

Expression of *Bacillus thuringiensis* insecticidal protein gene in transgenic oil palm

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Abbreviations: DGT: direct gene transfer
IEs: immature embryos

Oil palm, like all other crops, is susceptible to attack from several insect pests causing significant reduction in productivity. In the past, *cry* genes from *Bacillus thuringiensis* have been reported to be effective in conferring resistance towards insect pests in crops such as corn and rice. One of the advantages of these toxin proteins is their specificity towards certain harmful insects. A rapid and efficient method was developed for the transformation and evaluation of *CryIA(b)* expression in oil palm. A recombinant vector was introduced into immature embryos (IEs) of oil palm via the biolistic method. More than 700 putative transformed IEs from independent transformation events were generated. Transient transformation efficiency of 81-100 % was achieved. We found that pre-treatment of target tissues with phytohormones is essential for increasing the transformation efficiency. This finding could enable higher transformation rate in

oil palm that was previously difficult to transform. PCR analysis further confirmed the presence of the *CryIA(b)* in the transformed tissues. Expression of *CryIA(b)* from PCR-positive samples was further confirmed using a rapid gene expression detection system. This novel and rapid detection system could serve as a good opportunity to analyze the impact of transgenes upon transfer to the new environment, especially for crops with long generation cycle, such as oil palm.

Oil palm (*Elaeis guineensis* Jacq.) is the source of the most sought-after edible oil in the world market. With the advent of new biotechnology tools, in particular gene manipulation, palm oil monopoly as a commodity is challenged by other oil-bearing crops such as soybean (Kinney et al. 2001), sunflower seed (Osorio et al. 1995) and rape seed (Ramachandran et al. 2000; Dehesh et al. 2001) that have been successfully modified genetically.

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developing techniques for genes transfer into oil palm, both using direct gene transfer (DGT) and *Agrobacterium*-mediated approaches. Both techniques were then used to transfer useful genes such as *cowpea trypsin inhibitor* (*CpTI*) (Abdullah et al. 2003) and *Bacillus thuringiensis* (*Bt*) crystal insecticidal protein genes, to address problems related to insect pests (Sharma et al. 2000; Sharma et al. 2002) and *chitinase* (to address problems related to basal stem rot).

Transgenic plants such as tomato, tobacco, cabbage and rice containing *CryIA(b)* gene have been obtained using both DGT and *Agrobacterium* transformation methods (Cheng et al. 1998; Jouanin et al. 1998; Robinson, 1999; Bhattacharya et al. 2002). In this article, we report successful transformation of oil palm using particle bombardment for expression of an agronomic trait. We have carried out the construction of the *CryIA(b)* gene cassette (Sardana et al. 1996) which have been modified to express highly in plants, particularly oil palm. In the investigation, the synthetic *CryIA(b)* was introduced into oil palm, data for successful transformation and expression of the gene within the oil palm tissue were also presented. The *CryIA(b)* gene when expressed produced proteins which upon crystallization are highly toxic to Lepidoptera (Masson et al. 1999; Reardon et al. 2004), among which *Metisa plana* is a major insect pest for oil palm (Wood, 1968). Using our established DGT methods for oil palm, we then developed a rapid detection system for evaluating transgene expression among putative transformed tissues. The procedure developed is very sensitive, rapid and eliminates the long waiting period for transgenic plants to reach maturity.

MATERIALS AND METHODS

Transformation vector

Escherichia coli strain DH5 α was used as plasmid host for the construction of transformation vectors. Standard cloning techniques (Sambrook et al. 2001) were used to construct the recombinant plasmid, pRMP as shown in Figure 1. The coding sequence for *CryIA(b)* gene, a derivative of plasmid pUBB (Prof. Illimaar Altosaar, University of Ottawa, Canada), and known to produce lepidopteran-specific δ -endotoxin, was inserted into pCAMBIA 1301 cloning vector (Dr. Richard A. Jefferson, CAMBIA, Australia) using the *Bam*HI site. The *Hind*III site was then deleted through the excision of the 3' end of the cassette. Subsequently, rubisco promoter, a derivative of pBRC73 (Dr. J. K. Kim, Myongyi University, Korea) was fused to the *CryIA(b)* coding sequence using the remaining *Hind*III site. The terminator sequence was then inserted into the vector using the *Eco*RI site, giving rise to a recombinant plasmid pRMP. The integrity and integration of *CryIA(b)* was confirmed using restriction mapping, Southern blotting and sequencing. Apart from those present in the original parent plasmid pCAMBIA1301, the recombinant plasmid pRMP now also contains a chimeric

CryIA(b) gene under the transcriptional control of a rubisco promoter and terminated by a polyadenylation signal from the *nopaline synthase* gene (*nos T*). pRMP was then used in all subsequent transformation experiments.

Plant material

Oil palm (*E. guineensis* Jacq. var. *tenera*) samples were obtained from Pamol Plantations Sdn. Bhd. IEs were isolated from oil palm fruit bunch harvested 9-11 weeks after anthesis (WAA) according to protocols described earlier (Abdullah et al. 2005). Extracted IEs were cultured on N₆ media with or without 2.5 mg/l 2,4 D and incubated either in the dark at 28 \pm 1°C for 4 weeks prior to bombardment.

Transformation and GUS assay

Pre-cultured IEs were bombarded using the PDS-1000/He particle delivery system (BioRad) following the procedure established elsewhere (Wright et al. 2001) with some modifications (Abdullah et al. 2005). All components of the chamber were surface-sterilized using 70% (v/v) ethanol prior to bombardment. Plasmid DNA (2.5 μ g) were coated onto 3 mg of resuspended gold particles. This was followed by the addition of 2.5 M CaCl₂ and 0.1 M spermidine. The mixture was vortexed and centrifuged for 1 min. The DNA-coated particles were then washed with 100% (v/v) ethanol. Target samples were placed 6 cm from the stopping screen and bombardment was performed at 900 psi. Non-bombarded IEs and those bombarded without DNA were used as control. Treatments were replicated three times. After bombardment, IEs were cultured on regeneration media. The embryos were sub-cultured every 4 weeks. The cultures were maintained at 24°C in a culture room under a 16 hrs photoperiod. The expression of the *gusA* gene was assayed histochemically (Su et al. 1998; Stangeland and Salehian, 2002) using X-Gluc (5-bromo-4-chloro-3-indole- β -glucuronide) as substrate. GUS activity was inactivated with the addition of FAA solution [42.5% (v/v) ethanol; 5% (v/v) glacial acetic acid; 10% (v/v) formalin].

Isolation of genomic DNA and total RNA

Genomic DNA and total RNA was isolated according to modified protocols of Doyle and Doyle (1990) and Verwoerd et al. (1989), respectively, using bombarded IEs 3 days after bombardment. Both DNA and RNA were quantified using a spectrophotometer.

Expression analysis of *CryIA(b)* gene in IEs

Oligonucleotides were designed to amplify region specific to the *CryIA(b)* coding sequence. For the purpose of evaluating transformation events, evidence of transgene presence is considered positive PCR or a positive expression assay for the gene of interest. All PCR and RT-PCR were carried out according to the manufacturer's instruction manual (Promega). The primers set used were as follows: CRYF1 5'~GGTTCGTTCTCGGACTAGTT and

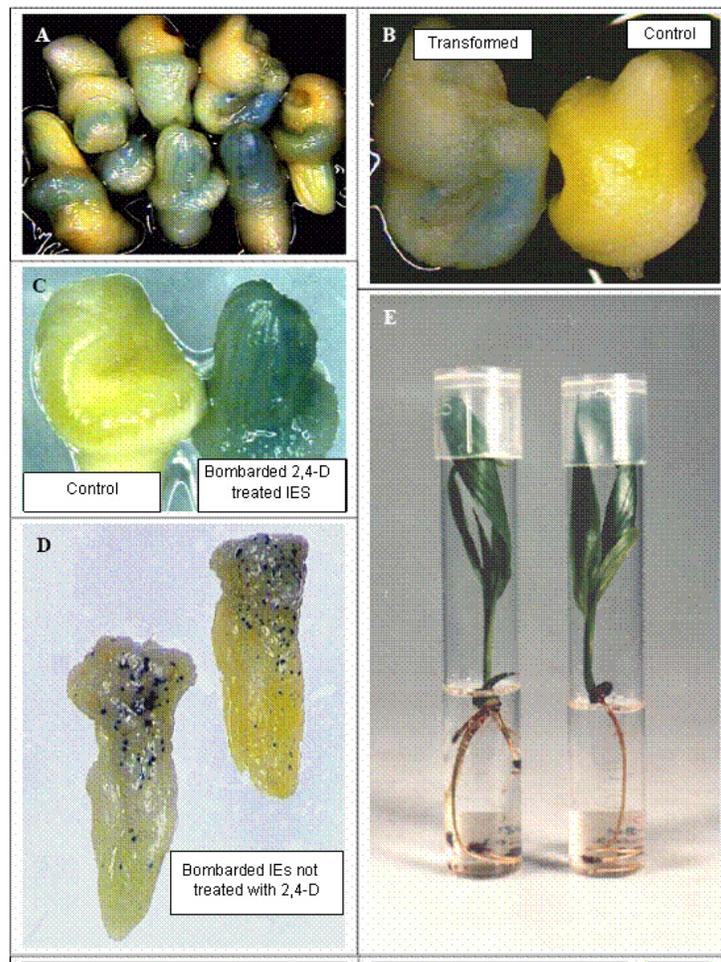


Figure 2. *gusA* expression patterns observed between transformed IEs treated with 2,4-D and those not treated with 2,4-D prior to bombardment.

(A) Randomly selected IEs assayed for *gusA* activity.

(B) In the presence of the *gusA* gene, substrate produced blue deposits as indicated by blue stains observed in successfully transformed cells and tissues. On the other hand, the absence of blue stain indicates the absence of *gusA* gene, which failed to produce blue deposit.

(C) Shades of blue stains (indicating *gusA* expression) were detected on transformed IEs spreading over the entire IEs treated with 2,4-D prior to bombardment. Prominent longitudinal blue stains probably along vascular bundles were also detected in 2,4-D treated IEs. No blue spot or stains were detected on all non-transformed sample (control).

(D) Only localized blue spots (distinct and isolated) were observed on IEs not treated with 2,4-D prior to bombardment. (E) Plantlets regenerated showed no difference in growth between those from double bombarded IEs (left) and from single bombardment (right).

CRYR1 5'~CTGGTAAGTTGGGACACTGT. A total of 0.1-0.4 µg genomic DNA was used as template in a 50 µl PCR reaction mix containing 25 pmol of each primer, 2.5 U Taq DNA polymerase, 1.5 mM MgCl₂, 1X buffer and 200 µM dNTP (200 µM each of dATP, dCTP, dGTP, dTTP) (Promega). The amplification conditions consisted of an initial DNA denaturation at 94°C for 5 min, followed by 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C for 30 cycles with a final extension cycle of 72°C for 7 min. PCR products were electrophoresed on 1% (w/v) agarose gel and analyzed under UV light after ethidium bromide staining. The presence of *CryIA(b)* gene was confirmed by Southern Blot analysis using the *CryIA(b)* gene as probe. Total RNA was isolated from tested PCR-positive samples. The same primer set (mentioned above) was used in RT-PCR. A total of 10 µg total RNA was used as template in a 50 µl PCR

reaction mix containing 50 pmol each of reverse and forward primers, 1X AMV/*Tfl* buffer, 0.2 mM dNTP, 1 mM MgSO₄, 0.1 U reverse transcriptase and 0.1 U *Tfl* DNA polymerase. Total RNA was reverse transcribed at 42°C, for 1 hr followed by PCR amplification as mentioned above. RT-PCR products were then separated on a 1.2% (w/v) agarose gel and blotted onto a nylon membrane. Hybridizations using DIG-labeled probes were then carried out according to Roche's instruction manual.

RESULTS AND DISCUSSION

Characterization of transformation vector

The open reading frame (ORF) of *CryIA(b)* was successfully cloned into pCAMBIA 1301 under the control

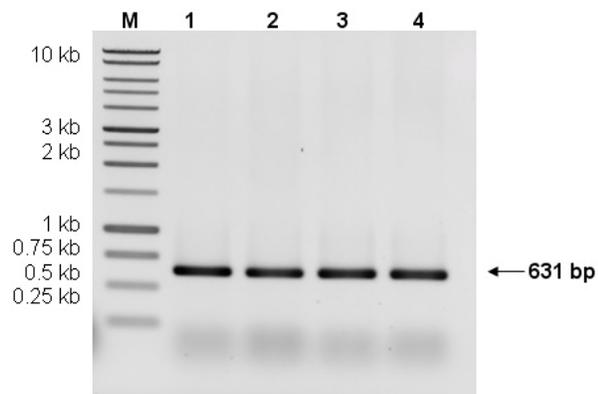


Figure 3. PCR analyses of putative transformed IEs for the presence of *CryIA(b)* gene. Genomic DNA was isolated from 3-day old putative transformed IEs and amplified using the CRYF1 and CRYR1 primer combination. All 15 samples tested showed the presence of the expected 631bp, thus confirming the presence of *CryIA(b)* gene in putative transformed IEs. Lane 1-4: examples of positive signals detected from all 15 samples tested. M, 1 kb DNA ladder marker.

of rubisco promoter in the sense orientation creating a new recombinant plasmid, pRMP (Figure 1). The orientation and integration of *CryIA(b)* in pRMP were confirmed through restriction mapping, Southern blotting and nucleotide sequencing. Southern blot analysis following multiple enzymatic digestions showed that *CryIA(b)* was successfully integrated into pRMP. Integration of *CryIA(b)* in pRMP was in-frame as confirmed through nucleotide sequencing with only the *Bam*HI site connecting the rubisco promoter with the *CryIA(b)* coding sequence. Besides the *CryIA(b)* gene which is under the control of a rubisco promoter, the newly constructed pRMP also contains *hptII* (selectable marker gene) and *gus* (reporter gene), from the original pCAMBIA1301. Since all three genes cassette are flanked by the T-DNA right and left border sequences, thus, pRMP is useful for both biolistic-mediated and *Agrobacterium*-mediated transformation experiments where they should be transferred in the process of successful gene transfer mediated by *Agrobacterium*.

Pre-treatment of target tissues

In our earlier work, we have shown that 2,4-D pre-treatment of target tissues prior to particle bombardment and *Agrobacterium* infection (Abdullah et al. 2005) was essential for increasing the number of transformation events in target cells or tissues. IEs pre-cultured on N₆ media supplemented with 2.5 mg/l 2,4-D prior to bombardment also showed different GUS expression pattern as compared to IEs maintained on hormone-free N₆ media (Figure 2). In contrast, no blue spot or shade was observed on all control or non-bombarded tissues. The GUS-positive blue spots on IEs cultured on hormone-free N₆ media were distinct, isolated and localized, whereas the GUS stains on 2,4-D pre-treated IEs spread over a larger area. There are two

possibilities that may result in the expression of GUS that produced the blue stains over a larger area. First, the spread could be a consequence of a possible leakage between cells following damage caused during the process of bombardment. However, if this were the case, similar GUS expression pattern would also be observed on IEs pre-treated on hormone-free N₆ media since both samples were treated the same. Second, the spread of blue stains may be from relatively more cells that may have received the *gusA* gene either through gene transfer (bombardment) or daughter cells derived from transformed cells undergoing cell divisions. This larger population of cells now possessing the *gusA* gene may have resulted in larger area of the tissues being stained blue following the expression of the transferred gene on the substrate. Therefore, it is more likely that the 2,4-D present in pre-treatment media must have influenced the expression pattern of the *gusA* gene transferred as observed in Figure 2. Being an auxin (Jain and Minocha, 2000; Pasternak et al. 2002), 2,4-D induces rapid cell division. Since the target tissues were pre-treated with 2,4-D prior to bombardment, therefore, upon successful transfer and integration, the *gusA* gene could easily be amplified together during subsequent cell divisions, thus giving rise to a wider spread of the blue stains observed (Abdullah et al. 2005). On the other hand, without 2,4-D, IEs pretreated on hormone-free N₆ media did not undergo rapid cell division, but following the path of differentiation instead. Thus, the *gusA* stains detected in untreated IEs were isolated and distinct. In addition the stains were mainly localized in cells that may have received the transgene from bombardment only. Similar observations were also reported in rice transformation using *Agrobacterium*, where only target tissues cultured on 2,4-D-containing media were capable of expressing GUS whereas those untreated tissues did not (unpublished data).

Yang et al. (1999) also claimed that cell divisions correlate with maximum transformation events, suggesting cell division is essential for cell competency. Competent cells have been associated to its susceptibility to receive foreign DNA during the process of transformation (Tang and Tian, 2003). Though we observed similar results in the case of oil palm, the reasons still remain unclear. However, it is believed that during cell division, active replication of DNA strand takes place. This is when chromosomes unwind and the double helix denatures (Gerald, 2002), hence exposing the fragile DNA. It is assumed that it was at this time that cells are most vulnerable to the invasion of foreign DNA. Illegitimate recombination between the vulnerable DNA and transgenes may have resulted in stable integration (Tzfira and Citovsky, 2002; Hohe and Reski, 2003). Therefore, factors that induce rapid cell division could play major role in ensuring higher transformation events.

Transformation of oil palm

Under our new experimental design, putatively transformed IEs were assayed 3 days after transformation. 750 IEs

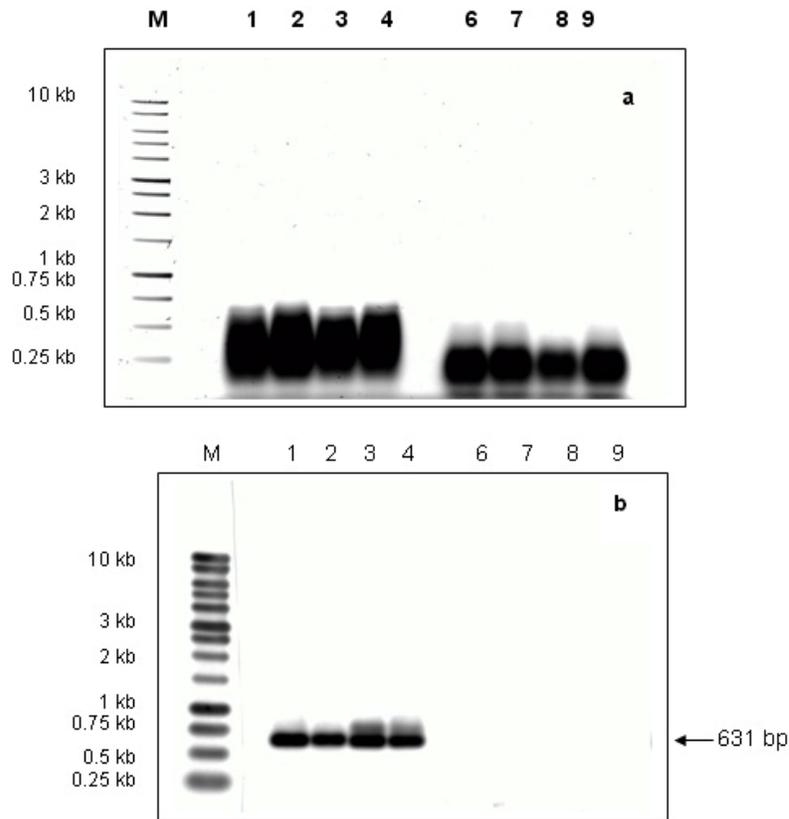


Figure 4. RT-PCR-Southern blot analysis of *CryIA(b)* transcripts in putative transformed IEs of oil palm.

(a) Lane 1-4: Electrophoresis of RT-PCR products on agarose gel. Probable bands corresponding to *CryIA(b)* transcripts were clouded with RNA and primers. Lane 6-9: Total RNA from putative transformed IEs subjected to PCR amplification without reverse transcription also produced no band, thus, further ascertained the absence of genomic DNA contamination that could produce a false positive signal. (b) Lane 1-4: Hybridization of RT-PCR products with *CryIA(b)* probe showing signals corresponding to the expected 631bp bands. Lane 6-9: Again, no signal was detected from all controls, thus confirming successful expression of *CryIA(b)* transgene in putative transformed IEs.

bombarded with pRMP were randomly selected and analyzed through histochemical staining (Figure 2). Transient GUS assay was used to evaluate the efficiency of gene transfer, where transformation efficiency is defined as the number of independent event recovered per explant bombarded (Wright et al. 2001). This was determined using result from the GUS assay. GUS-positive blue spots or stains were detected on randomly selected IEs assayed, suggesting gene transfer had taken place. In contrast, no blue spot was observed in all control samples assayed. Here, observation of one or more blue spots on a single IE is considered as one unit of expression (Klein et al. 1988). On average, the transient transformation efficiency of IEs bombarded once was recorded at 81% as compared to 100% for those subjected to double bombardment. Double or multiple bombardments is not normally practiced in other crops, as it would cause excessive wounding to target tissues (Jiang et al. 2000) resulting in eventual death. However, we believed that oil palm being a *hardy* plant could withstand certain amount of wounding to its tissues. Post bombardment observation showed that there was no excessive accumulation of phenolic compound in the

culture medium, which would be the case for severely injured or dying oil palm tissues.

IEs from both treatments were cultured on hormone-free N_6 media and regenerated to complete plantlets (Figure 2). Apart from about 3% losses due to contamination, all bombarded IEs were regenerated into complete plantlets. The main objective of direct plant regeneration was to evaluate the impact of double bombardment on the plant as compared to single bombardment. Although the construct contained the *hptIII* gene, antibiotic selection was not performed, as this would cause cell death to untransformed cells, thus inflicting additional stress to the limited transformed cells present. During antibiotic selection, dying cells inhibit nutrient supply to transgenic cells or excrete toxic compounds, which further impedes proliferation of transgenic cells to differentiate into transgenic plants (Ebinuma et al. 2001). This adverse effect would then influence IEs growth thus causing difficulties in data collection. Preliminary observation showed that there were no morphological differences between plantlets derived from IEs bombarded once or twice (Figure 2e). Similar

observations were also noted in the formation of shoots and roots of plants from both groups. Plants from both groups also exhibited similar growth rate. Therefore, based on these observations, oil palm IEs can withstand double bombardment and would still be able to regenerate into healthy plantlets. However, these qualitative observations need further detailed study, especially on the plants performance upon maturity.

Detection of transgene using PCR

Many techniques have been developed to detect transgenes in T_0 plants. These include PCR-based techniques followed by gel electrophoresis and detection, and are routinely used to detect transgenes in plants (Higuchi et al. 1992; Abedinia et al. 1997). Other techniques such as the use of molecular beacon assays (Kota et al. 1999) and FISH (Jin et al. 2002) had also been used. But this was not the case for slow growing plants like oil palm where detection of transgenes has always been a major problem. In this study, preliminary analyses on transformants were carried out by PCR analyses. Two primers, CRYF1 (nt 170-189) and CRYR1 (nt 800-781) designed based on the coding sequence of *CryIA(b)* from plasmid pUBB with an expected PCR product of 631 bp were used in subsequent PCR analyses. Genomic DNA isolated from fresh tissues of putative transformed and non-transformed (negative control) plants were used as templates in the analyses. In all cases, the expected 631 bp bands were present in all 15 samples tested (Figure 3), indicating the presence of *CryIA(b)* in the genomic DNA of putative transformed plants. In contrast, no band was observed for all control samples. The experiments were repeated three times, giving the same result, suggesting successful gene transfer into oil palm via particle bombardment. The 100% transient transformation efficiency observed following PCR of *CryIA(b)* was similar to those observed earlier from GUS assay carried out using the same sample. Therefore, this further confirmed successful transfer of transgenes from pRMP into the oil palm tissues. However, the presence of *CryIA(b)* in putative transformed plants is no indication of its functionality in its new environment.

Expression of *CryIA(b)* gene in oil palm

Evaluation of transgene expression in T_0 transgenic plants has always been difficult, especially when the transgenes undergoes various modifications in its new environment (Stam et al. 1998; Francis and Spiker, 2005). Many reports have shown that transgenes may be present in its new environment but were not expressed or expressed at a very low level making it undetectable. Failure for transgene to express may be due to mutations in the transgenes (Tinland, 1996), post-transcriptional gene silencing (Mitsuhashi et al. 2002; Szittyta et al. 2003), or chromatin-related transgene silencing (Francis and Spiker, 2005). In this study, although PCR analyses could easily detect the presence of *CryIA(b)* in the putative transformed plants, attempts to detect its expression and functionality could not be easily carried out.

Several attempts using Northern blots to detect specific RNA sequences corresponding to the *CryIA(b)* gene was unsuccessful (data not shown). Subsequently, however, RT-PCR analyses produced signals but could not be discriminated between those of the transgene [*CryIA(b)*] or contaminating primers and RNA (Figure 4a). However, when the RT-PCR products were blotted onto nylon membrane and hybridized against the *CryIA(b)* probe, it gave rise to positive signals with the expected size (631 bp, Figure 4b). The RT-PCR products were subsequently sequenced and showed that they were 100% homologous to the *CryIA(b)* from the original plasmid pUBB (Prof. Illimaar Altosaar, University of Ottawa, Canada). Thus, the presence of *CryIA(b)* mRNA transcripts shows that it is fully functional in oil palm, at least at the transcriptional level. Therefore, it can be concluded that the DNA and RNA specific to *CryIA(b)* were actually present in putative transformed oil palm (as also shown in PCR analyses) but were below detectable level. However, once amplified (using RT-PCR), these could easily be detected as RT-PCR allows for reverse transcription of mRNA and amplification of the transcripts (Pfaffl and Hageleit, 2001). Combining Southern blot with RT-PCR further substantiate evidence on the functionality of *CryIA(b)* in oil palm. These observations are the first evidence on the functionality of a transgene in oil palm following transformation and could also serve as a sensitive detection assay as shown by the positive signals produced.

Most published papers on oil palm transformation (Chowdhury, 1997) used *gusA*, *bar* or *ppt* as reporter genes, which are easy to assay. In addition, there has been no conclusive report on transgenes expression in oil palm to date. In this regard, the fact that the *CryIA(b)* transcripts were detected directly using the RT-PCR-Southern Blotting method, it shows that not only the gene has been successfully transferred but is also successfully transcribed in its new environment. The combination of RT-PCR-Southern Blotting method is therefore useful to detect and study the fate of transgenes upon transfer in its new environment. This is essentially more important when multi-cellular tissues were used as target tissues, where only a minute fraction of cells within the tissues would be transiently transformed per bombarded explant. In such cases, it is quite impossible to determine the fate of the newly transferred transgene in its new environment. Similarly, it is often difficult to quantify transgene copy number in its new environment (Mason et al. 2002; Bubner et al. 2004). However, this would not be the case for transgenic plants that are derived from single cells, where every cell in the plant would have the stably integrated transgene. Similar findings have also been observed in maize transformed with *Bt* gene, where the expression of reporter genes at the protein level is indirectly implied. This led to the development of a direct detection method for protein expression of the gene of interest (Sardana et al. 1996). However, our approach gives a more accurate picture on the of transcription level of the gene of interest in oil palm. For example, it was found that, the *CryIA(b)*

transcripts were relatively little compared to the GUS protein expression observed in the transformed IEs. This approach could also serve as an important tool in promoter study, as it would be more precise to evaluate promoter efficiency at the transcription level as compared to the protein level, since it will not be influenced by translational factors.

In conclusion, this paper provides the first evidence on the functionality of a transgene in oil palm following transformation. In addition, this detection technique that combines RT-PCR and Southern blotting is highly sensitive in detecting minute traces of transgenes expression in putative transformed plants. It is a useful tool that enables closer expression studies of individual genes at the cellular level where transcript analyses could be carried out immediately following transformation. This is especially important for crops with slow growth rate such as oil palm. In addition, the system also allows for the detection of mRNA expression reflecting biological phenomena in minute amount of cells and also for accurate evaluation of essential but lowly expressed mRNA in transgenic plants.

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