

MOLECULAR CLONING AND REGULATION OF OIL PALM (*E. guineensis* Jacq.) PHYTOENE DESATURASE IN DEVELOPING MESOCARP TISSUES

O A RASID*; W S WAN NUR SYUHADA*; A NOR HANIN*; S S MASURA* and G K A PARVEEZ*

ABSTRACT

Oil palm is one of the richest natural sources of carotene and therefore it is an interesting target for genetic modification for high value carotenoid products. Phytoene desaturase (PDS) catalyses the formation of one of the double bonds during the conversion of phytoene into lycopene. Introduction of an additional copy of pds could potentially increase plant carotenoid content. Moreover, pds is considered as a potential target of bleaching herbicide action and as a determinant of geometric isomer states of carotenoids. This article describes the isolation of cDNA clones coding for pds from oil palm. In this work, we have successfully obtained a 865 bp fragment through reversed transcriptase PCR (RT-PCR) using degenerate primers. The sequence information of this initial fragment was subsequently used to obtain full-length coding region of oil palm pds. The clone is moderately identical to other plant pds sequences at about 80% identity. Real-time PCR analysis was carried out to study the expression of the gene in the developing oil palm mesocarp tissues. Results indicate that the gene is highly regulated during the course of oil palm fruit development. The expression is relatively high in the 19-week mesocarp tissues as well as in mature leaves at about 1.97- and 2.42-fold, respectively compared to the calibrator. A moderate expression level of about 1.5-fold was observed in 5- and 15-week mesocarp and young leaf (spear leaf) tissues. The results suggest that oil palm pds is highly regulated during fruit development probably to meet the demand for growth or storage.

Keywords: oil palm, carotenoid, phytoene desaturase, cDNA, expression.

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INTRODUCTION

Carotenoids are a widespread class of naturally occurring pigments found in plants, algae, and some fungi and bacteria. In photosynthetic tissues, carotenoids function as ancillary pigments and photoprotectants. They also fulfil an additional important

purpose in higher plants as colorants of flowers and fruits. The potential health benefits of carotenoids as anti-cancer and antioxidant agents have been demonstrated. In particular, lycopene and β -carotene have recently been shown to be able to reduce the risk of chronic conditions of coronary heart disease, certain cancers and macular degeneration. In addition, β -carotene, a provitamin A carotenoid, has been shown to be critical for normal development of children. Vitamin A deficiency may cause premature death, abnormal body growth, xerophthalmia and

* Malaysian Palm Oil Board, 6 Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.
E-mail: omar@mpob.gov.my

night blindness. The findings have led to rapid development in the field aimed at understanding the biosynthesis pathway and ultimately engineering the carotenoid content (Bartley and Scolnik, 1995; Hirschberg, 1999; Canfield, 1995; Mayne, 1996).

In plants, carotenoids are synthesised and accumulated in plastids. The first committed step in carotenoid synthesis is the formation of the first C40 compound, phytoene by condensation of two molecules of geranylgeranyl diphosphate (GGDP). This reaction is catalysed by phytoene synthase. A series of four dehydrogenation reactions convert this colourless compound into lycopene. The first two desaturation steps are catalysed by phytoene desaturase (PDS). Zeta carotene desaturase catalyses the following two desaturation steps. The formation of β -carotene and its derivative xanthophylls (zeaxanthin, antheraxanthin, violaxanthin and neoxanthin) is catalysed by lycopene β -cyclase. Alternatively, lycopene can be converted into α -carotene and its derivatives such as lutein. The cDNA for most of the major enzymes have been cloned from both plant and microbial sources (Cunningham and Gantt, 1998; Bramley, 1997).

Carotene desaturation is the key step in the biosynthesis of coloured carotenoids. Changes in the property of the intermediates are obtained by the addition of the conjugated bonds. The changes in the intermediate property result in changes in their colour as well as antioxidant property. This critical role has led to the high interest in this particular reaction step. It has also received much attention as target for bleaching herbicide action (Chamovitz *et al.*, 1993). PDS inhibition results in accumulation of phytoene and concurrent bleaching of organism due to destruction of chlorophyll. The protein has become a target for herbicide active ingredient. Certain mutations in *pds* gene resulted in resistance to the herbicide. This unique property leads to possible uses of the gene as a selectable marker in plant transformation (Arias *et al.*, 2006; Huang *et al.*, 2008).

The regulatory control of PDS in plants has not been documented. However, manipulation of desaturation activity in plants can produce transgenic plants with increased β -carotene contents. An example is the introduction of *E. uredovora pds* gene (*crt1*) into tomato under the control of CaMV 35S promoter by *Agrobacterium*-mediated transformation. The β -carotene content in *crt1*-transformed tomato fruit increased three-fold, up to 45% of the total carotenoid content. Determination of gene expression by RT-PCR showed that endogenous PDS, ZDS and lycopene β -cyclase were concurrently upregulated, converting lycopene to β -carotene (Rohmer *et al.*, 2000). In contrast to the results obtained by Rosati

et al. (2000), the total carotenoid content in the transgenic tomatoes was lower than that in the wild type. This result suggested a feedback inhibition of the pathway.

Crude palm oil is one of the richest natural sources of carotenoids (500-700 ppm). The α -carotene and β -carotene are the two major components, which make up about 90% of total carotenoid content (Choo, 1995). Therefore, this plant is a potential system to study the regulation of carotenoids synthesis. In addition, it is also a potential target for the genetic manipulation and improvement of carotenoid content. A number of recent studies have reported the progress to shed some light on the oil palm carotenoid biosynthesis. To date, isolation and characterisation of genes coding for a few of the enzymes in the pathway in oil palm (*Elaeis guineensis*) have been reported. They include 1-deoxy-D-xylulose 5-phosphate synthase (Khemvong and Suvachittanont, GenBank Accession No. AY58378 and AY611205), zeaxanthin epoxidase (Rasid *et al.*, 2005) and lycopene cyclases (Rasid *et al.*, 2007; 2003). The partial cDNA encoding the oil palm phytoene synthase (PSY) has recently been described (Rasid *et al.*, 2008). In this article, we report the isolation of cDNA clone that codes for oil palm phytoene desaturase and its regulation during the developmental course of oil palm fruits.

MATERIALS AND METHODS

Isolation of Total RNA

Total RNA was isolated from mesocarp tissues of oil palm fruit and leaves according to Hosein (2001) with modification. The cDNA generated from total RNA from 17 WAA (week after anthesis) mesocarp tissues of *E. guineensis* were used in RTPCR (reverse transcriptase PCR). First strand cDNA was synthesised using 1 μ g of the total RNA using SMART RACE kit (BD Biosciences) according to the manufacturer's protocol.

RTPCR and Cloning

Oligonucleotide primers used in this study were synthesised based on the conserved regions of plant PDS. Plant PDS sequences available at the GenBank were retrieved and aligned for the identification of the conserved regions. The plant *pds* cDNA that were available and used in the sequence alignment for the identification of possible conserved blocks were from *A. thaliana* (GenBank Accession # AY05769), *L. esculentum* (GenBank Accession # X59948), *C. annuum* (GenBank Accession # X68058), *C. sinensis* (GenBank Accession # AJ319761), *O. sativa* (GenBank Accession # AF049356) and

Z. mays (GenBank Accession # U37285). The fidelity of these identified conserved regions was evaluated using BLAST search at the Genbank databases. Only regions with good fidelity were used for the primer synthesis. The two sense primers are PDS1 (5'-TAY GAG ACW GGY YTG CAY ATW TTC-3') and PDS2 (5'-YAA YGA RAT GCT KAC WTG GCC RGA R -3') while the antisense primers are PDS3 (5'-CAT RGT ACA RCA RTT YTG MGG YTC Y -3') and PDS4 (5'-CGR CCH CTR ATG TGY TTT GTC TTY A -3'). The amino acid sequences for the PDS1, PDS2, PDS3 and PDS4 are YETGLHIF, NELMLTWPE, KYHVVKTP and AGDYTKQK, respectively. The degenerate genetic codes are according to the Sambrook *et al.* (1989).

RT-PCR was carried out using the Advantage 2 Kit (BD Biosciences) with 35 sequential cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 3 min on PTC-100 Programmable Thermal Controller (MJ Research). The PCR products were electrophoresed, and the amplified bands were excised and purified using QIAEXII Gel Extraction Kit (QIAGEN) and finally ligated into pCRII-TOPO vector (Invitrogen). Plasmid DNA from several selected colonies was isolated using QIAGEN Plasmid Preparation kit (QIAGEN) according to the manufacturer's protocol. Confirmation of insert was performed with *Eco* RI digestion and followed by agarose gel electrophoresis.

DNA Sequence Analysis

Plasmid DNA for sequencing was prepared using QIAGEN miniprep Kit (QIAGEN). DNA sequencing was carried out on ABI Prism 377 automated DNA sequencer (Pharmacia).

Analysis of DNA sequences was carried out using VectorNTI software (Invitrogen). The analysis included the removal of unreadable and vector sequences, sequence alignment, ORF identification and contig analysis and assembly. DNA and protein homology search against the GenBank databases was performed using BLAST 2.0 (Altschul *et al.*, 1997).

PCR Amplification and Cloning of 3' and 5' Regions

Based on the sequence obtained above, new sets of gene specific primers (sense and antisense) were synthesised to facilitate the amplification of the 3'-end and 5'-end regions of the genes. Two primers were designed and synthesised for each end. The outer most primers were located between 50 bp to 100 bp from the end of the sequence. This provided the overlapping sequence between the newly amplified end regions and the amplified middle region above. The sequence of these primers is given in *Table 1*.

RT-PCR amplifications of 3'- and 5'- end regions were performed using first strand cDNAs generated using SMART RACE kit (BD Biosciences) and a combination of a gene specific primer (*Table 1*) and universal primer or nested universal primer supplied with the kit. Amplifications were carried out using the Advantage 2 Kit (BD Biosciences) with 35 sequential cycles of denaturation at 94°C for 30 sec, annealing for 30 s and extension at 72°C for 3 min on PTC-100 Programmable Thermal Controller (MJ Research). The annealing temperature was empirically determined for each primer combination. The PCR products were electrophoresed, and the amplified bands were excised, purified, cloned and sequenced.

Real-time PCR

The expression study of oil palm *pds* was performed using real-time PCR. Total RNA from mesocarp of 5, 7, 9,11,13,15, 17 and 19 WAA fruits was used in the analysis. Total RNA from kernel, spear and green leaves was also included for comparison. Removal of DNA contamination in total RNA samples was carried out using the QIAGEN RNase-free DNase Set according to the manufacturer's protocol. The concentration of the RNA samples was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). The RNA samples were subjected to integrity analysis using Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit) (Agilent). Intact RNA was converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems).

Analysis for conserved regions that were used for primer and probe synthesis was performed using VectorNTI (Invitrogen). Real-time PCR (TaqMan assay) was carried out in mixtures containing 1X TaqMan Universal PCR Master Mix, 1X Assay Mix (containing specific primers and probe) and 45 ng of template cDNA. PCR cycling parameters were set at one cycle at 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Real-time detection of fluorescence was performed on the ABI PRISM 7000 Sequence Detection System.

The efficiency of the real-time assays for each gene was determined by performing the amplification using five serial 10-fold dilutions of a template (Toplak *et al.*, 2004). The real-time PCR standard curve was obtained by plotting the CT values against log₁₀ template concentrations. The slope (S) of the standard curve was used to calculate the PCR efficiency using the following equation: PCR efficiency (%) = (10^{-1/S}-1) × 100. A standard curve of -3.32 indicates a PCR reaction with 100% efficiency.

TABLE 1. PRIMERS FOR THE AMPLIFICATION OF 3'-END AND 5'-END REGIONS OF OIL PALM *pds*

Primer	Target region	Nucleotide sequence
PDS5	3'	TGCAAGGAATACTATGATCC
PDS6	5'	ACTTCATCATTGACTCGATC
PDS7	3'	GAAATCATTGATGCTACTATG
PDS8	5'	ATGGCAGGCAAAAGTCCAATAGC

Note: Primers PDS5 and PDS7 were for the 3'-end region. Primers PDS6 and PDS8 were for the 5'-end region. The sequences are from 5' to 3'.

RESULTS AND DISCUSSIONS

RTPCR and Cloning of Oil Palm Phytoene Desaturase

The present work was aimed at the isolation and characterisation of a number of genes that code for the key enzymes of the oil palm carotenoid biosynthesis. One of the genes is PDS. The enzyme is responsible for the first two initial desaturation steps of phytoene. In certain plant species, the enzyme has been shown to have a rate limiting role. For example, Shewmaker *et al.* (1999) showed that the carotenoid content in *Brassica napus* seed was increased 50-fold by overexpression of bacterial *pds*.

Primers used in this work were designed based on the known sequences of PDS from other plants available on the GenBank. Their amino acid sequences were aligned to allow the identification of possible conserved regions within the gene. The degenerate primers were then designed based on the sequence of the conserved regions. The specificity of each primer was verified by BLAST search (Altschul

et al., 1997). Primers which hit any sequence with more than 70% identity were discarded. This is to reduce the possibility of obtaining unspecific products during the RTPCR amplification. Four degenerate oligonucleotide primers were shown to have a good specificity and used in this work.

Our effort to amplify the full-length sequence by coupling the gene specific degenerated primers to the universal primer (provided in the SMART™ RACE cDNA Amplification Kit) was unsuccessful. The product has either multiple bands or smearing, indicating unspecific binding of the primers. Alternatively, RTPCR amplification was carried out using combinations of two gene specific degenerate primers. Combinations of PDS2 and PDS3, and PDS2 and PDS4 were successfully optimised to give a single band product. The size of the amplified fragment was estimated to be about 0.6 kb and 0.9 kb for the above primer combinations, respectively (Figure 1). Amplification using the other primer combinations usually gave multiple band products indicating unspecific binding of the primers.

The amplified fragments were purified and cloned into pCRII-TOPO vector for further analysis. The presence of the DNA insert in the transformed cells was confirmed by *Eco RI* digestion followed by agarose gel electrophoresis. Clones that originated from the 0.6 kb fragment were designated as pEPDS40(1-5) and those that originated from the 0.9 kb fragment were designated as pEPDS50(1-5).

The complete DNA sequence was obtained for representative clones originated from each RTPCR fragment. DNA sequence analysis result showed that the insert size of pEPDS40 is 675 bp. Clone pEPDS50 contains a slightly longer insert of 865 bp. However, further analysis showed that the two fragments are very similar to each other. Clones pEPDS40 was

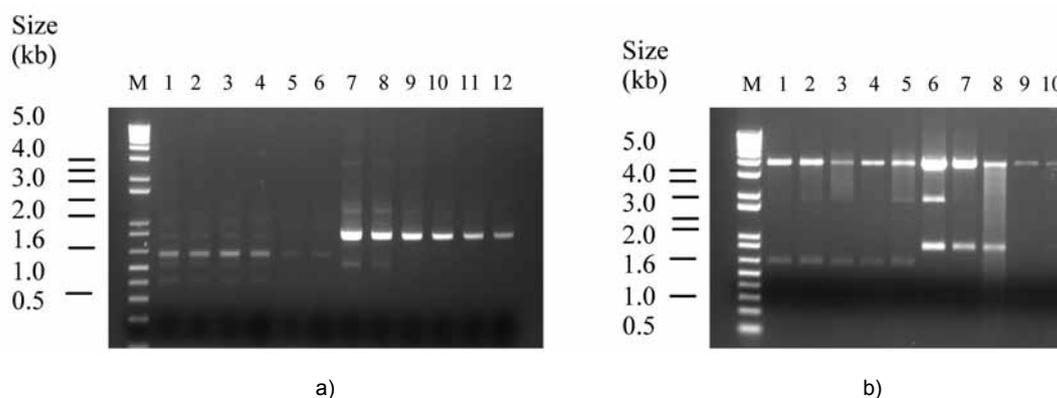


Figure 1. (a) Reverse transcriptase PCR (RTPCR) amplification of oil palm *pds* using degenerate oligonucleotide primers. Combination of PDS2/PDS3 primers gave a product of about 0.6 kb fragment (lanes 1 to 6). Combination of PDS2/PDS4 gave a slightly larger fragment of about 0.8 kb (lanes 7 to 12). (b) *Eco RI* digestion and electrophoresis of plasmid DNA obtained from representative clones for insert confirmation. All clones derived from 0.6 kb fragment were shown to carry the insert (lanes 1 to 5). Only three of the clones derived from the 0.8 kb fragment were shown to carry the insert (lanes 6 to 8). M is 1 kb plus DNA ladder.

shown to lack about 200 bp at the 3' end compared to pEPDS50.

Before the work was carried into the next stage, the identity of the amplified fragments was first established. The pEPDS50 sequence identity was analysed using BLAST homology search against the GenBank databases (Altschul *et al.*, 1997). Results indicated that the DNA sequence of the amplified fragment is highly identical to other plant *pds* at about 80%. Comparatively, the pEPDS50 sequence aligned to the region between 630 bp to 1495 bp of *Oryza sativa pds* sequence. Based on the observation, it was concluded that the cDNA clone obtained indeed codes for a partial oil palm PDS.

Isolation of 3'- and 5'-End Regions of Phytoene Desaturase

The availability of the sequence information of the middle fragment provides the necessary tool that could facilitate the cloning of the complete cDNA for oil palm *pds*. Based on the sequence obtained, gene specific primers (sense and antisense) were designed for the amplification of the 3'- and 5'-end regions of oil palm *pds*. At least two primers were designed and synthesised for each end.

The primary RTPCR amplification of the 3'-end region was carried out using a combination of PDS5/UPM. The primer combination was successfully optimised to give a major amplified product of about 0.7 kb (Figure 2a). Thus, for this particular set of experiment, the secondary RTPCR was not necessary and not carried out. The amplified product was subsequently cloned into pCRII-TOPO vector and representative clones containing the fragments were identified using *EcoRI* restriction analysis. The clones containing the PDS5/UPM fragment were designated as pEPDS59.

The primary RTPCR amplification of the 5'-end region was carried out using a combination of PDS6/UPM. In contrast to the results of 3'-end reactions, the 5'-end primary RTPCR reactions gave multiple fragments. Therefore, the primary RTPCR product was subsequently used as template in secondary PCR reactions using either PDS8/NUPM primer combination. The amplification was successfully optimised to give a major amplified product of about 0.9 kb (Figure 2b). The clones containing the PDS8/NUPM fragment were designated as pEPDS80.

DNA sequences were completely determined for both fragments. The size of the fragments was shown to be 687 bp and 904 bp for 3'- and 5'- end fragments, respectively. Results from BLAST homology search indicated that a part of the fragment sequences shared a high identity to their corresponding regions of other plant desaturases. The results suggested that these fragments contained the non-coding region.

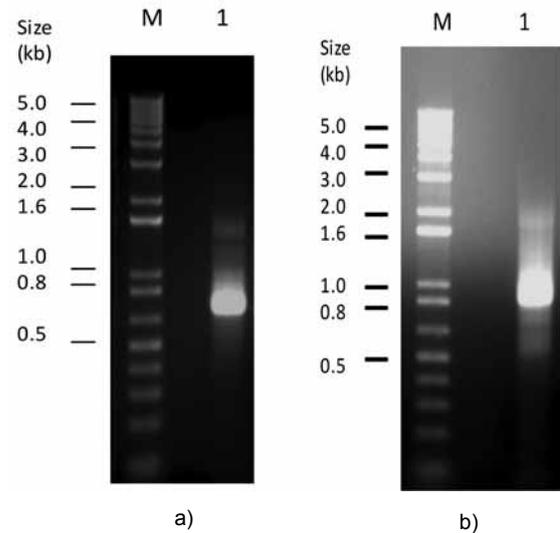


Figure 2. Agarose gel electrophoresis of reverse transcriptase PCR (RTPCR) products of 3'- and 5'-end regions of oil palm *pds*. a) A fragment of about 0.7 kb obtained from the 3'-end RT-PCR amplification of oil palm *pds* using the combination of PDS5/UPM (lane 1). b) A fragment of about 0.9 kb obtained from the 5'-end RTPCR amplification of oil palm *pds* using the combination of PDS8/NUPM (lane 1). M is 1 kb Plus DNA Ladder (Invitrogen).

Generation and Analysis of Full-length Sequence for Oil Palm Phytoene Desaturase

A consensus DNA sequence for oil palm *pds* was generated by combining the sequences of the three amplified fragments above (*i.e.* 3'-end, middle and 5'-end regions). This was feasible as part of the amplified fragments contained the overlapped regions of at least 50 bp to the middle region. These regions provided an anchor to which the end fragments could be joined to the middle fragment. Consensus sequences of 2155 bp, excluding the polyA tail, was subsequently generated for oil palm *pds* from the sequences of three fragments.

The identification of a possible open reading frame (ORF) for oil palm *pds* was carried out using ORF VectorNTI software (Invitrogen). The longest ORF of 1749 bp was obtained from this analysis. The 1749 bp ORF starts at nucleotide 168 from the 5'-end and ends at nucleotide 1916. It codes for 583 deduced amino acid residues. The size of the ORF is comparable to other plant PDS, which are in the range between 500 to 600 amino acid residues. For examples, the size of maize and persimmon PDS are 571 and 586 amino acid residues, respectively (Li *et al.*, 1996; Zhao *et al.*, 2011).

To confirm the identity of the amplified fragment, the consensus sequence generated was analysed for similarity using BLASTn search at the GenBank (Altschul *et al.*, 1997). The BLAST results indicated that the oil palm *pds* is highly identical to other plant *pds*. It is about 86%, 85% and 83% identical to the *pds*

Oil palm PDS	(1)	MNTIGFISTLSSTVQRHYWDPNLQRYSLGIDSGCRILKAFGNSEFMG
A. Thaliana PDS	(1)	-----VVFNVCAANLPYNGFLEALS SGG -----CELMGH
O. sativa PDS	(1)	-----MTASARYAGLPTHRCF SS -----IQALKGQHVE
Z. mays PDS	(1)	-MDTGCLSSNIT ASQT S AG LPPQRCF SS -----HYTE
Z. mays ZDS	(1)	-----M ASVA
Oil palm PDS	(51)	CHLEQASLFGTSSHKVSPLOVVCMYFPELENTVNFLEAQLSSFF
A. Thaliana PDS	(34)	ERVPSQALKTRTARRSTAGPLQVVCVDFPELENTVNFLEAASLSSAS
O. sativa PDS	(34)	FGVSLVLRN KGK FRRLLGALQVVCQDFPELENTVNFLEAQLSSFF
Z. mays PDS	(38)	FAYRKLVRN KGR SHRHPALQVVCQDFPELENTVNFLEAQLSSFF
Z. mays ZDS	(7)	TTTLAPALAPRA PGTG IVP PRRASAVAA ST TSPTWRQR QR FPPE
Oil palm PDS	(101)	<u>ENCARPEKGLVYIAGAGLACSTAKYLADAGHKPILLEARDV</u> LGGLGVA
A. Thaliana PDS	(84)	<u>ESAPRAFKVYIAGAGLACSTAKYLADAGHKPILLEARDV</u> LGGLIAA
O. sativa PDS	(84)	<u>ENSEQTKPQVYIAGAGLACSTAKYLADAGHKPILLEARDV</u> LGGLIAA
Z. mays PDS	(88)	<u>ENSEKPEVQVYIAGAGLACSTAKYLADAGHKPILLEARDV</u> LGGLVAA
Z. mays ZDS	(57)	PEHYGPK IKVA I CAGLAC ST AV ELLQ CH EV DL Y ES RP FG GV GS
Oil palm PDS	(151)	NKVEDGDWYETGLHIFPGAYFQVQNLFGELGINDHLQNKESMIFAMPNK
A. Thaliana PDS	(134)	NKVEDGDWYETGLHIFPGAYFQVQNLFGELGINDHLQNKESMIFAMPNK
O. sativa PDS	(134)	NKVEDGDWYETGLHIFPGAYFQVQNLFGELGINDHLQNKESMIFAMPNK
Z. mays PDS	(138)	NKVEDGDWYETGLHIFPGAYFQVQNLFGELR EDHL QNKESMIFAMPNK
Z. mays ZDS	(106)	EV IR Q NHI EM SL EV FP GCYS NI FR M KK V QADNN LV KE R HT VN --K
Oil palm PDS	(201)	FGEFSRPOFPELLPAPLNGIWAIRNNEMLTWPEKVF FAIGL IPAMVGGQ
A. Thaliana PDS	(184)	FGEFSRPOFPELLPAPLNGIWAIRNNEMLTWPEKVF KFAIGL IPAMVGGQ
O. sativa PDS	(184)	FGEFSRPOFPELLPAPLNGIWAIRNNEMLTWPEKVKF AGL IPAMVGGQ
Z. mays PDS	(188)	FGEFSRPOFPELLPAPLNGIWAIRNNEMLTWPEKVF FAIGL IPAMVGGQ
Z. mays ZDS	(154)	G T I G E L D R F P P V G A P L H Q A P L T N Q L K V D KARNAVALA SP V R A L
Oil palm PDS	(251)	A-----YEAQGLTVSEWKKQVFPDRVNDVFIAMSKALNFINPDE
A. Thaliana PDS	(234)	A-----YEAQGLSVK HEKQ V RV TDEVFLAMSKALNFINPDE
O. sativa PDS	(234)	A-----YEAQGLF TVSE WKKQVFPDRVNDVFIAMSKALNFINPDE
Z. mays PDS	(238)	P-----YEAQGLTVSEWKKQVFPDRVNDVFIAMSKALNFINPDE
Z. mays ZDS	(204)	VDPDGALQQ RDLD DIS FD W FMSK GTRESITR MDP VRY AL G F DC DN
Oil palm PDS	(294)	LSNQCIIIALNR LQENHGS KMAFLDGNP PERLCM PIVDH RSL GGEVRL
A. Thaliana PDS	(277)	LSNQCIIIALNR LQENHGS KMAFLDGNP PERLCM PIVDH IRSL GGEVQL
O. sativa PDS	(277)	LSNQCIIIALNR LQENHGS KMAFLDGNP PERLCM PIVDH RSL GGEVRL
Z. mays PDS	(281)	LSNQCIIIALNR LQENHGS KMAFLDGNP PERLCM PIVDH IR GGEVRL
Oil palm PDS	(522)	RFLQRSPIEGFYLAGDYTKQY YLA SMEGAVLSGKLCACQ IV ODYDLIVAR
A. Thaliana PDS	(504)	RFLQRSPIEGFYLAGDYTKQY YLA SMEGAVLSGKLCACQ IV ODYDLIVAR
O. sativa PDS	(504)	RFLQRSPIEGFYLAGDYTKQY YLA SMEGAVLSGKLCACQ IV ODYDLIVAR
Z. mays PDS	(508)	RFLQRSPIEGFYLAGDYTKQY YLA SMEGAVLSGKLCACQ IV ODYDLIVAR
Z. mays ZDS	(499)	R P D K T P V K N F E L S S Y T K Q D I D S M E G R T L S C R T A Y CGAG E L L A L
Oil palm PDS	(572)	NQSSPAEMTL-----
A. Thaliana PDS	(554)	GF K SEAT SS -----
O. sativa PDS	(554)	SL S I Q S E VPVAS-----
Z. mays PDS	(558)	SO S I Q S E VPVPS-----
Z. mays ZDS	(549)	RK L I D D G E K L G N V Q V L Q A S

Figure 3. Deduced amino acid sequence of oil palm pds compared to its counterparts from *O. sativa* (GeneBank # AF049356), *Z. mays* (GenBank # U37285) and *C. sativus* (GeneBank # AY183118), as well as *Z. mays* zeta-carotene desaturase (ZDS)(GenBank Accession # AF047490). The dinucleotide-binding (GAGLACSTAKYLADAGHKPILLEARDV) and carotenoid domains (AGDYTKQYLA~~SM~~EGAVLSGKL) are underlined. Yellow colour indicates the identical residue, blue colour denotes greater than 50% match and green colour indicates weak similarity.

sequences from *Crocus sativus*, *Zea mays* and *Oryza sativa*, respectively. A slightly lower identity (80%) was observed between oil palm *pds* sequence to its corresponding gene from *Arabidopsis thaliana*.

Amino acid sequence alignment between oil palm PDS and other plant PDS indicated that the gene is highly conserved throughout higher plants (Figure 3). The results also indicated the presence of two conserved domains, namely dinucleotide-binding and carotenoid-binding domains. This may suggest the crucial role of the gene in the carotenoid biosynthesis pathway, particularly in the formation of lycopene and its derivatives.

The deduced amino acid sequences of these clones were shown to be highly identical to their respective counterparts from other plants. In particular, PDS was shown to be highly conserved across plant species. In addition, results from amino acid sequence analysis also indicated that oil palm PDS shared a relatively high identity to its cyanobacterial counterparts at about 60%. The similarity to bacterial and yeast phytoene desaturase (*crtI*) was shown to be very much lower. The only significant similarity was only observed in the nucleotide-binding motif. This result may suggest that the plant and cyanobacterial phytoene desaturase shared no common ancestor to the bacterial and yeast PDS. This could be due to the fact that plant and cyanobacterial PDS catalyses the introduction of two double bonds into phytoene to form ζ -carotene, whereas *crtI* gene product introduces four double bonds into phytoene to form lycopene. Interestingly, a similar level of identity was also observed between oil palm PDS to bacterial amine oxidase, a FAD dependent oxidoreductase. In contrast, oil palm PDS, like other plant PDS, shared a little resemblance to a related carotene desaturase enzyme, ζ -carotene desaturase (ZDS). The oil palm PDS was shown to have about 30% identity to plant ζ -carotene desaturases. Similarly, pepper ZDS has been indicated to be about equidistant from plant and cyanobacterial PDS (33%-35% identity). This observation has led to the suggestion that these three proteins originated from a common ancestor (Albrecht *et al.*, 1995).

To this point, the complete coding region has only been generated based on the consensus sequences obtained from the combination of sequences of the three PCR amplified fragments. However, further analysis will definitely require the clone, at least the coding region of the gene, to be in one piece. In order to do this, a pair of primers flanking the coding region of this gene was synthesised. The sense and antisense primers were 5' -ACA CTA GGG TAG AAC TGC AAC- 3' and 5' -ACA CCA CTA TGCAAG AGT CA- 3', respectively. The primers were successfully used to amplify about 1.7 kb fragments (Figure 4). The fragment was subsequently cloned into pCRII-TOPO vector. The clones carrying the insert were verified by digestion with EcoR I followed by agarose

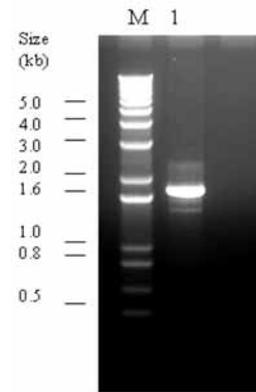


Figure 4. End to end reverse transcriptase PCR (RT-PCR) amplification of the coding region of oil palm *pds*. The full coding region of oil palm phytoene desaturase was amplified using combination of PDS13/PDS14 primers (lane 1).

gel electrophoresis. Finally, the identity of the cloned fragment was determined by DNA sequencing. The results from the DNA sequence obtained indicated that the clones indeed carry the full-length coding region of the gene.

Expression and Regulation of Phytoene Desaturase in Developing Mesocarp Tissues

The expression of the gene was studied using real-time PCR. RNA samples from a number of oil palm tissues, namely mesocarp (M) 5, 7, 9, 11, 13, 15, 17 and 19 WAA, kernel (K) 10 WAA, spear leaves and green matured leaves were used in the real-time PCR analysis. The primers and the probes used in the real-time PCR were designed from highly conserved regions of the genes. These regions were identified by VectorNTI sequence alignment and BLAST Search at the GenBank. Sequence of primers and probes are shown in Table 2.

The relative quantification of the genes was carried out using the comparative $\Delta\Delta C_T$ method (Livak and Chmittgen, 2001). The real-time PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was also included in the analysis as the internal control. Since the analysis is comparative, it is important to standardised the equal amounts and integrity of template. In this study, the concentration and quality of these samples were determined by using NanoDrop® ND-1000 Spectrophotometer and Agilent 2100 bioanalyser, respectively.

The amplification efficiency of the TaqMan assay is shown in Figure 5. The quantitation was linear over a range of 5 log units of *pds* and GAPDH genes indicating a wide dynamic range and high reliability. The efficiency of PCR reaction for *pds* and GAPDH was 103.3% and 97.8%, respectively. The threshold cycle (C_T) values obtained from the amplification plot were used to estimate the relative abundance of the genes. The value marks the first cycle at which the signal is significantly above the background.

TABLE 2. LIST OF PRIMERS AND TaqMan PROBES SPECIFIC FOR OIL PALM *pds* AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)

Genes	Primer's Name	Sequence (5' – 3')
Phytoene desaturase	PDS For	F: GGTTTAACTGTAGCAGAGTGGATGA
	PDS Rev	R: GCTTTAGACATGGCAACGAAAACCT
	PDS Probe	FAM-CCTGATCGAGTCAATGAT-NFQ
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH For	F: ACTGCTACTCAGAAGACTGTIGATG
	GAPDH Rev	R: TGCTGCTAGGAATGATGTTAAAGCT
	GAPDH Probe	FAM-ACCCCTCCAGTCCTTG-NFQ

Note: FAM and NFQ are the reporter dye and quencher, respectively.

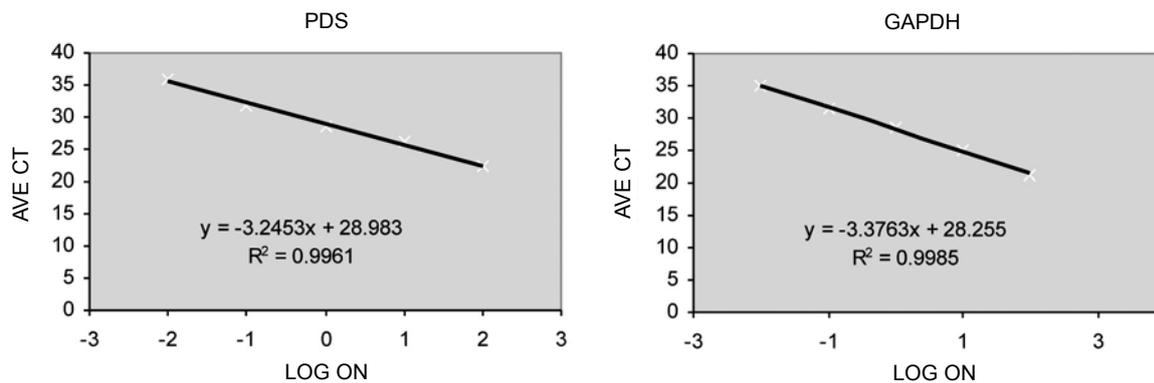


Figure 5. Real-time PCR amplification efficiency curve generated from 10-fold serial dilutions of cDNA for *pds* (a) and GAPDH (b). The average C_T value is plotted against the log of input template amount. The C_T value decreased log-linearly with increasing amount of template.

Then, the average C_T values for the target gene were normalised to the average C_T values of the internal control (GAPDH). The difference in C_T values (ΔC_T) is equal to the difference in expression after being normalised to the endogenous control. Finally, the comparative C_T values ($\Delta\Delta C_T$) were obtained by subtracting the ΔC_T of calibrator sample (K10) from ΔC_T of test sample. The calibrator is normally an untreated sample. However for expression studies, the calibrator can be any one of the samples used but normally the sample with the lowest C_T value. These $\Delta\Delta C_T$ values represent the difference in the expression after being normalised to internal control and relative to a calibrator. The relative expression (RQ) of the target genes was calculated using the $\Delta\Delta C_T$ values according to the equation $2^{-\Delta\Delta C_T}$. Fold-differences were expressed as log values of RQ values.

The expression of *pds* was shown to be relatively high in the young mesocarp tissue (M5) (Figure 6). It was about 1.5-fold higher than in K10 (calibrator). However, the expression decreased to about one-fold from 7th week to 13th week (M7 to M13). The expression further decreased to the lowest level in the 15th week (M15). The expression then was increased to about two-fold in 17th and 19th WAA tissues (M17

and M19). Overall, the expression of oil palm *pds* gene seemed to be highly regulated at the different developmental stages of mesocarp tissue. This may be needed for the accumulation of carotene in fruit tissues. According to Tay and Gwendoline (2006), the accumulation of α - and β -carotenes in oil palm fruits increased to a significantly high level at 18 WAA fruit. In this study, the expression of oil palm *pds* was indicated to increase to almost two-fold at the 17th week of fruit development. The relatively high level of *pds* expression was maintained in the 19 WAA fruit. The high level of *pds* expression at these stages of oil palm fruit may be correlated to the increase of α - and β -carotenes. In their study, Tay and Gwendoline (2006) also indicated that a relatively high amount of lutein was present in young mesocarp (1 WAA) tissues. This carotenoid remained high through the 8 WAA of mesocarp development. The formation of this carotenoid at a relatively high amount at these stages may partly explain the moderate expression level of *pds* gene in M5 through M13 WAA fruits observed in this work.

In leaves, the expression of *pds* in spear and green leaves was at about 1.3- and 2.4-fold, respectively, compared to the calibrator. The expression of *pds* in green leaves was higher than

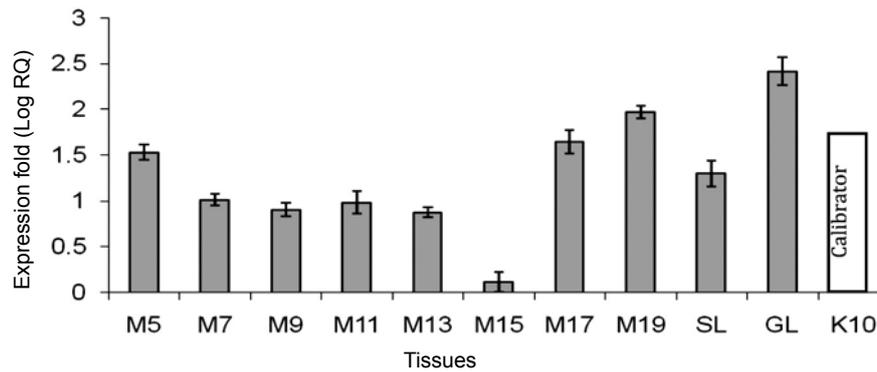


Figure 6. The expression of oil palm *pds* gene in developing oil palm fruits (M5-M19) obtained by real-time PCR analysis. A relatively high level of *pds* expression was observed in the early and late developmental stages of oil palm fruits. A comparable level of expression was also observed in spear (SL) and green (GL) leaves. M5, M7, M9, M11, M13, M15, M17 and 19 represent mesocarp tissues at 5, 7, 9, 11, 13, 15, 17 and 19 weeks after anthesis (WAA), respectively. K10 is kernel tissue at 10 WAA and referred as the calibrator.

its expression level in mesocarp tissues and spear leaves. The high expression of this gene in the green leaves could reflect the large requirement for carotenoids including lutein and other xanthophylls in photosynthesis. In young leaf tissues, carotenoids are also needed for tissue development. This requirement may explain the moderate expression level of *pds* in the spear leaves.

CONCLUSION

In this study, we have isolated and completely sequenced a cDNA clone coding for oil palm *pds*. Based on the sequence comparison either at the nucleic acid or deduced amino acid level, we concluded that the clone obtained indeed codes for oil palm *pds* as indicated by the high identity to other plant *pds* genes. The expression study showed that the pattern of oil palm *pds* expression was regulated during the mesocarp tissue development. The regulation of oil palm *pds* may be necessary for the accumulation of carotenes in mesocarp tissue for storage. This finding may be useful for developing strategies to genetically modify the carotenoid content in oil palm fruit.

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