Molecular Characterization of an Unknown Protein (Acc. No. EU795363) from the ESTs of Oil Palm (*Elaeis guineensis* Jacq.) Cell Suspension Culture

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ABSTRACT

A large quantity of ESTs is available from various cDNA libraries of oil palm. The information from oil palm EST databases has been utilized to identify several interesting sequences for molecular characterization. In this study, we report molecular characterization of clone 583 (Acc. No. EU795363) isolated from cell suspension culture of oil palm. This clone is predicted to encode a single major open reading frame for a polypeptide of 177 amino acids with a predicted molecular mass of 19.6 kDa. The predicted amino acid sequence does not contain any signal peptide and transmembrane region. Based on Kyte-Doolittle hydropathy profile, this protein is predicted to be a soluble protein. The predicted ORF of clone 583 was 59% identical to an amino acid sequence of an unknown protein from *Oryza sativa* (Acc. BAD25663). Southern analysis showed that this clone might be a member of a multigene family in the oil palm genome. Gene expression study by real time quantitative RT-PCR showed that transcripts of clone 583 might be present in low abundance.

Keywords: Molecular characterization, unknown protein, oil palm, *Elaeis guineensis*

INTRODUCTION

Oil palm is an economically important plant as it is the second largest source of edible oil in the world. The priority of Malaysia at present is to ensure that the yearly surplus of palm oil is exported to satisfy the growing market demand of oils and fats worldwide which is expected to rise to about 58 million tonnes by 2020 (Yusof and Chan, 2004). The area planted with oil palm in this country increased from 0.06 million hectares in 1960 to 3.5 million hectares in 2001 and is predicted to increase to up to 4.72 million hectares in 2010. With the expansion of the oil palm cultivation areas, palm oil production rose from 91,793 tonnes in 1960 to 11.80 million tonnes in 2001 and is expected to reach 18.81 million tonnes in 2020 (MPOB, 2001). Although there has been an increase in production with the expansion of planted areas, the national yield of oil per hectare does not reflect the technological and scientific advances made in the oil palm industry, especially in breeding, agronomy and other estate management practices (Jalani *et al.* 2002). As the breeding cycle of the oil palm is very long and the progeny of crosses shows

large variations in yield, the palm oil industry has invested in the development of new techniques for multiplying superior parents for seed production and elite progeny palms for commercial planting (Basri *et al.,* 2005). Clonal propagation of oil palm has been studied for many years as a potential way to develop high-yielding collections while circumventing the long generation time required with traditional breeding techniques. The oil palm, *Elaeis guineensis* Jacq., is amenable for vegetative propagation by means of somatic embryogenesis. Cell suspension cultures, initiated at an early stage in embryo development, have been extensively utilized in oil palm micropropagation (Gorret *et al.* 2004; Tarmizi *et al.,* 2004). Tissue culture of oil palm has resulted in the production of elite palms that are uniform with desirable traits and optimum yield. Nevertheless, *in vitro* propagation of oil palm frequently induces a somaclonal variant called the 'mantled' abnormality (Syed-Alwee *et al.,* 2006) which is observed only in palms produced by tissue culture (Jouannic *et al.,* 2005). In addition, the rate of callogenesis from oil palm explants remains low, at about 19% (Corley and Tinker, 2003), while the average rate of embryogenesis from proliferating callus cultures has been reported to be only 6% (Wooi, 1995). To alleviate these problems, markers could be developed at the molecular level to detect callogenesis, embryogenesis and abnormality, making large-scale propagation viable (Low *et al.,* 2006). In this aspect, a large quantity of Expressed Sequence Tags (ESTs) would be an effective first step towards gene discovery and the characterisation of transcription patterns. The EST databases from oil palm projects will facilitate the identification of many important genes. However, there are many unknown genes involved in the regulation of various developmental processes. Unknown genes can represent a significant portion of ESTs, for example, Cooke *et al.* (1996) estimated that only a third of the 5000 non-redundant ESTs corresponded to unknown proteins. In any case, ESTs may be an important means of discovering new biochemical and regulatory pathways (Fristensky *et al.,* 1999). Thus, the aim of this study is to characterize an unknown protein from the ESTs of oil palm cell suspension cultures which may have implications in the tissue culture processes of oil palm.

MATERIALS AND METHODS

Materials

The EST clone 583 was previously isolated from oil palm cell suspension cultures (Ho *et al.,* 2007). The GenBank accession number for this sequence is EU795363. The *in vitro* cultures of oil palm and clone 583 were kindly provided by the Malaysian Palm Oil Board (MPOB). Chemicals used in all experiments were generally obtained from Sigma, Biosciences and MERCK. Primer synthesis and sequencing of DNA were done by First Base Laboratories Sdn. Bhd., Kuala Lumpur, Malaysia.

Sequence Analysis

Sequence analysis was carried out using BLAST 2.0 (Altschul *et al.,* 1997), accessible from the internet (http://www.ncbi.nlm.nih.gov.BLAST). A homology search using the BLASTX algorithm, at the protein level, was carried out by comparing the translated protein sequence with other protein sequences available in the databases. Alignment of the protein sequence with several closely related genes was carried out using the CLUSTAL W (Thompson *et al.,* 1994). Other sequence analyses were conducted using Biology Workbench version 3.2 available at http://biowb.sdsc.edu/CGI/BW.cgi to compute

predicted molecular weight (MW) and isoelectric point (pI) while prediction of transmembrane topology was done using Localizome (Lee *et al.*, 2006). A phylogenetic tree was constructed using the neighbour joining method via MEGA4 package (Tamura et al. 2007). The reliability of the neighbour joining tree was estimated by bootstrap analysis of 1000 replicates.

Southern Blot

Genomic DNA was extracted from young leaves according to the cetyltrimethylammonium bromide method (Murray and Thompson, 1980) and digested with 4 restriction endonucleases, *Hin*dIII, *Not*I, *Eco*RI and *Taq*I (Fermentas), in respective optimal reactions. Digested genomic DNA (30 μg/lane) was separated on 0.7% (w/v) agarose gel in 1 × Tris-borate buffer and then transferred onto a positive-charged nylon membrane Hybond N$^+$ (Amersham Biosciences) by capillary blotting with 0.4 M NaOH and hybridized overnight with DNA probe. The radioactive DNA probe was prepared using the High Prime reaction mix (Roche), purified 3'UTR PCR product as a template, forward primer (5'-AGAATCTAATCAAGCACCGGCTAC-3') and reverse primer (5'-CCTACCCAGAAA GGAACCGTTTTTA-3') according to the manufacturer's instructions. The hybridization protocol used for Southern analysis was based on the method by Church and Gilbert (1984). The pre-hybridization and hybridization temperature was set at 60°C and hybridizations were carried out for 20 hours. Stringency washes were performed twice in 40mM sodium phosphate buffer (pH 7.4), 1% (w/v) SDS for 5 minutes each at room temperature. The blots were then washed in 40mM sodium phosphate buffer (pH 7.4), 5% (w/v) SDS at the hybridization temperature for 15-20 minutes. The blots were exposed to an imaging plate (FujiFilm) and scanned using a PhosphorImager (FujiFilm FLA5100).

Real Time RT- PCR

Total RNA was extracted from all tissues sampled (leaves, meristems, roots, female flowers, suspension cultures, non-embryogenic calli and embryogenic calli) according to the SDS-phenol/LiCl method (Shizadegan *et al.,* 1991). Two micrograms of total RNA was used for reverse transcription into first-strand cDNA using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. All oligonucleotides for TaqMan were designed by Sigma-Proligo (Sigma-Genosys, Sigma-Aldrich Co.). The primer sequences are given in Table 1. All reactions were carried out in three replicates using the ABI Prism 7900 Sequence Detection System and software (PE Applied Biosystems, USA). The PCRs were performed under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 57°C in 384-well optical reaction plates (Applied Biosystems, USA). A validation graph was generated and PCR efficiency was estimated for each primer pair by using a serial dilution of reverse transcription products and plasmid DNA. The quantity of gene expression in every experimental tissue is expressed relative to the calibrator, suspension culture. PCR reactions contained all components: 100 nM forward and reverse primers, 250 nM TaqMan probe (Table 1), TaqMan Universal PCR Master Mix (Applied Biosystems, USA), in a final volume of 20 μl. The quantification of the relative transcript levels was performed using the comparative C_T method (Livak and Schmittgen, 2001). The transcript levels of the target genes were normalized against the GAPDH gene, an endogenous control, as described in the ABI PRISM 7700 Sequence Detection System user Bulletin #2 (Applied Biosystems).

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TABLE 1
Primers and probe sequences for real time RT-PCR

Name	Sequences 5'-3'
GAPDH For	5' - GCCAGCTTTAACATCATTCCTAGC-3'
GAPDH Rev	5' - AGCTTTCCATTTAAGGCAGGAAG-3'
GAPDH PRO	5'-(Tet)CAACAGCCTTGGCAGCACCAGTGC(Tamra)
Clone 583 For	5'-GTTCTTGTGCTTCTTCCGATTGC- 3'
Clone 583 Rev	5'-TGTTAGTAGGGGGAACCGTTAAGG- 3'
Clone 583 PRO	5'-(6-Fam)AGCAGCAGCAGCAAACCCCCAGC(Tamra)

Notes: For: Forward primer, Rev: Reverse primer; PRO: probe

RESULTS AND DISCUSSION

Sequence Analysis

The full sequence of clone 583 is 833 bp in length. This sequence was translated into six possible frames, and the longest ORF was a start codon at position $A_{57}TG$ and a stop codon at position $T_{588}AG$ as shown in *Fig. 1,* which encodes 177-aa (amino acids, aa) protein. The statistics based on amino acid abundance of the predicted protein of clone 583 showed that it consists of lysine (11.79%), serine (11.67%), leucine (9.47%), arginine (8.34%), asparagine (7.29%), phenylalanine (6.64%), glutamine (6.66%), proline (6.44%), glycine (6.29%), threonine (6.60%) and tyrosine (5.08%). The deduced polypeptide had a predicted molecular mass of 19.6 kDa and a theoretical isoelectric point of 7.6. Four potential functional sites are present in this sequence (*Fig. 1*). These are Protein kinase C phosphorylation site, N-myristoylation site, cAMP-and cGMP-dependent protein kinase phosphorylation site and Casein kinase II phosphorylation site. The presence of the potential site for casein kinase II phosphorylation which has a role in the regulation of transcription factor activity is noteworthy (Lin and Hiscott, 1999). The casein kinase II phosphorylation is probably involved in the regulation of metabolic pathways and in the process of cell division and proliferation (Meisner and Czech, 1991). The activities of casein kinase II protein require cAMP-dependent protein kinase as a substrate and a frequent feature of casein kinase II protein phosphorylations is that they are apparently 'silent' as they do not promote any sharp and obvious change of activity (Pinna, 1990).

The results from BLASTX showed that the amino acid sequence of clone 583 is 59% (55/93) identical to an amino acid sequence of an unknown protein (BAD25663), 46% (54/116) identical to a cold induced protein-like (BAB55503) from *Oryza sativa* and 49% (44/89) identical to an unknown cold induced protein from *Deschampsia antarctica* (AAM11916) (*Fig. 2*).

Conserved domain database (CDD, NCBI) search recognized a SIN3 domain from the deduced amino acid sequence of clone 583 (*Fig. 3*). The SIN3 domain is located towards the N'- terminus of the amino acid sequence i.e. from amino acids 43 to 70. SIN3 domain functions as a global transcriptional regulator for a diverse and ever growing set of cellular processes (Silverstein and Ekwall, 2005). It also behaves as a platform for multiple protein complexes that acts as a transcription silencer (Kurita *et al.,* 2007). In yeast, the SIN3 gene encodes a global regulatory factor that has been shown to affect expression of numerous unrelated genes (Slekar and Henry, 1995). In Arabidopsis, a

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3     CAC GAG AGG CTC CAT ATC CTT CAT CTC TTA GGT TCC AAA GAC CAG    47

48    AAA CCA GGC ATG GGT GAA GAT CTC AGC CAG AGG TTT CAT GGG ATC    92
                      M   G   E   D   L  |S   Q   R| F   H   G   I    12

93    CTA CCT TGT GAC GAG GAT GAT GAT GAG CGA CGA GAA TCT TCC TCC    137
13     L   P   C   D   E   D   D   D   E  |R   R   E   S| S   S      27

138   TTC TCC GGC GAC TCC GAA GAC GAA GAT TTC AGG GAG GAG GAA ACT    182
28     F   S   G   D  |S   E   D   E| D   F   R   E   E   T          42

183   TAT TCT CCA AAA TTT CCA CCA CCT TTG GAC AAA ACA ACC TCC TTG    227
43     Y  |S   P   K| F   P   P   P   L   D   K   T   T   S   L      57

228   CCA TCA AAA TTC GAT GCA ACT GGG CCT CTC TAC GAT TTA TCC TCC    272
58     P  |S   K   F   D| A   T   G   P   L   Y   D   L   S   S      72

273   CTC ACG GCG CAA CTT CCT AGC AAG AGG GGG CTA TCA AAG TAC TAT    317
73     L   T   A   Q   L   P   S   K   R   G   L   S   K   Y   Y     87

318   CAA GGG AAG TCC CAG TCC TTC ACC TCC CTG TCC GAT GTG AGG TGC    362
88     Q   G   K   S   Q   S   F   T  |S   L   S   D| V   R   C     102

363   CTC CAG GAT CTG CCT AAG AAA GAG ACT CCC TTC AGA AGG AAG ATG    407
103    L   Q   D   L   P  |K   K   E   T| P   F   R   R   K   M     117

408   AAG TCA TGC AAG AGC TAT GCA GGA GGT CTA GAT GGA AGC ACA GAA    452
118    K  |S   C   K| S   Y   A  |G   G   L   D   G   S   T| E      132

453   TCT AAT CAA GCA CCG GCT ACT TCC AGG AAG ACC AGA TCA AAG AAG    497
133    S   N   Q   A   P   A  |T   S   R| K   T   R  |S   K   K|    147

498   GCG TCA AGA GGT TCT TGT GCT TCT TCC GAT TGC AAG CAG CAG CAG    542
148    A   S   R  |G   S   C   A   S| D   C   K   Q   Q   Q         162

543   CAA ACC CCC AGC TAT TCC TGT ACG AAA GGG ATT TTT GTC TCC ACT    587
163    Q   T   P   S   Y   S   C   T   K  |G   I   F   V   S   T|   177

588   TAA ATT TCA TGG ACA TAA AAA AGC CCT TAA CGG TTC CCC CTA CTA    632
       *

633   ACA TTA TAT TTT AGG ATT TTT AAA AGA AGA AGA AAG CCG GGG AGG    677

678   GAT GCA TGG TGG TCC GAG GTT TTC CTT TTG TAA TTC GGT GGG CCA    722

723   ATC CTT TAT TTA AAA ACG GTT CCT TTC TGG GTA GGG GTA AAA AAA    767

768   AAA AAA AAA ATT TTT TGG GGG GGG AAA GGT TTT TTC CAA GGC CAA    812

813   AAA AAA AAA AAA AAA AAA AAA    833
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Fig. 1: The nucleotide and deduced amino acid sequences of clone 583. Predicted start and stop codons are in bold. Putative functional sites are indicated by underlines/italics and box: Protein kinase C phosphorylation site (aa6-8, aa44-46, aa79-81, aa119-121, aa139-141, aa140-142, aa145-147), N-myristoylation site (aa11-16, aa125-130, aa126-131, aa129-134, aa151-156, aa172-177), cAMP-and cGMP-dependent protein kinase phosphorylation sites (aa22-25, aa108-111, aa146-149), Casein kinase II phosphorylation site (aa32-35, aa59-62, aa96-99). The polyadenylation signal is shaded
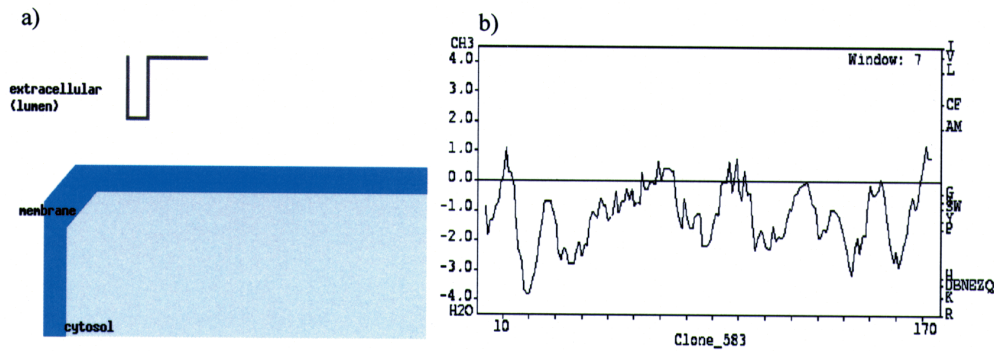
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Clone 583         MSEDLSQRFHG--ILPQDED---DIERPESSSFSGD------SEDEDFRE
O. sativa         MRSACAPVPEGAHEEASIEQ---SLURSFDGSFSGSSIGSSCSSASDISD
O. sativa         -----MGEFGGAAVLPVMSBEEDSDLFETSSSISGD-----------SD
D. antarctica     -----MGRFGGAIVLPAMPE--DFDLFETSSSISFD-----------SD
Clustal Consensus                 *      .  .:   .*   ..*:*.          :

Clone 583         E-ETYSP-KFPPFLDKTTSLPS---KFDATGPLYDLSSLTAQLPSKRGLS
O. sativa         DGSSMPEGDYIEESSSSSSASSSTLQLDSEGPLQDLSSLIAQLPIFRGLS
O. sativa         DEAQFSDSEEAEAQED--QFAQQPARRMNSDSLYDLSSMKAQLPMVKKGLS
D. antarctica     DEAQLSEFEAQERNQFGLQVAPKIVRRLNSDSVYDMSSVKAFLPIKKGLS
Clustal Consensus     :            .      .  .   :       .: *:**: *:.** ::***

Clone 583         KYYQGKSQSFTSLSDVRCLQDLPKKEIEPRRKMKSCKSYAGSLDGSTESN
O. sativa         NYYQGKSQSFTSISDATHVQDLAKPLT-NNPRMKACKSYAAGLDMNQPSN
O. sativa         KYYDGKSQSFACMSEVRCLEDLPKKENPYRK-IKSSKSYWALLGNQEAC
D. antarctica     RYYDGKSQSFACMSEVRCLEDLPKH-SPYRR-IKACNSFVDLDDGNQEAC
Clustal Consensus  .**:******:..*:.  *::** **  .:.: :*:.:*:.      . .:

Clone 583         QAEATSPITRSPKASRGSCASSDCK-------QQQQTESYSCTRGIFVS
O. sativa         PLEKPCNMMAKPPSKGSFTCILSPESSTSLLCSSAKPPTHQSKKDVQMH
O. sativa         ELPGANSTSHAK-RSGSSCANLMARNNTRS---MLYRPBEIPVNRSGYHC
D. antarctica     PSEGPNSRSLGPI-ESGSSCANLMARNNAAAN--MLYRPBAIPVNSSYHC
Clustal Consensus   *   .   .*   * .*  :     .:          ::.*.    .*.

Clone 583         T--
O. sativa         INS
O. sativa         ---
D. antarctica     ---
Clustal Consensus
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Fig. 2: Amino acid sequence alignment of clone 583 with an unknown protein (BAD25663; second sequence from top), a cold induced protein-like (BAB55503; third sequence) from O.sativa and an unknown cold induced protein (AAM11916) from D. antarctica. Dashed lines are gaps introduced to maximize alignments. The conserved residues are highlighted. * represent those that are highly conserved

Fig. 3: Location of SIN3 domain (from amino acids 43 to 70) that was recognized based on deduced amino acid sequence of clone 583 using NCBI-CDD program

Sin3-like protein was indicated to effect on a transcription factor which may be a part of a transcriptional repressor complex (Song *et al.*, 2005).

The Localizome program predicts the deduced protein of clone 583 to be an extracellular protein (*Fig. 4a*). According to Bishop-Hurley *et al.* (2003) some extracellular proteins may be involved in the process of initiation and termination of cell wall expansion that occurs during somatic embryogenesis. The Kyte Doolitle hydropathy profile for clone 583 showed that it was predicted to be a soluble protein (*Fig. 4b*). Most of the soluble proteins found in carrot suspension culture were suggested to play important roles in regulating gene expression during somatic embryogenesis (Nomura and Komamine, 1986).

A neighbour-joining tree consisting of clone 583 and similar members identified from Genbank with scores above 80 is shown in *Fig. 5*. This predicted protein is clustered with the OsI006314 protein which was predicted to be involved in cell adhesion but bootstrap value was not determined. According to Doyle and Gaut (2000), a bootstrap value of 70-80% is often taken to indicate strong support for a cluster of sequences. This

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Fig. 4: a) Prediction of transmembrane topologies of clone 583 using Localizome
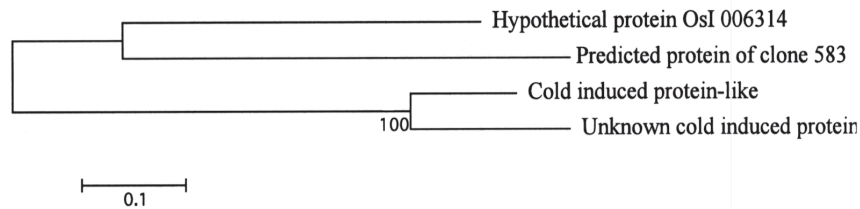b) Kyte Doolittle hydropathy profile of clone 583

Fig. 5: A neighbour-joining tree displaying the phylogenetic relationship of predicted protein of clone
583 to hypothetical protein OsI006314 (EAY85081) and cold induced protein-like (BAB55503)
from O. sativa and unknown cold induced protein (AAM11916) from D. antarctica.
Bootstrap values are indicated for each branch divergence of 1,000 replicates.
Undermined bootstrap values are not shown

gene is not the same cluster as cold induced protein-like from rice and unknown cold induced protein from *D. antarctica*.

Southern Hybridization

To examine the genomic organization of clone 583 gene in oil palm, genomic DNA blot analysis was performed using a 310 bp fragment at 3'UTR region as probe. Genomic DNA was isolated from oil palm leaves and digested with four restriction enzymes. These restriction sites were not found within the probe sequence, therefore, the expected results were one fragment hybridizing to the probe, should it be a single copy gene. However, there were two fragments each from *Taq*I-digestion and *Eco*RI-digestion that hybridized to the probe (*Fig. 6a*). These extra bands could be due to the presence of introns. To confirm that there is no intron within the 3'-UTR region, this region was amplified using oil palm genomic DNA as the template. The PCR product (*Fig. 6b*) and sequencing result (data not shown) verified that there is no intron within the 3'-UTR used as the probe for Southern hybridization. Therefore, the additional bands hybridizing to the probe were possibly due to the presence of multiple copies of clone 583 in the oil palm genome.
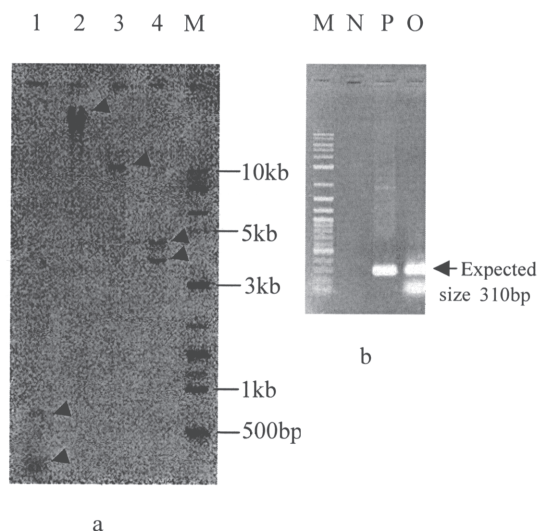
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Fig. 6: a) Southern blot analysis with 3'UTR region of clone 583 as probe on oil palm genomic DNA digested with TaqI (1), NotI (2), HindIII (3) and EcoRI (4), M: 2-log marker; b) PCR analysis of 3'UTR of clone 583 using cDNA (P) and oil palm genomic DNA (O) as templates, N: negative control

Real Time Quantitative RT-PCR

The results from *Fig. 7a* show that the housekeeping gene, GAPDH, was amplified with nearly 100% amplification efficiency (slope = - 3.35, R^2=0.99). These results indicated that the tested sample used in this experiment was qualified. However, using the same sample (first strand cDNA of cell suspension culture), amplification of clone 583 was not detected, although amplification of the target from the plasmid DNA of clone 583 showed nearly 100% amplification efficiency (slope = - 3.33, R^2=0.99) (*Fig. 7b*). Even though this cDNA was isolated from cell suspension cultures, the qPCR results suggested that the transcripts of clone 583 might be produced at other stages of cell suspension culture than the suspension culture used in this qPCR experiment or was present at levels too low to be detected. Most genes involved in the regulation of developmental pathways are normally expressed at very low abundance levels in the cells, such as DcSERK. DcSERK was found to be expressed at low levels during carrot embryogenesis with expression ceasing after the globular stage (Schmidt *et al.* 1997).

Gene expression studies by real time quantitative RT-PCR suggested that expression levels of clone 583 were either too low to be detected or absent in all samples tested (Table 2). Hence, this gene may be a low abundant gene in oil palm or requires a specific induction signal for expression. According to Zhang (2003) plant regulatory factors are mainly responsible for selectivity in gene regulation, and are often expressed in a tissue-specific, developmental-stage-specific or stimulus-dependent manner. This could be the scenario for clone 583 transcripts.
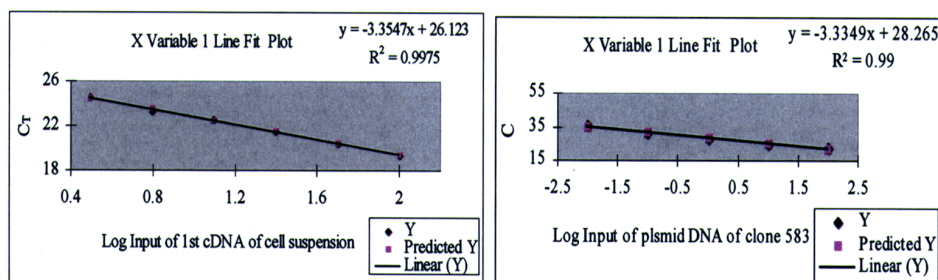
Fig. 7: Real time PCR results indicating the PCR efficiencies of: (a) the housekeeping GAPDH primers in a dilution series of first strand cDNA of cell suspension culture and (b) the target 583 primers in a dilution series of plasmid DNA of clone 583

TABLE 2

C_T values of GAPDH and target 583 gene on different tissues

Tissues	C_T values	
	GAPDH	Target gene
E	19.99557 ± 0.097	Undetermined
L	19.82735 ± 0.150	Undetermined
FF	19.61802 ± 0.147	Undetermined
M	20.10332 ± 0.108	Undetermined
N	19.77532 ± 0.091	Undetermined
SC	19.45607 ± 0.139	Undetermined
R	20.00530 ± 0.102	Undetermined

Notes: E: embryogenic calli, L: leaves, FF: female flowers, M: meristem, N: non-embryogenic calli, SC: suspension culture and R: roots

CONCLUSIONS

In this study, we report the characterization of a novel protein of clone 583, which is highly similar to an unknown protein from rice. Clone 583 might be a transcription factor involved in gene regulation in cell suspension cultures of oil palm. This gene may be a member of a multigene family in the oil palm genome. In future, the functional study of clone 583 should be conducted to understand the role of this gene in the cell suspension process.

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