AN INVESTIGATION OF ORANGE SPOTTING DISORDER IN OIL PALM

By

GANESAN VADAMALAI

M.Agric.Sc. (Universiti Putra Malaysia, Serdang, Malaysia)

Discipline of Plant and Pest Science

Waite Campus

The University of Adelaide

South Australia

Thesis submitted to the University of Adelaide in fulfilment of the requirement for the degree of Doctor of Philosophy

August, 2005

TABLE OF CONTENTS

Table of contents .... **i**

Summary .... **vii**

Statement .... **x**

Acknowledgements .... **xi**

Abbreviations.. .... **xii**

CHAPTER 1: GENERAL INTRODUCTION **1**

1.1 THE OIL PALM INDUSTRY 1

1.2 THE OIL PALM INDUSTRY IN MALAYSIA 1

1.3 ORANGE SPOTTING (OS) OF OIL PALM 2

1.3.1 History and prevalence 2

1.3.2 Symptoms and effects on growth 4

1.3.3 Infectivity and spread 4

1.3.4 Distribution and epidemiology 5

1.4 VIROIDS 6

1.4.1 Biological properties 7

1.4.1a Host range 7

1.4.1b Symptomatology 7

1.4.1c Ecology and epidemiology 8

1.4.2 Viroid classification and structure 9

1.4.3 Replication 11

1.4.4 Movement 13

1.4.5 Pathogenesis 13

1.4.5a Structural implications in pathogenesis 13

1.4.5b Association with host components 14

1.4.5c Association of gene silencing with pathogenicity 15

1.5 *COCONUT CADANG-CADANG VIROID* (CCCVd) 15

1.5.1 Variations of CCCVd molecule 16

1.5.2 Diagnostic methods 17

1.6 SCOPE OF THIS THESIS 18

CHAPTER 2: MATERIALS AND METHODS 19

2.1 MATERIALS 19

2.1.1 Leaf Samples 19

2.1.2 Biochemicals and miscellaneous chemicals 19

2.1.3 Polyacrylamide and agarose gels, bacterial media, solvents and buffers 20

2.1.4 CCCVd 20

2.1.5 ^{32}P-labelled cRNA probe 20

2.2 METHODS 21

2.2.1 Nucleic acid extraction 21

2.2.1a PEG extraction 21

2.2.1b CF11 extraction 22

2.2.1c Total nucleic acid extraction 23

2.2.1d NETME extraction 23

2.2.1e Guanidine extraction 24

2.2.1f CTAB extraction 25

2.2.2 Gel electrophoresis 26

2.2.2a Polyacrylamide gel 26

2.2.2b Two-dimensional (2-D) PAGE 26

2.2.2c Agarose gel 27

2.2.2d Staining of polyacrylamide gels 27

2.2.3 Preparation of CCCVd and CCCVd-like RNA 28

2.2.3a Two-dimensional (2-D) PAGE 28

2.2.3b Gel elution 28

2.2.4 Molecular hybridization 29

2.2.4a Capillary blotting 29

2.2.4b Electroblotting 29

2.2.4c Hybridization assay 30

2.2.4d Autoradiography 30

2.2.5 PCR assay 31

2.2.5a Amplification of viroids by RT-PCR 31

2.2.5b PCR for detection of phytoplasma 32

2.2.6 Molecular cloning of PCR products 33

2.2.6a Purification of PCR products 33
2.2.6b Ligation of ds-cDNA into a plasmid vector 34
2.2.6c Transformation by electroporation 34
2.2.6d Selection for recombinants 35
2.2.7 Mini-preparation of cloned plasmids 35
2.2.8 Analysis of inserts in the recombinant plasmids 36
2.2.9 Sequencing 36
2.2.9a Preparation of dsDNA template 36
2.2.9b Sequencing reaction 37
2.2.9c Sequence analysis 37

CHAPTER 3: SEARCH FOR VIROID-LIKE RNAS IN OIL PALM USING POLYACRYLAMIDE GEL ELECTROPHORETIC (PAGE) ANALYSIS AND HYBRIDIZATION ASSAYS 38

3.1 INTRODUCTION 38
3.2 MATERIALS AND METHODS 40
3.2.1 Materials 40
3.2.2 Methods 40
3.2.2a Total nucleic acid extraction 40
3.2.2b Polyacrylamide gel electrophoresis 41
3.2.2c Blotting of fractionated nucleic acids to membrane 41
3.2.2d Hybridization assay 41
3.3 RESULTS 42
3.3.1 Comparison of extraction methods to isolate CCCVd-like RNAs from oil palm leaves 42
3.3.2 PAGE analysis 42
3.3.3 Hybridization assay with CCCVd probe 43
3.4 DISCUSSION 47

CHAPTER 4: RIBONUCLEASE PROTECTION ASSAY (RPA) TO DETECT CCCVd-LIKE RNAS IN OIL PALM 50

4.1 INTRODUCTION 50
4.2 MATERIALS AND METHODS 51

4.2.1 Materials 51
4.2.2 Ribonuclease Protection Assay 51
4.2.2a *In vitro* transcription of CCCVd probe 52
4.2.2b Hybridization 52
4.2.2c RNAse digestion 52
4.2.2d PAGE analysis 53
4.3 RESULTS 54
4.3.1 RPA analysis of CCCVd from coconut 54
4.3.2 RPA analysis of RNA extracted from oil palms 55
4.3.3 RPA analysis of RNA extracted from an asymptomatic oil palm (SRH2) at different stages of growth 57
4.3.4 RPA analysis of RNA extracted from a symptomatic palm (SRD6 f12) 58
4.3.5 RPA analysis of RNA extracted from coconut samples from Sri Lanka 58
4.4 DISCUSSION 59

CHAPTER 5 : REVERSE-TRANCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) AMPLIFICATION OF CCCVd-RELATED NUCLEIC ACIDS 62

5.1 INTRODUCTION 62
5.2 MATERIALS AND METHODS 64
5.2.1 Materials 64
5.2.2 Methods 64
5.2.2a Purification of CCCVd-related RNAs 64
5.2.2b Design of primers for RT-PCR 65
5.2.2c RT-PCR assay 65
5.3 RESULTS 67
5.3.1 PAGE analysis 67
5.3.2 Evaluation of the primer sets for amplification of circular and linear CCCVd from coconut 68
5.3.3 RT-PCR analysis of guanidine and PEG extracted total RNA 68
5.3.4 RT-PCR of sample from SRH 2 f3 and SRH 2 f10 69

5.3.5 RT-PCR of sample from SRH 2 f20 prepared by 1D PAGE 70
5.3.6 Analysis of RT-PCR amplified products in agarose gel electrophoresis 70
5.4 DISCUSSION 71

CHAPTER 6: CLONING AND SEQUENCING OF CCCVd-RELATED RT-PCR PRODUCTS 73

6.1 INTRODUCTION 73
6.2 MATERIALS AND METHODS 74
6.2.1 Materials 74
6.2.2 Methods 74
6.2.2a Synthesis of cDNA from CCCVd-like RNA by RT-PCR and cloning of the PCR products 74
6.2.2b Sequence analysis 75
6.3 RESULTS 76
6.3.1 Selection of recombinant clones 76
6.3.2 Sizes of cloned inserts 76
6.3.3 Analysis of primary sequences 77
6.4 DISCUSSION 80

CHAPTER 7: DETECTION OF siRNA's IN CCCVd INFECTED COCONUT PALM 83

7.1 INTRODUCTION 83
7.2 MATERIAL AND METHODS 84
7.2.1 Material 84
7.2.2 Methods 86
7.2.2a Total nucleic acid extraction 86
7.2.2b PAGE 86
7.2.2c Hybridization assay 86
7.3 RESULTS 87
7.4 DISCUSSION 88

CHAPTER 8: STUDIES OF RELATIONSHIP BETWEEN OIL PALM ORANGE SPOTTING AND PHYTOPLASMAS 89

8.1 INTRODUCTION 89
8.2 MATERIAL AND METHODS 91
8.2.1 Materials 91
8.2.1a Leaf samples 91
8.2.2 Methods 91
8.2.2a DNA extraction... 91
8.2.2b PCR assay 91
8.2.2c Restriction Fragment Length Polymorphism (RFLP) 94
8.3 RESULTS 94
8.3.1 PCR analysis using primers P1/P7 and R16F2n/R16R2 94
8.3.2 RFLP analysis and comparison with AGYp.................................... 95
8.3.3 PCR analysis using Coconut lethal yellowing phytoplasma (LYp) specific primers.. 95
8.4 DISCUSSION 96

CHAPTER 9: GENERAL DISCUSSION 97

APPENDICES

APPENDIX A: Leaf Materials Used In This Study.. 106

APPENDIX B: Biochemicals And Miscellaneous Chemicals.......................... 111

APPENDIX C: Polyacrylamide And Agarose Gels, Bacterial Media, Buffers And Solvents.. 114

APPENDIX D: Components Of Kits Used In This Thesis 118

APPENDIX E: CCCVd Probe 120

APPENDIX F: Staining Solutions 121

APPENDIX G: Primers Used In This Thesis 122

APPENDIX H: Sequences Of Clones Of Oil Palm CCCVd Variants.............. 123

REFERENCES...127

SUMMARY

Molecular hybridization of Northern blots of single (1D) and two-dimensional polyacrylamide gels (2D-PAGE) with a ^{32}P-labelled full length $CCCVd_{246}$ cRNA probe demonstrated the presence of *Coconut cadang-cadang viroid* (CCCVd)-like RNAs in nucleic acid extracts of both symptomatic (orange spotted) and asymptomatic oil palms in commercial plantations in Malaysia. Compared with CCCVd in coconut these CCCVd-like RNAs seemed to be present at low concentration in the oil palm samples as shown by the weak hybridization signals observed in the oil palm samples even when large amounts of nucleic acid extract (leaf fresh weight equivalent of 20-100 g) were loaded onto the gel.

Ribonuclease protection assay (RPA) was found to be more sensitive in detecting low concentrations of the CCCVd-like RNAs in the oil palm samples than Northern blots as shown by the higher percentage of positive samples. RPA showed that 90 % of the symptomatic and 50 % of asymptomatic palms from Malaysia had RNAs which protected the ^{32}P-labelled full length CCCVd antisense probe and produced a similar RPA pattern to that of CCCVd. RPA results also indicated that there were mismatches in the sequence of the CCCVd-like RNAs in the oil palms compared to CCCVd from coconut.

RT-PCR amplification of CCCVd-like RNAs from an asymptomatic palm was only successful when nucleic acids were partially purified using 1D or 2D-PAGE. RNAs eluted from the circular region of 2D-gels of the asymptomatic palm were amplified to a low concentration using CCCVd-specific primers but re-amplification of these first round RT-PCR products was needed for detection of the amplicons by

ethidium bromide staining. No amplified product was obtained from a symptomatic palm.

Cloning and sequencing of the RT-PCR products from the asymptomatic oil palm produced 20 clones of five sizes comprising 297 nt (OP_{297}), 293 nt (OP_{293}), 270 nt (OP_{270}), 232 nt (OP_{232}) and 165 nt (OP_{165}). 71 % of the clones were OP_{297}. Comparison of OP_{297}, OP_{293}, and OP_{270} with genome database sequences showed high sequence similarity with $CCCVd_{296}$. OP_{297}, OP_{293}, OP_{270} had 98 %, 97 % and 90 % sequence similarity with $CCCVd_{296}$ respectively. OP_{232} and OP_{165} also had high sequence similarity with parts of $CCCVd_{246}$ with which they were aligned. Because an arbitrary level of 90 % sequence similarity is accepted as separating viroid species from variants, OP_{297}, OP_{293} and OP_{270} can be considered as variants of CCCVd. No variants of the 'fast' $CCCVd_{246}$ form were obtained.

The consensus OP_{297} sequence had single base substitutions or additions at 5 sites, OP_{293} had substitutions, additions or deletions at 8 sites, and OP_{270} had substitutions at 4 sites as well as deletion of a 26 nt repeat at the right terminus, producing a predicted branched secondary structure. Compared with $CCCVd_{296}$, all variants substituted ($C \rightarrow U$) at nt 31 in the pathogenicity domain and ($A \rightarrow C$) at nt 175 in the right hand terminal domain. The presence of sequences similar to OP_{232} and OP_{165} has not been reported for CCCVd.

Analysis of DNA extracted from both symptomatic and asymptomatic oil palms from Malaysia by nested PCR using universal primers sets to amplify the 16S rRNA operon showed the presence of phytoplasma-like DNAs in both sets of samples. They were also detected in DNA extracted from oil palm seedlings maintained at the Waite campus but not in the other palm species maintained in the glasshouse. RFLP analysis of phytoplasma-like DNAs gave a different pattern than that expected for Australian

grapevine yellows phytoplasma. The phytoplasma-like DNAs were also not related to lethal yellowing phytoplasma (LYp) as PCR analysis with LYp specific primers did not produce any amplicon. No association with OS was found and so they were not characterised further.

CCCVd-infected coconut leaf collected in the Philippines contained two short interfering RNAs (siRNA) approximately 20 nt and 25 nt in size. A high stringency wash of the Northern blots failed to remove the hybridisation signal suggesting that these siRNAs had sequences closely similar to CCCVd. The siRNAs were present in all stages of the cadang-cadang and also samples with the ‘brooming’ symptom. siRNAs are regarded as a marker for post-transcriptional gene silencing (PTGS) in plants infected by viroids but the results obtained were insufficient to determine whether PTGS regulates the accumulation of CCCVd.

This is the first report that a viroid closely related to CCCVd occurs in oil palm, and in a region outside the Philippines, the country where CCCVd is thought to be contained. The implications for quarantine matters are discussed.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Ganesan Vadamalai

August, 2005

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Prof. J.W. Randles, for his patience, encouragement, interest and critical discussion throughout the course of this study. I also wish to thank to Dr. M.A. Rezaian and Dr. D. Hanold for their support and critical discussions.

I thank all the members of the Plant Virology group at the Waite Institute and CSIRO for their support and helpful discussions, in particular Dr. Akbar Behjatnia for his advice on molecular cloning and Dr. Nuredin Habili for the phytoplasma work. Thanks are also due to Dr. Satish Dogra for his support, valuable advice and assistance in cloning and sequence analysis, Mrs. Kylie Cook for her friendship and help in preparing this thesis and all of my presentations.

I would like to acknowledge Consolidated Plantations Bhd Malaysia for providing the leaf material for this study, in particular Mr. Tang Men Kon, for allowing me to collect the oil palm samples, Mr. Chung Gait Fee and Mr. Shaji Thomas for their help in collection of the oil palm leaf samples.

I am grateful to the Goverment of Malaysia for a scholarship and Universiti Putra Malaysia, my employer, for granting me study leave. I thank, in particular, Prof. Yusof Hussein and Prof. Norani A. Samad, for encouraging me to undertake this study.

I extend my appreciation to Mr. K. Ganisan, Dr. Lau Wei Hong, Mr. S. Siva Kumar and Ms. Lim Chia Ying for their support throughout my study. A special thanks to Ms. Wang Yi-Chieh (Jay) for her encouragement and support.

Finally, I gratefully acknowledge the never ending love and support of my parents, sisters, brothers, brother-in-law, uncle, relatives and friends.

ABBREVIATIONS

Acryl	Acrylamide
amp	Ampicillin
Amp	Ampere
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APS	Ammonium persulphate
bp	Base pair
Bis	Bisacrylamide
CA	chloroform:iso-amyl alcohol mix
cDNA	Complementary deoxyribonucleic acid
cRNA	Complementary ribonucleic acid
CF11	Fibrous medium cellulose
cpm	Count per min
CTAB	N-Cetyl-N,N,N-trimethyl-ammonium bromide
dATP	2'-Deoxy-adenosine-5'-triphosphate
dCTP	2'-Deoxy-cytosine-5'-triphosphate
dGTP	2'-Deoxy-guanosine-5'-triphosphate
dTTP	2'-Deoxy-thymidine-5'-triphosphate
dNTP	Mixture of deoxynucleoside-triphosphates in equimolar amounts
DDW	Double distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ds-	Double stranded
EDTA	Ethylenediamine tetra acetic acid
EtBr	Ethidium bromide
FD	farred
g	Gram
g	Centrifugal force
HBO_3	Boric Acid
HCl	Hydrochloric acid
IPTG	*iso*-Propyl-β-D-thiogalactopyranoside
k	kilo
kb	kilo base
L	Litre
LiCl	Lithium chloride
M	Molar
μ-	Micro- (10^{-6})
m-	Milli- (10^{-3})
n-	nano- (10^{-9})
$Na_3C_6H_50_7.2H_2O$	trisodium citrate
NaAc	Sodium acetate
NaCl	Sodium chloride
Na_2EDTA	di-Sodium ethylenediamine tetra acetic acid
$NaH_2PO_4.2H_20$	Sodium dihydrogen orthophosphate dehydrate
$Na_2HPO_4.2H_2O$	di-Sodium hydrogen orthophosphate
nt	nucleotides

O/N	Overnight
p-	pico
PAGE	Polyacrylamide gel electrophoresis
PCA	Phenol:chloroform:iso-amyl alcohol mix
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
ppm	parts per million
PIPES	Piperazine-N,N'-bis(2-ethane-sufonic acid)
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer RNA
RPA	Ribonuclease protection assay
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDDW	Steriled double distilled water
SDS	Sodium dodecyl sulphate
ss-	Single stranded
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TEMED	N,N,N'-N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethtyl)aminomethane
u	Unit
UV	Ultra violet
V	Voltage
vol	Volume
v/v	volume per volume
w/v	weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactosidase