UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF WOUND-RESPONSE PROTEINS ASSOCIATED WITH GAHARU FORMATION IN Aquilaria malaccensis Lam VIA PROTEOMIC ANALYSIS

MUHAMMAD SYAHMI BIN HAJI HISHAMUDDIN

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By

MUHAMMAD SYAHMI BIN HAJI HISHAMUDDIN

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

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July 2018

Chairman: **Professor Rozi Mohamed, PhD**
Faculty: **Forestry**

Aquilaria malaccensis is a native tree in Malaysia that produce aromatic oleoresin known as *gaharu* in response to biotic and abiotic stress. *Gaharu* is a highl-value non-timber product due to its important role in fragrance, aromatherapy, medicines and religious activities. Little is known about *gaharu* formation in the wood. Mechanical wounding is a major trigger for the formation of *gaharu* in the tropical tree taxa *Aquilaria*. To understand the molecular mechanism by which *Aquilaria* reacts to wounding, we applied the proteomic approach using liquid chromatography coupled with mass spectroscopy (LC/MS-MS). Proteins were extracted from wounded area on the stems of 5-year old trees as a result of a drilling method. Changes in the *A. malaccensis* stem tissue proteomes were examined at 0, 2, 6, 12, and 24 hours after wounding treatment and led to the identification of 2227 differentially expressed proteins. The identified proteins were then undergone Venn diagram analysis resulting with 564 time-point specific proteins with 25% overlap among the five time points. All of the 564 time-point specific proteins were then grouped into biological process categories by using Gene ontology and REViGO analysis and based on these analyses, 21 wound-response proteins were successfully identified. Identification of a set of wound-response proteins that is part of the plant defense system and proteins that are involved in *gaharu* formation in *A. malaccensis* tree provides a valuable reference for further studies.

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

PENGENALAN TINDAK BALAS PROTEIN TERHADAP LUKA DIKAITKAN DENGAN PEMBENTUKAN GAHARU DALAM *Aquilaria malaccensis* Lam MELALUI ANALISIS PROTEOMIK

Oleh

MUHAMMAD SYAHMI BIN HAJI HISHAMUDDIN

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Pengerusi: **Professor Rozi Mohamed, PhD**
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Aquilaria malaccensis adalah pokok asli di Malaysia yang menghasilkan oleoresin aromatik yang dikenali sebagai gaharu sebagai tindak balas kepada tekanan biotik dan abiotik. Gaharu adalah produk bukan kayu yang sangat komersil kerana peranannya penting dalam wangian, aromaterapi, ubat-ubatan dan aktiviti keagamaan. Tidak banyak yang diketahui tentang pembentukan gaharu di dalam kayu. Luka mekanikal adalah pencetus utama pembentukan gaharu di dalam taksonomi tropika *Aquilaria*. Untuk memahami mekanisme molekul di dalam *Aquilaria* yang bertindak balas terhadap luka, kami menggunakan pendekatan proteomik menggunakan kromatografi cecair dan spektroskopi jisim (LCMS/MS). Protein diekstrak dari kawasan yang telah di cederakan pada batang pokok berusia 5 tahun menggunakan kaedah penggerudian. Perubahan dalam proteom tisu *A. malaccensis* diperiksa pada 0, 2, 6, 12, dan 24 jam selepas penggerudian dan membawa kepada identifikasi 2227 protein yang dinyatakan secara berbeza. Protein yang dikenal pasti kemudiannya menjalani analisis gambarajah Venn yang menghasilkan 564 protein yang spesifik terhadap masa yang tertentu dengan 25% bertindih di antara lima titik masa. Semua 564 masa spesifik protein kemudian dikumpulkan ke dalam kategori proses biologi dengan menggunakan analisis ontology Gene dan analisis REViGO dan berdasarkan analisis ini terdapat 21 protein telah dikenal pasti yang bertindak balas dengan luka. Kajian ini menyediakan satu set protein yang bertindak balas terhadap luka yang merupakan sebahagian daripada sistem pertahanan tumbuhan dan protein yang terlibat dalam pembentukan gaharu dalam *A. malaccensis*, menyediakan rujukan berharga untuk kajian seterusnya.

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SPECIALLY DEDICATED TO MY LATE FATHER,
Haji Hishamuddin Bin Haji Kadir

I certify that a Thesis Examination Committee has met on 24 July 2018 to conduct the final examination of Muhammad Syahmi Bin Haji Hishamuddin on his thesis entitled Identification of wound-response proteins associated with *gaharu* formation in *Aquilaria malaccencis* via proteomic analysis 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

BLAST	Basic Local Alignment Research tool
β-ME	B-Mercaptoethanol
cDNA	Complimentary deoxyribonucleic acid
CITES	Convention on International Trade In Endangered Species of Wild Flora and Fauna
Cq	Cycle threshold
Ct	Number of PCR cycles to a threshold fluorescence value
DTT	Dithiothreitol
EC	Enzyme commission
FDPM	Forestry Department Peninsular Malaysia
FDR	False discovery rate
GOI	Gene of interest
GOI_{norm}	Normalized gene of interest
GO	Gene Ontology
HR	Hypersensitive response
HSP	Heat shock protein
IPPs	Isopentenyl diphosphate
IAM	Iodoacetamide
KEGG	Kyoto Encyclopedia of Genes and Genomes
LSU	large subunit
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
MVA	Mevalonic acid pathway
MEP	methylerythritol phosphate pathway
mRNA	messenger RNA
MJ	Methyl Jasmonate
MPAKK	Mitogen-activated protein kinase kinase
NCBI	National Centre for Biotechnology Information
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PCD	Programmed cell death
PID	Percentage of identity
Q-TOF	Quadrupole-time of flight
qPCR	Quantitative real time RT-PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
REViGO	Reduce and Visualize Gene Ontology
RPL	Ribosomal protein
SYBR	Syber green
SD	Standard deviation
SE	Standard error
TMV	Tobacco mosaic virus
TAE	Tris acetate EDTA
TPS	Terpene synthase
UPM	University Putra Malaysia
VOCs	Volatile organic compounds
2-DE	Two-dimensional gel electrophoresis

CHAPTER 1

INTRODUCTION

1.1 General

Agarwood or known as *gaharu* in Malaysia is a non-wood fragrant product found in several parts of *Aquilaria* tree, a genus which is taxonomically belongs to the family of Thymeleaceae (Lee and Mohamed, 2016). *Gaharu* (highly fragrant wood) which is commonly dark in color, with the resins usually derived from the wounded stems of *Aquilaria* species as a result of fungal infection under natural condition (wind, lightning, invaded by the microorganism) or mechanical wounding. *Gaharu* actually have been used for over two thousand years, and mainly used as incense in Buddhist, Hindu and Islamic traditional ceremonies. Planters nowadays choose to plant *Aquilaria* because of its application in medicines, perfume and incense and also due to increasing demand of *gaharu* in the industries which provide substantial economic returns (Eurlings *et al*., 2010). Due to illegal harvesting by *gaharu* smugglers and illegal logging, many species of *Aquilaria* are being threatened with extinction. To control the trade of *gaharu* around the world, all species of *Aquilaria* were placed on CITES Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora in 1995.This matter has attracted the attention and interest of scientists around the world to find a solution to produce sustainable *gaharu* resources and to increase its production from an infected wood by intentionally wounding the trees. Conventional *gaharu* inducing techniques such as fungal inoculation, partial trunk pruning and burning chisel drilling were often used by traders to wound *Aquilaria* tree (Liu *et al*., 2013). Other traders and researcher also have developed several *gaharu* inducing methods such as nailing, drilling, aeration, and agar-wit to serve as a way to reserve *gaharu* and to conserve of wild *Aquilaria* stock. However, the current methods used by the planters are time-consuming, with low quality and yield. Thus better understanding of the *gaharu* production mechanism is needed.

According to Liu *et al*., (2013), *gaharu* formation is a result of activation plant defense responses of the *Aquilaria* tree upon wounding. Sesquiterpenes and phenylethyl chromone derivatives which are the main compounds in *gaharu* resin will be produced once the defense response is activated. Sesquiterpenes in *gaharu* was reported to have antimicrobial and anti-disease activity (Liu *et al*., 2013). The *gaharu* resin will play a role as a barrier to avoid damages towards the tree in place of a hypersensitive response (HR), a rapid death at the infection site which cause further necrotic lesions. Previously, the biosynthesis of both sesquiterpenes and chromone in the formation of *gaharu* were studied to understand the mechanism that occurs during *gaharu* formation (Xu *et al.,* 2013). It was also found that jasmonic acid is a crucial signal transducer that can induce sesquiterpenes formation in *Aquilaria* tree (Xu *et al*., 2016).

Gaharu formation is a result of defense-associated responses (Rasool & Mohamed, 2016), and based on the previous research, plant will produce proteins that have toxic, repellent and/or anti-nutritional effect to protect themselves (War *et al*., 2012). In the case of *Aquilaria*, secondary metabolites or specialized morphological structure will be produced in response to herbivore attack (War *et al*., 2012). Researchers believed that wound-response protein in *Aquilaria* is responsible for the activation of the plants defense (Chen & Pramanik, 2009; Azzarina *et al*., 2016; Rasool & Mohamed, 2016). Study on wound-response proteins by using proteomic approaches has been reported to be useful in term of understanding the relationship between the proteins and stress in plant for example, commercially exploited plants such as rice, where four proteins related to wound-response have been found (Shen *et al*., 2003). Several wound-response proteins have also been identified in papaya by using proteomic approach which includes trypsin inhibitor, a class-II chitinase and a glutaminyl cyclase (Azarkan *et al*., 2004). Not only that, tomato also produces wound-response proteins that have been identified as BofuT4_P108020 (Shah *et al*., 2012).

1.2 Problem Statement

At present, very little is known about proteins that involve in *gaharu* formation, this study will emphasize the use of proteomics to better understand the mechanism behind *gaharu* formation. *Gaharu* also has been listed as an endangered species on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora in 1995 due to decreasing in number of *Aquilaria* tree in the natural. The hypothesis for H_0 is there is a constant number and types of protein within *Aquilaria* tree. While, for H_1 there is significant different in number and types of proteins in pre and post wounding in *Aquilaria* tree.

1.3 Justification

The downturn in natural *Aquilaria* populations has raised the alarm among conservationists and researchers to conserve and protect this tree in its natural environment. In order to shorten the time for *gaharu* formation and to increase the yield and quality of the *gaharu* in *Aquilaria* tree, studies on the wound-response proteins that involve in the *gaharu* formation is an important from the point of understanding the mechanism of how the formation of the *gaharu* occurs.

Proteomics analysis that studies large-scale of proteins has always been the first choice for most researchers in investigating wound-response proteins. Protein-based studies on *Aquilaria* species begun to pick-up from 2005 onwards when TRAFFIC (the wildlife trade monitoring network) had pointed out the need to secure the natural *Aquilaria* resources in the wild (Heuveling & Philiphs, 1999). Proteomics analysis has developed rapidly over the years and

has been applied commonly in protein identification. The advantages of using protein in analyzing the plant samples is that they provide rich, reliable and high information regarding the biological process that occurs in the plant. Due to protein diversity, it can take on many different configurations and can play varied roles in cells and tissues.

1.4 Research Objectives

i) To compare the protein expression profiles in wounded stems of *Aquilaria malaccensis* at different time frames using Nanoflow-ESI-LC-MS/MS.

ii) To identify the differentially expressed proteins involved in wound-response in *Aquilaria malaccensis*.

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