UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF FUNCTIONALLY IMPORTANT SEQUENCE VARIANTS IN PROMOTER SEQUENCE OF HOMOGENTISATE GERANYLGERANYL TRANSFERASE FROM Elaeis guineensis Jacq. GERMPLASM MATERIALS

MOHD SHAHRUL NIZWANSHAH BIN KARIM

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MOHD SHAHRUL NIZWANSHAH BIN KARIM

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DEDICATION

This thesis is dedicated to my wife, Tuan Syaripah Najihah, who instilled in me the virtues of perseverance, determination and relentlessly encouraged me to strive for excellence. She's been a constant source of support and encouragement during the challenges of graduate school and life. This work is also dedicated to my mother Norminah Atim who has always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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

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Chairman: Professor Datin Siti Nor Akmar Abdullah, PhD
Faculty : Institute of Tropical Agriculture & Food Security

Vitamin E consists of tocopherols and tocotrienols which are lipid-soluble compounds produced by plants with various beneficial medicinal properties. Tocotrienols have cholesterol-lowering and anticancer properties and alpha-tocotrienols can prevent inducible neurodegeneration. There is high variability in the level of vitamin E in the *E. guineensis* germplasm materials from Angola and Tanzania ranging from 300-1600 ppm, while the level is 500-1000 ppm in the commercial variety. However, the development of markers for tocotrienols rich oil palm is still very scarce and the understanding of the regulation of vitamin E biosynthesis is important for genetic improvement specifically nutritionally vitamin E rich oil palm. Therefore, this study aimed to identify the SNPs in the promoter of HGGT that associate with the high vitamin E in *E. guineensis* germplasm materials. *Homogentisate geranylgeranyl transferase* (*HGGT*) is an important vitamin E biosynthetic enzyme that catalyses the first committed step for tocotrienols biosynthesis. Sequence alignment of 14 accessions from Angola and Tanzania with varying levels of vitamin E content showed the presence of an SNP (-454) that associate with high vitamin E Angolan (AH) and Tanzanian (TH) palms and two SNPs, -781 and -113 unique to AH. Functional characterisation to determine the roles of SNPs in influencing HGGT promoter activity was carried out by reporter gene assay in mesocarp tissues bombarded with four different HGGT promoter constructs. The pBGWFS7 vector used for cloning the HGGT promoter fragments contains two reporter genes, a green fluorescent protein (GFP) and GUS. The promoters were commercial DXP (COM), AH1, TH96 and a mutated derivative of COM (CM, g.-454A>G) produced by introducing the variant found in common in AH1 and TH96. The quantitative fluorometric GUS assay on GFP positive bombarded tissues shows that the lowest expression level was obtained from COM with GUS expression of 0.27 pmol MU min^{-1} mg $protein^{-1}$ per copy numbers. The expression level obtained for COM is about the same as the constitutive CaMV *35S* promoter that was used for comparison. There was no significant difference in the GUS expression level between the COM with the CM (g.-454A>G). The results showed that AH1 gave the highest GUS expression of 0.82 pmol MU min^{-1} mg $protein^{-1}$ per copy numbers. It

suggested that the CAAT-box unique to AH at the SNP (-113), a well-known proximal promoter element may enhance HGGT promoter activity and the tocotrienols content in AH1. Taken together, these studies have provided valuable information on the vitamin E biosynthetic regulatory mechanism in oil palm germplasm as these results suggest that the HGGT promoter is potentially useful for engineering of high vitamin E markers through SNPs association and it is essential for the future genetic improvement effort to produce high vitamin E oil palm.

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

PENENTUAN KEPELBAGAIAN JUJUKAN YANG PENTING DALAM JUJUKAN PROMOTER KEPADA HOMOGENTISATE GERANYLGERANYL TRANSFERASE GEN DARIPADA KUMPULAN GERMPLASMA *Elaeis guineensis Jacq.*

Oleh

MOHD SHAHRUL NIZWANSHAH BIN KARIM

Oktober 2020

Pengerusi: Professor Datin Siti Nor Akmar Abdullah, PhD
Fakulti : Institut Pertanian Tropika & Sekuriti Makanan

Vitamin E terdiri daripada tokoferol dan tokotrienol yang merupakan sebatian terlarut lipid yang dihasilkan oleh tumbuh-tumbuhan dengan ianya mempunyai pelbagai ciri-ciri perubatan yang bermanfaat. Tokotrienol mempunyai kebolehan menurunkan kolesterol dan bersifat anti-kanser manakala alfa-tokotrienol mampu mencegah degenerasi-saraf. Germplasma *E. guineensis* seperti Angola dan Tanzania mempunyai variasi kandungan vitamin E yang tinggi dan pelbagai iaitu bermula dari 300 sehingga 1600 ppm. Manakala dapat dilihat pula pada kadar 500 sehingga 1000 ppm dalam germplasma *E. guineensis* komersil. Walau bagaimanapun, pembangunan penanda kelapa sawit yang kaya kandungan tokotrienol masih terhad dan pemahaman mengenai pengaturan biosintesis vitamin E adalah penting demi penambahbaikkan genetik khasnya kelapa sawit yang kaya vitamin E. Oleh itu, kajian ini dijalankan dengan tujuan untuk mengenal pasti SNP yang terdapat pada jujukan promoter HGGT yang mempunyai hubung kait dengan kandungan vitamin E yang tinggi dalam bahan germplasma *E. guineensis*. *Homogentisate geranylgeranyl transferase* (*HGGT*) adalah enzim biosintetik vitamin E yang penting dalam memangkinkan langkah pertama dalam proses biosintesis tokotrienol. Justeru itu, kajian ini bertujuan untuk mengenal pasti SNP dalam promoter HGGT yang menjadi faktor penyumbang kadar kandungan vitamin E yang tinggi. Urutan penjajaran daripada 14 aksesi Angola dan Tanzania dengan kadar kandungan vitamin E yang berbeza-beza menunjukkan terdapat kehadiran SNP pada kedudukan (-454) yang berpotensi menyumbang kepada kandungan vitamin E yang tinggi pada populasi germplasma kelapa sawit Angolan (AH) dan Tanzanian (TH) dan dua SNP lagi iaitu pada kedudukan (-781) dan (-113) adalah unik dan hanya terdapat pada germplasma kelapa sawit AH. Analisa pencirian fungsi untuk mengenal pasti peranan SNP tersebut dalam mempengaruhi aktiviti promoter HGGT dilakukan dengan menggunakan assai gen pelapor yang dibombardir ke dalam tisu mesokap yang terdiri daripada empat binaan promoter HGGT yang berbeza. Vektor pBGWFS7 telah digunakan untuk pengklonan promoter HGGT dimana ia mengandungi dua gen pelapor, iaitu protein berpendarfluor

hijau (GFP) dan GUS. Para promoter itu adalah DXP (COM), AH1, TH96 dan mutan terbitan COM (CM, g.-454A>G) yang dihasilkan dengan memasukkan varian yang biasa ditemukan dalam AH1 dan TH96. Analisa kuantitatif fluorometrik GUS pada tisu positif GFP menunjukkan bahawa tahap ekspresi terendah adalah pada COM dengan ekspresi GUS sebanyak 0.27 pmol MU $minit^{-1}$ mg $protein^{-1}$ per nombor salinan. Manakala, Tahap ekspresi yang diperoleh untuk COM adalah hampir sama dengan promoter konstitutif CaMV *35S* yang digunakan sebagai perbandingan. Dapat dilihat, tidak terdapat perbezaan yang signifikan dalam tahap ekspresi GUS antara COM dengan versi bermutasi, CM (g.-454A>G). Tahap ekspresi tertinggi adalah dari promoter AH1 dan diikuti oleh TH96. Keputusan menunjukkan bahawa AH1 memberikan aktiviti GUS tertinggi dengan kadar ekspresi GUS sebanyak 0.82 pmol MU $minit^{-1}$ mg $protein^{-1}$ per nombor salinan . Justeru itu, kami mencadangkan bahawa kotak CAAT yang terdapat pada AH di SNP (-113) adalah unik kepada AH sahaja di mana ia merupakan unsur promoter proksimal yang telah dikenal pasti mampu meningkatkan aktiviti promoter HGGT dan kandungan tocotrienol dalam AH Secara keseluruhan, kajian ini telah memberikan maklumat yang bernilai mengenai mekanisma pengawalseliaan biosintetik vitamin E dalam germplasma kelapa sawit dan hasil kajian telah menunjukkan bahawa promoter HGGT berpotensi untuk digunakan dalam kejuruteraan penanda bagi kandungan vitamin E yang tinggi melalui hubung kait SNP dimana ia juga penting dalam pembangunan genetik pada masa hadapan dalam usaha menghasilkan kelapa sawit yang mempunyai kandungan vitamin E yang tinggi.

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

Siti Nor Akmar binti Abdullah, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Noor Azmi bin Shaharuddin, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name of
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Supervisory
Committee: Siti Nor Akmar Abdullah, PhD

Signature: _____
Name of
Member of
Supervisory
Committee: Noor Azmi Shaharuddin, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

AH	Angola High Vitamin E
AL	Angola Low Vitamin E
Bp	base pair
$CaCl_2$	calcium chloride
CM	Commercial Mutated DXP
COM	Commercial DXP
Ct	threshold cycle
CTAB	hexadecyl trimethyl ammonium bromide
DNA	deocyribonucleic acid
EDTA	ethylene diamine tetraacetate
E. coli	*Escherichia coli*
E. guineensis	*Elaeis guineensis*
GUS	β-glucuronidase
GGDP	geranylgeranyldiphosphate
HCL	hydrochloric acid
HGGT	homogentisate geranylgeranyl transferase
HPT	homogentisate phytyltransferase
IPTG	isopropyl β-D-I-thiogalactopyranoside
Kb	kilobase
LB	Luria-Bertani
M	molar
4-MU	4-methyllumbelliferone
4-MUG	4-methylumbelliferyl-β-D-glucuronide
$MgCl_2$	magnesium chloride

Min	minutes
NaCl	sodium chloride
Ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RT-PCR	real time PCR
SDS	sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TH	Tanzania High Vitamin E
TL	Tanzania Low Vitamin E
TSS	transcription start site
UTR	untranslated region
v/v	volume per volume
V	volts
w/v	weight per volume
X-gal	5-bromo-4-chloro-indoyl-β-D-galactopyranoside
µg	microgram
µM	micromolar
µl	microliter
g	relative centrifugal force
°C	degree celcius

CHAPTER 1

INTRODUCTION

Oil Palm (*Elaeis guineensis*) or locally known as "kelapa sawit" originated from Africa. It is planted in almost 43 countries today in the tropical belt ranging from South East Asia, Africa and South America. Oil palm was introduced to Malaya Peninsular (now known as Malaysia) by the British Government in early 1875 as an ornamental plant for landscaping (Cramb & McCarthy, 2016), laying the foundation for the industry in Malaysia with the first commercial planting in Tennamaran Estate in Selangor in 1917 (Nambiappan et al., 2018). Today, Malaysia has now become one of the major oil palm producers in the world accounting for 28% of oil palm production (Malaysian Palm Oil Counsel [MPOC], 2020a). The industry has emerged as one of the most highly organized sectors in the Malaysian agricultural system and has contributed significantly in sustaining the Malaysian economy (Kushairi et al., 2017).

Red palm oil extracted from oil palm mesocarp contain a mixture of different antioxidants and phytonutrients such as Vitamin E (tocopherol/tocotrienols), pro-vitamin A (alpha and beta carotene), phytosterol complex and coenzymes. It is found, red oil palm contains high levels of vitamin E especially tocotrienols, with crude palm oil containing up to 800 mg/kg of total vitamin E (Zainal et al., 2019). The nanomolar concentration of α-tocotrienol prevents inducible neurodegeneration (Sen et al., 2010; Khanna et al., 2003). Moreover, there is increasing interest in using tocotrienols as nutraceuticals in cancer treatment within the last few years due to their anticancer properties. Accumulating evidence on the anti-cancer potency of vitamin E showed that γ-tocotrienols and δ-tocotrienols have the highest anti-cancer activities (Constantinou et al., 2019). Malaysian Palm Oil Board (MPOB) located in Kluang, Johor has assembled the largest *E. guineensis* germplasm materials in the 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 variety (Wahid et al., 2005; Rajanaidu et al., 2000). This variation may arise due to the molecular setup of the *Homogentisate geranylgeranyl transferase* gene which catalyses the first committed step of tocotrienols biosynthesis in plants.

Single nucleotide polymorphisms (SNP) are increasingly becoming the marker of choice in genetic analysis and agricultural breeding programs (Pootakham et al. 2015; Babura et al. 2017). They are widely used due to their abundance and possibilities for automation. SNPs in genes encoding proteins are of interest as they may be directly associated with the traits being studied. The SNPs can be in the coding or the noncoding regions such as the promoters and introns (Uppu et al., 2018). Genetic characterization of diverse rice germplasm was performed based on SNPs at the promoter region from a rice sucrose synthase 3 (RSUS3) gene involves in starch biosynthesis (Lee et al., 2009). SNPs have also been used to assess interspecific differences between *E. guineensis* and

E. oleifera, the two most important oil palm species (Riju & Arunachalam 2009). Crop intervention through genetic modification and engineering of the oil palm vitamin E biosynthesis will provide a crucial understanding of how the expression of a key enzyme involved in the oil palm vitamin E biosynthesis is regulated. Moreover, further understanding of the association of SNP in the promoter of the *HGGT* gene with high vitamin E content is important. The identification of the sources of vitamin E content variation among the oil palm germplasm accessions would become a valuable study and provide useful strategies for oil palm genetic improvement through marker-assisted selection.

Therefore, it would be interesting to study functional nucleotide variants in the vitamin E *HGGT* biosynthetic gene as the regulatory sequence or the promoter for controlling expressions of the downstream gene will be the key factor in the success of oil palm genetic engineering programme (Sambanthamurthi et al., 2002).

The objectives of this study are:

1. To identify variant nucleotide in the promoter sequence of *homogentisate geranylgeranyl transferase* (*HGGT*) gene from oil palm germplasm materials associating with the high vitamin E trait.
2. To produce promoter reporter constructs designed to determine the effects of identified sequence variant on HGGT promoter activity.
3. To identify the effect of Single Nucleotide Polymorphism (SNPs) on the activity of the HGGT promoter through transient reporter gene assay of bombarded mesocarp tissues slices.

It was hypothesised that the variation in vitamin E content found among *E. guineensis* germplasm materials could be associated with functionally variant nucleotides (SNPs) in the promoter sequence of *homogentisate geranylgeranyl transferase* (*HGGT*) gene which catalyses the first committed step of tocotrienols biosynthesis in plants.

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BIODATA OF STUDENT

The student, Mohd Shahrul Nizwanshah Bin Karim was born in Sandakan, Sabah on June 4th, 1991. He received his primary education in Sekolah Kebangsaan Rancangan Suan Lamba from 1998 to 2003. He then continued his secondary school in SMK Elopura (Bestari) from 2004 to 2006 (PMR) and in SM Sains Sabah from 2007 to 2008 (SPM). In 2009, he enrolled at Labuan Matriculation College for his matriculation and completed his bachelor's degree in Agricultural Science at Universiti Putra Malaysia, Serdang in November 2014. After a year working as an Assistant Estate Manager with IOI Group, he pursued his studies in Master of Science at the Institute of Tropical Agriculture and Food Security, UPM in 2015 under MyBrain15 sponsorship program. In October 2019, he then decided to join the Malaysian Agricultural Research and Development Institute (MARDI) as a Research Officer to develop his passion for research in agriculture.

Experiences gained when preparing the thesis made him a better person, he learned patience, friendship, courage, and discipline. He is very thankful for the whole period of his study that he has been bestowed by supportive and encouraging persons from his family and friends. The success of his studies is above all attributed to his wife's love, prayers, and care.

PUBLICATIONS

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