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Original Article

Molecular cloning and expression of *EgTCTP*, encoding a calcium binding protein, enhances the growth of callus in oil palm (*Elaeis guineensis*, Jacq)

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Abstract

The translationally controlled tumor protein (TCTP) has now been identified in evolutionarily diverse organisms and is thought to play an important role in cell growth and cell division. We have identified an *EgTCTP* gene from *Elaeis guineensis* Jacq. It is a putative protein of 168 amino acids with a calculated molecular mass of 19.2 kDa. EgTCTP has a high homology (84% - 91% identity) at the amino acid level to other plant TCTPs from *Hevea brasiliensis*, *Arachis hypogaea* and *Glycine max*. The recombinant EgTCTP protein is a calcium binding protein. Transgenic embryonic calli overexpressing *EgTCTP* have a faster growth rate than non-transformed and empty vector transformed calli. The results show that the enhancement of *EgTCTP* gene expression in oil palm embryogenic calli may result in faster multiplication of the embryogenic calli. EgTCTP acts as another Ca^{2+}-modulated protein that is involved in the cell cycle progression.

Keywords: *Elaeis guineensis* Jacq, oil palm, transgenic, cell division, growth rate, embryogenic, translationally controlled tumor protein, real-time PCR

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a monocotyledonous perennial plant and is the highest yielding oil-producing crop in the world. Palm oil has multiple uses, including the production of cooking oils, margarines, soaps, detergents and biodiesel. Any improvement of oil yield and quality through genetic modification is desirable in order to meet the ever-increasing global demands. The conventional breeding of oil palm is a low-efficiency and time-consuming process due to the long reproductive cycle of the trees and the slow maturation of the seeds (Mayes *et al.,* 2000). Approaches using modern biotechnology, such as tissue culture, genetic transformation and marker-aided selection, may be a faster way to continue to improve the commercial value and productivity of oil palm (de Touchet *et al.,* 1991; Chowdhury *et al.,* 1997; Mayes *et al.,* 2000).

The translationally controlled tumor protein (TCTP) has been identified as a growth- and development-related protein encoded by abundant mRNA species (Yenofsky *et al.,* 1982; Chitpatima *et al.,* 1988). Despite being implicated in various functions, and its wide range distribution and high level of conservation among many organisms, the true function of TCTP still remains unclear (Kim *et al.,* 2000; Gnanasekar, *et al.,* 2002; Yan *et al.,* 2000; Bommer and Thiele, 2004).

In plants, TCTP was first identified in rapidly dividing pea root cap cells where it interacted specifically with an active *Pisum sativum* small GTPase (*pra3*) (Nagano *et al.,* 1995; Woo and Hawes, 1997; Kang *et al.,* 2003). It may be an important gene for the formation of storage roots in cassava (*Manihot esculentan* Crantz) (de Souza *et al.,* 2004). TCTP transcripts were also the most abundant sequences found in a cDNA library from the developing castor endosperm (Lu *et*

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al., 2007). Knockdown of *Arabidopsis* TCTP led to impaired pollen tube growth and root hair development, slowed vegetative growth and the plant produced dwarf leaves (Berkowitz *et al.,* 2008). The silencing of cabbage (*Brassica oleracea* L.) BoTCTP by RNA interference slowed the growth of the plant (Cao *et al.,* 2010).

Based on the above studies, there is every indication that TCTP has a growth related function in plants. We are therefore interested in attempting to manipulate the oil palm growth rate via genetic modification, of the TCTP target gene. This study reports the first cloning and characterisation of an *EgTCTP* gene from *E. guineensis* and the transformation of the putative gene into a callus using the micro-projectile bombardment method (biolistics™). The expression of this putative gene was controlled by a Cauliflower Mosaic Virus (CaMV35S) promoter and the transformants were observed for their growth characteristics**.** This work is a crucial first step in the application of advanced technology to control oil palm growth and to also validate the potential function of EgTCTP in oil palm and other monocotyledonous plants for the future.

2. Materials and Methods

2.1 Plant materials

In this study, the tenera varieties of *E. guineensis* were used as the source of the embryos to produce callus. The oil palm fruits were harvested 12 weeks after anthesis (waa) by detaching them from the spikelet stalk, then sterilised by washing in a solution of soap and Clorox, soaked in absolute ethanol for 1-hrs, and air-dried in a laminar flow chamber. The sterilised fruits were halved and the embryos were extracted. In order to establish callus cultures, these embryos were cultured on MS (Murashige and Skoog, 1962) media supplemented with 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The cultures were incubated at 25±2 °C in a culture room with a 16-hrs photoperiod and subcultured onto fresh media every month. After one month of culture, small calli were observed. However, the zygotic embryos were eliminated after the callus induction step. The small calli were maintained in the same media until embryogenic calli were formed within four to eight weeks, and then used for transgenic studies.

2.2 Isolation of RNA and cloning of *EgTCTP* from *E. guineensis* by reverse transcription (RT-PCR)

Total RNA was isolated from 100 mg of young *E. guineensis* leaves by extraction with the RNeasy extraction Kit (Qiagen). Fragments of *EgTCTP* were cloned using an RT-PCR reaction mixture, according to the manufacturer's instructions (Qiagen). A degenerate primer set was designed from the plant TCTP according to the conserved amino acids of the N-terminal, MLVYQDL. The specific primers used are as follows: the TCTP forward primer was 5′-ATG TTG GTB TAY CAR GAT CTT-3′ and the reverse primer was the oligo-dT primer (Invitrogen). An *18S* rDNA primer set was used for an internal marker in the RT-PCR experiments. Protein alignments were performed using ClustalX 2.0 and GENEDOC software (Larkin *et al.,* 2007) and the alignment was used as an input file to the PHYLIP 3.57c package. The phylogenetic analysis, performed by distance matrix, was computed by PRODIST and NEIGHBOR and used to construct trees with support for the inferred groups obtained by bootstrap analysis from 1000 replications of the data set using the SEQBOOT and CONSENSE programs. The phylogenetic tree was displayed using TreeView version 1.0 (Felsenstein, 1997). The search for different motifs in EgTCTP was performed with the Prosites software (see http://www.expasy.ch/tools / scnpsit1.html).

2.3 Expression and purification of recombinant EgTCTP in *E. coli* M15

The open reading frame (ORF) of *EgTCTP* was amplified by PCR using specific primers that corresponded to the beginning of the ORF of *EgTCTP*, with the addition of an upstream in-frame *Bam*HI restriction site (5′- CGG GAT CCA TGT TGG TTT ATC AGG -3′). The reverse primer corresponding to the 3′ end of the coding region was flanked by a *Pst* I restriction site (5′- GCC TGC AGT TAA CAC TTG ATC TCC -3′). The PCR products were cloned, in-frame, with 6 x Histidine tags into the *Bam*HI and *Pst* I restriction sites of pQE40 (Qiagen), in order to obtain pQE-*EgTCTP*, and were then transformed into the *E. coli* strain M15 (pRep4). The *E. coli* strain M15 (pRep4), harboring the recombinant DNA, was grown and induced by 0.1 mM IPTG to produce the recombinant protein. The histidine-tagged fusion protein was purified by Ni-NTA column chromatography and was analysed for purity on 12.5% SDS-PAGE gels.

2.4 ^{45}Calcium-overlay assay

The calcium overlay assay was performed according to Garrigos *et al.* (1991). The purified recombinant EgTCTP protein, a positive control protein (porcine tropomyosin, Sigma) and two negative control proteins (bovine serum albumin and non-calcium binding recombinant protein) were separated by 12.5% SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was briefly rinsed with assay buffer (60 mM KCl, 5 mM $MgCl_2$, 10 mM immidazole, pH 6.8) and then incubated with the same buffer containing 40 µCi/ml $^{45}CaCl_2$ (Amersham Bioscience, USA) for 1-hrs at room temperature with gentle agitation. The radioactive solution was removed, and the membrane was washed in assay buffer or 50% ethanol for 20 min, completely air-dried, and subsequently placed into a film cassette with an autoradiography film (X-ray film from Kodak, USA). The radioactive associated protein signal was developed using a developing machine (Kodak, USA).

2.5 Construction of recombinant TCTP for plant transformation

The forward PCR primer corresponding to the beginning of the ORF with the addition of an upstream in-frame *Nco* I restriction site (5′-CCA TGG TAA TGT TGG TTT ATC AGG ATC TTC-3′), and the reverse primer corresponding to the 3′ end of the coding region flanked by a *Bgl* II restriction site (5′-AGA TCT ACA CAC TTG ATC TCC TTC AGC CCA-3′), were used to amplify *EgTCTP*. The PCR products were ligated to a similarly digested pCAMBIA 1302 (www.cambia.com.au), carrying the *GFP* reporter gene controlled by a Cauliflower Mosaic Virus (CaMV35S) promoter. The inserted DNA fragments were sequenced using the 3730 DNA sequencer to ensure the authenticity of the cloned nucleotide sequence.

2.6 Plant transformation

The calli, approximately 3 mm in size and at least two months old, were selected from the same callus line and bombarded using the PDS-1000/He particle delivery system (BioRad), according to the optimal conditions that were modified from Ahlandsberg *et al.* (2001). The conditions for biolistic-mediated gene transfer for oil palm were optimized using callus and the pressure of 1100 psi was found to be best for bombardment and the frequency of transient transformation. Bombardments without DNA and with an empty vector were used as experimental controls. After bombardment, the calli were selected for transformants in media containing hygromycin (50 mg/ml). The expression of the *GFP* gene in transgenic calli were visualized with a confocal laser scanning microscope (FV300, Olympus) fitted with a GFP filter set for excitation between 455 nm and 490 nm and emission above 543 nm (Kanchanapoom *et al.,* 2008).

2.7 Histological analysis

For histological studies, the control and bombarded calli at the same development stages were fixed in a Navashin solution containing 90 ml of 70% ethyl alcohol, 5 ml of glacial acetic acid and 5 ml of formalin solution. These tissues were dehydrated through an ethanol-tertiary butanol series for 48-hrs and embedded in paraplast. The specimens were sectioned at 8 µm and stained with safranin and haematoxylin. All the sections were mounted with permount and were viewed with bright-field illumination and an Olympus microscope. Cell density was determined as the number of cells per surface unit counted from digital images (Brioudes *et al.,* 2010).

2.8 DNA and RNA analysis of transgenic calli

To analyse the integration of the *EgTCTP* gene into transgenic oil palm, genomic DNA was isolated and the DNA primers 5′-ATG CCA TCA TTG CGA TAA AGG AAA GG-3′ and 5′-AGA TCT ACA CAC TTG ATC TCC TTC AGC CCA-3′, were used for PCR amplification of an 879 bp *EgTCTP* fragment. The GFP primers set; GFP forward primer: 5′-TCA GTG GAG AGG GTG AAG GTG ATG-3′ and GFP reverse primer: 5′- GAA TAC AAC TAC AAC TCC CAC AAC G -3′, was used as a positive control to ensure a complete transgenic calli at a 363 bp of *GFP* amplicon size. An *18S* rDNA primer set of 540 bp fragment size; 18SF: 5′- AAA CGG CTA CCA CAT CCA AG-3′ and 18SR: 5′-AGG GCAT CAC AGA CCT GTT A-3′ was used for an internal positive control in the PCR reactions.

To measure the expression level of *EgTCTP*, RNA was isolated from control and *EgTCTP* transgenic calli, and real-time PCR was conducted by amplifying 0.5 µg of cDNA with the iQ™ SYBR® Green Super Mix (BIO RAD) on the MX3000P™.

The efficiency of real-time PCR reactions was determined by performing the reaction using 5 serial 10-fold dilutions of a DNA standard in the range of a 10^6-10^9 copy numbers and the Ct values were plotted against the log10 concentration of template (Nakkaew *et al.,* 2008). The PCR reaction was performed to amplify a 507-bp of *EgTCTP* using the gene-specific primers set ATG TTG GTT TAT CAG GAT CTT CTC A-3′ and 5′-TTA ACA CTT GAT CTC CTT CAG CC-3′ and an amplified 540-bp fragment of the oil palm *18S* rDNA as internal control using 18SF and 18SR primers as described above.

3. Results

3.1 Isolation of *EgTCTP* from oil palm leaves: sequence analysis

An *EgTCTP* (GenBank accession number GU014812) fragment of 722 bp, corresponding to an ORF of 507 bp (GenBank accession number AAQ87663.1), was cloned (Figure 1A). The predicted 168 amino acid polypeptide had a calculated molecular mass of 19.2 kDa. BLAST analysis of the encoded polypeptide sequence showed that EgTCTP is highly homologous at the amino acid level to several plant TCTPs. EgTCTP displayed an 84-91% identity with TCTPs from *Hevea brasiliensis* (Q9ZSW9.1), *Arachis hypogaea* (ABI84255.1) and *Glycine max* (ACU13569.1). A Clustal X alignment of EgTCTP with other TCTP homologous proteins of plants confirmed this remarkable degree of protein identity, and indicated that their highly conserved nature and the phylogeny of EgTCTP was very closely related to the TCTP from *H. brasiliensis* (Q9ZSW9.1) and *J. curcas* (ABO25950.1) (Figure 1B). A number of potential modification sites and functional motifs similar to other TCTPs have been found in this sequence. It also contains the highly conserved Ca^{2+}-binding site (Figure 1A).

3.2 Ca^{2+} binding property of recombinant TCTP

EgTCTP cloned in pQE40 was expressed as a histidine-tagged fusion protein. The recombinant fusion protein

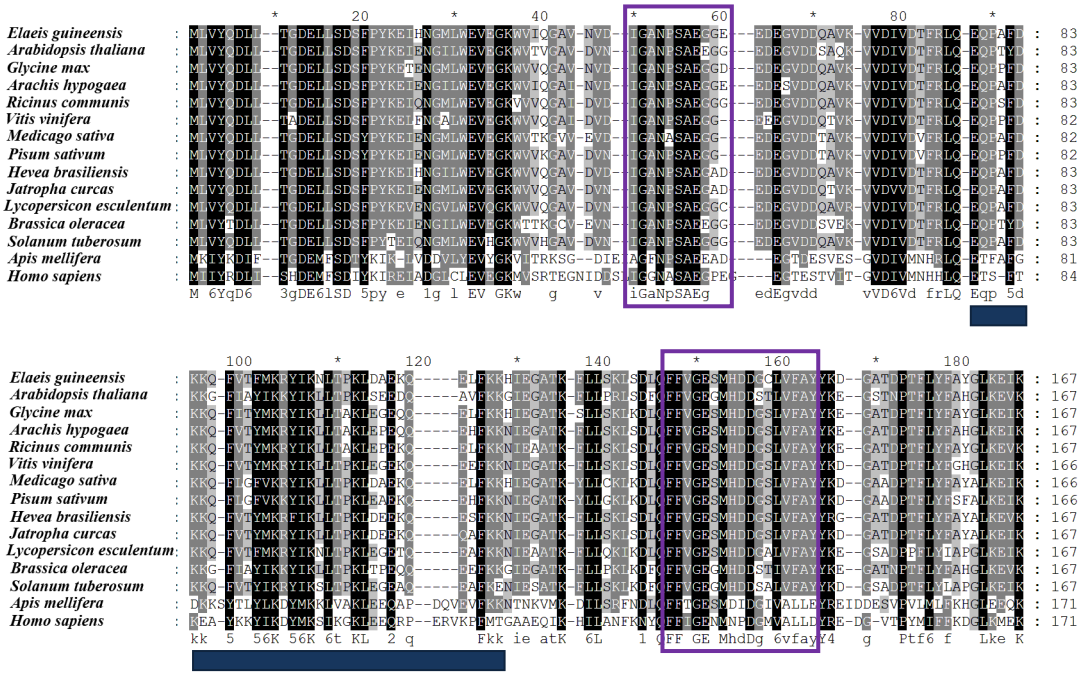

Figure 1A. Multiple alignments (ClustalX 2.0) of the amino acid sequences of the EgTCTP from oil palm *Elaeis guineensis* (AAQ87663.1) with that of other TCTPs and visualized with Genedoc program, such as *Arabidopsis thaliana* (AAM66134.1), *Glycine max* (ACU13569.1), *Arachis hypogaea* (ABI84255.1), *Ricinus communis* (XP_002512437.1), *Vitis vinifera* (XP_002271111.1), *Medicago sativa* (P28014.2), *Pisum sativum* (P50906.2), *Hevea brasiliensis* (Q9ZSW9.1), *Jatropha curcas* (ABO25950.1), *Lycopersicon esculentum* (Q6DUX3.1), *Brassica oleracea* (Q944W6.1), *Solanum tuberosum* (CAA85519.1), *Apis mellifera* (XP_395299.2), and. *Homo sapiens* (AAQ01550.1). The dash represents a gap at the indicated proteins. The translationally controlled tumor protein signatures and the Ca^{2+}-binding region are indicated by a violet box and blue bar, respectively.

with the histidine-tag has a molecular mass of 22.9 kDa. After being purified with a Ni-NTA column, the protein was used to analyse its calcium binding property (Figure 2). A ^{45}Ca-overlay assay was carried out as described by Rao *et al.* (2002). The autoradiography result shows a strong dark band on the recombinant EgTCTP (lane 5), as did porcine tropomyosin (lane 4), while there was no signal from the negative control (lane 2, 3). Lane 1 is molecular weight protein markers. This result demonstrates that EgTCTP binds calcium. Two negative control proteins were used, lane 2 is bovine serum albumin and lane 3 is a non-Ca^{2+} binding recombinant protein prepared by the same protocol as for the recombinant EgTCTP.

3.3 Does the expression of recombinant EgTCTP in callus affect the growth rate?

One day prior to bombardment, the calli were sub-cultured to fresh media to ensure the highest frequency of stable transformation (Parveez and Christou, 1998). After bombardment, the transformants were selected using 50 µg/ml hygromycin and were sub-cultured onto fresh selective media every month. The results showed that the hygromycin resistant explants were a healthy yellow, similar to normal calli, whereas the control calli became black and died within 1 month. The successful gene transformation and expression in the bombarded callus was examined with a confocal laser-scanning microscope, fitted with a GFP filter set. *GFP* gene expression was seen as a bright-green fluorescence caused by GFP accumulation in the cells (Figure 3). Monitoring was carried out at random on the bombarded calli, 1 to 6 months after bombardment. GFP expression was still detectable more than 6 months after bombardment. The appearance of the *EgTCTP* transformants was compared with the control calli that had recovered from bombardment without DNA or with an empty vector. Every observation showed that the calli bombarded with DNA containing *EgTCTP* had bright green spots from the GFP fluorescence inside the cells in comparison with bright field image (Figure 3B), there was none in the non-transgenic controls (Figure 3A). The presence of recombinant *EgTCTP* DNA was also confirmed by PCR using specific primers, and produced the expected band at 879 bp (Figure 4). The expression level of *TCTP* was measured by real time PCR and revealed a high copy number in the *EgTCTP* transformed calli ($1.5x10^8$ copies) compared with the control calli ($4.3 x10^6$ and $3.7 x10^6$ copies) as shown in Figure 5B and 5C in which each sample had three replicates and all reactions were independently repeated twice to ensure the reproducibility of the results and the Figure 5A shows the standard curve generated by serial dilutions from the cDNA of *EgTCTP* in the range of 10^6-10^9 copy numbers.

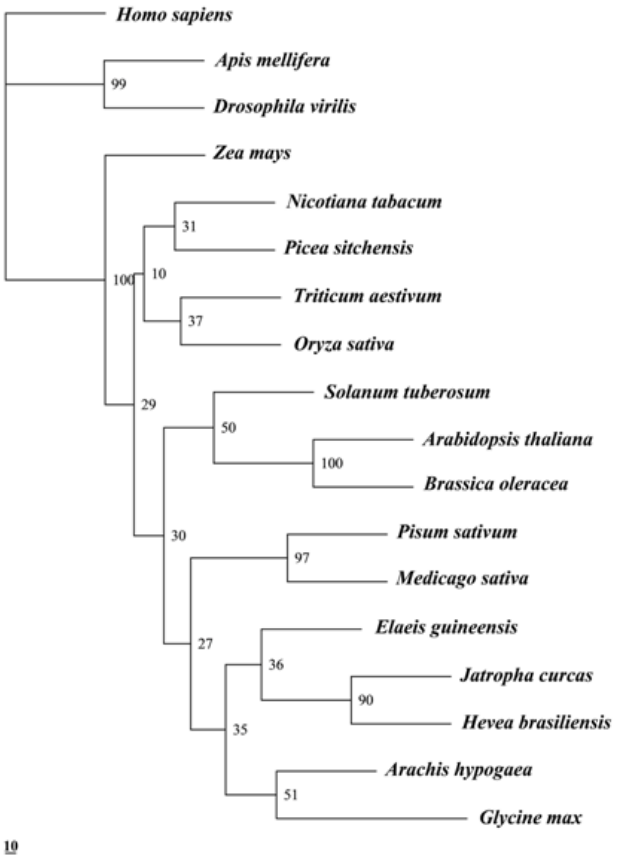

Figure 1B. Sequence comparison of EgTCTP and other related TCTP proteins. The predicted sequences of TCTP proteins were aligned using ClustalX 2.0. The alignment was analyzed using PRODIST and NEIGHBOR programs. Support for the inferred groups was obtained by bootstrap analysis from 1,000 replications of the data set using the SEQBOOT in PHYLIP packages. The accession number and species corresponding are as follows: *Homo sapiens* (AAQ01550.1), *Apis mellifera* (XP_395299.2), *Drosophila virilis* (XP_002056319.1), *Zea mays* (ACG24638.1), *Nicotiana tabacum* (Q9XHL7.1), *Picea sitchensis* (ABK22509.1), *Triticum aestivum* (Q8LRM8.1), *Oryza sativa* (NP_001068405.1), *Solanum tuberosum* (CAA85519.1), *Arabidopsis thaliana* (AAM66134.), *Brassica oleracea* (Q944W6.1), *Pisum sativum* (P50906.2), *Medicago sativa* (P28014.2), *Elaeis guineensis* (AAQ87663.1), *Jatropha curcas* (ABO25950.1), *Hevea brasiliensis* (Q9ZSW9.1), *Arachis hypogaea* (ABI84255.1), and *Glycine max* (ACU13569.1).

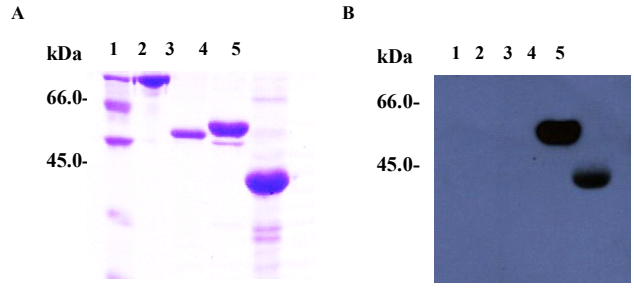

Figure 2. ^{45}Ca-overlay assay of purified recombinant EgTCTP. The purified protein (5 µg) was resolved by 12.5% SDS–PAGE, and the proteins were transferred to a nitrocellulose membrane and probed with radioactive $^{45}CaCl_2$. (A) Coomassie-blue staining of the 12.5% SDS-PAGE. (B) X-ray film after autoradiography; lanes 1: protein markers; lanes 2 and 3: bovine serum albumin and non-Ca^{2+} recombinant protein as negative control; lanes 4: porcine tropomyosin as positive control; and lanes 5: purified recombinant EgTCTP.

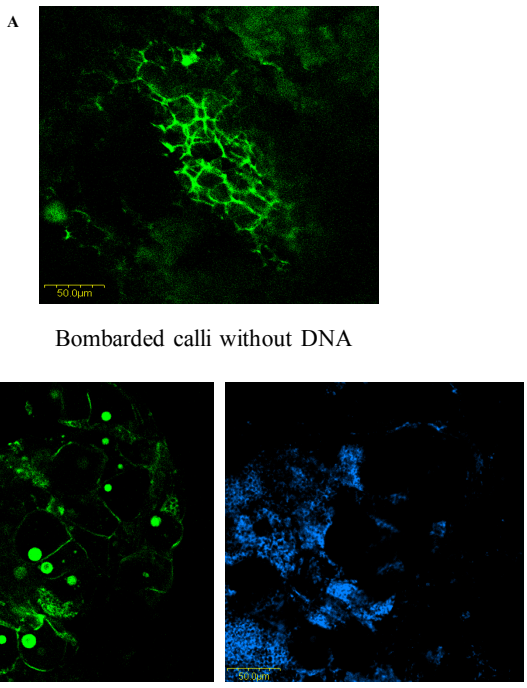

Figure 3. Confocal images that localise GFP in oil palm callus. (A) The bombarded calli without DNA, (B) the transgenic calli bombardment with pC-*EgTCTP*, green spot of green fluorescence protein is visualized using a GFP filter (left) in comparison with bright field images using a blue filter (right).

The transgenic embryonic calli that were overexpressing *EgTCTP* had a faster growth rate and were larger than the non-transformed and empty vector transformed calli and the average size of each callus from one to six months old was measured growth rate as shown in Figure 6A. The number of cells were counted from several sections of the histologically stained tissue isolated from the calli, and clearly demonstrated an increase in cell numbers with 23.3±2.3 cell/mm^2 in the non-transformed control compared to 98±1.8 cell/mm^2 in the *EgTCTP* containing calli (Figure 6B).

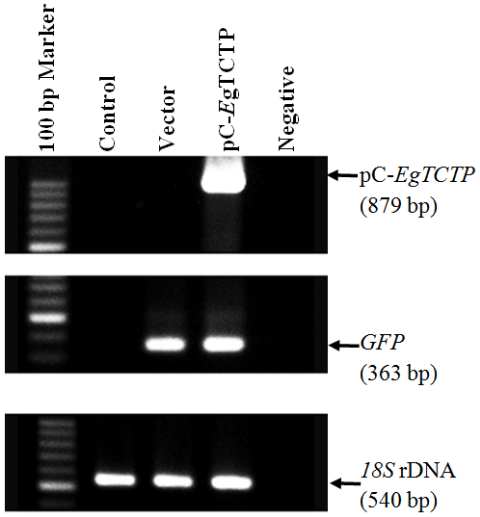

Figure 4. Gel-electrophoresis of the product obtained from the PCR reaction using specific primers to amplify the pC-*EgTCTP*, *GEP* and *18S* rDNA genes from the chromosomal DNA from bombarded calli without DNA (Control), transgenic calli bombarded with an empty vector (Vector) and transgenic calli bombarded with pC-*EgTCTP*; M is a 100 bp DNA marker.

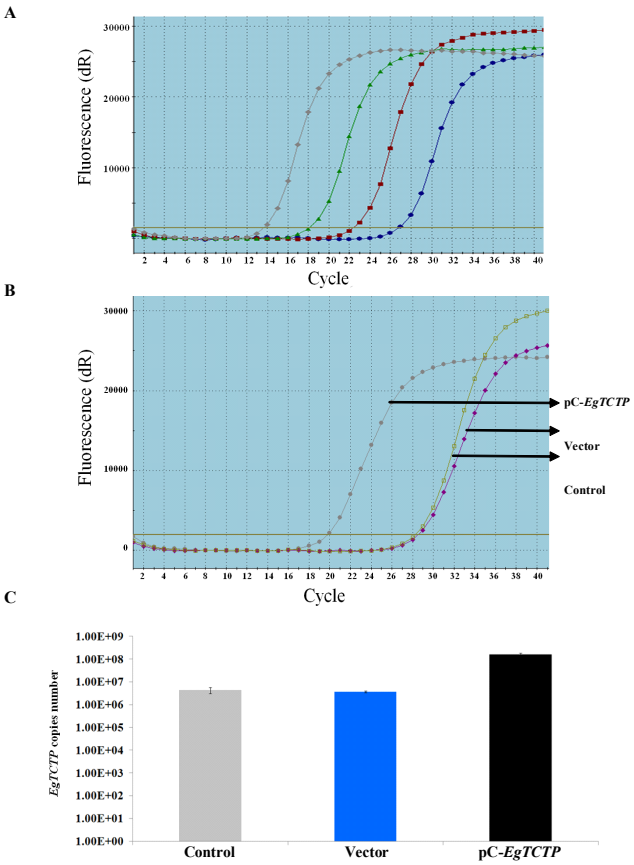

Figure 5. The observed of *EgTCTP* gene expression level by real-time PCR in the samples of *EgTCTP* transgenic calli (pC-*EgTCTP*) compared with a non-transgenic control (Control) and empty vector transgenic calli (Vector) by using *18S* rDNA as endogenous control. (A) Concentration-dependent amplification of an *EgTCTP* cDNA standard, serially diluted by factors of 10. A linear relationship was consistently observed with cDNA that ranged from 10^6 to 10^9 copies. (B) The *EgTCTP* gene expression of three sample groups by real-time quantitative PCR, the crossing point of each reaction was plotted against the initial concentration of nucleic acid in the reaction. (C) The observed expression of *EgTCTP* mRNA in three sample groups in which each sample had three replicates of calli.

4. Discussion

TCTP was discovered approximately twenty years ago. A series of recent reports has highlighted the importance of TCTP for cell cycle progression (Bommer and Thiele, 2004). In this study, we cloned a *TCTP* homologue from *E. guineensis* using PCR primers designed from previously isolated plant TCTP sequences. The putative polypeptide has high homology, with an average identity of 90%, to similar proteins from *H. brasiliensis*, *A. hypogaea* and *G. max*. This remarkable degree of protein identity indicates their highly conserved nature, and also that they may play an important role in the plant cell.

An attempt to determine the presence of probable secondary structures of the protein coded by the *EgTCTP* found that the predicted protein has potential modification sites, for example there are two TCTP signatures, the possibility of a site for protein-protein interactions, in which the TCTP signature 1 site can also play a functional role. The middle of the loop contains the highly conserved Ca^{2+}-binding site, which is similar to other TCTP proteins. Sanchez *et al.* (1997) have shown the presence of at least three TCTP isoforms that have similar molecular mass but different isoelectric points. This indicates that this protein could be phosphorylated. This is in agreement with the presence of phosphorylation signature sequences found in the EgTCTP sequence. This suggests that EgTCTP may have a role in cell growth and the cell cycle through TCTP phosphorylation and interaction with regulatory proteins, similar to other TCTP proteins (Brioudes *et al.,* 2010). The most interesting characteristic of the TCTP protein is its relationship to the growth condition of cells. In plants, the TCTP protein has been isolated from rapidly growing tissue, callus tissue, the apical stem, young leaves and the root meristem (Pay *et al.,* 1992; Woo and Hawes, 1997; Kang *et al.,* 2003; de Souza *et al.,* 2004). The TCTP protein associates with cytoskeletal microtubular networks and is an important component of the target of the rapamycin signal pathway, the major regulator of cell growth in animal, fungi and plants (Gachet *et al.,* 1999; Brioudes *et al.,* 2010).

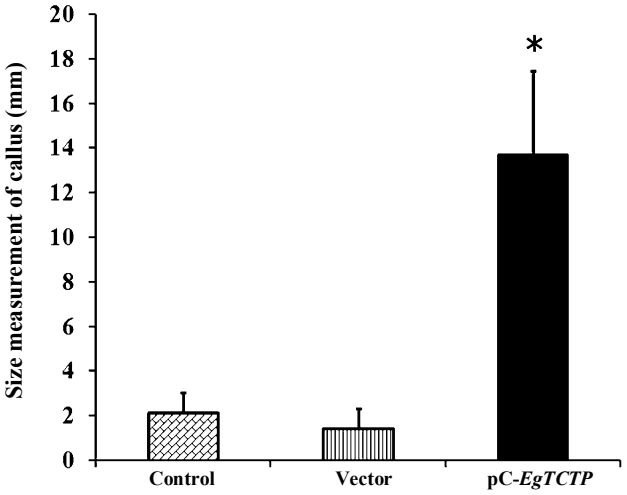

Figure 6A. Size measurements of the 6-month old callus from non-transgenic calli (control, n = 7), transgenic calli using empty vector (vector, n=10) and *EgTCTP* transgenic calli (pC-*EgTCTP*, n=12).* Significant different at P < 0.01 was analyzed by one-way ANOVA using SPSS for Windows.

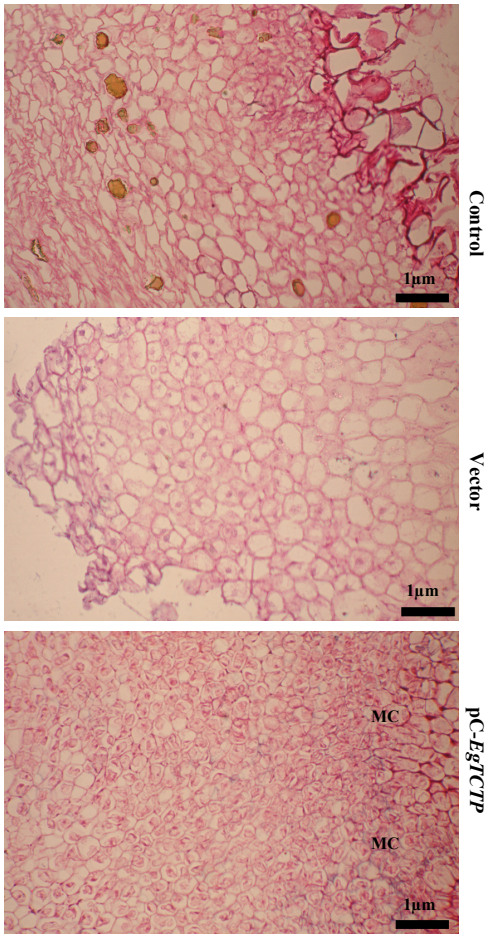

Figure 6B. Comparison of cell morphology from tissue isolated from transgenic and non-transgenic calli. All sections were mounted with permount and were viewed under bright-field illumination with an Olympus microscope (100X, scale bars =1 µm). Control was Bombarded calli without DNA, Vector as transgenic calli bombarded with empty vector and pC-*EgTCTP* as transgenic calli with *EgTCTP* that showed the meristematic cell (MC) formation by cell division of calli.

A pea TCTP gene is expressed at a high level in rapidly dividing cells within the root cap (Woo and Hawes, 1997). TCTP has a role in the light regulated growth of the Japanese morning glory plant (*Pharbitis nil* cv. Violet) (Sage-Ono *et al.,* 1998). The expression of strawberry TCTP increased in correlation with strawberry fruits (Lopez and Franco, 2006). Interestingly, transgenic tobacco plants overexpressing *TCTP* grow about 30% faster than the parental plants during the juvenile growth stage (Kang *et al.,* 2003). Moreover, the use of the expression vector with the TCTP promoter provides efficient transformation and makes plants more accessible to genetic engineering. Taken together, it would seem that *EgTCTP* is a good candidate gene to assist with the transformation of oil palm, and to provide benefits for plant growth and regeneration.

Our oil palm TCTP was found to be a calcium-binding protein. This work is the first report that targets the TCTP gene into the embryonic oil palm callus and produces TCTP transformants. The gene was stably transformed into the plant cell, as demonstrated by the high number of GFP spots that were retained for more than 6 months after gene bombardment, and by the presence of an integrated *EgTCTP* fragment as visualised by PCR. The presence of the transgenic callus containing TCTP did indeed have an accelerated growth rate when compared to the non-transformed callus. The reason that the transgenic calli were a larger size than the non-transgenic calli was due to an increase in the cell numbers, as demonstrated by histological staining. A higher number of meristematic cells were observed in the *EgTCTP* transgenic calli when compared to the non-transgenic calli and this might explain the larger calli size. Within the *EgTCTP* transgenic calli, there are differences in cell size that ranged from small to normal size, and this also indicates that the growing cell is probably controlled by a limited number of other factors. The numbers of division and the maintenance of meristematic cells is an important mechanism that mediates organ growth and cell proliferation. Cell growth and cell cycle progression are separable processes that are determined by internal developmental factors and the growth is rate-limiting (review by Mizukami and Fischer, 2000). It is shown here that in the presence of EgTCTP there is an increase in the cell number but not the cell size. These results indicate that EgTCTP might control the cell cycle progression but not directly to the cell growth.

Calcium has been implicated as an important regulatory component for cell cycle progression. The molecular

mechanisms of Ca^{2+} in the dividing cell have been intensively studied (Berridge *et al.,* 2000; Whitaker and Larman, 2001; Carafoli, 2002; Ikura *et al.,* 2002; reviewed by McKinsey *et al.,* 2002; Kahl and Means, 2003). Briefly, endogenous Ca^{2+} is released from internal calcium stores to generate the cytoplasmic Ca^{2+} transients required for the mitotic events. At anaphase there is a sudden increase in cytosolic calcium and this causes spindle dissolution. These Ca^{2+} signals act to regulate cellular pathways by a direct binding of Ca^{2+} to targets or by Ca^{2+} to an intracellular binding protein, such as CaM, which transduces the signal to Ca^{2+}/CaM-dependent targets. The strong binding of EgTCTP to Ca^{2+} demonstrated in this work implies that EgTCTP may be another Ca^{2+}- modulated protein. The increasing number of Ca^{2+} binding proteins has been recently identified and most have been implicated in the regulation of gene transcription. Their molecular and structural regulatory processes differ from one Ca^{2+}-binding protein to another.

The importance of the Ca^{2+} and Ca^{2+} binding proteins for the engineering of plants to obtain desired traits has become more evident (Yang *et al.,* 2007). The knowledge obtained from our work will also contribute to further benefit the development of research into improving the productivity of oil palm. Future work will be directed towards studying the mechanism of phosphorylation and the involvement of EgTCTP in cytoplasmic Ca^{2+} transients in the regulation of cell proliferation.

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