UNIVERSITI PUTRA MALAYSIA

***Agrobacterium*-MEDIATED TRANSFORMATION OF *Carica papaya* L. var 'Eksotika' WITH IMPROVED RESISTANCE TO DIEBACK DISEASE**

ROSLINDA A. RAZAK

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By

ROSLINDA A. RAZAK

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Agrobacterium-MEDIATED TRANSFORMATION OF _Carica papaya_ L. var 'Eksotika' WITH IMPROVED RESISTANCE TO DIEBACK DISEASE

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Chair: Assoc. Prof. Janna Ong Abdullah, PhD
Faculty: Biotechnology and Biomolecular Sciences

Papaya or *Carica papaya* L. is a tropical fruit that is popular worldwide. The *Carica papaya* L. var 'Eksotika' introduced by the Malaysian Agricultural Research and Development Institute (MARDI) in 1987, is a highly sought after cultivar grown in Malaysia. Unfortunately the Malaysian papaya industry in recent years has been afflicted by many diseases. One of the major diseases identified is the papaya dieback disease caused by the bacteria *Erwinia mallotivora*. This disease was first reported in Batu Pahat, Johor in 2003. The disease can affect the whole papaya plantation via soil, airborne and environmental conditions. Current method to address this disease is by demolishing the infected area. However, this method is ineffective because the infected area will be destroyed. Therefore, this project was aimed to combat the disease by genetically engineering a papaya dieback resistance gene into the papaya plant genome via *Agrobacterium*-mediated transformation. Throughout the years, development of transgenic plants frequently relies on antibiotic as a selectable marker, which raises public concern. Hence, in this study marker-free transgenic papaya plants were created via positive selection using the *phosphomannose isomerase* (*pmi*) gene. Phosphomannose isomerase (PMI) is an enzyme that converts mannose-6-phosphate to fructose-6-phosphate, a glycolysis intermediate that supports the growth of plant cells. To establish a marker-free positive selection system using this PMI, the effects of mannose on the growth and development of embryogenic 'Eksotika' papaya callus was evaluated. One-month old embryogenic calli were cultured on Murashige and Skoog (MS) medium in which 60 g/L sucrose in the original recipe was replaced with different concentrations of mannose and sucrose. Mannose was supplied as the sole carbon source or in combination with sucrose at 0, 5, 10, 15, 20, 25 or 30 g/L. Embryogenic calli cultured on medium supplemented with a ratio of 0:60 g/L mannose:sucrose was used as a control. The result after six sub-cultures showed that mannose at 30 g/L mannose was effective for screening transformed embryogenic calli. Evaluation of papaya transformation efficiency using this positive selection system was pursued using one-month-old embryogenic *Agrobacterium*-transformed calli harbouring pNOV2819 carrying the *pmi* gene. Only transformed cells were capable of utilizing mannose as a carbon source to grow. After five months on mannose selection,

all 70 putative transformants obtained were PCR-positives for the *pmi* gene. Having established an antibiotic-free selection system, the next stage was targeted at combating the dieback disease. Plant disease caused by bacteria had been related to quorum sensing; whereby the bacteria monitors each other by secreting a small signal molecule known as autoinducer. It was hypothesized that interference of the signalling molecule of bacteria pathogen during infection could starve off infection. Hence, in this study, *homoserine lactone (AHL)-lactonase* gene isolated from *Bacillus* sp. strain 240B1 was used as a quorum quencher against the signalling molecule, AHL. To investigate the resistance of the 'Eksotika' papaya against *Erwinia*, the anti-pathogenic gene cassette constructed as pNOV2819:*pmi:AHL-lactonase* was transformed into the 'Eksotika' papaya embryogenic callus using *Agrobacterium tumefaciens* strain LBA4404. Mannose at 30 g/L was used during the selection process. Stable *in vitro* transgenic papaya plants were successfully obtained within 7 to 8 months on De Fossard medium during regeneration. A total of 154 transgenic plants harbouring *pmi* and *AHL-lactonase* were obtained out of 1740 transformed calli. The overall transformation frequency of pNOV2819:*pmi* was 10.8 % and pNOV2819:*pmi*:*AHL-lactonase* was 8.1%. PCR analysis verified to confirm that the transgene was successfully integrated into the T_0 'Eksotika' papaya plants and the transgene was not detected in the control. In order to assess the level of resistance of T_0 plants produced against dieback, T_0 plants aged 5 to 6 months were injected with a needle at the stem area by bacteria *Erwinia mallotivora*. The result showed the control plant began to experience dieback symptoms as early as 3 days after infection. The symptoms then spread and infected other plant parts. While for transgenic plant lines, the shoots wilted and fortunately the symptoms did not spread to the other parts of the plant. The results of screening between this pathogen and *AHL-lactonase* proved that the transformation successfully disrupted the communication of the pathogen and able to reduce the spread of bacteria.

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

TRANSFORMASI *Carica papaya* L. var 'Eksotika' PENGANTARAAN-*Agrobacterium* DENGAN RINTANGAN YANG BERTAMBAH BAIK TERHADAP PENYAKIT MATI ROSOT

Oleh

ROSLINDA A. RAZAK

April 2017

Pengerusi: Prof. Madya. Janna Ong Abdullah, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Betik atau *Carica papaya* L. adalah buah-buahan tropika yang popular di seluruh dunia. *Carica papaya* L. var 'Eksotika' yang diperkenalkan oleh Institut Penyelidikan dan Kemajuan Pertanian Malaysia (MARDI) pada tahun 1987, adalah kultivar yang dicari selepas ditanam di Malaysia. Malangnya industry betik Malaysia pada sejak kebelakangan ini telah ditimpa pelbagai penyakit. Salah satu penyakit utama yang dikenal pasti adalah penyakit betik mati rosot yang disebabkan oleh bakteria *Erwinia mallotivora*. Penyakit ini pertama kali dilaporkan di Batu Pahat, Johor pada tahun 2003. Penyakit ini boleh menjejaskan keseluruhan ladang betik melalui tanah, udara dan keadaan persekitaran. Kaedah terkini untuk menangani penyakit ini adalah dengan melupuskan kawasan yang dijangkiti. Walaubagaimanapun, kaedah ini tidak berkesan kerana kawasan yang dijangkiti akan dimusnahkan. Oleh itu, projek ini bertujuan untuk memerangi penyakit secara kejuruteraan genetik gen rintang betik mati rosot ke dalam genom tumbuhan betik melalui transformasi pengantaraan-*Agrobacterium*. Sepanjang tahun, perkembangan tumbuhan transgenik kerap bergantung kepada antibiotik sebagai penanda boleh pilih, yang menimbulkan kebimbangan orang ramai. Oleh itu, dalam kajian ini pokok betik transgenik bebas penanda telah diwujudkan melalui pemilihan positif menggunakan gen *phosphomannose isomerase* (*pmi*). Phosphomannose isomerase (PMI) merupakan enzim yang menukarkan mannosa-6-fosfat kepada fruktosa-6-fosfat, glikolisis perantaraan yang menyokong pertumbuhan sel-sel tumbuhan. Untuk mewujudkan satu sistem pemilihan positif penanda percuma menggunakan PMI ini, kesan mannosa kepada pertumbuhan dan pembangunan embriogenik 'Eksotika' kalus betik telah dinilai. Kalus embriogenik yang berusia satu bulan telah dikulturkan pada medium Murashige dan Skoog (MS) yang mana 60 g/L sukrosa dalam resipi asal telah digantikan dengan kepekatan mannosa dan sukrosa yang berbeza. Mannosa telah dibekalkan sebagai sumber karbon tunggal atau dalam

kombinasi dengan sukrosa pada 0, 5, 10, 15, 20, 25 atau 30 g/L. Kalus embriogenik yang dikulturkan pada medium dengan nisbah 0:60 g/L mannosa:sukrosa telah digunakan sebagai kawalan. Hasil selepas enam kali sub-kultur menunjukkan bahawa mannosa pada 30 g/L berkesan untuk saringan berubah kalus embriogenik. Penilaian kecekapan transformasi betik menggunakan sistem pemilihan positif ini seterusnya menggunakan kalus embriogenik yang berusia satu bulan yang ditransformasikan dengan *Agrobacterium* yang mengandungi pNOV2819 pembawa gen *pmi* itu. Hanya sel-sel yang telah ditransformasi mampu menggunakan mannosa sebagai sumber karbon untuk berkembang. Selepas lima bulan pada pemilihan mannosa, ke semua 70 transformansi diperoleh adalah PCR-positif untuk gen *pmi*. Setelah menetapkan sistem pemilihan bebas antibiotik, peringkat seterusnya disasarkan untuk memerangi penyakit mati rosot ini. Penyakit tumbuhan yang disebabkan oleh bakteria telah berkaitan dengan penderiaan kuorum; dimana bakteria memantau antara satu sama lain dengan merembeskan molekul isyarat kecil yang dikenali sebagai molekul pengesan (*autoinducer*). Hipotesis telah menunjukkan bahawa gangguan isyarat molekul bakteria patogen semasa jangkitan boleh menghalang jangkitan. Oleh itu, dalam kajian ini, *homoserine lactone* (*AHL*) *-lactonase* gen diambil daripada *Bacillus* sp. strain 240B1 telah digunakan sebagai kuorum pelindapkejutan terhadap isyarat molekul, AHL. Untuk menguji kerintangan betik 'Eksotika' terhadap *Erwinia*, gen kaset anti-patogenik dibina sebagai pNOV2819:*pmi:AHL-lactonase*, telah ditransformasikan ke dalam kalus embrogenik betik 'Eksotika' dengan menggunakan *Agrobacterium tumefaciens* strain LBA4404. Mannosa pada 30 g/L digunakan semasa proses pemilihan. Tanaman *in vitro* betik transgenik telah berjaya diperolehi dalam masa 7-8 bulan keatas medium De Fossard semasa pertumbuhan semula. Sebanyak 154 tumbuhan transgenik mengandungi *pmi* dan *AHL-lactonase* diperoleh daripada 1740 kalus yang telah ditransformasikan. Kadar keseluruhan transformasi pNOV2819:*pmi* adalah 10.8% dan pNOV2819:*pmi*:*AHL-lactonase* adalah 8.1%. Analisis PCR telah mengesahkan bahawa transgen itu berjaya disepadukan ke dalam T_0 'Eksotika' dan transgen itu tidak dikesan dalam kawalan. Untuk menilai tahap ketahanan tumbuhan T_0 yang dihasilkan terhadap mati rosot, pokok-pokok T_0 berumur 5 hingga 6 bulan telah disuntik dengan jarum di kawasan batang dengan bakteria *Erwinia mallotivora*. Hasilnya menunjukkan pokok kawalan mula mengalami gejala mati rosot seawal 3 hari selepas jangkitan. Tanda-tanda itu kemudiannya merebak dan telah menjangkiti ke kawasan yang lain. Manakala bagi barisan pokok transgenik, pucuk didapati layu dan bernasib baik ia tidak merebak ke bahagian-bahagian pokok yang lain. Keputusan saringan antara patogen ini dan *AHL-lactonase* membukti kan bahawa transformasi telah berjaya mengganggu komunikasi daripada patogen dan dapat mengurangkan penyebaran bakteria.

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I certify that a Thesis Examination Committee has met on 14 March 2017 to conduct the final examination of Roslinda binti A Razak on her thesis entitled "*Agrobacterium*-Mediated Transformation of *Carica papaya* L. var 'Eksotika' with Improved Resistance to 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.

Members of the Thesis Examination Committee were as follows:

Muhajir bin Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Christina Yong Seok Yien, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Tee Chong Siang, PhD
Associate Professor
Universiti Tunku Abdul Rahman
Malaysia
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 2 June 2017

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:

Janna Ong Abdullah, PhD
Associate Professor
Faculty of Biotrechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Noor Azmi bin Shaharuddin, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Rogayah Sekeli, PhD
Research Officer,
Agri-Omics and Bioinformatics (BT1), Biotechnology and Nanotechnology Research Centre
Malaysian Agricultural Research and Development Institute (MARDI)
(Member)

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Signature: _____
Name of Member of Supervisory Committee: Noor Azmi bin Shaharuddin

Signature: _____
Name of Member of Supervisory Committee: Rogayah Sekeli

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2,4 D	2,4 dichlorophenoxyacetic acid
35S	cauliflower mosaic virus
AHL	Acyl-homoserine lactone
AHL-lactonase	Acyl-homoserine lactonase
BAP	6-Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
bp	base pair
cm	centimeter
DMSO	dimethylsulphonyl oxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Etbr	ethidium bromide
et al.	Et alia (and other)
g	gram
gusA	beta-glucuronidase
IBA	indole-3-butyric acid
LB	Luria-Bertani
M	molar
MARDI	Malaysian Agricultural Research and Development Institute
min	minute
mM	millimolar
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog basal medium

NAA	α-naphthaleneacetic acid
NCBI	National Centre for Biotechnology Information
OD	optical density
PCR	Polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
Sec	second
TAE	Tris boric acid EDTA
TE	Tris-EDTA
µg	microgram
µm	micromolar
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Papaya or *Carica papaya* L. is a member of the dicotyledonous plant from the family Caricaceae. It consists of four genera such as *Carica*, *Jacaratia*, *Jarilla* and *Cylicomorpha*. According to Department of Statistics Malaysia, papaya is an important horticultural crop in Malaysia, with self-sufficiency ratio of about 165.4% in 2015. 'Eksotika' papaya, a cross between the 'Subang 6' and the 'Hawaiian Sunrise Solo', is a popular papaya variety grown in Malaysia. It was released by the Malaysian Agricultural Research and Development Institute (MARDI) in 1987 (Chan, 1987). The sweet taste and pleasant aroma of 'Eksotika' papaya resulted in it to be highly demanded in the local and export markets, such as China, Hong Kong, Singapore, the Europe and Middle East (Sew *et al*., 2011).

The 'Eksotika' papaya variety is the most popular grown cultivar and one of the main varieties grown for export in Malaysia. Currently, one of the major diseases that attack the Malaysian papaya industry is the papaya dieback disease. This disease could destroy the whole papaya plant, and thereby, can result in 100% production losses. Papaya dieback was first reported in Batu Pahat, Johor in 2003. It spread to other papaya plantations in Perak, Selangor, Penang, Kedah and Pahang, affecting about 800 ha and resulting 100 % in the destruction (Roshidi, 2010). The total yield lost was estimated at 200,000 metric tons, equivalent to US$ 58 million. The affected varieties besides 'Eksotika' are such as 'Solo', 'Hong Kong' and 'Sekaki'. The pathogen that causes the disease has been determined as *Erwinia mallotivora*, from the family *Enterobactericeae* (Noriha *et al*., 2011).

Currently the only solution is by destroying the infected plant. Therefore, development of an 'Eksotika' papaya plant that harbours an *acyl-homoserine lactonase (AHL-lactonase)* gene conferring resistance to dieback through genetic engineering technique is seen as an alternative method to overcome and control this disease, besides upholding good management practice in the plantations. The current method for papaya transformation relies on a good antibiotic selectable marker gene for selecting the putative transformed tissues. Unfortunately, such use of antibiotic had resulted in widespread public concern, as it has potential consequences on the environment and human health (Wang *et al*., 2000). Therefore, in order to eliminate the use of antibiotic for selection of transformed tissues, an alternative efficient selection method was developed in this project. The strategy involved using a positive selection system such as phosphomannose isomerase (PMI) to select genetically transformed tissues capable of metabolising and growing on mannose supplemented medium. It was hoped that the usage of such antibiotic-free selection can help reduce the negative perception among the public on genetically engineered products, which were created to increase crop productivity.

Therefore, the objectives for the present study were:

1. to establish a non-antibiotic selectable marker system for *Carica papaya* L. var 'Eksotika',
2. to construct a gene transformation cassette harbouring *acyl-homoserine lactonase* and *phosphomannose isomerise* for *Agrobacterium*-mediated transformation,
3. to transfer the *AHL-lactonase* and *pmi* genes into *Carica papaya* L. var 'Eksotika' callus via *Agrobacterium*-mediated transformation, and
4. to challenge the characterised T_0 transformed papaya plantlets with *Erwinia mallotivora*.

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