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Molecular and Expression Analysis of Cowpea Trypsin Inhibitor (*CpTI*) Gene in Transgenic *Elaeis guineensis* Jacq Leaves

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Abstract

Cowpea trypsin inhibitor (CpTI) has been reported to have insecticidal properties against a wide range of insects. An 8-year-old insect resistant oil palm, *Elaeis guineensis* Jacq. variety Tenera assigned as CpTI P8 was the first transgenic oil palm tree generated by the co-transformation of pDM 402 and pCAMBIA 1301 plasmids into the immature embryos of the oil palm *via* particle bombardment. PCR analyses had confirmed that 100% of the tested CpTI P8 frond leaves and root tissue genomic samples showed the presence of the *CpTI* transgene. The partial pDM 402 expression cassette consisting of the actin promoter sequence (*Act1-5'*), the whole *CpTI* coding sequence and the *Nos-3'* terminator sequence was fully recovered from the CpTI P8 genome using the Act1F1/NosTR1 primer pair in subsequent PCR analysis. Moreover, Southern blotting analysis showed the presence of two hybridized fragments, 0.96 kb and 1.2 kb in size in the *Bam*HI-digested genomic DNA and also demonstrated the stable integration of the transgene into the CpTI P8 genome. The amplification of a 425 bp RT-PCR product using the *CpTI* gene-specific primer pair has clearly implicated the expression of CpTI transcript. Quantitative study on the expression of *CpTI* gene was carried out using SYBR Green Quantitative Real Time-Polymerase Chain Rection (QRT-PCR) assay and revealed a remarkably higher expression of CpTI in the CpTI P8 tree compared to non-transformed oil palm tree. Together, these data have proven the successful gene transfer and a 7-fold increase in CpTI transcript accumulation in the leaves of CpTI P8 tree which could ultimately confer greater resistance against insect pest invasion. Interestingly, the data in present study also suggest that a simple integration pattern contributes significantly to the stability and high transgene expression in transgenic plants.

Keywords: Bagworm, biolistic, insect resistance, leaf defoliation, Oil palm transformation, protease inhibitor

Abbreviations: CpTI_Cowpea Trypsin Inhibitor; CpTI P8_8-year-old transgenic oil palm, *Elaeis guineensis* Jacq. variety Tenera harboring *CpTI* transgene; MPOB_Malaysia Palm Oil Board; MYR_Malaysian Ringgit; QPCR_Quantitative Polymerase Chain Reaction; RT_Reverse Transcription

Introduction

The oil palm industry has continuously contributed to strong economic growth and is one of the most economically important crops in Malaysia. In 2008 alone, the total exports of oil palm products from Malaysia stood at 21.75 million tons and was worth 65.19 billion MYR or 18.11 billion USD (Malysian palm oil counsil, 2009). The oil palm is, therefore, the most valuable commercial crop in Malaysia. Currently, Malaysia is the largest exporting nation for palm oil in the world with, over 60% of the global share of palm oil, which is the second largest source of edible oil, after soybean oil (Basri et al., 2004). The oil palm industry is an important source of revenue for Malaysia. Thus, it is justifiable that significant emphasis should be given to all aspects pertaining to this industry, specifically the problems which can possibly affect the production of oil palm , such as insect invasion.

The oil palm industry in Malaysia is always threatened by the invasion of the bagworm (*Metisa plana* Walker), from the family Psychidae and Limacodidae larvae (*Darna diducta*). It is estimated that the invasion of insect pests has caused an approximately 20% reduction in the productivity of oil palm (Gatehouse et

al., 1992). The problem of insect invasions is tackled using chemical insecticides such as the use of trichlorfon to control the bagworm and Limacodidae larvae invasion (Khoo et al., 1991). Besides the spraying of insecticides through the air and soil, stem injection and biological control are also applied (Basri et al., 1988). However, these proved to be less efficient and have caused many undesirable side effects, including environmental pollution and the emergence of pesticide-resistant insects. In addition, these conventional methods take a longer time, are indirect and required a systematic method of selection (Koziel et al., 1993). Therefore, the generation of insect-resistant transgenic plants will be a better alternative to overcome this problem.

Genetic engineering is a technique that enables the transfer of target gene from various sources into the genome of plant or animal cells. The discovery of genes responsible for insect resistance has opened up the possibility of creating transgenic plants that are resistant to insect invasion. These genes can originate from either bacteria or higher order plant systems.

An endotoxin gene from *Bacillus thuringiensis,* which codes for the Bt toxin, has been frequently used to generate insect resistant plants. de Maagd et al. (1999) reported that transgenic crops carrying different types of Bt toxins in corn, tobacco, potato and rice are being developed and that some have been successfully generated at a commercial scale. However, the presence of these transgenic crops has increased the incidence of Bt toxin resistance in targeted insects (Heckel et al., 1999). In addition, the use of non-plant origin insecticidal genes in transgenic plants has raised concerns over the negative implications against other non-targeted insects (Hodgson, 1999; Wraight et al., 2000).

Naturally, plants have the ability to protect themselves from insect invasion via the synthesis of specific macromolecules, such as protease inhibitor (Gatehouse et al., 1980), α-amylase inhibitor (Ishimoto and Chrispeels, 1996), lectin (Gatehouse et al., 1991) and phenolic compounds. Plants also have the capacity to defend against pathogen infections by recognizing molecules produced by the attacking pathogen and mounting an effective defense response (Jones and Takemoto, 2004). Recently, Ismanizan et al. (2009) elucidated a higher *Elaeis oleifera* sesquiterpene synthase promoter activity, which is essential for the activation of plant natural defense mechanisms in the transgenic oil palm when it was subjected to elicitor induction.

The biotechnological potentials of natural protease inhibitors in conferring resistance against insects and other pests in plants have been reviewed (Mosolov and Valueva, 2008). Protease inhibitor is commonly present in plant storage organs such as seeds and tubers (Ryan and Walker-Simmons, 1981), does not inhibit plant endogenous protease and specifically acts against microorganism and animal enzymes (Boulter et al., 1989). The *CpTI* gene isolated from the cowpea plant (*Vigna unguiculata*) has been extensively used in the generation of insect resistant plants (Gatehouse et al., 1980; Gatehouse et al., 1991; Xu et al., 1996; Gatehouse et al., 1997; Golmirizaie et al., 1997; Graham et al., 1997; Ghoshal et al., 2001; Lv et al., 2005; Zhao et al., 2006). This is the first plant-originated insect resistance gene to be successfully transferred into other plants species (Hilder et al., 1987). CpTI is a member of the Bowman-Birk superfamily of protease inhibitors (Gatehouse et al., 1980) and possesses the insecticidal properties against the insect groups of Lepidoptera, Coleoptera and Orthoptera (Gatehouse et al., 1991).

Table 1. Sequences of primers used in the experiments.

Primer	Sequence 5' – 3'
CpTIF	TGA TGG TGC TAA AGG TGT GTG TGC TG
CpTIR	CAT CTT CAT CCC TGG ACT TGC AAG GT
Act1F1	CCC TCA GCA TTG TTC ATC GGT
NosTR1	AAT CAT CGC AAG ACC GGC AAC
NADF	TAG CCC GAC CGT AGT GAT GTT AA
NADR	ATC ACC GAA CCT GCA CTC AGG AA
CpTIF2	AAA GAG CAG TGA GAC TAA GCC AA
CpTIR	CAT CTT CAT CCC TGG ACT TGC AAG GT
rtNadF	TCG GGT CGT TTT ACT CTC TTT C
rtNadR	AGT ATG CGA TCC TAT CTG TGC
rtCpTIF	GGT GTG TGT GCT GGT ACT TTT C
rtCpTIR	GGC ATT GAG GAG GTA TTG ATT TAG

The CpTI protein functions to inhibit the insect proteases involved in digestive processes. Consequently, this will result in a depletion of essential amino acids required for growth, which in turn cause the larvae to die. The *CpTI* gene is heritable to the next generation (Hilder et al., 1990) and does not have negative effects against mammalians (Pusztai et al., 1992). Based on these discoveries, the *CpTI* gene was regarded as the most suitable candidate for the generation of insect-resistant transgenic plants.

This research focused on the expression analysis of the transgenic oil palm tree, which was labeled CpTI P8. The transgenic plant was generated through the co-transformation of pDM 402 and pCAMBIA 1301 plasmids into the genomic DNA of oil palm immature embryos by using the biolistic-mediated gene transfer approach (Leaw, 1998). CpTI P8 has been confirmed to be the first successful transgenic oil palm tree ever generated (Lee, 2000). Several bioassay analyses have also been conducted on the CpTI P8 tree since its development (Yap, 1999; Lim, 2002; Chari, 2003).

Bioassay analyses against bagworm larvae showed the differences in the level of leaf defoliation between control plants and transgenic oil palm trees that harbor the *CpTI* transgene (Yap, 1999). In addition, protease assays have indicated that proteolytic activities were higher in the rough extract samples of bagworms fed control leaves compared to extract samples of bagworms fed CpTI P8 leaves (Chari, 2003). Several bioassay analyses have demonstrated that the level of defoliation caused by bagworm and Limacodidae larvae in control leaves was worse than that in the leaves transformed with the *CpTI* gene (Chari, 2003).

In the generation of insect-resistant transgenic plants, the main factors that required major study are the integration patterns and the stable expression of the transgene, from the time when it was inserted into the genome of the host plant to the time when it is able to be inherited and function normally in subsequent generations. In addition, the transgene expression must

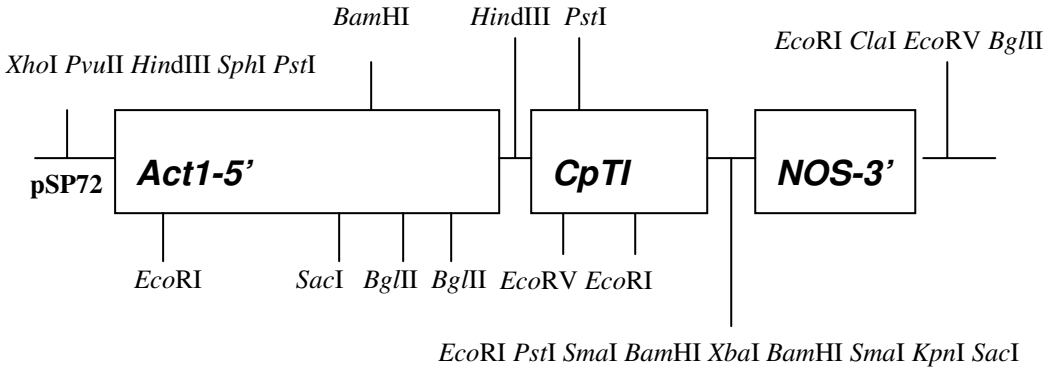

Fig 1. Schematic diagram of the transformation cassette of pDM 402 plasmid.

reach a certain level so that the desired traits can be observed in the transgenic plant. Therefore, the characterization of the *CpTI* transgene at the molecular level is crucial in order to evaluate the stability of transgene integration and expression. Most importantly, the novel findings in the expression analysis of the *CpTI* transgene in the 8-year-old CpTI P8 tree will provide new insights for the development and enhancement of insect resistant-transgenic plants.

Materials and methods

Starting material

These experiments were carried out at the Center for Plant Biotechnology, Institute of System Biology, Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor, Malaysia. The CpTI P8 oil palm tree (*Elaeis guineensis* Jacq. var Tenera) that had been transformed with the *CpTI* transgene was used in this study. This transgenic plant was generated *via* the co-transformation of pDM 402 and pCAMBIA 1301 plasmids into the immature embryo tissues of the oil palm (Leaw, 1998) using the particle bombardment method.

Vector construct

The pDM 402 plasmid was provided by Cornell University, Ithaca, USA (a gift from Dr. Ray Wu). This plasmid was designated as transformation construct with a size of 4.56 kb and contains a 441 bp *CpTI* gene, which was placed under the control of the actin promoter (*Act1-5'*) and flanked by a 260 bp 3' *Nos* terminator gene (Fig. 1). The backbone of this plasmid was derived from the pSP72 plasmid (Promega, USA), and it also harbors the selection marker for the *E. coli* ampicillin resistance gene.

DNA extraction

Preliminary analyses of DNA from the transgenic oil palm were carried out using genomic DNA samples extracted from approximately 1 g of CpTI P8 leaf and root tissues, as previously described by Doyle and Doyle (1990).

Polymerase Chain Reaction analysis

Gene-specific PCR primers for *CpTI* and *Act-1/nos* were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by the First Base Malaysia Sdn Bhd. As an internal control for DNA quality, the *nad5* gene was amplified in separate reactions. Amplification of the *CpTI* gene was carried out using the primer pair of CpTIF/CpTIR (Table 1), which amplifies an expected fragment size of 315 bp. For *Act-1/nos* analysis, the primers used were Act1F1/NosTR1 (Table 1), which amplifies an expected fragment size of 690 bp. For the *nad5* gene amplification, the primer pair of NADF/ NADR (Table 1) was used and is expected to amplify a fragment of 832 bp. PCR reactions (25 µL) were set up using 200 ng of DNA extract, 1 µM of each primer, 50 mM KCl, 10 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega, USA) and 0.2 U of Taq DNA polymerase (1U/µL) (Biotools, Spain). Temperature profiles (for all the evaluated genes) consisted of 94°C for 45 s, 58°C for 45 s, and 72°C for 2 min, applied for 30 cycles, with an additional extension step at 72°C for 5 min. Amplified DNA samples were size separated by conventional electrophoresis using 1% (w/v) agarose gels and ethidium-bromide staining (Sambrook and Russell, 2001).

Southern blotting analysis

Genomic DNA (30 µg) was digested with *Bam*HI (New England Biolabs, Ipswich, MA, USA), electrophoresed on a 1% (w/v) agarose gel, and then transferred to a positively charged nylon membrane (Amersham Bioscience, UK). The membrane was hybridized with α^{32}P-dCTP (Amersham, Bioscience, UK) radioisotope labeled probe of 690 bp PCR products that were amplified using the Act1F1/NosTR1 primer pair (Table 1). The membrane was washed, partially air-dried, wrapped in plastic wrap and exposed to X-ray film for 16 h at -70°C.

RNA extraction

Total RNA was prepared from leaf and root tissues of CPTI P8 using a modified phenol-SDS technique (Ausubel et al., 1987). After extraction, RNA was

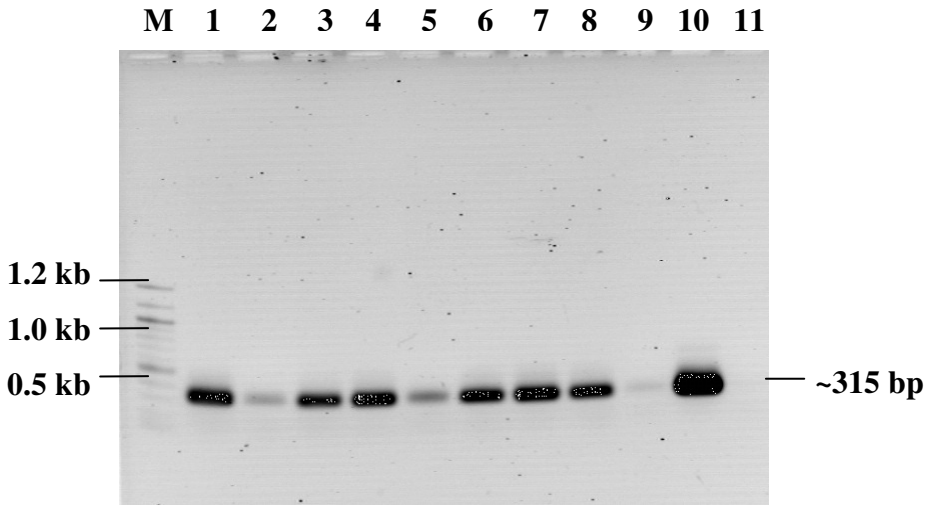

Fig 2. PCR amplification of CpTI gene in the CpTI P8 genome. CpTI gene was presence in the genome of every tested frond leaves and root samples. Lanes 1-8: CpTI P8 fronds and root genomic DNA; Lane 9: negative control (genomic DNA of non-transformed oil palm tree); Lane 10: positive control (pDM 402); Lane 11: negative control (without template); M: 100 bp DNA marker.

treated with DNase I (RNase-Free) (New England Biolabs Inc., Ipswich, MA, USA) at 10 U/ l g of total RNA, as recommended by manufacturer. The quality of RNA extraction was determined by the OD_{260} and OD_{280} reading and formaldehyde agarose gel electrophoresis.

Qualitative expression analysis: reverse transcription-PCR (RT-PCR)

Reverse transcription reactions were run with 2 µg of RNA as the template from which single-stranded cDNA was synthesized using the M-MuVL Revert-Aid™ reverse transcriptase (Promega, Madison, WI, USA) and 1 µL of oligo-dT primer (Invitrogen, Breda, The Netherlands), as described in the manufac- turer's protocol. Ten percent of the final volumes generated in the single-stranded cDNA synthesis reactions were used for the detection of CpTI trans- cripts by PCR amplification using the CpTIF/CpTIR and CpTIF2/ CpTIR (Table 1) primer pairs, with the expected PCR product sizes of 315 bp and 425 bp respectively.

Quantitative expression analysis: real-time quantitative PCR

Real-time qPCR primers were designed using the primer designing program, Beacon Designer 4 (Premier Biosoft International, Palo Alto, CA). Two sets of primer pairs, rtNadF/rtNadR and rtCpTIF/rtCpTIR (Table 1) were designed for the *nad5* and *CpTI* genes, respectively. The expected size of the PCR product for the *nad5* gene amplification is 174 bp, whereas the expected size for the *CpTI* gene is 165 bp.

Single-stranded cDNAs have been used as templates for real-time RT-PCR reactions. One tenth of the total volume of the PCR mix was taken from the reverse transcription product and was added to the PCR mixture that contained 1 X Brilliant® SYBR® Green qPCR Master Mix (Strategene, USA), 100 nM forward primer, 100 nM reverse primer and PCR-grade distilled water. PCR conditions for amplifying DNA from the *nad5* and *CpTI* genes were 95°C for 10 min to activate the DNA polymerase, 95°C for 30 s for denaturation, 60°C for 30 s for annealing, 68°C for 30 s for elongation. This cycle was repeated 40 times. Each DNA sample was analyzed two times in separate reactions and the average of the three replicates was used for analysis. The expression level of the *CpTI* gene was normalized to the Nad5 transcripts expression level, which acted as the standard internal control.

Results and discussion

Internal control

PCR amplification of endogenous genes was conducted to ensure the integrity and purity of the isolated genomic DNA before amplification of the target transgene was performed. Amplification of endogenous genes is used as an internal control for PCR analysis of transgenes. It is aimed at eliminating the possibility of inefficient transgene amplification resulting from impure DNA samples rather than the absence of target transgene (Mannerlöf and Tenning, 1997). To serve these purposes, the *nad5* gene (NADH dehydrogenase) from *Beta vulgaris* mitochondria (Ecke et al., 1990); *Vicia faba* (Scheepers et al., 1997) and *Taxus canadensis* (Jaramillo-Correa et al., 2002) have been used as internal controls because they exist naturally in the plant cells and are involved in the mitochondrial respiratory pathway.

Amplification of the *nad*5 gene was performed with the NADF/NADR primer pairs, (Table 1). Genomic DNA isolated from the CpTI P8 and the non-transformed oil palm trees was used as the template. The resultant PCR amplification of an expected band size of 832 bp (data not shown), which corresponds to the presence of the *nad5* gene, has confirmed the purity of the extracted genomic DNA. Thus, this extracted DNA sample could subsequently be used for the detection of *CpTI* gene *via* PCR analysis.

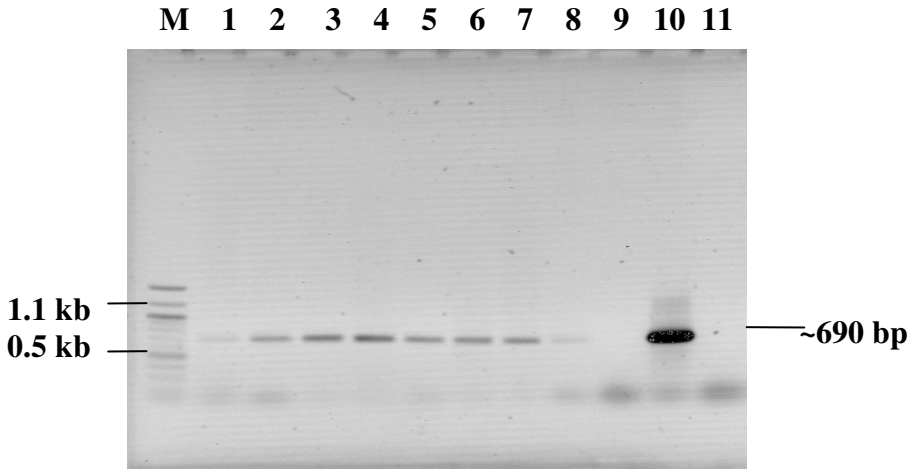

Fig 3. PCR amplification of Act1-5'-CpTI-NOS-3' fragment by using Act1F1/NosTR1 primer pair. lanes 1-7: CpTI P8 leaves genomic DNA; Lane 8: CpTI P8 root genomic DNA; Lane 9: negative control (genomic DNA of non-transformed oil palm tree); Lane 10: positive control (pDM 402); Lane 11: negative control (without template); M: 100 bp DNA marker.

PCR analysis for the CpTI gene

The presence of the *CpTI* gene in every tested genomic DNA sample from frond leaves and root tissues of CpTI P8 plants was verified by PCR analysis. Two sets of primer pairs, CpTIF/CpTIR and Act1F1/NosTR1 (Table 1), were used in the analysis. The CpTIF/CpTIR primer pair was designed based on the *CpTI* gene sequence in the pDM 402 plasmid. This pair of primers was utilized to amplify a DNA fragment of 315 bp in the genomic DNA sample from the CpTI P8 tree. Meanwhile, the genomic DNA sample from the non-transformed oil palm tree was set as the negative control in the PCR amplification.

Figure 2 shows the PCR amplification of leaf and root genomic DNA samples, with an expected band size of 315 bp, when the PCR products were electrophoresed in 1% (w/v) agarose gel. The template for the positive control, pDM 402 plasmid gave a positive result, whereas no band was detected for the negative control (without template). A negative control for the DNA sample from the non-transformed oil palm tree showed the presence of a faint band with a size of 315 bp. This may due to the presence of DNA sequences in the oil palm genome that can produce the PCR product.

The positive results of PCR analyses confirmed the stable integration of the *CpTI* gene into the genome of CpTI P8 during the transformation event conducted in eight years ago. The oil palm tree is a monocotyledonous plant, and, therefore, each part of the plant is derived from the same meristematic cell. Hence, transformation of a single immature embryo tissue will give rise to a uniformly transformed oil palm tree.

PCR and Southern blotting analysis of the Act1-5'-CpTI-NOS-3' fragment

The presence of the CpTI expression cassette in the genome of CpTI P8 was verified through PCR amplification. The primer pair of Act1F1/NosTR1 was designed based on the transformation cassette of CpTI (Fig. 1) and was used to amplify the Act1-5'-CpTI-NOS-3' fragment with an expected size of 690 bp, from the isolated genomic DNA. This DNA fragment comprises a partial actin promoter (*Act1-5'*) sequence, a *Nos* termination sequence and the whole coding sequence of the *CpTI* gene from the pDM 402 plasmid.

In the PCR analysis, the genomic DNA from the CpTI P8 frond leaves and root samples were used as a template, whereas the genomic DNA sample from the non-transformed oil palm tree was used as the negative control. Resultant amplification of the CpTI P8 genomic DNA samples produced a DNA fragment with an expected band size of 690 bp (Fig. 3). In contrast, no fragment was observed in the resultant amplification of the non-transformed oil palm DNA sample. This is because none of the expression cassette, which contains the Act1-5'-CpTI-NOS-3' fragment, was transformed into the control plant. Moreover, it is impossible for the non-transformed oil palm genome to have the *Act1* intron 1 sequence which is part of the Act1-5'-CpTI-NOS-3' fragment.

The successful amplification of the 690 bp Act-5'-CpTI-NOS-3' fragment in the CpTI P8 genomic sample and the resultant negative amplification of the non-transformed oil palm genomic sample have proven that the whole expression cassette of the *CpTI* gene was stably transferred into the genome of CpTI P8 without truncation. This type of integration pattern is required for the stable expression of the transgene and to ensure its heritability to subsequent generations. Furthermore, the *Act1* intron 1 sequence was present in the amplified 690 bp PCR product following nucleotide sequence analysis. This result was in line with subsequent Southern blotting analysis, where the Act1-5'-CpTI-NOS-3' fragment was made as a probe.

The *Bam*HI restriction enzyme was chosen and used for the CpTI P8 genomic DNA digestion (Fig. 4a). The Act1-5'-CpTI-NOS-3' expression cassette was restricted at two sites by enzyme and produced an internal hybridization fragment with a size of 0.96 kb, containing a partial *Act1-5'* sequence and the whole *CpTI* gene. This internal fragment was used as a probe to identify the integration of the Act1-5'-CpTI-NOS-3'

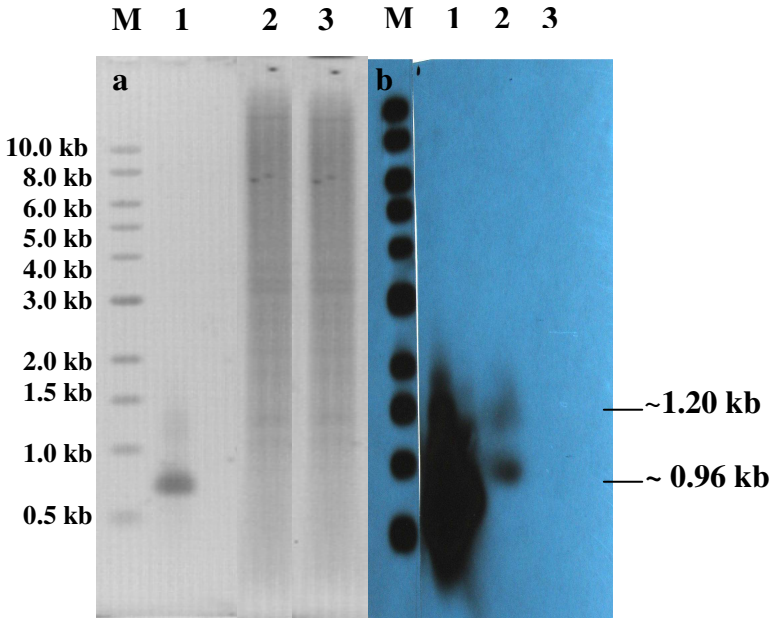

Fig 4. Southern blotting analysis on the isolated genomic DNA samples. **a** BamHI-digested genomic DNA of CpTI P8 and the non-transformed oil palm tissues. lane 1: positive control; Lane 2: digested CpTI P8 genomic DNA; Lane 3: digested non-transformed oil palm genomic DNA; M: 1 kb DNA marker. **b** Autoradiogram generated from Southern hybridization. No hybridization band was detected in the non-transformed oil palm genome sample.

fragment in the CpTI P8 genome. The resultant hybridization indicated the presence of two hybridization bands that each corresponded to a size of 0.96 kb and 1.2 kb, respectively, on the *Bam*HI-digested CpTI P8 genomic DNA (Fig. 4b). The whole expression cassette was found to be stably inserted into the genomic DNA. In contrast, none of the digested genomic DNA of the non-transformed oil palm tree produced a hybridization band.

The amplified internal fragment, with an expected size of 0.96 kb, consisted of the 3' end of the actin promoter sequence and the full coding region of the *CpTI* gene. This further elucidates our finding that the integration of the Act1-5'-CpTI-NOS-3' fragment into the CpTI P8 genome was stable and without any truncation or deletion process during the transformation event. The occurrence of another hybridization band of 1.2 kb might suggest the presence of a sequence that contains the additional *Nos-3'* sequence and a partial genomic DNA sequence flanked by a *Bam*HI restriction site. This 1.2 kb hybridization fragment could be the result of a possible transgene rearrangement in the CpTI P8 genome. Similar integration patterns have been previously reported (Pawlowski and Somers, 1998; Lee et al., 1999; Fu et al., 2000).

The integration pattern of a transgene into the plant genome *via* the direct gene transfer method is difficult to predict. The complex nature of transgene insertion structures, such as the inverted repeat, concatamer, truncation (Smith et al., 2001; Bubner and Baldwin, 2004), rearrangement, tandem repeat and interspersed forms, has frequently occurred during transformation events (Kohli et al., 1998; Kohli et al., 1999; Kohli et al., 2003). Characterization of transgene locus organization is essential gaining insights into the mechanisms of transgene integration into the host genome. The stability of transgene integration and the hereditary of the integrated transgene are very dependent on the transgene locus organization. Thus, many studies are actively being carried out to understand the mechanisms of transgene integration and the relationship between the transgene locus structure and its expression in transgenic plants (Kohli et al., 1998; Kohli et al., 2003).

The structure and organization of the transgene can also be studied using fluorescent in-situ hybridization (FISH) analysis (Abranches et al., 2000; Jackson et al., 2001) in addition to the conventional Southern blotting analysis. Furthemore, the locus on integration site could be easily isolated by performing numerous molecular-based techniques, including inverse PCR (Fu et al., 2000), TAIL-PCR (Liu et al., 1995), long range PCR (Kohli et al., 1998; Hernández et al., 2003), cytogenetic analysis and 5' nuclease real-time Taqman QRT-PCR (Terry and Harris, 2001; Holck et al., 2002; Hernández et al., 2003).

Southern blotting analysis has verified the insertion and the presence of only one to two integration sites of the *CpTI* gene in the CpTI P8 genome. Our findings are supported by the hypothesis that states that at low copy number of a transgene (one to three) or a simple integration pattern will usually contributes to the stable expression of the transgene in the host plant (Agrawal et al., 2005). The simple integration of the *CpTI* transgene has also been previously documented by Newell et al., (1995).

Qualitative analysis of CpTI gene expression

The presence of CpTI transcripts in the leaf and root tissues of CpTI P8 was validated using RT-PCR analy-

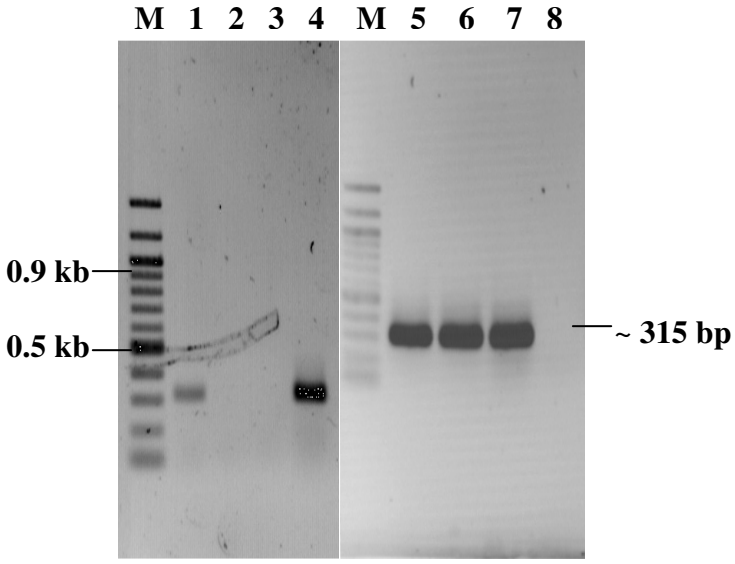

Fig 5. RT-PCR amplification of CpTI transcripts by using CpTIF/CpTIR primer pair. Lanes 1,4,5-7: The amplified 315 bp RT-PCR product from the cDNA samples of CpTI P8 leaf and root tissues; Lane 8: negative control (without template); Lane 2: control for non-RT leaf samples; Lane 3: control for non-RT root samples; M: 100 bp DNA marker

sis. The *CpTI* gene is regulated by a constitutive actin promoter, and, accordingly, the gene mRNA transcripts were constitutively expressed in all the transformed tissues of CpTI P8. Two sets of primer pairs, CpTIF/CpTIR and CpTIF2/CpTIR (Table 1) were used in the RT-PCR analysis, and they produced the expected band sizes of 315 bp and 425 bp respectively.

Both RT-PCR and non RT-PCR were simultaneously run on the RNA samples of CpTI P8 leaf and root tissues using the CpTIF/CpTIR primer pair for the detection of CpTI transcripts. All RNA samples from CpTI P8 showed positive RT-PCR results and produced a single DNA fragment with an expected size of 315 bp (Fig. 5). This indicates the presence of the 315 bp CpTI transcripts in CpTI P8 tissues. In contrast, none of the controls for the non-RT samples produced any DNA fragments. These results coincided with the fact that both positive RT-PCR amplifications must be derived from the cDNA template of the CpTI P8 leaf and root RNA samples and not from genomic DNA samples.

In the RT-PCR amplification using the CpTIF2/CpTIR primer pair, a similar positive RT-PCR amplification was obtained for the RNA samples from CpTI P8 leaf and root tissues. The production of a 425 bp DNA fragment (Fig. 6) demonstrated the presence of CpTI transcripts in the CpTI P8 tree. Nevertheless, there was unexpected amplification of this 425 bp DNA fragment in the RNA samples of the non-transformed oil palm tree and this might imply the presence of endogenous transcripts that resemble the *CpTI* gene. This result has been verified in the nucleotide sequence analysis and will be clarified further in the following discussion.

Quantitative analysis of CpTI gene expression

In this study, the relative quantification of CpTI transcripts was conducted for different tissues of the CpTI P8 tree and the non-transformed oil palm tree (control) and was compared to the Nad5 transcripts. The same amount of relative template was used in this experiment. Both the amplification of the *nad5* gene (endogenous gene) and the *CpTI* gene (targeted gene) were performed simultaneously in the same experiment by using a set of different tubes so that the changes in *CpTI* gene expression normalized to *nad5* gene expression could be observed instantaneously.

The cDNA samples from the non-transformed oil palm tree were used for calibration. This calibrator sample was a reference point for the relative quantification of the *CpTI* gene expression level, which had been normalized to Nad5 transcripts. The calibrator value was set at 1. Therefore, the level of *CpTI* gene expression in each of the tested samples is stated as a comparison to the calibrator value. The endogenous *nad5* gene expression was used for normalization of the quantitative PCR (qPCR) reaction based on the amount of cDNA increments.

All qPCR reactions were prepared in three replicates to reduce the intra-sample variation (coefficient of variation, CV) and to ensure the reproducibility of real-time qPCR results. The percentage of CV obtained from the relative quantification study was less than 2.5%. Most of the qPCR reactions had a small standard deviation (SD) value, which was less than 0.4, as shown in the error bar in Fig. 7 and Fig. 8. Both the calculated CV and SD values demonstrated a high accuracy and reproducibility in real-time qPCR results obtained in this study.

The changes in threshold cycle (ΔCt) between the *CpTI* gene and the *nad5* gene in different tissue samples were compared against the calibrator tissue sample by using the Mx-Pro™ qPCR (Stratagene, USA) program. Thus, the collected data were displayed as the number of fold changes in *CpTI* gene expression that had been normalized to *nad5* gene expression and were relative to the calibrator value. The number of fold changes in the CpTI expression and its relativity against the calibrator value are shown in two charts (Fig. 7 and Fig. 8).

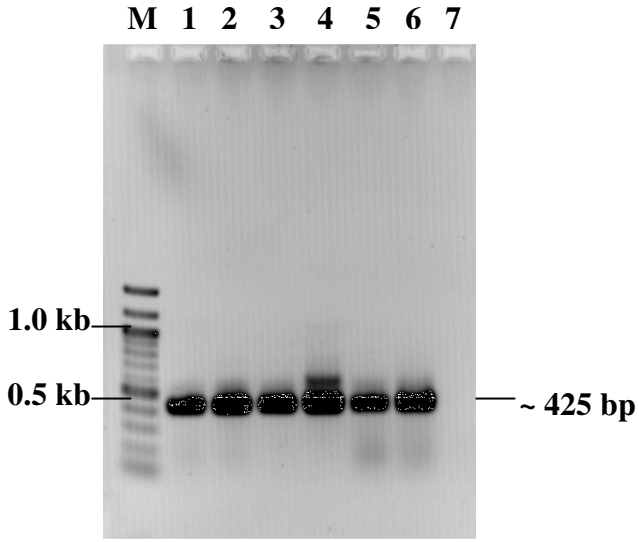

Fig 6. RT-PCR amplification of CpTI transcripts by using CpTIF2/CpTIR primer pair. Lane 1: The amplified 425 bp RT-PCR product from the cDNA samples of non-transformed oil palm tissues; Lanes 2-6: The amplified 425 bp RT-PCR product from the cDNA samples of CpTI P8 leaf and root tissues; Lane 7: negative control (without template); M: 100 bp DNA marker

Figure 7 shows that the expression of the *CpTI* gene in the CpTI P8 leaves was 7.46-fold higher compared to the calibrator tissue sample. Meanwhile, the expression level of the *CpTI* gene in the CpTI P8 roots was 0.846-fold relative to the calibrator tissue sample. These results indicate that the expression of CpTI transcripts occurred at different level in various CpTI P8 tissues. Data showed the presence of CpTI expression at 1.09-fold in the leaves of control plants, which may due to the expression of endogenous genes in the control plant that resembles the *CpTI* gene. Confirmation of endogenous gene expression that resembles the *CpTI* gene can only be done if the completed gene sequence was successfully isolated. Nonetheless, no studies thus far have documented the isolation of genes coding for trypsin or serine protease inhibitor from oil palm.

The nucleotide sequence of the RT-PCR product amplified by the primer pair CpTIF2/CpTIR in the RNA sample from the non-transformed oil palm tree (control) showed a 98% similarity to the nucleotide sequence of *Vigna unguiculata* fIV trypsin inhibitor RNA (GenBank accession: X51617). These results indicate the presence of a putative serine protease inhibitor or transcript that closely resembles the oil palm trypsin inhibitor. The discovery of expressed sequence tag (EST) clones encoding for a putative oil palm Bowman-Birk serine protease inhibitor (MPOB accession no. E0004917 and E0005950) in the MPOB PalmGenes database (2009) is consistent with our findings in this study. However, the gene which codes for the putative serine protease inhibitor is expressed at low level in the control plant (Fig. 8).

The relative quantification data of the CpTI expression level is shown in the log-fold-change difference in Fig. 8. The fold-change difference was used to determine whether the *CpTI* gene expression was up-regulated or down-regulated in different tissues of transgenic oil palms. The expression of the *CpTI* gene was up-regulated in the leaves and was down-regulated in the roots of CpTI P8 when both samples were compared to the calibrator value. There were differences in the CpTI expression level in different tissues of CpTI P8, notwithstanding the same constitutive *Act1* promoter sequence that regulates its expression.

Differences in the CpTI expression level may be due to the influence of other factors. Several reports had identified the expression of protease inhibitor and/or trypsin inhibitor induced in vegetative tissues such as leaves, in response to mechanical wounding (McGurl et al., 1995; Tamayo et al., 2000; Qu et al., 2003), and these were related to increased resistance against insect invasion in plants (Koiwa et al., 1997; Stotz et al., 1999; Ussuf et al., 2001). High levels of protease inhibitor accumulation was discovered in the leaves of tomato, potato and tobacco plants following wounds infliction (Green and Ryan, 1972) and pathogen infections (Geoffroy et al., 1990). In order to comprehend the effect of Lepidoptera insect invasion of the CpTI P8 tree, studies on *CpTI* transgene expression induced by *Metisa plana* Walker invasion at different periods of time were simultaneously conducted by other researchers.

To analyse the expression of an endogenous gene that closely resembles the *CpTI* gene, the relative quantification experiment was conducted using the samples from control plant. As seen in Figure 7, the rise of the CpTI expression level was 7-fold higher in CpTI P8 compared to control plants. The expression of CpTI in transgenic oil palm was regulated by the constitutive actin promoter (*Act1*), which is able to produce a high level of CpTI transcript accumulation.

Act1-based expression vectors contain the *Act1* intron 1 promoter sequence that is able to elevate the expression level of foreign genes in most of the transformed monocotyledonous cells (McElroy et al., 1991). High levels of CpTI protein accumulation and an enhanced resistance to insects in transgenic rice has been reported by Xu et al. (1996). Similar patterns of potato proteinase inhibitor (PIN II) protein accumula-

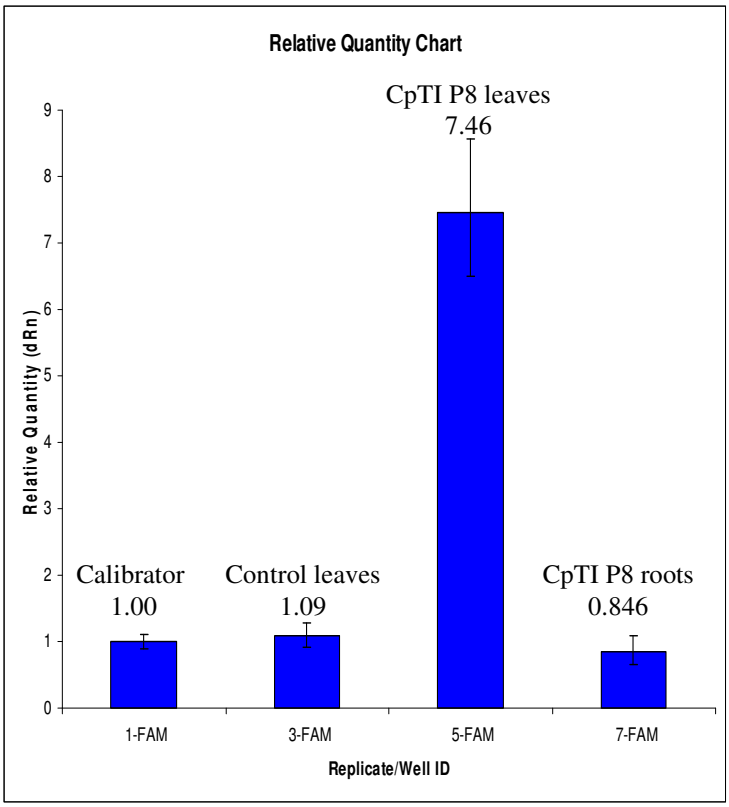

Fig 7. Relative quantification data for normalized CpTI expression level in different tissues of CpTI P8 compared to the non-transformed oil palm tree (control). The data shows the fold-change differences in CpTI expression level compared to the calibrator value which has been set at 1.

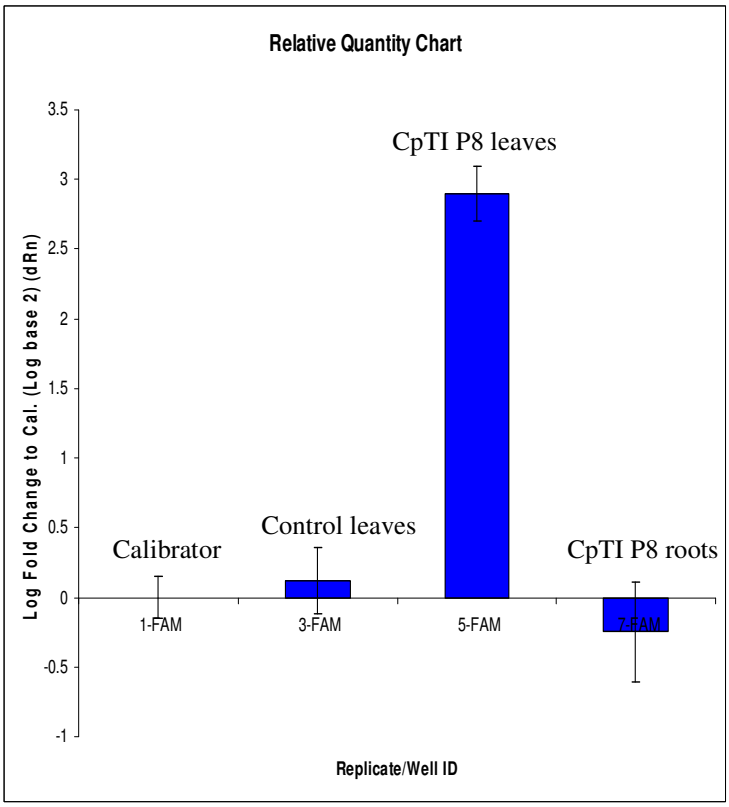

Fig 8. Relative quantification data for normalized CpTI expression level in different tissues of CpTI P8 compared to the non-transformed oil palm tree (control). The data shows the log-fold-change differences in CpTI expression level compared to the calibrator value.

tion in transgenic rice has also been documented by Duan et al. (1996) when the experiment was conducted using the *Act1*-based expression vector.

The relative quantification results clearly demonstrate that the expression level of the *CpTI* gene was 7-fold higher in the leaves of CpTI P8 compared to the non-transformed oil palm tree. This provides evidence that underpins the successful transfer of the *CpTI* gene into the targeted leaf tissues of CpTI P8 tree, which are vulnerable to Lepidoptera insect invasion. The high level of CpTI transcript accumulation in the leaves is believed to reduce the impact of CpTI P8 leaf defoliation and to ultimately confer resistant against bagworm insect invasion (specifically the *Metisa plana* walker). It is interesting to note that the increased expression level of the *CpTI* gene in transgenic plants has led to greater resistance against insect pests, including those from the orders of Coleoptera, Orthoptera and Lepidoptera (Gatehouse et al., 1991). In-depth bioassay analyses have shown that the level of leaf defoliation resulting from bagworm invasion was strikingly higher in control plants than in transgenic oil palm trees, which harbor the *CpTI* gene.

The findings presented in this paper have augmented our understanding of the molecular basis of insect-resistant plant development. In addition, this study has shed light on the importance of a simple integration pattern for stability and higher CpTI transcript expression levels in conferring resistance against major insect invasion from *Metisa plana* walker, in the transgenic oil palm leaves. Nevertheless, the impact of the CpTI P8 tree on the environment and other non-targeted insects will need to be thoroughly explored and evaluated in subsequent experiments and field trials before the concept of the insect-resistant transgenic plant reported in this article could be adopted for future commercial use in the oil palm industry.

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