UNIVERSITI PUTRA MALAYSIA

ISOLATION AND SEQUENCE ANALYSIS OF CANDIDATE cDNA INVOLVED IN CAROTENOID BIOSYNTHESIS IN THE FLAVEDO TISSUE OF PUMMELO (*CITRUS GRANDIS* L. OSBECK)

UMMI KALTHUM BINTI HANAPI

FBSB 2008 6

ISOLATION AND SEQUENCE ANALYSIS OF CANDIDATE cDNA INVOLVED IN CAROTENOID BIOSYNTHESIS IN THE FLAVEDO OF PUMMELO (*CITRUS GRANDIS* L. OSBECK)

UMMI KALTHUM BINTI HANAPI

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2008

ISOLATION AND SEQUENCE ANALYSIS OF CANDIDATE cDNA INVOLVED IN CAROTENOID BIOSYNTHESIS IN THE FLAVEDO TISSUE OF PUMMELO (*CITRUS GRANDIS* L. OSBECK)

By

UMMI KALTHUM BINTI HANAPI

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

January 2008

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

ISOLATION AND SEQUENCE ANALYSIS OF CANDIDATE cDNA INVOLVED IN CAROTENOID BIOSYNTHESIS IN THE FLAVEDO TISSUE OF PUMMELO (*CITRUS GRANDIS* L. OSBECK)

By

UMMI KALTHUM BINTI HANAPI

January 2008

Chairman : Associate Professor Dr. Norihan Mohd Saleh, PhD

Faculty : Biotechnology and Biomolecular Sciences

Citrus grandis L. Osbeck (pummelo) was recognized as one of the potential fruit to be commercialized under the goal of the Third National Agricultural Plan's (DPN3, 1998-2010). However, the pale yellowish green colour of *C. grandis* fruit flavedo rendered is unattractive and has less market demand compared to other citrus vaireties. Carotenoid biosynthesis has been well studied and recognized to be the main pathway which contributes to the formation of colour in the flavedo of most citrus fruit. However, there is still limited information currently available on carotenoid biosynthesis in green pigmented citrus fruit flavedo. To increase the market demand, locally and overseas, there is a need to modify the colour of *C. grandis* from pale yellowish green to more attractive colour like other citrus. Therefore, the study was initiated to explore the possibility of modifying fruit flavedo colour through the use of molecular techniques. Thus, it is necessary to isolate and sequence the genes involved in carotenoid pathway of green flavedo of citrus fruit. In this study, comparative analysis of the sequences isolated from the

gene of green flavedo will be compared with orange pigmented citrus, other plants and bacteria which contain high carotenoids. The study began with tagging of open pollinated flowers (that showed indication of fruit formation to ensure the right stage of maturation), followed by genomic DNA and total RNA isolation prior to further molecular analyses, such as PCR and RTPCR. The primers used in this study were designed based on the sequence information obtained from the Genbank database. The four genes which are involved in carotenoid biosynthesis and responsible for the orange pigmentation in citrus fruit *viz. phytoene synthase* (*psy*, 0.46 kb), *phytoene desaturase* (*pds*, 1.11 kb), *lycopene β-cyclase* (*lyc*, 0.70 kb) and *β-carotene hydroxylase* (*chx*, 0.23 kb), were isolated *via* PCR and RTPCR. From sequence analysis, *psy*, *pds*, *lyc* and *chx* showed high similarities with the corresponding genes in citrus varieties, *Prunus armeniaca* and *Capsicum annuum*. A full sequence of *chx* gene was isolated from cDNA library of *C. grandis* fruit flavedo. The 1268 bp of *chx* gene consisted of an open reading frame (ORF) of 271 codons (mass of 65.8 kD) and 170-nucleotide 5' untranslated sequences was isolated. Compared to other organisms, seven histidine residues which were present in *C. grandis chx* were also found to be conserved in *Alcaligenes sp* (accession no. D58422), *Agrobacterium aurianticum* (accession no. D58420), *Erwinia herbicola* (accession no. M87280) and *Pseudomonas putida* (accession no. KT2440 NP_745389). 'Motif 1' and the predicted 'TM helix' regions were also observed in *C. grandis* when compared to *Prochlorococcus marinus* MED4 *CrtL-ε* (CAE19092), *Lycopersicon esculentum Lcy-ε* (O65837), *Tagetes erecta Lcy-ε* (AAG10428), *Capsicum annuum Lcy-β* (Q43415) and *Arabidopsis thaliana Lcy-β* (AAA81880). In conclusion, *psy*, *pds*, *lyc* and a full length of *chx* have been successfully isolated the flavedo of *C. grandis*.

The sequences of these genes were found to be highly conserved with the corresponding gene in other plants especially in citrus varieties.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMENCILAN DAN ANALISIS JUJUKAN cDNA TERPILIH YANG TERLIBAT DALAM BIOSINTESIS KAROTENOID DALAM TISU FLAVIDO LIMAU BALI (*CITRUS GRANDIS* L. OSBECK)

Oleh

UMMI KALTHUM BINTI HANAPI

Januari 2008

Pengerusi : Profesor Madya Dr. Norihan Mohd Saleh, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Citrus grandis L. Osbeck (limau bali) telah dikenalpasti sebagai salah satu daripada buah-buahan yang mempunyai potensi untuk dikomersilkan di bawah Dasar Pertanian Negara Ketiga (DPN3 1998-2010). Walaubagaimanapun, warna hijau kekuningan yang pucat pada permukaan kulit (flavido) buah *C. grandis* menjadikannya tidak menarik untuk dieksploitasi secara komersil. Laluan biosintesis karotenoid telah dikaji dengan mendalam dan dikenalpasti sebagai laluan biosintesis utama yang menyumbang kepada pembentukan warna flavido pada kebanyakan buah limau. Walaupun banyak informasi berkenaan biosintesis karotenoid di dalam flavido buah-buahan limau yang berwarna oren dan kuning, tiada informasi mengenai flavido buah berwarna hijau. Untuk meningkatkan permintaan pasaran di dalam dan di luar negara, adalah perlu untuk mengubah warna flavido *C. grandis* daripada warna hijau kekuningan yang pucat kepada warna yang lebih menarik sebagaimana warna buah limau yang lain. Maka, penyelidikan dimulakan untuk mengkaji kemungkinan mengubah warna flavido buah tersebut melalui penggunaan teknik-teknik molekul. Justeru itu, adalah penting untuk memencilkan dan membuat

analisis jujukan terhadap gen-gen yang terlibat dalam laluan karotenoid di dalam flavido hijau *C. grandis*. Analisis perbandingan jujukan yang diperolehi daripada gen-gen flavedo berwana hijau tersebut akan dibandingkan dengan limau berwarna oren, tumbuhan lain dan bakteria yang mempunyai kandungan karotenoid yang tinggi. Kajian ini bermula dengan menanda bunga yang tersenyawa yang telah menunjukkan tanda pembentukan buah untuk memastikan peringkat kematangan yang betul, diikuti dengan pemencilan DNA dan RNA total sebelum analisis molekul seterusnya seperti PCR dan RTPCR. Primer yang digunakan di dalam teknik tersebut direkabentuk berdasarkan maklumat jujukan yang diperolehi daripada pengkalan data GenBank. Empat gen yang terlibat di dalam biosintesis karotenoid dan bertanggungjawab terhadap pembentukan pigmen oren pada buah limau iaitu; *fitoene sintase* (*psy*, 0.46 kb), *fitoene disaturase* (*pds*, 1.11 kb), *likopene β-siklase* (*lyc*, 0.70 kb) dan *β-karotene hidroksilase* (*chx*, 0.23 kb), telah dipencilkan melalui kaedah PCR dan RTPCR. Daripada analisis jujukan, *psy*, *pds*, *lyc* dan *chx,* persamaan yang tinggi dengan variati limau, *Prunus armeniaca* dan *Capsicum annuum*. Satu jujukan lengkap gen *chx* telah dipencilkan daripada kulit buah *C. grandis.* Jujukan lengkap 1268 bp gen *chx* tersebut mengandungi 271 kodon bacaan terbuka (ORF) (jisim 65.8 kD) dan 170-nucleotida 5' jujukan yang tidak ditranslasikan telah dipencilkan. Dengan membuat perbandingan terhadap organisma lain, tujuh residu histidin yang terdapat di dalam gen *chx C. grandis* juga ditemui dalam gen yang sama pada organisma *Alcaligenes sp* (no. akses D58422), *Agrobacterium aurianticum* (no. akses D58420), *Erwinia herbicola* (no. akses M87280) dan *Pseudomonas putida* (no. akses KT2440 NP_745389). Bahagian 'Motif 1' dan 'Predicted TM helix' juga terdapat di dalam *lcy-β C. grandis* apabila dibandingkan dengan *Prochlorococcus marinus* MED4 *CrtL-ε* (no. akses

CAE19092), *Lycopersicon esculentum Lcy-ε* (no. akses O65837), *Tagetes erecta Lcy-ε* (no. akses AAG10428), *Capsicum annuum Lcy-β* (no. laluan Q43415) dan *Arabidopsis thaliana Lcy-β* (no. akses AAA81880). Kesimpulannya, *psy, pds, lyc* dan jujukan lengkap *chx* telah berjaya dipencilkan daripada flavido *C. grandis*. Persamaan jujukan *psy, pds, lyc* dan *chx* adalah sangat tinggi dengan gen-gen yang berpadanan pada tumbuhan lain terutama sekali variati limau.

ACKNOWLEDGEMENTS

Most of all, my greatest gratitude goes to my supervisor, Assoc. Prof. Dr. Norihan Mohd Saleh for giving me the opportunity to pursue my studies in the field of Plant Molecular Biology and Genetic Engineering.

My greatest debt of gratitude is, however, due to Dr. Marzukhi Hashim, Biotechnology Centre (MARDI). His encouragement stimulated me to work in this project and his continued unstinting help during the research has been invaluable.

I am also grateful beyond words to Assoc. Prof. Dr. Foo Hooi Ling for her considerable help during the writing of this thesis and made many valuable suggestions.

Special thanks are extended to members of the genetic lab (Department of Cell and Biomolecular, UPM) as well as the MARDI research group; it should be emphasized, however none can be considered in any way, responsible for any peculiarities which may exist in the thesis.

Last but no least, I wish to thank my family for loving me, all the constant support, words of encouragements and advices for the past few years.

I certify that an Examination Committee has met on 28th January 2008 to conduct the final examination of Ummi Kalthum Hanapi on her degree in Master of Science thesis entitled "Isolation and Sequencing Analyses of cDNAs Involved in Carotenoid Biosynthesis in the Flavedo Tissue of Pummelo (*Cirus grandis* (L.) Osbeck)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

Members of the Examination Committee were as follows:

Dr. Ho Chai Ling, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Chairman)

Mohd Puad Abdullah, PhD
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Internal Examiner)

Suhaimi Napis, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Internal Examiner)

Ismanizan Ismail, PhD
Associate Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

_____
HASANAH MOHD. GHAZALI, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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:

Norihan Mohd. Saleh, PhD
Associate Professor
Department of Molecule and Cell Biology
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Chairman)

Foo Hooi Ling , PhD
Associate Professor
Department of Molecule and Cell Biology
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

Marzukhi Hashim, PhD
Biotechnology Center
Malaysian Agriculture Research and Development Institute (MARDI)
(Member)

_____

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 July 2008

DECLARATION

I declare that the thesis is my original work except for equations 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 UPM or at any other institutions.

_____

UMMI KALTHUM BINTI HANAPI

Date: 13/5/2008

TABLE OF CONTENTS

LIST OF TABLES

128

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	-	alpha
β	-	beta
ε	-	epsilon
γ	-	gamma
λ	-	lambda
μ	-	micro
ξ	-	zeta
ABA	-	abscisic acid
BLAST	-	Basic Local Alignment Search Tool
bp	-	basepair
BSA	-	bovine serum albumin
βCHX	-	β-carotene hydroxylase
εCHX	-	ε-carotene hydroxylase
CCD	-	carotenoid cleavage dioxygenase
cDNA	-	complementary deoxyribonucleic acid
Ci	-	Curie
CoA	-	coenzyme A
C-terminal	-	carboxyl terminal
CTRB	-	bacterial phytoene synthase
CrtB	-	bacterial phytoene synthase gene
CrtE	-	bacterial gene encodes for enzyme responsible for the conversion of DMAPP to GGPP
CrtI	-	bacterial *carotenoid desaturase* gene

CrtL-b	-	*Cyanobacterium synechococcus* PCC 7942 *lycopene ε-cyclase* gene
CrtL-e	-	tomato *lycopene β-cyclase* gene
CRTISO	-	carotenoid isomerase
CrtN	-	bacterial *carotenoid desaturase* gene
CRTY	-	bacterial lycopene β-cyclase
CrtY	-	bacterial lycopene β-cyclase gene
DEPC	-	diethyl pyrocarboate
dH_2O	-	distilled water
DMAPP	-	dimethylallyl pyrophosphate
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
DNase	-	nuclease
dNTPs	-	deoxynucleosides triphosphate
DOPA	-	L-3,4-dihydroxyphenylalanine
DXP	-	deoxyxylulose 5-phosphate
DXR	-	deoxyxylulose-5-phosphate reductoisomerase
DXS	-	deoxyxylulose-5-phosphate synthase
EDTA	-	ethylene diamine tetraacetate
EtBr	-	ethidium bromide
FAD	-	Flavin adenine dinucleotide
GA	-	gibberellin acid
GGPP	-	geranylgeranyl pyrophosphate
HCl	-	hydrochloric acid
IPP	-	isopentenyl pyrophosphate
kb	-	kilobase

kD	-	kilodalton
LB	-	Luria-Bertani
LDL	-	low density lipoprotein
LiCl	-	Lithium chloride
LCYβ	-	lycopene β-cyclase
LYCε	-	lycopene ε-cyclase
NCBI	-	National Center for Biotechnology Information
NTES	-	Natrium chloride-Tris-EDTA-SDS
MCS	-	multiple cloning site
MEP	-	2-*C*-methyl-D-erythritol 4-phosphate
$MgCl_2$	-	magnesium chloride
$MgSO_4$	-	magnesium sulphate
mRNA	-	messenger RNA
MVA	-	mevalonic acid
NaCl	-	natrium chloride
NAD	-	nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
NaOAc	-	sodium acetate
NaOH	-	Natrium hydroxide
NCBI	-	National Centre for Biotechnology Information
nced	-	9-*cis*-epoxy-carotenoid dioxygenase gene
NSY or NXS	-	neoxanthin synthase
N-terminal	-	amino-terminal
OD	-	optical density
PCR	-	Polymerase Chain Reaction

PCI	-	phenol:chloroform:isoamyl alcohol [25:24:1 (v/v)]
PDS	-	phytoene desaturase
pfu	-	plaque forming unit
PPPP	-	prephytoene pyrophosphate
poly A^+ RNA	-	polyadenylated RNA
PSY	-	phytoene synthase
RNA	-	ribonucleic acid
mRNA	-	messenger RNA
rRNA	-	ribosomal ribonucleic acid
RNase	-	ribonuclease
ROS	-	reactive oxygen species
rpm	-	revolution per minute
RTPCR	-	Reverse Transcriptase Polymerase Chain Reaction
SA-PMPs	-	Streptavidin-Paramagnetic Particles
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	SDS-polyacrylamide gel electrophoresis
TE	-	tris-EDTA
TLC	-	thin layer chromatography
TM	-	transmembrane
Tris	-	tris[hydroxymethyl]amino methane
Tris-HCl	-	tris hydrochloride
U	-	unit
UV	-	ultraviolet
VDE	-	violaxanthin de-epoxidase
v/v	-	volume per volume

w/v	-	weigh per minute
x	-	times
xg	-	times gravity force
ZDS	-	ζ-carotene desaturase
Zds	-	ζ-carotene desaturase gene