UNIVERSITI PUTRA MALAYSIA

VITAMIN E PRODUCTION IN AMARANTHUS SP. AND ALLIUM PORRUM BY CO-SUPPRESSION OF HOMOGENTISATE PHYTYLTRANSFERASE AND TOCOPHEROL CYCLASE GENES FROM ELAEIS GUINEENSIS

UMAIYAL MUNUSAMY

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By

UMAIYAL MUNUSAMY

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

August 2013

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Specially Dedicated

In

Loving Memory

Of

My Uncle

Mr VEERIAH PERUMAL

&

Mr RAJANDREN GOVINDASAMY

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

VITAMIN E PRODUCTION IN *AMARANTHUS* SP. AND *ALLIUM PORRUM* BY CO-SUPPRESSION OF HOMOGENTISATE PHYTYLTRANSFERASE AND TOCOPHEROL CYCLASE GENES FROM *ELAEIS GUINEENSIS*

By

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August 2013

Chair: Professor Datin Siti Nor Akmar Abdullah, PhD

Faculty: Agriculture

Vitamin E is a fat-soluble vitamin that consists of four different tocopherol and tocotrienol isomers (α, β, γ and δ). Medical evidence suggests that vitamin E especially α-tocotrienol protects the cells against cancer, is a strong antioxidant and an effective neuroprotector. However, α-tocotrienol can only be found in seeds/cereal grain. In this study α-tocotrienol was produced by co-suppression of α-tocopherol by silencing the expression of two key vitamin E biosynthetic genes, homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC) in *Amaranthus* sp. and *Allium porrum* leaves by transient transformation. HPT catalyses the condensation of homogentisate (HGA) and phytyl diphosphate (PDP). While TC forms the chromanol headgroup of the various tocopherol isomers. Gene silencing was performed using short sense conserved gene sequences of HPT and TC isolated from *Elaeis guineensis.* Through bioinformatics analysis, both isolated HPT and TC cDNAs were successfully verified to possess similar characteristics of the HPT and TC from other plant species. These isolated genes were predicted to encode a protein of 151 and 207 amino acid residues and they fell under the UbiA superfamily and tocopherol_cyc1 superfamily, respectively. Five gene constructs were generated p5b5, p5d9, p5f7, p4a11 and p4c9 driven either by the maize ubiquitin promoter (Ubi1P) or the *Elaeis guineensis* leaf-specific promoter (LHCB), in pDRB6b expression vector. All five recombinant vector constructs were successfully cloned, giving the expected size bands in gel electrophoresis analysis. The PCR product of the recombinant vector shared 78% identity with HPT from *Zea mays* (NM001112407.1). While the TC cDNA showed 79% identity with *Vitis vinifera* (XM002281388.2). In addition, plasmid stability in the transformed AGL1 showed stability ranging from 40% to 60%, identical to pDRB6b vector in AGL1. Optimised HPLC analysis showed that α-tocopherol was suppressed with different trend among the constructs (from minimum 4% up to 100%) in all transiently transformed plants. The p5f7 construct (LHCB-Ubi1intron-HPT-NosT) worked best in *Allium porrum* to suppress α-tocopherol in the range 62%-86%. While, p4c9 construct (LHCB-TC-NosT) showed suppression up to 100% in *Amaranthus* sp. The results confirmed that the usage of LHCB showed better silencing than UbiP. In addition, with the usage of HPT gene, α-tocopherol was suppressed better (up to 86%) in *Allium porrum* than in *Amaranthus* sp. (75%). However, with TC gene the suppression was better in *Amaranthus* sp. (100%) than *Allium porrum* (57%). The production of α-tocotrienol was observed upon suppression of α-tocopherol using all recombinant vector constructs. With HPT gene the level of α-tocotrienol production was not dependent but with TC gene it was dependent on the level of α-tocopherol suppression. Overall, α-tocotrienol was produced more in *Allium porrum* than in *Amaranthus* sp. with p5d9 construct (Ubi1P-Ubi1intron-HPT-NosT). Thus, *Amaranthus* sp. and *Allium porrum* which do not accumulate α-tocotrienol naturally had shown to produce α-tocotrienol after suppression in the α-tocopherol production through transgenic manipulation. Finally, *Agrobacterium -mediated* transformation of inflorescence of *Amaranthus* sp. was carried out through drop by drop technique for producing transformed seeds. Nineteen transgenic plants were successfully obtained with transformation efficiency from 0.1% up to 2% for each constructs. This could serve as a potential system to engineer production of α-tocotrienol in transgenic *Amaranthus* sp.

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

PENGHASILAN VITAMIN E DALAM *AMARANTHUS* SP. DAN *ALLIUM PORRUM* MELALUI KOSUPRESI GEN HOMOGENTISATE PHYTYLTRANSFERASE DAN TOCOPHEROL CYCLASE DARI *ELAEIS GUINEENSIS*

Oleh

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Vitamin E adalah vitamin larut lemak yang terdiri daripada empat isomer tokoferol dan tokotrienol yang berbeza (α, β, γ dan δ). Bukti-bukti perubatan menunjukkan bahawa vitamin E terutamanya α-tokotrienol melindungi sel-sel terhadap kanser, adalah antioksidan yang kuat dan neuroprotector yang berkesan. Walau bagaimanapun, α-tokotrienol hanya boleh ditemui dalam biji/bijirin. Dalam kajian ini α-tokotrienol dihasilkan oleh penindasan bersama α-tokoferol dengan penyenyapan pengekspresan dua gen utama biosintesis vitamin E, homogentisate phytyltransferase (HPT) dan tokoferol cyclase (TC) dalam daun *Amaranthus* sp. dan *Allium porrum* melalui transformasi transien. HPT memangkin penggabungan homogentisate (HGA) dan phytyl difosfat (PDP). Manakala TC membentuk kumpulan kepala kromanol untuk pelbagai isomer tokoferol. Penyenyapan gen telah dilakukan dengan menggunakan jujukan pendek gene HPT dan TC yang terpulihara yang dipencilkan daripada *Elaeis guineensis*. Melalui analisis bioinformatik, kedua-dua cDNAs HPT dan TC yang terpencil telah berjaya disahkan mempunyai ciri-ciri yang sama dengan HPT dan TC dari spesis tumbuhan yang lain. Gen terpencil telah diramalkan mengekod protein 151 dan 207 residu asid amino dan masing masing jatuh di bawah superfamili UbiA dan superfamili tocopherol_cyc1. Lima vektor konstruk yang telah dibina p5b5, p5d9, p5f7, p4a11 dan p4c9 adalah didorong sama ada oleh promoter ubiquitin jagung (Ubi1P) atau promoter khusus daun *Elaeis guineensis* (LHCB), dalam vektor pengekpresan pDRB6b. Kesemua lima konstruk vektor rekombinan telah berjaya diklon, memberi jalur dengan saiz yang dijangkakan dalam analisis gel elektroforesis. Produk PCR vektor rekombinan berkongsi identiti 78% dengan HPT dari *Zea Mays* (NM001112407.1). Manakala, TC cDNA menunjukkan identiti 79% dengan *Vitis vinifera* (XM002281388.2). Di samping itu, kestabilan plasmid dalam AGL1 yang ditransformkan menunjukkan kestabilan dalam lingkungan 40% ke 60%, menyamai dengan vektor pDRB6b dalam AGL1. Analisis HPLC yang telah dioptimumkan menunjukkan α-tokoferol telah ditindas dengan tren yang berlain diantara konstruk (dari minimum 4% sehingga 100%) dalam semua tumbuhan yang ditransform secara transien. Konstruk p5f7 (LHCB-Ubi1intron-HPT-NosT) paling berkesan dalam *Allium porrum* untuk menindas α-tokoferol dalam lingkungan 62%-86%. Manakala, konstruk p4c9 (LHCB-TC-NosT) menunjukkan penindasan sehingga 100% dalam *Amaranthus* sp. Keputusan

ini mengesahkan bahawa penggunaan LHCB menunjukkan penyenyapan lebih baik daripada UbiP. Di samping itu, dengan penggunaan gen HPT, α-tokoferol telah ditindas lebih baik (sehingga 86%) dalam *Allium porrum* berbanding *Amaranthus* sp. (75%). Walau bagaimanapin dengan gen TC kadar penindasan adalah lebih baik dalam *Amaranthus* sp. (100%) daripada *Allium porrum* (57%). Penghasilan α-tocotrienol telah diperolehi melalui penindasan α-tokoferol menggunakan kesemua konstruk vektor rekombinan. Dengan gen HPT, tahap penghasilan α-tocotrienol tidak bergantung tetapi dengan gen TC ia adalah bergantung kepada tahap penindasan α-tokoferol. Secara keseluruhan, α-tocotrienol dihasilkan lebih dalam *Allium porrum* berbanding *Amaranthus* sp. dengan konstruk p5d9 (Ubi1P-Ubi1intron-HPT-NosT). Oleh itu, *Amaranthus* sp. dan *Allium porrum* yang tidak mengumpul α-tocotrienol secara semulajadi telah menunjukan penghasilan α-tocotrienol selepas penindasan dalam pengeluaran α-tokoferol melalui manipulasi transgenik. Akhir sekali, transformasi pengantaraan *Agrobacterium* ke atas jambak bunga *Amaranthus* sp. telah dijalankan melalui teknik ‘drop by drop’ untuk menghasilkan biji benih tertransform. Sembilan belas tumbuhan transgenik telah berjaya diperolehi dengan kecekapan transformasi dari 0.1% hingga 2% bagi setiap konstruk. Ini boleh di jadikan sebagai satu sistem yang berpotensi untuk kejuruteraan pengeluaran α-tocotrienol dalam *Amaranthus* sp. transgenik.

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I certify that a Thesis Examination Committee has met on 27 AUGUST 2013 to conduct the final examination of UMAIYAL MUNUSAMY on her thesis entitled " Vitamin E Production in *Amaranthus* sp. and *Allium porrum* By Co-Suppression of Homogentisate Phytyltransferase and Tocopherol Cyclase Genes from *Elaeis Guineensis*" in accordance with the Universities and University Colleges 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 Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotation and citations which have been dully 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.

UMAIYAL MUNUSAMY

Date: 27 August 2013

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LIST OF SYMBOLS AND ABBREVATIONS

%	percentage
$(NH_4)_2SO_4$	ammonium sulfate
°C	degree celsius
a.u	absorbance unit
aa	amino acid
Act	rice actin 1
Adh-1	maize alcohol dehydrogenase 1
Ala	alanine
Amp^r	ampicillin resistant
ANOVA	analysis of variance between groups
ATP	adenosine triphosphate
bar	phosphinothricin acetyltransferase
BHA	butylated hydroxyanisole
BHT	butylated hydroxytolune
BlastP	basic local alignment search tool for protein
bp	base pair
Bspred	protein-protein binding site prediction
Buffer EB	elution buffer
Buffer PE	wash buffer
Buffer QG	neutralization and binding buffer
CaMV	cauliflower mosaic virus
CaMV35S	cauliflower mosaic virus 35S
CAT	chloramphenicol acetyltransferase
cDNA	copy deoxy nucleic acid
CFU/g	colony forming unit per gram
cfu/µg	colony forming unit per microgram

CHS	chalcone synthase
cm	centimetre
Co	company
CO_2	carbon dioxide
ColE Ori	origin of replication
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C-term	carboxyl-terminus
cTP	chloroplast transit peptide
CV	coefficient variance
dH_2O	distilled water
DMGGBQ	2-3-dimethyl-5-geranylgeranyl-benzoquinol
DMPBQ	2-methyl-6-phytylbenzoquinol
DNA	deoxyribonucleic acid
dNTP	deoxynucleotides
dsRNA	double stranded rna
DXP	1-deoxy-$_{D}$-xylulose-5-phosphate
E8 gene	fruit ripening gene
EDTA	ethylenediaminetetra acetic acid
et al	and others
EtBr	ethidium bromide
F	forward
FASTA	format for representing either nucleotide sequences or peptide sequences
g/L	gram per litre
GGDP	geranylgeranyl diphosphate
GGDP	geranylgeranyl diphosphate reductase

reductase

Gly	glycine
Gmubi	glycine max polyubiquitin
GOR	secondary prediction sturcture
Gt 1	seed storage protein
GUS	beta-glucuronidase
h	hour
HGA	homogentisate
HGGT	homogentisate geranylgeranyl
Hph	hygromycin B resistance
hph^R	hygromycin resistant
hph^S	hygromycin sensitive
HPLC	High Performance Liquid Chromatography
HPP	ρ-hydroxyphenylpyruvate
HPPD	4-hydroxyphenylpyruvate dioxygenase
HPT	homogentisate phytyltransferase
Ile	isoleucine
IPTG	isopropyl-thio-galastoside
ISO	International Organization for Standardization
K_2HPO_4	dipotassium phosphate
Kac	potassium acetate
kb	kilo base pair
kDa	kilodalton
KH_2PO_4	monopotassium phosphate
KOH	potassium hydroxide
LB	Luria Bertani
LB	left border

LDL	low density lipid
Len	length
Leu	leucine
LHCB	leaf specific promoter
LiCl	lithium chloride
LOQ	limit of quantification
LSD	least significant different
M	molar
Mardi	Malaysian Agricultural Research and Development Institute
MCS	multiple cloning site
MEGA5	molecular evolutionary genetics analysis
MES	2-ethanesulfonic acid
mg	milligram
mg/ kg	milligram per kilogram
mg/g	milligram per gram
MGGBQ	2-methyl-6-geranylgeranylbenzoquinol
$MGSO_4$	magnesium sulfate
min	minute
ml	millilitre
ml/min	millilitre per minute
mm	millimeter
mM	millimolar
MMLV	RNA-dependent DNA polymerase
MODPIPE	software tool for large-scale comparative modeling
MODWEB	server for comparative protein structure modeling
MPBQ	2-methyl-6-phtyl-1,4benzoquinol

MPBQ MT	2-methyl-6-phytylplastoquinol methyltransferase
MPN	minimal plate count
MPN/g	most probable number per gram
MPN/g	minimal plate number per gram
MPOB	Malaysian Palm Oil Board
mRNA	messenger RNA
MSA	multiple sequence alignment
MSBQ	methyl-6-solanylbenzoquinone methyltransferase
NaCitrate	sodium citrate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng/µL	nanogram per microlitre
nm	nanometer
No	number
nos	termination site
np	nested PCR
nptII	neomycin phosphotransferase II
N-term	amino-terminus
NY	New York
OD	optical density
ORF	open reading frame
Ori RK2	vegetative replication origin
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDP	phytyl diphosphate
PHD	Predict Heidelberg Deutscland method
PPT	phosphinotricin

PrBQMT	2-methyl-6-prenylbenzoquinol methyltransferase
PTFE	polytetrafluoroethylene
PTGS	post transcriptional gene silencing
R	reverse
RACE	rapid amplification of cdna ends
RB	right border
RISC	RNA-induced silencing complex
RK2	origin of replication
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
rpm	revolution per unit
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
SAM	s-adenosylmethionine
SD	standard deviation
SDS	sodium dodeocyl sulphate
SE	standard error bar
sec	second
siRNA	small interfering RNA
SOPMA	self optimised prediction methods alignments
Spec R	spectinomycin resistant gene
SPSS	statistical program for social sciences
TAE	tris acetate EDTA
TAP	tobacco acid pyrophosphatase
TC	tocopherol cyclase
T-DNA	transfer deoxyribonucleic acid

TGS	transcriptional gene silencing
T_m	melting temperature
TMHMM	transmembrane hidden markov models
TMT	tocopherol methyltransferase
trfA	replication initiation protein
Tris-HCl	tris hydrochloride
tRNA	translate ribonucleic acid
U/µL	unit per microlitre
Ubi1-intron	ubiquitin intron
Ubi1P	ubiquitin promoter
UbiA	ubiquitin superfamily
Uep1	ubiquitin extension protein
uidA	beta-glucuronidase enzyme
UniProt	universal protein resource
UPM	University Putra Malaysia
USA	United States of America
USDA	United State Department of Agriculture
UV	ultra violet
Val	valine
vc	vector confirmation
VIGS	virus induced gene silencing
w/v	weight per volume
X-gal	5-bromo-4-chloro-indolyl-β-d-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
α	alpha
β	beta
γ	gamma

δ	delta
λ	lambda
μg	microgram
μg/mL	microgram per micro litre
μL	microlitre
μM	micromolar

CHAPTER 1

INTRODUCTION

Plants produce a wide range of metabolites that are beneficial to human as food, medicines and industrial raw materials (Dudareva and Pichersky, 2008; Oksman-Caldentey and Saito, 2005). Plant-derived vitamins are of great interest because of their impact on human health (Asensi-Fabado and Munne-Bosch, 2010). Even though plant is nature's best production system (Kishore and Shewmaker, 1999), due to the exponential growth in world population particularly in developing countries, they may not be able to cater for future demand of important nutrients especially the different types of vitamins (Bouis, 2003a). In order to reduce dietary deficiencies, (Bouis, 2003b), one of the strategies that can be applied is through metabolic engineering (Storozhenko et al., 2005) can be carried out through over-expressing or silencing of a particular gene to channel the metabolite flux of the biosynthetic pathway or via decreasing the amount of a competitive compound (Weckwerth and Fiehn, 2002). α-tocopherol and α-tocotrienol (component of vitamin E and chemically known as tocochromanols) (Horvath et al., 2006a) are produced by two metabolic pathways, the shikimate and non-mevalonate pathways (Collakova and DellaPenna, 2001). In plants, vitamin E plays various roles in plant growth and development (Horvath et al., 2006b). While, in human and animals, vitamin E can protect membranes from photo oxidation involving several signal transduction pathways (Ren et al., 2011b). In addition, α-tocotrienol is considered as a promising anticancer agent due to its potent effects against a wide range of cancers, as a cholesterol reducing agent and as a better neuro-protective agent compared to α-tocopherol (Ling et al., 2012; Viola et al., 2011a). Given the important functions of α-tocopherol and α-tocotrienol in plants, human and animals, in addition to its accumulation in plants, studies on its biosynthetic pathways in plants have been actively pursued. These studies resulted in the identification of the different enzymes involved in the production of α-tocochromanols (Fujita et al., 2009; Abbasi et al., 2007).

α-tocochromanols content can be manipulated either by over-expression or silencing of the biosynthetic enzymes homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC) (Ren et al., 2011a; Seo et al., 2011). Vitamin E engineering involving manipulating expression of HPT and TC gene resulted in the changes of α-tocopherol and α-tocotrienol. Homogentisate phytyltransferase gene is involved in the condensation of homogentisate (HGA) and phytyl diphosphate (PDP) to produce MPBQ (2-methyl-6-phytyl-1, 4-benzoquinol) (Collakova and DellaPenna, 2003.) This reaction product is the first intermediate for the production of vitamin E and when it is further cyclised and methylated by subsequent enzymes in the biosynthetic pathway, other tocopherol isoforms are produced (Endrigkeit et al., 2009). Tocopherol cyclase is involved in the formation of chromanol headgroup of δ and γ-tocochromanols (Hunter and Cahoon, 2007). Methylation of δ and γ-tocopherols by tocopherol methyltransferase produces β and α–tocochromanols.

Since, α-tocopherol are synthesised only in the photosynthetic tissues of plants (Falk and Munne-Bosch, 2010), while α-tocotrienol accumulation are mainly in plant seeds (Abbasi et al., 2007; Tan, 2005; Ajjawi and Shintani, 2004), vegetable leaves that contain tocotrienol can be regarded as an extra source of this valuable vitamin due to the added nutritional value in the leaves (Matringe et al., 2008). Thus far, there is only one report on a successful attempt in increasing α-tocotrienol production via metabolic engineering. This was achieved by introducing a barley cDNA encoding another enzyme of the biosynthetic pathway, homogentisate geranylgeranyl transferase (HGGT) resulting in its over-expression in corn seeds (Cahoon et al., 2003). Therefore, in the present study, leaves were chosen to manipulate the content of α-tocochromanols with an *Elaeis guineensis* HPT and TC gene. The cDNA of the HPT gene was isolated from the leaves, and the cDNA of the TC gene was isolated previously by another student from the mesocarp tissues. Both genes were isolated from *Elaeis guineensis* since it is the richest source of vitamin E (Ebong et al., 1999).

1.1 Objectives

The aim of this study was to produce α-tocotrienol by co-suppression of α-tocopherol by silencing homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC) gene expression using partial genes (gene sequences from conserved region) isolated from *Elaeis guineensis.*

The specific objectives were:

1. Transient transformation by agroinfiltration and *Agrobacterium*-mediated transformation of green *Amaranthus* sp. using partial HPT and TC genes from *Elaeis guineensis.*
2. Transient transformation by agroinfiltration of *Allium porrum* using partial HPT and TC genes from *Elaeis guineensis.*

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