UNIVERSITI PUTRA MALAYSIA

MOLECULAR CLONING AND CHARACTERIZATION OF TRANSCRIPTS ENCODING TERPENE SYNTHASE GENES FROM Aquilaria malaccensis Lam

WONG MUN THENG

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By

WONG MUN THENG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillments of the Requirements for the Degree of Doctor of Philosophy

August 2017

DEDICATION

TO MY LATE GRANDMOTHER,
Ng Yoon Hup

MY LATE GRANDAUNTY,
Ng Phaik Choo

BELOVED PARENTS,
Wong Yeen Seong & Lor Chow Thye

BROTHER,
Wong Leong Kit

AND FRIENDS.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MOLECULAR CLONING AND CHARACTERIZATION OF TRANSCRIPTS ENCODING TERPENE SYNTHASE GENES FROM *Aquilaria malaccensis* Lam

By

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August 2017

Chairman : Associate Professor Rozi Mohamed, PhD
Faculty : Forestry

Aquilaria malaccensis Lam. is an endangered tropical tree that produces agarwood, a natural product well-known for its fragrance and medicinal properties, in response to external stimuli. Agarwood contains a wide variety of terpenes and phenylethyl chromone derivatives. However, sesquiterpene was the only terpene synthase (TPS) gene that was being studied in *Aquilaria*. Therefore, both monoterpene and diterpene synthase genes which are equally important as sesquiterpene in agarwood formation from *A. malaccensis* were identified in this study. The aim of this study described here was to determine candidate TPS transcripts from *A. malaccensis* stems, to analyze the transcript expression profile in different tissues of *A. malaccensis* and then use this information to express terpene synthase in an *Escherichia coli* expression system. Using an in-house transcriptome assembled from *A. malaccensis* RNA sequencing project, specific primers targeted for TPS genes were designed. Three TPS candidate genes were selected based on their sequence similarities to other known plant TPSs and named *AmTPS01*, *AmTPS*02 and *AmTPS03*. The partial-length and full-length cDNAs were amplified from stem tissues using the RACE approach and cloned into the pSTBlue-1 vector. Only *AmTPS03* is a full-length cDNA (3027 nucleotides), while the *AmTPS01* and *AmTPS02* sequences included only the 3'-ends (2014 and 1601 nucleotides, respectively). Gene annotation analysis indicates that the deduced peptide sequences of AmTPS01, AmTPS02 and AmTPS03 had 603, 421 and 786 amino acids, respectively. BLAST searches against the GenBank revealed that AmTPS01 had 72% similarity to myrcene synthase from *Morus notabilis*, AmTPS02 to 3R-linalool synthase from *Theobroma cacao* (62%) and AmTPS03 to ent-kaurene synthase from *Castanea mollissima* (81%). From phylogenetic analyzes, the three TPSs are found clustered within the functional homologs of the terpene synthase subfamilies. *AmTPS01* and *AmTPS02* are clustered with the monoterpene Tps-b subfamily, while *AmTPS03* with the diterpene Tps-e subfamily. For the transcript expression analysis, qPCR experiments were carried out using different tissues and treatments. In tissue culture plantlets of *A. malaccensis*, *AmTPS01* and *AmTPS02* transcripts were highly expressed in the roots and

stems, respectively, compared to leaves. The preference of expression in wood tissues is supported from the experiment using nursery-grown tree, where *AmTPS02* also showed high expression in both stem (450-fold) and roots (100-fold) compared to leaves, while *AmTPS01* showed the highest expression in stem (10-folds). *AmTPS01* and *AmTPS02* in calli responded to methyl jasmonate (MJ), a known trigger to production of fragrance constituents in agarwood with the highest expression detected 6 hours after treatment. Drill-wounding and application of a liquid inducer were carried out on plantation trees in a time-course experiment (0, 2, 4, 6, 12 and 24 hours). The liquid inducer apparently induced expression of *AmTPS01* (4 hours) and *AmTPS03* (6 hours) to the highest level (10-30-folds) compared to drill-wounding. *AmTPS03* transcript was abundant in induced-plantation trees but was lowly expressed in MJ-treated calluses, tissue culture plantlets and nursery-grown trees, suggesting its expression is related to the tree's physiological age and a strong inducer. To functionally characterize the three genes, the ORFs were ligated into pET-28a vector and expressed in the *Escherichia coli* BL21 (DE3) strain. Expression of the three His-tag fused proteins, AmTPS01 (70.90 kDA), AmTPS02 (50.29 kDa) and AmTPS03 (90.55 kDa) in bacteria resulted in the accumulation of the protein in insoluble forms. In conclusion, the cloned *A. malaccensis* TPS genes are wood-specific and respond differently to triggers of agarwood induction. In future, studies should be conducted to understand their actual roles in transforming basic isoprene building blocks into terpene compounds. This study provides a foundation for further elucidating the role of TPS genes in the biosynthesis of agarwood compounds in *A. malaccensis*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGKLONAN MOLEKUL DAN PENCIRIAN TRANSKRIP YANG MENGEKOD GEN TERPENE SYNTHASE *Aquilaria malaccensis* Lam

Oleh

WONG MUN THENG

Oktober 2017

Pengerusi : Profesor Madya Rozi Mohamed, PhD
Fakulti : Perhutanan

Aquilaria malaccensis Lam. merupakan sebuah pokok terancam yang menghasilkan gaharu apabila bertindak balas dengan perangsang luaran. Gaharu, terkenal dengan aroma dan ciri-ciri perubatannya. Gaharu mengandungi pelbagai sesquiterpenes dan derivatif phenylethyl chromone. Walaubagaimanapu, sesquiterpene merupakan satu-satunya gen sintesis terpene (TPS) yang dikaji dalam *Aquilaria*. Oleh itu, kedua-dua monoterpen dan diterpen sistesis gen yang sama pentingnya dengan sesquiterpene dalam pembentukan agarwood dari *A. malaccensis* telah dikenal pasti dalam kajian ini. Tujuan kajian ini adalah untuk menentukan transkrip TPS dari batang *A. malaccensis*, menganalisis profil ekspresi transkrip dalam tisu-tisu *A. malaccensis* dan menggunakan maklumat ini untuk mengekspres sintesis terpene dalam sistem ekspresi *Escherichia coli*. Menggunakan transcriptomik dalaman dari projek penjujukan RNA *Aquilaria malaccensis*, primers khusus disasarkan untuk gen TPS telah direka. Tiga gen terpene terpilih berdasarkan persamaan jujukannya dengan tumbuhan-tumbuhan TPS yang telah dikenalpasti dan dinamakan *AmTPS01*, *AmTPS02* dan *AmTPS03*. Kepanjangan separa serta kepanjangan penuh cDNA semua gen telah diamplifikasikan dari batang pokok menggunakan kaedah RACE dan kemudiannya diklonkan ke dalam vektor pSTBlue-1. Daripada tiga gen, hanya *AmTPS03* mempunyai kepanjangan cDNA yang lengkap (3027 nukleotida), manakala, *AmTPS01* dan *AmTPS02* hanya mempunyai hujung 3' dengan jumlah kepanjangan 2014 nukleotida dan 1601 nukleotida, masing-masing. Merujuk kepada setiap satu peptida AmTPS01, AmTPS02 dan AmTPS03, masing-masing mempuyai saiz 603 acid amino, 421 acid amino dan 786 acid amino. Analysis BLAST memaparkan AmTPS01 mempunyai 72% persamaan dengan myrcene synthase dari *Morus notabilis*, AmTPS02 mempunyai 62% persamaan dengan 3R-linalool synthase dari *Theobroma cacao* dan AmTPS03 mempunyai 81% persamaan dengan *ent*-kaurene synthase dari *Castanea mollissima*. Analisis filogenetik menyatakan bahawa tiga gen berkumpulan dengan subfamili homologs berfungsi terpene synthase. *AmTPS01* dan *AmTPS02* berkelompok di bawah Tps-b subfamili, manakala *AmTPS03* dikelompok ke

dalam Tps-e subfamili. Untuk kajian expresi transkrip, experimen qPCR telah dijalankan dengan menggunakan pelbagai tisu pokok serta rawatan yang berlainan. Dalam tisu kultur tumbuhan *A. malaccensis*, transkrip *AmTPS01* and *AmTPS02* menunjukkan expresi yang tinggi dalam akar serta batang, masing-masing berbanding dengan daunnya. Kenyataan mengenai keutamaan expresi gaharu dalam batang pokok disokong dengan experimen yang menggunakan pokok yang dibesarkan dalam tapak semaian, di mana *AmTPS02* menunjukkan expresi di kedua-dua batang (450 kali ganda) serta akar (100 kali ganda) berbanding dengan daun, manakala *AmTPS01* menunjukkan expresi yang tinggi dalam batang (10 kali ganda). *AmTPS01* dan *AmTPS02* dalam callus bertindak balas dengan methyl jasmonate (MJ), sejenis peransang hasil aroma dalam gaharu, dengan puncak ekspresinya pada 6 jam selepas rawatan. Pengerudian dan aplikasi cecair peransang pada masa tertentu (0, 2, 4, 6, 12 and 24 jam) telah dijalankan di atas pokok-pokok ladang. Cecair peransang mencetuskan tahap expresi paling tinggi (10-30 kali ganda) *AmTPS01* (4 jam) dan *AmTPS03* (6 jam) berbanding dengan penggerudian. Transkrip *AmTPS03* amat banyak dalam pokok yang telah diransangkan. Akan tetapi, expresinya amatlah rendah dalam callus yang dirawat dengan MJ, tumbuhan tisu kultur dan pokok-pokok yang dibesarkan dalam tapak semaian. Ini mencadangkan expresi ini berkait rapat dengan umur fisiologi pokok serta peransang yang kuat. Untuk pencirian fungsi ketiga-tiga gen, ORF mereka disatukan dengan vektor pET28a dan diekspreskan dalam *Escherichia coli* BL21 (DE3). Pengekspresan ketiga-tiga protein His-tag, AmTPS01 (70.90 kDA), AmTPS02 (50.29 kDa) dan AmTPS03 (90.55 kDa) dalam bakteria menyebabkan acumulasi protein dalam bentuk yang tidak larut. Kesimpulannya, klon TPS gen *A. malaccensis* adalah specifik kepada batangnya dan bertindak balas dengan cara berbeza untuk menghasilkan gaharu. Pada masa depan, penyelidikan perlu dijalankan untuk mengetahui peranan sebenar dalam pembentukan sebatian terpene daripada isoprene. Penyelidikain ini menyediakan asas kepada kefahaman peranan gen-gen TPS dalam biosintesis gaharu di dalam *A. malaccensis* pada peringkat molekul.

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I certify that a Thesis Examination Committee has met on 18 August 2017 to conduct the final examination of Wong Mun Theng on her thesis entitled "Molecular Cloning and Characterization of Transcripts Encoding Terpene Synthase Genes from *Aquilaria malaccensis* Lam." 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 Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AcC	*Aquilaria crassna* clone
AmTPS	*Aquilaria malaccensis* terpene synthase
ASS	*Aquilaria sinensis sesquiterpene*
BAP	6-benzylaminopurin
BCD	Burning-chisel-drilling
BLAST	Basic Local Alignment Search Tool
CDP	Copalyl diphosphate
CDS	Coding sequence
cDNA	complementary DNA
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CPS	Copalyl diphosphate synthase
cp	chloroplast
Cq	Threshold cycle
DEPC	Diethyl pyrocarbonate
DMAPP	Dimethylallyl diphosphate
dNTPs	Deoxynucleotides
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DXP	1-deoxy-D-xylulose 5-phosphate
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene
FPP	Farnesyl diphosphate
FPS	FPP synthase
gDNA	genomic DNA

GGPP	Geranylgeranyl diphosphate
GOI	Gene of interest
GOI_{norm}	Normalized gene of interest
GPP	Geranyl diphosphate
GST	Glutathione-S-transferase
EtBr	Ethidium bromide
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HgCl2	Mercury chloride
HMGR	Hydox-3-methylglutaryl-coenzyme A reductase
H_2O_2	Hydrogen peroxide
IPP	Isopentenyl diphosphates
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IUCN	International Union for Conservation of Nature
JA	Jasmonic acid / Jasmonate
KEGG	Kyoto Encyclopedia of Genes and Genomesd
KS	*ent*-kaurene synthase/ Kaurene synthase
LB	Luria Bertani
MAPK	Mitogen-activated protein kinase
MEP	Methylerithritol phosphate
mRNA	messenger RNA
miRNA	MicroRNAs
MJ	Methyl jasmonate
MS	Murashige and Skoog
MTIB	Malaysia Timber Board Industry
MVA	Mevalonate
NAA	Naphthaleneacetic acid

NCBI	National Centre for Biotechnology Information
NJ	Neighbour-joining
NPK	Nitrogen (N), Phosphorus (P) and Potassium (K)
NTFP	Non-timber forest product
NR	Non-redundant
OD	Optimal density
ORF	Open Reading Frame
PCD	Programmed cell death
PCR	Polymerase Chain Reaction
qPCR	Quantitative real-time PCR
RACE	Rapid amplification of cdna ends
RIN	RNA integrity number
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SA	Salicylic acid
SOC	Super optimal broth with catabolite repression
SPME	Solid phase microextraction
SS	Sesquiterpene synthase
TB	Terrific Broth
TF	Transcription factor
Tp	Transit peptide
TPS	Terpene synthase
UTR	Untranslated region
UPM	Universiti Putra Malaysia

UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
β-ME	β-Mercaptoethanol

CHAPTER 1

INTRODUCTION

1.1 General

Aquilaria, a member of the Thymelaeaceae is an evergreen tree found mostly in Southeast Asia region. Agarwood is formed mainly in the stems or main branches of *Aquilaria* after being initiated by wounding or infected by biotic disease (Pojanagaroon and Kaewrak, 2003). It is a high quality non-timber product used in perfumery, medicinal and as incense across South Asia, Middle East and Europe (Yagura et al., 2005). Besides that, interior decoration using agarwood sculpturing has been generating a lot of income in Asia. Owing to the economic value, overexploitation has resulted in serious depletion of wild agarwood. Consequently, nine endangered species has been listed in the IUCN Red List of Threatened Plants of the International Union for Conservation of Nature (IUCN) since 1998. Moreover, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has been regulating all *Aquilaria* species since 2004. Nowadays, cultivation of *Aquilaria* are being practiced in countries like China, India, Indonesia, Malaysia, Thailand and Vietnam. Still, they require considerable time for agarwood formation and do not promise in high yield and good quality.

The biosynthetic pathway responsible for formation of agarwood fragrant constituents is only recently revealed (Gao and Wei, 2016; Rasool and Mohamed, 2016) but previous studies have shown that wounding and microbial infection are able to stimulate the resin formation. Different methods such as fungi or chemical inoculation, trunk pruning and burn-chisel-drill (BCD) have been practiced in plantations. In general, agarwood is known to be produced upon wounding regardless of mechanical or biological as a result of plant defense mechanism (Pojanagaroon and Kaewrak, 2003; Nobuchi and Siripatanadilok, 1991). When damage signals are induced once wounded, it will be transmitted to activate the defense gene and later defensive substance, agarwood will be produced to hinder further invasion (Gao and Wei, 2016). This resin is known as agarwood which is mostly found in the parenchyma cells of the xylem (Mohamed et al., 2013).

Aquilaria malaccensis Lam., a member of the Thymelaeaceae family from the order Myrtales, is one of the 21 species from the genera *Aquilaria* from the Indomalesia region (The Plant List, 2013; Mabberly, 2008). Up to now, as many as five species of *Aquilaria* have been found in Malaysia, which are *A. malaccensis*, *Aquilaria beccariana* Van Teigh., *Aquilaria hirta* Ridl., *Aquilaria microcarpa* Baill *and Aquilaria rostrata* Ridl. (Chua, 2008). More than 3.5 milllion *Aquilaria* trees are estimated to be scattered throughout the natural forests of Peninsular Malaysia (Forestry Department Peninsular

Malaysia, 2005). Planted *Aquilaria* is estimated at 1, 300 hectares with 1.2 million trees in Peninsula Malaysia (MTIB, 2016).

Agarwood is widely used in traditional medicines, such as digestive, sedative and antiemetic drug. The main compounds related to the medicinal properties of agarwood are terpenes and phenylethyl chromone derivatives (Liu et al., 2013; Chen et al., 2012; Ueda et al., 2006), which can be highly variable in content and composition among different agarwood-producing tree species. Previous studies had focused on sesquiterpenes, the most abundant terpenes compound in agarwood (Xu et al., 2013; Kumeta and Ito, 2010). However, little is known about monoterpene and diterpene in agarwood.

The most widely represented class of hydrocarbons in essential oils is the terpenes. Their compound, terpenoid plays important roles in direct and indirect plant defense against biotic and abiotic stresses or they are treated as signal molecules reproduction by attraction of pollinators and seed disseminators (Dudareva et al., 2006). Besides that, volatile terpenoids are often used as natural flavor and aroma compounds which had proven to be benefical to humans' health (Wagner and Elmada, 2003). Common monoterpenes found in essential oils are limonene, pinene, terpinene, and cymene (Salehi-Arjmand et al., 2014; Adukwu et al., 2012; Giatropoulos, 2012; Spadaro, 2012). Diterpenes are rarely found in most essential oils due to their high molecular weight. Diterpenes that may be found in essential oils include camphorene, cafestol, kahweol and cambrene (Rosa et al., 2016; Rajagopal et al., 2013; Rani and Mishra, 2013). The study on these compounds and their biosynthetic pathways is thus an important field in terpene-rich agarwood research.

In all, 69 sesquiterpenes have been isolated from *Aquilaria* plants (Chen et al., 2012); however, to date, only a few sesquiterpene synthase genes involved in sesquiterpene biosynthesis have been identified from *Aquilaria* plants (Xu et al., 2013; Kumeta and Ito, 2010). Many genes and gene family members are still unknown.

1.2 Problem statement

Despite terpene synthase (TPS) genes being studied thoroughly in many different plant species over the decades, those that encode for monoterpene and diterpene groups are yet to be discovered in *Aquilaria*. Previous studies have only focused on TPS genes encoding for sesquiterpenes production, the most abundant terpenes in agarwood. In contrast to recent progress in the understanding of the regulation of agarwood formation, reports on other terpenes which are equally as important as sesquiterpene are not known. Thus, in this study both monoterpene and diterpene synthase genes were identified from available transcriptomic data of *A. malaccensis* study.

1.3 Hypothesis

The current study postulates that wounding regulates expression of TPS transcripts, which are associated with agarwood formation in *Aquilaria*. Different tissue types as well as different treatments conducted on it will alter the transcripts expression. Stem should has a significant expression as agarwood was observed to be formed in stem. The key to testing this hypothesis is to discover the candidate transcripts that are involved in terpene synthesis pathway of agarwood formation and verify their functions.

1.4 Objectives

The general objective of this study was to characterize TPS genes from *A. malaccensis*. The specific objectives of this research were:

1. To determine transcripts encoding terpene synthases from stems of nursery grown wound-induced *Aquilaria malaccensis* stem.
2. To analyze the expression profile of transcripts encoding terpene synthase in selected tissue types and subjected to different treatments.
3. To express terpene synthase in an *Escherichia coli* expression system.

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