UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF SEQUENCE VARIANTS IN KEY VITAMIN E GENES FROM Elaeis guineensis Jacq. GERMPLASM FOR DEVELOPMENT OF DNA-BASED MARKERS

BABURA SULAIMAN RUFA'I

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By

BABURA SULAIMAN RUFA'I

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

February 2018

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This thesis is dedicated to my loves ones, especially to my parents, wife, children (Aisha and Asma'u) and all my family members who have given me enormous support and prayers since the beginning of my studies.

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of
the requirement for the degree of Doctor of Philosophy

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BABURA SULAIMAN RUFA'I

February 2018

Chairman: Professor Datin Siti Nor Akmar Abdullah
Faculty: Agriculture

Vitamin E possesses important nutritional attributes that play various roles in human disease protection. The most well-known function of this noble compound is that of chain breaking antioxidant activity that scavenge free radical ions and reduce lipid peroxidation in membrane systems. Homogentisate geranylgeranyl transferase (HGGT) and homogentisate phytyl transferase (HPT) that catalyse the first committed step of tocotrienol and tocopherol biosynthesis, respectively are important in determining plant vitamin E composition. In *Elaeis guineensis,* there is high variability in the level of vitamin E among the germplasm materials from Angolan and Tanzanian origins. Therefore, the first objective of this study was to determine important sequence variants in these key vitamin E genes from *E. guineensis* germplasm materials that can be used for the development of DNA-based markers. The second objective was to analyse the effects of the sequence variants on vitamin E content and composition by overexpression of the *HGGT* gene and its mutant derivatives in *Arabidopsis thaliana*. Sequence analysis reveals no important variants in *HPT* gene that could be associated with low and high vitamin E content. However, the analysis reveals four SNPs at positions 193, 2225, 2429 and 6932 in the coding region of the *HGGT* gene that are associated with the vitamin E content. SNPs at 193 and 2429 positions lead to non-conservative amino acid changes in the sequence from Proline (CCT) in low vitamin E to Serine (TCT) in high vitamin E and from Methionine (ATG) in low to Isoleucine (ATA) in high vitamin E palms, respectively. SNP markers 193F/413R and 2225F/2429R were developed at these SNP locations for selection of high and low vitamin E germplasm materials in *E. guineensis*. Fourty one germplasm materials with different vitamin E level were screened to validate these two functional SNP markers using designed PCR-based mismatch primers. The results showed 100% success of the SNP-based markers in differentiating low and high vitamin E accessions. Furthermore, single nucleotide mutagenesis was successfully carried out to generate three cDNA sequence variants (*193SNPHGGT*, *2429SNPHGGT* and *HighSNPHGGT*) with one or both SNP variants incorporated into the sequence of the commercial D×P genotype (*LowSNPHGGT*). The variant *HGGT* cDNA sequences together with the unmodified cDNA were successfully transformed into *Arabidopsis*

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thaliana. The relative expression levels of *HGGT* in T_3 homozygous lines having the four different constructs separately showed significant ($P \leq 0.005$) up-regulated expression compared with untransformed wild type *Arabidopsis*. However, there was no significant difference observed in the expression among transgenic *Arabidopsis* plants harbouring the different *HGGT* constructs. This demonstrated that the different variants of the *E. guineensis HGGT* gene was expressed at about the same levels in the transgenic *Arabidopsis*. HPLC analysis indicates significant increase ($p \leq 0.05$) in the total tocotrienol content between wild type and all the four transgenic lines (1.50 – 1.82-fold increase). Similarly, significant difference ($p \leq 0.05$) in total tocotrienol was also recorded within the transgenic lines specifically between the two lines that harboured the two SNPs changes (*HighSNPHGGT)* and the one harboring the unmodified gene (*LowSNPHGGT*), which showed 1.22-fold increase. According to these results the two SNP variants introduced into the *HGGT* sequence of low vitamin E commercial variety affect the tocotrienol content and composition when analysed by functional characterization in *Arabidopsis thaliana*.

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

PENGENALAN VARIAN JUJUKAN DALAM GEN UTAMA VITAMIN E DARIPADA BAHAN GERMPLASMA *Elaeis guineensis* Jacq. UNTUK PEMBANGUNAN PENANDA BERASASKAN DNA

Oleh

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Vitamin E mempunyai ciri nutrisi penting yang memainkan pelbagai peranan dalam ketahanan penyakit manusia. Fungsi yang paling dikenali bagi sebatian adi ini adalah aktiviti antioksidan pemutus rantai yang mengaut ion radikal bebas dan mengurangkan pemperoksidaan lipid dalam sistem membran. Homogentisat geranilgeranil transferase dan homogentisat fitil transferase yang memangkin langkah berkomitmen pertama daripada biosintesis tokotrienol dan tokoferol adalah penting dalam menentukan komposisi vitamin E tumbuhan. Dalam *Elaeis guineensis*, terdapat variabiliti yang tinggi dalam paras vitamin E di antara bahan germplasma yang berasal dari Angola dan Tanzania. Dengan itu, objektif pertama kajian adalah untuk menentukan varian jujukan penting dalam gen vitamin E utama dari bahan germplasma *E. guineensis* yang boleh digunakan untuk pembangunan penanda berasasakan DNA. Objektif kedua adalah untuk menganalisis kesan varian jujukan keatas kandungan dan komposisi vitamin E melalui pengekspresan melampau gen HGGT dan mutan yang terhasil darinya dalam *Arabidopsis thaliana*. Analysis jujukan mendedahkan tiada varian dengan fungsi yang penting dalam gen *HPT* yang boleh dikaitkan dengan kandungan vitamin E yang rendah dan tinggi. Walau bagaimanapun, analisis mendedahkan empat SNPs pada kedudukan 193, 2225, 2429 dan 6932 dalam kawasan pengekodan gen *HGGT* yang dikaitkan dengan kandungan vitamin E. SNPs pada kedudukan 193 dan 2429, masing-masing membawa kepada perubahan non-konservatif asid amino dalam jujukan dari Prolin (CCT) dalam vitamin E yang rendah kepada Serine (TCT) dalam vitamin E yang tinggi dan dari Methionein (ATG) dari yang rendah kepada Isoleusin (ATA) dalam palma yang bervitamin E tinggi. Penanda SNPs 193F/413R dan 2225F/2429R telah dibangunkan pada lokasi SNPs untuk pemilihan bahan germplasma vitamin E yang tinggi dan rendah dalam *E.guineensis*. Empat puluh satu bahan germplasma dengan paras vitamin E yang berbeza telah disaring untuk mengesahkan dua penanda SNP fungsional menggunakan primer berasasakan PCR yang tidak sepadan. Keputusan menunjukkan 100% kejayaan penanda SNP tersebut dalam membezakan aksesi vitamin E yang rendah dan tinggi. Tambahan pula, kaedah mutagenesis nukleotida tunggal telah berjaya dilakukan untuk menjana tiga varian jujukan cDNA (*193SNPHGGT*, *2429SNPHGGT* dan

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HighSNPHGGT) dengan satu atau dua varian SNP dimasukkan ke dalam jujukan cDNA genotip D×P komersial (*LowSNPHGGT*). Varian jujukan cDNA bersama dengan cDNA yang tidak diubah suai telah bejaya ditransformkan kedalam *Arabidopsis thaliana*. Paras ekspresi relatif *HGGT* dalam titisan homozigot T_3 yang mempunyai empat konstruk berbeza yang berasingan menunjukkan ekspresi menaik yang ketara ($P \leq 0.05$) berbanding *Arabidopsis* jenis liar yang tidak diubahsuai. Walau bagaimanapun, tiada perbezaan ketara yang diperhatikan dalam ekspresi antara tumbuhan *Arabidopsis* transgenik yang memiliki konstruk *HGGT* yang berbeza. Ini menunjukkan varian gen *HGGT E. guineensis* yang berbeza telah diekspres pada paras yang sama dalam *Arabidopsis* transgenik. Analisis HPLC menunjukkan peningkatan yang ketara ($P \leq 0.05$) dalam kandungan tokotrienol keseluruhan antara jenis liar dan empat titisan transgenik (1.5-1.82 kali ganda meningkat). Begitu juga, perbezaan ketara ($P \leq 0.05$) dalam keseluruhan tokotrienol juga direkodkan diantara titisan transgenik terutama di antara dua titisan yang mempunyai dua perubahan SNPs (*HighSNPHGGT*) dan yang mempunyai gen tidak diubahsuai (*LowSNPHGGT*), yang mana menunjukkan peningkatan 1.22 kali ganda. Berdasarkan penemuan ini, kedua varian SNPs yang dimasukkan kedalam *HGGT* varieti komersial dengan vitamin E rendah memberi kesan terhadap kandungan dan komposisi vitamin E apabila dianalisis melalui pencirian kefungsian dalam *Arabidopsis thaliana*.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of requirement for degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µl	Micro-liter
A.thaliana	*Arabidopsis thaliana*
A.tumefaciens	*Agrobacterium tumefaciens*
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
CDS	Coding Region
CTAB	Hexacetyltrimethyl Ammonium Bromide
D x P	Dura x Pesifera
DEPC	Diethyl Pyrocarbonate
DLD-1	Colorectal anticancer agent
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP$_S$	Deoxynucleotides
EDTA	Ethylenediaminetetraacetic acid
EgHGGT	Elaeis guineensis HGGT
EtBr	Ethidium Bromide
FFB	Fresh fruit bunches
Gb	Giga bite
GBS	Genotyping by sequencing
GGDP	Geranylgeranyl diphosphate
GWAS	Genome-wide association study
HGA	Homogentisic acid
HGGT	Homogentisate geranylgeranyl transferase
HPLC	High performance liquid chromatography
HPPD	Hydroxyphenyl pyruvate dioxygenase
HPT	Homogentisate petyltransferase
InDels	Insertion Deletion
KASP	Competitive allele specific PCR
kb	Kilo Base-Pair
Km	Michaelis constant
LB	Luria-Bertani
LD	Linkage disequilibrium
LiAc	Lithium Acetate
LiCl	Lithium Chloride
MAS	Marker-assisted selection
MPOB	Malaysian palm oil board
mRNA	Messenger RNA
MYB	Myeloblastosis gene
NaCl	Sodium Chloride
NaOH	Natrium Hydroxide
NCBI	National Center For Biotechnology Information
ng	Nanogram
NGS	Next-generation sequencing
OD	Optical Density

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OFR	Open reading frame
PacBio	Pacific Bioscience
PCR	Polymerase Chain Reactions
PDP	Phytyl diphosphate
pH	potential of hydrogen
PTGs	Post transcriptional gene silencing
PVP	Polyvinylpyrrolidone
qPCR	Quantitative real-time PCR
QTLs	Quantitative trait loci
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Round per minute
rtITP	reversibly terminated deoxyinosine triphosphate
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S.O.C	Super Optimal Broth
SDS	Sodium Dodecyl Sulphate
SGS	Second generation sequencing
SMRT	Single-molecule real-time
SNP	Single nucleotide polymorphism
SSRs	Simple sequence repeats
TAE	Tris-acetate EDTA
TE buffer	Tris-EDTA buffer
TGS	Third generation sequencing
Tm	Melting temperature
UTR	Untranslated region
UV	Ultraviolent
w.a.a	Week After Anthesis
WT	Wild Type
193SNPHGGT	*HGGT* with mutated SNP at 193 position
2429SNPHGGT	*HGGT* with mutated SNP at 2429 position
HighSNPHGGT	*HGGT* with two mutated SNPs at 193 and 2429 position
LowSNPHGGT	Non Mutated *HGGT*

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CHAPTER 1

INTRODUCTION

The term vitamin E is used to describe eight lipophilic, naturally occurring compounds that include four tocopherols designated as α-, β-, γ- δ- tocopherols and their four corresponding tocotrienols (Peh et al., 2015). Tocopherols and tocotrienols isomers are distinguished based on the number and position of the methyl groups on their chromanol ring. Tocopherols have saturated phytyl tail that differentiates them from tocotrienols, which possess unsaturated tail containing three double bonds. Tocopherols are important lipid soluble antioxidants that protect cell membrane from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Hunter and Cahoon, 2007). Like tocopherols, tocotrienols are good antioxidants that tend to guard plant cells against any biochemical stress especially those arising from the breakdown of unsaturated fatty acids is the seeds (Das et al., 2005). Compared with tocopherols, tocotrienols are sparsely studied, but the current research direction is starting to give more attention to the tocotrienols, the lesser known but more potent antioxidant in vitamin E. Tocotrienols are believed to possess greater ability than tocopherols in scavenging free radical ions and reducing peroxidation of lipids in membrane system (Shahidi et al., 2010). In addition, some studies suggested that tocotrienols have specialized role in protecting neurons from damage (neurodegradation) (Sen et al., 2006) and cholesterol reduction properties (Das et al., 2005). Oral consumption of tocotrienols protects against stroke-associated brain damage *in vivo* (Khanna et al., 2005). Generally, most reports on vitamin E have shown that many of the properties in tocotrienols are not present in tocopherols. Tocotrienols are commercially produced from extracts of rice and oil palm and can be purchased in different forms (Cahoon et al., 2003).

Crude red palm oil is a unique vegetable oil obtained from the fruits of oil palm tree (*Elaeis guineensis*). The only natural oil produces a mixture of different antioxidants and phytonutrients such as tocopherol/tocotreinol (vitamin E), alpha and beta-carotene (pro-vitamin A) in high level, phytosterol complex and coenzymes. No other vegetable oil has this natural combinations of phytonutrients (Corley, 2007). Malaysian Palm Oil Board (MPOB) has most member of *Elaeis guineensis* germplasm materials in the whole world (Zaki et al., 2012). Among the *E. guineensis* germplasm materials, the Angolan and Tanzanian materials were observed to have high variability in the level of vitamin E content ranging from 300 – 1600 ppm, while the level is 500 – 1000 ppm in the commercial (D×P) variety (Wahid et al., 2005). This variation may arise from the molecular set up of the genes responsible for the production of vitamin E in the plant. Thus, it would be interesting to study functional nucleotide variants in the key vitamin E biosynthetic genes (*HGGT* and *HPT*), which catalyses the first committed step of tocotreinol and tocopherol biosynthesis, respectively.

Identification of potentially functionally important sequence variants in the form of SNPs or indels as well as functional analysis of *HGGT* responsible for tocotrienol biosynthesis

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and *HPT* gene responsible for tocopherol biosynthesis in oil palm would be very valuable. It could help in identifying the sources of vitamin E content variation among the accessions, and in the production of nutritionally rich palm oil in the long term. Since such studies have not been done so far, the results obtained will serve as valuable background information for genetic improvement of the oil palm. The objectives of this study are:

1) To determine the sequence variations in *homogentisate geranylgeranyl transferase* (*HGGT*) and *homogentisate phytyltransferase* (*HPT*) genes that differentiates accessions producing high and low vitamin E in *Elaeis guineensis* germplasm materials.
2) To develop SNPs/Indels markers for high and low vitamin E germplasm materials identification based on potential functional nucleotide variants.
3) To produce different expression vector constructs by introducing different combination of variant nucleotides into the *HGGT* sequence from D×P variety for functional studies.
4) To analyse the effects of sequences variant on vitamin E composition and content by overexpression of the *HGGT* gene and its mutant derivatives in transgenic model plant (*Arabidopsis thaliana)*.

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