bp) and rtNad5F/rtNad5R for *nad*5 (174 bp) amplicons. Prior to gene expression analysis, the primers should be tested to verify their specificity and sensitivity. In this case, the melting curve analysis and agarose gel electrophoresis were carried out in order to verify the homogeneity of PCR products. The emergence of single peak melting curve in every real-time PCR reaction confirmed specific amplification for *tdc*B amplicon (87.6°C) and *nad*5 amplicon (81.0°C) (data not

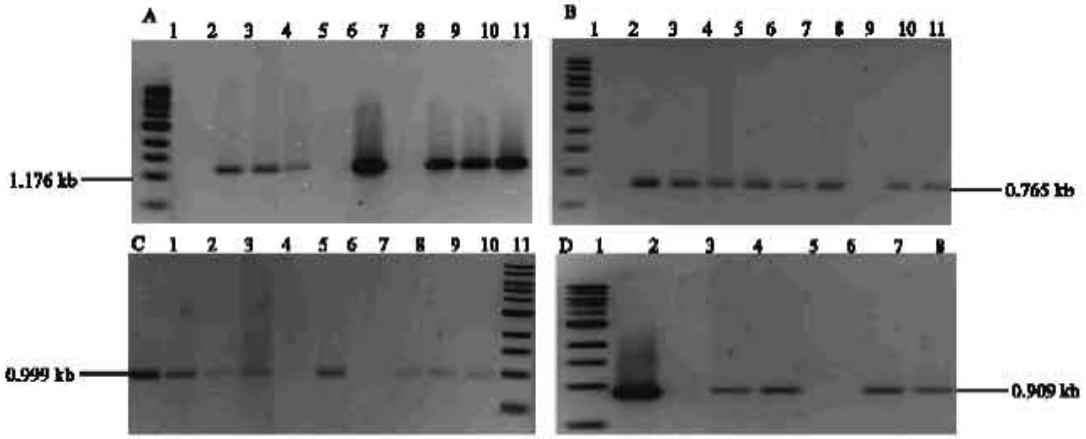

Fig. 5: PCR analysis of transformed immature embryos (IEs) for detection of PHBV genes using specific primers (A). Amplification of 1.176 kb fragment of *bkt*B gene. Lane 3-4: pFA2 transformed IEs, lane 5 and 9: pFA3 transformed IEs, lanes 5 and 10: pMR505 transformed IEs, lane 7: Positive control; lanes 2 and 6 negative control, lane: 8 blank; lane 1: ladder 1 kb. B) amplification of 0.765 kb fragment of *pha*B gene. Lanes 4-6: pFA2 transformed IEs; lanes 7-8: pFA3 transformed IEs; lanes 10-11: pMR505 transformed IEs; lane 3: positive control; lane 9: negative control; lane 1: 1 kb ladder. C) amplification of 0.999 kb fragment of *pha*C gene. Lane 1: positive control; lanes 2-3: pFA2 transformed IEs; lanes 4 and 6: pMR505; lane 7: negative control; lanes 8-10: pFA3 transformed IEs; lane 11: 1 kb ladder D). Amplification 0.909 kb fragment of *tdc*B gene. Lane 1: 1 kb ladder; lane 2: positive control; lane 3: negative control; lane 5: pFA2 transformed IEs; lane 6: pFA3 transformed IEs; lane 7: negative control; lane 8-9: pMR505 transformed IEs

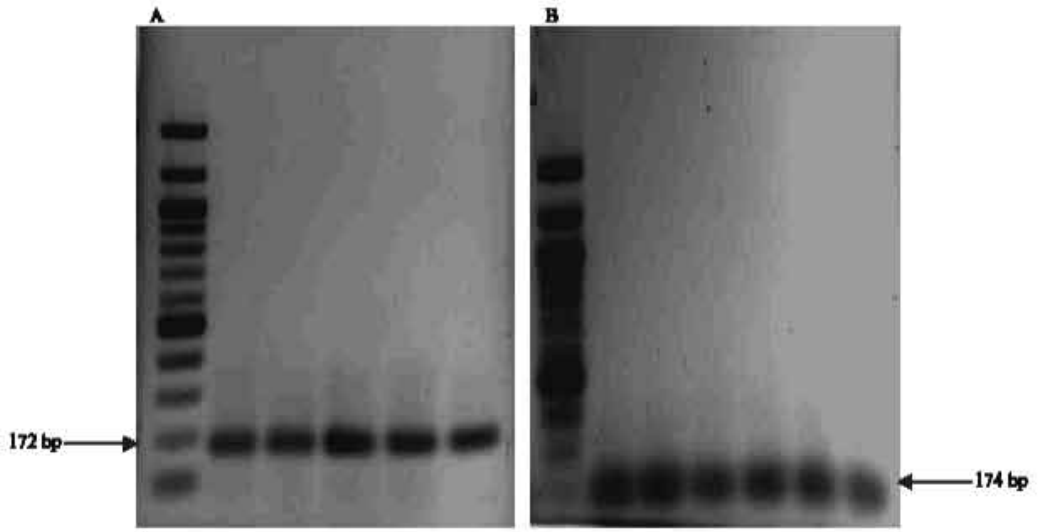

Fig. 6: Primers validation. Agarose gel analysis showed the presence of (A) ~172 bp fragment of *tdc*B gene with T_m 87.6°C. (B) ~174 bp fragment of *nad5* gene with T_m 81.0 °C

shown). Electrophoresis gel agarose also showed specific amplification with the presence of single DNA b and which was 172 bp and 174 bp for *tdc*B amplicon and *nad5* amplicon respectively (Fig. 6A, B).

To compare the efficiency of binary and super binary vectors in affecting gene expression, relative quantification through $2^{-\Delta\Delta}$Ct method has been performed. This method is the most common mRNA quantification in real-time PCR analysis, especially in quantitating relative gene expression changes (Livak and Schmittgen, 2001). The analysis can be performed without the need to generate standard curves in every single experiment. In contrast, this method applied arithmetic formulae based on ΔCt and transcript amplification efficiency. This

method can be applied only if the PCR efficiency for both endogenous and target genes are the same or nearly the same. Relative quantification for *tdc*B transcript has been carried out and compared between pFA2, pFA3 and pMR505 transformed immature embryos together with the calibrator (non-transformed immature embryos), compared to *nad*5 transcript that acted as the normalizer.

*tdc*B expression in pMR505 transformed immature embryos is 7.41 fold greater than the calibrator. In contrast, *tdc*B expression in pFA3 transformed immature embryos is 0.665 fold greater than the calibrator, even though both vectors used were super binary vectors. However, pFA2 transformed immature embryos showed 2.45 fold greater than the calibrator (Fig. 7). These results showed that *tdc*B expression level in oil palm immature embryos varied with different transformation vectors used. Log fold difference indicated whether *tdc*B expression was upregulated or downregulated and in this study, pFA2 and pMR505 samples have shown upregulated expression. pFA3, however, showed downregulated expression level. These results were obtained even though the promoters used for the three constructs were the same, which was CaMV35S (Fig. 8).

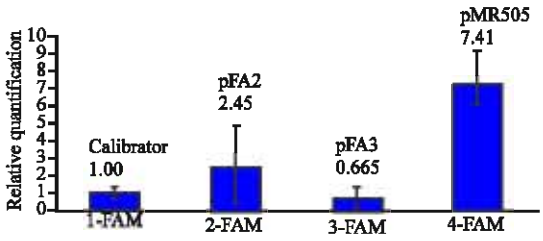

Fig. 7: Relative quantification data for the expression level of *tdc*B gene in pFA2, pFA3 and pMR505 transformed immature embryos compared to non-transformed control after normalization. The data was shown in fold change compared to the calibrator

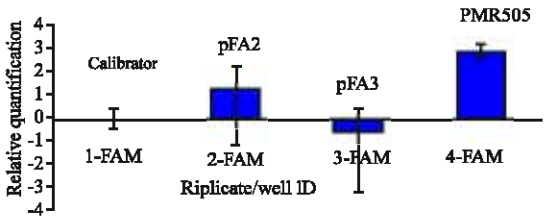

Fig. 8: Relative quantification data for the expression level of *tdc*B gene in pFA2, pFA3 and pMR505 transformed immature embryos compared to non-transformed control after normalization. The data was shown in log-fold difference compared to the calibrator

In plants, it has been reported that the variety of transgene expression level were influenced by positional effect, which is where the T-DNA integrates in a plant genome. T-DNA may integrate near or far from the transcription activating element or enhancer, which resulted in transgene activating or inactivating (Campisi *et al*., 1999 ; Springer *et al*., 1995). Apart from that, the T-DNA may also integrate in transcription competent areas or where it tends to cause gene silencing. This factor can be related to *tdc*B expression in pFA3 immature embryos which were very low and downregulated, compared to the expression of the same gene in pFA2 and pMR505 immature embryos, which were quite high and upregulated. Meins (2000) reported that gene silencing phenomena occurs post-transcription, which means that the transgenes express, but the resulting RNA is not stable. This is caused by multiple copies of transgenes that presents in the plant cells. Although *Agrobacterium*-mediated transformation resulted in low copy number of transgenes, tandem copies of T-DNA that integrates in single loci also has been reported. Transgene silencing may occur even though the plant produces single-integrated T-DNA (Jorgensen *et al*., 1987). In this case, the variety of transgene expression level failed to correlate with the number of stably integrated transgenes (Peach and Velten, 1991). However, a significant correlation between transgene copy number and the expression level has been reported by Gendloff *et al*. (1990).

The effect of genomic position and copy number towards transgene expression has been reported in numerous plants (Allen *et al*., 2000). Foreign DNA integrates in random non-homologous sites; hence some of the transgenes may integrate in chromatin-active areas or stiff-chromatin transcription areas. Thus, transgenes that integrate in heterochromatic areas, such as centromere, tend to experience gene silencing, which resulted in low gene expression level (Mengiste and Paszkowski, 1999). Over the years, numerous reports have been made on the correlation between copy numbers and expression levels in plants. Allen *et al*. (1993) and Linn *et al*. (1990) have reported inverse correlation between copy numbers and transgene expression. On the contrary, Shirstat *et al*. (1989) and Van der Krol *et al*. (1990) demonstrated that there were no significant correlation between transgenes expression and copy numbers. McCabe *et al*. (1999) and Voelker *et al*. (1996) however, reported that the increment of transgene expression is correlated directly to high copy numbers of transgenes. Apart from that, it has been reported that plants with multiple copies of transgenes may express higher rate of proteins than plants with lower copies of

transgenes. Based on all these studies, we can conclude that transgene expression or silencing may attribute to specific gene contructs, where in this present study, refers to transformation vectors used during gene transfer into the oil palm genome.

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