UNIVERSITI PUTRA MALAYSIA

CLONING, CHARACTERIZATION AND EXPRESSION OF *SESQUITERPENE SYNTHASE 1* AND *DELTA GUAIENE SYNTHASE 1* GENES ASSOCIATED WITH 'GAHARU" FORMATION IN *Aquilaria malaccensis* Lam

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By

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

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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July 2015

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'Gaharu' is a valuable fragrant resin produced by several *Aquilaria spp.*, many of which are considered threatened. The product is highly recognized for its vast medicinal values as a digestive, sedative and antiemetic drug and also popular as perfumes and incenses in the Middle East, South Asia, Japan and China. Additionally, 'gaharu' sculpturing for interior decoration is another important of its value which generates an income in Asia. Phytochemical studies revealed that sesquiterpenes are one of the main components in gaharu. Therefore, in this study, two genes, *sesquiterpene synthase 1* and *δ-guaiene synthase 1* in the terpenoid biosynthesis pathway have been successfully cloned from *A. malaccensis*. These encoding genes were successfully isolated by rapid amplification of cDNA ends. They were designated as *SesTPS1* and *GuaiS1*, respectively. In the course of obtaining high quality RNA for gene cloning experiments, callus was successfully induced from leaf tissues of *in vitro A. malaccensis* on Murashige & Skoog medium supplemented with 2.2 µM 6-Benzylaminopurine (BAP) and 1.1 µM naphthaleneacetic acid (NAA). Cell line A2 and A4 developed into healthy calli with cremish yellow color and the increased in diameter measurement. *SesTPS1* and *GuaiS1* were cloned from callus RNA, using specific primers derived from transcriptomic data, in a reverse transcription-PCR reaction. The results, the full-length cDNA sequence of *SesTPS1* was 1632 bp encoding for 544 amino acids, while *GuaiS1* was 1644 bp encoding for 547 amino acids. Sequence alignment analysis showed that *SesTPS1* shared between 99% to 100% identity with sesquiterpene synthase from *Aquilaria sinensis* while *GuaiS1* shared between 95% to 99% identity with δ-guaiene synthases from *Aquilaria crassna* and *A. sinensis*. The genes were functionally characterized in a time-course wounding experiment using 3-year-old living trees. Two types of wood samples were collected: 1) from wounded area (S1) and, 2) from 5 cm

below the wounded area (S2). *SesTPS1* was highly expressed after 6 hours post wounding for both S1 and S2, at a level 3-to 6-folds higher than that of control (0 h). The expression of *SesTPS1* was downregulated between 8 h to 24 h after which it climbed to between 1-to 2-fold at 48 h. The pattern of expression of *GuaiS1* was not very different when compared to *SesTPS1*. *GuaiS1* was drastically induced after just 2 h of wounding to 2.8-folds. The expression levels fell back to lower levels (1-fold of control) but increased to 2-folds at 24 h. The expression patterns of *SesTPS1* and *GuaiS1* prevealed that they both responded similarly to wounding. In conclusion, full-length *SesTPS1* and *GuaiS1* genes were successfully cloned from *A. malaccensis*. This is the first report on sesquiterpene genes from this species. It can be deduced that wounding triggers genes in the sesquiterpene synthesis pathway and that might lead to 'gaharu' formation.

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

PENGKLONAN, PENCIRIAN DAN EKSPRESI GEN *SESQUITERPENE SYNTHASE 1* DAN *DELTA GUAIENE SYNTHASE 1* DALAM PEMBENTUKAN GAHARU *Aquilaria malaccensis* Lam.

Oleh

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Gaharu adalah resin wangi yang bernilai dihasilkan dari beberapa *Aquilaria spp.* di mana ianya dianggap terancam. Produk ini telah dikenali dengan nilai perubatan yang luas dalam pencernaan, sedatif dan ubat antimetik, juga terkenal dalam pembuatan minyak wangi serta kemenyan di Timur Tengah, Selatan Asia Jepun dan China. Tambahan pula, pengukiran kayu gaharu untuk hiasan dalaman adalah satu nilai yang penting dalam menambah pendapatan di Asia. Kajian Fitokimia mendedahkan bahawa sesquiterpenes adalah merupakan salah satu komponen dalam gaharu. Oleh kerana demikian, dalam kajian ini, dua calon gen yang terlibat, *sesquiterpene synthase 1* dan *δ-guaiene synthase 1* dalam laluan sintesis terpenoid telah berjaya diklonkan dari *A. malaccensis*. Gen yang mengkodkan gen ini telah berjaya diklonkan melalui 'rapid amplification of cDNA ends' yang masing-masing dinamakan sebagai *SesTPS1* and *GuaiS1*. Bagi mendapatkan RNA yang berkualiti tinggi untuk pengklonan gen, kalus telah berjaya dihasilkan dari tisu daun *in vitro A. malaccensis*. Kalus rapuh telah didorong dengan menggunakan medium Murashige & Skoog dengan penambahan 2.2 μM 6-Benzylaminopurine (BAP) dan 1.1 μM Naphthaleneacetic Acid (NAA). Selepas lebih kurang 6 bulan, kalus yang terbentuk dikeluarkan dari eksplant dan dipindahkan ke media baru. Setiap kalus memberikan satu garis sel yang spesifik. Garis sel ini telah dilabelkan sebagai A1 hingga A4. Hanya garis sel A2 dan A4 hidup dan menunjukkan perkembangan kepada kalus sihat boleh diperhatikan dari warna krim kekuningan dan juga pertambahan ukuran diameter. *SesTPS1* dan *GuaiS1* telah diklonkan dari RNA kalus, menggunakan primer spesifik yang diperolehi daripada data transkriptom melalui tindakbalas PCR transkripsi berbalik. Keputusannya, Jujukan penuh *SesTPS1* cDNA ialah 1632 bp mengkodkan 544 amino asid manakala *GuaiS1* ialah 1644 bp mengkodkan 547 asid amino. Analisis penyelarasan jujukan menunjukkan *SesTPS1*

adalah di antara 99% hingga 100% identiti dengan sesquiterpene synthase dari *Aquilaria sinensis* manakala *GuaiS1* adalah di antara 95% hingga 99% identiti dengan δ-guaiene synthases dari *Aquilaria crassna* dan *A. sinensis*. Pencirian pengelasan fungsi gen dalam tempoh masa kecederaan telah dijalankan dengan menggunakan pokok berusia 3 tahun. Dua jenis sampel kayu yang dikumpulkan: 1) dari kawasan cedera (S1) dan, 2) dari 5 cm daripada kawasan cedera (S2). *SesTPS1* diekspres tinggi selepas 6 jam dicederakan untuk kedua-dua S1 dan S2, tahap 3 hingga 6 kali ganda lebih tinggi dari kawalan (0 j). Di antara 8 jam hingga 24 jam, ekspresi *SesTPS1* menunjukkan penurunan regulasi di mana ekspresi meningkat semula di antara 1 hingga 2 kali ganda pada 48 jam. Corak ekspresi δ-guaiene synthase adalah tidak jauh berbeza apabila dibandingkan kepada *SesTPS1*. *GuaiS1* teraruh secara drastik hanya selepas selepas 2 jam kecederaan (2.8 kali ganda). Tahap ekspresi mengalami penurunan kepada tahap rendah (1 kali ganda kawalan) tetapi meningkat semula kapada 2 kali ganda pada 24 jam. Corak ekspresi *SesTPS1* dan *GuaiS1* mendedahkan bahawa kedua-duanya bertindakbalas sama kepada kecederaan. Kesimpulannya, jujukan penuh *SesTPS1* and *GuaiS1* telah berjaya diklonkan dari *A. malaccensis*. Ini merupakan laporan pertama gen sesquiterpene dari spesis ini. Boleh disimpulkan bahawa kecederaan merupakan pencetus gen dalam aliran sintesis sesquiterpene dan ini membawa kepada pembentukan gaharu. Kedua-dua gen yang bertindakbalas di awal kecederaan mungkin mempunyai peranan dalam sintesis gaharu.

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TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
CHAPTER	
1. INTRODUCTION	1
2. LITERATURE REVIEW	**3**
2.1 Taxonomy and Botanical Description of *Aquilaria malaccensis*	3
2.2 Uses of 'Gaharu'.	4
2.3 'Gaharu' Formation	5
2.3.1 Roles of Biotic Agents Involved in 'Gaharu' Formation	6
2.3.2 Roles of Abiotic Agents Involved in 'Gaharu' Formation	7
2.4 Biosynthetic Pathway of Terpenoids	7
3. CLONING OF TERPENE SYNTHASE ENCODING GENES FROM *AQUILARIA MALACCENSIS*	11
3.1 Introduction	11
3.2 Materials and Methods	11
3.2.1 Plant Materials	11
3.2.1.1 Preparation of Explant	12
3.2.2 RNA Extraction	12
3.2.3 Determination of RNA Concentration and Quality	13
3.2.3.1 Quantification of RNA Samples By Spectrophotometer	13
3.2.4 Synthesis of first-strand of cDNA	14
3.2.5 Primer Design for *Sesquiterpene synthase 1* and *δ-guaiene synthase 1* genes	14
3.2.6 PCR Amplification of *Sesquiterpene synthase 1* and *δ-guaiene synthase 1* genes	15
3.2.7 Cloning of *Sesquiterpene synthase 1* and *δ-guaiene synthase 1* genes	15
3.2.8 Purification of PCR Product	17
3.2.9 Cloning of *SesTPS1* and *GuaiS1*	17
3.2.10 Isolation of Recombinant Plasmid	18
3.2.11 Cloning Full-Length cDNA Sequence	18
3.2.12 Analysis of Sequence Data	19

3.3 Results and Discussions 20
3.3.1 Callus Induction 20
3.3.2 RNA Extraction and Determination of RNA Concentration and Purity 25
3.3.3 Amplification of Partial Terpene Synthase (TPS) Encoding Genes by PCR 26
3.3.4 Analysis of cDNA Sequence 28
3.3.5 Isolation of Full-Length of *SesTPS1* and *GuaiS1* Using RACE 32
3.3.6 Analysis of Full-length cDNA Sequence of *SesTPS1* 33
3.3.7 Isolation of Full-Length cDNA of AmSesTPS1 and AmGuaiS1 Clones Using PCR 43
3.3.8 Analysis of Full-length cDNA Sequences Using PCR 43

4. CHARACTERIZATION OF TERPENE SYNTHASE GENES IN WOUNDED *AQUILARIA MALACCENSIS* OVER TIME 51
4.1 Introduction 51
4.2 Materials and Methods 52
4.2.1 Plant Materials 52
4.2.2 Wounding Treatment 52
4.2.3 RNA Extraction 54
4.2.4 First-Strand cDNA Synthesis 55
4.2.5 Primer Design 55
4.2.6 Quantitative Real-Time RT-PCR (qRT-PCR) 55
4.2.7 Analysis of qRT-PCR Data 57
4.3 Results and Discussions 57
4.3.1 Isolation of RNA 57
4.3.2 Quantitative Real-time qRT-PCR 61
4.3.2.1 Standard Curves 61
4.3.2.2 Dissociation Curves 64
4.3.2.3 Amplification Plots 67
4.3.2.4 Gene Expression 70
4.4 Conclusion 76

5. SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 77

BIBLIOGRAPHY 79
APPENDICES
A: Height, Stem Length And Tree Diameter 92
B: Calculation 92
C: The Expression Level 92
BIODATA OF STUDENT 119

LIST OF TABLES

Table		Page
3.1	Specific primers for amplifying sesquiterpene synthase (*SesTPS1*) and δ-guaiene synthase (*GuaiS1*) encoding genes from *Aquilaria malaccensis*	14
3.2	Thermal cycle parameters for amplifying the candidate genes.	15
3.3.	Gene specific primers used in the RLM-RACE.	16
3.4	Thermal cycle parameters used in RLM-RACE.	17
3.5	Specific primers for amplifying full-length cDNA sequences.	19
3.6.	Thermal cycling condition for amplifying full-length cDNA sequences.	19
3.7	List of web servers applied for full-length cDNA analysis.	20
3.8	Absorbance reading of RNA sample from stem tissue, before and after DNase treatment.	25
3.9	The number and concentration of *AmSesTPS1* nucleotide.	35
3.10	The percentage of amino acid composition of the AmSesTPS1 protein.	36
3.11	BlastX analysis of *AmSesTPS1 cDNA sequence*. The table summarizes four of the most significant matches.	37
3.12	The number and concentration of *AmGuaiS1* nucleotide.	40
3.13	The percentage of amino acids composition of the AmGuaiS1 protein.	41
3.14	BlastX analysis of *AmGuaiS1* cDNA sequence.	42
3.15	BlastX analysis of *SesTPS* cloned 2 sequence.	46
3.16	BlastX analysis of *SesTPS* cloned 3 sequence.	46
3.17	BlastX analysis of *GuaiS* clone 2.	49
3.18	BlastX analysis of *GuaiS* clone 3.	49
4.1	List of qRT-PCR primers.	56
A	Height, Stem Length And Tree Diameter	92
B	Calculation	93
C.1	T1S1	95
C.2	T1S2	99
C.3	T2S1	103
C.4	T2S2	107
C.5	T3S1	111
C.6	T3S2	115

LIST OF FIGURES

Figure		Page
2.1	*Aquilaria malaccensis* tree	4
2.2	Biosynthetic pathway of terpenoids.	9
3.1	Callus development in Plate A.	22
3.2	Callus induction from leaf explant in plate A.	23
3.3	Development of cell line A1, A2, A3 and A4.	24
3.4	Denaturing gel electrophoresis of RNA isolated from stem tissues of *Aquilaria malaccensis.*	26
3.5	PCR product using the SesTPS1 primers amplified from cDNA template of *Aquilaria malaccensis.*	27
3.6	PCR product using the GuaiS1 primers amplified from cDNA template of *Aquilaria malaccensis.*	27
3.7	Nucleotide sequence of *SesTPS1* gene fragment.	28
3.8	Similarity of translated *SesTPS1* gene fragment from *Aquilaria malaccensis* (Query) to the amino acid sequence of sesquiterpene synthase from *Aquilaria sinensis.*	29
3.9	Similarity of translated *SesTPS1* gene fragment from *Aquilaria malaccensis* (Query) to the amino acid sequence of putative (-) germacrene-D synthase from *Aquilaria sinensis.*	29
3.10	Nucleotide sequence of *GuaiS1* gene fragment.	30
3.11	Similarity of translated *GuaiS1* gene fragment from *Aquilaria malaccensis* (Query) to the amino acid sequence of δ-guaiene synthase from *Aquilaria crassna.*	31
3.12	Similarity of translated *GuaiS1* gene fragment from A*quilaria. malaccensis* (Query) to the amino acid sequence of δ-guaiene synthase (ASS3) from *Aquilaria sinensis.*	31
3.13	Agarose gel (2%) electrophoresis of RACE PCR products of *SesTPS1* gene from *Aquilaria malaccensis.*	32
3.14	Agarose gel (2%) electrophoresis RACE PCR products of *GuaiS1* gene from *Aquilaria malaccensis.*	33
3.15	Full-length sequence of *AmSesTPS1.*	34
3.16	The number and concentration of *AmSesTPS1* nucleotides.	35
3.17	Deduced amino acids of AmSesTPS1.	35
3.18	Multiple sequence alignment of sesquiterpene synthase.	37
3.19	Full-length sequence of *AmGuaiS1.*	39
3.20	The number and concentration of *AmGuaiS1* nucleotides.	40
3.21	Deduced amino acids of AmGuaiS1.	41
3.22	Multiple sequence alignment of delta-guaiene synthase.	42
3.23	Agarose gel (2%) electrophoresis of full-length sequence products from *Aquilaria malaccensis.*	43
3.24	Partial sequence of *AmSesTPS2* gene fragment.	44
3.25.	Deduced amino acids of AmSesTPS2.	44
3.26	Partial sequence of *AmSesTPS3* gene fragment.	45
3.27	Deduced amino acids of AmSesTPS3	45
3.28.	Full-sequence of *AmGuaiS2* gene fragment.	47
3.29	Deduced amino acids of AmGuaiS2.	47
3.30	Full-sequence of *AmGuaiS3* gene fragment.	48

3.31	Deduced amino acids of AmGuaiS3.	48
3.32	Multiple sequence alignment of δ-guaiene synthases.	50
4.1	Tree replicates for the wounding experiment and time of collection.	53
4.2	Schematic drawing of wounded stem showing the location of wounds and the collected samples (S1 and S2).	54
4.3	Gel electrophoresis of RNA samples from wounded *A. malaccensis*.	60
4.4	Standard curve generated for each gene in qRT-PCR.	63
4.5	Dissociation curve for (A) *SesTPS1*; (B) *GuaiS1*; (C) *RPL*; (D) *TUA*.	66
4.6	Amplification plots for (A) *AmSesTPS1*; (B) *AmGuaiS1*; (C) *RPL*; (D) *TUA*.	69
4.7	The expression level of (A) *AmSesTPS1* and (B) *AmGuaiS1* in wounded stem.	74
4.8	Interaction of expression level between *AmSesTPS1* and *AmGuaiS1* in (A) S1 and (B) S2 based on time-course of wounding.	75

LIST OF ABBREVIATIONS

A	adenine
Acetyl	acetoacetyl
BLAST	Basic Local Alignment Research Tool
β-ME	β-Mercaptoethanol
bp	base pairs
C	cytosine
cDNA	complimentary deoxyribonucleic acid
CIP	Calf Intestine Alkaline Phosphatase
CITES	Convention on International Trade In Endangered Species of Wild Flora and Fauna
CoA	coenzyme A
Cq	cycle threshold
Ct	number of PCR cycles to a threshold fluorescence value
DDBJ	DNA Data Bank of Japan
DEPC	diethyl pyrocarbonate
DMADP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
dNTPs	2'-Deoxy-adenosine-5'-triphosphate
dsDNA	double-stranded deoxyribonucleic acid
EDTA	ethyletediaminetetraacetic acid
FDP	farnesyl diphosphate
FPS	farnesyl diphosphate synthase
G	guanine
GGDP	geranylgeranyl diphosphate
GOI	gene of interest
GOI_{norm}	normalized gene of interest
GPP	geranyl diphosphate
HMG	3-hydroxy-3-methyl-glutataryl
HMGR	HMG-CoA reductase
IDP	isopentenyl diphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
KD	kilo daltons
LB	Luria Bertani
MeJa	methyl jasmonate
MEP	2-C-methylerythritol-4-phosphate
MEV	mevalonate
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
MVA	mevalonic acid
NaCl	sodium chloride
NCBI	National Centre for Biotechnology Information
ORF	open reading frame
PCR	polymerase chain reaction
PDA	potato dextrose agar
qPCR	Quantitative real time RT-PCR
RLM-RACE	RNA Ligated Mediated Rapid Amplification of cDNA ends
RNA	ribonucleic acid
ROX	6-Carboxyl-X-Rhodamine

RPL	ribosomal protein
RT	reverse transcriptase
RT-PCR□RACE	rapid amplification of cDNA ends□RNA- ribonucleic acid □
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SOC	Super Optimal broth with Catabolite repression
STS	sesquiterpene synthase
SYBR	Sybre green
T	thymine
TAE	Tris-acetate EDTA
TAP	Tobacco Acid Phyrophosphatase
TPS	terpene synthase
TUA	tubulin
UPM	Universiti Putra Malaysia
UTR	untranslated region (of mRNA)
X-Gal	5-bromo-4-chloro-3-indoyl-beta-D- galactopyranoside

CHAPTER 1

INTRODUCTION

Aquilaria is a genus of tropical evergreen tree distributed mainly in Asia. It is precious for producing highly valued 'gaharu' which is broadly used in traditional medicine as a digestive, sedative, and antiemetic, and also in incense and perfume across Asia, Middle East, and Europe (Persoon 2008; Takemoto *et al.*, 2008; Kakino *et al.*, 2010; Ito and Honda, 2008). The most important species for producing 'gaharu' are *Aquilaria malaccensis*, *Aquilaria crassna*, *Aquilaria sinensis* and *Aquilaria rugosa* (Santisuk, 2007).

As a result of the defense mechanism to deter off pathogens, *Aquilaria* species produce 'gaharu' known as agarwood, aloeswood, eaglewood, jinkoh and agalloch. 'Gaharu' has been used in traditional medicines over many generation and recently has been included in pharmaceutical products to treat many illnesses, including coughs, acroparalysis, asthma and as an anti-histamine (Bhuiyan *et al.*, 2009). The price of 'gaharu' ranges from a few US$ per kilo for the lowest quality, to over US$30,000 (RM 104,000) per kilo for top quality oil and resinous wood (Reekruelee, 2008). In attempt to find the resin 'gaharu', *Aquilaria* trees are indiscriminately cut in the natural forest. Due to the extensive harvesting, *Aquilaria* trees have become very rare in the wild and for this reason, *Aquilaria* spp. are now protected in most countries and listed in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Biosynthesis of the resin 'gaharu' in *Aquilaria* is commonly associated with the tree's defense system against injury and attacks by biological agents such as fungi and insects. Other factors such as the age of the tree, differences in the tree caused by seasonal variation, environment variation and genetic variation of *Aquilaria spp* may also play important roles in 'gaharu' formation. In the wild, 'gaharu' production happens naturally in old trees. In other trees like spruce, fir and poplar, resin production has been successfully induced via wounding, fungal inoculation, insects attack and treatment with methyl jasmonate (MeJA)(Martin *et al.*, 2002; Nagy *et al.*, 2000). The formation of resin is a result of massive response at the site of damage, which forms a 'barrier zone' to seal off the wound or the infective agent and to protect new cells from decay. In *Aquilaria*, 'gaharu' is composed of many chemical substances, most importantly the group known as sesquiterpene and phenylethyl chromone (Chen *et al.*, 2012), Thus, it is critically important to understand the biosynthesis and regulation of sesquiterpenes in determination of 'gaharu' formation.

The goal of this study was to examine the biosynthesis mechanism of sesquiterpene in *A. malaccensis*, a major 'gaharu' producer, by isolating several candidate genes in resin synthesis pathway and by determining their expression patterns in woody tissues after wounding.

The main objectives of this research were:

1. To isolate and characterize the *sesquiterpene synthase 1* and *δ-guaiene synthase 1 genes*
2. To determine the expression of the two genes based on time-course of wounding
3. To determine the interaction of these two genes on time-course of wounding

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