UNIVERSITI PUTRA MALAYSIA

SEQUENCE VARIATION ANALYSIS AND CHARACTERIZATION OF DEFENCE AND STRESS-RELATED GENES IN *Coconut Cadang-Cadang Viroid* INOCULATED OIL PALM SEEDLINGS

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FP 2019 13

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By

NURULATIKA BINTI MINHAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

November 2018

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

SEQUENCE VARIATION ANALYSIS AND CHARACTERIZATION OF DEFENCE AND STRESS-RELATED GENES IN *Coconut Cadang-Cadang Viroid* INOCULATED OIL PALM SEEDLINGS

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December 2018

Chairman : Associate Professor Ganesan Vadamalai, PhD
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Coconut cadang-cadang viroid (CCCVd) is associated with an orange spotting (OS) disorder, which is an emerging problem recently reported affecting oil palms in Malaysia. CCCVd variants were shown to contain low concentrations of several sequence variants of CCCVd in both OS and asymptomatic palms. Knowledge of interaction between CCCVd-infected oil palm is lacking and no study on defence and stress genes upon infection of CCCVd to date. Plant response has become an important approach to understand the host-pathogen interaction. The study was undertaken to study sequence variation of CCCVd oil palm variants from inoculated oil palm seedlings using RT-PCR, cloning and sequencing. Subsequently, detection and characterization of defence and stress genes in CCCVd inoculated oil palm seedlings were investigated. A total of 30 oil palm seedlings comprising ten healthy seedlings, ten seedlings inoculated with plasmid of $CCCVd_{246OP}$ and $CCCVd_{293OP}$ were used. Total nucleic acid was extracted using modified NETME extraction method. The presence of CCCVd variants were detected by RT-PCR using CCCVd specific primers, cloning and sequencing. For genes study, ten seedlings inoculated with $CCCVd_{293OP}$ and ten seedlings as control were used to express total of 11 oil palm genes consisting of 4 stress, 4 defence and 3 reference genes. Optimization using gradient PCR and validation of genes were carried out for real-time PCR reaction. The detection of genes were preceded with quantitative real-time PCR using SYBR Green-I. For sequence variation study, RT-PCR analysis showed that all seedlings inoculated with plasmid $CCCVd_{246OP}$ and $CCCVd_{293OP}$ were detected and generated amplicons at approximately 300 nt on 2% agarose gel electrophoresis. There was no OS symptoms observed for both CCCVd variants. Results of sequencing showed that none of the clones of $CCCVd_{246OP}$ were positive with CCCVd, whereas seedlings inoculated with $CCCVd_{293OP}$ had 99-100% sequence similarity to CCCVd variant 293 nt (DQ097184). Five clones had two to three base substitutions in their sequence compared with the consensus sequence of $CCCVd_{293OP}$ which indicate that there were minor sequence variation. However, there are no quasispecies occurs in this study. For genes study, stress and defence genes in CCCVd-inoculated oil palm seedlings were successfully detected and characterized using conventional PCR and real-time PCR.

Real-time PCR was found to be more sensitive than conventional PCR. The validation of genes through DNA sequencing was successful as the sequencing results showed 86-100% sequence similarity to respective genes. The study showed that all the genes involved were significantly different with the control seedlings. Stress and defence genes were significantly different with the time of post-inoculation. In conclusion, there are minor sequence variation present in oil palm seedlings inoculated with $CCCVd_{293OP}$ and no quasispecies observed. Defence and stress-related genes were successfully detected and characterized through real-time PCR and conventional PCR in oil palm seedlings inoculated with $CCCVd_{293OP}$ plasmid.

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

ANALISIS VARIASI JUJUKAN DAN PENCIRIAN GEN BERKAITAN PERTAHANAN DAN STRES DALAM ANAK-ANAK BENIH KELAPA SAWIT YANG DI INOKULASI *Coconut Cadang-Cadang Viroid*

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Coconut cadang-cadang viroid (CCCVd) dikaitkan dengan penyakit *orange spotting* (OS) kelapa sawit, yang mana akhir-akhir ini muncul penyakit OS dan dilaporkan menjejaskan pokok-pokok kelapa sawit di Malaysia. Varian-varian CCCVd menunjukkan bahawa beberapa varian jujukan CCCVd mempunyai kepekatan yang rendah di dalam OS dan pokok kelapa sawit yang tidak bersimptom. Pengetahuan antara interaksi kelapa sawit yang dijangkiti CCCVd adalah kurang dan tiada kajian sehingga kini mengenai gen pertahanan dan gen stres ketika jangkitan CCCVd. Tindak balas tumbuhan terhadap penyakit telah menjadi pendekatan penting untuk memahami interaksi antara hos-patogen. Kajian ini dilaksanakan untuk mengkaji variasi jujukan CCCVd daripada pokok kelapa sawit yang telah di inokulasi menggunakan ujian RT-PCR, pengklonan dan penjujukan. Selepas itu, pengesanan dan pencirian gen pertahanan dan gen stres dalam anak-anak benih kelapa sawit yang di inokulasi CCCVd di siasat. Sebanyak 30 anak benih kelapa sawit yang terdiri daripada sepuluh anak-anak benih sihat, sepuluh anak benih yang di inokulasi dengan plasmid $CCCVd_{246OP}$ dan $CCCVd_{293OP}$ telah digunakan. Jumlah asid nukleik di ekstrak menggunakan kaedah pengekstrakan NETME. Kehadiran varian-varian CCCVd di kesan melalui ujian RT-PCR menggunakan primer-primer spesifik CCCVd, pengklonan dan penjujukan. Bagi kajian gen-gen, sepuluh anak benih yang di inokulasi $CCCVd_{293OP}$ dan sepuluh anak benih sebagai kawalan digunakan untuk mengekspres sebanyak 11 gen-gen pokok kelapa sawit yang terdiri daripada 4 gen stres, 4 gen pertahanan dan 3 gen rujukan. Pengoptimuman menggunakan *gradient* PCR dan pengesahan gen-gen dijalankan untuk tindakbalas *real-time* PCR. Pengesanan gen-gen di dahului dengan kuantitatif *real-time PCR* menggunakan SYBR *Green-I*. Bagi kajian variasi jujukan, analisis RT-PCR menunjukkan kesemua anak-anak benih yang di inokulasi dengan plasmid $CCCVd_{246OP}$ and $CCCVd_{293OP}$ dikesan dan menghasilkan amplikon anggaran 300 nt pada 2% gel agaros elektroforesis. Tiada simptom OS diperhatikan untuk kedua-dua varian CCCVd. Keputusan penjujukan menunjukkan tiada klon $CCCVd_{246OP}$ yang positif dengan CCCVd, sedangkan anak-anak benih yang di inokulasi dengan $CCCVd_{293OP}$ mempunyai

99-100% persamaan jujukan dengan variann CCCVd 293 nt (DQ097184). Lima klon yang mempunyai dua hingga tiga penggantian dalam jujukannya dibandingkan dengan jujukan konsensus $CCCVd_{293OP}$ menunjukkan bahawa terdapat jujukan variasi kecil. Walau bagaimanapun, tiada *quasispecies* berlaku dalam kajian ini. Bagi kajian gen, gen pertahanan dan gen stres dalam anak-anak benih kelapa sawit yang di inokulasi CCCVd telah berjaya di kesan dan dicirikan menggunakan PCR konvensional dan *real-time PCR*. *Real-time PCR* didapati lebih sensitif berbanding PCR konvensional. Pengesahan gen-gen melalui penjujukan DNA telah berjaya kerana keputusan penjujukan menunjukkan 86-100% persamaan jujukan terhadap gen masing-masing. Kajian menunjukkan bahawa kesemua gen-gen yang terlibat berbeza dengan ketara terhadap anak-anak benih kawalan. Gen stress dan gen pertahanan juga menunjukkan perbezaan yang ketara terhadap masa selepas inokulasi. Kesimpulannya, terdapat sedikit variasi jujukan dalam anak-anak pokok yang di inokulasikan dengan $CCCVd_{293OP}$ dan tiada *quasispecies* diperhatikan. Gen stress dan gen pertahanan berjaya di kesan dan dicirikan menggunakan *real-time PCR* dan konvensional PCR dalam anak-anak pokok kelapa sawit yang di inokulasikan dengan plasmid $CCCVd_{293OP}$.

ACKNOWLEDGEMENTS

I would like to extend my deepest appreciation and thank you to Assoc. Prof. Dr. Ganesan Vadamalai for his knowledge, guidance, advice, motivation and willingness to allocate time to guide me in my research whenever necessary. I would like to thank my supervisory committee members, Dr. Kong Lih Ling for her endless guidance, positivity and opinions as well as Assoc. Prof. Dr. Wong Mui Yun for her intellectual contribution towards my research plan.

My special thanks to Dr. Sathis Sri Thanarajoo for her invaluable comments, suggestions and guidance. My heartfelt thanks to my lab mates cum my best friend, Nurul Najwa Che Roslan and Khoo Ying Wei for their existence, assistance and always together during hard and good times throughout my research journey. Thanks also to Muhammad Buhari for his guidance in statistical analysis.

I extend my appreciation to the Ministry of Higher Education for the scholarship (SLAB) and my employer, Universiti Teknologi Mara (UiTM) for sponsoring my research. My sincere gratitude to Institute of Tropical Agriculture, Institute of Plantation Studies and Faculty of Agriculture for providing the research facilities.

Finally, I owe a considerable debt of gratitude to my family members (Minhad, Rabiah, Norliza, Muhammad Suhaimi, Nazrul, Suzana and Aina Darwisyah) for giving me so much love, strength, encouragement and support throughout my study and always be there for me during my hard time, obstacles and tears. I am grateful to Allah for giving me the strength and patience to complete this study. Alhamdulillah.

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 Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AMV-RT	Avian myeloblastosis virus reverse transcriptase
ASBVd	Avocado sunblotch viroid
bp	Base pair
BLAST	Basic local alignment search tool
C	Central
CA	Chloroform: iso-amyl alcohol mix
CCCVd	Coconut cadang-cadang viroid
CCR	Conserved central region
CTiVd	Coconut tinangaja viroid
CEVd	Citrus exocortis viroid
cDNA	Complementary deoxyribonucleic acid
Cq	Quantification cycle
CRD	Completely randomized design
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Mixture of deoxynucleoside-triphosphates in equimolar amounts
dTTP	Deoxythymidine triphosphate
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EG5	Elaeis guineensis genome
EtBr	Ethidium bromide
EtOH	Ethanol
GC	Guanine-cytosine
GOS	Genetic orange spotting'
g	Gram
g	Centrifugal force
ha	Hectare
HCl	Hydrochloric acid
L	Litre
LB	Lysogeny broth
LiCl	Lithium chloride
M	Moles/ liter (Molarity)
M	Marker of DNA ladder
MnT	Million tonnes
MgCl2	Magnesium chloride
mRNA	Messenger RNA
min	Minute
m-	Milli- (10-3)
ml	Milliliter
mM	Millimolar
mg	Milligram
mpi	Month post infection

µ-	Micro- (10-6)
µl	Microliter
µM	Micromolar
NETME	Natrium Chloride EDTA Tris-HCL Mercaptoethanol
NGS	Next-Generation Sequencing
NCBI	National Center for Biotechnology Information
NaCl	Sodium chloride
Na2EDTA	di-sodium ethylenediamine tetra acetic acid
NTC	Non-template control
nt	Nucleotides
n-	Nano- (10-9)
ng	Nanogram
OS	Orange spotting
pmol	Picomole
P	Pathogenicity
PAGE	Polyacrylamide gel electrophoresis
PCA	Phenol: chloroform: iso-amyl alcohol mix
PCR	Polymerase chain reaction
PSTVd	Potato spindle tuber viroid
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAsin	RNase inhibitor
RT-PCR	Reverse transcriptase polymerase chain reaction
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
SOC	Super optimal broth
s	second
TBE	Tris-borate EDTA
TCH	Terminal conserved hairpin
TCR	Terminal conserved region
Tris	Tris(hydroxymethyl) aminomethane
Tris-HCl	Tris(hydroxymethyl) aminomethane hydrochloride
TuMV	Turnip mosaic virus
TL	Terminal left
TR	Terminal right
UV	Ultraviolet
u	Unit
V	Variable
V	Voltage
vol	volume
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-Indolyl –D-galactosidase

CHAPTER 1

INTRODUCTION

The oil palm tree (*Elaeis guineensis Jacq.*) originated from West Africa and established in Malaysia in early 1870's and now, oil palm tree has been developed into an agricultural crop (MPOC, 2012a). Oil palm is the most productive oil-bearing crop as Malaysia supplies 10% of the world market oils and fats by utilizing only 4 million hectare of land (Casson, 2003).

The oil palm production is affected by pests and diseases. The most severe disease affects palms in Indonesia and Malaysia is basal stem rot (BSR) disease which is caused by *Ganoderma boninense* (MPOC, 2012b). In addition, threats such as an orange spotting (OS) disorder caused by variants of *Coconut cadang-cadang viroid* (CCCVd) have been reported affecting oil palms in Malaysia (Vadamalai, 2005; Vadamalai *et al*., 2006).

CCCVd is the causal agent of the lethal cadang-cadang disease of coconut palms (*Cocos nucifera* L.) in the Philippines, which have been estimated to kill over 40 million coconut palms when it was first recognized (Zelazny *et al*., 1982; Hanold & Randles, 1991; Randles & Rodriguez, 2003). Although OS was recognized as a disorder in the early 19th century in West Africa, but only recently it was associated with variants of CCCVd (Hanold & Randles, 1991; Vadamalai *et al*., 2006). Since OS is associated with CCCVd, it is crucial to determine the extent of similarity between CCCVd and the CCCVd-like molecules in oil palm with OS (Vadamalai, 2005; Vadamalai *et al*., 2006, Wu *et al*., 2013). However, several aspects of its epidemiology in both coconut and oil palm still demand more researches and the principal mode of natural spread of CCCVd is still unknown (Randles & Rodriguez, 2003; Vadamalai *et al*., 2009).

CCCVd variants associated with OS in oil palm in Malaysia have been reported to propagate in their hosts as populations of closely related sequence variants (quasi-species) (Flores *et al*., 2005a). Nevertheless, there is lack of study on sequence variation within the host induced by plasmid. The distribution, accumulation and translocation of CCCVd within the oil palm has been studied previously (Thanarajoo, 2014). However, there is still lack of knowledge in the epidemiological aspects and mainly in the host pathogen interaction. In oil palm, several sequence variants of CCCVd reported to be present in low concentration and difficult to detect (Vadamalai, 2005; Mohammadi *et al*., 2010). Previous study on CCCVd include reverse-transcription polymerase chain reaction (RT-PCR), hybridization assay, polyacrylamide gel electrophoresis (PAGE), ribonuclease protection assay (RPA), reverse transcription loop-mediated isothermal amplification (RT-LAMP), real-time PCR (qPCR) and sequencing (Vadamalai, 2005; Vadamalai *et al*., 2006; Vadamalai *et al*., 2009; Cheong, 2012; Joseph, 2012; Wu *et al*., 2013; Thanarajoo, 2014; Thanarajoo *et al*., 2014).

Plant response has become a decisive way to gain knowledge of host-pathogen interaction. The interactions of plants and microbial pathogens are among the most

complex phenomena in nature. Relative quantification was used to compare the level of gene expression of a particular gene of interest in a chemically-treated sample relative to the level of gene expression in an untreated sample. The changes are either; up-regulated to strengthen the host defence against pathogen invasion, or down-regulated due to the suppression of the host defence system by the pathogen (Applied Biosytem, 2010). Gene expression studies have been used in virus or viroid-host interaction such as in Turnip mosaic virus (TuMV) (Yang *et al*., 2007) and *Citrus exocortis viroid* (CEVd) (Vidal *et al*., 2003). The expression of genes involved in defence and stress responses were widely studied in oil palm infected by *Ganoderma boninense* but no study on genes involved in oil palm infected by CCCVd to date. Therefore, there is an inadequate information and lack of study on host-pathogen interaction of oil palm and CCCVd. Therefore, rapid and sensitive method is needed to study the host-gene interaction of CCCVd. Real-time PCR (qPCR) is used due to its highly sensitivity, able to quantify certain transcripts and changes in gene expression as well as produces reliable and rapid quantification results (Pfaffl, 2001).

In view of this, the objectives of this study are:

1. To study sequence variation of CCCVd oil palm variants from inoculated oil palm seedlings using RT-PCR, cloning and sequencing.

2. To detect and characterize defence and stress-related genes in CCCVd inoculated oil palm seedlings.

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