UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND MOLECULAR CHARACTERISATION OF VASCULAR-RELATED DEFENSE GENES AGAINST PAPAYA DIEBACK DISEASE

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FBSB 2017 26

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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

May 2017

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

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Chairperson : Associate Professor Janna Ong Abdullah, PhD
Faculty : Biotechnology and Biomolecular Sciences

Malaysia is one of the world's main exporters of papaya with an export value worth RM120 million per year. An outbreak of papaya dieback disease on 2003 lowered the market value up to 60% from the previous year, and remains affected since. *Erwinia mallotivora*, the causal bacteria for papaya dieback disease, enters the papaya plant either through natural openings or wounds, to penetrate into the petioles and stem, and subsequently colonizes the entire vascular system. This results in the appearance of water-soak lesion at the infected region and disruption of the plant's upper meristem region, which produces papaya fruits. To date, the only solution to this problem is by demolishing all the infected papaya plants as it can easily be transmitted to other plants nearby. An alternative approach such as genetic engineering to avoid mass destruction of matured papaya plants is deemed critical. Hence, the aims of this study were to identify potential vascular-related defense genes using bioinformatics approach, isolate and characterize the genes from Eksotika papaya and subsequently to assess the functionality of the genes in transformed papaya seedlings challenged with *E. mallotivora*. This study has successfully identified vascular-related defense genes against papaya dieback disease using bioinformatics approach. After data mining of bioinformatics databases including The Arabidopsis Information Resources (TAIR), *Arabidopsis thaliana* Trans-factor and cis-element Prediction Database (ATTED), Phytozome, and National Center for Biotechnology Information (NCBI), identified five potential genes were then mapped onto the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of Arabidopsis model plant. Orthologs of the Glycerol kinase (*NHO1*), Pathogenesis-related protein 1 (*PR1b*), Leucine-rich repeat receptor-like serine/threonine-protein kinase (*EFR*), RPM1 interacting protein 4 (*RIN4*) and Mitogen-activated protein kinase 4 (*MPK4*) genes were isolated and fully characterized from *Carica papaya* var. Eksotika I. *NHO1* gene with an estimated size of 1572 base pair (bp) was hypothesized as defense-related gene against pathogen. The similar role was predicted with *PR1b* gene (492 bp), while *EFR* gene (741 bp) was postulated to be involved in pathogen signaling. In addition, *RIN4* gene (540 bp) was suggested to be a member of resistance inducer upon pathogen invasion. *MPK4* gene (1125 bp) was estimated to work in a cascade of mediating various responses against pathogens through different signaling pathway of defense responses. These genes were then sub-

cloned, in a sense orientation, into the pEAQ virus vector for subsequent transformation into papaya seedlings via *Agrobacterium tumefaciens* strain GV3101. The constructs were designated as pEAQ.NHO1, pEAQ.PR1, pEAQ.EFR, pEAQ.RIN4 and pEAQ.MPK4. Leaves of two months old papaya seedlings were infiltrated with *A. tumefaciens* strain GV3101 (OD_{600} = 1.5) harbouring each respective constructs. The transformed papaya seedlings were then challenged with *E. mallotivora* (cfu = 10^6) and exhibited disease symptoms development as early as day two after infection. Further profiling of the transgene expression in the papaya seedlings via real-time polymerase chain reaction (qRTPCR) analysis resulted in functional expression against the causal bacteria of this papaya dieback disease. Overexpression of *PR1b* and *NHO1* genes resulted in high expression level in infected plants compared to the uninfected plants. While overexpression of *MPK4* and *RIN4* showed high fold changes compared to control plants, expression of *MPK4* exhibited a repressed defense response via earlier development of symptoms in both infected and uninfected plants. In addition, overexpression of *EFR* showed a down-regulation expression for both infected and uninfected plants. These findings will lay the foundation for subsequent studies in developing a conceivable solution against this papaya dieback disease.

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

PENGENALPASTIAN DAN PENCIRIAN MOLEKUL GEN – GEN PERTAHANAN BERKAITAN VASKULAR TERHADAP PENYAKIT MATI ROSOT BETIK

Oleh

MUHAMMAD HANAM BIN HAMID

Mei 2017

Pengerusi : Profesor Madya Janna Ong Abdullah, PhD
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Malaysia merupakan salah satu daripada pengeksport utama betik di dunia dengan nilai eksport bernilai RM120 juta setiap tahun. Serangan wabak mati rosot betik pada tahun 2003 telah mengurangkan nilai pasaran betik sehingga 60% berbanding tahun sebelumnya, dan masih terkesan sehingga sekarang. *Erwinia mallotivora*, bakteria penyebab kepada penyakit mati rosot betik, memasuki pokok betik sama ada melalui bukaan semulajadi atau luka kecederaan, dengan menembusi petiol dan batang, seterusnya mengkoloni keseluruhan sistem vaskular tumbuhan. Proses ini mengakibatkan keadaan lecuh basah pada kawasan yang dijangkiti dan gangguan terhadap kawasan meristem atas tumbuhan, yang menghasilkan buah betik. Sehingga kini, satu – satunya penyelesaian terhadap masalah ini ialah dengan memusnahkan keseluruhan kawasan pokok betik yang dijangkiti disebabkan oleh penyakit ini yang senang merebak ke pokok – pokok betik berdekatan. Pendekatan alternatif seperti kejuruteraan genetik bagi mengelakkan kemusnahan besar kepada pokok – pokok betik matang adalah keperluan yang kritikal. Objektif kajian ini adalah untuk mengenal pasti gen – gen pertahanan berkaitan vascular berpotensi melalui pendekatan bioinformatik, memencil dan mencirikan gen – gen daripada betik Eksotika dan seterusnya melalui menilai kefungsian gen – gen di dalam anak pokok betik terubah suai yang dijangkitkan dengan *E. mallotivora*. Kajian ini telah berjaya mengenal pasti gen – gen pertahanan berkaitan vaskular terhadap penyakit mati rosot betik melalui pendekatan bioinformatik. Melalui pencarian data pengkalan - pengkalan data bioinformatik seperti *The Arabidopsis Information Resources* (TAIR), *Arabidopsis thaliana Trans-factor and cis-element Prediction Database* (ATTED), *Phytozome*, dan *National Center for Biotechnology Information* (NCBI), lima gen berpotensi telah ditemui dan dipetakan pada tapakjalan *Kyoto Encyclopedia of Genes and Genomes* (KEGG) bersandarkan tumbuhan model Arabidopsis. Gen – gen ortolog *Glycerol kinase (NHO1), Pathogenesis-related protein 1 (PR1b), Leucine-rich repeat receptor-like serine/threonine-protein kinase (EFR), RPM1 interacting protein 4 (RIN4)* dan *Mitogen-activated protein kinase 4 (MPK4)* telah dipencilkan dan dicirikan sepenuhnya daripada *Carica papaya* var. Eksotika I. Gen *NHO1* dengan saiz anggaran 1572 pasangan bes (bp) dihipotesiskan sebagai gen berkait pertahanan melawan patogen. Fungsi yang serupa diramalkan dengan gen *PR1b* (492 bp), sementara gen

EFR (741 bp) terlibat dalam fungsi pengisyaratan patogen. Gen *RIN4* (540 bp) dicadangkan sebagai sebahagian pengaruh kerintangan terhadap serangan patogen. Gen *MPK4* (1125 bp) pula dianggarkan untuk berfungsi di dalam lata pengantara kepada pelbagai gerak balas melawan patogen melalui tapakjalan pengisyaratan gerak balas pertahanan. Gen – gen ini seterusnya disub-klonkan dalam orientasi *sense* ke dalam vektor virus pEAQ untuk transformasi ke dalam anak pokok betik melalui *Agrobacterium tumefaciens* strain GV3101. Konstruk berkenaan dinamakan sebagai pEAQ.NHO1, pEAQ.PR1, pEAQ.EFR, pEAQ.RIN4 dan pEAQ.MPK4. Daun anak pokok betik berusia dua bulan disusupkan dengan *A. tumefaciens* strain GV3101 (OD_{600} = 1.5) yang membawa konstruk secara berasingan. Anak – anak betik yang diubah suai kemudiannya dijangkitkan dengan *E. mallotivora* (cfu = 10^6) dan menunjukkan simptom penyakit seawal dua hari selepas jangkitan. Analisis pemprofilan tahap pengekspresan transgen dalam anak pokok betik melalui kaedah tindakbalas berantai polimerase masanyata (qRTPCR) mencadangkan tahap pengekspresan transkrip gen – gen pertahanan yang dikaji adalah berkesan terhadap bakteria penyebab penyakit mati rosot betik. Pengekspresan gen – gen *PR1b* dan *NHO1* menunjukkan tahap pengekspresan yang tinggi di dalam pokok yang dijangkiti pathogen berbanding dengn pokok yang tidak dijangkiti. Sementara pengekspresan gen – gen *MPK4* dan *RIN4* menunjukkan perubahan (*fold change*) yang tinggi berbanding pokok kawalan, pengekspresan *MPK4* menunjukkan ketindasan (*repressed*) gerak balas pertahanan melalui perkembangan gejala penyakit lebih awal dalam kedua – dua pokok yang dijangkiti dan yang tidak dijangkiti patogen. Pengekspresan *EFR* pula menunjukkan pengekspresan yang menurun (*down-regulated*) bagi kedua – dua pokok yang dijangkiti dan yang tidak dijangkiti. Kajian ini memberi asas kepada penyelidikan berterusan bagi membangunkan suatu penyelesaian yang berpotensi melawan penyakit mati rosot betik ini.

ACKNOWLEDGEMENTS

Creating a Master degree is not an individual experience; rather it takes place in a social context and includes several persons, whom I would like to thank sincerely.

First to all, I would like to express my deepest gratitude to my main supervisor, Assoc. Prof. Dr. Janna Ong Abdullah, for her constant guidance, advice, and efforts to ensure this study was very well sailed. She is a great thinker and a coach who never tires of her passion for looking for new insights and ways to comprehend the reality around us. She has done many great things during supervising my study in a way inspired me to achieve a lot more than I could have. For that, I am very thankful.

Secondly, I also wish to genuinely thank my committee members, Dr. Wee Chien Yeong (MARDI) and Dr. Noor Baity Saidi, who throughout my graduate study have been so helpful in providing me treasured counsels and assistances any possible ways they could have. Dr. Wee's generosity to lend me the research facilities and allocate her project's funding to support my research and copious amount of workshops and scientific conferences, is forever be thankful for. Dr. Noor Baity, together with other committee members patiently read my texts, asked inspiring questions and assisted me with her skills in structuring papers.

My gratitude also goes to the staffs and my colleagues in Biotechnology and Nanotechnology Research Centre, MARDI, namely Dr. Rogayah Sekeli, Mohd Redha Abdullah Zawawi, Suhaina Supian, Norhidayu Che Asari and Azlinda Erny Yunos for not only stimulating research community; but for their aids, references, peer supports and great fun. A special thanks to Lina Rozano, Mohd Waznul Adly Mohd Zaidan, and Noriha Mat Amin, for providing good advice and acted as invaluable sounding boards when formulating my Master arguments and ideas.

Finally, I wish to acknowledge two most significant figures behind my study, my parents, Hamid Salleh and Zainu Hussin, as well as my beloved sister, Siti Nadira Hamid and her family who always give their best to support me emotionally and personally throughout my study. One I could never thank enough. Thank you.

I certify that a Thesis Examination Committee has met on 23 May 2017 to conduct the final examination of Muhammad Hanam bin Hamid on his thesis entitled "Identification and Molecular Characterisation of Vascular-Related Defense Genes Against Papaya Dieback Disease" 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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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

ASGPB	Advanced Studies in Genomics, Proteomics and Bioinformatics
ATTED	*Arabidopsis thaliana* Trans-factor and cis-element Prediction Database
ATP	Adenosine triphosphate
bp	Base pair
ca.	*circa* (around or approximately)
CDS	Coding sequence
cfu	Colony-forming unit
C_t	Threshold cycle
DEPC	Diethylpyrocarbonate or Diethyl dicarbonate
DMSO	Dimethyl sulfoxide
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EEO	Electroendoosmosis
EMBL	European Molecular Biology Laboratory
gDNA	Genomic deoxyribonucleic acid
HiFi	High fidelity
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KEGG	Kyoto Encyclopedia of Genes and Genomes
MARDI	Malaysian Agricultural Research and Development Institute
mmHg	Millimeter mercury
OD	Optical density (absorbance unit)
PEG 8000	Polyethylene glycol 8000
RNase	Ribonuclease
rpm	Rotation per minute
RT	Reverse-transcriptase
TA	Transcript assembly
TAE	Tris-acetate-EDTA
TAIR	The Arabidopsis Information Resource
TIGR	The Institute for Genomic Research
Tris	Tris(hydroxymethyl)aminomethane
TSS	Transformation-Storage solution
x g	Relative centrifugal force (times gravity)
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
YEB	Yeast extract buffer

CHAPTER 1

INTRODUCTION

Papaya (*Carica papaya* L.), a member of dicotyledonous family of Caricaceae is an important tropical fruit in the international market with global production of 9.1 million metric tonnes in 2015 (FAOSTAT, 2015). Found widely in tropical and subtropical countries, papaya is also known as *papaw* or *paw paw* in Australia, and Mamao in Brazil. Other than being eaten fresh, papaya is also used occasionally in making jams, preserves and canned in fruit cocktails. The young fruit is eaten as a vegetable or pickled whereas, papain, an enzyme found in the white latex, is used as a meat tenderiser.

Malaysia has been reported as one of the world's main exporters of papaya, ranked at second place after Mexico, with an export value of about RM100-120 million per year (FAOSTAT, 2015). Eksotika papaya cultivar is the main variety grown for commercialization purposes and especially for export market, with a yield of about 50-70 tonnes per hectare over a two-year crop cycle (Chan, 1987; FAOSTAT, 2015).

Papaya industry faced various problems which affected its annual production including the outbreak of the papaya dieback disease in late 2003, and fruit fly quarantine restrictions from China which resulted in 60% reduction of Malaysia papaya export market (Chan and Baharuddin, 2010). The varieties affected by papaya dieback disease include the Eksotika, Solo, Hong Kong and Sekaki (Maktar *et al.,* 2008). Eksotika papaya, a variety known for its export value, dropped to 42,000 tonnes in 2007 as compared to 71,000 tonnes in 2003 upon the incident of papaya dieback disease took place. As papaya dieback disease resulted in enormous quantity loses in the papaya industry, countless resolutions are taken by researchers and farmers to reduce its implications towards papaya production.

The infection of papaya dieback disease was reported to be fast and easily transmitted within a farm from one particular infected tree to another, and this could also be further transmitted to nearby farms in a short amount of time. To date, many treatments reported are ineffective in controlling the disease, and the only advised procedure to stop the spread is by demolishing the whole papaya plantation. This, however, is a massive cost for the farmers to bear.

Hence, in this study, identification and assessment of potential vascular-related defense genes in papaya at molecular level is essential to strategize a solution against papaya dieback disease with the following objectives:

1. to identify potential vascular-related defense genes using bioinformatics approach,
2. to isolate and characterize the vascular-related defense coding sequences (CDS) from Eksotika papaya, and

3. to assess the functionality of the vascular-related defense genes in transformed Eksotika papaya seedlings challenged with *Erwinia mallotivora*.

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