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ACTIVITY STUDIES, GENE CHARACTERIZATION AND MANIPULATION OF β-KETOTHIOLASE OF OIL PALM (*Elaeis guineensis* Jacq.) MESOCARP

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ABSTRACT

*The enzyme β-ketothiolase plays a major role in isoprenoid metabolism as well as in polyhydroxybutyrate (PHB) biosynthesis. PHB is a member of the polyhydroxyalkanoate (PHA) family of polymers with potential as biodegradable replacements for the current petrochemical plastics used. A coupled assay system for β-ketothiolase activity in oil palm mesocarp crude extracts was designed and optimized. The highest levels of β-ketothiolase specific activity were seen in oil palm fruits 8 to 11 weeks after anthesis (WAA) with ripe fruits showing lower activity. A cDNA coding for oil palm (*Elaeis guineensis *Jacq.) β-ketothiolase was isolated through RT-PCR and RACE techniques. The longest reading frame encoded a protein of 415 amino acids with a predicted relative molecular weight of 43 217 Da, and considerable similarities to the gene/enzyme in other plant thiolases and, to a lesser extent, prokaryotic thiolases. There is no evidence for the presence of a signal peptide, suggesting that the β-ketothiolase cDNA encodes a cytosolic protein.*

Genomic DNA gel blot analysis suggested a small family of β-ketothiolase isogenes. Northern analysis revealed that β-ketothiolase mRNA transcripts are present in higher quantities in the riper (13, 17, 20 WAA) than younger fruits at 6, 8 and 11 WAA, contradicting the biochemical activity profile. This discrepancy may be caused by interfering substances in the oil palm crude extract such as lipids or competition for substrates by other enzymes. Other explanatory factors include genotype dependency, thiolase mRNA transcripts of the same size and post-translational modification. The expression profile obtained in the Northern analysis is in agreement with that of sterol and carotenoid accumulation during fruit ripening. Sequence analysis with biocomputing tools showed that β-ketothiolase cDNA is relatively lowly expressed in oil palm mesocarp throughout its development. The gene was used in an intervention strategy to substitute for bacterial β-ketothiolase by redesigning the PHB transformation vector driven by oil palm mesocarp-specific promoter (MSP1), for synthesizing biodegradable plastics in oil palm. The new vector, designated pMS35, was later transformed into oil palm embryogenic calli using the biolistics approach. Currently, Basta-resistant embryogenic calli have been obtained and are undergoing proliferation and regeneration.

Keywords: β-ketothiolase, oil palm, gene isolation, intervention strategy, polyhydroxybutyrate.

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INTRODUCTION

Oil palm is an important commercial oil crop in Southeast Asia and equatorial Africa. In Malaysia, about two-thirds of its agricultural area, *i.e*., over 4 million hectares, are under the crop, which produces more oil per unit land area than any other crop (Poku, 2002). Palm oil is an important vegetable oil as well as a raw material for the oleochemicals industry. With its high productivity, there is great potential to genetically engineer the palm to produce novel higher value products (Sambanthamurthi *et al*., 2000), for example, bioplastics, by diverting its assimilates from oil production to plastic biosynthesis. These novel plastics are known as polyhydroxyalkanoates (PHA), of which polyhydroxybutyrate (PHB) is the most common member.

Several plants have already been transformed to produce PHB (Poirier *et al*., 1995; Bohmert *et al*., 2000; Nawrath *et al*., 1994), a non-toxic thermoplastic with chemical and physical properties not unlike polypropylene. It has been used as a biodegradable and eco-friendly substitute for petroleum plastics, notably in the automobile and construction industries (Foulk *et al*., 2002; Heijenrath and Peijs, 1996).

PHB was first discovered by M Lemoigne in the bacterium *Bacillus megaterium* in 1926. It has since been found in other bacteria where it functions as the carbon and energy source. The most studied model system for PHB synthesis is *Ralstonia eutropha* (Houmiel *et al*., 1999), in which PHB is synthesized in a three-step pathway. First β-ketothiolase catalyzes condensation of two acetyl-CoA molecules into acetoacetyl-CoA which is then reduced to β-hydroxybutyryl-CoA by the action of NADPH-dependent acetoacetyl-CoA reductase. PHB synthase then polymerizes β-hydroxybutyryl-CoA to PHB (Steinbuchel and Fuchtenbusch, 1998). Currently, PHB is produced by costly bacterial fermentation and is thus more expensive than conventional petroleum plastics. If the highly productive oil palm can be made to produce PHB, then the cost would most likely fall, and this has beckoned biotechnologists in recent years. The best attempt so far has been with *Arabidopsis thaliana* which was made to produce PHB to 42 mg g^{-1} of the plant fresh weight. But, the growth of the plants was severely reduced (Bohmert *et al*., 2000).

The β-ketothiolase was suggested to be a rate-limiting enzyme for PHB production (Daae *et al*., 1999). It is also known as acetoacetyl-CoA thiolase and occurs in both eukaryotes and prokaryotes. Biochemical purification has been carried out from bacteria (Nishimura *et al*., 1978; O' Connell *et al*., 1990; Senior and Dawes, 1973) as well as from chicken (Clinkenbeard *et al*., 1973) and rat peroxisomes (Antonenkov *et al*., 2000). The Claisen condensation catalyzed by β-ketothiolase is the first step in most biosynthetic pathways. Specifically, the enzyme plays a major role in the biosyntheses of ketone bodies, cholesterol, steroid hormones and isoprenoids in higher eukaryotes. Most of the literature on β-ketothiolase are on non-plants.

Constitutive expression of the β-ketothiolase gene, however, was reported to decrease the transformation rate, inhibiting the recovery of transformants and preventing the analyses of transgenic tobacco, potato and *Arabidopsis* plants (Bohmert *et al*., 2002). The problem can be partly solved using an inducible instead of constitutive promoter. When all the three PHB genes were transformed directly into the plastid (chloroplast) of tobacco, the negative transformation effect of the β-ketothiolase gene was not observed, but instead complete male sterility (Ruiz and Daniell, 2005). Due to the ketothiolase gene in plant systems and the possible negative effect of the bacterial β-ketothiolase gene, an intervention strategy was proposed for oil palm, *i.e*. redesign the PHB transformation vector driven by the oil palm mesocarp-specific promoter (MSP1) by substituting the bacterial β-ketothiolase (*phbA*) gene with the oil palm β-ketothiolase gene. The PHB production efficiency of both types of transgenic plants, derived from bacterial and oil palm gene, could then be compared. In addition, substituting the bacterial β-ketothiolase gene with oil palm β-ketothiolase gene will minimize the non-oil palm genes in the subsequent transgenic palms, the better for plant regeneration and transformation efficiency.

The aims of this study were three. First, to study β-ketothiolase activity during oil palm fruit ripening, by assaying the extracts from fruits of 6 to 20 WAA. Second, to isolate and characterize the cDNA using expressed sequenced tag (EST) pOP-CA90 which exhibits high sequence similarity to other β-ketothiolases. Finally, the gene is used to replace the bacterial gene for synthesizing biodegradable plastics in oil palm.

MATERIALS AND METHODS

Plant Material

Oil palm (*Elaeis guineensis* var. *tenera*) fruits from 6 to 20 WAA were obtained from the Malaysian Palm Oil Board (MPOB) – Universiti Kebangsaan Malaysia (UKM) Research Station in Bangi, Selangor.

Tagging the Oil Palm Inflorescences

Clusters of female oil palm flowers, also known as inflorescences, were tagged and labelled to obtain fruits at specific stages of ripening. Inflorescence that

were yellowish-white in colour were assumed to be two days old after anthesis.

Expressed Sequenced Tags

Phage stock carrying the pOP-CA90 clone was obtained from the Genomics Lab, Advanced Biotechnology and Breeding Centre, MPOB. This expressed sequenced tag (EST) clone had an intact 3′ poly(A) tail, obviating the requirement for 3′ RACE.

Crude Extract from Oil Palm Mesocarp

The mesocarp is the fleshy outer layer of the oil palm fruit just beneath the skin. Two different sets of crude extracts were prepared. Fresh mesocarp (50 g) was sliced and placed in 400 ml ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5; 10% glycerol; 2% PVPP; 5 mM DTT; 1 mM PMSF). This mix was blended in a Waring blender at high speed for 2 min. The homogenate was filtered through seven layers of muslin cloth and the filtrate centrifuged at 20 000 g for 15 min at 4°C. Three layers were formed, of which the middle one was carefully removed and filtered through glass wool. The filtrate was again centrifuged at 20 000 g for 15 min at 4°C, and the supernatant (crude extract) stored at -80°C until use.

β-ketothiolase Assay

The assay components in 1.5 ml final volume were in the concentrations: 0.106 M Tris-HCl, pH 7.3; 0.67 mM DTT; 0.806 mM acetyl-CoA; 1.88 mM β-NADH; 2.5 U β-hydroxyacyl-CoA dehydrogenase (auxillary enzyme). The mixture was pre-incubated at 30°C for 2 min before assay initiation by adding 100 µl of the crude extract. The rate of NAD^+ formation at 340 nm was measured by an UVIKON 923 Double Beam Spetrophotometer (Kontron Instruments). Protein content was determined by the method of Lowry *et al*. (1951).

Total RNA and Genomic DNA Isolation

Total RNA was isolated from the mesocarp by the method of Rochester *et al*. (1986). For genomic DNA isolation from young oil palm leaves, a CTAB-based extraction method was used. Briefly, 5 g fresh leaf were ground to powder in liquid nitrogen. Then, DNA extraction buffer [20 mM EDTA, pH 8.0; 0.1 M Tris-Cl, pH 8.0; 1.4 M NaCl; 2% (w/v) CTAB; 50 mM ascorbic acid; 40 mM DIECA; 286 mM β-mercaptoethanol] was heated to 60°C to facilitate dissolving the CTAB, and the solution then pre-equilibrated to 37°C. It was added to the powdered leaf and mixed well. PVPP was added in the ratio of 0.1 g per g leaf tissue. This was followed by incubation at 65°C for 25 min before being left to cool to room temperature. After that, the mix was inverted several times, and centrifuged at 10 000 rpm for 20 min. The aqueous phase was extracted twice with chloroform:isoamyl alcohol (24:1) followed by ethanol precipitation and quantification.

***In vivo* Excision**

The ExAssist™/SOLR system (Stratagene) was used to excise the pBluescript phagemid from the Uni-ZAP XR vector according to the protocol by the manufacturer.

Isolation of Full Length β-Ketothiolase cDNA

The 5′ RACE was performed using the Generacer™ (Version E) kit according to the manufacturer's instructions to isolate the 5′ end of cDNA. This 5′ end was amplified using touchdown PCR. The components in a 50 µl reaction were: 8 µl 10 x PCR reaction buffer; 1 µl 5′ race 1 primer (10 µM, 5′ATGTATGTATTGTTGGCGTTGC 3′); 3 µl Generacer 5′ primer (10 µM, 5′CGACTGGAG CACGAGCACACTGA3′); 1 µl RT-cDNA; 1 µl dNTP mix (10 mM each); 2 µl 50 mM $MgSO_4$; 5 U *Taq* DNA polymerase (Invitrogen, USA) and 33 µl sterile water.

Amplification was carried out using a GeneAmp 9700 thermocycler (Applied Biosystems, USA). The PCR programme was: 94°C for 2 min followed by 5 cycles of 94°C for 5 s and 72°C for 32 s. The next five cycles consisted of 94°C for 5 s, 70°C for 10 s, 72°C for 32 s followed by 25 cycles of 94°C for 5 s, 68°C for 10 s and 72°C for 32 s.

The PCR product was sub-cloned into the pCR®4-TOPO vector, transformed into TOP10 cells using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) and sequenced.

Northern Blotting and Hybridization

The oil palm mesocarp RNA was transferred to nylon membranes using the method described by Chomczynski (1992). ULTRAHyb (Ambion Inc., USA) was the hybridization buffer of choice with the EST clone pOP-CA 90 as radioactive probe. Labelling was done with the Megaprime DNA Labelling Systems kit (Amersham). Briefly, 25 ng probe were labelled with 4 µl [α-^{32}P]dCTP, 3000 Ci/mmol by adding 2 U Klenow polymerase. A Spehadex G-75 column was used to purify the radio-labelled probe. The heat-denatured probe was added to 1 ml ULTRAHyb and hybridization carried out at 42°C overnight with gentle shaking. The nylon membrane was washed for 15 min with 4X SSC, 0.1% SDS at 42°C. This washing step was repeated with 2X SSC, 0.1% SDS and the semi-dry membrane

exposed to X-ray film at -70°C and the film developed.

Southern Blotting and Hybridization

Genomic DNA was digested with four enzymes - *Eco*RI, *Eco*RV, *Bgl*II and *Rsa*I (30 μg DNA for each enzyme) - and subsequently loaded onto agarose gels. Transfer of the digested DNA from gel to Hybond-N^+ nylon membrane (Amersham International, UK) was achieved with vacuum transfer (Vacugene XL, Pharmacia LKB) and 0.4 M NaOH as transfer buffer. After 30 min of the transfer, the semi-dry membrane was UV crosslinked. The probe labelling and hybridization steps were identical to those described above. The washing solutions were pre-equilibrated at 42°C, and all the washing steps done for 15 min each with gentle shaking. The first wash was with 2X SSC, 0.1% SDS and this was repeated once. The final washing used 1X SSC, 0.1% SDS. The semi-dry membrane was exposed to X-ray film at -70°C and the film developed.

Sequence Analysis

The ORF Finder online software (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to obtain the open reading frames. Hydrophobicity and signal peptide analyses were done with Protscale (http://www.expasy.ch/cgi-bin/protscale.pl) and SignalP (http://www.cbs.dtu.dk/services/SignalP/), respectively.

DNA Manipulation

The plasmids used in this study are listed in *Table 1*. DNA manipulations were carried out using the standard protocols (Sambrook *et al*., 1989). Plasmid DNA isolation was carried out using the Plasmid Mini Kit (QIAGEN). Fragments generated by restriction endonuclease digestions were separated on agarose gel and purified by QIAquick Gel Extraction Kit (QIAGEN). Sticky-end ligations were carried out using T4 DNA ligase from Research Biolabs. Blunt-end ligations were carried out by filling the cohesive ends in a reaction volume using 2.5 units of the Platinum *Pfx* DNA polymerase (Invitrogen), 1 mM dNTP mixture, 1X *Pfx* amplification buffer and 1 mM $MgSO_4$, followed by incubation at 72°C for 30 min. *Escherichia coli* strain DH5α and strain STBL4 (Life Technologies) were used for the cloning experiments.

Polymerase Chain Reaction (PCR) Amplification

PCR was carried out in a 25 μl volume containing 100 ng plasmid DNA, 10 μM dNTPs mixture and 1.75 units Expand High Fidelity PCR System (Roche). The buffer solution contained 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.75), 20 mM $MgSO_4$, 1% Triton X-100 and 1000 μg BSA ml^{-1} supplemented with 1 μM of the respective primers. The primers were based on the sequence of the gene of interest and contained convenient cloning sites. Amplification reactions were carried out in a MJ Research Inc. Programmable Thermal Controller (PTC-100™ or PTC-200™). The amplified DNA fragments were later digested with the appropriate restriction endonuclease and cloned into pFC2 (Lonsdale *et al*., 1995) cloning vector for DNA sequencing and further cloning procedures.

Vector Construction

The DNA sequence encoding the transit peptide (*Tp*) was fused to the 5′ end of the β-ketothiolase gene sequence by PCR amplification using the primers TP2-F (5′-GGGGCGCCTTCCATGGCTTC-3′), ACCT1-R (5′-*CCAGATGGTGCC ATCTGGT TTTG*CAGCACA-3') and ACCT2-F (5'-CAAAACCAGATGGCACCATCTGGGGTTTATGA-3′) and ACCT3-R (5′-GGGGCGCGCCTCATAGCAAAGAGCGTTCGAT-3′). ACCT2-

TABLE 1. PLASMIDS USED IN THIS STUDY

Plasmid	Description	Reference or source
pGWI	Carrier of coding region of oil palm β-ketothiolase gene	MPOB
pACP3	Carrier of full length oil palm *ACP* gene with the transit peptide	MPOB
pFC2	Cloning vector with multiple cloning sites of pBlueSkript SK- flanked by unique rare cutter enzymes sites for cloning of useful genes	Lonsdale *et al*. (1995)
pMS3	Carrier of the *MSP1-Nos* cassette	MPOB
pMB41	Binary Ti vector with *MSP1-Nos* cassette sites flanked by RB7MAR	MPOB
pMS29	PHB expression vector driven by MSP1 promoter	MPOB
pMS31	PHBV expression vector driven by MSP1 promoter	MPOB
pMS23	*phbB* expression cassette	MPOB
pMS24	*phbC* expression cassette	MPOB

F and ACCT3-R were designed to recognize the 5′ and 3′ ends of the β-ketothiolase sequence, respectively, and TP2-F synthesized based on the sequence at the 5′ end of the *Tp* sequence. Primer ACCT1-R was designed to contain sequences complementary to the 3′ end of the *Tp* and 5′ end of the β-ketothiolase (the italicized sequences of primer ACCT1-R) genes. The amplified gene fusion was digested with *Asc*I, gel purified and cloned into the *Asc*I site of pFC2 to generate pACCT. The *TpACCT* fusion was released from pACCT by *Asc*I digestion, gel purified and ligated into the *Asc*I site of pMB41, and then transformed into DH5a cells. Positive clones were selected by *Asc*I digestion and the sense orientation of the ketotiolase gene confirmed by PCR amplification using primers ACCT2-F and NOS2-R (5 ' - G G A C T A G T G C T A G C G A T C T AGTAACATAGAT-3'). The plasmid generated from this cloning procedure was used as an intermediate plasmid to replace the bacterial *phbA* gene with the oil palm β-ketothiolase gene in plasmid pMS29 (carrying the 3 bacterial PHB genes driven by oil palm mesocarp-specific promoter and Basta-resistant gene driven by the maize ubiquitin promoter) (Masani *et al*., 2008).

RESULTS AND DISCUSSION

Determination of β-ketothiolase Activity

The β-ketothiolase activity was measured in a condensation reaction. The literature indicates that the commonly used Mg^{2+}-enolate assay which measures the activity by the thiolysis reaction is prone to underestimation (Antonenkov *et al*., 1999).

A coupled assay was therefore used instead, the primary reaction of which was formation of acetoacetyl-CoA from acetyl-CoA catalyzed by β-ketothiolase. Acetoacetyl-CoA was then reduced by β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) to L(+)-β-hidroxybutyryl-CoA concurrent with the oxidation of NADH to NAD^+ (Lynen *et al*., 1952).

The β-ketothiolase activity levels were measured in crude extracts of mesocarp at 6 to 20 WAA. The highest activity of 0.1085 U mg^{-1} was found at 8 WAA while 19 WAA showed the lowest activity of 0.0292 U mg^{-1}. There was a general decrease in β-ketothiolase activity from 8 WAA to 19 WAA after which it recovered slightly to 0.0799 U mg^{-1} (*Figure 1*). Thus, the younger fruits show higher β-ketothiolase activity. A *t*-test was conducted and no significant difference found between the two β-ketothiolase activity data sets used to generate the profile.

The lower β-ketothiolase activity in older fruits can be explained by lipid biosynthesis. Lipid biosynthesis begins in the fruits at about 15 WAA, with decreasing β-ketothiolase levels in the mesocarp. Acetyl-CoA is the raw substrate for isoprenoid and lipid biosyntheses in oil palm, so increasing lipid production would require more of it in the plastids, 'crowding out' the cytosolic acetyl-CoA pool for other metabolic pathways, such as the isoprenoid pathway involving β-ketothiolase.

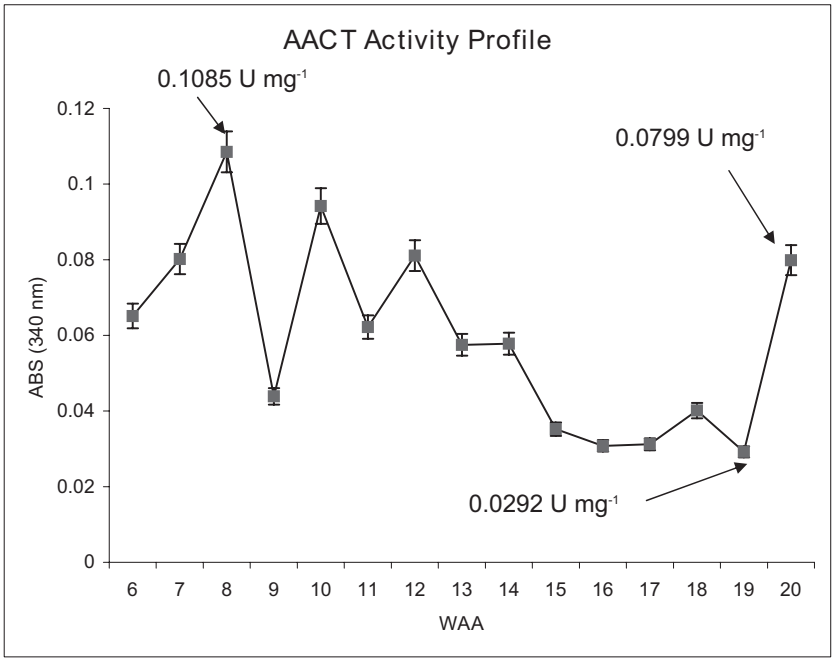

Figure 1. Activity profile of β-ketothiolase in crude extracts from 6 to 20 weeks after anthesis (WAA) oil palm fruit mesocarp.

Generation of Full Length β-ketothiolase cDNA

The pOP-CA 90 EST clone was completely sequenced, and gene-specific RACE primers designed based on this sequence. *Figure 2* shows the sequencing reactions conducted to obtain the complete sequence of pOP-CA 90.

The pOP-CA 90 EST clone had a poly(A) tail at the 3′ end, obviating the need for 3′ RACE to isolate the 3′ end of oil palm β-ketothiolase cDNA. A pair of gene-specific, high T_m (>70°C) 5′ RACE anti-sense primers were designed using the Primer Premier 5 software (Premier Biosoft International, USA). The primers are listed below:

5′ race 1 (5′ CATTCGACATGCTTTCCATGCCACCAGC 3′)
5′ race 2 (5′ CGACATGCTTTCCATGCCACCAGCC 3′)

These primers had a high GC content of 50% – 70%, making them high T_m primers. These characteristics enabled their use in touchdown PCR to increase the specificity of amplification.

The RACE products were analysed on agarose gel (*Figure 3*). Through comparison with DNA standards, both the RACE products were found to be ~500 bp. No positive result was seen in the negative control. The distances of the priming sites for 5′ race 1 and 5′ race 2 to the 5′ end of pOP-CA 90 were 443 bp and 439 bp, respectively. This showed the pOP-CA 90 clone to be almost full length with only a slight truncation at the 5′ end. The RACE products were cloned into pCR®4-TOPO vectors, transformed and later sequenced. It was found that the full length β-ketothiolase sequence had an extra 22 bases compared to the truncated pOP-CA 90 clone.

Northern Analysis

Despite the advent of powerful techniques like the nuclease protection assay and real-time PCR, Northern analysis has remained the standard method for detection and quantitation of mRNA. This is probably because Northern analysis is the only method that provides information about the mRNA size, and that it is a relatively simple and straight-forward procedure. The Northern membrane is hybridized to a labelled probe complementary to the desired mRNA, and the signals generated through probe detection then used to determine the size and abundance of the target mRNA (Alwine *et al*., 1977).

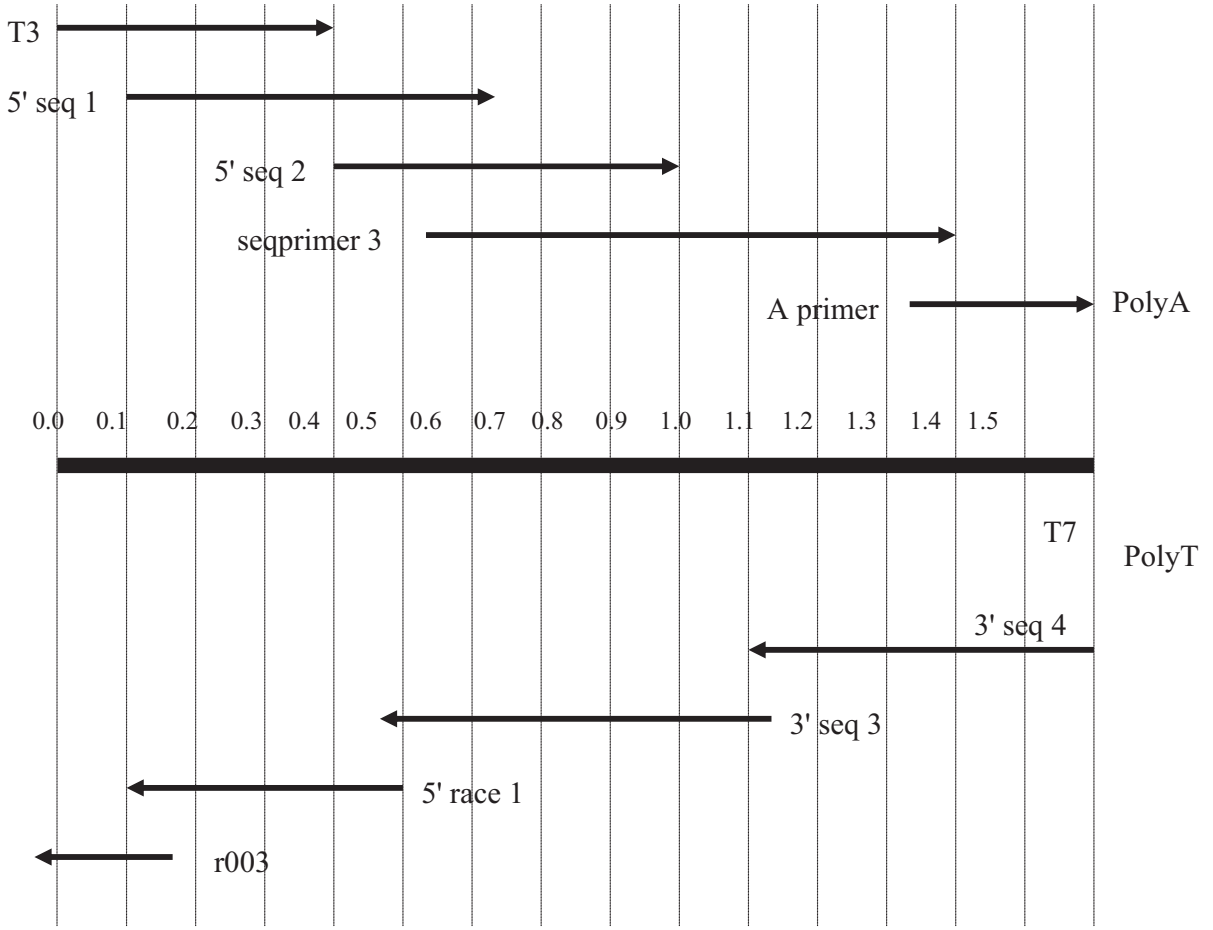

Figure 2. Complete sequencing of the pOP-CA 90 clone through primer walking.

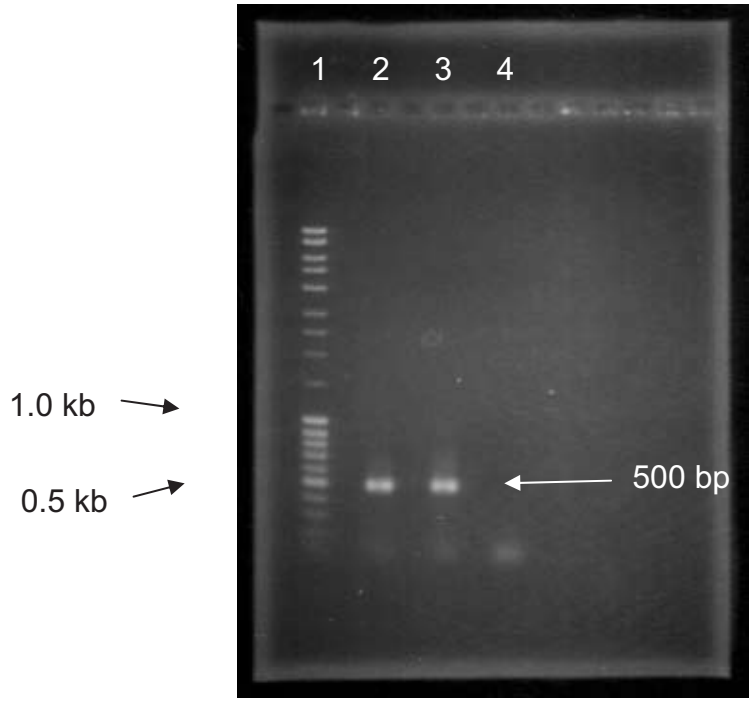

Figure 3. The 5' RACE products produced using the primers 5' race 1 and 5' race 2. Lane 1: MassRuler DNA Ladder Mix (Fermentas); lane 2: 5' RACE product using 5' race 1 primer; lane 3: 5' RACE product using 5' race 2 primer; and lane 4: negative control (no template).

Total RNA was isolated from 6, 8, 11, 13, 17 and 20 WAA oil palm mesocarp. The yield and purity of the isolated RNA were determined prior to preparing the Northern blot. With the EST clone pOP-CA 90 as radioactive probe, a single hybridization signal was detected in all the sample lanes (*Figure 4*). The size of this signal was ~1.5 kb, in close agreement to the estimated full length

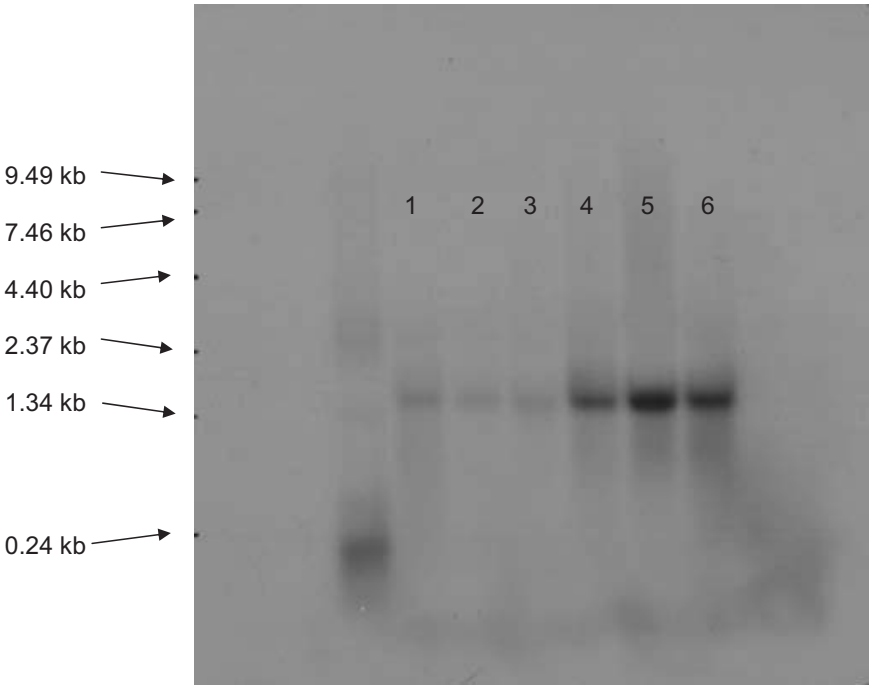

Figure 4. Northern analysis result for β-ketothiolase cDNA in oil palm with two weeks' exposure time. A signal of size ~1.5 kb was detected in all the sample lanes. Lanes 1 - 6 represent 10 ug total RNA from 6, 8, 11, 13, 17, 20 weeks after anthesis (WAA) mesocarp, respectively.

β-ketothiolase cDNA isolated through PCR-RACE. It was clearly shown on the autoradiograph that β-ketothiolase was more highly expressed in the 13, 17 and 20 WAA RNA by the more intense signals. Weak signals were seen in the 6, 8 and 11 WAA RNA samples. From this result, it is concluded that β-ketothiolase expression is up-regulated during fruit ripening.

Vollack and Bach (1996) isolated a full length radish β-ketothiolase transcript of 1.5 kb, similar in size to the β-ketothiolase cDNA isolated in this study. However, Vollack and Bach found no difference in β-ketothiolase expression in radish with maturity. Oxoacyl-CoA thiolase (OACT) is close to β-ketothiolase in sequence similarity and reaction mechanism. Studies on OACT isolated from mango have found its expression to be related to the fruit ripening, as with oil palm. Further exposure of the autoradiograph revealed a faint band in the RNA samples from young fruit (Bojorquez and Gómez-Lim, 1995).

Southern Analysis

Genomic DNA of satisfactory yield and quality was isolated from young oil palm leaf. Four restriction enzymes - *Eco*RI, *Eco*RV, *Bgl*II and *Rsa*I - were used to digest the genomic DNA. The pOPCA 90 clone was once again used as radioactive probe in Southern analysis. *Figure 5* shows the autoradiograph of the Southern hybridization. About five to six signals were seen in every oil palm RNA sample lane. The result concurs with that of Vollack and Bach (1996) on radish β-ketothiolase. Based on this result, it can be deduced that oil palm β-ketothiolase occurs as a single gene or a small family of genes. Mango OACT is only present in single copy (Bojorquez and Gómez-Lim, 1995), as also in cucumber (Preisig-Müller and Kindl, 1993). All eukaryotic thiolases are encoded by genomic DNA (Igual *et al*., 1992).

Sequence Analysis

The β-ketothiolase full length cDNA obtained was subjected to BLAST (basic local alignment search tool) (Altschul *et al*., 1997) similarity search. Both blastx and blastn searches revealed *Hevea brasiliensis* b-ketothiolase cDNA and amino acid sequence as the most significant hits. Other significant hits included thiolases from *Oryza sativa*, *Arabidopsis thaliana* and *Raphanus sativus*.

The most probable open reading frame (ORF) encoded by the β-ketothiolase full length sequence was determined by the online ORF Finder application (available at http://www.ncbi.nlm.nih.gov), encoded

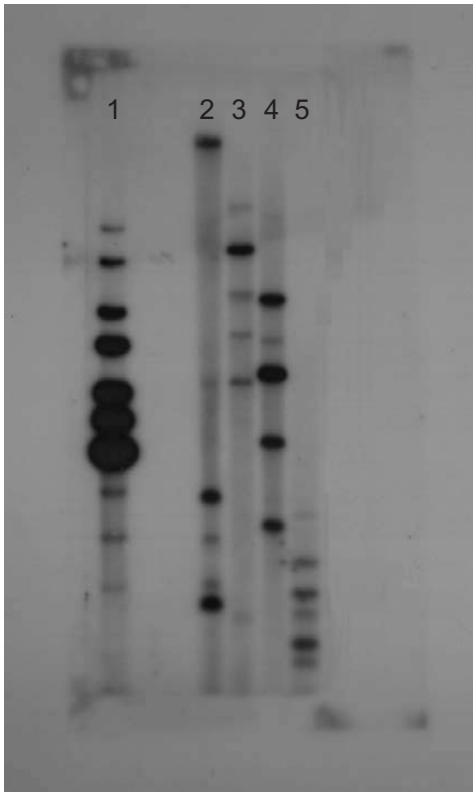

Figure 5. Result of Southern analysis for the β-ketothiolase gene in oil palm with exposure of six days. Multiple signals are seen in every lane. Lane 1: 1 kb DNA ladder (Fermentas). Lanes 2 - 5 represent genomic DNA samples digested with EcoRI, EcoRV, BglII and RsaI, respectively.

by the +2 frame. It began with the ATG starting codon and ended with the stop codon, TGA. The ORF started at base 50 and lasted until base 1297, encoding a polypeptide of 415 amino acids (*Figure 6*). The length of the 5' UTR was 49 bp while the 3' UTR was much longer by 240 bp.

The sequence CCATTTATGGC containing the start codon (in bold) at the border of the 5' UTR and

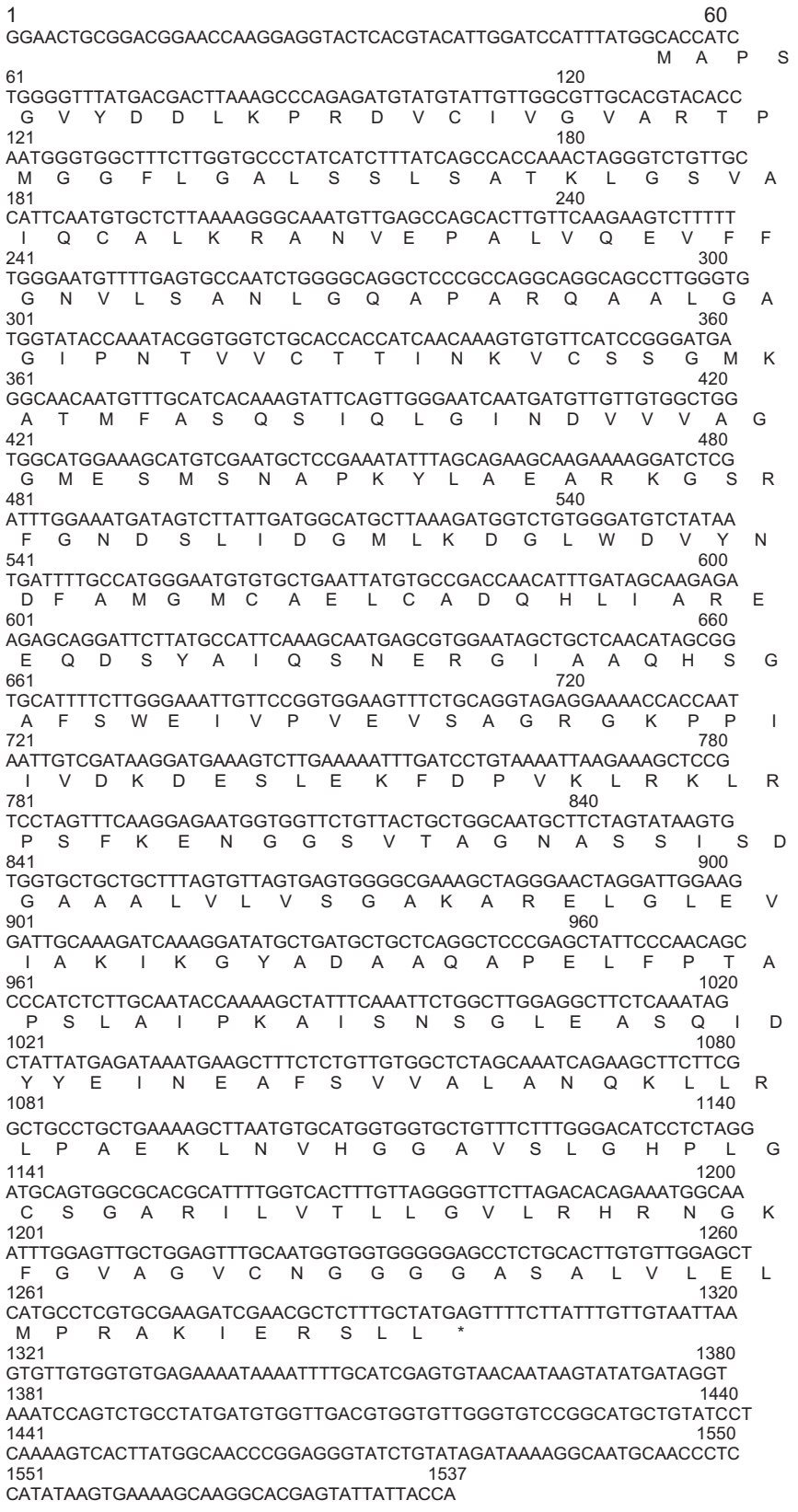

Figure 6. Complete sequence of the oil palm β-ketothiolase cDNA enzyme.

Note: * represents the stop codon.

the ORF did not fulfil the requirements of a consensus sequence for translation initiation based on previous work. In most plant mRNAs, the sequences TAAACATGGCT (Joshi, 1987) and AACAATGGC (Lutcke *et al*., 1987) have been identified as the consensus sequences. The bases surrounding the start codon play an important role in the efficiency of translation initiation (Futterer and Hohn, 1996). When these bases differ from the consensus, a part of the 40S ribosome subunit is unable to identify the ATG codon as the starting point for translation, and this will lower the rate of polypeptide synthesis. However, Kirsi and Williams (1990) showed that consensus sequences do not play major roles in translation.

The 5' UTR sequence was sent for a translation initiation efficiency test (available online at http://wwwmgs.bionet.nsc.ru/programs/act/mo_mRNA.htm) based on Kochetov *et al*. (1999). The results showed that oil palm β-ketothiolase cDNA is expressed at low levels.

Hydrophobicity analysis was conducted using the Kyte and Doolittle (1982) algorithm. A total of 42 peaks in the sequence exceeded the hydrophobic threshold of +1.22 (*Figure 7*). These peaks were scattered evenly throughout the sequence with none of the 20 to 23 consecutive hydrophobic regions forming a transmembrane domain. It was thus concluded that the protein encoded by oil palm β-ketothiolase is not a *trans*-membrane protein, and more likely a cytosolic protein. Although no *trans*-membrane region was found, there were several small regions with high hydrophobicity, for example, in residues 28 - 33, 266 - 273 and 370 - 376. It is likely that these small hydrophobic regions form inner core domains that function in ligand binding. The β-ketothiolase amino acid sequence was subjected to signal peptide analysis using SignalP v2.0.b2 (Nielsen *et al*., 1997) with neural network and Hidden Markov Model parameters (Nielsen and Krogh, 1998), and no signal peptide was detected.

Activity Profile *vs*. Northern Analysis

The activity profile obtained through biochemical assays did not correlate with the Northern analysis result. By the profile, the highest activity occurred in eight WAA mesocarp, followed by 10 WAA. The expression of β-ketothiolase mRNA was strongest at 17 WAA, 13 WAA and 20 WAA, with only faint expressions seen in six, eight and 11 WAA mesocarp total RNA.

Previous work on the bacterium *Candida tropicalis* revealed the cytosolic and peroxisomal β-ketothiolase to be encoded by the same gene (Kanayama *et al*., 1997). The mRNA transcripts of these two thiolases were of similar sizes as well at ~1.7 kb. Currently, there is no evidence of this phenomenon occuring in plants. However, more research is needed to conclusively rule this out. It is widely believed that there are a few thiolase isozymes in eukaryotes with a similar size and function, and Northern analysis may not be able to differentiate closely-related mRNA transcripts of similar sizes.

The biochemical assay for β-ketothiolase activity profile was done on crude and not purified extracts, so there may well be interfering substances present. More sensitive biochemical methods, such as quantitative immunoblotting [used by Fukao *et al*. (1997)] to study the levels of the human β-ketothiolase enzyme and its mRNA transcripts) may need to be used.

Post-translational modification on the β-ketothiolase enzyme by CoA has been documented. Both b-ketothiolase and OACT in the rat mitochondrial matrix undergo modification that determines the half-life of the mature enzyme and

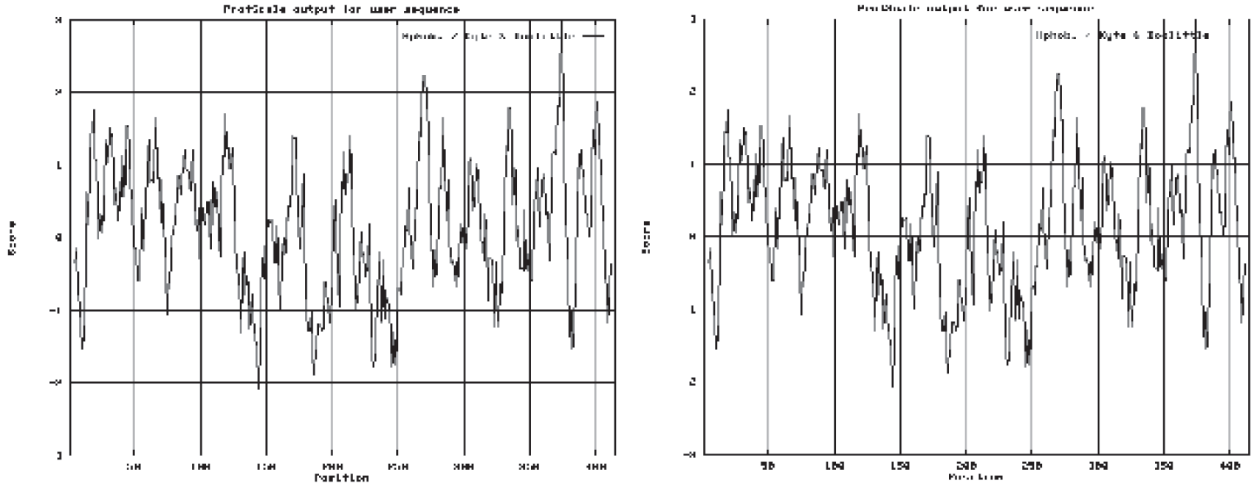

Figure 7. Hydrophobicity of the amino acid sequence of β-ketothiolase.

its subsequent turnover rates (Huth *et al*., 1988; 1991; Quandt and Huth, 1984; Schwerdt and Huth, 1993). Modification by CoA may make β-ketothiolase more susceptible to proteolytic cleavage, but, conversely, also protect β-ketothiolase from degradation by matrix proteases (Huth *et al*., 2002). So far, there has been no study on β-ketothiolase post-translational modification in plants.

A lipase study has revealed genotype dependency in the oil palm population. Sambanthamurthi *et al*. (2000) found that lipase activity differed greatly between palms. This could have happened in this study as two oil palm populations were used in both the β-ketothiolase assay and Northern analysis, and may have contributed to the conflicting results.

The β-ketothiolase enzyme plays a role in the early isoprenoid pathway. Isoprenoid compounds are the oldest biomolecules known to man when hopanoid derivatives was discovered from 2.5 billion-year-old sediment (Summons *et al*., 1999; Brocks *et al*. 1999). The isoprenoid family consists of about 30 000 members and derivatives (Buckingham, 1998) and has various biological functions as listed below:

- quinone compounds in the electron transport chain;
- membrane components (prenyl lipids in archaebacteria and sterols in eubacteria and eukaryotes);
- sub-cellular regulation and targeting (protein prenylation);
- photosynthetic pigments (carotenoids and the chlorophyll side chain);
- hormones (gibberellin, brassinosteroids and abcissic acid); and
- defense compounds in plants (monoterpenes, sesquiterpenes and diterpenes).

As currently known, isoprenoids are formed via two pathways. The classical mevalonate pathway (MVA) involves the formation of mevalonate as an intermediate, catalysed by β-ketothiolase. The non-mevalonate pathway (DxP) was more recently discovered and is characterized by condensation of pyruvate and glyceraldehydes-3-phosphate to 1-deoxyxylulose-5-phosphate (DxP). There has been no conclusive evidence which pathway (or both) operate in the oil palm.

Studies incorporating ^{14}C-labelled substrates into oil palm 20 WAA mesocarp and TLC analysis showed that isopentenyl pyrophospate (IPP) is the main intermediate in carotenoid synthesis. The ^{14}C-G3P (glyceraldehyde-3-phosphate) and ^{14}C-pyruvate were not incorporated into α- and β-carotenes, suggesting that carotenoid synthesis in the oil palm is through the MVA (Jane Sonia Kaur, 2002).

Studies on the carotenoid content in oil palm mesocarp by Ilango (1987) have shown that the level rises sharply after 15 WAA and continues until 20 WAA. This supports the Northern analysis result of concomitant rises in the carotenoid content and β-ketothiolase mRNA expression, consistent with the hypothesis that oil palm carotenoids are produced via the MVA pathway. The process of fruit ripening involves increased cell division which would indirectly require more sterol production (Chapell, 1995) and, in turn, higher carbon flux through the MVA pathway.

Isoprenoid compounds usually accumulate slowly over a rather long time, suggesting that the enzymes involved in their biosynthesis are present only in low quantities or have a low turnover rate (rate of transformation of substrate to product per enzyme molecule per unit time).

For example, sterol accumulates slowly in immature fruits but when a tissue reaches maturity, sterols contribute to about 0.1% - 0.4 % dry weight. The sterol biosynthetic machinery in plants is not very large. Other isoprenoids synthesized at low rates include sesquiterpenes, monoterpenes and diterpenes, depending on the tissue or cell (Chapell, 1995). This is in agreement with the current study where the analysis of translation efficiency showed the β-ketothiolase mRNA to be lowly expressed. Besides, the sequence that flanked the starting codon in the β-ketothiolase mRNA did not agree with the designated consensus sequence and may lower the translation efficiency. A combination of all or some of the factors mentioned above may explain the low expression of β-ketothiolase mRNA in six, eight and 11 WAA RNA samples as well as the long exposure time required in the Northern analysis.

In photosynthetic eukaryotes, *e.g Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella fusca isoprenoids*, are only produced via the DxP pathway. Other organisms like the rodophytes *Cyanidium caldarum* and *Ochromanas danica* have both the MVP and DxP pathways. In contrast, *Euglena gracilis* has just the MVA pathway (Disch *et al*., 1998). In higher plants, all the cell machinery required for sterol production are in the cytosol (Bach *et al*., 1999). Much more work is required to investigate the intricacies of plant isoprenoid production, especially the metabolite flux through both the pathways.

Construction of the PHB Vector with Oil Palm β–Ketothiolase Gene (pMS35)

The oil palm β-ketothiolase was subsequently used in an intervention strategy by replacing the bacterial gene with it. As PHB synthesis occurs in the plastids, a transit peptide sequence was attached to the oil palm β-ketothiolase gene at the 5' end to target the gene product to the plastid. A technique

involving PCR was employed to join the frame of the oil palm transit peptide sequence (*Tp*) of the acyl-carrier-protein (ACP) gene to the β-ketothiolase gene. The linking involved three PCR reactions. The first PCR added a small portion of the 5' end of the β-ketothiolase gene to the 3' end of the *Tp* sequence. The second added a small portion of the 3' end of the *Tp* to the 5' end of β-ketothiolase gene. The third used the overhangs produced in the first and second PCR to extend across the junction and produce a full length *Tp* linked in frame with the β-ketothiolase gene. For the first PCR, the template DNA was pACP3 and the primers TP2-F and ACCT1-R. The resulting PCR product of 0.2 kb fragment was gel purified (*Figure 8a*). In the second PCR, the template DNA and primers used were pGWI, ACCT2-F and ACCT3-R. The product of the reaction was again run on agarose gel and the expected 1.1 kb band gel purified (*Figure 8a*). The third PCR used the DNA isolated from the two preceding PCRs as template with primers TP2-F and ACCT3-R. As shown in *Figure 8a*, the reaction produced a 1.3 kb product corresponding to the fusion of *Tp* and the β-ketothiolase gene flanked by an *Asc*I site (underlined sequences in the primers TP2-F and ACCT3-R).

Replacing the β-ketothiolase (*phbA*) gene in pMS29 with the plastid targeted oil palm β-ketothiolase (*TpACCT*) gene to create the pMS35 expression vector required the construction of pMS34 as an intermediate expression vector (*Figure 9*). The 1.3 kb *Asc*I *TpACCT* fragment of pACCT was cloned into the *Asc*I site of pMS3 to give an approximately 9.8 kb pACCTMSP expression vector. *Spe*I digestion of pACCTMSP was performed to cleave an approximately 2.5 kb *MSP1-TpACCT-Nos* for ligation to the *Avr*II site of pMS23 (*Figure 8b*). The resulting vector containing the *MSP1-TpACCT-Nos-MSP1-TpphbB-Nos* expression cassette (confirmed by the *Spe*I digestion) was designated pMS34. To clone the *MSP1-TpphbC-Nos* into pMS34 for the construction of pMS35, pMS24 was first digested

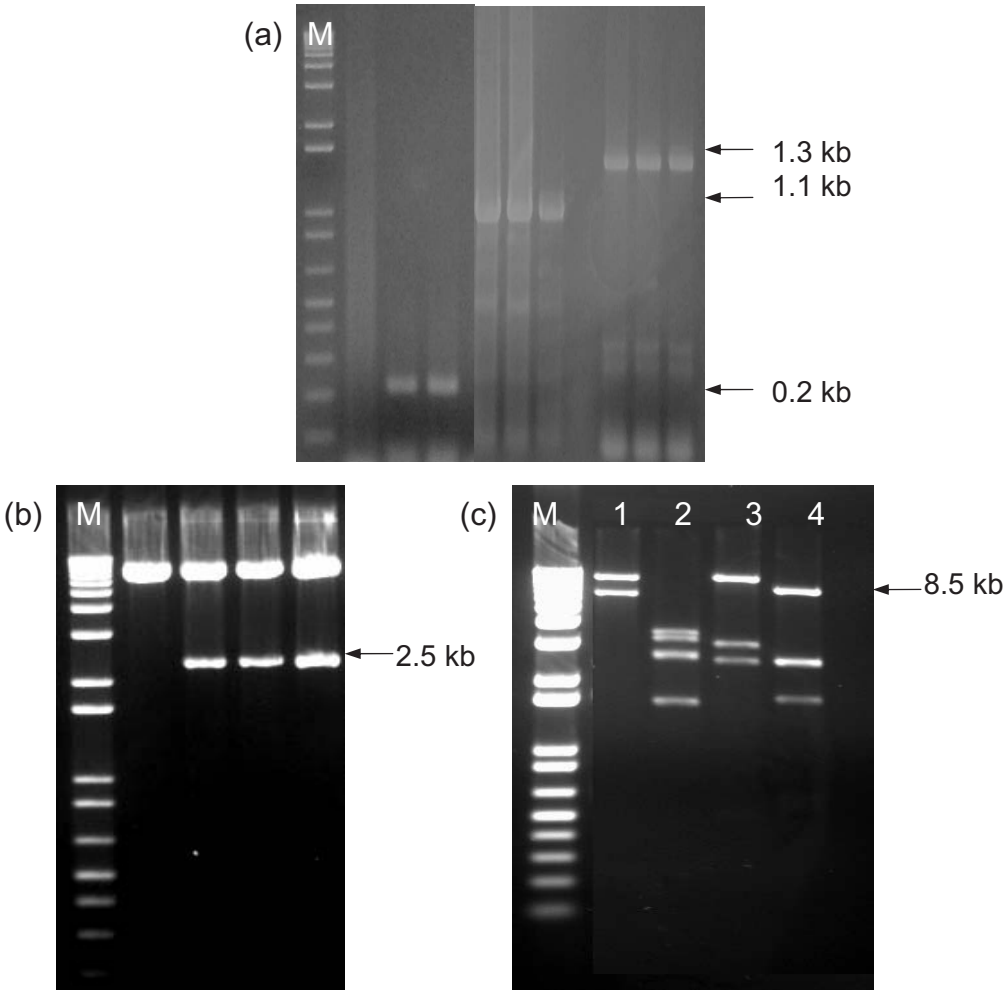

Figure 8a. PCR for fusion of Tp and β-ketothiolase (arrows indicate the PCR products of 0.2 kb, 1.1 kb and 1.3 kb using primers TP2-F and ACCT1-F, ACCT2-F and ACCT3-F, and TP2-F and ACCT3-F, respectively). b. Digestion of pACCTMSP with SpeI (arrow indicates the 2.5 kb DNA fragment of MSP1-TpACCT-Nos). c. Restriction enzymes analysis of pMS35 (lane 1: SpeI, lane 2: BglII, lane 3: BamHI, lane 4: SalI, and arrows indicate the 8.5 kb DNA fragment of MSP1-TpACCT-Nos-MSP1-TpphbB-Nos-MSP1-TpphbC-Nos).

with *Spe*I and the 3.4 kb DNA fragment of *MSP1-TpphbC-Nos* purified and ligated to the *Avr*II site of pMS34. The final pMS35 clones containing the *MSP1-TpACCT-Nos-MSP1-TpphbB-Nos-MSP1-TpphbC-Nos* expression cassette were identified by *Spe*I digestion for the expected DNA fragment of ~8.5 kb (*Figure 8c*).

The pMS35 plasmid was later used to transform oil palm embryogenic calli using the biolistic approach as described by Parveez (1998). The bombarded calli were later subjected to selection using the herbicide Basta. Many Basta-resistant transformed calli were obtained and are currently undergoing proliferation and regeneration to produce transgenic oil palm. It would require several more years before the effect of this oil palm gene vis-à-vis the bacterial gene can be evaluated - at least two years to produce plantlets from the transformed calli, one year in the nursery and then three years after field planting for the palms to fruit and produce/not produce the products expected.

As reported earlier, constitutive expression of the bacterial β-ketothiolase gene has had a detrimental effect on the transformation process of a number plants (Bohmert *et al*., 2002), as well as caused male sterility (Ruiz and Daniell, 2005). Using the plant β-ketothiolase gene in oil palm may overcome the adverse effects suffered. Furthermore, it is hoped that the endogenous gene may be more efficient than the bacterial gene, resulting in more PHB synthesized in oil palm.

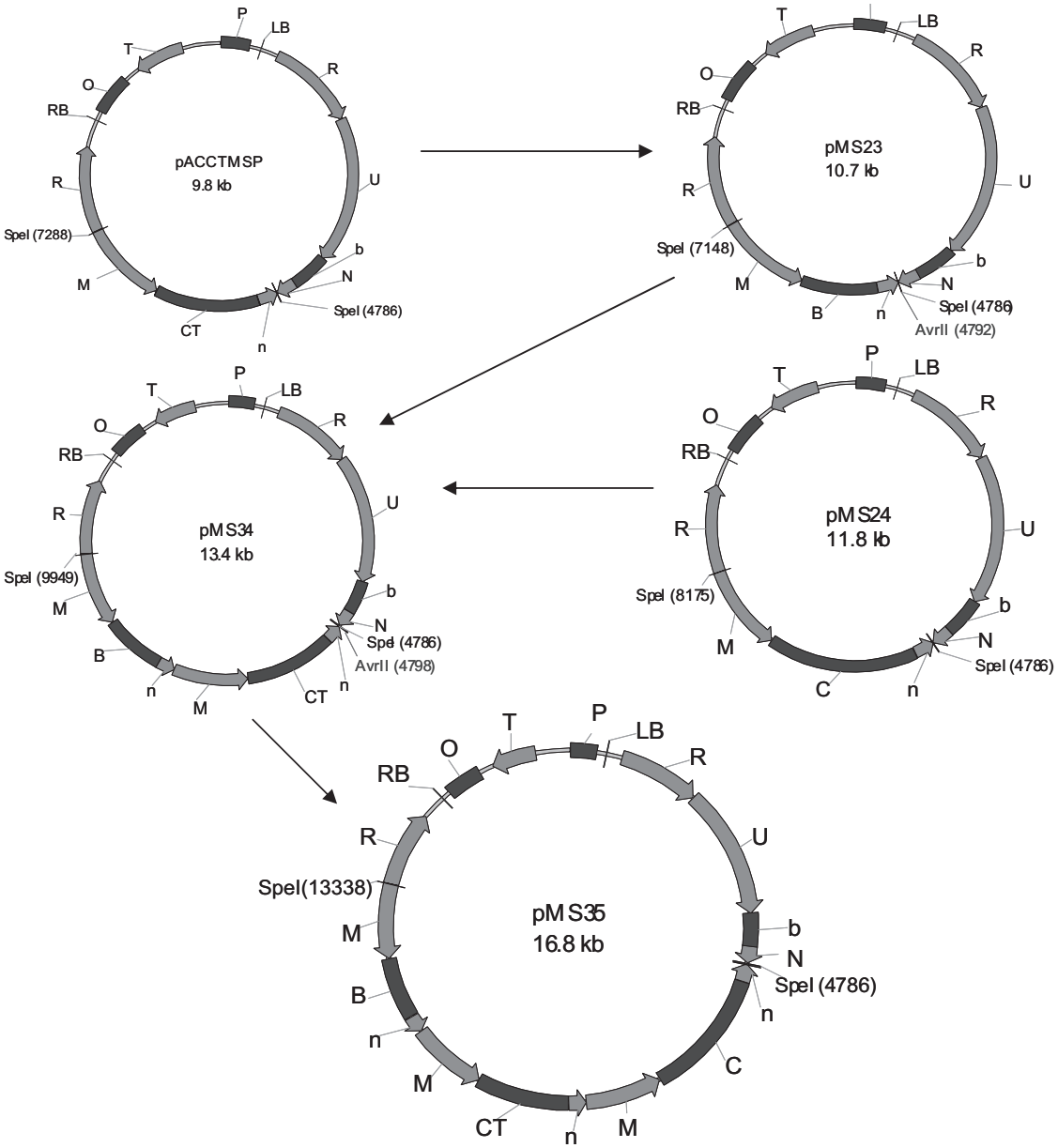

Figure 9. Construction of pMS35 for production of PHB. The restriction sites (cloning sites) and the numbers indicate the approximate position in the vectors. CT: TpACCT, B: TpphbB, C: TpphbC, M: MSP1, N and n: Nos, O: CoIE1 ori: P: pSa Ori: R: RB7MAR, T: npt1, U: UbiPro, b: bar. LB: left border, RB: right border.

CONCLUSION

Highest levels of β-ketothiolase specific activity in oil palm mesocarp crude extracts were observed in oil palm fruits 8 to 11 WAA. A ~1.5 Kb fragment of β-ketothiolase gene was successfully isolated using RT-PCR and RACE approaches. A encodes for a protein of 415 amino acids with a predicted relative molecular weight of 43 217 Da. Northern analysis revealed that β-ketothiolase mRNA transcripts are present in higher quantities in the riper (13, 17, 20 WAA) than younger fruits at 6, 8 and 11 WAA, contradicting the biochemical activity profile. The gene was later successfully used in an intervention strategy to substitute for bacterial β-ketothiolase by redesigning the PHB transformation vector driven by oil palm mesocarp-specific promoter (MSP1), for synthesizing biodegradable plastics in oil palm. The new vector was finally been used to transform oil palm embryogenic and the bombarded embryogenic calli are undergoing proliferation and regeneration.

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