
**UNIVERSITI PUTRA MALAYSIA**

# *MOLECULAR CLONING, CHARACTERIZATION, AND PROMOTER ANALYSIS OF VITAMIN E BIOSYNTHETIC GENES FROM THE OIL PALM*

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**MASTER OF SCIENCE**
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# MOLECULAR CLONING, CHARACTERIZATION, AND PROMOTER ANALYSIS OF VITAMIN E BIOSYNTHETIC GENES FROM THE OIL PALM

**By**

**KONG SZE LING**



**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

**MOLECULAR CLONING, CHARACTERIZATION, AND PROMOTER ANALYSIS OF VITAMIN E BIOSYNTHETIC GENES FROM THE OIL PALM**

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**July 2013**

**Chairman: Prof. Datin Siti Nor Akmar Abdullah, PhD**

**Faculty: Institute of Tropical Agriculture**

Tocopherols and tocotrienols, commonly known as vitamin E, play a crucial role in human and animal nutrition. In recent years, tocotrienols have been reported as a powerful antioxidant agent and linked with various potential health benefits such as anti-angiogenic properties exhibited by palm tocotrienols. Therefore this brings the interest to carry out isolation and characterization of vitamin E biosynthetic genes from the oil palm (*E. guineensis* and *E. oleifera*) since crude palm oil has been well known to be the richest source of tocotrienols in nature. Homogentisate geranylgeranyl transferase (HGGT) and homogentisate phytyltransferase (HPT) are the two key enzymes that catalyse the condensation of homogentisic acid (HGA) with a prenyldiphosphate to produce tocotrienols and tocopherols in plants, respectively. The partial cDNAs encoding HGGT and HPT enzymes were successfully isolated from both oil palm species by PCR amplification using degenerate primers. Subsequently, full length cDNA sequences were obtained by rapid amplification of cDNA ends (RACE) using gene-specific primers. The full length deduced amino acid sequences were further analyzed using various bioinformatics tools available publicly. The analysis revealed the presence of an UbiA prenyltransferase conserved domain in all four protein sequences and suggested that oil palm HGGT and HPT are more evolutionarily related with their counterparts from other monocot plant species based on the result from homologous alignment and phylogenetic analysis. Next, quantitative gene expression analysis was carried out to elucidate the transcript profiles of the oil palm HGGT and HPT genes in different tissues and at different developmental stages of the mesocarp by real-time PCR. Two reference genes that showed to be stably expressed in each experimental set were identified using geNorm software. The expression level of each target gene in each experimental sample was subsequently determined by normalizing to the two validated reference genes. Overall result showed that the oil palm HGGT and HPT transcript production is spatially and temporally regulated. The HPT gene was constitutively expressed in all tested tissues except in 15 w.a.a kernel whereas oil palm HGGT gene showed preferential expression in mesocarp and kernel tissues and highly expressed when active oil deposition occurred in 17 w.a.a mesocarp. This indicates that HGGT expression is regulated by the oil synthesis process in palm fruits. Lastly, genome-

walking PCR successfully amplified the HGGT promoter region of both oil palm species. By searching in PLACE, PlantCARE and PlantPAN databases, a number of important *cis*-regulatory elements were found and comparison between these data has resulted in the identification of several common motifs which may be involved in coordinating expression of these genes. The motifs basically can be divided into four main groups including phytohormone-responsive, light-responsive, abiotic factor-responsive and endosperm specificity. This suggests that the regulation of HGGT expression in *E. guineensis* and *E. oleifera* involved many similar factors. Further characterization of the potential important motifs would facilitate better understanding on the regulatory mechanism of tocotrienol synthesis in oil palm at the molecular level.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains

**PENGKLONAN, PENCIRIAN, DAN ANALISIS PROMOTER GEN-GEN BIOSINTESIS VITAMIN E DARIPADA KELAPA SAWIT**

Oleh

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Tokoferol dan tokotrienol yang dikenali secara am sebagai vitamin E memainkan peranan penting dalam pemakanan manusia dan haiwan. Beberapa tahun kebelakangan ini, tokotrienol telah dilaporkan sebagai agen antioksidan yang hebat dan dikaitkan dengan pelbagai manfaat kesihatan yang berpotensi seperti ciri-ciri anti-angiogenik yang dipamerkan oleh tokotrienol sawit. Oleh itu, perhatian diberikan untuk memencilkan dan mencirikan gen-gen biosintesis vitamin E daripada minyak sawit (*E. guineensis* dan *E. oleifera*) memandangkan minyak sawit mentah merupakan sumber asli yang terkaya dengan tokotrienol. Homogentisat geranilgeranil transferase (HGGT) dan homogentisat fitiltransferase (HPT) adalah dua enzim utama yang menjadi pemangkin dalam pemeluwapan asid homogentisik (HGA) dengan prenildifosfat untuk menghasilkan tokotrienol dan tokoferol dalam tumbuhan. Amplifikasi PCR menggunakan pencetus degenerasi telah berjaya memencilkan cDNA separa lengkap yang mengekod enzim HGGT dan HPT daripada kedua-dua spesis kelapa sawit. Seterusnya, jujukan lengkap cDNA telah diperoleh melalui amplifikasi pantas hujung cDNA dengan penggunaan pencetus spesifik. Jujukan asid amino yang dijangka telah dianalisis dengan menggunakan perisian bioinformatik awam. Analisis tersebut mendedahkan kehadiran domain terpelihara UbiA preniltransferase dalam kesemua jujukan protein sementara keputusan penjajaran homolog dan analisis filogenetik mencadangkan HGGT dan HPT sawit lebih mempunyai pertalian evolusi dengan tumbuhan monokot lain. Seterusnya, analisis kuantitatif pengekspresan gen dijalankan untuk mendapatkan profil transkrip HGGT dan HPT dalam tisu kelapa sawit yang berlainan dan juga pelbagai peringkat perkembangan dalam mesokarpa melalui kaedah "real-time" PCR. Dua gen rujukan yang menunjukkan tahap pengekspresan yang stabil dalam setiap set eksperimen telah dikenalpasti oleh perisian geNorm. Tahap pengekspresan untuk gen sasaran masing-masing dalam setiap sampel kemudiannya dinormalisasikan oleh kedua-dua gen rujukan yang telah disahkan tersebut. Keputusan keseluruhan menunjukkan bahawa pengekspresan HGGT dan HPT sawit telah dikawalatur dalam tisu dan peringkat perkembangan. HPT sawit diekspreskansecara konstitutif dalam semua tisu kecuali kernel (15 m.s.a) manakala gen HGGT sawit hanya dapat dikesan dalam tisu tertentu, iaitu mesokarpa dan kernel serta menunjukkan tahap pengekspresan yang tinggi semasa pemendapan minyak berlaku secara aktif dalam mesokarpa (17 m.s.a). Ini menunjukkan bahawa pengekspresan HGGT dikawal oleh proses sintesis minyak dalam buah sawit. Akhir sekali, jujukan promoter HGGT sawit telah berjaya diamplifikasikan menggunakan teknik "genome-walking PCR". Pencarian menggunakan pangkalan data

PLACE, PlantCARE serta PlantPAN, telah mengenalpasti beberapan elemen cis-pengawalatur yang mungkin terbabit dalam mengkoordinasi pengekspresan. Secara am motif tersebut dapat dikategorikan kepada empat kumpulan utama termasuk yang respon terhadap fitohormon, cahaya, faktor abiotik dan endosperm spesifik. Ini menunjukkan bahawa pengawalaturan transkripsi HGGT dalam *E. guineensis* dan *E. oleifera* melibatkan banyak faktor yang sama. Pencirian lanjut untuk motif penting yang berpotensi akan memudahkan pemahaman yang lebih baik mengenai mekanisme pengawalaturan sintesis tokotrienol dalam kelapa sawit pada peringkat molekul.

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I certify that a Thesis Examination Committee has met on 8 July 2013 to conduct the final examination of Kong Sze Ling on her thesis entitled "Molecular Cloning, Characterization and Promoter Analysis of Vitamin E Biosynthetic Genes from the Oil Palm" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A). 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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| | |
|---|---|
| AP | Adaptor Primer |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair |
| $CaCl_2$ | calcium chloride |
| cDNA | complementary deoxuribonucleic acid |
| Ct | threshold cycle |
| CTAB | hexadecyl (or cetyl) trimethyl ammonium bromide |
| Da | Dalton |
| DNA | deoxyribonucleic acid |
| DNase I | deoxyribonuclease I |
| dNTP | deoxynucleoside triphosphate |
| EDTA | ethylene diamine tetracetate |
| *E. coli* | *Escherichia coli* |
| GA | gibberillin |
| GAPDH | glyceraldehydes-3-phosphate dehydrogenase |
| GGDP | geranylgeranyldiphosphate |
| GGPP | geranylgeranyl pyrophosphate |
| GSP | gene-specific primer |
| HCl | hydrochloric acid |
| HGA | homogentisic acid |
| HGGT | homogentisate geranylgeranyl transferase |
| HPP | p-hydroxyphenylpyruvate |

| | |
|---|---|
| HPPDase | p-hydroxyphenylpyruvate dioxygenase |
| HPT | homogentisate phytyltransferase |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| kb | kilobase |
| LB | Luria-Bertani |
| LiCl | lithium chloride |
| M | molar |
| $MgCl_2$ | magnesium chloride |
| min | minutes |
| mRNA | messenger ribonucleic acid |
| NaCl | sodium chloride |
| NaOAc | sodium acetate |
| ng | nanogram |
| NCBI | National Center for Biotechnology Information |
| OD | optical density |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| PDP | phytyldiphosphate |
| phytyl-PP | phytyl pyrophosphate |
| pI | isoelectric point |
| PrDP | prenyldiphosphate |
| PVP-40 | polyvinylpyrrolidone-40 |
| RACE | Rapid Amplification of cDNA End |

| | |
|---|---|
| RNA | ribonucleic acid |
| $R^2$ | correlation coefficient |
| RT-PCR | reverse transcription PCR |
| SDS | Sodium dodecyl sulfate |
| sec | seconds |
| SNP | Single nucleotide polymorphism |
| TAE | tris-acetate-EDTA |
| TC | tocopherol/tocotrienol cyclase |
| TE | Tris-EDTA |
| TG | triacylglycerols |
| TMT | tocopherol/tocotrienol methyltransferase |
| TRF | tocotrienol-rich fraction |
| TSS | transcription start site |
| UTR | untranslated region |
| v/v | volume per volume |
| w/v | weight per volume |
| w.a.a | week after anthesis |
| µg | microgram |
| µM | micromolar |
| µl | microliter |
| *g* | relative centrifugal force |
| ℃ | Degree Celsius |

# CHAPTER 1

# INTRODUCTION

Since it was first planted as a commercial crop in 1917, oil palm cultivation has shown rapid expansion and it is now the main commodity crop of Malaysia. The palm oil industry has contributed significantly to Malaysia economic development and foreign exchange earnings. Palm oil is presently the world's major source of vegetable oil and Malaysia is second only to Indonesia as the world leading exporter of palm oil (CME Group, 2010).

As the world population increases, the demand from the oil palm industry also increases. Among the immediate challenges is the decrease in land availability and labour shortage. Besides, Malaysian palm oil industry also face great competition from other palm oil producing countries especially Indonesia. Thus, appropriate strategies need to be planned in order to ensure agricultural sustainability and to stay competitive in the future. An effective approach is to improve the oil yield per unit area of land with the view of maximizing returns. In addition, improvement of palm oil quality has also been set as one of the priority areas for oil palm research. Oil palm, being naturally rich in the minor components such as carotenoids and vitamin E, offers a great potential to be exploited as a value-added vegetable oil which is an important advantage over other vegetable oils and fats.

Tocochromanols, commonly known as vitamin E, play a crucial role in human and animal nutrition. Belonging to the amphipatic tocochromanol group of molecules, the eight structurally related tocopherols and tocotrienols forms (α-, β-, γ- δ- tocopherols and α-, β-, γ- δ-tocotrienols) collectively constitute the content of vitamin E (Kamal-Eldin and Appelqvist, 1996). Tocotrienols in vitamin E have been reported to possess powerful antioxidant and anti-cancer activities (Ebong *et al.*, 1999). Moreover, palm tocotrienols exhibit anti-angiogenic properties that may inhibit tumour progression (Selvaduray *et al.*, 2012). Besides contributing to human health, tocochromanols are also linked with a number of beneficial properties for cereals which include extending their storage life and contributing to the nutritive value of cereal grains in human and livestock diets.

Oil palm is one of the richest source of vitamin E especially tocotrienols which are not normally present in other edible oil. Crude palm oil extracted from the fruits of *Elaeis guineensis* particularly contains a high amount of tocotrienols (up to 800 mg/kg), mainly consisting of γ-tocotrienol and α-tocotrienol (Sen *et al.*, 2006). While *E. oleifera* also been reported to contain significantly higher amount of tocotrienol (Choo & Yusof, 1996). Clearly, it has great advantage compared to other plants for genetic manipulation of vitamin E. However, the knowledge on oil palm vitamin E biosynthesis pathway

which is one of the basic requirements for genetic manipulation is quite limited. This will definitely become an impediment to improve oil palm vitamin E content through genetic engineering, development of molecular markers for cross species breeding and other biotechnological approaches. This work is an initial effort towards the understanding of oil palm vitamin E biosynthetic pathway. This includes molecular characterization of the cDNAs encoding homogentisate geranylgeranyl transferase (HGGT) and homogentisate phytyltransferase (HPT), that catalyse the first commited step in tocotrienol and tocopherol production, respectively. The promoter sequences which regulate the expression of these genes will certainly contribute to the basic platform required for the oil palm vitamin E content and composition improvement.

Therefore the objectives of this study are

1. To isolate and characterize full length cDNA sequences encoding homogentisate geranylgeranyl transferase (HGGT) and homogentisate phytyltransferase (HPT) from oil palm (*E. guineensis* and *E. oleifera*).

2. To characterize the transcript expression profile of HGGT and HPT in different oil palm tissues and at different developmental stages through real-time quantitative PCR method in both oil palm species.

3. To isolate and analyze the promoter region of oil palm HGGT and to compare the presence of known *cis*-acting regulatory elements in both promoters.

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