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ISOLATION AND IN SILICO CHARACTERIZATION OF TWO ANTHOCYANIN TRANSCRIPTS CHS AND F3H FROM CALYX OF Hibiscus sabdariffa L. VAR. UMKL

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By

MARYAM ROSHANI

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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

ISOLATION AND *IN SILICO* CHARACTERIZATION OF TWO ANTHOCYANIN TRANSCRIPTS *CHS* AND *F3H* FROM CALYX OF *Hibiscus sabdariffa* L. VAR. UMKL

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Flavonoids represent a large class of secondary metabolites. These compounds possess many pharmacological and nutritional properties that are beneficial to human, as well as performing important biological roles in the growth and development of plants. Anthocyanin is one of the most abundant and important flavonoids. The calyx of *Hibiscus sabdariffa* (roselle) is rich in anthocyanin and phenolic compounds. In spite of the importance of Chalcone synthase (*CHS*) and Flavonone 3-hydroxylase (*F3H*) genes in the biosynthesis of flavonoids in plants, these genes have not been investigated in roselle. In this study, the transcripts of *HsCHS* and *HsF3H* were isolated from the calyx of roselle for the first time. *HsCHS* transcript sequence with a total length of 1167 bp and *HsF3H* with the total length of 1107 bp were isolated using PCR approaches. In addition, the start and stop codons, poly-A signal, poly-A tail, 3´ UTR and 5´ UTR regions for both isolated transcripts were also identified using *in-silico* analyses. Several important domains were predicted in the HsCHS deduced amino acid sequence, including CHS-like, fabH, BcsA, Chal-sti-synt-N and Chal-sti-synt-C, which indicates that the isolated cDNA sequence is probably a *CHS* transcript belonging to the polyketide synthase family. On the other hand, predictions of 2OG-FeII_oxy, Isopenicillin N synthase-like, DIOXN and PLN02515 domains in HsF3H deduced amino acid sequence, supports that the isolated cDNA sequence is an *F3H* transcript related to the large gene family of 2-oxoglutarate-dependent dioxygenases (2-ODDs). BLAST and phylogenetic analyses predicted the putative functions of the isolated *HsCHS* in the of condensation reaction of one 4-coumaroyl-CoA with three acetate molecules to produce naringenin; whereas an involvement of the *HsF3H* in catalyzing the reaction that transforms naringenin to dihydrokaempferol. However, further investigations must be carried out to confirm the function of *HsCHS* and *HsF3H*. This study predicted the putative functions of the two central genes (*CHS* and *F3H*) governing the flavonoid pathway of *H. sabdariffa,* which leads to anthocyanin production. It has also provided primary evidences on the predicted structures of the *HsCHS* and *HsF3H* transcripts, which is critical for getting a deeper insight into understanding the function of these genes in roselle.

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

PENGASINGAN DAN PENCIRIAN DALAM SILICO DUA TRANSKRIP ANTOSIANIN *CHS* DAN *F3H* DARIPADA KALIKS *HIBISCUS SABDARIFFA* L. VAR. UMKL

Oleh

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Jun 2015

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Flavonoid mewakili satu kelas metabolit sekunder yang besar. Sebatian-sebatian ini mempunyai banyak ciri-ciri farmakologikal dan nutrisi yang membawa kebaikan kepada manusia, dan juga menjalankan peranan biologi yang penting di dalam pertumbuhan dan perkembangan tumbuhan. Antosianin adalah salah satu daripada flavonoid yang paling banyak dan penting. Kaliks *Hibiscus sabdariffa* L. (roselle) adalah kaya dengan antosianin dan sebatian-sebatian fenolik. Walaupun gen-gen Chalkon sintase *(CHS*) dan Flavonon 3-hidrosilase *(F3H*) adalah gen-gen yang mustahak dalam biosintesis flavonoid di dalam tumbuhan, gen-gen ini belum dikaji di dalam roselle. Di dalam kajian ini, gen-gen *HsCHS* dan *HsF3H* telah diasingkan daripada kaliks roselle untuk kali pertama. Jujukan lengkap transkrip cDNA *HsCHS* dengan kepanjangan 1167bp, dan *HsF3H* dengan kepanjangan 1107bp telah diasingkan. Di samping itu, kodon-kodon permulaan dan hentian, isyarat poli-A, ekor poli- A, dan kawasan-kawasan 3´ UTR dan 5´ UTR untuk kedua-dua transkrip yang diasingkan ini telah dikenalpasti menggunakan analisi *in-silico*. Beberapa domain mustahak telah diramalkan di dalam jujukan asid amino HsCHS yang disimpulkan, termasuklah CHS-like, fabH, BcsA, Chal-sti-synt-N, dan Chal-sti-synt-C, domain ini menunjukkan bahawa gen yang diasingkan kemungkinan adalah transkrip *CHS* yang tergolong dalam keluarga polyketide synthase. Selain itu, domain-domain 2OG-FeII_oxy, Isopencillin N synthase-like, DIOXN, dan PLN02515 yang diramalkan di dalam jujukan asid amino HsF3H yang disimpulkan menyokong bahawa gen *F3H* yang diasingkan adalah tergolong dalam keluarga gen 2-oxoglutarate-dependent dioxygenases (2-ODDs). Analisis BLAST dan filogenetik meramalkan fungsi putatif *HsCHS* yang dipencilkan di dalam reaksi kondensasi satu 4 koumaril-CoA dengan tiga molekul asetat untuk menghasilkan naringenin; manakala penglibatan *HsF3H* di dalam mengkatalisi reaksi yang menukar neringenein kepada dihidrokaempferol. Walaubagaimanapun, penyiasatan lanjut harus dijalankan untuk mengesahkan fungsi-fungsi *HsCHS* dan *HsF3H* di dalam rosel. Penyelidikan ini meramal fungsi putatif untuk gen-gen *CHS* dan *F3H* yang mengawal laluan flavonoid *H. sabdariffa* dan membawa kepada pengeluaran anthocyanin. Penyelidikan ini juga menerangkan

struktur gen-gen *HsCHS* dan *HsF3H*, maklumat yang kritikal untuk pemahaman fungsi gen-gen tersebut dalam roselle.

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I certify that a Thesis Examination Committee has met on 16 June 2015 to conduct the final examination of Maryam Roshani on her thesis entitled "Isolation and *In silico* Characterization of Two Anthocyanin Transcripts *CHS* and *F3H* from Calyx of *Hibiscus sabdariffa* L. var. UMKL" 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 Master of Science.

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LIST OF ABBREVIATIONS

µL	Microliter
%	Percentage
^{0}C	Centigrade Celsius
1X	One time
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
ß- ME	Beta-Mercaptoethanol
CTAB	Hexadecyl (or cetyl) trimethyl ammonium bromide
cDNA	Complementary deoxyribonucleic acid
CHS	Chalcone synthase
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
DEPC	Diethyl pyrocarbonate
DNase	Nuclease
EDTA	Ethylene diamine tetra acetic Acid
EtBr	Ethidium bromide
g	Gram
GSP	Gene-specific primer
G-force	Gravity force
HCL	Hydrochloric
kb	Kilobasepair
kD	Kilo Daltons
L	Liter
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Milliliter
ng	Nanogram
nr	non-redundant
NJ	Neighbor Joining
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NaOAc	Sodium acetate
NCBI	National Center for Biotechnology Information
OD	Optical density
ORF	Open Reading Frame
OTUs	Operational taxonomic units
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room Temperature
rRNA	Ribosomal ribonucleic acid
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
U	Units
UPM	Universal Primer
UTR	Untranslated region
V	Volt
V/V	Volume per volume
W/V	Weight per volume
X	Times

CHAPTER 1

INTRODUCTION

The genus *Hibiscus* belongs to the family Malvaceae includes over 300 species distributed in tropical and sub-tropical regions. Roselle (*Hibiscus sabdariffa* L.), an annual erect shrub growing up to 2-2.5 m tall is a tetraploid (2n = 4x = 72) medicinal plant. The field longevity of the plant is approximately six to seven months. Roselle is vastly cultivated in Malaysia, India and Africa, mostly because of its fleshy shiny calyx, which is the commercially important part of the shrub (Abdel-Moniem and Abd El-Wahab, 2006; Mohamed *et al*., 2012).

Roselle extract is used in drugs, food and the cosmetic industries (Abdel-Moniem and Abd El-Wahab, 2006). Its fiber content is also of an eminent importance commercially. *H. sabdariffa* possesses a wide range of beneficial properties, including antioxidant, anti-cancer, antihypertensive, hypolipidaemic, hepatoprotective, anti-stress, antispasmodic, diuretic and antidiarrheal activities (Salem *et al*., 2014).

Flavonoids are natural products that widely distributed as a group of polyphenols present in the diet. To date, more than 4000 different flavonoids have been identified and characterized. According to their phenolic structures, flavonoids are categorized into a few main groups including flavonols, flavanones, flavones, catechin, isoflavone, chalcones and anthocyanin (Labib *et al*., 2004).

Anthocyanin is a kind of flavonoid that derives from a flavylium nucleus. These pigments are the source of different colors such as pink, violet, blue and purple in flowers, fruits, vegetables and different parts of plants (Cisse *et al.,* 2009). Six different classes of anthocyanins including delphinidin, cyanidin, malvidin, pelargonidin, petunidin and peonidin have been found in different vegetables with great antioxidant activities (Aurelio *et al.,* 2008; Kähkönen and Heinonen, 2003).

The main medicinal characteristic of anthocyanin is protecting DNA integrity as well as anti-ageing activities. Additionally, anthocyanins serve as cardio protective and anti-inflammatory factors (Bagchi *et al.,* 2004). Subsequently, they have been the center of attention for the last decade for their desirable act in minimizing the risk of cancer, stroke and coronary heart disease (Clifford, 2000; Scalbert and Williamson, 2000; Wrolstad, 2004). Many edible plants, including roselle, are rich sources of anthocyanins and flavonoids. Significant amounts of anthocyanins, as much as 2.5 g/100 g DW, can be found in roselle calyx, mainly two kind of anthocyanins: delphinidin and cyanidin (Cisse *et al.,* 2009; Du and Francis, 1973).

In line with the above-mentioned importance of the flavonoid-based compounds, the priority of conducting researches on the genetic aspect of the case is extremely tangible. In this regard, gene isolation and identification are conceivable approaches towards fortification of the potential gene transformation programs.

CHS is a member of the polyketide synthase gene family, and *F3H* belongs to the 2-oxoglutarate-dependent dioxygenases (2-ODDs) gene family. Nevertheless, both genes are native to endoplasmic reticulum (ER) (Lepiniec *et al*., 2001). *CHS* is a cardinal enzyme of a biosynthetic pathway that leads to the production of different types of flavonoids (de Meaux *et al*., 2005). This enzyme catalyzes the first step of flavonoid biosynthesis that catalyzes three molecules of malonyl-CoA and 4-coumaroyl-CoA, and produces 4, 2′, 4′, 6′-tetrahydroxy chalcone (She *et al*., 2013). *F3H* is a key branch-point enzyme necessary for the synthesis of 3-hydroxylated flavonoids including dihydroflavonols, flavonols, anthocyanins, flavon-3-ols, and proanthocyanidins (Shih *et al*., 2008).

The calyx of the roselle plant has long been recognized as a source of anthocyanin, but flavonoid biosynthesis pathway and its genetic mechanism, as well as the genes involved with the pathway have not been investigated in roselle.

To fill a part of the aforementioned lacunae in roselle, two anthocyanin genes namely *HsCHS* and *HsF3H* of the *Hibiscus sabdariffa* var. UMKL were investigated here. This variety is one of the most commonly planted varieties in Malaysia for its potential as a great source of natural antioxidants.

The objectives of this study were to:
1) Isolate the *CHS* and *F3H* transcripts from young calyx tissue of roselle
2) Analyze the *CHS* and *F3H* transcript sequences using *in silico* appro

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