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MOLECULAR CHARACTERISTICS AND METABOLIC FINGERPRINTING OF GINGER (*Zingiber officinale* ROSCOE)

BY

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// ABSTRACT

The genetic polymorphisms among (*Zingiber officinale* Roscoe.) from Bukit Tinggi, Tanjung Sepat and Sabah cultivars were studied using three single microsatellite oligo-nucleotide primers: $(CATA)_5$, $(GATA)_5$ and $(GAC)_6$ as DNA molecular markers in the polymerase chain reaction (PCR). Seven polymorphic bands were obtained from the PCR products, with in average about 2.334 polymorphic bands per primer, leading to a polymorphic rate of 17.9 %. Jaccard's similarity coefficient varied from 0.562 to 0.875, indicative of close genetic relatedness among the genotype studied. Un-weighted pair group method with arithmetic average (UPGMA) cluster analysis indicated that the Bukit Tinggi ginger is more related genetically to the Tanjung Sepat ginger compared to the Sabah ginger. A putative new gene was observed from the DNA sequencing of the polymorphic bands of Tanjung Sepat cultivar, with the upstream region of DNA sequence contained of a guanine–rich core sequence (GGGCGG); enhancer (CCAAT); promoters (TATA box) and starting site (ATG). Our results showed the presence of genetic diversity among three Malaysian ginger cultivars by using microsatellites DNA. In the second part of this study a comprehensive metabolic fingerprinting of three micro propagated ginger explants, Bukit Tinggi, Tanjung Sepat and Sabah cultivars, was carried out using gas chromatography coupled with mass spectrometry (GC-MS). The ginger leave tissues were fractionated in a polar (methanol) and non polar (chloroform) solvents, subsequently methoximated and silylated prior to GC-MS analysis. By applying this technique, over 300 metabolites (polar and non-polar) in total were detected in each ginger cultivar. However, only about 25% of these compounds can be definitely characterised by using the Wiley7n.1 and the National Institute of standards and Technology (NIST) mass spectra libraries for the best hit of the molecular ion peaks and the fragmentation patterns. Fatty acids and sugars (mono and disaccharides) as the main constituents of the ginger leaf tissues besides a small amount of essential amino acids as well as some organic acids. In addition, a distinct GC-MS metabolic fingerprinting in each of the ginger cultivar can be used as "unequivocal pattern recognition" among the ginger phenotype derived from Bukit Tinggi, Tanjung Sepat and Sabah. Thus, we strongly suggest that the most likely differences in the chemical fingerprinting due to the genetic diversity which exist among the ginger cultivars rather than the environmental factors.

ملخص البحث

تمت دراسة التباين الجيني لثلاثة زروع زنجبيل(بوكيت تينغّي, تنجونغ سبت و صباح) في ماليزيا بأستخدام ثلاثة كبسولات مايكروستلايت أحادية النيوكليوتايد هي $(CATA)_5$, $(GATA)_5$ و$(GAC)_6$ كدليل جزيئي للحامض النووي الرايبوزي منقوص الاوكسجين في تفاعل البوليمريز المتسلسل. تم تسجيل 7 حزم متنوعة بمعدل 2.334 حزمة متنوعة لكل كبسولة تضخيم وهذا ادى الى معدل تباين جيني بمستوى 17.9 %. وبحساب معامل التشابه لجاكارد (Jaccard) حصلنا على مستوى تشابه يتراوح بين 0.562 الى 0.875 مما يدل على التقارب الجيني الشديد بين النماذج المدروسة. أظهر تحليل التكتل بأستخدام طريقة مجموعة الازواج غير الموزونه مع المعدل الرياضي(UPGMA) أن الزنجبيل من منطة بوكت تنجي أقرب جينيا الى الزنجبيل من منطقة تانجونغ سبت مقارنة مع الزنجبيل من منطقة صباح. بعد تحديد تسلسل النيكليوتايدات للحزم المتباينه المأخوذة من زنجبيل تانجونغ سبت لوحظ وجود العلامات المميزة لكونها جين جديد مفترض لوجود تسلسل غني بنيوكليوتايد الجوانين (GGGCGG) و تسلسل المعزز (enhancer) (CCAAT) و تسلسل المحفز (promoter) (TATA box) ووجود تسلسل موقع البداية (ATG). أظهرت نتائجنا وجود تباين جيني بين زروع الزنجبيل من أصل جغرافيّ مختلف (ضمن ماليزيا).

في الجزء الثاني من الدراسة,. تم عمل تحليل شامل للبصمة الايضية لأوراق ثلاثة مستنبتات زنجبيل مختبرية هي بوكيت تينغّي, تنجونغ سبت و صباح بأستخدام الفصل اللوني للغاز-طيف الكتله. تم اولا تجزاءة المكونات الكميائية الى جزء قطبي (ميثانول) وجزء غير قطبي (كلوروفورم) وبعد ذلك تم عمل (methoxaminated) ثم (silylated) لكل الاجزاء قبل تحليلها بجهاز الفصل اللوني للغاز-طيف الكتله. بتطبيق هذه الطريقة حصلنا على اكثر من 300 مركب من كلا الجزئين القطبي وغير القطبي لكل نموذج من الزنجبيل. حوالي 25% من هذه المركبات أمكن التعرف عليها من خلال نمط طيف الكتله لها ومقارنتها مع طيف الكتله المتوفر في قاعدة البيانات المتوفرة في (Wiley 7n.1) و (NIST webbook) وذلك بأختيار افضل مطابقه للجزيئات الايونية ونمط التجزئة. اظهرت النتائج ان المكونات الرئيسية الموجودة في نسيج اوراق الزنجبيل هي الاحماض الدهنية والسكريات (الاحادية والثنائية) بالاضافة الى كمية قليلة من الاحماض الامينية وبعض الاحماض العضوية. كذلك أظهرت النتائج أمكانية استخدام تقنية الفصل اللوني الغازي- طيف الكتلة كوسيلة واضحه للتميز بين زروع الزنجبيل الثلاث. وبناءا على ذلك نستنتج ان الاختلاف الكيميائي بين زروع الزنجبيل ناتج عن وجود تباين جيني وليس بسبب العوامل البيئية فقط.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Science

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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MOLECULAR CHARACTERISTICS AND METABOLIC FINGERPRINTING OF GINGER (*Zingiber officinale* ROSCOE)

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To my beloved parents…………To my supportive wife

To my compassionate brothers and sisters

I would like to dedicate this work

Without the understanding of the motivation of the smiling face that think Daddy can do anything, this work would not have been possible.

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LIST OF SYMBOLS

A	Adenine
$AFLP_S$	Amplified Fragment Length Polymorphisms
AP-PCR	Arbitrarily Primer- Polymerase Chain Reaction
BAC_S	Bacterial Artificial Chromosomes
BSA	N,O-bistrimethyl Silyl Acetamide
BSTF	N, O-bismethyl Silyl Trifluoro Acetamide
bp	Base Pair
C	Cytosine
Da	Dalton
DAF	DNA Amplified Fingerprinting
dNTP	Deoxynucleotide Triphosphate
ECD	Electron Capture Detector
EST	Expressed Sequence Tag
FEN_S	Flap Endonucleases
G	Guanine
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
GSC	Gas-Solid Chromatography
ISSR	Inter Simple Sequence Repeat
Kb	Kilo-Base
LC	Liquid Chromatography

LD_{50}	Lethal Dose 50
Mb	Mega-Base
MMR	DNA Mismatch Repair
MP-PCR	Microsatellite Primer-Polymerase Chain Reaction
MS	Mass Spectrometry
MSTFA	N-methyl-N-trimethyl Silyl Trifluoro Acetamide
m/z	Mass-To-Charge Ratio
ORF_S	Open Reading Frames
PCR	Polymerase Chain Reaction
pg	Pictogram
ppm	Part Per Million
QTL	Quantitative trait loci
$RAHM_S$	Random Amplified Hybridization Microsatellites
$RAMPO_S$	Random Amplified Microsatellite Polymorphisms
RAM_S	Randomly Amplified Microsatellites
$RFLP_s$	Restriction Fragment Length Polymorphisms
RAPD	Random Amplified Polymorphic DNA
SNP_S	Single Nucleotide Polymorphisms

SSCP	Single-Strand Conformation Polymorphism
SSLP$_S$	Simple Sequence Length Polymorphisms
SSR	Simple Sequence Repeat
STR	Simple Tandem Repeat
T	Thymine
TMS	Trimethyl silyl
UPGMA	Un-weighted pair group method with arithmetic average
UTR$_s$	Un-Translated Regions
VSSM$_S$	Variable small Sequence Markers

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

History and science have shown repeatedly that almost all things are cyclical. Currently, we find ourselves in an era of resurgent interest in natural products as medicine. Almost every culture around the world has noted its individual contributions to pharmacognosy and use of foods as medicine. The oldest "prescription" found on Babylonian clay tablets and the hieratic writing of ancient Egyptians on papyrus, archive numerous ancient pharmaceutical and medical uses of hundreds of botanicals and foods (Newall et al., 1996).

Plants have been used through unwritten and written history as a source of medicines, fragrances, spices, and colorants. Plant secondary metabolites have been the successful source of drugs and provided many therapeutic entities to human health problems. Their impact has been huge, for example, 25% of all commonly prescribed pharmaceuticals are directly or indirectly (via semi-synthesis) derived from plants (EPSO, 2005). The world health organization records the fact that 80% of the world's population still relies on botanical medicines (ICMR Bulletin, 2003). With continuous advancements in molecular biology, cellular biology and genomics, the number of molecular targets has been significantly increased in high throughput screening. Innovative biotechnologies and the latest achievements in metabolic engineering and genetic modification should significantly improve the sustainability of plant metabolites in drug development.

One of the most widely used plant as a medicine worldwide is Ginger. Medicinal use of ginger dates back to ancient China and India; ginger use are found in Chinese pharmacopoeias, the Sesruta scriptures of Ayurvedic medicine as well as Sanskrit writing (Garner-Wizard et al., 2006; The review of natural products, 2005). Once ginger culinary properties were discovered in the 13th century, use of this herb became widespread throughout Europe. Traditionally, ginger is used as an acrid bitter to strengthen and stimulate digestion. Modern uses include prophylaxis for nausea and vomiting, dyspepsia, lack of appetite, anorexia, colic, bronchitis, and rheumatic complaints.

Ginger now is in the official pharmacopoeias of Austria, China, Egypt, Great Britain, India, Japan, the Netherlands, Switzerland, and united state (The review of natural products, 2005). It approved as a non-prescription drug in Germany (Kemper, 1999) and as a dietary supplement in the united state (Moore et al., 2008).

Despite their importance in cuisine and especially in medicine, very little information has been available about the genome of ginger, comparing with plants like *Arabidopsis thaliana* or rice, no tools such as bacterial artificial chromosome (BAC) libraries or molecular marker-based genetic maps have been produced (Moore et al., 2008), or at least, no such resources have been released or even mentioned in the literatures because this plant is not amenable to production of genetic maps. Within the last two years, several additional genes have been identified from ginger, including: a cysteine protease, a chalcone sythase, a polyphenol oxidase, a germacrene D sythase, violaxanthine de-epoxidase, and NBS-LRR disease resistance protein (Moore et al., 2008).

Over the past decade, there has been a growing interest in using evolutionary analyses to identify genes that control phenotypes of biological, agronomic, or

medical importance. Crop plants offer a special opportunity to identify such genes because they have been through recent and strong selective sweeps targeted at phenotypes that improve agronomic performance, palatability, or nutritional quality. Thus, by scanning crop genomes for genes or genomic regions that show the signature of selection, one can identify candidates for genes that control phenotypes of agronomic importance. Genomic scans for the signature of selection offer a means of identifying new genes of agronomic importance even when gene function and the phenotype of interest are unknown.

Microsatellites, or simple sequence repeats (SSR) markers belong to the family of repetitive non-coding DNA sequences. Microsatellites are DNA sequences characterized by short (1 - 6 nucleotides) tandem arranged repeats with a total length not exceeding 200 bp. They are highly polymorphic and widely distributed in the eukaryote genome, therefore generating a unique genotypic profile that permits individual identification and relationship categorization. Consequently, assays of microsatellite loci have rapidly become established as a powerful tool for the analysis of mating systems and population structure. Microsatellites are used for analysis of population genetic diversity, either natural or artificial, in gene flow studies, parent-ship analysis, or construction of linkage maps. Microsatellites have been used for the study of the genetic structure of human populations, mammals, birds, fungi, plants, and numerous other species.

In recent years new technologies have allowed gene expression, protein and metabolite profiles in different tissues and developmental stages to be monitored. This is an emerging field in plant science and is applied to diverse plant systems in order to elucidate the regulation of growth and development. The plant metabolites are extracted and analyzed using various sensitive analytical techniques, usually mass

spectrometry (MS) in combination with chromatography. The goal in plant metabolomics is to analyze, identify and quantify all low molecular weight molecules of plant organisms.

The genes, transcripts and proteins are regulated and organized in a complex network that controls plant development. To be able to apply these approaches to plant biology on a routine basis, appropriate methodology has to be developed. For example, metabolomic analysis must be performed using highly sensitive analytical instruments (e.g. mass spectrometry, MS, in combination with chromatography) to give interpretable results.

Primary metabolites are affected by, and affect the physiological processes in the developmental processes in the plant and can be seen as effect of end products of gene expression and enzymatic activity. Thus**,** metabolomics has been proposed as a useful tool for studying gene function. Metabolomics can be used to explain the biochemical function of annotated genes. It can also be used to define phenotypes and to bridge the genotype-to-phenotype gap.

Three ginger cultivars from Bukit Tinggi, Tanjung Sepat and Sabah, in Malaysia, were first collected from its original plantation places and micro-propagated to get a plant leaves grown in identical condition to exclude and discarded any factors that may effect in the quality and quantity of chemical constituents of the ginger like environment, geography, sun light, humidity and else. Ginger leaves were used for DNA extraction for the molecular characterisation tests and also leaves were used for the GC-MS metabolomic fingerprinting test.

In this study we identified the polymorphic DNA of three of ginger cultivars using microsatellite DNA, extraction of the polymorphic bands, re-amplification of the extracted bands, and sequencing to detect the nature of the polymorphic bands. In

the second part we are going to extract the metabolites, chemically derivatize the metabolites and characterize the chemical constituents to get metabolic fingerprinting for each ginger cultivar using GC-MS.

1.2 OBJECTIVES AND HYPOTHESIS

1.2.1 Primary Objectives

- To investigate DNA polymorphisms among ginger plants grown in Bukit Tinggi, Tanjung Sepat and Sabah in Malaysia.
- To apply microrosatellites DNA as a molecular marker for the detection of DNA polymorphism of ginger plants.

1.2.2 Hypothesis

Microsatellites DNA [$(GATA)_5$, $(CATA)_5$, and $(GAC)_6$] can be use as molecular marker for find out the genetic diversity as well as discriminate any differences among the ginger cultivars.

1.2.3 Secondary Objective

To examine the effect of genetic diversity on the chemical profile of three micropropagated ginger explants, namely Bukit Tinggi; Tanjung Sepat and Sabah cultivars using a metabolomic approach and to set a framework for the authentication of the gingers.

1.2.4 Hypothesis

The distinct metabolic fingerprinting of each ginger explants can be observe by using Gas Chromatography – Mass Spectrometry (GC-MS) technique.