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ISOLATION AND CHARACTERISATION OF CPRGA3 IN PAPAYA INDUCED BY Erwinia mallotivora

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ISOLATION AND CHARACTERISATION OF CPRGA3 IN PAPAYA INDUCED BY *Erwinia mallotivora*

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Papaya dieback disease is rapidly progressing and has been negatively affecting Malaysian papaya export industry. Due to the complex nature of plant disease resistance mechanisms, development of papaya variety that is resistant to dieback disease has remained a great challenge. Thus, characterisation of resistance gene analogs (RGAs) in papaya could be an alternative to understand disease resistance in papaya. RGAs are potential plant resistance (R) genes that mainly encode for nucleotide binding site (NBS) and leucine rich repeat (LRR) domains. Although NBS-LRR gene is essential in pathogen recognition and defense signaling, NBS-LRR gene can be also be found mediating plant disease susceptibility. In this study, CpRGA3 sequences of dieback tolerant papaya, Viorica and dieback susceptible papaya, Eksotika was isolated and analyzed following infection by *Erwinia mallotivora*. Sequencing results revealed a single nucleotide polymorphism (SNP) at three positions in CpRGA3 of Viorica papaya resulting in amino acid changes. Interestingly, a change from lysine to arginine at the first position lies in the conserved LRR domain. On the other hand, phylogenetic analysis showed CpRGA3 was closely related with RGA2 and RGA3 from other plant species. Structural analysis showed that there were differences in the secondary structure where CpRGA3-Viorica have 15 α-helix while CpRGA3-Eksotika have 13 α-helix and amino acid residues involved at the ligand binding surface of CpRGA3 from Viorica and Eksotika varies. The SNP at LRR domain might contribute to the structural differences of CpRGA3 protein observed in Viorica and Eksotika, which then resulting in the tolerant and susceptible phenotype respectively. However, when superimposed, the two proteins did not have any structural differences. Next, the gene expression pattern of CpRGA3 in response to *E. mallotivora* was determined through RT-PCR analysis. Despite having the characteristic features of an RGA, the result showed that CpRGA3 was up-regulated at 48 hpi with relative band intensity at 8.7 in Eksotika papaya but with low expression (less than 0.5) in Viorica papaya. Furthermore, disease symptoms development was observed in transgenic papaya over-expressing CpRGA3. Transient overexpression of CpRGA3 in Eksotika papaya expedited the onset of dieback disease symptoms

following inoculation with *E. mallotivora* by three days. Overall, this study suggests that CpRGA3 encoding NBS-LRR mediates disease susceptibility to *E. mallotivora* in papaya. This study provides new insight into the role of NBS-LRR genes in disease susceptibility that could implicates the enhancement of plant disease resistance and the overall strategy for plant disease management in the future.

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

PENGASINGAN DAN PENCIRIAN CPRGA3 DI DALAM BETIK TERARUH OLEH *Erwinia mallotivora*

Oleh

NUR SYAZANA BINTI MOHAMED ABU BAKAR

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Penyakit mati rosot betik sedang berlaku dengan pesat dan telah menyebabkan kesan negatif terhadap industri eksport betik di Malaysia. Namun, penghasilan varieti betik yang mempunyai rintangan terhadap penyakit mati rosot telah menjadi satu cabaran yang besar disebabkan oleh mekanisme kerintangan tumbuhan yang bersifat kompleks. Oleh itu, pencirian analog gen rintangan (RGA) di dalam betik adalah pendekatan alternatif bagi memahami kerintangan penyakit di dalam betik. RGA adalah calon-calon gen kerintangan dalam tumbuhan yang kebanyakannya mempunyai domain nucleotide binding site (NBS) dan leucine-rich repeat (LRR). Walaupun gen NBS-LRR penting untuk mengenali pathogen dan isyarat pertahanan, gen NBS-LRR juga telah ditemui terlibat di dalam tumbuhan yang mudah terdedah kepada penyakit. Di dalam kajian ini, jujukan CpRGA3 telah diasingkan dan dianalisis daripada betik toleran mati rosot, Viorica dan betik mudah terdedah kepada mati rosot, Eksotika selepas jangkitan *Erwinia mallotivora*. Jujukan CpRGA3 telah menunjukkan bahawa polimorfisme jujukan tunggal (SNP) dijumpai pada tiga posisi CpRGA3 Viorica yang menyebabkan perubahan asid amino. Lebih menarik lagi, perubahan daripada lisin kepada arginina pada posisi pertama berada di dalam domain LRR. Selain itu, analisis filogenetik menunjukkan CpRGA3 mempunyai kaitan rapat dengan RGA2 dan RGA3 dari spesis tumbuhan yang lain. Analisis struktur menunjukkan bahawa terdapat perbezaan dari segi struktur sekunder di mana CpRGA3-Viorica mempunyai 15 heliks alfa dan CpRGA3-Eksotika mempunyai 13 heliks alfa dan asid amino yang terlibat di permukaan ikatan ligan pada protein CpRGA3 daripada Viorica dan Eksotika berbeza. SNP di domain LRR mungkin menyumbang kepada perbezaan struktur protein CpRGA3 dari Viorica dan Eksotika, sekaligus menyebabkan fenotip toleran dan mudah terdedah kepada mati rosot. Namun, apabila disuperimposisikan, kedua-dua protein tidak mempunyai sebarang perbezaan struktur. Seterusnya, corak pengekspresan gen CpRGA3 selepas infeksi *E. mallotivora* telah dilihat menggunakan analisis RT-PCR. Walaupun mempunyai ciri-ciri RGA, pengekspresan CpRGA3 di dalam Eksotika adalah sangat tinggi berbanding Viorica. Selain itu, perkembangan symptom penyakit juga telah dipantau pada betik transgenik yang tinggi pengekspresan

CpRGA3. Pengekspresan tinggi secara sementara di dalam betik Eksotika telah mempercepatkan simptom penyakit selepas infeksi *E. mallotivora* selama tiga hari. Secara keseluruhannya, kajian ini mencadangkan bahawa CpRGA3 telah mengakibatkan betik lebih mudah terdedah kepada penyakit mati rosot. Kajian ini mendedahkan pandangan baru menunjukkan fungsi gen NBS-LRR di dalam kecenderungan tumbuhan kepada penyakit yang dapat digunakan untuk meningkatkan kerintangan penyakit dalam tumbuhan dan juga strategi keseluruhan dalam pengurusan penyakit tumbuhan pada masa akan datang.

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"Nothing in this world can take the place of persistence. Talent will not: nothing is more common than unsuccessful man with talent. Genius will not: unrewarded genius is almost a proverb. Education will not: the world is full of educated derelicts. Persistence and determination alone are omnipotent"- Calvin Coolidge.

This thesis was submitted to the Senate of the 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

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BTH	benzothiadiazole
CaM	Calmodulin protein
CDPK	Calcium-dependent protein kinase
dpi	Day Post Inoculation
EDTA	ethylenediaminetetraacetic acid
EIF	Eukaryotic initiation factor 4A
ET	Ethylene
ETI	Effector-triggered immunity
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
hpi	Hour post inoculation
HR	Hypersensitive response
hrp	Hypersensitive reaction and pathogenicity
INA	2,6-dichloroisonicotinic acid
JA	Jasmonic acid
LB	Luria-Bertani
LPS	lipopolysaccharides
LRR	Leucine-rich repeat
MAFC	Malaysia Agrifood Corporation Berhad
MAPK	Mitogen-activated protein kinase

NBS	Nucleotide-biding site
NCBI	National Centre of Biotechnology Information
NGS	Next generation sequencing
NPR1	NON EXPRESSOR OF PR1
PAMP	Pathogen associated molecular pattern
PCD	Programmed cell death
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
RGA	Resistance gene analog
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RLK	Receptor like kinase
RLP	Receptor like protein
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcriptase polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired response
SNP	Single nucleotide polymorphism
TAE	Tris-Acetate-EDTA
T3SS	Type III secretion system
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Papaya (*Carica papaya* L.) is a tropical fruit tree that belongs to the *Caricaceae* family. It is one of the fruits known with outstanding nutritional properties and delightful taste, making it grow in demand. In 2016, the global production of papaya was 13.05 million metric tonnes, where large portion of the production were heavily contributed by developing countries including Malaysia (FAOSTAT, 2016). However, the production of papaya in Malaysia has been on decline due to the outbreak of papaya dieback disease more than a decade ago. Total yield losses were estimated at 200,000 metric tonnes, which is equivalent to US$ 58 million. The papaya varieties that were susceptible to this disease were Eksotika, Solo, Hong Kong and Sekaki (Maktar et al., 2008). The causal agent of papaya dieback disease is gram-negative bacteria called *Erwinia mallotivora* (Mat Amin et al., 2011). *E. mallotivora* infected papaya plant through open wounds of leaf, moving to the petiole and stem (Mat Amin et al., 2011), followed by the blockage of vascular system, leading to plant death.

Back in 2012, MARDI has discovered a papaya variety tolerant to this pathogen, named Viorica to help manage this disease (Sarip et al., 2015). Recently, next generation sequencing (NGS) and proteomics analysis on tolerant (Viorica) and susceptible (Eksotika) papaya varieties infected by E. malotivora (Ling et al., 2016; Supian et al., 2016) has identified one resistance gene analog (RGA) responsive to *E. mallotivora* infection, CpRGA3 (Rozano, unpublished data). In the case of papaya and *E. mallotivora* pathosystem, the function of the RGA in papaya following the pathogen infection is unknown and never been revealed.

Resistance gene analogs (RGA) are candidates of resistance (R) genes that mediated plant disease resistance. Plant R genes are mainly encoded by NBS-LRR protein, consist of nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Marone et al., 2013). Each domain plays important role where NBS domain is involved in signal transduction after pathogen recognition, while LRR domain is involved in protein-protein interactions and for pathogen binding (Sharma and Pandey., 2016). The interaction of plant NBS-LRR protein with pathogen effectors occur when the effector is directly recognized by the NBS-LRR protein, which will induce conformational changes of the protein, exchange of ADP to ATP, and activates downstream signalling resulting in hypersensitive response (HR) (Flor et al., 1971). Alternatively, the NBS-LRR protein will guard any changes of other plant proteins that was initially targeted by pathogen effector (McHale., 2006).

Many experimental evidences showed that NBS-LRR gene was involved in disease resistance response (Lawrence et al., 2007; Zhang et al., 2017). There are also considerable number of studies that suggest NBS-LRR gene was involved in disease susceptibility response (Meng et al., 2006; Yang et al., 2006; Lorang et al., 2007). Here in the present work, the function of CpRGA3 in response to *E. mallotivora* infection was elucidated to better understand the papaya-*E. mallotivora* interaction. It is hypothesized that CpRGA3 in papaya was involved in defense response against *E. mallotivora*.

1.2 Objectives of The Study

The objectives of this study are stated as below:

i. To isolate and analyze the sequence of CpRGA3 encoding NBS-LRR gene from Viorica and Eksotika papaya varieties,
ii. To determine the expression profile of CpRGA3 in Viorica and Eksotika papaya varieties in response to *E. mallotivora* infection,
iii. To functionally characterize CpRGA3 encoding NBS-LRR gene via transient overexpression in papaya.

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