**UNIVERSITI PUTRA MALAYSIA**

# *TRANSCRIPTOME PROFILING OF Hibiscus sabdariffa L. AT TWO MATURATION STAGES OF CALYX TISSUE*

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# TRANSCRIPTOME PROFILING OF *Hibiscus sabdariffa* L. AT TWO MATURATION STAGES OF CALYX TISSUE

By

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**TRANSCRIPTOME PROFILING OF *Hibiscus sabdariffa* L. AT TWO MATURATION STAGES OF CALYX TISSUE**

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**March 2020**

**Chairman : Christina Yong Seok Yien, PhD**
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Roselle (*Hibiscus sabdariffa* L.) is a non-model plant species whose calyces have been studied progressively in science for their metabolite composition and pharmacological potentials in the treatment of hypertension, diabetes, cancer, hyperlipidemia and hyperglycemia. The genetic mechanism that governs the production of potent phytochemicals found in roselle calyx tissues such as anthocyanin are yet to be deciphered and understood. The purpose of this study is to construct a transcriptome dataset for *H. sabdariffa* calyx tissues during the last two stages of maturation (stages three and four) using next-generation sequencing (NGS) technologies. These two maturation stages are critical as they may affect the quality of the calyx produced. A series of wet lab experiments were conducted prior to sequencing which included RNA-extractions, rRNA-depletion and cDNA sequencing library constructions. The Illumina NextSeq 500 sequencer platform was employed for sequencing; while data analysis was orchestrated using a number of software that included Trinity version 2.2.0 and CLC Genomic Workbench version 10.1.0. A combined total of more than 200 million good quality paired-end reads were generated from sequencing that resulted in a *de novo* assembled reference transcriptome consisting of 221,334 transcripts, of which 92,974 transcripts (42%) were successfully annotated. Twelve anthocyanin-related genes were effectually annotated; chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavanoid 3'-monoxygenase, flavonoid 3',5'-hydroxylase, dihydroflavonol 4-reductase, leucoanthocyanidin dioxygenase, flavonoid 3-*O*-glucosyltransferase, anthocyanidin 3-*O*-glucoside 2"-*O*-glucosyltransferase, coumaroyl-CoA:anthocyanidin 3-*O*-glucoside-6"-*O*-coumaroyltransferase, malonyl-CoA:anthocyanidin 5-*O*-glucoside-6"-*O*-malonyltransferase and flavonoid 3',5'-methyltransferase in this dataset. Differential expression analysis had identified a total of 504 significant differentially expressed genes (SDEGs) that were effectively mapped onto 193 KEGG pathway maps. The secondary metabolites biosynthesis category had attained a relatively high number of SDEGs (40) mappings. To name a few: the phenylalanine biosynthesis pathway, isoquinoline alkaloid biosynthesis pathway, diterpenoid biosynthesis pathway, and stilbenoid,

diarylheptanoid and gingerol biosynthesis pathway. This study represents the first time the transcriptome of *H. sabdariffa* calyx tissues were sequenced using NGS technologies. The novel transcriptomic data produced in this research provides an expansion of information on the genetics alongside their dynamics in the calyx tissues of *H. sabdariffa* throughout the third and fourth maturation stages, which is useful for future studies on functional analysis and marker development.

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

**PEMPROFILAN TRANSKRIPTOM TISU KALIKS *Hibiscus sabdariffa* L. PADA DUA FASA MATURASI**

Oleh

**NUR ATHEEQAH BINTI HAMZAH**

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Rosel (*Hibiscus sabdariffa* L.) merupakan spesies tumbuhan bukan model yang mempunyai kaliks yang telah diselidiki secara berperingkat di dalam sains untuk komposisi metabolit dan potensi farmakologinya dalam merawat hipertensi, diabetes, kanser, hiperlipidemia dan hiperglikemia. Mekanisma genetik yang mengawal produksi fitokimia yang ampuh terdapat di dalam tisu kaliks rosel seperti antosianin masih belum ditafsir dan difahami. Tujuan penyelidikan ini adalah untuk menghasilkan set data transkriptom bagi kaliks tisu *H. sabdariffa* pada dua fasa terakhir kematangannya (fasa tiga dan empat) menggunakan teknologi jujukan-generasi seterusnya (JGS). Beberapa siri eksperimen makmal basah telah dijalankan sebelum jujukannya termasuklah pengekstrakan-RNA, penipisan-rRNA dan pembinaan perpustakaan jujukan cDNA. Platform jujukan Illumina NextSeq 500 telah digunakan untuk penjujukan manakala analisis data telah diatur menggunakan beberapa jenis perisian termasuklah Trinity versi 2.2.0 dan CLC Genomic Workbench versi 10.1.0. Sebanyak 200 juta jujukan berpasangan yang berkualiti baik telah dihasilkan dari penjujukan yang membawa kepada pembentukkan transkriptom rujukan secara *de novo* yang mempunyai sebanyak 221,334 transkrip di mana 92,974 (42%) transkrip tersebut telah berjaya dianotasi. Sebanyak 12 gen berkaitan dengan antosianin telah dianotasi secara berkesan di dalam set data ini; chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavanoid 3'-monoxygenase, flavonoid 3',5'-hydroxylase, dihydroflavonol 4-reductase, leucoanthocyanidin dioxygenase, flavonoid 3-*O*-glucosyltransferase, anthocyanidin 3-*O*-glucoside 2"-*O*-glucosyltransferase, coumaroyl-CoA:anthocyanidin 3-*O*-glucoside-6"-*O*-coumaroyltransferase, malonyl-CoA:anthocyanidin 5-*O*-glucoside-6"-*O*-malonyltransferase dan flavonoid 3',5'-methyltransferase. Analisis perbezaan ekspresi telah mengenalpasti 504 gen yang mempunyai perbezaan ekspresi signifikan (GPES) yang secara efektif dipetakan kepada 193 laluan peta KEGG. Kategori biosintesis metabolit sekunder telah mencapai bilangan pemetaan SPEGs (40) yang agak tinggi. Antaranya ialah, laluan biosintesis fenilalanin, laluan biosintesis alkaloid isoquinoline, laluan biosintesis diterpenoid dan laluan biosintesis stilbenoid, diarylheptanoid dan gingerol. Penyelidikan ini merupakan kali pertama penjujukan transkriptom tisu kaliks

*H. sabdariffa* dilakukan mengunakan teknologi JGS. Data novel transkriptomik yang dihasilkan di dalam penyelidikan ini menyediakan peningkatan penambahan ilmu dan perkembangan informasi genetik serta perubahan dinamiknya di dalam tisu kaliks *H. sabdariffa* sepanjang fasa ketiga dan keempat kematangannya, di mana ia akan berguna untuk penyelidikan analisis fungsi dan penghasilan penanda genetik.

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

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| 18S/25S | Svedberg Unit |
| AA | Ascorbate |
| ACE | Angiotensin-converting Enzyme |
| Acetyl-CoA | Acetyl Coenzyme A |
| ADP | Adenosine Diphosphate |
| BLAST | Basic Local Alignment Search Tool |
| BLASTn | Nucleotide Basic Local Alignment Search Tool |
| BLASTx | Protein Basic Local Alignment Search Tool |
| bp | Base Pair |
| CA | Caffeic Acid |
| cDNA | Complementary DNA |
| CLCGWB | CLC Genomic Workbench |
| Cq | Quantification Cycle |
| DAB | Days After Blossoming |
| DEG | Differentially Expressed Genes |
| dNTP | Deoxynucleotide Triphosphate |
| dsDNA | Double Stranded DNA |
| EDGE | Empirical Analysis of Differential Gene Expression |

| | |
|---|---|
| FAMA | Federal Agricultural Marketing Authority |
| FAO | The Food and Agriculture Organization of the United Nations |
| FDR | False Discovery Rate |
| FPKM | Fragments per Kilobase per Million |
| FU | Fluorescent Unit |
| GO | Gene Ontology |
| *HSPs* | Heat Shock Protein |
| IUCN | International Union for Conservation of Nature |
| kb | Kilobase |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| L. | Linnaeus |
| M | Molar |
| mRNA | Messenger RNA |
| NADH | Reduced Nicotinamide Adenine Dinucleotide |
| NCBI | National Center for Biotechnology Information |
| ng | Nanogram |
| NGS | Next-Generation Sequencing |
| nM | Nanomolar |
| nt | Nucleotide |
| NTC | No Template Control |
| Oligo-dT | Oligo-Deoxythymine |
| p | Probability |
| PCA | Principal Component Analysis |

| | |
|---|---|
| PCR | Polymerase Chain Reaction |
| QC | Quality Check |
| Q | Phred Quality Score |
| r | Pearson's Coefficient of Correlation |
| RNA | Ribonucleic Acid |
| $R^2$ | Pearson's Coefficient of Determination |
| RIN | RNA Integrity Number |
| RNA-Seq | RNA Sequencing |
| rRNA | Ribosomal Ribonucleic Acid |
| RT-PCR | Reverse-Transcription Polymerase Chain Reaction |
| qPCR | Quantitative Real-Time Polymerase Chain Reaction |
| SDEGs | Significant Differentially Expressed Genes |
| TAE | Tris-Acetate-Ethylenediamine Tetraacetic Acid |
| t | Time |
| TPM | Transcripts per Million |
| U | Unit |
| UDP | Uridine Diphosphate |
| Uniprot | Universal Protein Resources |
| USDA | United States Department of Agriculture |
| V | Volts |
| ΔΔCt | Comparative Threshold Cycle |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

*Hibiscus sabdariffa* L. is an annual, tropical shrub that grows abundantly in warm climates such as Malaysia. Locals in Malaysia call this plant asam paya though it is also widely known as roselle. Historically, *H. sabdariffa* has been consumed by locals in Myanmar and West Africa for their fibre-rich leaves and nutritious calyx. To date, the main *H. sabdariffa* producing countries include Thailand, China, Tanzania, Senegal and Egypt. The deep purple to reddish colour of the roselle's calyx gives a strong indication of the production of highly dense anthocyanin present especially in its mature stages. The calyx of roselle typically undergoes four stages of maturation according to the Federal Agriculture Marketing Authority (FAMA, 2006), whereby changes in colouration and phytochemical profile are prevalent and could be a result of differential gene expressions that take play over the course of flower development and fruit ripening (Yu et al., 2012).

The *Hibiscus sabdariffa* L. is an underrated plant species within the scientific community and the general public. However, in recent years, more interest and focus have been shifting towards understanding the potential of this plant in relation to science, pharmacology, and the food industry. Studies investigating the beneficial natural compounds in this plant have seen an increasing trend in the past two decades. Phytochemical screening of its calyx's extract confirmed the presence of flavonoids (Olaleye & Tolulope, 2007) and anthocyanin derivatives such as delphinidin and cyanidin (Ojeda et al., 2010). Numerous studies have also evinced the therapeutic effects of anthocyanin extracts from the calyces. It was discovered to act as a prophylactic and is capable of inducing phase II drug detoxification (Ajiboye et al., 2011). The anthocyanins extract was found to exert antihypertensive property by inhibiting the activity of Angiotensin-converting enzyme, an enzyme responsible for hypertension (Ojeda et al., 2010). Peng and colleagues (2011) also displayed the potential of roselle polyphenolic extract as an adjuvant in diabetic therapy and its potential in reducing hyperglycemia and hyperlipidemia. Clearly, these studies with exhaustive focus on the phytochemistry of *H. sabdariffa* have highlighted its potential as a plant that produces medicinal compounds to improve human health.

Nevertheless, genetic information about this plant is very limited. Till to date, there are only 454 nucleotide sequences and 174 protein sequences related to *H. sabdariffa* reported in the National Centre for Biotechnology Information database (NCBI, January 2020). Despite anthocyanins being the main component contributing to roselle calyx's antioxidant activity, only three sequences of anthocyanin-related genes of *H. sabdariffa* have been reported thus far: *CHS* (chalcone synthase), *F3H* (flavanone 3-hydroxylase) and *ANS* (anthocyanidin synthase). In terms of transcriptomics, no studies have been done specifically on the transcriptome of roselle calyx *per se*. Transcriptome is simply a

set of the total RNA molecules also known as transcripts in an individual cell or a group of cells at any particular developmental stage or physiological state (Wang, Gerstein & Snyder, 2009). In the light of transcriptome, it is feasible to initiate a comprehensive spectrum on genes' functions at variegated stages of development.

## 1.2 Problem-Statement and Significance of Study

Calyx is the most economically important part of the *Hibiscus sabdariffa* L. plant. Harvesting of calyces at the suitable maturation stage has critical association with its quality. The quality of overripe calyx was found to be less desirable in other past studies. Thus, it is important to understand the maturation process and the genetics that govern the maturation process. This study's research problem is pivoted on the certainty that no genetic work with main emphasis on transcriptomics have yet been conducted on the calyx tissue of roselle. The current absence of transcriptome studies associated with the roselle's calyx tissue makes it difficult to have a comprehensive understanding of the gene expression profiles during the maturation stages of roselle. Without a doubt, further downstream application will continue to be restricted. Such examples include a clearer understanding of the genetic regulation and biosynthesis of flavonoids particularly anthocyanins in roselle calyx that could possibly enable future work in genetic engineering to enhance the metabolite quantity and quality. The latter is rather crucial as calyx colour is a factor of quality and taste in the worldwide commercial trade of roselle calyces.

## 1.3 Hypothesis

Anthocyanin related genes could be identified from the calyx transcriptome of *Hibiscus sabdariffa* L. There are differences in the gene expression profiles of calyx tissues between the two maturation stages.

## 1.4 Objectives

(1) To sequence the transcriptome of the calyx tissue of roselle in its third and fourth maturation stages.
(2) To identify the gene or group of genes involved in the biosynthesis of anthocyanin throughout the two maturation stages of roselle's calyx.
(3) To compare the transcriptome profiles of roselle's tissue calyx between both stages of maturation.

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## BIODATA OF STUDENT

Nur Atheeqah Binti Hamzah was born in Penang in the year 1993. She was the youngest out of three siblings. At the young age of two years old she moved to Bath, England where she spent the next five years of her childhood days building snowmen, dancing to Spice Girls, and eating fish and chips on a regular basis. Ironically, as a teen, science was her least favourite subject in school. She had inspired to pursue the performing arts alongside linguistics, but her interests were quickly redirected. Only when she started to contemplate on the meaning of life, the purpose of creation and how creations itself came about did she begin to develop deep interest in the field she is actively pursuing today, science. Nur Atheeqah received her Bachelor's (Honors) Degree in Pure Sciences (Biology) at Universiti Sains Malaysia, Penang. As a science student, she found Allah SWT's creations amazing and the more she dived deeper into understanding the mechanisms behind it, the more she was left in awe with the might and power of God. How God effortlessly brings things into existence while leaving behind a trail of methodology for humans to comprehend and manipulate for their own benefit. Out of all the branches of biology, Atheeqah found genetics to be among her favourites, as it had further strengthened her belief in the existence of a Creator. "Chance" could not have possibly been responsible for the construction of a superior, highly complexed, and well-preserved molecule that has unquestionably great competency like the DNA. The DNA carries the genetic material to create and sustain organisms. Every instruction needed to create life is stored within the DNA, just like a manual book. And as this book is a testimony of a writer, the DNA is a testimony of a Creator. Nur Atheeqah hopes to continue her journey to make it apparent that science is not the source of creation rather, a means for it.

## PUBLICATION

Atheeqah-Hamzah, N., Yong, C. S. Y., & Yusuf, U. K. (2019). Extraction of High-quality RNA from Metabolite and Pectin Rich Recalcitrant Calyx Tissue of *Hibiscus sabdariffa* L. *Pertanika Journal of Tropical Agriculture Science, 43*(4).

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