THE ISOLATION AND CHARACTERISATION OF OIL PALM (Elaeis guineensis Jacq.) 
\(\beta\)-KETOACYL-ACYL CARRIER PROTEIN (ACP) SYNTHASE (KAS) II cDNA

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ABSTRACT
Modulating endogenous levels and/or producing novel fatty acids of oils have gained significant attention in recent years to meet the demand for oils for specific markets. The commodity palm oil is composed mainly of four fatty acids: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2). The percentages of these fatty acids in palm oil average 44%, 4%, 39% and 10%, respectively, with trace amounts of other fatty acids. Metabolic engineering may be used to produce oil crops with desired fatty acid compositions. We have isolated and characterised \(\beta\)-ketoacyl ACP-synthase II (KASII) cDNA from oil palm (Elaeis guineensis) which is one of the main components for the oil palm genetic engineering programme. KAS II is associated with the accumulation of palmitic acid in oil palm, and its role in controlling the ratio of C16:C18 has been previously determined. We isolated KAS II cDNA from oil palm, and functionally characterised the same in Escherichia coli and Arabidopsis. Partial length KAS II cDNA was first obtained by the reverse transcriptase-polymerase chain reaction (RT-PCR). Rapid amplification of cDNA ends (RACE) was then used to isolate both the 5’ and 3’ ends of the KAS II sequences. Assembly of the partial length sequence fragments, including the 5’ and 3’ ends, allowed for the full-length sequence information on the KAS II cDNA to be obtained and used in the gene isolation. Expression studies in E. coli resulted in an increase in oleic acid at the expense of palmitic acid. Arabidopsis thaliana was also used to further confirm the functional activity of the oil palm KAS II. A significant decrease in C18:0 and accumulation of C16:0 were detected in the plants that had been transformed with the antisense KASII construct. This suggests that the substrate specificity of the oil palm KAS II is similar to that of KAS II from other plants which preferentially elongate palmitic to stearic acids. The oil palm KAS II may, therefore, be useful in providing new opportunities in the genetic engineering programme for the production of high-value products such as an oil with a high content of monounsaturated fatty acids from the transgenic oil crops.

Keywords: oil palm (Elaeis guineensis), \(\beta\)-ketoacyl-acyl carrier protein synthase II, gene isolation.

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INTRODUCTION
Plant oils are used in a variety of industrial and edible applications. Novel vegetable oil compositions and/or improved means to obtain such oil compositions, from biosynthetic or natural resources, are needed to meet the demand for expanding the range of high-energy and nutritious foods. Palm oil, a well-balanced healthy edible oil,
is now an important energy source for mankind. Today, it is widely acknowledged to be a versatile and nutritious vegetable oil, trans fat-free, with a rich content of vitamins and antioxidants. Palm oil extracted from the mesocarp layer of the oil palm fruit contains about 50% saturated, 40% monounsaturated and 10% polyunsaturated fatty acids. Its fatty acid composition consists of 44% palmitic acid (C16:0), 5% stearic acid (C18:0), 39% oleic acid (C18:1) and 10% linoleic acid (C18:2) (Tan and Oh, 1981).

Malaysia being the second largest producer of palm oil after Indonesia, has proposed many strategies to maintain profitability and to combat potential threats, such as shortages of labour and land resources. Biotechnology offers vast opportunities for energising and revolutionising the oil palm industry, and one of the initiatives is through genetic engineering to modify the fatty acid composition of palm oil to suit specific applications (Sambanthamurthi et al., 2002). However, modification of biochemical processes would require a large initial outlay to provide the means to change fatty acid composition. For instance, the means to transfer genetic material to the plant in a stable, heritable manner and nucleic acid capable of producing the desired phenotypic result must be available.

Higher plants appear to synthesise fatty acids via a common metabolic pathway. In developing seeds, de novo production of fatty acids takes place in the proplastids. These fatty acids are attached to triacylglycerides and stored as a source of energy for further germination. The first step is the formation of acetyl-ACP (acyl carrier protein) from acetyl-CoA and ACP catalysed by the enzyme, acetyl-CoA:ACP transacylase. Elongation of acetyl-ACP to 16- and 18-carbon fatty acids involves a series of four reactions:

- condensation with a two-carbon unit from malonyl-ACP to form a \( \beta \)-ketoacyl ACP (KAS II);
- reduction of the keto-function to an alcohol (\( \beta \)-ketoacyl ACP reductase);
- dehydration to form enoyl-ACP (\( \beta \)-hydroxyacyl-ACP dehydrase); and finally
- reduction of the enoyl-ACP to form the elongated saturated acyl-ACP (enoyl-ACP reductase).

Typically, KAS II operates in the plastid together with other enzymes of the fatty acid synthase (FAS) system, acting on acyl-ACP to produce fatty acids with carbon chain lengths of <18 carbons (Browse and Sommerville, 1991; Harwood, 1998). Of the three known classes of plant KAS enzymes, KAS I, KAS II and KAS III, only KAS II (EC 2.3.1.179) shows the highest activity towards elongating palmitoyl-ACP (C16:0-ACP) to oleoyl-ACP (C18:0-ACP) via Claisen condensation (Carlsson et al., 2002). KAS II mutants partially deficient in KAS II activity with increased levels of C16:0-ACP (Wu et al., 1994), and a direct association between KAS II activity and palmitate levels were demonstrated in Arabidopsis (Carlsson et al., 2002) and soybean seeds (Aghoram et al., 2006).

Over the last several years, biochemical studies and cloning of genes encoding several fatty acid biosyntheses have been reported in Arabidopsis thaliana (Post-Beittenmiller et al., 1989; Carlsson et al., 2002), Brassica napus (Kater et al., 1992; Simon and Slabas, 1998), Cuphea sp. (Klein et al., 1991; Voetz et al., 1994; Slabaugh et al., 1995), Spinacia oleracea (Scherer and Knauf, 1987; Schmid and Ohlrogge, 1990; Tai and Jaworski, 1993), Hordeum vulgare (Hansen and von Wettstein-Knowles, 1991), Coriandrum sativum (Mekhedov et al., 2001), and Allium ameloprasum (Chen and Post-Beittenmiller, 1996). The crystal structure determination of E. coli type II FAS enzymes has been completed (White et al., 2005), but only the crystal structure of B. napus has been determined in higher plants (Fisher et al., 2000). The altered expression levels of KAS II and KAS III lead to a change in oil content and quality in A. thaliana (Abbadi et al., 2000; Dehesh et al., 2001). More recently, a seven-fold increase in C16:0 levels compared to the wild type was observed by expressing a hairpin RNAi construct that down-regulated KAS II activity (Pidkowich et al., 2007).

High oleate soybean oil and transgenic cotton plants with increased seed oleic acid content have been successfully developed (Chapman et al., 2001). In oil palm, considerable efforts have been made towards producing transgenic oil palm (Sambanthamurthi et al., 1999; Parvez et al., 2005; Ramli et al., 2009), specifically for palm oil with a higher oleate content. Ideally, the genetic modification of the oil palm is anticipated for an increased oleic acid (a monounsaturated fatty acid) content over conventionally grown oil palm, and subsequent decreased saturated palmitic acid content. Increasing the oleic acid content of palm oil is required for two main reasons. Firstly, the price of oleic acid is generally about 10% higher than palm oil (Abdullah, 2005). Secondly, high oleate palm oil is a useful feedstock for the oleochemical industry, and will serve to fill the increase in demand by the industry (Basri et al., 2004).

In order to tailor-make the fatty acid composition of palm oil, the regulation of fatty acid biosynthesis in the oil palm has to be clearly defined. Biochemical studies on oil palm fatty acid biosynthesis indicate that KAS II activity correlates positively with total C18 saturated and unsaturated fatty acids (Sambanthamurthi et al., 2002). In addition, results from metabolic control analysis of fatty acid biosynthesis indicate that KAS II activity plays a role in controlling the balance between palmitate
and stearate products of fatty acid synthase (Ramli et al., 1996). Gene isolation studies were also carried out for important enzymes required for the production of specific fatty acids in oil palm (Siti Nor Akmar et al., 2001). In this article, we describe the efforts aimed at cloning and characterising the cDNA clone of KAS II from oil palm. These findings should benefit the oil palm industry by providing a key gene involved in the regulation of fatty acid biosynthesis which may be useful for creating new opportunities in the genetic engineering programme of oil palm and other crops.

MATERIALS AND METHODS

Plant Materials

Oil palm (Elaeis guineensis Jacq.) samples for the preparation of total RNA and Northern blot analysis were obtained from the Malaysian Palm Oil Board (MPOB) Research Station, Bangi, Selangor. These include fruits at various stages of development [8, 12, 15, 17 and 20 weeks after anthesis (WAA)], kernel (10, 11, 12, 15 and 17 weeks after anthesis) and spear leaf tissues. Germinated seedlings and root tissues were obtained from MPOB Kluang Research Station, Johor.

Isolation of Total RNA

Total RNA was isolated from the mesocarp tissues of oil palm fruits according to the method described by Hosein (2001) with modification. The cDNA generated from total RNA from mesocarp tissues at 17-18 WAA were used in RT-PCR (reverse transcriptase PCR).

Amplification of Partial Length KAS II Sequence by Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

The first partial fragment of the oil palm KAS II cDNA was isolated by a three-step PCR procedure which included an asymmetric PCR with a P1 primer, and two exponential amplification steps towards the polyA tail with the P1 and P2 primers, respectively, as described by van der Linden et al. (2002).

One µg of oil palm total RNA was used to synthesise first strand cDNA from the polyA tail. A sense gene-specific primer P1 (5'-GCC ACA TCC TGA AGG TAG AG-3') which was designed based on the 5'-end of the highly conserved region of rice KAS II gene (AU031953) was used to synthesise second strand cDNA in a linear amplification procedure. For this, 1.5 µl of 0.5 pmol P1 primer was used in a total reaction volume of 25 ml containing 2.5 µl 10X Advantage® PCR buffer (Clontech Laboratories, Inc.), 0.5 µl of 50X Advantage® polymerase mix and 0.5 µl of 10 mM each of dNTP mix. The PCR mixture was heated at 95°C for 3 min in a PE GeneAmp System 9600 (Perkin Elmer). Thirty cycles of linear amplification were performed as follows: denaturation for 30 s at 94°C, annealing for 90 s at 55°C and extension for 2 min at 68°C. Immediately following the linear amplification, PCR was performed using the P1 primer and an anchored poly-dT primer with non-specific 5'-tail. For this, 22.5 µl reaction mixture containing 0.75 µl of 20 µM poly-dT primer, 2 µl of 5 mM dNTP mix, 2.25 ml of 10X Advantage® PCR buffer and 0.75 µl of 50X Advantage® polymerase mix was added to the linear amplification reactions. Two PCR cycles (94°C for 30 s, 40°C for 2 min and 68°C for 3 min) were applied to perform proper annealing of the poly-dT primer to the poly-A sequence. Subsequently, 1.5 µl of 10 pmol P1 primer in 2.5 µl was added to the PCR mix, and cycling was immediately continued for 30 cycles at a higher annealing temperature using the linear amplification cycling conditions described above. One µl of this PCR product was used in a second PCR with identical conditions, but with an antisense gene-specific primer P2 (5' TGA GTT ATG CCC ACC GAA TC-3'). PCR products from the second exponential amplification were electrophoresed on agarose gel to confirm the production of amplicon. The band of interest was excised from the gel, eluted and cloned directly into PCR 2.1-TOPO® vector (Invitrogen, CA), and positive clones were analysed by sequencing.

Isolation of 3' - and 5' End Sequences by Rapid Amplification of cDNA Ends

Rapid amplification of cDNA ends (RACE) was carried out to obtain the 3' and 5' ends of the full-length KAS II. A RACE-ready cDNA template was synthesised from oil palm mesocarp total RNA according to the method provided by the manufacturer (GeneRacer Kit, Invitrogen). To prepare the RACE-ready cDNA template, 1 µl of GeneRacer Oligo dT primer was used in a 12 µl reaction mixture containing 10 µl of RNA and 1 µl of dNTP mix. The mixture was incubated at 65°C for 5 min to remove any secondary structure, followed by chilling on ice for 2 min. Subsequently, the following reagents were introduced into the reaction mixture: 4 µl of 5X first strand buffer, 2 µl of 0.1 mM DTT, 1 µl of RNaseOUT (40 U ml⁻¹) and 1 µl of Superscript II RT (200 U ml⁻¹) in a total volume of 20 ml. The reaction was incubated at 42°C for 50 min. The reaction was terminated by heating up the samples to 70°C for 15 min, followed by 2 min cooling on ice. Subsequently, 1 µl of RNaseH (2U) was added to the mixture followed by incubation at 37°C for 30 min.
The RACE-ready cDNA served as the template in PCR to amplify either the 3’ or 5’ cDNA ends of KAS II gene. The 3’ RACE approach was used to amplify 3’ cDNA ends of the KAS II gene and the transcription termination site. The PCR reaction was prepared in a total volume of 50 µl containing 1 µl of 10 µM of sense gene-specific primer GKAS20F (5’-CTC TCG CAG AGA AAT AGT GAT CGC ACT AAA GCG TCA CGG CCT-3’), 1 µl of cDNA template, 5 µl of 10X high fidelity PCR buffer, 3 µl of 10 µM GeneRacer 3’ primer provided with the kit, 1 µl of dNTP mix (10 mM each), 2 µl of 50 mM MgSO4 and 0.5 µl of Platinum high fidelity Taq DNA polymerase (5 U µl-1). PCR conditions were as follows: one cycle at 94°C for 2 min, followed by five cycles at 94°C for 30 s and 72°C for 1 min. Another five cycles of PCR were performed as follows: 94°C for 30 s and 70°C for 1 min. Next, 20 PCR cycles were performed as follows: 94°C for 30 s, 65°C for 30 s and 72°C for 1 min, followed by one cycle of PCR at 72°C for 10 min. One µl of the PCR product was used as the template in the secondary PCR reaction adopting identical conditions, but with the nested GeneRacer 3’ primer provided with the kit and a nested gene-specific primer GKAS21F (5’-GGG CTG GCG TGC TTC TAC TGG AAG AAT TAG AGC ATG CT-3’). The secondary PCR was carried out using the following PCR programme: one cycle at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 65°C for 30 s and 68°C for 2 min, and finally one cycle at 68°C for 10 min. The PCR product was analysed and purified from the agarose gel using a gel extraction kit (Nucleospin) and cloned into PCR 2.1 TOPO® vector (Invitrogen).

For 5’ RACE, two sets of primers were designed for PCR (GKAS7 5’-CAA CAT CCC AGG GGC GAG AAG CTT TCA CTG CTG-3’) and (GKAS8 5’-GTC AAA GTG CTC CGC ATG CCA AAA AAC C-3’). PCR products from the first and second amplifications were electrophoresed on agarose gel to confirm the production of amplicons. The bands of interest were cut out of the gel, eluted and cloned into PCR 2.1 TOPO® and the recombinant clones were analysed by sequencing.

Amplification of the Full-length KAS II cDNA from Oil Palm

The entire open reading and some of the untranslated regions at each end were amplified using primers designed using the consensus sequence. Two sense primers GKAS27 (5’-ATG CTT TTT TGT TGT TAC GCC CAT AGC TGA TGC-3’) and GKAS28 (5’-TTC CAT ATG ATC AAG ATG TTT GTG GG-3’) were designed that bound upstream of the start codon while the antisense primers primers GKAS29 (5’-TGA GAT TGA CGA TGC CAT TCT GCA TCA TCC CTT TCG G-3’) and GKAS30 (5’-TCT GGG CCT CTG CTC TCT CAA CTT C-3’) were located downstream of the stop codon. Preparation of cDNA and PCR amplification were carried out using the SMART RACE cDNA amplification kit and the Expand high fidelity PCR system (Roche). The cDNA used was derived from RNA isolated from the oil palm mesocarp tissue. The PCR parameters were optimised with all combinations of the sense and antisense primers. A specific product of the expected size was obtained using two primer combinations, namely GKAS27/GKAS29 and GKAS27/GKAS30. This resulted in two PCR products of 2.1 kb and 2.0 kb, respectively. The amplification was carried out using 35 cycles of 30 s at 94°C, 30 s at 58°C and 2 min 30 s at 72°C. Fragments were purified and subsequently cloned into PCRII-TOPO vector (Invitrogen) for sequence analysis.

Northern Blotting

Northern blot analysis was carried out using RNA from oil palm mesocarp and kernel at various stages of development, germinated seedlings, spear leaves, as well as root and flower tissues. For these analyses, mRNA were denatured in 18 µl of solution containing 78% v/v deionised formamide, 16% v/v deionised glyoxal and 10 mM NaH2PO4/Na2HPO4 (pH 7.0) by heating for 15 min at 55°C followed by immediate cooling. Denatured mRNA was separated on 1.2% w/v agarose gel (3 hr) using 40 mM tris-acetate (pH 7.0) as the electrophoresis buffer. The lane containing RNA Millenium markers (Ambion) was excised from the gel and stained with etidium bromide and the markers were visualised by UV trans-illumination. Transfer to a nylon membrane (Hybond-N Amersham) was carried out using a VacuGene XL vacuum blotter (Amersham Pharmacia Biotech) (60 cm H2O, 4 hr) in 20X SSC (1X SSC is 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0). The membrane was rinsed in 2X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH2PO4, pH 7.5, 1 mM EDTA) before continuing with pre-hybridisation. The cDNA insert from a KAS II clone was purified and subsequently labelled with [α-32P]dCTP (Amersham Pharmacia Biotech) using the Megaprime DNA labelling system (Amersham Pharmacia Biotech) according to the method of the manufacturer. The unincorporated [α-32P]dCTP was removed from the probe by gel filtration using a Chromaspin TE-10 column (Clontech). Pre-hybridisation of the membrane was performed at 65°C for 2 hr in 5X SSPE, 5X Denhardt’s solution (1X Denhardt’s solution is 0.02% v/v Ficoll 400, bovine serum albumin and polyvinylpyrrolidone), 0.5% w/v SDS and 100 g ml-1 denatured herring sperm DNA. This is followed by hybridisation of the membranes with 1 x 106 to 5 x 108 cpm ml-1 radiolabelled probe overnight.
Expression Studies of Oil Palm KAS II in Escherichia coli

EcoRI-digested pET-29 was dephosphorylated with 1 U shrimp alkaline phosphatase (Promega, USA). The dephosphorylation cocktail consisted of 2 µl digested DNA (200 µg total), 1 µl 10X SAP buffer, 5 ml water and 2 ml SAP (1 U ml\(^{-1}\)). This was incubated at 37°C, followed by incubation at 65°C for 15 min each. The excess alkaline phosphatase was eliminated using the Qiaquick PCR purification kit (Qiagen, Germany). Linearised pET vector and KAS II were ligated with T4 DNA ligase according to the manufacturer’s directions (Fermentas, USA). The ligation mixture was then incubated overnight at 16°C, followed by inactivation at 65°C for 10 min. The ligation mixture was added to the E. coli BL21 (DE3)pLysS cells and the mixture was swirled gently to mix. The cells were incubated for 5 min on ice, followed by heat shock for 30 s at 42°C. Eighty microlitres of SOC media was added to the cells followed by further incubation for 30 min at 37°C with shaking. The cells were evenly spread onto Luria broth (LB) media supplemented with kanamycin (30 µg ml\(^{-1}\)) and chloramphenicol (34 µg ml\(^{-1}\)) and incubated overnight at 37°C. Screening for positive transformants was carried out by PCR using the gene-specific primers, GKA527 and GKA530. Positive transformants were identified by the production of a 2.0 kb PCR product. Transformants confirmed by PCR were induced with IPTG. For this, a single colony was grown in 5 ml LB with appropriate antibiotics [kanamycin (30 µg ml\(^{-1}\)) and chloramphenicol (34 µg ml\(^{-1}\))] overnight at 37°C with shaking at 200 rpm. The next day, 1 ml culture was transferred into 49 ml of new LB containing antibiotics, and incubated until the OD\(_{600}\) reached 0.4 to 0.6 (mid log phase, approximately 6 hr). Half of the culture was saved to serve as a control before IPTG induction. IPTG (1 mM final concentration) was added to the rest of the culture, and incubation continued overnight. Cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The pellet was then re-suspended in 0.25 volume with 20 mM Tris-HCl, pH8.0, and stored at -20°C for SDS-PAGE analysis. Fatty acid analysis of E. coli cells containing the KAS II construct and of control cells was conducted by gas chromatography (GC).

Expression Studies of Oil Palm KAS II in Arabidopsis thaliana

The pCB302-1 vector (~6.8 kb), which includes the CaMV 35S promoter, was digested with EcoRI. The full-length KAS II cDNA (~2.0 kb), derived from KasEg2C, was digested with EcoRI and purified using the Qiaquick gel extraction method. Cloning of the full-length KAS II into pCB302-1 was carried out by sticky end ligation into the EcoRI restriction site. The vector (pCB302-1) and insert (KAS II gene) were ligated using a 1:3 vector to insert end ratio. Transformation of E. coli DH5α competent cells was carried out using heat shock (Hanahan, 1983). Kanamycin (50 mg litre\(^{-1}\)) was used as a selection agent. Putative transformants were inoculated into 10 ml LB supplemented with 50 mg litre\(^{-1}\) kanamycin. After incubation, the plasmids were isolated from the putative transformants using alkaline lysis (Sambrook et al., 1989). The positive clones were selected by re-digestion of the plasmid with EcoRI. The orientation of the insert was confirmed by digestion with BamHI to yield approximately 0.3 kb and 8.8 kb fragments for sense orientation, and approximately 1.2 kb and 7.9 kb fragments for antisense orientation. Two final plasmids were designed, pCBKASII-7 (sense KAS II) and pCBKASII-11 (antisense KAS II) (Figure 1).

Agrobacterium tumefaciens Transformation

Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983) was transformed with either plasmid pCBKASII-7, a binary vector carrying oil palm sense KAS II and bar genes driven by CaMV 35S promoters, or plasmid pCBKASII-11, a binary vector carrying oil palm antisense KAS II and bar genes driven by CaMV 35S promoters, via electroporation using the Bio-Rad Gene Pulser. A. tumefaciens was grown overnight at 27°C in 2XYT medium (1% w/v NaCl, 1% w/v tryptone and 0.5% v/v yeast extract, pH 7). Two 1.5 ml aliquots of bacteria were centrifuged. The bacterial pellets were then resuspended in 0.5 ml ice-cold 10% v/v glycerol and re-centrifuged. The pellets were again resuspended in 20 µl of 10% v/v glycerol, and the contents of both tubes were combined. One µl of each plasmid was added into 2X 40 µl aliquots of Agrobacterium, and left on ice for 2 min. The mixture was pulsed in an ice-cold 0.2 cm Bio-Rad cuvette. The Gene Pulser was set at 25 µF capacitance and 2.5 KV charge, and the pulse controller at 400 W resistance. Immediately after the pulse, 1 ml of SOC broth was added into each tube. The mixture was incubated at 27°C for 4-6 hr before plating on 2XYT agar containing 10 µg ml\(^{-1}\) chloramphenicol and 50 µg ml\(^{-1}\) [2XYT(10C50K)]. The plates were incubated overnight, and 10 resistant colonies were selected randomly and inoculated into 10 ml 2XYT medium.
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Small-scale Plasmid Isolation

Ten microlitres of the Agrobacterium culture were inoculated into 10 ml of 2XYT (10C50K) medium. The overnight culture was transferred into microfuge tubes and the bacteria was pelleted by centrifugation at 4000 rpm for 5 min at 4°C. Plasmid isolation was carried out using the alkaline lysis method according to Sambrook et al. (1989). The bacterial pellet was resuspended in 200 ml of ice-cold solution I (25 mM Tris-HCl, 10 mM Na₂-EDTA, 50 mM glucose, pH 8.0, 5 mg ml⁻¹ lysozyme), and the mixture was kept at room temperature for 5 min. Three hundred µl of solution II (0.2 M NaOH, 1% w/v SDS) was added, mixed by gentle inverting and incubated on ice for 5 min. Two hundred and fifty µl of solution III (5 M KAc) was added, mixed vigorously and incubated on ice for 5 min. The lysate was centrifuged (15 000 rpm, 5 min, 4°C). The aqueous phase was then transferred to 600 µl of chloroform, mixed for 2 min and centrifuged (15 000 rpm, 5 min, 4°C). The aqueous phase was transferred to a new tube, one volume of isopropanol and 100 µl of 10 M NH₄Ac were added, mixed gently and incubated at room temperature for 15 min. Nucleic acids were collected by centrifugation (15 000 rpm, 15 min, room temperature), rinsed with 70% v/v ethanol and dried at room temperature for 30 min. The final pellet was resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), gently mixed and treated with RNase [75 µl (5 mg ml⁻¹, 15 min, 37°C)].

Arabidopsis thaliana Floral-dip Transformation with Agrobacterium tumefaciens LBA4404

Arabidopsis plants were transformed according to the method developed by Clough and Bent (1998). Agrobacterium tumefaciens strains LBA4404 carrying the binary plasmid pCBKASII-7 or pCBKASII-11 were used. Unless otherwise noted, the bacteria were grown to stationary phase in liquid culture at 25°C-28°C and 250-280 rpm in sterilised 2XYT (10C50K) medium. The cells were
harvested by centrifugation (5500X g) for 20 min at room temperature, and then resuspended in a floral-dip medium to a final OD<sub>600</sub> of approximately 0.80 prior to use. The floral-dip inoculation medium contained 5.0% w/v sucrose and 0.05% w/v Sliwet L-77. The inoculum was transferred to a beaker before the plants were immersed into this suspension in such a way that all the above-ground tissues were submerged. The dipped plants were removed from the beaker after 3-5 s of gentle agitation, placed on a plastic tray and covered with a tall clear plastic dome to maintain humidity. The plants were left in a low-light or dark location overnight and returned to the growth chamber the next day. The domes were removed at approximately 12-24 hr after treatment, and the plants were grown for a further three to five weeks until the siliques were brown and dry. Seeds were harvested by gently pulling grouped inflorescences through the fingers over a piece of clean paper, and stored in a microcentrifuge tube prior to screening.

Screening of Transformants

The seeds were germinated on wet compost in plastic pots. The compost was wetted with water containing Basta (final 10 ppm). Selection with the herbicide was more efficient (in eliminating escape plants) as compared with using antibiotics. The pots were placed at 4°C for stratification. The pots were later placed in a Conviron growth chamber (Model TC30) with a 16-hr day photoperiod, a temperature of 20°C-25°C, with additional artificial light (105 E/m<sup>2</sup>/s) and sub-irrigation with a layer of tap water containing Basta. When the plantlets produced normal green cotyledons and two true leaves, they were sprayed with Basta solution (final 50 ppm). The plantlets were sprayed again with the same Basta solution after two weeks. The resistant plantlets were transferred into individual pots when they were sufficiently developed (4-5 leaf stage) to prevent cross-pollination and/or seed contamination. The surviving plants were grown until the siliques were brown and dry. The T2 seeds were harvested as described above and stored in a microcentrifuge tube.

Fatty Acid Analysis

Total fatty acids were extracted from <i>E. coli</i> using solvent extraction consisting of water:2% HCl-acidified methanol:chloroform (0.8:0.2, v/v). The suspension was centrifuged at 4000 rpm for 15 min. A mixture of 5 ml water and chloroform (0.5:0.5, v/v) was added to the supernatant to extract the organic bottom phase. Fatty acid methyl esters (FAME) were prepared from the total fatty acids using 2 ml toluene and 2 ml 1% H<sub>2</sub>S<sub>4</sub>-acidified methanol, and the reaction was incubated at 80°C for 2 hr. FAME were extracted by the addition of 5% NaCl and 2 ml hexane to the esterified mixture, and the upper layer phase was collected after centrifugation. FAME extract was concentrated under a nitrogen gas stream and reconstituted in 0.5 ml hexane. GC/FID analysis were performed at 250°C on Agilent GC6890N (Agilent, USA) that was equipped with a splitless injector. Around 1 µl of the solution of FAME in hexane (1:5 v/v) was applied into GC. An Agilent autosampler, AOL series (Agilent, 7683 Series), and FID at 300°C were used (H<sub>2</sub> flow 40 ml min<sup>-1</sup>, air flow 350 ml min<sup>-1</sup> and make-up He 45 ml min<sup>-1</sup>). Data acquisition was performed by MSD and MSD data analysis software (Agilent, USA). A column, DB-23 (J & W P/N:123-2332) (0.25 mm x 30 m x 0.20 mm film), was used for the analysis. The temperature programme was as follows: 50°C initial temperature for 2 min, ramp 1 from 50°C–180°C at 10°C min<sup>-1</sup>, ramp 2 at 180°C for 5 min, with the final temperature from 180°C-240°C at 5°C min<sup>-1</sup> (total run time 37 min). Helium was used as the carrier gas (1 ml min<sup>-1</sup>). FAC was calculated as normalised percentages from the peak areas.

RESULTS AND DISCUSSION

Multi-step PCR and RACE Experiments for Amplifying Full-length cDNA Sequence of KAS II

The first amplification of the partial length cDNA of KAS II was achieved by RT-PCR which produced a single amplified fragment of ~450 bp nucleotide sequence (Figure 2), and the clone was designated as KasU26RM. The nucleotide sequence possessed >80% homology to KAS II in the database, and was used to design primers for amplification of both the 3'- and 5'-end sequences. The 3'-RACE using a gene-specific primer (GKAS20F) produced non-specific primary PCR products as seen from the several bands obtained. Therefore, 1 ml of the primary PCR product was used in a second-round PCR reaction using a 38-mer nested gene-specific primer (GKAS21F), which finally amplified a single fragment of 1.0 kb. The band was purified from the agarose gel and cloned into PCR 2.1 TOPO vector, and one of the recombinant clones designated KasU86m (Figure 2) was sequenced for both directions using M13 forward and reverse primers. The nucleotide sequence of clone KasU86m showed significant homology with other KAS II sequences and had a stop codon and a further 300 bp of the 3' untranslated region (3' UTR).

In order to obtain the 5'-end sequence of the oil palm KAS II, 5' RACE was used. Several primers were designed based on the partial clones of KAS II to maximise the number of putative KAS
II sequences that might be amplified by these primers. Three independent RACE were conducted using different primer combinations. One of the longest PCR products of ~1.5kb was amplified using the primers GKAS7/GKAS8. A positive clone, designated as KasU24, was completely sequenced on both strands using the M13 forward and reverse primers (Figure 2). Alignment analysis showed that the nucleotide sequence of KasU24 overlapped (82% identical) with the 5’-end of the KasU26RC sequence (approximately 485 bp). Therefore, KasU24 provided the remaining 5’-end sequence of KAS II. The sequence of the clone KasU24 contained 400 bp of the 5’-UTR. The three clones, KasU86m, KasU26RC and KasU24, were thus used to produce a final consensus sequence of the 5’-end for the KAS II gene (Figure 2).

Amplification of the Full-length Oil Palm KAS II cDNA by PCR

The entire open reading of the oil palm KAS II was amplified using different primer combinations designed against the 2.0 kb consensus sequence of the oil palm KAS II. In this work, one of the primer combinations (GKAS27/GKAS30) was successfully optimised to produce a single amplified fragment of about 2.0 kb (Figure 3). Based on the positions of the primers relative to the known sequences for KAS II and the consensus nucleotide sequence of the oil palm KAS II obtained by RT-PCR and RACE, this fragment was of the expected size. Thus, the amplified fragment was subsequently purified and cloned into the PCRIITOPO vector for further analysis. The complete DNA sequence was obtained for representative clones, and a BLAST search indicated that the clone’s DNA sequence was highly identical to KAS II from other plants at about 90%-95% identity. Based on a comparison to the amino acid sequence of KAS II from other plants, a possible deduced amino acid sequence for the oil palm KAS II was obtained. It was shown that the clone coded for a 1650 bp open reading frame coding for 549 amino acid residues. The identity of the deduced amino acids was further verified by a BLAST search of the Genbank database. The results show that the sequence shared a very high level of identity to KAS II from other plants including C. wrightii (accession number U67317), A. thaliana (accession number NM106154), G. max (accession number AF244518) and O. sativa (accession number NM001066809) (Figure 4).

Northern Blotting

Northern blot analysis was carried out using the full-length KAS II as the probe to study the expression of the KAS II gene. RNA samples from a number of oil palm tissues including fruit
mesocarp at various stages of development, viz., 8, 12, 15, 17 (WAA), and kernel at 10, 11, 12, 15, 17 (WAA), germinated seedlings, roots, flowers and spear leaves. The full-length coding sequence of the oil palm KAS II was used as the probe. The results demonstrate that the KAS II encoding genetic sequence was expressed at low levels in the various oil palm tissues, including the mesocarp and kernel at different stages of development, leaves, germinated seedlings and flowers, but almost none in roots. Overall, the KAS II expression pattern detected by Northern blot analysis was shown to be relatively high in 17- and 20-week mesocarp, 12-week kernel and in germinated seedlings (Figure 5). However, expression of KAS II was low in younger mesocarp tissues (8- and 12-week) and in the flowers, and almost negligible in the tissues of roots and spear leaves. KAS II plays a role in the synthesis of C18:0 saturated fatty acids which undergo further unsaturation reactions to C18:1, C8:2 and C18:3. These fatty acids are required as components of plant membranes, and this may explain the high expression of KAS II in germinated seedlings. The expression pattern of the oil palm KAS II seemed to be well correlated to the accumulation of TAG synthesis in the fruit tissues. It was previously shown that the synthesis of TAG started around 15 WAA and reached a maximum around 20 weeks, after which the content of TAG started to decline (Oo et al., 1985). A similar pattern was also observed for KAS II enzyme activity that was detected to begin at 15 weeks and reached a maximum around 18 weeks, with the enzyme activity declining after 20 weeks (Sambanthamurthi et al., 1999). Thus, it should be expected that the KAS II gene required for the formation of TAG must be present at a relatively high amount at these stages. The high level of KAS II both at the later stages of fruit development and in germinated seedlings could reflect its high requirement for development in the younger kernel and for lipid accumulation for storage in the older mesocarp tissues.
Functional Analysis of Oil Palm KAS II in *Escherichia coli*

Higher plants synthesise fatty acids via a common pathway. To determine the enzymatic function of the oil palm KAS II, first, we used *E. coli* to transform the KAS II construct. Previously, Garwin *et al.* (1980) reported on the enzymatic activities of *E. coli* KAS II that were significantly enhanced at temperatures lower than 37°C. Results in Table 1 demonstrate the percentages of fatty acids from transformed and untransformed *E. coli* cells. The percentage of C18:1 increased upon expression of the oil palm KAS II protein in *E. coli*. Therefore, it is expected that increasing the activity of this enzyme will decrease the palmitic acid (C16:0) and increase the oleic acid (C18:1) content in palm oil. Thus, the expression studies in *E. coli* demonstrated proof of principle on the functional activity of the oil palm KAS II.

Transgenic Expression of Oil Palm KAS II in Plants

The levels of particular fatty acids in plants are modulated by altering the expression of the genes involved in the biosynthesis of the fatty acids. Up-regulation or down-regulation of any of these genes potentially alters the production of particular fatty acids. Specifically, in one strategy, the relative or absolute quantities of C18 and/or unsaturated fatty acids are increased in a plant by the expression of a KAS II genetic sequence. In order to effect this expression, *Arabidopsis* plants have been transformed with antisense nucleotide sequences of KAS II in order to reduce palmitic acid production and increase production towards 18 carbon acyl chains. In this study, an antisense KASII construct was cloned into the pCB302-1 vector and transformed into *Arabidopsis thaliana*. It is expected that down-regulation of KAS II in the transformed plants would result in the accumulation of palmitic acid. This was confirmed by comparing the levels of fatty acids produced by the transformed and untransformed recombinant plants. Significant decreases in C18:0 and accumulation of C16:0 were detected in the plants that had been transformed with the antisense KASII construct (Table 2).

**CONCLUSION**

KAS II is an interesting candidate for exploitation as its role in controlling the ratio of C16/18 carbon acyl chains has been well demonstrated. Oil palm KAS II cDNA was isolated from mesocarp tissue, and the sense and antisense nucleotide sequences were heterologously transformed into *E. coli* and *Arabidopsis*, respectively. Consistent with our hypothesis, higher oleate was observed in *E. coli* expressing the oil palm KAS II, thus demonstrating that the full-length cDNA encoded an active KAS II. To gain further insight into the function of

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**Table 1. Analysis of Fatty Acids in Sense-KAS II Transformed E. coli**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control (n=2)</th>
<th>Sense KAS II (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 Myristic</td>
<td>7.0</td>
<td>12.5 ± 1.0</td>
</tr>
<tr>
<td>C16:0 Palmitic</td>
<td>58.2</td>
<td>26.2 ± 1.9</td>
</tr>
<tr>
<td>C18:0 Stearic</td>
<td>11.2</td>
<td>6.7 ± 3.0</td>
</tr>
<tr>
<td>C18:1 Oleic</td>
<td>18.6</td>
<td>47.5 ± 5.2</td>
</tr>
<tr>
<td>C18:2 Linoleic</td>
<td>4.8</td>
<td>6.1 ± 0.7</td>
</tr>
</tbody>
</table>

**Table 2. Analysis of Fatty Acids in Antisense-KAS II Transformed A. thaliana**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control (n = 7)</th>
<th>Antisense KAS II (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 Myristic</td>
<td>4.8 ± 1.8</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>C16:0 Palmitic</td>
<td>37.8 ± 8.6</td>
<td>66.3 ± 7.1</td>
</tr>
<tr>
<td>C16:1 Palmitoleic</td>
<td>4.6 ± 1.1</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>C18:0 Stearic</td>
<td>21.2 ± 5.0</td>
<td>8.6 ± 1.8</td>
</tr>
<tr>
<td>C18:1 Oleic</td>
<td>21.9 ± 5.0</td>
<td>21.6 ± 7.0</td>
</tr>
<tr>
<td>C18:2 Linoleic</td>
<td>4.0 ± 0.6</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>C18:3 Linolenic</td>
<td>5.7 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

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Figure 5. Northern blot analysis for determination of KAS II expression pattern using oil palm KAS II or ribosomal DNA as probes. The mRNA samples were from mesocarp and kernel tissues, as well as spear leaves, germinated seedlings, roots and flowers. The blot was hybridised with a 32P-labelled probe prepared using the full-length KAS II. The membrane was reprobed with ribosomal DNA to check for equal loading of RNA samples.

**Note:** WWA = weeks after anthesis; F = flower; L = leaf; R = roots and GS = germinated seedlings.
KAS II and into the regulation of carbon flow, expression studies using Arabidopsis mutants were conducted. The results demonstrate that reducing KAS II expression provides an effective strategy for increasing 16-carbon fatty acid accumulation. The full-length KAS II cDNA is now being used in the genetic engineering programme for modifying fatty acid composition in palm oil.

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