



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR CLONING AND CHARACTERIZATION OF cDNA
ENCODING FOR ENZYMES IN THE CAROTENOID BIOSYNTHETIC
PATHWAY OF OIL PALM (*Elaeis guineensis* Jacq.)**

OMAR BIN ABD RASID

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By

OMAR BIN ABD RASID

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Philosophy**

2008



Abstract of thesis presented to the Senate of Universiti Putra
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ENCODING FOR ENZYMES IN THE CAROTENOID BIOSYNTHETIC
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Chairman: Associate Professor Suhaimi Napis, PhD

Faculty: Biotechnology and Biomolecular Sciences

The potential health benefits of carotenoids, in particular as anticancer and antioxidant agents, have recently been highlighted. Extensive studies have been conducted to elucidate the plant carotenoid pathway. Oil palm is known to be the richest natural source of carotene. However, to date, there has been no work carried out to elucidate the pathway in this species. The lack of the knowledge could restrain the potential advantages of the plant for further improvement through genetic modifications. This work is the first effort towards the understanding of the oil palm carotenoid biosynthesis pathway. The aim was to isolate cDNA clones encoding the oil palm lycopene β -cyclase (LCYb), lycopene ϵ -cyclase (LCYe), phytoene desaturase (PDS) and zeaxanthin epoxidase (ZEP). The first two enzymes have been suggested to have a regulatory control over the formation of carotene. Thus, their genes are believed to have an important and urgent biotechnological application. Fragments containing partial sequences of these genes were successfully generated through reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers. The complete DNA sequence of these



fragments were determined. Primers were then designed based on these sequences to facilitate the amplification of the 3' and 5' end regions of the transcripts. Both ends were successfully obtained for both cyclases. A consensus sequence of 1962 bp and 1759 bp was generated for oil palm *lcye* and *lcyb*, respectively. An open reading frame (ORF) of 1617 bp encoding 539 amino acid (AA) residues was identified for *lcye*. Similarly, an ORF of 1509 bp encoding for 503 AA residues was also identified for *lcyb*. Deduced AA sequences were shown to be highly identical to their respective counterparts from other plants at about 80% identity. Although the enzymes were functionally equivalent, they were shown to share little resemblance at about 30% identity. However, oil palm LCYb was shown to share a relatively high identity to plant neoxanthin and capxanthin-capsorubin synthases, suggesting the common ancestor of the cyclases and synthases.

RTPCR amplifications using degenerate primers were also successfully used to generate fragments of 865 bp and 567 bp for oil palm *pds* and *zep*, respectively. Subsequently, the deduced AA sequence for both fragments was identified based on comparison to peptide sequences of their counterparts from other plants. Both oil palm PDS and ZEP were shown to be highly identical to their respective counterparts from other plants at about 85%.

The regulation of these four carotenogenic genes as well as phytoene synthase was studied in developing mesocarp tissues using real-time PCR analysis. The results indicated that all of the carotenogenic genes were expressed at a low level in the tissues tested. *psy* and *pds* were shown to be expressed at a relatively higher level in young and late developing mesocarp tissues, as well as in leaves. A similar



expression level was observed for the cyclases, although at a relatively lower level than the *psy* and *pds* level. Nevertheless, the expression of these genes seemed to be correlated and thus believed to be regulated for the accumulation of carotenoids in the tissues both for developmental process and storage.

The copy number of the two oil palm cyclase genes was examined using Southern analysis. The results indicated that there was at least one of the restriction enzymes used gave a single hybridized band. This finding strongly suggested that the two cyclase genes are present in a single copy in oil palm.

In conclusion, the full length cDNAs coding for lycopene β -cyclase and lycopene ϵ -cyclase and partial cDNAs for phytoene desaturase and zeaxanthin epoxidase were successfully obtained and characterized. This work provides the required genetic material for the modification of oil palm carotenoid content, especially for the production of high lycopene transgenic oil palm. Furthermore, the results of the expression study provide very valuable information for formulating an effective strategy for oil palm carotenoid genetic engineering especially toward the increase of lycopene by down-regulating the two cyclase genes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah



**PENGLONAN DAN PENCIRIAN cDNA YANG MENKOD ENZIM-
ENZIM DI DALAM TAPAK JALAN SINTESIS KAROTENOID SAWIT
(*Elaeis guineensis* Jacq.)**

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Karotenoid merupakan sebatian semulajadi yang dianggap berpotensi sebagai agen antikanser dan antioksidan. Banyak kajian telah dijalankan untuk memahami tapak jalan sintesis karotenoid di dalam tumbuhan. Buah sawit merupakan sumber asli yang terkaya dengan karoten. Walaubagaimanapun, tidak terdapat banyak kajian yang dijalankan untuk memahami tapak jalan sintesis karotenoid di dalam species ini. Ini pastinya akan menyebabkan kesukaran untuk mengeksplotasi kelebihan yang terdapat pada tumbuhan ini untuk penambahbaikan melalui kejuruteraan genetik. Justeru, kajian ini merupakan usaha pertama untuk memahami tapak jalan sintesis karotenoid sawit. Kajian ini bertujuan untuk memencilkan klon cDNA yang mengkod enzim likopen β -siklase (LCYb) dan likopen ϵ -siklase (LCYe) serta fitoene desaturase (PDS) dan zeaxanthin epoksidase (ZEP) daripada sawit. Kedua-dua likopen siklase tersebut dipercayai penting dalam pengawalaturan biosintesis karoten. Amplifikasi RTPCR menggunakan pencetus degenerasi telah berjaya menjana serpihan yang mengandungi jujukan separa gen-gen tersebut. Jujukan lengkap DNA klon-klon tersebut telah diperolehi. Pencetus-pencetus untuk amplifikasi kawasan hujung 3' dan hujung 5' kemudiannya dihasilkan berdasarkan jujukan DNA yang diperolehi. Kedua-dua kawasan hujung telah berjaya

diampifikasi untuk kedua-dua likopen siklase. Kombinasi jujukan DNA daripada ketiga-tiga serpihan telah berjaya menjana jujukan konsensus untuk *lcye* dan *lcyb* yang masing-masing bersaiz 1962 pb dan 1759 pb. Rangka bacaan terbuka (ORF) yang bersaiz 1617 pb dan 1509 bp telah dikenalpasti masing-masing untuk *lcye* dan *lcyb*. Terjemahan ORF tersebut menghasilkan jujukan peptida yang bersaiz 539 residu asid amino (AA) untuk *lcye* dan 503 residu AA untuk *lcyb*. Jujukan peptida kedua-dua gen menunjukkan tahap identiti yang tinggi iaitu melebihi 80% terhadap jujukan protein masing-masing daripada tumbuhan lain. Bagaimanapun, tahap persamaan di antara kedua-dua siklase sawit ini adalah rendah, iaitu pada tahap 30% identiti, walaupun pada hakikatnya kedua-dua peptida tersebut mempunyai fungsi yang hampir serupa. Sebaliknya, jujukan LCYb sawit menunjukkan tahap identiti yang agak tinggi terhadap jujukan peptida neoxanthin dan kapxanthin-kapsorubin sintase. Keputusan ini mencadangkan gen siklase dan sintase berasal dari leluhur yang sama.

Amplifikasi RTPCR juga telah berjaya digunakan untuk menghasilkan serpihan untuk *pds* dan *zep* sawit yang masing-masing bersaiz 865 pb dan 567 pb. Berdasarkan jujukan AA daripada tumbuhan lain, jujukan terjemahan AA untuk kedua-dua klon tersebut telah dikenalpasti. Jujukan peptida separa untuk PDS dan ZEP sawit menunjukkan tahap identiti yang tinggi terhadap jujukan peptida enzim masing-masing daripada tumbuhan lain iaitu pada tahap 85%.

Profil pengekspressan keempat-empat gen karotenoid di atas serta fitoene sintase sawit telah diperolehi menggunakan kaedah “real-time” PCR. Keputusan menunjukkan bahawa kesemua gen karotenoid sawit ini diekspres pada tahap yang

rendah. Bagaimanapun, gen *psy* dan *pds* diekspres pada tahap yang agak tinggi di dalam tisu mesokarpa yang muda dan pada peringkat lewat perkembangan, serta di dalam tisu daun. Tahap pengekspresan yang agak serupa juga diperolehi untuk kedua-dua gen siklase. Bagaimanapun, profil pengekspresan gen-gen tersebut adalah berkolerasi dengan kandungan karotenoid di dalam tisu-tisu tersebut. Ini menunjukkan pengawalaturan gen-gen tersebut di dalam penghasilan karotenoid untuk proses perkembangan dan pensetoran.

Seterusnya, analisis penghibridan Southern telah dilakukan untuk menentukan salinan kedua-dua gen siklase di dalam sawit. Keputusan yang diperolehi menunjukkan sekurang-kurangnya satu daripada enzim pembatas yang digunakan menghasilkan satu jalur penghibridan. Ini menunjukkan kedua-dua gen ini berkemungkinan besar hadir sebagai salinan tunggal di dalam sawit.

Sebagai kesimpulan, klon cDNA lengkap yang mengekod likopen β -siklase dan likopen ϵ -siklase serta klon separa untuk fitoene desaturase dan zeaxanthin epoksidase telah berjaya diperolehi dan dicirikan. Kajian ini telah berjaya menghasilkan bahan genetik yang diperlukan untuk pengubahsuaian kandungan karotenoid sawit, terutamanya untuk penghasilan sawit transgenik yang mempunyai kandungan likopen yang tinggi. Keputusan kajian profil pengekspresan gen-gen tersebut juga telah menyediakan maklumat yang penting untuk membangunkan strategi yang berkesan untuk meningkatkan kandungan likopen melalui penurunan pengekspresan kedua-dua gen likopen siklase.

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I certify that an Examination Committee has met on 25 March 2008 to conduct the final examination of Omar bin Abd Rasid on his Doctor of Philosophy thesis entitled "Molecular Cloning and Characterization of cDNAs Encoding for Enzymes in the Carotenoid Biosynthetic Pathway of Oil Palm (*Elaeis guineensis* Jacq.)" in accordance with Universiti Pertanian Malaysia (Higher Degree Act 1980 and



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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.



OMAR BIN ABD RASID

Date: 25 March 2008

TABLE OF CONTENTS

Page



ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xxi

CHAPTER		
		1
I	INTRODUCTION	
II	LITERATURE REVIEW	10
	Biological Roles of Carotenoids	11
	Roles of Carotenoids in Human Health and Consumption	14
	Precursors of Vitamin A	14
	Anticancer Agents and Antioxidants	16
	β -carotene	16
	Lycopene	19
	Xanthophylls	21
	Biosynthetic Pathway for Plant Carotenoids	22
	Genes and Enzymes of Plant Carotenoid Synthesis	29
	IPP Formation	31
	Lycopene Formation	33
	Cyclization of Lycopene	35
	Xanthophyll Formation	36
	Control and Regulation of Plant Carotenoid Biosynthesis	37
	Regulation in Green Plastids (chloroplast)	37
	Regulation in Non-Green Plastids	39
	Formation of IPP	40
	Roles of Phytoene Synthase	41
	Cyclization of Lycopene	43
	Modification of Carotenoid Contents in Plants	44
	Carotenoids in Oil Palm	48
III	MATERIALS AND METHODS	51
	Plant Tissues	51
	Chemicals, Enzymes and Kits	51
	Gene Isolation, Cloning and Sequencing	53
	Design and Generation of Degenerate Primers	53
	Preparation of Total RNA	53
	Generation of First Strand cDNAs	55
	Primary RTPCR Amplification Using Degenerate Primers	55
	Agarose Gel Electrophoresis of RTPCR Products	56
	Secondary RTPCR	57
	Purification of PCR Fragments	57
	Ligation into PCRII-topo Vector	58



Transformation into One Shot® Chemically Competent <i>E. coli</i>	58
Rapid Preparation of Plasmid DNA	59
Restriction Analysis of Putative Clones	60
Preparation of Plasmid DNA for Sequencing	60
DNA Sequence Analysis	60
End to End RTPCR and Product Cloning	61
Northern Analysis	62
RNA Isolation	62
Electrophoresis and Northern Blotting	62
Preparation of DNA Probe	63
Hybridization	64
Real-time PCR	64
Removal of DNA Contaminant and Determination of RNA Concentration	64
Estimation of RNA Integrity	65
Reverse Transcription	67
Real-time PCR amplification	67
Southern Hybridization	69
Isolation of Genomic DNA	69
Restriction and Electrophoresis of Genomic DNA	70
Southern Hybridization	71
IV RESULTS	72
Cloning of Oil Palm Lycopene ϵ -cyclase	72
Degenerate Primers for Lycopene ϵ -cyclase	72
Primary RTPCR and Cloning of Partial Clones	75
DNA Sequence Analysis of Primary RTPCR Products	79
Isolation of 3' and 5' Regions of Lycopene ϵ -cyclase	82
Generation and Analysis of Full Length Sequence for Lycopene ϵ -cyclase	87
End to End PCR for Lycopene ϵ -cyclase	95
Cloning of Oil Palm Lycopene β -cyclase	97
Degenerate Primers for Lycopene β -cyclase	97
Primary RTPCR and Cloning of Partial cDNA Clones	100
Amplification of 3'- and 5'- Regions of Lycopene β -cyclase	103
Generation and Analysis of Full Length Sequence for Lycopene β -cyclase	107
End to End PCR for Lycopene β -cyclase	116
Cloning of Partial cDNA Clone for Oil Palm Phytoene Desaturase	117
Degenerate Primers	117
RTPCR Amplifications	119
DNA Sequence Analysis	119
Cloning of Partial cDNA Clone for Oil Palm Zeaxanthin Epoxidase	126
Degenerate Primers	126



	RTPCR Amplifications	128
	DNA Sequence Analysis	128
	Expression of Oil Palm Carotenoid Genes	133
	Northern Analysis	133
	Real-time PCR Analysis	135
	Southern Analysis	149
V	DISCUSSION	157
	RTPCR Using Degenerate Primers	158
	Selection of Tissues for Source of RNA	160
	Isolation of Oil Palm Lycopene Cyclases	161
	Cloning of Partial cDNA Clones Coding for Oil Palm Phytoene Desaturase and Zeaxanthin Epoxidase	164
	Southern Analysis	166
	Expression and Regulation of Oil Palm Carotenoid Genes	167
VI	CONCLUSION	172
	REFERENCES	175
	BIODATA OF STUDENTS	192
	List of Publications	193

List of Tables

Table		Page
4.1	Degenerate primers used in the RTPCR amplification of oil palm lycopene ϵ -cyclase.	76



4.2	Primers for the amplification of 3'-end and 5'-end regions of oil palm lycopene ϵ -cyclase.	84
4.3	Degenerate primers used in the RTPCR amplification of oil palm lycopene β -cyclase.	99
4.4	Primers for the amplification of 3'-end and 5'-end regions of oil palm lycopene β -cyclase.	104
4.5	Degenerate primers used in the RTPCR amplification of oil palm phytoene desaturase.	120
4.6	Degenerate primers used in the RTPCR amplification of oil palm zeaxanthin epoxidase.	127
4.7	List of primers and probes used in the real-time PCR analysis.	138
4.8	The summarized results of real-time PCR analysis for oil palm <i>lcy</i> in mesocarp tissues of different developmental stages (M5-M19 WAA), kernel (K10 WAA) and spear (SL) and green (GL) leaves.	142
4.9	The summarized results of real-time PCR analysis for oil palm <i>lcyb</i> in mesocarp tissues of different developmental stages (M5-M19 WAA), kernel (K10 WAA) and spear (SL) and green (GL) leaves.	143
4.10	The summarized results of real-time PCR analysis for oil palm <i>pds</i> in mesocarp tissues of different developmental stages (M5-M19 WAA), kernel (K10 WAA) and spear (SL) and green (GL) leaves.	144
4.11	The summarized results of real-time PCR analysis for oil palm <i>zep</i> in mesocarp tissues of different developmental stages (M5-M19 WAA), kernel (K10 WAA) and spear (SL) and green (GL) leaves.	145
4.12	The summarized results of real-time PCR analysis for oil palm <i>psy</i> in mesocarp tissues of different developmental stages (M5-M19 WAA), kernel (K10 WAA) and spear (SL) and green (GL) leaves.	146

List of Figures

Figure		Page
2.1	Schematic diagram depicting the formation of IPP via MEP and MVA pathways in plants.	23



2.2	Schematic diagram depicting the carotenoid synthetic pathway in plants.	27
3.1	A schematic diagram of 16 well RNA 6000 Nano chip used in RNA integrity analysis using Agilent 2100 Bioanalyzer.	66
4.1	Identification of conserved regions within plant lycopene ϵ -cyclases for synthesis of degenerate primers.	74
4.2	Relative positions of degenerate primers for lycopene ϵ -cyclases to the coding region of <i>A. palaestina lcy1</i> .	76
4.3	Electrophoresis of RTPCR amplification products of oil palm lycopene ϵ -cyclase gene using degenerate oligonucleotide primers.	78
4.4	Electrophoresis of representative clones obtained from the transformation of amplified fragments using combination of degenerate primers for lycopene ϵ -cyclase.	80
4.5	Complete cDNA sequence (1104 bp) of fragment encoding the middle region of oil palm lycopene ϵ -cyclase obtained from amplification using combination of CRE2/8 primers.	81
4.6	Agarose gel electrophoresis of RTPCR product and restriction digest (<i>EcoR</i> I) of plasmid DNA containing 3'-end region of oil palm lycopene ϵ -cyclase.	84
4.7	Agarose gel electrophoresis of RTPCR product and restriction digest (<i>EcoR</i> I) of plasmid DNA containing 5'-end region of oil palm lycopene ϵ -cyclase.	86
4.8	The 1962 bp consensus cDNA sequence of oil palm lycopene ϵ -cyclase generated by combining the sequences from the amplified 3'-end, 5'-end and middle regions.	88
4.9	Comparison of amino acid sequence of oil palm lycopene ϵ -cyclase (LCYEOP) to representative lycopene ϵ -cyclase sequences from other plants.	91
4.10	End to end RTPCR amplification of the coding region of oil palm lycopene ϵ -cyclase.	96
4.11	Alignment of amino acid sequences of plant lycopene β -cyclases for the identification of possible conserved regions for generation of degenerate primers.	98



4.12	The relative position of degenerate primers for RTPCR amplification of oil palm lycopene β -cyclase to the coding region of <i>C. annum</i> lycopene β -cyclase.	99
4.13	Agarose gel electrophoresis of RTPCR products and restriction digest (<i>Bst</i> X I) of plasmid DNA containing oil palm lycopene β -cyclase.	101
4.14	Agarose gel electrophoresis of RTPCR product and restriction digest (<i>Bst</i> X I) of plasmid DNA containing 3'-end region of oil palm lycopene β -cyclase.	106
4.15	Agarose gel electrophoresis of RTPCR product and restriction digest (<i>Bst</i> X I) of plasmid DNA containing 5'-end region of oil palm lycopene β -cyclase.	106
4.16	The 1759 bp consensus cDNA sequence of oil palm lycopene β -cyclase.	108
4.17	Comparison of deduced oil palm LCYb sequence to representative LCYb sequences from other plants.	112
4.18	Comparison of deduced oil palm LCYb sequence to oil palm LCYe, capxanthin-capsorubin synthase (CCS) from <i>C. annum</i> and <i>C. sinensis</i> and neoxanthin synthase (NXS) from <i>L. esculentum</i> and <i>S. tuberosum</i> .	114
4.19	End to end RTPCR amplification of the coding region of oil palm lycopene β -cyclase.	118
4.20	The relative position of degenerate primers for the amplification of oil palm <i>pds</i> to the coding region of <i>O. sativa pds</i> .	120
4.21	Agarose gel electrophoresis of RTPCR products and restriction digest (<i>Eco</i> R I) of plasmid DNA containing oil palm phytoene desaturase.	121
4.22	The complete cDNA sequence of pEPDS50 coding for a partial region of oil palm phytoene desaturase.	122
4.23	Comparison of deduced amino acid sequence of oil palm PDS (pEPDS50) to representative PDS sequences from other plants.	124
4.24	Comparison of deduced oil palm PDS sequence to ζ -carotene desaturase from <i>A. thaliana</i> and <i>Z. mays</i> , a bacterial phytoene desaturase from <i>Synechococcus</i> and a bacterial amine oxidase from <i>A. variabilis</i> .	125



4.25	The relative position degenerate primers for the amplification of oil palm <i>zep</i> to the coding region of <i>C. unshiu zep</i> .	127
4.26	Agarose gel electrophoresis of RTPCR products and restriction digest (<i>EcoR</i> I) of plasmid DNA containing oil palm zeaxanthin epoxidase.	129
4.27	The complete cDNA sequence of clone pEZEP11 encoding a partial region of oil palm zeaxanthin epoxidase.	130
4.28	Alignment of the deduced amino acid sequence of oil palm zeaxanthin epoxidase clone to its counterparts from other plants.	132
4.29	Agarose and formaldehyde gel electrophoresis and autoradiogram of total RNA samples isolated from different tissues of oil palm.	134
4.30	Determination of RNA quality using Bioanalyzer.	137
4.31	The efficiency test of real-time PCR assays for oil palm lycopene β -cyclase (<i>lcyb</i>), lycopene ε -cyclase (<i>lcye</i>), phytoene synthase (<i>psy</i>), phytoene desaturase (<i>pds</i>), zeaxanthin epoxidase (<i>zep</i>) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).	139
4.32	Agarose gel electrophoresis of real-time PCR products for <i>lcyb</i> and GAPDH.	141
4.33	The expression of oil palm <i>lcyb</i> , <i>lcye</i> , <i>pds</i> , <i>zep</i> and <i>psy</i> genes in developing mesocarp tissues of different developmental stages and spear and green leaves relative to kernel.	148
4.34	Electrophoresis of oil palm total DNA samples digested with <i>Sca</i> I and <i>Bst</i> N I.	151
4.35	Southern hybridization of oil palm total DNA digested with <i>EcoR</i> I, <i>Rsa</i> I and <i>Taq</i> I to oil palm lycopene β -cyclase cDNA (<i>lcyb</i>).	152
4.36	Southern hybridization of oil palm total DNA digested with <i>Dra</i> I and <i>Sca</i> I to oil palm lycopene β -cyclase cDNA (<i>lcyb</i>).	153
4.37	Southern hybridization of oil palm total DNA digested with <i>Rsa</i> I, <i>Taq</i> I and <i>Bst</i> N I to oil palm lycopene ε -cyclase cDNA (<i>lcye</i>).	155
4.38	Southern hybridization of oil palm total DNA digested with <i>Sst</i> I and <i>Sca</i> I to oil palm lycopene ε -cyclase cDNA (<i>lcye</i>).	156

List of Abbreviations

AA	Amino acid
ABA	Absciscic acid
ACCase	Acetyl-CoA carboxylase



ACP	Acyl carrier protein
CCS	Capxanthin-capsorubin synthase
CDPME	4-diphosphocytidyl-2-C-methyl-D-erythritol
CDPME2P	4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate
CHYb	Carotene β -hydroxylase;
CHYe	Carotene ϵ -hydroxylase;
CMK	CDPME kinase
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	DXP reductoisomerase
DXS	DXP synthase
FAD2	Oleoyl-CoA desaturase
FPP	Farnesyl diphosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGPP	Geranylgeranyl diphosphate
HDR	HMBPP reductase
HDS	HMBPP synthase
HYB	Carotene hydroxylase
IPP	Isopentenyl diphosphate
K10	Kernal at 10 WAA
KAc	Potassium acetate
KAS	β -ketoacyl-ACP synthase
LCYb	Lycopene β -cyclase
LCYe	Lycopene ϵ -cyclase
M5-M19	Mesocarp tissues at 5-19 WAA
MCS	ME2,4cPP synthase
MEP	2-C-methyl-D-erythritol 4-phosphate
MPOB	Malaysia Palm Oil Board



MVA	Mevalonic acid
MVAP	Mevalonic acid 5-phosphate
MVAPP	Mevalonic acid 5-diphosphate
NaAc	Sodium acetate
NPQ	Nonphotochemical quenching
NXS	Neoxanthin synthase
NUPM	Nested universal primer mix
PDS	Phytoene desaturase
PSY	Phytoene synthase
PVP	Polyvinylpyrrolidone
RTPCR	Reverse transcriptase polymerase chain reaction
SAD	Stearoyl-ACP desaturase
SDS	Sodium dodecyl sulfate
UPM	Universal primer mix
VAD	Vitamin A deficiency
VDE	Violaxanthin de-epoxidase
WAA	Week after anthesis
ZDS	(zeta) ζ -carotene desaturase
ZEP	Zeaxanthin epoxidase



CHAPTER 1

INTRODUCTION

Oil palm is the main commodity crop of Malaysia. Since it was first introduced as a commercial crop in 1917, the planted area and palm oil production have continuously expanded and reached 4.17 million hectares (ha) and 15.88 million tonnes in 2006, respectively (Mohd Basri, 2007; Basiron and Chan, 2004b; Jalani *et al.*, 2002). The palm oil industry has contributed continuously and significantly to the country's economic development and foreign exchange earnings. In 2007, the total export value of palm oil and its products was recorded at RM 45.1 billion, representing 41.8% increase from the previous year (Basiron and Mohd Arif, 2005; Basiron and Chan, 2004a; Mohd Basri, 2008; Mohd Basri, 2007).

Despite the strong contribution to the country's economic development, Malaysian palm oil industry is facing several challenges. Among the immediate challenges are the decrease in land availability for further expansion and labor shortage (Khoo and Chandramohan, 2002; Cheah, 2000; Parveez *et al.*, 2000; Sambanthamurthi *et al.*, 2000a). In addition, Malaysian palm oil industry is also faced with competition from other oils such as soybean oil, rapeseed oil and sunflower seed oil as well as other palm oil producers. The challenge for oil palm industry is to ensure the demand for its oil continues to increase. However, due to the fact that agriculture is one of the most protected and heavily subsidized industries, it will be difficult for palm oil to maintain its competitiveness and market share (Mohd Nasir *et al.*, 2005; Basiron, 2001). Currently, the most prominent competitor for Malaysia palm oil

producers is Indonesia. Although the Indonesian palm oil industry was only recently established, it has shown an impressive expansion in both the cultivated areas and production. With the lower production cost and improvements in performance, the Indonesian oil palm industry is believed to be able to continue its expansion and emerge as the leading supplier of palm oil in the world market (Barlow *et al.*, 2003).

In the past, the competitive edge has been the driving force to the rapid growth and tremendous performance of the Malaysian palm oil industry. The factor will remain as a vital factor for its future development. Therefore, proper strategies should be put in place in order to meet the current challenges and to remain competitive in the future. The industry needs to increase its productivity, explore the opportunities to diversify its income base and widen the end-use base for palm oil. A straightforward and effective approach towards the productivity of the oil palm industry is to improve the oil yield for each unit planted area. In the past, the introduction of tenera hybrid as the commercial planting materials has been responsible for the 30% increase of the oil yield compared to dura (Soh *et al.*, 1994). Nevertheless, the level of oil palm productivity has been static for a considerable time period (Basiron, 2001; Baskett *et al.*, 2007). Thus, effort has to be vigorously made to improve the oil yield. Recent work through advanced breeding technology has been able to produce new tenera varieties with a higher oil yield potential (8 to 10 t/ha/yr). It is believed that further selection of newer elite duras and pisiferas derived from selected germplasm materials can further improve the tenera oil yield in the future (Jalani *et al.*, 2002; Rajanaidu *et al.*, 2007; Sharifah Shahrul Rabiah and Abu Zarin, 2007).