
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 Malaysia, in fulfilment of the requirements for the Degree of Doctor of Philosophy**

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**2008**

**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 dusaturase (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 (RTPCR) 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

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

Oleh

**OMAR BIN ABD RASID**

**2008**

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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

diamplifikasi 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

Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the degree of Doctor of Philosophy.

Members of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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Date: 10 July 2008

## 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**

## List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| AA | Amino acid |
| ABA | Abscisic acid |
| ACCase | Acetyl-CoA carboxylase |

| | |
|---|---|
| ACP | Acyl carier protein |
| CCS | Capxanthin-capsorubin synthase |
| CDPME | 4-diphosphocytidyl-2-C-methyl-D-erythritol |
| CDPME2P | 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate |
| CHYb | Caritene 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 tones 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).