AN INVESTIGATION OF ORANGE SPOTTING DISORDER IN OIL PALM

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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→U) at nt 31 in the pathogenicity domain and (A→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

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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

CHAPTER 1

GENERAL INTRODUCTION

1.1 The oil palm industry

The oil palm, *Elaeis guineensis*, is native to Africa (Pippet, 1987). Its commercial value lies mainly in the oil, which can be obtained from the mesocarp of the fruit (palm oil) and the kernel of the nut (palm kernel oil). Palm oil is used mainly in food stuffs such as cooking oil and margarine, as well as in soap, detergent and cosmetics.

The importance of oil palm has increased considerably during the last 40 years. Currently, it comprises about 22 % of the total world market of edible oils. There are indications that the importance of this product will continue to increase and that it will remain competitive with other edible oils and fats (MPOPC, 2005).

1.2 The oil palm industry in Malaysia

The Malaysian oil palm industry has seen unprecedented growth in the last four decades to emerge as the leading agricultural industry in the country. From a mere 55,000 hectares in 1960, the area under the crop has grown more than fifty fold to 3.38 million hectares in 2000. Approximately 60,000 hectares of old oil palms are replanted annually. Presently about half of the agricultural land in Malaysia is used for oil palm production and the area is expanding (Gurmit *et al.*, 1999). Malaysia is today the world's largest producer and exporter of palm oil accounting for 51 % of world production and 62 % of world exports in 2003 (MPOPC, 2005).

1.3 Orange spotting (OS) of oil palm

1.3.1 History and prevalence

Orange spotting (OS) was recognised as a disorder of oil palm in the early 19th century in Africa (Coulter and Rosenquist, 1955; Smilde, 1962; Smilde, 1963; Forde and Leyritz, 1968; Robertson *et al*., 1968). The term 'orange spotting' was first used by Waterston (1953) to describe symptoms of necrotic spotting in oil palms. It has also been described as 'confluent orange spotting', which was a reference to the type of orange spotting in which the background of affected leaves remains green but with small or large fused orange spots of an irregular pattern appearing on the leaflets (Forde and Leyritz, 1968).

The disorder was first associated with nutrient deficiency (Coulter and Rosenquist, 1955). It was always associated with a lower leaf potassium level than that in healthy palms, which suggested that the disease might be due to a deficiency in potassium (Hale, 1947). Coulter and Rosenquist (1955) also found a significant relationship between low potassium content and OS in selfed progenies of oil palms. However, spots were found to appear in treated palms as well as in the controls when potassium deficiency conditions were relieved in seedlings known to be susceptible to OS (Bull, 1958 cited in Forde and Leyritz, 1968). Ochs (1965, cited in Forde and Leyritz, 1968) demonstrated that the disorder was not related to potassium deficiency and indicated that the symptom most widely observed in plots low in potassium could be described as a diffuse discolouration of the lamina, not related to OS.

Forde and Leyritz (1968) carried out a detailed study of soil and leaf nutrient levels in OS palms and concluded that OS is not a deficiency symptom of any of the major elements; they suggested that it may be of genetic origin. Even when the general

fertility, and in particular the potassium status of the soil supporting palms was high, symptoms of OS still occurred. Further investigation showed that OS is specific to some selfed progenies and crosses. Hence, the disease was termed 'genetic orange spotting' (GOS) (Turner, 1981). However, the pattern of transmission of GOS through seeds from parent to progeny was found not to follow any of the predicted Mendelian segregation ratios, indicating that the disorder might be caused by a biotic agent (Gascon and Meunier, 1979; Hanold, 1998).

The unexpected detection of low molecular weight nucleic acids in GOS oil palms in the Solomon Islands in 1986 led to a wide spread survey in countries of the Pacific region (Hanold and Randles, 1991). Nucleic acids extracted from tissues of African oil palm and other monocotyledonous species from several areas of the south-west Pacific region demonstrated the presence of low molecular weight nucleic acids with nucleotide sequence similarity to *Coconut cadang-cadang viroid* (CCCVd) (Hanold and Randles, 1991), which is associated with cadang-cadang disease in coconut. The symptoms were also similar to those in coconut, where spotting and decline of leaflets had been observed (Randles, 1975) and in oil palm inoculated with CCCVd (Imperial *et al*., 1985).

The oil palms which contained CCCVd-related molecules showed orange leaf spots resembling those described for oil palm naturally infected with CCCVd in the Philippines and also characteristic of GOS (Hanold and Randles, 1991). Hanold and Randles (1991) suggested that GOS was probably an infectious disorder caused by a viroid allied to CCCVd. Surveys were done to further test the correlation between GOS and CCCVd-related molecules in other geographical areas, and preliminary results indicate that similar sequences are also present in oil palm in South America and West Africa (Hanold and Randles, 1991). For this study, the term 'orange spotting' will be

used to describe this disorder instead of 'genetic orange spotting' as these recent studies suggest that this disorder is not of genetic origin.

1.3.2 Symptoms and effects on growth

Oil palms with OS show bright orange spotting on leaflets and appear from a distance to be bronze-coloured to necrotic. The youngest fronds are free of spots, and the size and number of spots increase with increasing age of fronds (Hanold and Randles, 1991). In young fronds, the irregular shaped and non-necrotic orange spots are about 2-3 mm long and occur between the veins of the leaflets (Forde and Leyritz, 1968). As the age of the frond increases, the spots coalesce into large circular patches, and distal necrosis of leaflets is seen in the oldest fronds (Forde and Leyritz, 1968; Hanold and Randles, 1991).

OS palms are generally smaller than their neighbours (Hanold and Randles, 1991). Observations in an eight year old commercial plantation in the Solomon Islands showed significant stunting in OS palms compared to healthy palms when the height of the trunk from ground level to the lowest bunch of fruits was measured (Randles, 1998).

OS palms bear smaller fruit bunches and the palms are significantly less productive. The average yield of OS-affected palms was 25-50% lower than adjacent healthy palms (Forde and Leyritz, 1968; Hanold and Randles, 1991; Randles, 1998).

1.3.3 Infectivity and spread

Spread of OS and the OS-associated viroid-like molecules occurs naturally through seeds (Forde and Leyritz, 1968; Gascon and Meunier, 1979; Hanold and

Randles, 1991; Randles, 1998). A survey conducted by Hanold and Randles (1991) in a 12 year old commercial plantation from 1987 to 1991 in the Solomon Islands showed an increase to 85 % in the incidence of OS at one site surveyed. This finding provides strong evidence that natural spread occurs (Randles, 1998).

OS is seed transmissible (Hanold and Randles, 1991). Twenty percent of plantlets raised from seeds of symptomatic oil palms collected from the Solomon Islands showed OS after 18 months (Hanold and Randles, 1991; Hanold, 1998). This was also in agreement with earlier reports from other areas (Forde and Leyritz, 1968; Gascon and Meunier, 1979).

A survey of a three year old planting of oil palm near Medan, North Sumatra showed an incidence of OS in the range of 1-2 %. Detection at such an early stage in the field would be expected if the OS incidence was due to seed transmission (Randles, 1998). Hanold and Randles (1991) suggested that seed transmission might provide primary foci for secondary spread of the viroid-like molecule to adjacent palms. They also suggested that secondarily infected palms could be pre-symptomatic and may act as a source of infected seed for carryover of the putative viroid to the next generation. Five out of 10 asymptomatic trees that were observed in a trial plot in the Solomon Islands contained the viroid-like molecules. Alternatively, the viroid-like molecules could be spread into nurseries or plantations from an outside source (Hanold and Randles, 1991).

1.3.4 Distribution and epidemiology

OS was first recognised in West Africa (Forde and Leyritz, 1968) and since then the disorder has been observed in commercial oil palm plantations in Indonesia, Malaysia, Thailand, Papua New Guinea, the Philippines, the Solomon Islands and

Central America (Randles, 1998). OS has also been observed in oil palms in South America (Hanold and Randles, 1991). OS has been observed in plantations at an incidence between 0.1% and 10% (Hanold and Randles, 1991).

The widespread distribution of OS could be explained by vertical transmission of the causal agents and their distribution with seed. The narrow genetic base of oil palms could have contributed to this distribution (Randles, 1998). For example, oil palms with OS in the Solomon Islands are descendants of parents in a seed garden in Papua New Guinea, which in turn derived its germplasm from Malaysia. The Malaysian material was derived from Sumatra Deli dura palms. Currently growing West African and South American commercial oil palm plantations are also derived from Sumatran material (Randles, 1998). The epidemiology of the disorder is still unclear, and can only be elucidated if the causal agent can be isolated and characterised (Randles, 1998).

1.4 Viroids

Viroids are the smallest plant pathogens known, with single stranded, circular, covalently closed, autonomously replicating RNA as genomes. Viroid genomes are non-encapsidated molecules of 246-401 nucleotides, with extensive internal base-pairing resulting in a rod-like secondary structure (Sanger *et al*., 1976; Keese and Symons, 1985; Flores *et al*., 2005b). The first viroid, *Potato spindle tuber viroid* (PSTVd) (Diener, 1971a,b; 1972), was identified when attempting to characterize the virus presumed to cause a potato disease. Since then, additional plant diseases which were presumed to have a virus etiology, have been shown to be caused by viroids, thus establishing the viroid concept on solid experimental grounds (Sanger, 1972; Semancik and Weathers, 1972; Symons, 1981; Gross *et al*., 1982, Haseloff *et al*., 1982; Candresse *et al*., 1987; Keese *et al*., 1988; Rezaian, 1990).

1.4.1 Biological properties

1.4.1a Host range

Viroids have been found to be the causal agents of economically important plant diseases in spite of their extreme molecular simplicity. To date, nearly 30 different viroid species have been detected, the majority causing economically important diseases in crops including potato, tomato, coconut, hop, grapevine, citrus, avocado, peach, apple, pear and also ornamentals such as chrysanthemum and coleus (Flores *et al*., 2005b). CCCVd and *Coconut tinangaja viroid* (CTiVd) are the only known viroids that infect monocotyledons, while the others infect dicotyledons. Some viroids, especially *Hop stunt viroid* (HSVd) have wide host ranges but others such as *Avocado sunblotch viroid* (ASBVd) are mainly restricted to their natural host (Flores *et al*., 2005b). A single nucleotide substitution converts PSTVd from non-infectious to infectious for *Nicotiana tabacum* (Wassenegger *et al*., 1996).

1.4.1b Symptomatology

Viroid-induced symptoms cover the whole range from the slowly developing lethal disease in coconut palms caused by CCCVd (Hanold and Randles, 1991) to the symptomless infection of hops with *Hop latent viroid* (HLVd) (Puchta *et al*., 1988). Some host plants appear to recover from viroid infection but in fact become symptomless while maintaining a stable viroid population; an example is the ASBVd in avocado trees (Semancik and Szychowski, 1994).

Viroids produce a range of symptoms similar to those observed in viral diseases. Symptoms include stunting, epinasty, mottling, leaf distortion, veinal discolouration and

localised chlorotic or necrotic spots and death of whole plants (Diener, 1987; Hull, 2002). Cytopathic effects of viroid infections have been reported. Viroid-infected tissues have been reported to show changes in plasmalemmasomes (Semancik and Vanderwourde, 1976; Diener, 1987; Hull, 2002) pronounced corrugations and irregular thickness in cell walls (Momma and Takahashi, 1983) and degenerative abnormalities in chloroplasts (da Graca and Martin, 1981).

Absence of symptoms is common in naturally infected wild plants, which can act as reservoirs. Symptom expression is generally favored by high light intensity and high temperature (30-33°C) (Diener, 1987; Flores *et al*., 2005b).

1.4.1c Ecology and epidemiology

Viroids are readily transmitted by mechanical means in most of their hosts. For example, the spread of PSTVd was mainly through contact with farm implements (Diener, 1987). Several viroids have been shown to be pollen and seed transmitted (Hull, 2002). Transmission of PSTVd has been reported to occur through seed and pollen (Diener, 1987; Singh *et al*., 1992) while vertical transmission has been shown in ABSVd (Diener, 1987). Low frequency transmission by aphids has been reported in PSTVd (Hull, 2002) while *Tomato planta macho viroid* (TPMVd) was shown to be transmitted by an aphid (Galindo *et al*., 1989). Other viroids that spread by still unknown means are CCCVd and CTiVd (Hanold and Randles, 1991). Viroid diseases have only been recognised since 1971 (Diener, 1971a,b; Hull, 2002).

1.4.2 Viroid classification and structure

The scheme of viroid classification (Diener, 2001) is presented in Table 1.1. Viroids are classified into two families, the *Pospiviroidae* and the *Avsunviroidae*. Most of the nearly 30 viroids species known (Flores *et al*., 2005a) as listed in Table 1.1 belong to the family *Pospiviroidae*, type species PSTVd (Diener, 1972; Gross *et al*., 1978), and adopt a rod-like or quasi rod-like secondary structure of minimal free energy *in vitro* (Sanger *et al*., 1976; Dingley *et al*., 2003) with five structural-functional domains; the central (C), pathogenicity (P), variable (V) and right (TR) and left (TL) terminal domains (Keese and Symons, 1985, 1987; Sano *et al*., 1992; Flores *et al*., 2005a) (Figure 1.1). The central conserved region (CCR), within the C domain, is formed by two stretches of conserved nucleotides, in which those of the upper strand are flanked by an inverted repeat. The pathogenic domain is associated with symptom expression and is characterized by an oligo ($A_{5\text{-}6}$) sequence present in all pospiviroids. The variable domain shows the highest sequence variability between closely related viroids.

Both terminal domains are involved in intermolecular RNA exchange between viroids to give rise to new, chimeric viroid species (Keese and Symons, 1985; Keese *et al*., 1988; Flores *et al*., 2005a) and recently it has been suggested that these domains could play a role in viroid movement in plants (Hammond, 1994; Maniataki *et al*., 2003). Depending on the nature of the CCR and on the presence or absence of a terminal conserved region (TCR) and a terminal conserved hairpin (TCH), members of this family are allocated to five genera (Flores *et al*., 2005a).

Table 1.1 Viroid species with their abbreviations, sizes, genus and family as classified by Flores *et al*. (2005a).

Family *Pospiviroidae*			
Species name	Abbreviation	Size (nt)	Genus
Potato spindle tuber viroid	PSTVd	356, 359-360	
Chrysanthemum stunt viroid	CSVd	354, 356	
Citrus exocortis viroid	CEVd	370-375, 463	
Columnea latent viroid	CLVd	370, 372	
Iresine 1 viroid	IrVd	370	*Pospiviroid*
Mexican papita viroid	MPVd	360	
Tomato apical stunt viroid	TASVd	360-363	
Tomato chlorotic dwarf viroid	TCDVd	360	
Tomato planta macho viroid	TPMVd	360	
Hop stunt viroid	HSVd	295-303	*Hostuviroid*
Coconut cadang-cadang viroid	CCCVd	246-247, 287-301	
Coconut tinangaja viroid	CTiVd	254	*Cocadviroid*
Hop latent viroid	HLVd	256	
Citrus viroid IV	CVd-IV	284	
Apple scar skin viroid	ASSVd	329-330	
Apple dimple fruit viroid	ADFVd	306-307	
Australian grapevine viroid	AGVd	369	
Citrus bent leaf viroid	CBLVd	318	*Apscaviroid*
Citrus viroid III	CVd-III	294, 297	
Grapevine yellow speckle viroid-1	GYSVd-1	366-368	
Grapevine yellow speckle viroid-2	GYSVd-2	363	
Pear blister canker viroid	PBCVd	315-316	
Coleus blumei viroid-1	CbVd-1	248, 250-251	
Coleus blumei viroid-2	CbVd-2	301-302	*Coleviroid*
Coleus blumei viroid-3	CbVd-3	361-362, 364	
Family *Avsunviroidae*			
Species name	Abbreviation	Size (nt)	Genus
Avocado sunblotch viroid	ASBVd	246-250	*Avsunviroid*
Peach latent mosaic viroid	PLMVd	337	*Pelamoviroid*
Chrysanthemum chlorotic mottle viroid	CChMVd	399	
***Eggplant latent viroid*	ELVd	333	*Elaviroid*

- ** ELVd pending ICTV approval

- underlined species name indicates the type species for the genus

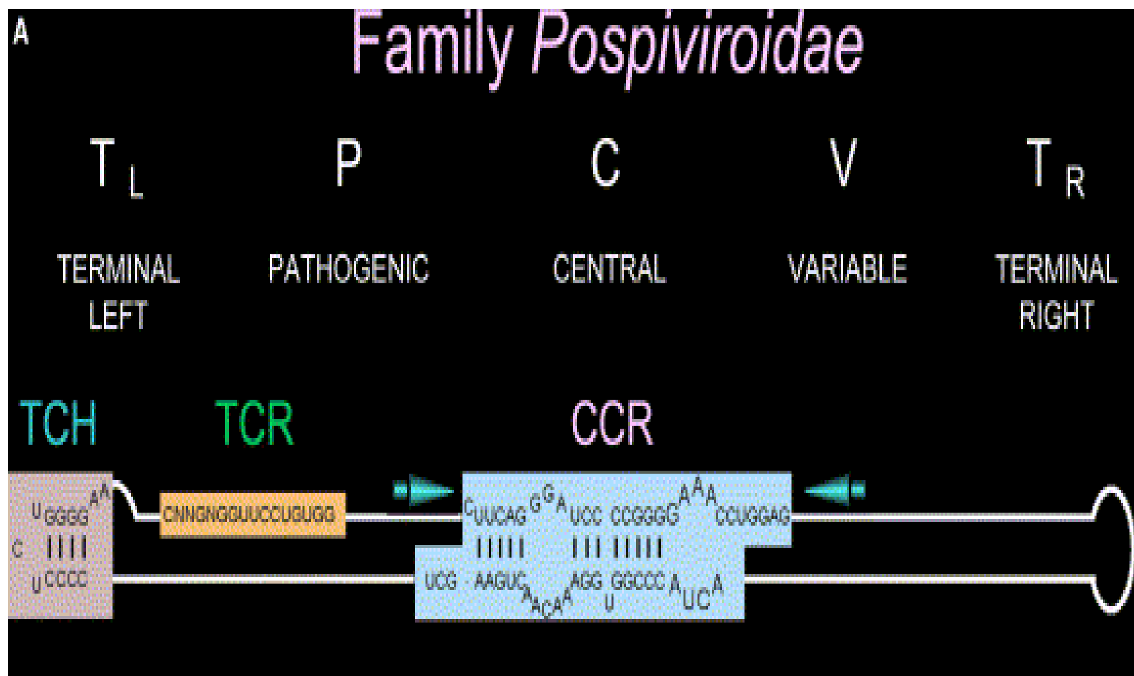


Figure 1.1 Model of viroid domains for the *Pospiviroidae* family members. The five domains, TL, P, C, V and TR were determined from sequence homologies between the viroids. The motifs CCR (of genus Pospiviroid), TCR (present in genera Pospi- and Apscaviroid, and in the two largest members of genus Coleviroid) and TCH (present in genera Hostu- and Cocadviroid) are shown. Arrows indicate flanking sequences that, together with the upper strand CCR, form imperfect inverted repeats that can form a stem loop. This figure was obtained from Flores *et al.* (2005a).

Viroids in the *Avsunviroidae* family, whose type species is ASBVd, do not have the conserved CCR, TCR and TCH motifs but they are able to self-cleave through hammerhead ribozymes (Flores *et al*., 2000). Other members of this family are *Peach latent mosaic viroid* (PMLVd) (Hernandez and Flores, 1992; Bussiere *et al*., 2000), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Navarro and Flores, 1997) and a new member, *Eggplant latent viroid* (ELVd) (Fadda *et al*., 2003). Apart from core nucleotides conserved in their hammerhead structures, no extensive sequence similarities exist between them, but PLMVd and CChMVd are grouped in one genus because of their branched secondary structure (Hernandez and Flores, 1992; Navarro and Flores, 1997; De la Pena *et al*., 1999) and their insolubility in 2M LiCl (Navarro and Flores, 1997). ASBVd is the only viroid with a high A + U content (62%) (Hutchins *et al*., 1986) and ELVd, whose properties fall between those of the members of the other two genera, has been proposed to constitute its own genus (Fadda *et al*., 2003).

Within each genus, the species are primarily defined on the basis of sequence data. An arbitrary level of 90% sequence similarity and distinct biological properties are accepted as separating species from variants. The presence and type of CCR serve to define the genus. Viroids, like viruses, propagate in their hosts as populations of closely related sequence variants (quasi-species), although one or more may predominate in the population (Flores *et al*., 2005b).

1.4.3 Replication

The actual replication site of viroids in the cell is still not known but reports show that PSTVd accumulates in the nucleolus (Harders *et al*., 1989), CEVd and CCCVd in the nucleus (Bonfiglioli *et al*., 1996), while ASBVd and PLMVd accumulate

in the chloroplast (Lima *et al*., 1994; Bonfiglioli *et al*., 1994; Bussiere *et al*., 1999). Although the accumulation of viroid in an organelle does not indicate the site of replication, the localization of the viroid complementary strands that are synthesized during replication support the conclusion that PSTVd replicates in the nucleus (Spiesmacher *et al*., 1983; Diener, 1991) while ASBVd (Bonfiglioli *et al*., 1994; Navarro *et al*., 1999) and PLMVd (Bussiere *et al*., 1999) replicate in the chloroplast.

Based on the detection of different replication intermediates in plants, replication of viroids has been proposed to occur by a rolling circle mechanism (Branch and Robertson, 1984: Symons *et al*., 1985; Diener, 2001). Two variations of the rolling circle mechanism have been suggested, asymmetric and symmetric. In the asymmetric pathway, the infectious circular (+sense) RNA is copied continuously by RNA-dependent RNA polymerase or DNA-dependent RNA polymerase (Schindler and Muhlbach, 1992) into a concatameric (-) strand. The concatameric (-) RNA strand then serves as a template for the production of concatameric (+)RNA strands that are cleaved to monomers and finally produce the circular progeny. PSTVd and presumably other members of the *Pospiviroidae* family replicate according to this pathway (Symons, 1991). In the symmetric pathway, the concatameric (-) RNA strands created as in the first mechanism are cleaved producing monomers that are circularized and then copied to yield concatameric (+) RNA molecules. Specific cleavage of these strands produce (+) monomers that are circularized to yield the progeny RNA. This symmetric pathway is proposed for the members of the *Avsunviroidae* family, ASBVd and PLMVd (Symons, 1991; Diener, 2001).

1.4.4 Movement

Systemic viroid infection consists of two major steps, replication in plant cells and movement throughout the whole plant. Cell-to-cell movement of PSTVd occurs through plasmodesmata and appears to be an active process mediated by specific sequence or structural motifs (Ding *et al*., 1997; Zhu *et al*., 2001). Viroids move rapidly through the plant, most probably through the phloem. Viroid RNA is relatively resistant to nucleases, so this probably facilitates the long-distance movement (Palukaitis, 1987; Zhu *et al*., 2001; Gafny *et al*., 1995; Hull, 2002). It is possible that the viroid molecules are translocated whilst bound to some host protein (Gomez and Pallas, 2001; Owens *et al*., 2001; Hull, 2002).

1.4.5 Pathogenesis

Despite their small size and lack of mRNA activity, viroids can induce disease symptoms similar to those induced by plant viruses by direct interaction between the viroid RNA and certain host constituents, which could be host proteins or nucleic acids. This interaction triggers a series of events, which are still poorly understood, and these eventually lead to the expression of symptoms (Flores *et al*., 2005b).

1.4.5a Structural implications in pathogenesis

With the 'domain' concept for the organization of the viroid genome, Keese and Symons (1985) introduced the 'P' or pathogenic domain, which is associated with symptom expression. Changes as little as one to three nucleotides were shown to affect

infectivity (Wassenegger *et al*., 1996) as well as symptom severity (Gross *et al*., 1981) by affecting the virulence modulating (VM) region (Schnolzer *et al*., 1985). However, characterization of several other viroid species has shown that pathogenicity encompasses additional domains (Sano *et al*., 1992; Rodriguez and Randles, 1993; Reanwarakorn and Semancik, 1998; Palacio-Bielsa *et al*., 2004).

Viroids generally exist as complex populations of variants (Gora *et al*., 1994; Gora-Sochacka *et al*., 1997) or quasi-species (Holland *et al*., 1992). Changes in a few nucleotides affect pathogenicity. For example, in ASBVd, reduction of the TR domain has been associated with symptom differences as shown by three variants of ASBVd, which have been found to predominate in localized bleaching, generalized variegation and asymptomatic tissues (Semancik and Szychowski, 1994).

1.4.5b Association with host components

Based on the sequence similarity between (+) and (-) viroid strands and various cellular RNAs, it was proposed that viroids may interfere with mRNA splicing or pre-rRNA processing (Diener, 1987). Induction of enzymatic activities such as the phosphorylation of certain host proteins (Hiddinga *et al*., 1988) or methylation of sequence-specific host DNA (Wassenegger *et al*., 1994) due to direct interaction of viroid RNA with host nucleic acid components was also suggested to result in pathogenic responses. However, further studies are needed to support these initial observations (Semancik, 2003).

1.4.5c Association of gene silencing with pathogenicity

The possibility that viroids could exert their pathogenicity through RNA-directed gene silencing was proposed by Sanger *et al*. (1996). This was supported by the recent identification of viroid-specific short interfering RNAs (siRNAs) in plants infected by members of the family *Pospiviroidae* (Itaya *et al*., 2001; Papaefthimiou *et al*., 2001; Markarian *et al*., 2004) and *Avsunviroidae* (Martinez de Alba *et al*., 2002; Markarian *et al*., 2004), together with the previous finding that replicating PSTVd induces *de novo* methylation of PSTVd sequences transgenically inserted into the plant genome (Wassenegger *et al*., 1994). Viroids were also shown to cause disease symptoms by directing RNA silencing against physiologically important host genes (Wang *et al.*, 2004). In addition to targeting RNA for degradation, siRNAs generated by viroids could also act like micro-RNAs (Ambros *et al.*, 2003) to form mismatched dsRNA complexes with cognate sequences of host gene mRNAs and thereby inhibit their translation and induce symptoms.

1.5 Coconut cadang-cadang viroid (CCCVd)

Several reviews provide a detailed description of the coconut cadang-cadang disease and its viroid agent, CCCVd (Randles, 1987; Randles *et al*., 1988; Hanold and Randles, 1991; Randles *et al*., 1992; Randles, 1998; Randles and Rodriguez, 2003). The following is a brief description of CCCVd.

1.5.1 Variations of CCCVd molecule

The basic form of CCCVd is a 246 nucleotide molecule, which is the smallest viroid known. In addition, CCCVd has a number of molecular forms, which vary at the right-hand end of the molecule. This variation is due to the reiteration of either 41, 50 or 55 nucleotides, producing larger forms of the viroid of between 287 and 301 nucleotides. A minor variation also occurs at position 197, where an additional cytosine may be inserted to produce a basic form comprising 247 nucleotides, and the corresponding large forms. Detected concurrently with the monomers are their respective dimers which are covalently linked forms of the monomers, incorporating the same sequence variations (Haseloff *et al*., 1982; Keese *et al*., 1988).

Variation in CCCVd is observed with disease progression. The small 246/247 variants (with their dimers) are observed in the early stages of infection and as the disease symptoms develop, the large 287/296/297/301 variants (also with their dimers) arise and eventually become the dominant population as the disease progresses (Imperial *et al*., 1981; Mohamed *et al*., 1982). A systematic progression has been observed where if infection starts with variant 246, then 247 appears next followed by 296, then 297 but if variant 247 appears first, then only 297 variant could be observed as the disease progressed (Imperial and Rodriguez, 1983). All variants are infectious and in the field the frequency of occurrence of the 246 variant was higher than that of variant 247 (Randles *et al*., 1992). The functional implications of the development of the larger CCCVd variants are not known but were suggested to be advantageous during replication by providing increased competition for binding of some host component important for replication (Keese *et al*., 1988).

There were reports of additional variation in the CCCVd molecules that caused a severe form of cadang-cadang disease called brooming (Rodriguez and Randles 1993). Sequencing of a number of these variants has shown that the mutations associated with brooming occurred at 2 or 3 sites in the molecule, and may be deletions, additions or substitutions of nucleotides (Rodriguez and Randles 1993). One of the important consequences of this report is that it shows that CCCVd is mutable, and that severe mutants can exist in palms growing in the field and possibly may lead to a range of disease symptoms in the affected areas.

1.5.2 Diagnostic methods

Diagnosis of CCCVd through symptoms is unreliable, thus molecular diagnostic methods have been developed. Polyacrylamide gel electrophoresis (PAGE) has been widely used for detection of CCCVd. Several modifications, from the nucleic acid extraction method to gel staining have been made to simplify the procedure without compromising its sensitivity (Imperial *et al.*, 1981; Imperial and Rodriguez, 1983). Two-dimensional (2-D PAGE) (Schumacher *et al.*, 1983), bi-directional gel electrophoresis and return gel electrophoresis (R-PAGE) (Schumacher *et al.*, 1986) and sequential PAGE (Semancik *et al.*, 1987) are useful for identifying CCCVd in mixtures when linear RNAs of similar mobility are present.

CCCVd is detected by PAGE based on size but hybridization assays using labelled complementary RNA or DNA probes have been developed for more sensitive and specific assay (Randles and Palukaitis, 1979; Imperial *et al.*, 1985; Hanold and Randles, 1991). RT-PCR using CCCVd specific primers for diagnosis have also been reported (Hodgson and Randles, 1997; Hodgson *et al.*, 1998).

1.6 Scope of this thesis

This thesis reports an investigation of the oil palm orange spotting disorder by nucleic acid studies. Oil palm is a crop of major importance in S.E. Asia, especially in Malaysia, and therefore the detection and control of oil palm diseases is of great importance. OS of oil palm is recognised as a disorder and its transmission through seeds poses a threat to the oil palm industry, as there is no screening procedure for this disorder in seeds. The causal agent has not been isolated or identified, and this will be the focus of this study. Since OS in oil palm is associated with small circular ssRNA related to CCCVd (Hanold and Randles, 1991), in the first instance the search for a causal pathogen in this study concentrated on the detection of viroid-like RNA molecules.

It is reported that a CCCVd-like RNA could be detected in the oil palm samples but only when hybridization assays were used, indicating that it had a very low concentration. Because of the low concentration of the molecules in the sample, ribonuclease protection assays and RT-PCR were developed for detection of this CCCVd-like RNA. Cloning and sequencing showed that the molecule present in the oil palm sample is a variant of CCCVd.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Leaf Samples

Appendix A lists sources of oil palm and coconut leaf samples used in this work. Symptomatic and asymptomatic oil palm trees were sampled on 6/2/02 and 9/6/04 from two different oil palm plantations in Malaysia; CEP Renggam plantation in Johor and Selangor River plantation in Selangor. Leaflets from fronds no. 3, 10 and 20 were cut and cleaned. Approximately 500 g of tissue from each frond was sealed in resealable polythene bags. The samples were transported to Adelaide within 24 hours of sampling, and stored at –70 ^{o}C on arrival. Brown, wilted or bruised tissues were discarded as this indicated that cell breakdown had occurred with likely RNA degradation.

Coconut cadang-cadang disease affected coconut leaf samples from the Philippines were provided by Dr Dagmar Hanold of Plant and Pest Science, School of Agriculture and Wine, University of Adelaide. Healthy oil palm and coconut seedlings were obtained from the live plant collection maintained in the glasshouse at Waite Campus.

2.1.2 Biochemicals and miscellaneous chemicals

The main biochemical and miscellaneous chemicals used in this study are listed in Appendix B.

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

Composition and preparation of polyacrylamide and agarose gels, preparation of bacterial media and their storage, and solvents and buffers are described in Appendix C.

2.1.4 CCCVd

Partially purified CCCVd used as a standard marker was prepared according to Section 2.2.1a from infected coconut leaf obtained from mid-stage diseased palms.

2.1.5 ^{32}P-labelled cRNA probe

The cRNA probe used in this study was supplied by Prof. J.W. Randles of Plant and Pest Science, School of Agriculture. Using the transcription kit from PROMEGA (Appendix D), transcription was done from a pGEM-T easy plasmid vector containing a monomeric insert of the 246 nucleotide form of CCCVd (Appendix E).

A 20 µl reaction was prepared for transcription of the probe using a Riboprobe® *in vitro* transcription system from Promega (Appendix D) according to the recommendation of the manufacturer. Linearised plasmid (approximately 1 µg) was mixed with a transcription reaction containing 1 X transcription buffer, 10 mM DTT, 20 U recombinant RNasin®Ribonuclease Inhibitor, 0.5 mM each rATP, rCTP, rGTP and 20 U of T7 RNA polymerase. α-^{32}P-UTP (3 µCi) was added to the mix and incubated at 37 ^{o}C for 1 hr. The transcription was stopped by adding 1 µl of RQ1 DNase (RNAse-free) and incubated at 37 ^{o}C for 20-30 min. The labeled probe was then purified using G-50 Sephadex spin columns (Roche).

2.2 METHODS

2.2.1 Nucleic acid extraction

2.2.1a PEG extraction

The PEG extraction method was carried out according to the method of Hanold and Randles (1991). 10 g – 20 g of leaf was cut into small pieces and blended in 120 ml of 100 mM Na_2SO_3. The slurry was then strained through cotton muslin and shaken vigorously for 30 min at 4 ^oC with 20 g/L PVPP. Fifty ml of chloroform was added and mixed vigorously for 5 min. The mixture was centrifuged at 10,000 g for 10 min. The aqueous supernatant was collected and PEG 6000 was added at 80 g/L. After stirring to dissolve the PEG, and 2 hr incubation at 4 ^oC, the resulting precipitate was collected by centrifugation at 10,000 *g* for 10 min. Nucleic acids were extracted from the precipitate by dissolving it in 2 ml of 10 g/L SDS, then adding 2 ml of aqueous phenol (900 g/L) containing 1 g/L 8-hydroxy quinoline and shaking vigorously for 1 hr. The aqueous supernatant was collected by centrifugation at 10,000 *g* for 10 min and re-extracted with 1 ml of phenol and 1 ml of chloroform for 5 min. NaCl was added to 0.1 M, followed by cetyl trimethylammonium bromide (CTAB) to 3.3 g/L and incubation for 30 min at 0 ^oC. The precipitate was collected by centrifugation for 30 min at 10,000 *g*. The pellet was washed 2 times with 0.1 M Na-acetate in 75 % ethanol and once with 100 % ethanol. The pellet was air dried and re-suspended with 500 µl of SDDW. An equal volume of 4 M LiCl was added. The mixture was incubated at 4 ^oC for 15-18 hr and centrifuged at 12,000 g for 15 min (Imperial *et al.*, 1981). The LiCl-soluble components were recovered and precipitated with 2.5 vol of ethanol for 2-3 hr and centrifuged at 12,000 g for 15 min to collect the pellet. The pellet was dissolved in 30 µl SDDW and stored at –20 ^oC.

2.2.1b CF11 extraction

Extraction was done according to R. Flores (J.W. Randles, pers. comm.). 10 g of leaf sample was ground to a fine powder in a mortar and pestle with approximately 150 ml of liquid nitrogen. The sample was transferred to a beaker and mixed with extraction buffer (10 ml 0.2 M Tris-Cl pH 8.9, 2.5 ml 0.1 M Na_2EDTA pH 7, 2.5 ml 5 % SDS, 1.25 ml monothioglycerol) and 40 ml water-saturated phenol (pH 7) for 20 min. Buffer and phenol was kept on ice before use. The mixture was centrifuged at 10,000 g for 10 min. The supernatant was re-extracted with 0.5 vol of neutralized phenol and shaken briefly before centrifugation at 10,000 g for 10 min. The supernatant was recovered and made up to 20 ml with SDDW, then mixed with 3.7 ml 10X STE, 13.4 ml 100 % ethanol and 1.25 g CF-11 cellulose for RNA binding. The mixture was vortexed and shaken for more than 2 hr or overnight. The pellet was collected by centrifugation at 3000 g for 3 min and washed 3X with 35 % ethanol in STE using 30 ml each time. The pellet was shaken or vortexed briefly (approx 1min) before each wash and centrifuged at 3000 g for 3 min for each wash. After each wash the aqueous phase was discarded, leaving only the RNA bound cellulose. The cellulose was then washed 3X with 3.3 ml of 10X STE. For each wash, the mixture was shaken or vortexed and centrifuged at 3000 g for 3 min for each wash. The supernatant was pooled on ice after each wash. The supernatant was then clarified by centrifugation for 10 min at 10,000 g and decanted into a new tube and mixed with 30 ml ethanol and left to precipitate at –20 ^oC for more than 2 hr or overnight. The pellet was collected by centrifugation at 12,000 g for 20 min and air-dried for approx 1-2 hr. The final pellet was resuspended in 500 µl SDDW and stored at –20 ^oC.

2.2.1c Total nucleic acid extraction

Method modified from Hodgson (1998). 1 g of leaf sample was mixed with 2 ml extraction buffer (0.1 M Tris-HCl pH 7.5, 1 % SDS, 0.25 % (v/v) 2-mercaptoethanol) and 2 ml 90 % phenol and ground with a mortar and pestle until fine. Acid-washed sand was added to the mix to aid grinding. Samples were incubated for 1 to 2 hr at room temperature and then transferred to a 10 ml tube and centrifuged at 10,000 g for 30 min. The supernatant was collected in a 2 ml eppendorf tube and re-extracted with 0.5 vol of 90 % phenol then centrifuged at 10,000 g for 10 min. The supernatant was transferred to a new 2 ml eppendorf tube and NaAc was added to 0.3 M before precipitation with 3 vol of ethanol at –20 ^{o}C for 1-2 hr. The pellet was collected by centrifugation at 10,000 g for 30 min. The pellet was washed with 1 ml of 75 % ethanol and air-dried for 30-60 min at room temperature. The pellet was dissolved in SDDW and stored at –20 ^{o}C.

2.2.1d NETME extraction

Method modified from Hodgson (1998). 1 g of leaf sample was mixed with 2 ml NETME (2.0) extraction buffer (2 M NaCl, 100 mM sodium acetate, 50 mM Tris-HCl pH 7.5, 20 mM EDTA pH 8.0, 20 % ethanol, 0.25 % (v/v) 2-mercaptoethanol) and ground with a mortar and pestle until fine. SDS was added to 1 % and the mixture was incubated for 30 min at room temperature. 900 µl of the slurry was transferred to a 1.5 ml centrifuge tube, mixed with 600 µl PCA mix and centrifuged at 10,000 g for 15 min at room temperature. Approximately 800 µl of the supernatant was transferred to a new tube, mixed with 600 µl CA and centrifuged at 10,000 g for 15 min. The supernatant was transferred to a new tube and mixed gently with 0.9 vol isopropanol. The solution

was incubated at –20 ^{o}C for 3 hr and centrifuged at 10,000 g for 10 min. The supernatant was discarded and the pellet was washed with 700 µl 70 % ethanol by gentle mixing (Rotomixer, 20 rpm) for about 2 hr. The pellet was collected by centrifugation at 10,000 g for 10 min and air dried. The pellet was dissolved in 150 ml of SDDW by overnight incubation at 4 ^{o}C and stored at –20 ^{o}C.

2.2.1e Guanidine extraction

Dr. Nuredin Habili from Waite Diagnostics, School of Agriculture and Wine, University of Adelaide, kindly provided the following extraction method.

0.02 g of leaf tissue was transferred to a long plastic bag (100 mm x 150 mm) containing 0.02 g sodium metabisulphite. 2 ml of lysis buffer (0.2 M NaAc pH 5.0, 25 mM EDTA, 4 M guanidine hydrochloride, 2.5 % PVP-40) was added to the bag and the tissue was crushed evenly with a hammer on a wooden board. 800-1000 µl of the leaf extract was transferred to Eppendorf tubes containing 70 µl 20 % Sarkosyl and incubated at 70 ^{o}C with constant agitation for 10 min. 300 µl of CA was added to the mixture and vortexed for 10 sec before centrifugation at 10,000 g for 10 min. About 360 µl of the resulting clear phase was transferred to a new tube containing silica milk (provided by Waite Diagnostics), vortexed, mixed with 320 µl absolute ethanol, vortexed again and centrifuged at 6000 g for 30 sec to allow binding of RNA to the silica. The supernatant was poured off and the pellet was washed with 600 µl of wash 1 (lysis buffer without PVP) by pipetting up and down to re-suspend the pellet and left for 5 min (or overnight) at room temperature. The solution was then centrifuged at 6000 g for 30 sec. The supernatant was discarded and the pellet was washed by centrifugation at 6000 g for 30 sec with 500 µl of wash 2 (80 % ethanol in TES, 10 mM Tris pH 7.5,

1 mM EDTA, 100 mM NaCl). The supernatant was discarded and the second wash was repeated. The resulting pellet was centrifuged again at 6000 g for 1 min to remove the remaining wash buffer. The pellet was then dried at 65 °C in a heating block for 2 min with the lids of the tube open to remove all traces of ethanol in wash buffer. The pellet was then mixed with 70 µl TE buffer (Appendix C) and incubated at 65 °C for 1 min with lids closed and centrifuged at 10,000 g for 10 min. The aqueous layer was collected in a new tube and stored at –20 °C.

2.2.1f CTAB extraction

The extraction method was kindly provided Dr. Nuredin Habili from Waite Diagnostics, School of Agriculture and Wine, University of Adelaide.

0.02 g of leaf tissue was placed in a thick plastic bag containing 0.02 g sodium metabisulphite (or alternatively added to the extraction buffer). 2 ml of CTAB extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 50 mM EDTA pH 8.0, 2.5 % CTAB, 2.5 % PVP-40) was added to the bag and the tissue crushed as above. 800 µl of the leaf extract was transferred to an Eppendorf tube containing 60 µl 20 % Sarkosyl and incubated at 65 °C with gentle shaking for 30 min. 600 µl of CA was added, mixed and centrifuged at 10,000 g for 10 min. 650 µl of the aqueous phase was transferred to a new tube and mixed with 400 µl CA, and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant of 500 µl was transferred to a new tube, mixed with 400 µl isopropanol, and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 600 µl of cold 76 % ethanol containing 100 mM ammonium acetate. After a short centrifugation the pellet was air dried to remove all traces of ethanol. The pellet was suspended in 200 µl TE pH 8.0 (Appendix C) and

then mixed with 500 µl of cold 100 % ethanol and 3 µl 3M NaAc (pH 4.6-5.2) and stored on ice for 10 min (or overnight at –20 ^{o}C). The precipitate was centrifuged at 12,000 g at 4 ^{o}C for 15 min. The supernatant was discarded and the pellet was washed with 1 ml cold 70 % ethanol at 12,000 g for 10 min. The pellet was then air-dried at room temperature for 15-30 min. The final pellet was suspended in 50 µl TE and stored at –20 ^{o}C.

2.2.2 Gel electrophoresis

2.2.2a Polyacrylamide gel

Analysis of nucleic acids was done on polyacrylamide gels buffered in TAE or TBE (Appendix C). Electrophoresis was in 140x120x1.5 mm gels (BioRad) under non-denaturing conditions at 75 mA for 2 hr or in 150x200x1.5 mm gels (BioRad) under denaturing conditions at 200 V for 4 hr. The gel was removed and stained with silver (Section 2.2.2.d).

2.2.2b Two-dimensional (2-D) PAGE

Separation of viroids from contaminating host nucleic acids by two dimensional PAGE was done as described by Schumacher *et al*., (1983). In the first dimension, nucleic acid extracts were electrophoresed in a non-denaturing 5 % slab gel in 1X TAE buffer at 75 mA for 2 hr. Vertical lanes of the samples were cut and placed across the top of the second dimension denaturing 5 % gel containing 8M urea buffered in 0.25X TBE. The gel was run at 25 mA/350 V for 90 min. The gel was stained with silver.

2.2.2c Agarose gel

Analytical agarose gels contained either 1.5 % or 2 % agarose buffered with either TAE or TBE (Appendix C). Agarose (final concentration 1.5 % or 2 % (w/v)) was dissolved by heating in a microwave oven in 100 ml buffer, cooled to 60 ^{o}C and EtBr was added to 0.5 µg/ml. Bands were visualized by UV fluorescence at 254 nm.

2.2.2d Staining of polyacrylamide gels

Gels were removed from the electrophoresis apparatus and stained in either EtBr or $AgNO_3$.

Gels were placed in contained with 200 ml of DDW. EtBr was added at 0.5 µg/ml and incubated for 20-30 min. The gel was visualized as described in Section 2.2.2c (Sambrook and Russell, 2001).

Polyacrylamide gels were fixed in 10 % (v/v) ethanol and 5 % (v/v) acetic acid for 10 min and then in 10 % (v/v) ethanol and 0.5 % (v/v) acetic acid for 10 min. Silver staining solution (Appendix F) was added. After incubation with gentle shaking for 1 hr, the silver solution was removed and the gel was washed for 1 min in DDW, twice. 200 ml of developing solution (Appendix F) was added. The colour was developed until bands were visible. The developing solution was removed and the gel was rinsed once with DDW. The development was stopped by adding 5 % (v/v) acetic acid. The gel was photographed and stored in a sealed plastic bag with 0.1 % (v/v) acetic acid (Randles *et al.*, 1986) or dried using the cellophane gel drying apparatus (BioRad).

2.2.3 Preparation of CCCVd and CCCVd-like RNA

2.2.3a Two-dimensional (2-D) PAGE

2D-PAGE was done as described in Section 2.2.2b. The location of zones expected to contain circular and linear CCCVd and CCCVd-like RNA was determined after staining the gel with (0.5 µg/ml). These zones were excised from the gel with a sharp blade.

2.2.3b Gel elution

Prior to adding the elution buffer, the gel piece was crushed in an eppendorf tube using a blue pipette tip. CCCVd and CCCVd-like RNA molecules were eluted by soaking the crushed gel pieces in 2 vol of elution buffer (0.5 M ammonium acetate, 0.1 % SDS, 1 mM EDTA pH 8.0) for 12-16 hr at 37 ^{o}C with constant shaking (Sambrook and Russell, 2001). The eluted sample was centrifuged at 12,000 g for 1 min at 4 ^{o}C. The supernatant was transferred to a fresh tube, carefully avoiding the transfer of fragments of polayacrylamide. 0.5 vol of elution buffer was added to the pellet, and it was vortexed and centrifuged again at 12,000 g for 1 min at 4 ^{o}C. The supernatants were combined and RNA was precipitated with 2 vol of ethanol at –20 ^{o}C for 6 hr. Following centrifugation at 12,000 g for 20 min, the pellet was collected and air-dried. The pellet was taken up in 10 µl of 1X TE pH 8.0 or SDDW.

2.2.4 Molecular hybridization

Blotting and hybridization were done according to Sambrook and Russell (2001) and Hanold and Randles (1991) with some modifications.

2.2.4a Capillary blotting

Gels were equilibrated in 200 ml of either 0.01 N NaOH / 3 M NaCl or 0.02 N NaOH blotting solutions (Appendix C). A Wettex® was laid on a horizontal surface and pre-wetted with the blotting solution. Three sheets of Whatman 3MM filter paper were over-laid and bubbles were removed with a glass rod. The gel was laid over the filter paper. Nylon membrane (Zeta Probe®, BioRad) was cut to the size of the gel and laid over it. A plastic transparency sheet cut to the gel size was laid on top of the nylon membrane to mask the gel. Three sheets of pre-wet Whatman 3MM filter paper were laid over the membrane. Bubbles were removed as above at each step. A 3-5 cm thick layer of paper tissue was placed on top and a weight of 500-1000 g was applied. The nucleic acids were transferred for 12-40 hr and the nucleic acids blotted to the nylon membrane were bound with a UV Cross Linker (BioRad) at 125 kJoule.

2.2.4b Electroblotting

Nucleic acid samples were fractionated by PAGE (Section 2.2.2a and 2.2.2b). After completion of the gel run, the gel was equilibrated in blotting buffer (1X $NaH_2PO_4.2H_20$ pH 6.5) for 5 min and assembled in contact with a pre-wetted nylon membrane (Zeta Probe®, BioRad) in an electroblot apparatus (BioRad) containing

blotting buffer. Current was passed through at 1 Amp, 20 V for 2 hr to transfer the nucleic acids to the nylon membrane (Hanold and Randles, 1991; J. Randles, pers.comm.). The nylon membrane was UV cross linked as above.

2.2.4c Hybridization assay

The capillary and electroblotted membranes (Zeta Probe®, BioRad) were pre-hybridized for 90 min at 45 °C in pre-hybridization buffer (Appendix C). The CCCVd probe (Section 2.1.5) was mixed with 200 µl pre-hybridization buffer and heated at 100 °C for 2 min and added to the hybridization solution (same as the prehybridization solution but containing ^{32}P labelled probe at about 10^6 cpm/ml). They membranes were incubated at 45 °C for 15-24 hr. They were washed twice in 0.5X SSC, 0.1 % SDS for 5 min at 25 °C. For low stringency washes, the membranes were incubated with agitation in 1X SSC, 0.1 % SDS for 60 min at 55 °C. High stringency washes were done in 0.1X SSC, 0.1 % SDS for 60 min at 60 °C.

2.2.4d Autoradiography

Radioactive nylon membranes were covered in plastic wrap and exposed to X-ray film (Kodak AR) for at least 72 hr at –70 °C using an intensifying screen. The films were developed using an automatic X-ray film developer (AGFA CP1000, Germany).

2.2.5 PCR assay

2.2.5a Amplification of viroids by RT-PCR

The first strand cDNA was synthesized according to Rodriguez (1993) and Hodgson *et al*. (1998). Reverse transcription was done using a reverse transcription kit from Promega (Appendix D). First strand cDNA was synthesized with AMV reverse transcriptase and a CCCVd specific antisense primer (Table 5.1, Appendix G) in a 20 µl reaction volume. According to the method described by Rodriguez (1993), 2 µl of sample nucleic acid (Section 2.2.1a,e and 2.2.3b) and 0.5 µM primer was added with 7.5 µl nuclease free water and incubated at 80 ^{o}C for 12 min and then chilled on ice for 5 min. The components required for reverse transcription (5 mM $MgCl_2$, 1X reverse transcription buffer, 1 mM each of dATP, dCTP, dGTP, dTTP, 20 U of RNAsin and 24 U of AMV reverse transcriptase) were then added to a final volume of 20 µl. The reverse transcription was done by incubating the mixture at room temperature for 5 min, then 55 ^{o}C for 30 min.

The components for the reverse transcription method of Hodgson *et al*., (1998) were as above except that the incubation of nucleic acid with the primer mix was done at 94 ^{o}C for 4 min and the reverse transcription was done at 50 ^{o}C for 20 min.

PCR was done using the PCR kit of Promega (Appendix D). The PCR mix contained 200 µM dNTPs, 1.5 mM $MgCl_{2,}$ 25 unit/ml *Taq* DNA polymerase, 0.5 µM of both forward and reverse CCCVd specific primers (Appendix G), and nuclease free water, in a 25 µl reaction volume. 5 µl of the first strand cDNA product was added to a final volume of 30 µl. Initial denaturation of the above mixture was done at 96 ^{o}C for 3 min as described by Rodriguez (1993). Amplification was done using a Perkin-Elmer

Analytical Gene Amp PCR System 2400 under the following conditions: 93 ^{o}C for 45 sec, 55 ^{o}C for 45 sec and 72 ^{o}C for 3 min, 40 cycles; 72 ^{o}C for 15 min.

The components for PCR by Hodgson *et al*., (1998) were as above. Initial denaturation was done at 94 ^{o}C for 2 min and amplification was done under the following conditions: 70 ^{o}C for 1 min, 94 ^{o}C for 30 sec for 5 cycles; 70 ^{o}C for 30 sec, 94 ^{o}C for 30 sec for 40 cycles; 72 ^{o}C for 5 min and 25 ^{o}C for 1 min.

The PCR products were analysed on a 1.5-2 % agarose gel (Appendix C) and 5 % non-denaturing PAGE (Appendix C).

2.2.5b PCR for detection of phytoplasma

Four sets of universal primers were used to amplify the 16S rRNA operon; P1 (Deng and Hiruki, 1991), P7 (Smart *et al*., 1996), R16F2n (Lee *et al*., 1993), and R16R2 (Lee *et al*., 1993) (Appendix G). Nested PCR was done according to conditions provided by Dr. Nuredin Habili of Waite Diagnostics.

PCR was done using a PCR kit by Promega (Appendix D). The PCR mix contained 200 µM dNTPs, 1.5 mM $MgCl_2$, 25 unit/ml *Taq* DNA polymerase, 0.5 µM P1 (forward) and 0.5 µM P7 (reverse) primers, nuclease free water, and 1 µl of CTAB extracted nucleic acid (Section 2.2.1d) in a final volume of 10 µl. Amplification conditions were: 94 ^{o}C for 1 min; 94 ^{o}C for 30 sec, 55 ^{o}C for 45 sec, 72 ^{o}C for 1 min for 34 cycles; final elongation at 72 ^{o}C for 5 min.

For nested PCR, 1 µl of the P1/P7 product was added to a PCR mix as above with 0.5 µM of Rf12n (forward) and 0.5 µM R12n (reverse) primers to a final volume of 10 µl and amplified using the above conditions. The PCR products were analysed on a 1.5 % agarose gel (Appendix C).

2.2.6 Molecular cloning of PCR products

2.2.6a Purification of PCR products

PCR mixtures were analysed by 2 % agarose gel electrophoresis in 1X TBE buffer, stained with EtBr and visualized under UV. Amplicons were excised from the gel using a sharp blade and extracted from gel fragments using MinElute Gel Extraction Kit (QIAGEN Australia Pty Ltd) according to the recommendation of the manufacturer.

Excised gel fragments were weighed and placed in 1.5 ml eppendorf tubes and mixed with 3 vol (w/v) of buffer QG. They were incubated at 50 ^{o}C for 10 min. Samples were vortexed every two minutes to dissolve the gel fragment. One gel vol of isopropanol was added to the sample and mixed by inverting the tube. The DNA was bound to a MinElute column by applying the sample to the column placed in a 2 ml collection tube and centrifuged at 10,000 g for 1 min. The flow-through was discarded and 500 µl of buffer QG was added to the column and centrifuged at 10,000 g for 1 min. The flow-through was discarded and the DNA was washed by adding wash buffer PE to the column and centrifugation at 10,000 g for 1 min. The flow-through was discarded and an additional centrifugation at 10,000 g for 1 min was done to remove any residual ethanol from buffer PE. The MinElute column was placed in a clean 1.5 ml tube and 10 µl SDDW was added to the centre of the membrane of the column and centrifuged at 10,000 g for 1 min to elute the DNA. The eluted DNA was stored at –20 ^{o}C.

2.2.6b Ligation of ds-cDNA into a plasmid vector

Ligation of DNA to the plasmid vector was done using a QIAGEN PCR Cloning kit (QIAGEN Australia Pty Ltd) according to the manufacturer's recommendation. The amplified DNA (Section 2.2.5a) was ligated into the pDrive cloning vector. 50-100 ng of DNA was ligated into 50 ng cloning vector with a Ligation master mix (QIAGEN Australia Pty Ltd) containing all reagents and cofactors required for ligation in a 10 µl reaction volume. The mixture was incubated at 16 ^{o}C for 24 hr. An aliquot of this ligation mix was used for transformation.

2.2.6c Transformation by electroporation

Transformation was done according to the method of Sambrook and Russell (2001) with some modifications. The competent cells used for this experiment were XL1-Blue (from Stratagene) provided by Dr. Satish Dogra. The competent cells were thawed on ice and mixed gently by swirling. 40 µl of the competent cells were added to 1 µl of the ligation reaction mix in a chilled 1.5 ml tube. The mixture was gently swirled and placed on ice. The mixture was then pipetted into a 1 mm cuvette and electroporated using the Bio Rad Gene Pulser with voltage set at 1.8 kV, capacitance at 25 µFD and resistance at 200 ohm. The mixture was pulsed for 4.1 to 5.0 milliseconds. Immediately, 960 µl of SOC medium (Appendix C) was added to the mixture in the cuvette and mixed thoroughly. The mixture was then transferred to a 1.5 ml tube and incubated at 37 ^{o}C for 60-90 min.

2.2.6d Selection for recombinants

As described by Sambrook and Russell (2001), an aliquot of 50-200 µl of the transformation mixture was spread gently (using a bent Pasteur pipette) on a LB plate containing ampicillin, IPTG and X-gal (Appendix C). The rest of the mixture was concentrated by centrifugation at 10,000 g for 1 min. The supernatant was poured off and the cells re-suspended in 200 µl SOC medium were also plated as above. The plates were incubated at 37 ^{o}C for 12-16 hr and the transformed white colonies were picked and used for preparation of plasmid.

2.2.7 Mini-preparation of cloned plasmids

Plasmid DNA was extracted with a QIAprep Spin Miniprep kit (QIAGEN Australia Pty Ltd) according to the manufacturer's recommendation.

The selected white colonies picked from the plates were transferred to 4 ml LB medium containing 60 µg/ml ampicillin. The cultures were incubated overnight (12-16 hr) at 37 ^{o}C with vigorous shaking. The cells were harvested from 1.5 ml aliquots of each culture by centrifugation at 10,000 g for 1 min at room temperature. The supernatant was removed with a Pasteur pipette and the bacterial pellet was re-suspended (by pipetting up and down) in 250 µl of Buffer P1. 250 µl of Buffer P2 was added and the tube was gently inverted a few times to mix the sample until the solution becomes viscous and slightly clear. 350 µl of Buffer N3 was added and the tube was inverted immediately to mix the contents of the tube. The sample was then centrifuged for 10 min at 10,000 g at room temperature to remove the cellular debris. The supernatant was transferred to the QIAprep spin column by pipetting and centrifuged

for 1 min at 10,000 g. The flow-through was discarded and the QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuging for 1 min at 10,000 g. The flow-through was discarded and the sample was centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml tube and 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of each QIAprep spin column and centrifuged for 1 min at 10,000 g to elute DNA. The eluate was stored at –20 ^{o}C.

2.2.8 Analysis of inserts in the recombinant plasmids

Minipreps of plasmids (Section 2.2.7) were analysed for the presence of the correct size of DNA insert by digestion with EcoRI according to the manufacturer's recommendation (Promega). An aliquot of the plasmid prep was digested with EcoRI restriction enzyme (0.5 U/µl) for 2 hr at 37 ^{o}C in a 20 µl reaction vol. The reaction mixtures were then fractionated on a 5 % non-denaturing polyacrylamide gel in TAE buffer.

2.2.9 Sequencing

2.2.9a Preparation of dsDNA template

The template plasmid DNAs from Section 2.2.7 were further purified by n-butanol precipitation. 50 µl of plasmid prep was mixed with 500 µl n-butanol (Butan-1-ol) and vortexed for 2-5 min. The sample was centrifuged at 10,000 g for 15 min. The supernatant was discarded and the pellet was air dried to remove traces of n-butanol. The pellet was dissolved in 10 µl SDDW.

2.2.9b Sequencing reaction

800-1200 ng of plasmid template was mixed with 6.4 pmoles of either forward or reverse sequencing primers (Appendix G) in an 8 µl reaction volume and sent for automatic cycle sequencing at the Australian Genome Research Facility (AGRF) in Brisbane.

2.2.9c Sequence analysis

Comparisons between sequenced nucleotides and sequence databases were done using BLAST program in the internet site of National Center for Biotechnology Information (NCBI) (http//www.ncbi.nlm.nin.gov). The sequence alignment was done with Clustal W (Chenna *et al.*, 2003) and the GENEDOC program (Nicholas *et al.*, 1997). Secondary structure was predicted by Mfold version 3.1 (Zuker, 2003).

CHAPTER 3

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

3.1 INTRODUCTION

Viroids produce no specific proteins thus immunological methods applied to viruses cannot be used for the diagnosis of disease caused by viroids. Similarly because no characteristic particles can be detected, electron microscopy techniques are inappropriate (Hull, 2002). For these reasons, diagnostic procedures have been confined to biological tests, gel electrophoresis assays, nucleic acid hybridization tests and polymerase chain reaction (PCR) assays (McInnes and Symons, 1991; Hanold, 1993).

Polyacrylamide gel electrophoresis (PAGE) has played a key role in viroid research. Viroids generally occur in very low concentrations in infected hosts. Thus some partial purification and concentration of the nucleic acids is required before analysis in an appropriate PAGE system. Detection of viroids is carried out by the separation of nucleic acids, total or partially fractionated, in an electric field before staining and visualisation of the viroid RNA. Among the more widely used techniques are two-dimensional (2-D) PAGE (Schumacher *et al*., 1983), bi-directional gel electrophoresis, return gel electrophoresis (R-PAGE) (Schumacher *et al*., 1986) and sequential PAGE (Semancik *et al*., 1987) followed by the detection of low levels of viroids by silver staining which was reported to be 3-100 fold more sensitive than ethidium bromide or toluidine blue (Igloi, 1983). Two-dimensional PAGE has been

widely used for routine viroid detection (Hanold, 1993). Most two-dimensional PAGE systems for RNA analysis are based on the effect of increasing the concentration of either acrylamide or urea between the first and second dimension (Schumacher *et al*., 1983). Feldstein *et al*., (1997) developed a 2-D PAGE system that relies on increase in the degree of crosslinking rather than acrylamide concentration to preferentially retard migration of circular molecules. This system was successfully used to resolve a complex mixture of circular and linear CCCVd. PAGE analysis remains integral to viroid research as it is simple to use and does not require nucleotide sequence information.

Methods for detection and diagnosis based on nucleic acid hybridization are increasingly important. The basis of all molecular hybridization is the specific interaction between complementary purine and pyrimidine bases. This will result in a stable hybrid formed by part or all of the nucleic acid sequence of the target (pathogen) and the labelled complementary sequence (probe) (Hull, 2002).

RNA probes are now used for routine detection of PSTVd in potato tuber flesh and sprouts (Salazar *et al*., 1988). Sano *et al*. (1988) used synthetic oligonucleotide probes to diagnose HSVd strains and CEVd. Dot blot hybridization, the simplest format for the hybridization procedure, in which the target sample is spotted on the membrane, is now widely used (Owens and Diener, 1981; Candresse *et al*., 1988). However, this format does not give any information on the size or number of species of the target nucleic acid. Such information can be obtained by Northern blotting where the nucleic acid of the target is electrophoresed in a gel and then transferred to a membrane (Hull, 2002). This procedure has been coupled with the use of non-radioactive DNA probes to provide a sensitive procedure for routine diagnosis of viroid in plant extracts (McInnes

et al., 1989; Roy *et al*., 1989). Several viroids including PSTVd and citrus viroids have been detected by tissue print hybridization (Palacio-Bielsa *et al*., 1999).

This chapter describes the use of PAGE and nucleic acid hybridization techniques to identify viroid-like nucleic acids associated with the orange spotting of oil palm.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Leaf samples from ten symptomatic and eight asymptomatic oil palms from Malaysia were used in this study (Appendix A). CCCVd extracted from coconut leaf samples from the Philippines were used as a standard. The CCCVd probe used for hybridization experiments is described in Section 2.1.5

3.2.2 Methods

3.2.2a Total nucleic acid extraction

Nucleic acids from the symptomatic and asymptomatic oil palm leaf samples were extracted using methods described in Section 2.2.1a, 2.2.1b, 2.2.1c and 2.2.1d. All chemicals used in these experiments are described in Appendix B.

3.2.2b Polyacrylamide gel electrophoresis

Total nucleic acids from the leaf samples were analysed using 1D and 2D-PAGE as described in Section 2.2.2a and 2.2.2b. The gels were analysed by silver staining as described in Section 2.2.2d.

3.2.2c Blotting of fractionated nucleic acids to membrane

Nucleic acids from 1D and 2D-PAGE were transferred to a positively charged Nylon membrane (Zeta Probe®, BioRad) either by capillary blotting as described in Section 2.2.4a or by electroblotting (Section 2.2.4b).

3.2.2d Hybridization assay

A full-length antisense probe was transcribed by T7 RNA polymerase from a plasmid vector containing the $CCCVd_{246}$ clone (Appendix E) and labelled with ^{32}P (Section 2.1.5). The transcribed probes were heated at 100 ^{o}C for 2 min and added to the pre-hybridization solution (Appendix C) at about 10^6 cpm/ml and used for the hybridization assay.

Capillary or electroblotted membranes (Zeta Probe®, BioRad) were hybridised according to the method of Hanold and Randles (1991) with some modifications as described in Section 2.2.4c.

3.3 RESULTS

3.3.1 Comparison of extraction methods to isolate CCCVd-like RNAs from oil palm leaves

Four extraction methods as described in Section 2.2.1a, 2.2.1b, 2.2.1c and 2.2.1d were compared for efficient isolation of CCCVd-like RNAs from the oil palm leaf samples. PEG (2.2.1a) extraction was found to be the most effective in retaining the fraction of the nucleic acids containing the CCCVd-like RNAs based on hybridization assays with CCCVd probe. PEG was found to precipitate and concentrate CCCVd molecules (Hanold, 1993) and this concentration step is not available in all the other extraction methods. Moreover, large amounts of leaf tissue (20-100 g) had to be extracted to detect the presence of CCCVd-like RNAs. PEG extracted nucleic acids were used in all the following experiments unless mentioned otherwise.

3.3.2 PAGE analysis

Analysis of nucleic acid extract from both symptomatic (Figure 3.1) and asymptomatic (Figure 3.2) oil palm leaf tissues using 5 % PAGE under denaturing conditions by silver staining showed no viroid-like molecules that corresponded with CCCVd extracted from coconut which was used as positive control.

2D-PAGE analysis also showed no indication of the presence of viroid-like molecules in the oil palm samples when stained with silver, as there were no clearly visible bands in the region where circular RNA would be expected to migrate (Figure 3.3).

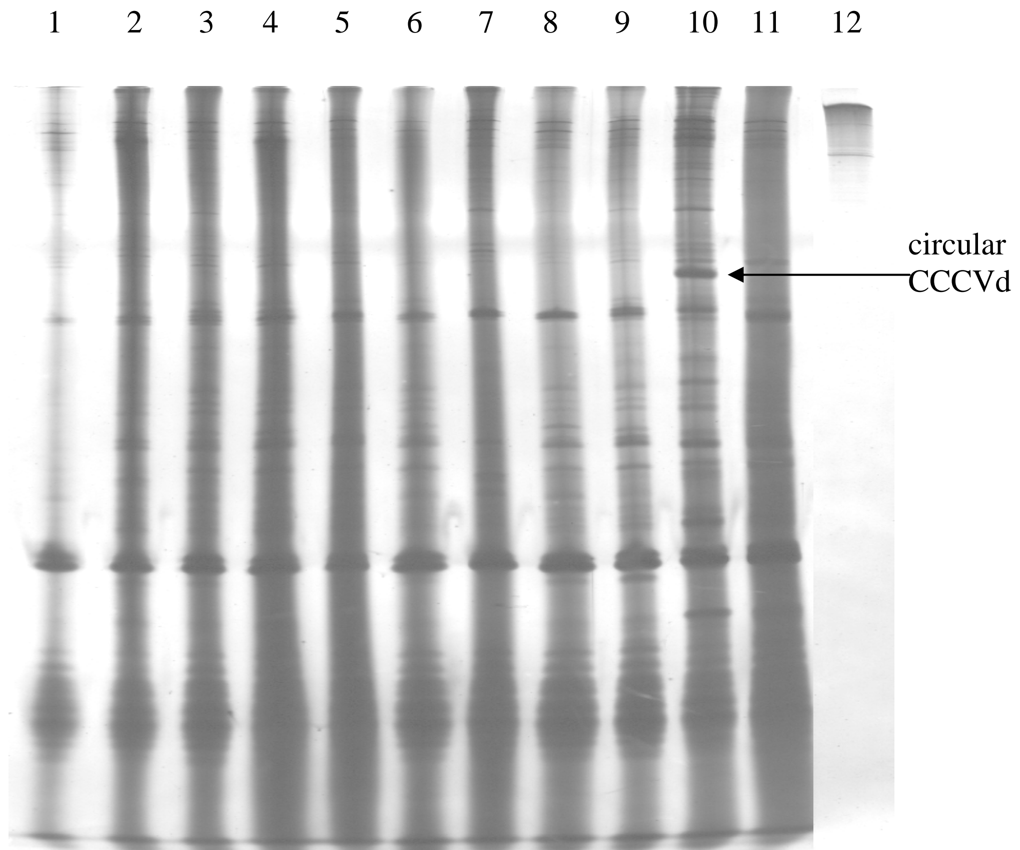


Figure 3.1 PAGE analysis of PEG extracted nucleic acids extracted from symptomatic oil palm on a 5% denaturing gel stained with silver. Arrow indicates the position of the circular CCCVd molecule.

Lane 1-9, 11	Samples from the symptomatic oil palm leaf
Lane 10	CCCVd infected coconut palm leaf
Lane 12	Total nucleic acid of Tobacco mosaic virus (TMV) extracted from TMV infected tobacco

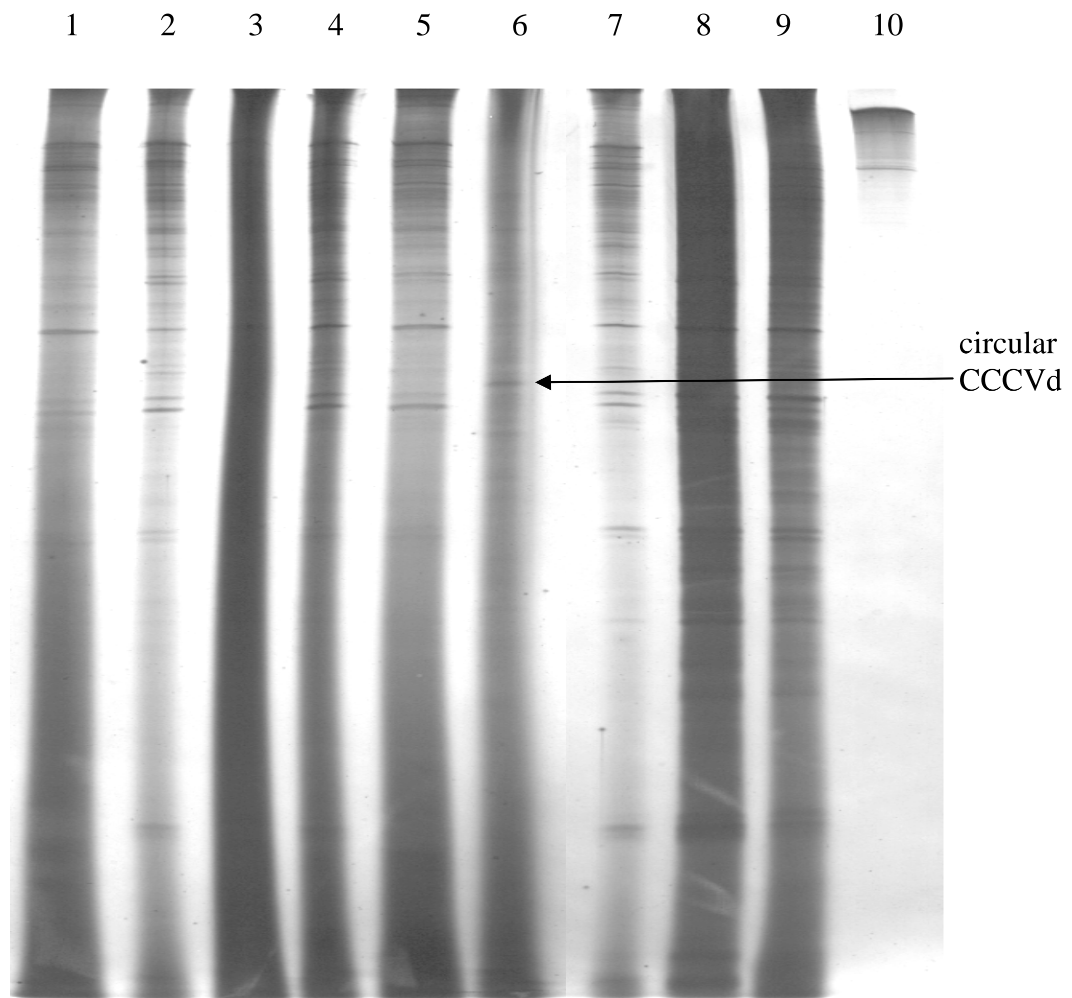


Figure 3.2 PAGE analysis of PEG extracted nucleic acids extracted from asymptomatic oil palms on a 5% denaturing gel stained with silver. Arrow indicates the position of the circular CCCVd molecule.

Lane 1-5, 7-9 Samples from the asymptomatic oil palm leaf

Lane 6 CCCVd infected coconut palm leaf

Lane 10 Total nucleic acid of Tobacco mosaic virus (TMV) extracted from TMV infected tobacco

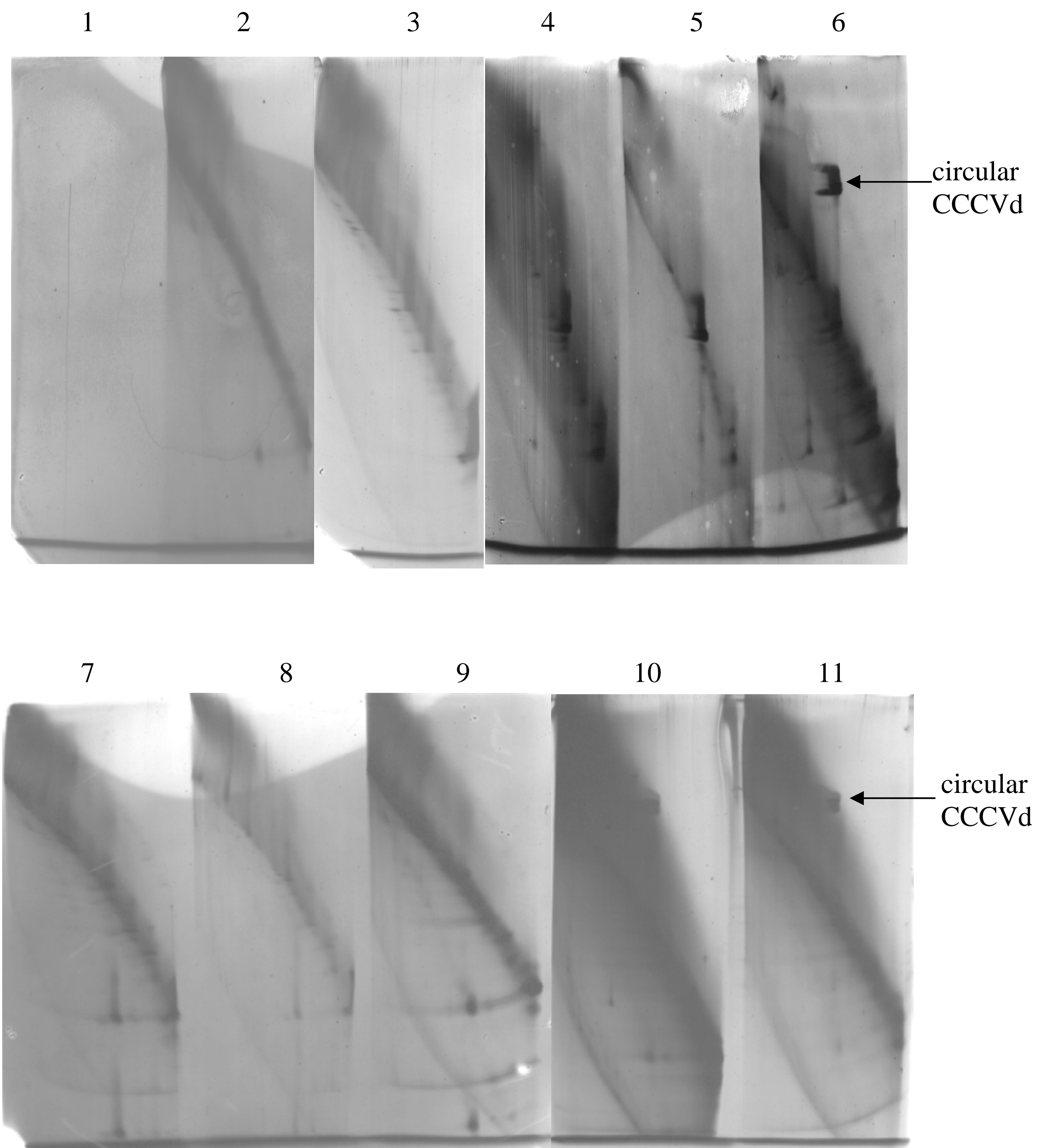


Figure 3.3 2D-PAGE analysis of PEG extracted nucleic acids from symptomatic and asymptomatic oil palms leaf samples stained with silver. Arrow indicates the circular band of the CCCVd molecule.

Gel 1-5 Samples from the symptomatic oil palm leaf

Gel 6 CCCVd infected coconut palm leaf

Gel 7-9 Samples from the asymptomatic oil palm leaf

Gel 10-11 Oil palm inoculated with cloned CCCVd

None of the oil palm samples showed detectable viroid-like molecules even after increasing the oil palm nucleic acid extract loading to ten fold compared with the amount required to detect CCCVd (Table 3.1)

3.3.3 Hybridization assay with CCCVd probe

Initial Northern blot analysis of PEG extracted total nucleic acid showed no signals when hybridised with a ^{32}P-labelled full-length $CCCVd_{246}$ cRNA probe. However, when the loading of the oil palm nucleic acid extract was increased to 5 fold of CCCVd, there was one oil palm (asymptomatic oil palm, SRH2 f20), which was positive for a viroid-like RNA. The nucleic acid loading of SRH2 f20 was from 10 g of leaf and fractionated on 5% non-denaturing PAGE showed a weak signal when hybridised with the CCCVd probe (Figure 3.4-lane 3) under high stringency wash conditions. This band corresponded to that of CCCVd.

2D-PAGE Northern blot analysis of nucleic acid extracted from both asymptomatic and symptomatic oil palms showed the presence of a CCCVd-like RNA in one asymptomatic (SRH2 f20) and two symptomatic (SRD6 f12 and SRD2 f20) oil palms. The CCCVd-like RNAs in SRH2 f20 (Figure 3.5-gel 2) and SRD6 f20 (Figure 3.5-gel 4) had the mobility expected for a circular structure of CCCVd but the hybridization signal was weak compared to CCCVd from coconut (Figure 3.5-gel 1). The other symptomatic palm (SRD2 f20; Figure 3.5-gel 5) showed mobility that corresponded to linear CCCVd molecules.

Table 3.1 Summary of the results from polyacrylamide gel electrophoresis (PAGE) assays. 1D and 2D-PAGE were used to detect the presence of CCCVd-like RNAs in nucleic acid samples extracted from symptomatic and non-symptomatic oil palms from Malaysia.

	No. of positive oil palms for CCCVd-like RNA	
Palms \ Method	1D-PAGE	2D-PAGE
Symptomatic	0/10	0/10
Asymptomatic	0/8	0/8

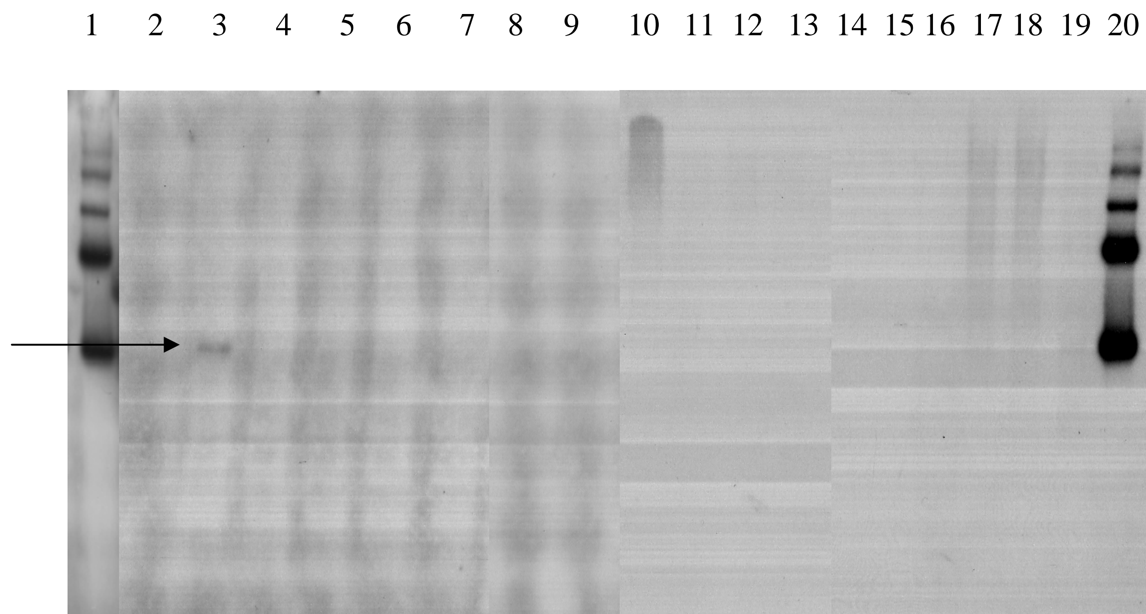


Figure 3.4 Northern blots of 5 % non-denaturing PAGE of PEG extracted nucleic acids from both symptomatic and asymptomatic oil palm leaf samples hybridised with full length ^{32}P-labelled CCCVd$_{246}$ cRNA probe and washed under high stringency conditions. An asymptomatic palm (SRH2 f20) in lane 3 (indicated by the arrow) showed a faint band similar in size to CCCVd.

Lane 1, 20	CCCVd infected coconut leaf samples
Lane 2-9	Samples from the asymptomatic oil palm leaf
Lane 10-19	Samples from the symptomatic oil palm leaf

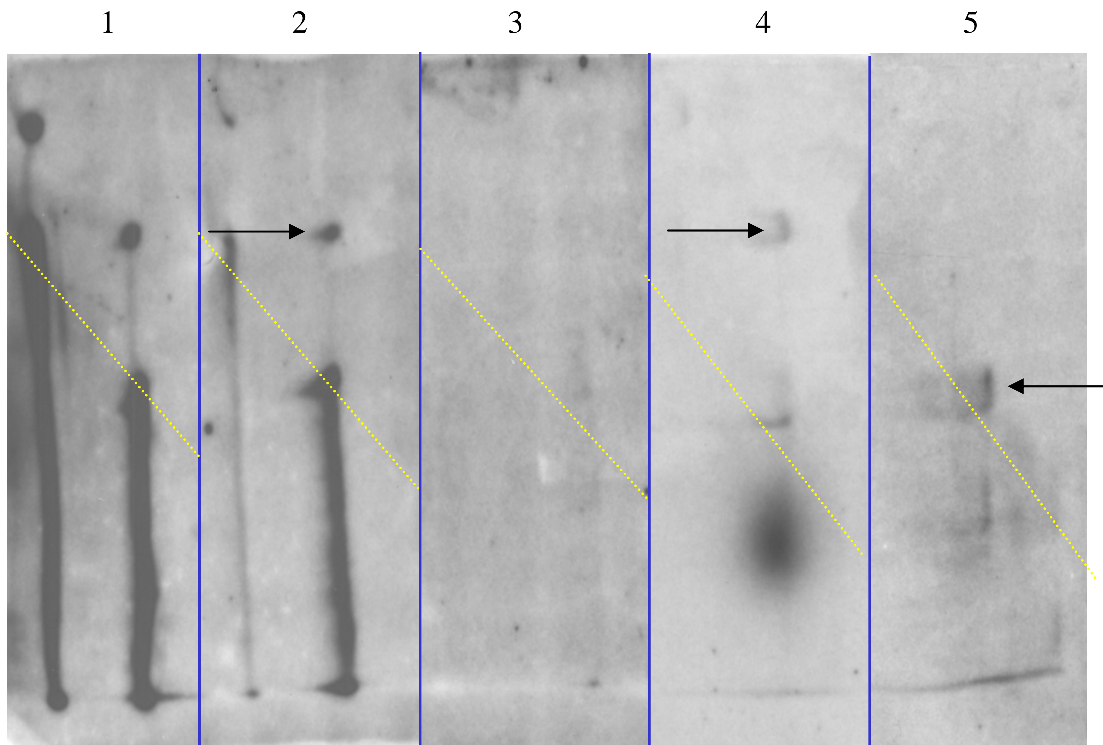


Figure 3.5 Northern blots of 2D-PAGE of PEG extracted nucleic acids from both symptomatic and asymptomatic oil palm samples hybridised with full length ^{32}P-labelled $CCCVd_{246}$ cRNA probe and washed under high stringency conditions. Arrows indicate CCCVd-like RNA in the oil palm samples that correspond to the circular (SRH2 f20 and SRD 6 f12) and linear molecule (SRD2 f20) of CCCVd. The dotted line indicates the position of the diagonal where corresponding linear molecules will migrate.

Gel 1	CCCVd infected coconut leaf sample
Gel 2	SRH2 f20 (asymptomatic oil palm leaf sample)
Gel 3	SRH7 f20 (asymptomatic oil palm leaf sample)
Gel 4	SRD6 f12 (symptomatic oil palm leaf sample)
Gel 5	SRD2 f20 (symptomatic oil palm leaf sample)

These molecules in the oil palm samples were only detectable when nucleic acid extracts from 20g (SRH2 f20) and 100g (SRD6 f12 and SRD2 f20) of leaf tissues were used for the analysis, which is 10X and 50X more than required for detecting CCCVd. No other oil palm samples showed a signal when hybridised with the CCCVd probe (Table 3.2).

Northern blots from three different fronds of SRH2; frond 3 (f3-third youngest), 10 and 20 showed that circular CCCVd-like RNAs which corresponded to the size of the circular CCCVd were present in all three fronds (Figure 3.6). In addition, 2D-PAGE northern blot of SRH2 f3, showed two additional bands with weak signal in the circular region (Figure 3.6-gel 4).

Table 3.2 Summary of results of hybridization assay of Northern blots using ^{32}P-labelled full-length $CCCVd_{246}$ cRNA probe to detect the presence of CCCVd-like RNAs in nucleic acid sample extracted from symptomatic and asymptomatic oil palms from Malaysia.

	No. of positive oil palms for CCCVd-like RNA	
Method / Palms	Northern blots of 1D-PAGE	Northern blots of 2D-PAGE
Symptomatic	0/10	2/10
Asymptomatic	1/8	1/8

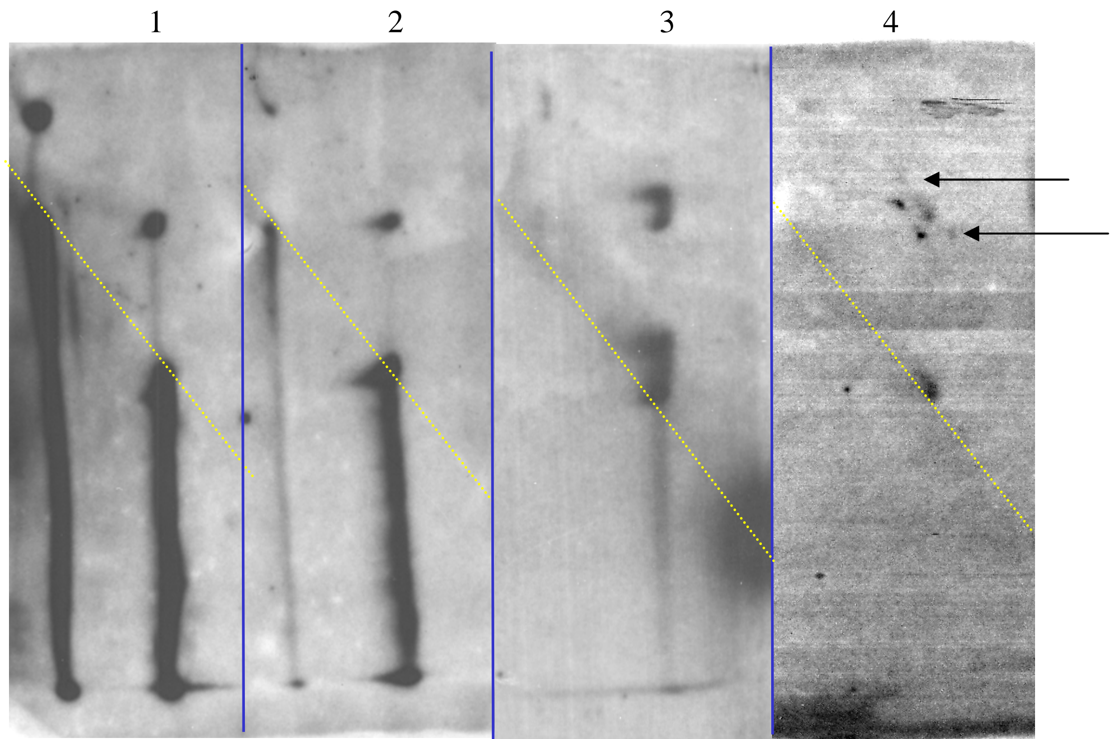


Figure 3.6 Northern blot of 2D-PAGE of PEG extracted nucleic acids from three fronds (f3, f10 and f20) of an asymptomatic oil palm (SRH2) hybridised with full length ^{32}P-labelled CCCVd$_{246}$ cRNA probe. Nucleic acid extract from all three fronds of SRH2 showed a band in the circular viroid region of the gel, which corresponded to the circular molecule of CCCVd. In addition, there were also two additional bands in the circular region (indicated by arrows) of SRH2 f3. The dotted line indicates the position of the diagonal where corresponding linear molecules will migrate.

Gel 1	CCCVd infected coconut leaf sample
Gel 2	SRH2 f20 (asymptomatic oil palm leaf sample; frond 20)
Gel 3	SRH2 f10 (asymptomatic oil palm leaf sample; frond 10)
Gel 4	SRH2 f3 (asymptomatic oil palm leaf sample; frond 3)

3.4 DISCUSSION

The results of this study show that there are molecules in the nucleic acids of both symptomatic (OS) and asymptomatic oil palm samples from Malaysia that bind a CCCVd probe. The size of the molecules (approximately 250-300 nt) based on the mobility in 5 % non-denaturing PAGE and the ability to hybridise with the cRNA probe suggest that the nucleic acid is most likely a RNA. The presence of the RNA in the circular viroid region of the 2D-PAGE shows that the RNA in the oil palm nucleic acid extracts has a circular structure. The presence of low molecular weight and circular RNA suggests that the RNAs in the nucleic acid extract of the oil palms have properties of a viroid RNA.

Hybridization assays of the RNA from the oil palms with a full-length ^{32}P-labelled $CCCVd_{246}$ cRNA probe shows that there is some degree of sequence similarity between these RNAs and CCCVd based on the ability of these RNAs to retain the hybridization signals after high stringency wash conditions. Moreover, the mobility of the RNAs were also similar to CCCVd suggesting that the RNAs in the nucleic acid extract of the oil palms may be closely related to CCCVd.

Northern blot analysis of 2D-PAGE with the CCCVd probe showed the presence of CCCVd-like RNAs in an asymptomatic palm (SRH 2 f20) and two symptomatic palms (SRD6 f12 and SRD2 f20). Three fronds of different ages (f3, f10 and f20) of the asymptomatic palm contained CCCVd-like RNAs. In addition, there appeared to be two additional bands with a weak signal in the circular viroid region of 2D-PAGE of SRH2 f3.

The disease stages of the OS could be related with age of the frond. Oil palm leafs grow from the apex of the palm where each leaf remains enclosed for about 2 years and then develops into a central spear and finally opens. After the leaf has opened

it is displaced centrifugally as leaves emerge (Hartley, 1988). The first opened spear is designated as frond no. 1 and when a new spear opens and displaces the old one, the newly opened leaf is designated as frond 1 and the previous frond will become frond 2. Thus, frond number increases as the age of the palm increases. Based on this, the oldest frond represents the early stage of OS disease while the younger frond represents the latter stages of the disease. Therefore, SRH2 f3, f10 and f20 represent the late, middle and early stages of the disease respectively. The observation of additional bands in f3 suggests that there are more than one CCCVd-like RNAs in the latter stage of the disease.

The weak hybridization signals observed in the oil palm samples suggest that the CCCVd-like RNAs in the oil palm samples are present in low concentrations compared to CCCVd. This may be due to the sequence variability between CCCVd and the CCCVd-like RNAs and the low concentration of the CCCVd-like RNAs in the oil palm samples. The ability to retain the hybridization signal after high stringency wash shows that the CCCVd-like RNA may have a high sequence similarity with CCCVd. Therefore, the low concentration of the CCCVd-like RNAs in the nucleic acid extract of the oil palms is more likely to be the cause of the weak hybridization signals.

Low efficiency of extraction from the oil palm samples could have contributed to this. A large amount of nucleic acid extract (leaf weight equivalent of 20-100 g) had to be loaded into the gel for these CCCVd-like RNAs to be detected by hybridization assays. Four extraction methods were tested for efficient isolation of CCCVd-like RNAs from the oil palm leaf samples and the PEG extraction method was preferred as it showed the best result. PEG extraction was also used in previous studies with CCCVd in coconut and oil palms (Hanold and Randles, 1991) and it was found to precipitate and concentrate CCCVd molecules (Hanold, 1993).

The low concentration of these CCCVd-like RNAs may explain the low incidence of oil palms positive to these CCCVd-like RNAs.

The weak signals obtained in the hybridization assays may indicate that these techniques may be at the limit of sensitivity. Therefore, a more sensitive method is needed for detection of this CCCVd-like RNAs in the oil palm. RPA was reported to be a sensitive procedure and provided more sequence information than nucleic acid hybridization (Palukaitis *et al*., 1994). An attempt was made to use RPA as a detection method for the CCCVd-like RNAs in the oil palm, which will be tested in the next chapter.

CHAPTER 4

RIBONUCLEASE PROTECTION ASSAY (RPA) TO DETECT CCCVd-LIKE RNAs IN OIL PALM

4.1 INTRODUCTION

The ribonuclease protection assay (RPA) has been widely used as a procedure to detect and quantify specific RNAs (Winter *et al.*, 1985; Kurath and Palukaitis, 1989b; Ahmad *et al.*, 1993; Aranda *et al.*, 1993) and to detect variations in the nucleotide sequence of a RNA population (Lopez-Galindez *et al.*, 1988; Kurath and Palukaitis, 1989a; Cabrera *et al.*, 2000). It is a sensitive procedure that provides more information than nucleic acid hybridization, but not as much information as nucleic acid sequencing (Palukaitis *et al.*, 1994). RPA is also much less labor intensive than cloning and sequencing of nucleic acid samples (Palukaitis *et al.*, 1994).

RPA offers distinct advantages over other RNA detection methods. In an RPA reaction, the probe and target mRNA are hybridized in a solution where target availability is maximal, affording increased detection of rare messages (Rosenau *et al.*, 2002). In addition, RPA allows probing for multiple targets in one sample of RNA. This "multiplexing" technique is not suited for RT-PCR, which rivals RPA sensitivity but also commonly requires extensive optimization. Northern blots, which are 10 times less sensitive than RPA, can be probed for multiple transcripts but require multiple stripping and re-probing of a single blot (Rosenau *et al.*, 2002).

RPA has been shown to be useful in plant virology (Palukaitis *et al.*, 1994). Specific applications of RPA include (a) detecting the expression of viral sequences in

transgenic plants, (b) quantitation of viral sequences present in nucleic acid extracts, (c) detecting and mapping nucleotide sequence variation in strains or isolates of a virus, and (d) detecting and mapping heterogeneity in RNA populations; for example, in studies involving virus evolution and virus epidemiology (Palukaitis *et al.*, 1994). In contrast, RPA has not been widely used for studies of viroids but it has been used for studying mutations in PSTVd (Lakshman and Tavantzis, 1992).

This chapter describes the use of RPA as a detection method for CCCVd-like RNAs in oil palm samples from Malaysia.

4.2 MATERIALS AND METHODS

4.2.1 Materials

PEG extracted nucleic acid extracts from ten symptomatic (orange spotted) and eight asymptomatic oil palms from Malaysia were used in this study (Appendix A). Healthy oil palm and coconut leaf samples obtained from seedlings at the Waite glasshouse were also used in this study as controls. t-RNA (Wheat Germ) was used as a negative control.

4.2.2 Ribonuclease Protection Assay

The RPA method was modified from that of Winter *et al.* (1985).

4.2.2a *In vitro* transcription of CCCVd probe

A RNA transcription kit (Riboprobe system, Promega) (Appendix D) was used to transcribe the ^{32}P-UTP labeled full length CCCVd$_{246}$ cRNA probe. As described in Section 2.1.5, 2.4 µl of 100 µM cold UTP was added to the reaction mix before transcription.

4.2.2b Hybridization

Dried pellets of nucleic acids extracted from 5 g (oil palm) or 1 g (coconut) of leaf tissue were suspended in 30 µl of hybridization buffer (80% formamide, 40 mM PIPES pH 6.5, 400 mM NaCl, 1mM EDTA pH 8.0, probe [2x10^5 cpm]), heated for 10 min at 95 ^{o}C and then incubated at 55 ^{o}C for 12-18 hr. A tube containing 100 µg tRNA was included as a negative control.

4.2.2c RNAse digestion

Tubes were cooled to room temperature and mixed with 350 µl of digestion buffer (300 mM NaCl, 10 mM Tris pH 7.5, 5 mM EDTA pH 8.0). 1 µl of 4 mg/ml RNAse A and 0.4 µl of 10 U/µl RNAse T1 were added to give final concentration 10 µg/ml and 10 U/ml respectively. Tubes were incubated at 30 ^{o}C for 1 hr.

The RNAse digestion reaction was stopped by adding 10 µl of 20 % SDS and 2.5 µl of 10 mg/ml Proteinase K to give final concentration of 0.5 % and 60 µg/ml respectively and the tubes were incubated at 37 ^{o}C for 20 min. tRNA was added to a final concentration of 0.025 µg/µl. 400 µl of PCA was added for a final extraction.

Approximately 300 µl of the supernatant was transferred to a new tube and mixed with 3 vol. of 100 % ethanol and incubated at –70 ^{o}C for 30 min. Pellets were collected by centrifugation at 12,000 g for 15 min, excess ethanol was removed from the tubes by pipetting and pellets were air-dried.

4.2.2d PAGE analysis

Pellets were re-suspended in 10 µl of denaturing loading buffer (Appendix C). They were heated for 5 min at 95 ^{o}C, immediately chilled on ice, then loaded onto a 5 % denaturing PAGE (8M urea) (Appendix C), which was run at 40 mA for 1 hr. The gel was fixed for 10 min in 0.5 % (v/v) acetic acid and 10 % (v/v) ethanol and washed once with DDW. The gel was placed on a wet sheet (rinsed with DDW) of 3MM Whatman filter paper (22 x 22 cm). The filter paper and gel was placed on a gel dryer (BioRad model 583) and dried for 2 hr at 80 ^{o}C. The gel was exposed to an X-ray film (Kodak) at –70 ^{o}C for 1-72 hr with an intensifying screen.

4.3 RESULTS

4.3.1 RPA analysis of CCCVd from coconut

The *in-vitro* synthesized ^{32}P-labeled full length $CCCVd_{246}$ cRNA probe was protected by RNA extracted from CCCVd-infected coconut leaves. Digestion with RNAse A and T1 produced three major protected fragments (Figure 4.1), which were approximately 50 nt, 125 nt and 250 nt in size. The 250 bp protected fragment corresponds to the full length $CCCVd_{246}$. The other two protected fragments together with some additional minor protected fragments observed in that sample indicate that there were single-stranded mismatches between the probe and some of the target RNAs.

The slightly slower mobility of the undigested probe (Figure 4.1-lane 1,6) and undigested probe hybridised with CCCVd (Figure 4.1-lane 2) is assumed to be due to additional viroid-nonspecific nucleotides transcribed from the polylinker region of the plasmid. The probe alone (Figure 4.1-lane 5) and the probe hybridised with t-RNA as negative control (Figure 4.1-lane 4) was completely digested.

Healthy coconut (Figure 4.2-lane 2) and healthy oil palm (Figure 4.2-lane 9) from the Waite Glasshouse contained no detectable CCCVd-like RNA. Nucleic acid extracts from two oil palm samples inoculated with cloned CCCVd, E21 (Figure 4.2-lane 4) and T31A (Figure 4.2-lane 5) showed a RPA pattern similar to that of CCCVd infected coconut (Figure 4.2-lane 2). Additional minor protected fragments were observed.

Small protected fragments approximately 30 nt and 50 nt were observed in the sample from PSTVd extracted from tomato (Figure 4.2-lane 11). Whereas, the RNA extracted from healthy tomato was completely digested (Figure 4.2-lane 10).

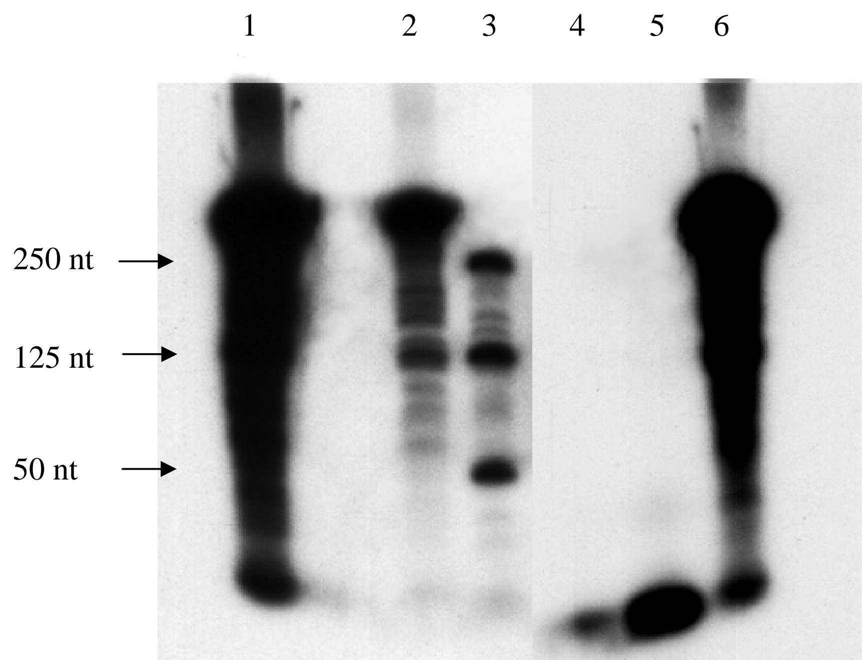


Figure 4.1 RPA analysis of PEG extracted nucleic acids from CCCVd-infected coconut leaves. Autoradiography of RNAse digested samples after 5% denaturing PAGE showed three major protected fragments of approximately 50 nt, 125 nt and 250 nt in size.

Lane 1 and 6 - probe alone not treated with RNAse

Lane 2 - probe with CCCVd sample not treated with RNAse

Lane 3 - probe with CCCVd sample treated with RNAse

Lane 4 - probe with tRNA treated with RNAse

Lane 5 - probe alone treated with RNAse

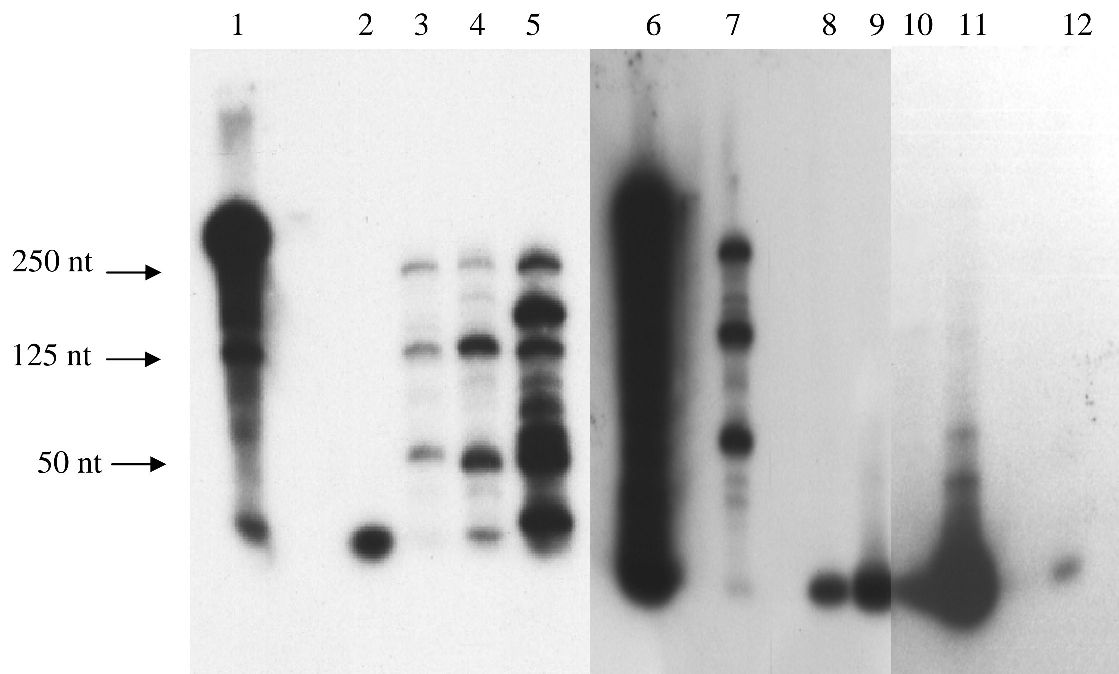


Figure 4.2 RPA analysis of nucleic acid extracted from a range of plants. The probe was hybridized with the samples before RNAse digestion.

Lane 1,6	probe alone not treated with RNAse
Lane 2,8	healthy coconut extract treated with RNAse
Lane 3,7	CCCVd from infected coconut treated with RNAse
Lane 4	E21 inoculated with a CCCVd clone treated with RNAse
Lane 5	T31A inoculated with a CCCVd clone treated with RNAse
Lane 9	Healthy oil palm extract treated with RNAse
Lane 10	Nucleic acid from healthy tomato treated with RNAse
Lane 11	PSTVd extracted from tomato treated with RNAse
Lane 12	tRNA treated with RNAse

4.3.2 RPA analysis of RNA extracted from oil palms

Analysis of nucleic acids extracted from symptomatic and asymptomatic oil palms showed a RPA pattern similar to that of CCCVd (Section 4.3.1) indicating the presence of CCCVd-like RNAs in both samples. Positive samples had three major protected fragments of approximately 50 nt, 125 nt and 250 nt in size, the largest of which corresponds to $CCCVd_{246}$, as well as several minor protected fragments. Some of the minor protected fragments differed from those observed for CCCVd.

90 % of the symptomatic palms were positive for the presence of CCCVd-like RNAs although the strength of the hybridization signal was weaker than that for a CCCVd infected coconut palm (Figure 4.3, Table 4.1). The symptomatic palm with the strongest hybridization signal was SRD 6 f12 (Figure 4.3-lane 12).

Analysis of the asymptomatic palms showed that 50 % of the samples were positive for CCCVd-like RNAs (Table 4.1, Figure 4.4). Two of the four positive samples showed protected fragments similar to CCCVd but SRH 2 f20 (Figure 4.4-lane 4) and SRH3 f20 (Figure 4.4-lane 5) showed additional protected fragments that were not present in CCCVd. As with the symptomatic samples, the intensity of the signals varied between samples (Table 4.1) with SRH2 f20 (Figure 4.4-lane 4) giving the strongest signal compared to the others.

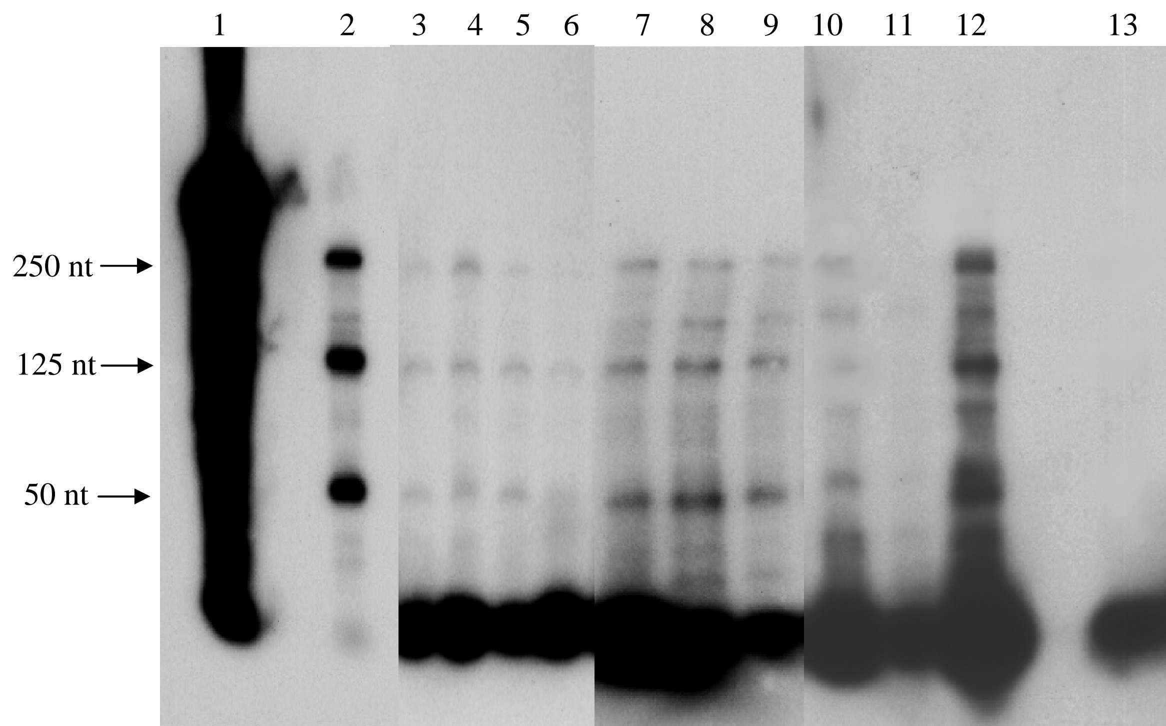


Figure 4.3 RPA analysis of PEG extracted nucleic acids from symptomatic oil palms from Malaysia showing that the protected fragments are similar to those for CCCVd infected coconut palm. Autoradiography was done for 12 hr and 72 hr at -70 ^{o}C for the CCCVd and oil palm samples respectively.

Lane 1	probe alone not treated with RNAse
Lane 2	probe with CCCVd from coconut treated with RNAse
Lane 3-12	probe with nucleic acid extracts from symptomatic oil palm samples treated with RNAse
Lane 13	probe with tRNA treated with RNAse

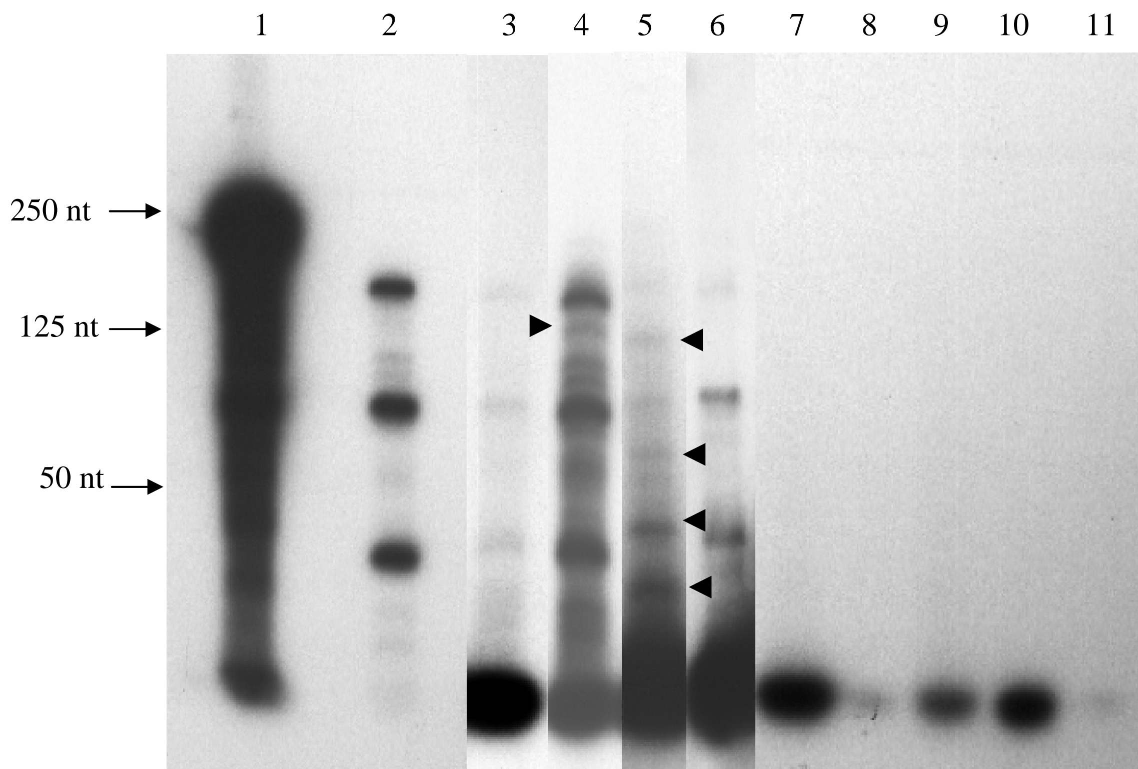


Figure 4.4 RPA analysis of PEG extracted nucleic acids from asymptomatic oil palms from Malaysia showing that the protected fragments are similar to those for CCCVd infected coconut palm. Autoradiography was done for 12 hr and 72 hr at -70 °C for the CCCVd and oil palm samples respectively. Arrows indicate protected fragments that were different from those for CCCVd infected coconut palm.

Lane 1	probe alone not treated with RNAse
Lane 2	probe with CCCVd from coconut treated with RNAse
Lane 3-10	probe with nucleic acid extracts from asymptomatic oil palm samples treated with RNAse
Lane 11	probe with tRNA treated with RNAse

Table 4.1 Results of RPA analysis of oil palm samples from Malaysia

Leaf sample	Positive for CCCVd-like RNA	* Strength of signal
A) Symptomatic palm		
CEP 1 f 20 (lane 3)[a]	yes	+
CEP 2 f 20 (lane 4)[a]	yes	+
CEP 3 f 20 (lane 5)[a]	yes	+
CEP 4 f 20 (lane 6)[a]	yes	+
SRD 1 f 20 (lane 7)[a]	yes	+
SRD 2 f 20 (lane 8)[a]	yes	+
SRD 3 f 20 (lane 9)[a]	yes	+
SRD 4 f 20 (lane 10)[a]	yes	+
SRD 5 f 20 (lane 11)[a]	no	-
SRD 6 f 12 (lane 12)[a]	yes	++
B) Asymptomatic palm		
SRH 1 f 20 (lane 3)[b]	yes	+
SRH 2 f 20 (lane 4)[b]	yes	++
SRH 3 f 20 (lane 5)[b]	yes	+
SRH 4 f 20 (lane 6)[b]	yes	+
SRH 5 f 20 (lane 7)[b]	no	-
SRH 6 f 20 (lane 8)[b]	no	-
SRH 7 f 20 (lane 9)[b]	no	-
SRH 8 f 20 (lane 10)[b]	no	-

* Strength of protected fragment from ribonuclease on the autoradiograph, +++: strong, ++: medium, +: weak, -: not protected compared to CCCVd from coconut

[a] lane numbering as shown in Figure 4.3

[b] lane numbering as shown in Figure 4.4

4.3.3 RPA analysis of RNA extracted from an asymptomatic oil palm (SRH2) at different stages of growth

RPA analysis was done to determine if RPA patterns varied between different stages of growth of an oil palm. Nucleic acids were extracted from three fronds of an asymptomatic oil palm (SRH2), which had given the strongest signal (Section 4.3.2). They were frond 3 (SRH2 f3), 10 (SRH2 f10) and 20 (SRH2 f20). SRH2 f20 represented the older fronds in the palm and SRH2 f3 the younger fronds of the palm.

RPA was done as described in Section 4.2.2 except that PAGE fractionation was done at 40 mA for 2.5 hr to get better separation of the protected fragments.

The RPA of SRH2 f20 showed similar sized protected fragments as CCCVd where the three major protected fragments of approximately 50 nt, 125 nt and 250 nt were present together with other minor protected fragments as shown in Figure 4.5 (lane 9, 12, 15). SRH2 f3 and f10 varied in the size of the protected fragments with several additional protected fragments observed as indicated by arrows in figure 4.5 (SRH2 f3- lanes 7, 11, 14; SRH2 f10- lanes 6, 10, 13, 16). The 250 nt fragment was present in the f10 sample but the hybridization signal was very weak suggesting that this form of CCCVd occurred less in the nucleic acid extract of that sample while there were no clear bands in that region observed in the f3 sample.

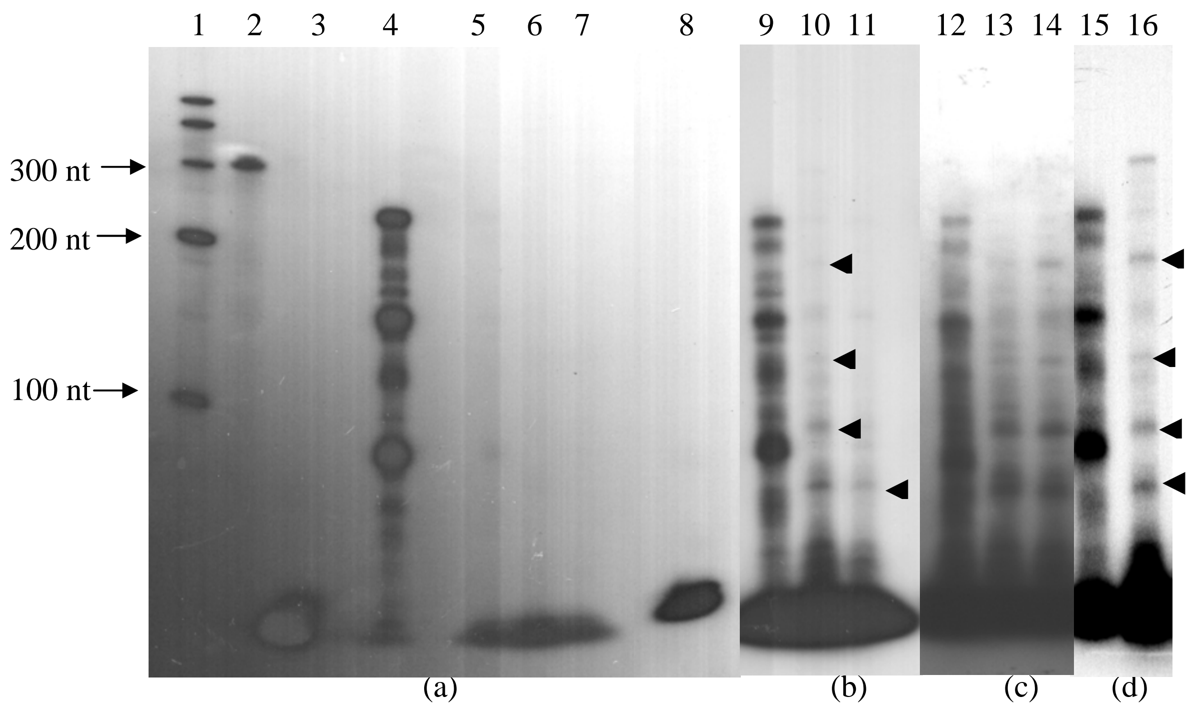


Figure 4.5 RPA analysis of PEG extracted nucleic acids from SRH2 f3, f10, and f20 showing protected fragments similar to those for CCCVd infected coconut palm. Autoradiography was done at (a) 1 hr (b) 12 hr and (c) 72 hr at -70 ^{o}C. (d) shows RPA of SRH2 f20 and f10 from another experiment. Arrows indicate protected fragments that were different from those for CCCVd infected coconut palm.

Lane 1	^{32}P labeled RNA size marker
Lane 2	probe alone not treated with RNAse
Lane 3	probe alone treated with RNAse
Lane 4	probe with CCCVd from coconut treated with RNAse
Lane 5, 9, 12, 15	probe with SRH2 f20 sample treated with RNAse
Lane 6, 10, 13, 16	probe with SRH2 f10 sample treated with RNAse
Lane 7, 11, 14	probe with SRH2 f3 sample treated with RNAse
Lane 8	probe with tRNA treated with RNAse

4.3.4 RPA analysis of RNA extracted from a symptomatic palm (SRD6 f12)

RPA of a symptomatic palm (SRD6 f12) was done using the method as described in Section 4.3.3 to see the degree of similarity between the protected fragments of CCCVd-like RNA and CCCVd from coconut. RPA of SRD6 was done using frond 12 (f12) as this was the only available leaf tissue from this palm. RPA analysis of SRD6 f12 shows that the CCCVd-like RNAs in that sample shows high similarity with CCCVd (Figure 4.6).

4.3.5 RPA analysis of RNA extracted from coconut samples from Sri Lanka

Four coconut leaf samples from asymptomatic palms; H1, H2, LSD and CRD from Sri Lanka (Appendix A) were also tested for the presence of CCCVd-like RNAs. Analysis of nucleic acids extracted from these samples showed the presence of CCCVd-like RNAs in one sample, H1 (Figure 4.7-lane 3). The other three samples showed no clear protected bands. The RPA patterns observed in H1 showed similar pattern with CCCVd infected coconut sample from the Philippines with the three main protected fragments observed.

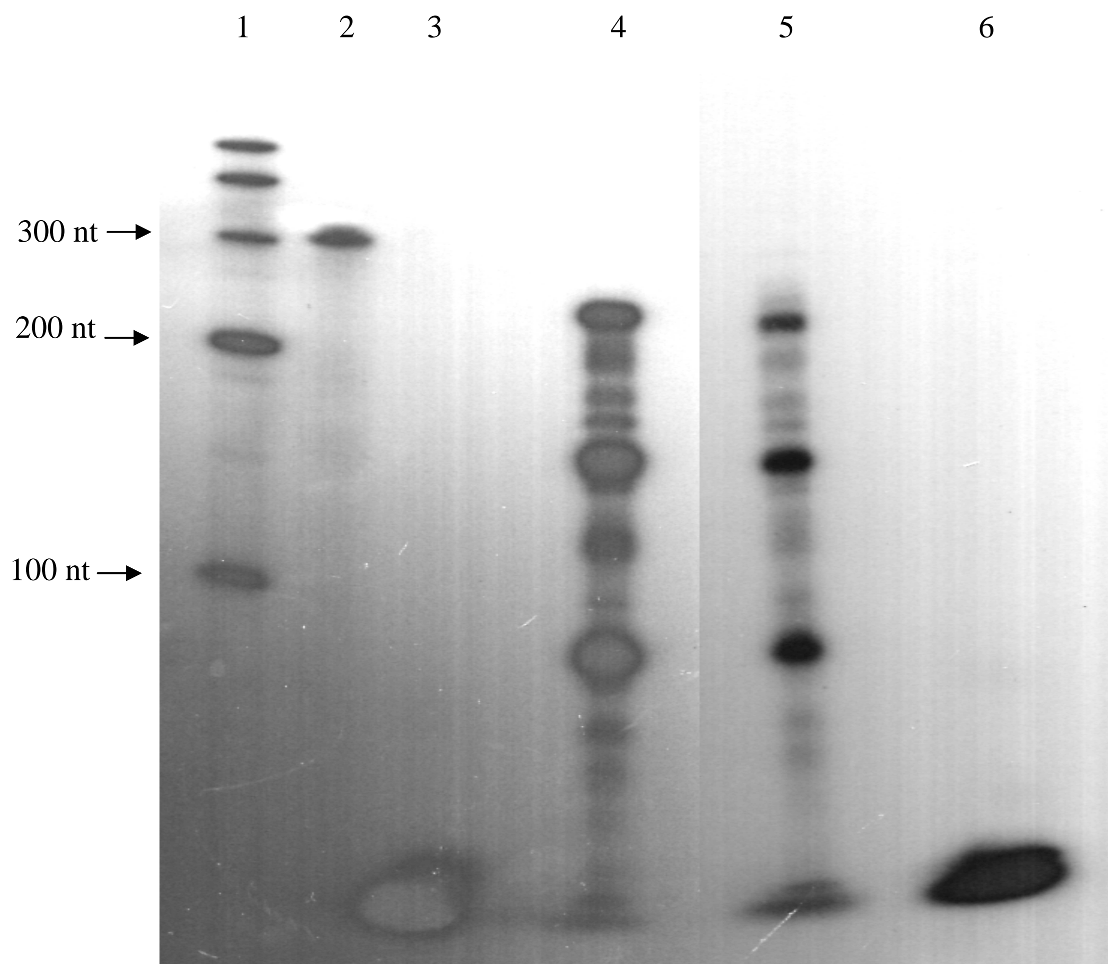


Figure 4.6 RPA analysis of PEG extracted nucleic acid from SRD6 f12 showing protected fragments similar to those for CCCVd infected coconut palm. Autoradiography was done for 1 hr at -70 ^{o}C

Lane 1	^{32}P labeled RNA size marker
Lane 2	probe alone not treated with RNAse
Lane 3	probe alone treated with RNAse
Lane 4	probe with CCCVd from coconut treated with RNAse
Lane 5	probe with SRD6 f12 sample treated with RNAse
Lane 6	probe with tRNA treated with RNAse

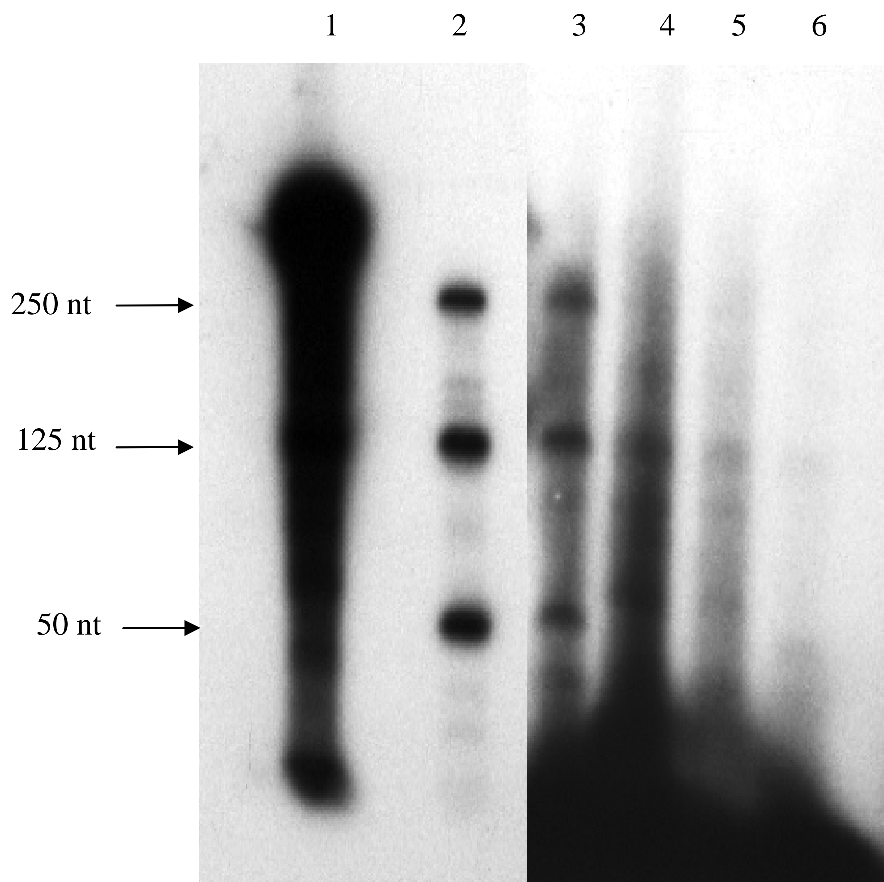


Figure 4.7 RPA analysis of nucleic acids extracted from coconut samples from Sri Lanka. Nucleic acids from sample H1 showing protected fragments similar to those for CCCVd infected coconut palm. Autoradiography was done for 12 hr and 72 hr at -70 ^{o}C for the CCCVd and coconut samples respectively.

Lane 1 probe alone not treated with RNAse

Lane 2 probe with CCCVd from coconut treated with RNAse

Lane 3 probe with H1 sample treated with RNAse

Lane 4 probe with H2 sample treated with RNAse

Lane 5 probe with LSD sample treated with RNAse

Lane 6 probe with CRD sample treated with RNAse

4.4 DISCUSSION

RPA analysis of CCCVd extracted from coconut showed that there were three major protected fragments. The largest corresponded to the 246 nt form of CCCVd. The presence of additional protected bands in the nucleic extract suggests that there are mismatches between the $CCCVd_{246}$ probe and the various forms of CCCVd reported to be found in infected coconut palm, 246, 247, 296 and 297 (Imperial *et al*., 1981; Mohamed *et al*., 1982; Haseloff *et al*., 1982).

CCCVd comprises two monomeric ‘fast’ ($CCCVd_{246}$ and $CCCVd_{247}$) and ‘slow’ ($CCCVd_{296}$ and $CCCVd_{297}$) electrophoretic forms as well as dimeric forms of each monomer (Randles, 1985). $CCCVd_{247}$ differs from $CCCVd_{246}$, and $CCCVd_{297}$ from $CCCVd_{296}$, by the insertion of a cytosine at position nt 197. The slow RNAs are directly derived from the corresponding ‘fast’ forms by a partial duplication of the right-hand terminus (TR) of the molecule (Haseloff *et al*., 1982). In addition, CCCVd also has a number of molecular forms due to the reiteration of 41, 50 or 55 nucleotides the right-hand end of the molecule, producing larger forms of the viroid of between 287 and 301 nucleotides (Haseloff *et al*., 1982).

Analysis of nucleic acids extracted from symptomatic and asymptomatic oil palms using RPA showed that 90 % of the symptomatic and 50 % of asymptomatic palms had RNAs that protected the CCCVd probe. The RPA patterns of these palms were similar to those for CCCVd infected coconut palm suggesting that CCCVd-like RNAs in oil palm were closely similar to those found in infected coconut palm. The oil palm samples possibly also contain a similar range of forms of CCCVd to that found in coconut palm.

RPA of nucleic acid extracted from the three fronds of the asymptomatic palm, SRH2, representing the different stages of the disease showed that there were variations

in the sequence of the CCCVd-like RNAs compared with CCCVd. For example, the protected fragments in SRH2 f3 (late stage) and f10 (mid stage) were unique to those samples. The protected fragments of SRH2 f20 (early stage) were similar to CCCVd suggesting that with the increasing time after infection, variation in the sequence of the CCCVd-like RNA increased. Variations in the forms of CCCVd with disease progression have been previously observed. The small 246/247 variants (with their dimers) are observed in the early stages of infection and as the disease symptoms develop, the large 287/296/297/301 variants (also with their dimers) arise and eventually become the dominant population as the disease progresses (Imperial *et al.*, 1981; Mohamed *et al.*, 1982).

The strength of the RPA signal varied between the oil palm samples suggesting that the concentration of the CCCVd-like RNAs can vary in populations of trees. Generally, the RPA signal was weak in the oil palm samples supporting earlier observations that the CCCVd-like RNAs may be present in low concentrations in the oil palm nucleic acid extracts. The fact that the RPA patterns were the same show that the weak signal is not due to sequence variability.

The detection of CCCVd-like RNAs using RPA in oil palms from Malaysia and coconut palms from Sri Lanka shows that CCCVd-like RNAs are present outside the cadang-cadang affected area in the Philippines.

In this study, RPA was found to be sensitive in detecting low concentrations of CCCVd-like RNAs in the oil palm samples compared to other methods including Northern blots. More positive samples were detected by RPA than Northern blots. Thus, RPA could be a useful method for viroid detection. The hybridization of the probe and target RNA in solution where target availability is maximal, afford increased sensitivity of detection of the target RNA (Rosenau *et al.*, 2002). The advantages of RPA as a

detection method for viroid-like molecules present in low concentrations *in vivo* are that it (i) detects mutation and mismatches in the nucleic acid sequence; (ii) works in crude mixtures of nucleic acids; (iii) detects dsRNA hybrids-suitable for RNA targets; (iv) is not sensitive to inhibitors of enzyme activity; (v) long hybridization periods maximizes chances for hybridising the targets and (vi) is less labor intensive than hybridsation assays. However, RPA does not give as much information on sequence as nucleic acid sequencing (Palukaitis *et al*., 1994).

These results showed that the CCCVd-like RNAs in the nucleic acid extract from both symptomatic and asymptomatic oil palms have high sequence similarity to CCCVd. Sequencing of these molecules would reveal the degree of sequence similarity between the oil palm and coconut variants. The high sequence similarity of the CCCVd-like RNAs in the oil palm nucleic acid extract should enable RT-PCR amplification of these RNAs using CCCVd-specific primers, which could be useful for detection purposes due to the low concentrations of these CCCVd-like RNAs in the oil palm nucleic extract. RT-PCR as an alternative diagnostic method is discussed in the next chapter. Moreover, RT-PCR amplified products can be used for cloning and sequencing.

CHAPTER 5

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

5.1 INTRODUCTION

Some viroids are present at low concentration in plant tissues, thus isolation, purification and molecular analysis becomes difficult. The introduction of the polymerase chain reaction (PCR) where cDNA of viroid molecules is amplified overcomes this problem (Puchta and Sanger, 1989). PCR is an *in vitro* method of amplifying sequences of DNA exponentially through repetitive cycles of DNA synthesis. The reaction can be achieved by annealing of specific primers to a target DNA followed by its extension using a heat stable DNA polymerase (Wetzel *et al.*, 1991). Since the initial reports by Hadidi and Yang (1990) of the adaptation of PCR for viroid detection, this technique had been used extensively in viroid research (Candresse *et al.*, 1998).

Viroids are circular single stranded RNA, and therefore, require an additional reverse transcription (RT) step to transcribe the viroid RNA to cDNA before amplification processes. The two most frequently used reverse transcriptases are isolated from either avian myeloblastosis virus (AMV-RT) or from Moloney murine leukemia virus (MMLV-RT). The RT-PCR assay has been shown to transcribe viroid RNA and amplify its cDNA with high specificity and fidelity, thus allowing the discrimination of sequences that differ by as little as single nucleotide mutations

(Hadidi and Yang, 1990; Hadidi *et al*., 1995; Candresse *et al*., 1998; Shamloul and Hadidi, 1999; Shamloul *et al*., 2002). The detection of viroids in very small amounts of total nucleic acid of infected tissues by RT-PCR is generally more sensitive than detection by hybridization using cRNA probes and return gel electrophoresis analysis (Hadidi and Yang, 1990), thus making it a good alternative diagnostic method. RT-PCR has been applied to the diagnosis of several viroids, for example ASBVd, ASSVd, CCCVd, CTiVd and CEVd (Hadidi and Yang, 1990; Yang *et al*., 1992; Schnell *et al*., 1997; Hodgson *et al*., 1998)

PCR amplification of the complete viroid genome has been successfully achieved from a variety of hosts (Hadidi and Candresse, 2003). Since viroids are circular RNA molecules, using adjacent primers that anneal within the central conserved region of the viroid with their 3' ends facing away from each other, allows full length copies to be successfully amplified in a fashion reminiscent of the inverse PCR technique (Hadidi and Yang, 1990; Yang *et al*., 1992). In addition to diagnostic applications, RT-PCR has been used for screening transgenic plants, molecular cloning and sequencing of viroids, and for studying host-viroid interactions (Hadidi *et al*., 1995; Candresse *et al*., 1998). This technique has also been used for studies of viroid infection (Zhu *et al.*, 1998).

This chapter describes the use of RT-PCR for amplifying CCCVd-like RNAs in the oil palm samples from Malaysia, for diagnosis and for application to molecular cloning as described in the next chapter.

5.2 MATERIALS AND METHODS

5.2.1 Materials

RNA extracted from both symptomatic and asymptomatic oil palms by the PEG and guanidine methods as described in Section 2.2.1a and Section 2.2.1e respectively were used for RT-PCR. RNA from healthy oil palm and coconut maintained in the Waite glasshouse were used as negative controls together with total RNA from *Nicotiana benthamiana*, extracted by the guanidine method. Total RNA from CCCVd-infected coconut palm was used as a positive control.

RT-PCR amplification was also done with PEG extracted RNA from the asymptomatic oil palm (SRH 2), which was partially purified by 2D-PAGE as described in Section 5.2.2a. 20-100 g of leaf tissue was used for extraction of nucleic acids from fronds 3 (f3) and 10 (f10) of SRH 2. Only 20 g of leaf tissue was used for the extraction from frond 20 (f20) of SRH 2 as there was insufficient leaf material remaining for this experiment. The palm SRH2 was selected for this experiment due to its strong positive results in previous experiments (Chapter 3 and 4). CCCVd extracted from coconut leaf samples was used as the positive control. RT-PCR could not be done with the symptomatic oil palms as there was not enough leaf material for the necessary large scale extractions.

5.2.2 Methods

5.2.2a Purification of CCCVd-related RNAs

Nucleic acids prepared by PEG extraction from the oil palm and coconut samples were fractionated by 2-D PAGE (Section 2.2.2b). Although laborious, this

method provided efficient fractionation of viroid-like molecules. The zones expected to contain the circular molecules and corresponding linear molecules were separately excised and eluted from the gel as described in Section 2.2.3b. Zones outside the expected viroid region were also excised to serve as a control.

5.2.2b Design of primers for RT-PCR

The sequence of $CCCVd_{246}$ (Haseloff *et al*., 1982) was used for designing the primers. The sequence of CCCVd was analysed for primer positions with the Oligo ™ program, and the primers were checked for possible hairpin structures and stability so that the most stable with the least self priming were selected. Altogether four sets of forward and reverse primers were designed in the size range of 18-25 nucleotides and another CCCVd-specific primer set from Hodgson and Randles (1997) were used for PCR (Table 5.1).

5.2.2c RT-PCR assay

The RT-PCR assay was carried out using all primer sets according to the method described in Section 2.2.4a. The PCR conditions of Hodgson *et al.* (1998) were applied for primer set I and the assay for other four sets of primer were according to Rodriguez (1993). The amplified products were analysed by 5 % non-denaturing PAGE buffered in 1X TAE and 1.5-2 % agarose gel buffered in 1X TBE.

Table 5.1 Properties of the CCCVd-specific primers used for reverse transcription (RT) and polymerase chain reaction (PCR)

Primer set	Sequence	Polarity	Position on $CCCVd_{246}$	Reaction	PCR product length
I[a]	5'-d(AGG TTT CCC CGG GGA TCC CTC AAG CGG CCT C)-3'	-ve	71-41	RT and PCR	240 nts
	5'-d(CGA ATC TGG GAA GGG AGC GTA CCT GGG TCG)-3'	+ve	76-105	PCR	
II	5'-d(GAT CCC TCA AGC GGC CTC TCC TG)-3'	-ve	58-36	RT and PCR	237 nts
	5'-d(ACG TCA AGC GAA TCT GGG AAG GGA G)-3'	+ve	68-92	PCR	
III	5'-d(AAG AGC CGC GTG AGT TGT AT)-3'	-ve	206-187	RT and PCR	198 nts
	5'-d(ATC TAC AGG GCA CCC CAA A)-3'	+ve	9-27	PCR	
IV	5'-d(CCC AGA TTC GCT TGA CGT TTC CCC G)-3'	-ve	85-61	RT and PCR	238 nts
	5'-d(GTA CCT GGG TCG ATC GTG CGC GTT G)-3'	+ve	94-118	PCR	
V	5'-d(TGT ATC CAC CGG GTA GTC TC)-3'	-ve	191-172	RT and PCR	246 nts
	5'-d(ACT CAC GCG GCT CTT ACC)-3'	+ve	192-209	PCR	

a – primer set from Hodgson and Randles (1997)

5.3 RESULTS

5.3.1 PAGE analysis

RNA from the CCCVd-infected coconut leaf, fractionated by 2D-PAGE showed a band in the zone expected for circular RNA when stained with EtBr (Figure 5.1- gel 1). 2D-PAGE analysis of RNA extracted from either 20 g or 100 g of SRH 2 f3 and f10 leaf samples showed no bands in the corresponding circular region (Figure 5.1- gel 2, 3). Gel fragments of approximately 1 x 1 cm were excised from the region of the gel which corresponded to the region expected to contain RNA the size of circular CCCVd (Figure 5.1). A gel fragment was also excised from the region of the gel expected to contain molecules the size of linear CCCVd. Portions of the gel outside the expected viroid region were also cut to serve as the controls (Figure 5.1). RNA from the excised gel pieces was eluted as described in Section 2.2.3b and used for amplification by RT-PCR.

Extracted nucleic acids from SRH2 f20 were fractionated by 5 % non-denaturing PAGE. No clear bands corresponding to CCCVd were observed when stained with EtBr. A gel piece of 1 x 1 cm was cut from the 200-350 bp region of the gel and the nucleic acids was eluted from the gel piece (Figure 5.2) for RT-PCR amplification.

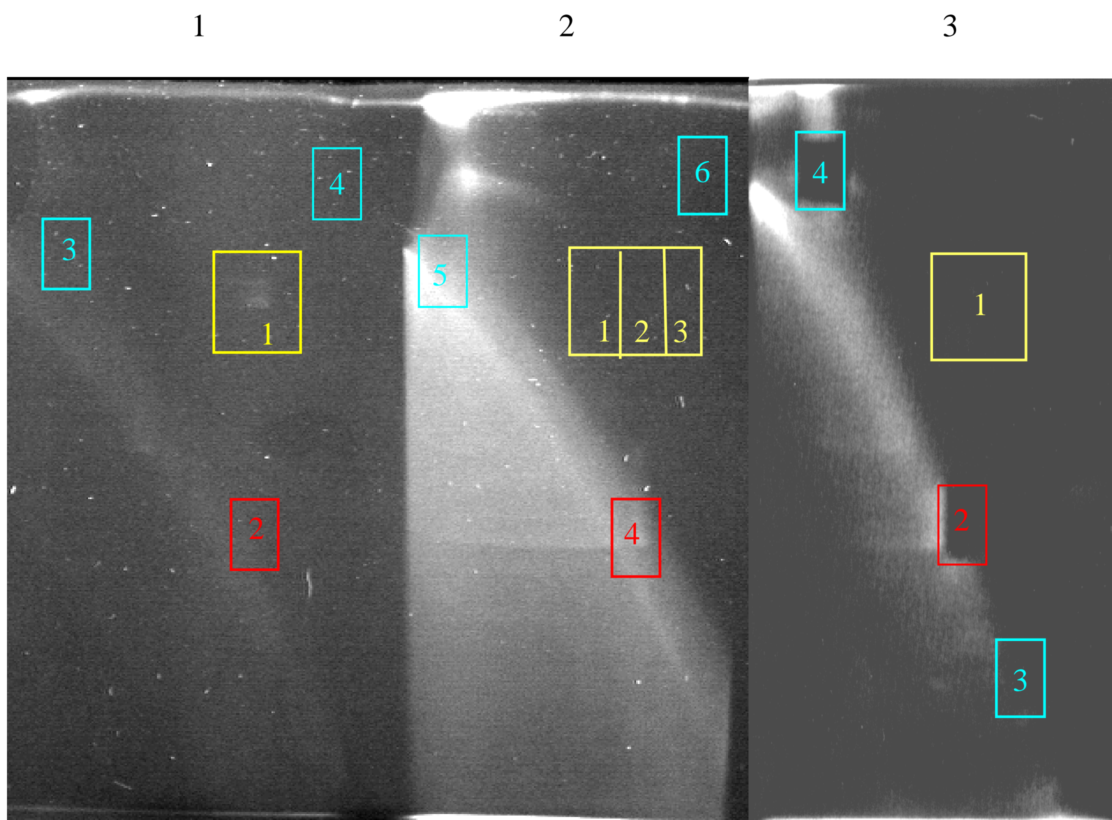


Figure 5.1 2D-PAGE of PEG extracted nucleic acids from CCCVd infected coconut sample, SRH 2 f3 and SRH2 f10 stained with EtBr. Gel pieces were cut from the expected circular RNA region (yellow), corresponding linear molecule region (red) and several control regions (green) as numbered above.

Gel 1	CCCVd infected coconut palm
Gel 2	SRH2 f10 (asymptomatic oil palm, frond 10)
Gel 3	SRH2 f3 (asymptomatic oil palm, frond 3)

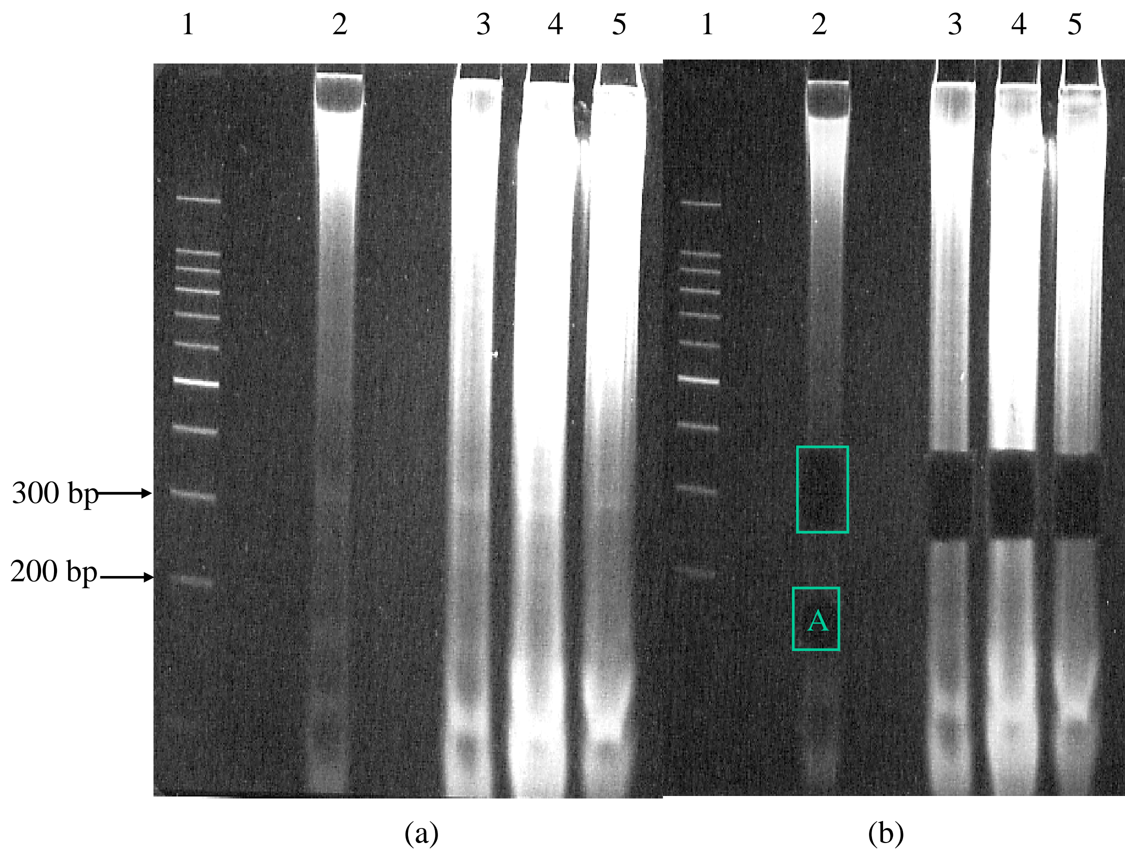


Figure 5.2 5% non-denaturing PAGE of PEG extracted nucleic acids from CCCVd infected coconut sample, SRH 2 f3, SRH2 f10 and f20 stained with EtBr. Gel pieces were cut from the 250 bp -300 bp region of the gel as shown in (b). The gel piece labeled A was used as a negative control for the RT-PCR experiments.

Lane 1	100 bp DNA ladder
Lane 2	CCCVd-affected coconut palm
Lane 3	SRH2 f3 (asymptomatic oil palm, frond 3)
Lane 4	SRH2 f10 (asymptomatic oil palm, frond 10)
Lane 5	SRH2 f20 (asymptomatic oil palm, frond 20)

5.3.2 Evaluation of the primer sets for amplification of circular and linear CCCVd from coconut

RT-PCR of RNA eluted from the circular region of the CCCVd gel (Figure 5.1-gel 1) showed an amplified PCR product of approximately 300 bp (Figure 5.3) in a 5 % non-denaturing PAGE using primer sets I, II, IV and V (Table 5.1). An additional band of approximately 150 bp was observed only when amplification was done with primer set V. Primer set II gave an amplified product of approximately 250 bp in size from both the circular (Figure 5.3-lane 3) and the linear region (Figure 5.3-lane 4) of the 2D-gel.

Based on the above results, further work with oil palm samples were done using using primer set I and V. Primer set I was used by Hodgson *et al.* (1998) for diagnosis of CCCVd and primer set V was specifically designed to amplify the full length of the CCCVd molecule.

5.3.3 RT-PCR analysis of guanidine and PEG extracted total RNA

RT-PCR amplification of guanidine extracted total RNA from the CCCVd-infected coconut palm using primer set I showed an amplified product of approximately 300 bp in size by 2 % agarose gel electrophoresis (Figure 5.4-lane 2). No amplified products were observed in any other samples including nucleic acids from both symptomatic and asymptomatic oil palms from Malaysia.

PEG extracted nucleic acids from the CCCVd-infected coconut palm and both symptomatic and asymptomatic oil palms from Malaysia gave no amplicons of the expected size when RT-PCR was done using primer sets I and V (Figure 5.5).

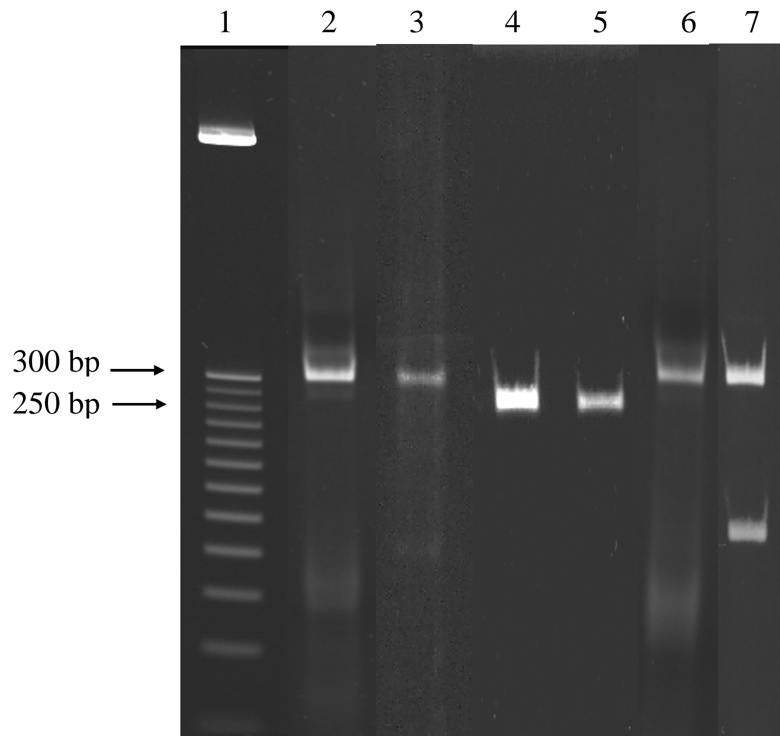


Figure 5.3 RT-PCR of RNA eluted from the circular CCCVd region from 2D-PAGE (Figure 5.1-gel 1). Amplification was done based on the protocols from Hodgson *et al*. (1998) for primer set I and Rodriguez (1993) for primer sets II, III, IV and V and analysed by 5 % non-denaturing PAGE.

Lane 1	25 bp DNA ladder
Lane 2	Primer set I
Lane 3	Primer set II
Lane 4	Primer set II (linear region)
Lane 5	Primer set III
Lane 6	Primer set IV
Lane 7	Primer set V

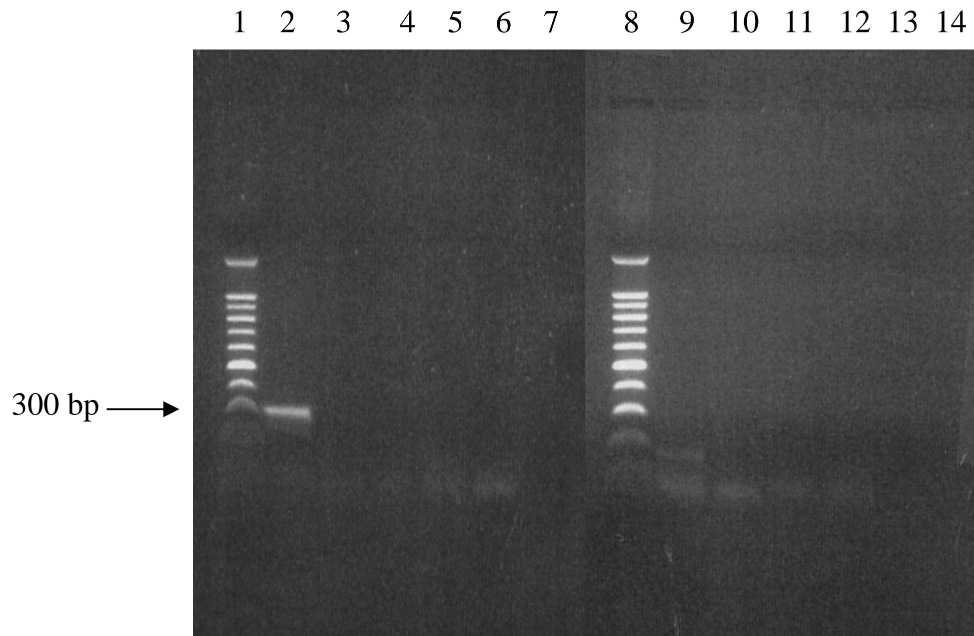


Figure 5.4 RT-PCR of guanidine extracted total RNA from coconut, oil palm and *Nicotiana benthamiana* and analysed by 2 % agarose gel electrophoresis. Amplification was done by primer set I based on the protocols from Hodgson *et al*. (1998).

Lane 1, 8	100 bp DNA ladder
Lane 2	CCCVd-affected coconut palm
Lane 3	Asymptomatic oil palm (SRH2 f20)
Lane 4	Symptomatic oil palm (SRD6 f12)
Lane 5	Asymptomatic oil palm (SRH7 f20)
Lane 6,7,13, 14	SDDW
Lane 9	Asymptomatic oil palm (SRH8 f20)
Lane 10	Healthy oil palm (from Waite glasshouse)
Lane 11	Health coconut palm (from Waite glasshouse)
Lane 12	*Nicotiana benthamiana*

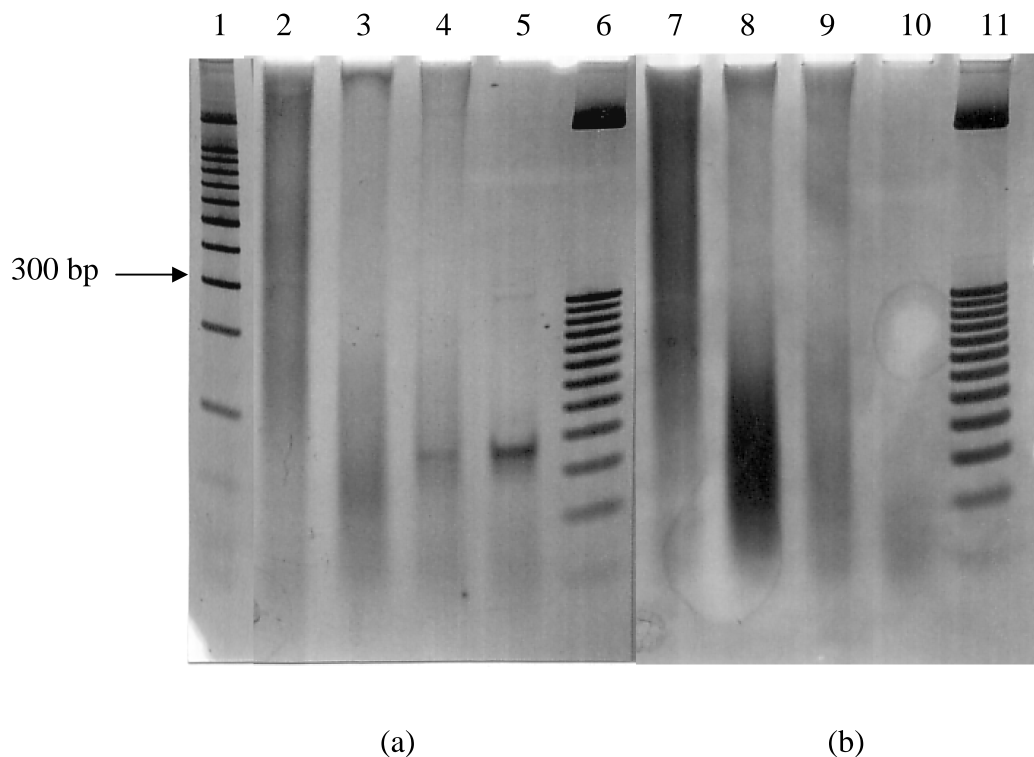


Figure 5.5 RT-PCR of PEG extracted RNA from coconut and oil palm samples analysed by 5 % non-denaturing PAGE and stained with silver. Amplification was done using primer sets I (a) and V (b) based on protocols by Hodgson *et al*. (1998) and Rodriguez (1993) respectively.

Lane 1	100 bp DNA ladder
Lane 2, 7	CCCVd-infected coconut palm
Lane 3, 8	Asymptomatic oil palm (SRH2 f20)
Lane 4, 9	Symptomatic oil palm (SRD6 f12)
Lane 5, 10	Healthy coconut palm (from Waite glasshouse)
Lane 6,11	25 bp DNA ladder

5.3.4 RT-PCR of sample from SRH 2 f3 and SRH 2 f10

RT-PCR of molecules from the circular region of the 2D-PAGE of SRH 2 f3 (Figure 5.1) analysed by 5 % non-denaturing PAGE showed an amplified PCR product of 300 bp using primer set V (Table 5.1) but it was only detectable by silver staining (Figure 5.6-lane 3). Hence, this region from the first cycle of RT-PCR was eluted and re-amplified by PCR using the same primer set. This second product was sufficient to be detected by EtBr staining (Figure 5.7-lane 7), which corresponded to the size of the amplified product of CCCVd (Figure 5.7-lane 2, 3). However, no additional 150 bp fragment was observed as in CCCVd from coconut.

No amplicon was observed when molecules from the linear region (Figure 5.1) were amplified using this primer set. No amplification was observed with eluates from the control regions of the SRH2 f3 and CCCVd samples.

RT-PCR amplification of RNA eluted from the circular region of 2D-PAGE of SRH 2 f10 also showed a similarly sized amplicon approximately 300 bp in size, using primer set I and V (Figure 5.8-lane 3, 4 and 14). As with SRH 2 f3, re-amplification was necessary for the detection of the amplified products by EtBr staining. No amplification was observed when eluates from the linear and control regions were used for amplification.

A comparison of the intensity of the EtBr stained bands from SRH2 f3 and f10 with CCCVd and the need for the re-amplification step, indicates that the CCCVd-like molecules were present at a much lower concentration in oil palm than was CCCVd in the coconut palm sample.

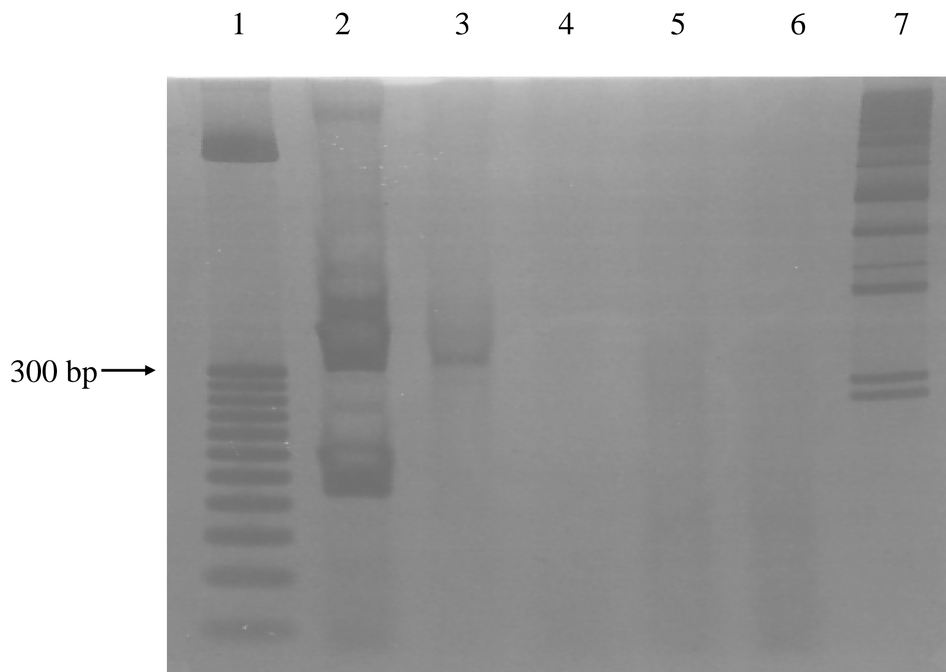


Figure 5.6 RT-PCR amplification of RNA eluted from 2D-PAGE of CCCVd and SRH2 f3 (Figure 5.1) and analysed by 5 % non-denaturing PAGE. Amplification was done using primer set V based on protocols by Rodriguez (1993).

Lane 1	25 bp DNA ladder
Lane 2	CCCVd circular region (1)*
Lane 3	SRH2 f3 circular region (1)*
Lane 4	SRH2 f3 linear region (2)*
Lane 5	SRH2 f3 control region (3)*
Lane 6	SRH2 f3 control region (4)*
Lane 7	1kb DNA marker

* numbering as shown in figure 5.1

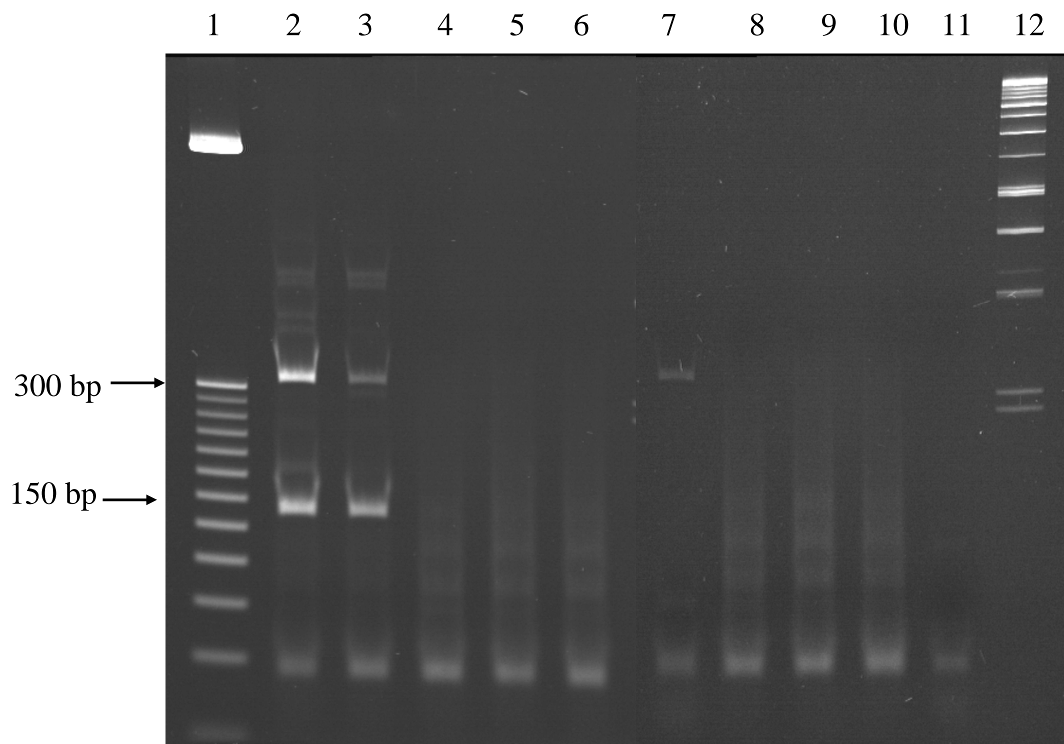


Figure 5.7 RT-PCR amplification of RNA eluted from 2D-PAGE of CCCVd and SRH2 f3 (Figure 5.1) and analysed by 5 % non-denaturing PAGE. Amplification was done using primer set V based on protocols by Rodriguez (1993).

Lane 1	25 bp DNA ladder
Lane 2, 3	CCCVd circular region (1) *
Lane 4	CCCVd linear region (2) *
Lane 5, 6	CCCVd control region (3 and 4) *
Lane 7	SRH2 f3 circular region (1) *
Lane 8	SRH2 f3 linear region (2) *
Lane 9, 10	SRH2 f3 control region (3 and 4) *
Lane 11	SDDW
Lane 12	1kb DNA marker

* numbering as shown in figure 5.1

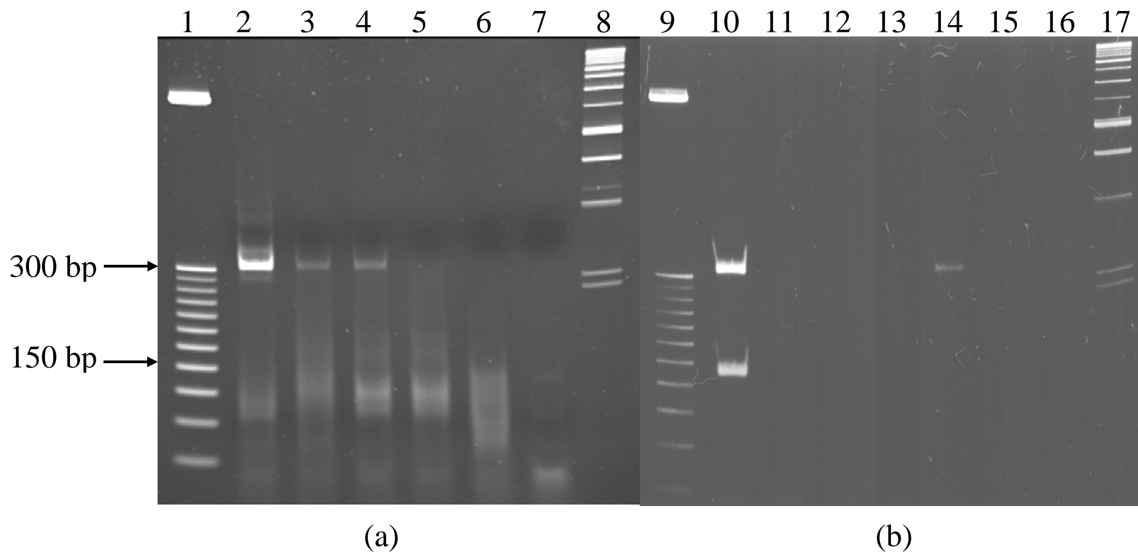


Figure 5.8 RT-PCR amplification of molecules eluted from 2D-PAGE of CCCVd and SRH2 f10 (Figure 5.1) and analysed by 5 % non-denaturing PAGE. Amplification was done using primer set I (a) and V (b) based on protocols by Hodgson *et al*. (1998) and Rodriguez (1993) respectively.

Lane 1, 9	25 bp DNA ladder
Lane 2	CCCVd circular region (1) *
Lane 3	SRH2 f10 circular region (1) *
Lane 4	SRH2 f10 circular region (2) *
Lane 5	SRH2 f10 circular region (3) *
Lane 6	SRH2 f10 linear region (4) *
Lane 7	SRH2 f10 control region (5) *
Lane 8, 17	1kb DNA marker
Lane 10	CCCVd circular region (1) **
Lane 11	CCCVd linear region (2)**
Lane 12	SRH2 f10 control region (6) **
Lane 13	SRH2 f10 circular region (1) **
Lane 14	SRH2 f10 circular region (2) **
Lane 15	SRH2 f10 circular region (3) **
Lane 16	SDDW

* numbering as shown in figure 5.1, amplification using primer set I

** numbering as shown in figure 5.1, amplification using primer set V

5.3.5 RT-PCR of sample from SRH 2 f20 prepared by 1D PAGE

RT-PCR of RNA eluted from gel pieces of SRH 2 f20 from a 5 % non-denaturing PAGE (Figure 5.2) showed an amplified PCR product of 300 bp (Figure 5.9-lane 5) using primer set I (Table 5.1), which corresponded to the size of the amplified product of CCCVd (lane 2). Re-amplification was done twice (as described in Section 5.3.4) to see the amplified fragment with EtBr staining indicating that the molecules were present in the sample at a low concentration. Using this method, RNA from SRH 2 f3 was also amplified but no amplification was observed using sample SRH 2 f10. Amplification was not observed from the control region (Figure 5.2).

5.3.6 Analysis of RT-PCR amplified products in agarose gel electrophoresis

The re-amplified products from SRH2 (f3, f10 and f20) analysed on 2 % agarose with EtBr staining showed a band of approximately 300 bp as observed for CCCVd. Additional bands around the 200 bp region were observed in the SRH2 samples (Figure 5.10 lane 2-13) but not in the CCCVd sample (Figure 5.10 lane 14-16). The bands appeared to differ in intensity in all three oil palm samples with SRH2 f3 showing the highest intensity followed by SRH2 f10 and the least in SRH2 f20.

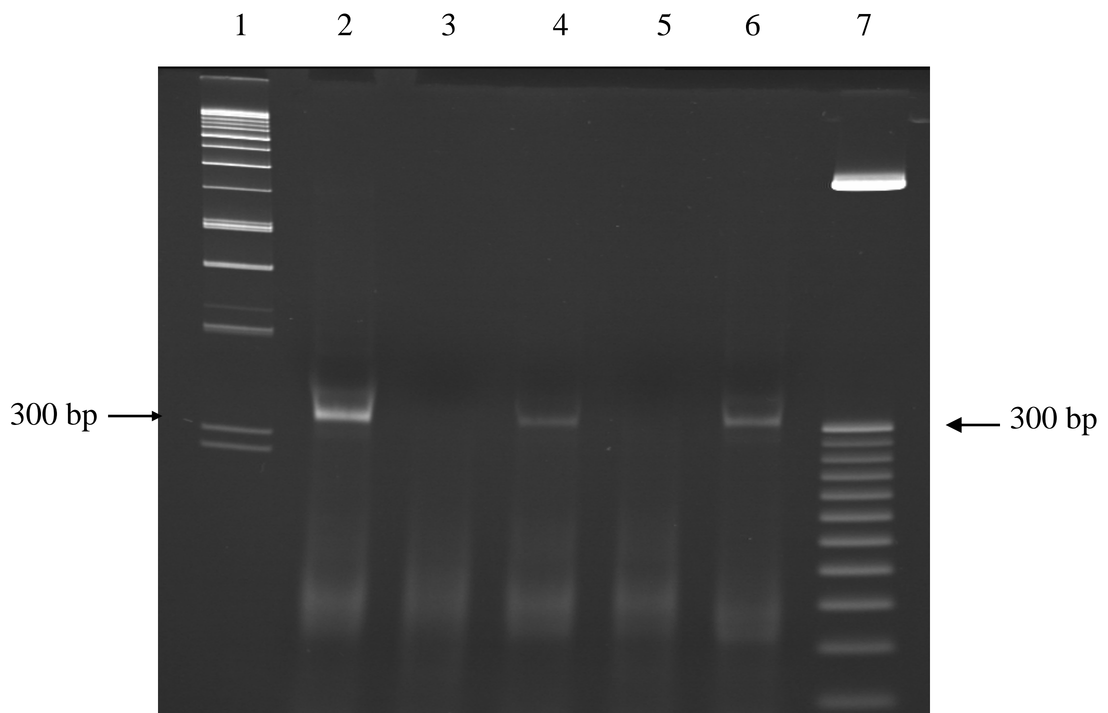


Figure 5.9 RT-PCR amplification of RNA eluted from 1D PAGE of CCCVd, SRH2 f3, f10 and f 20 (see Figure 5.2) and analysed by 5% non-denaturing PAGE. Amplification was done using primer set I based on protocols by Hodgson *et al*. (1998).

Lane 1	1 kb DNA marker
Lane 2	CCCVd
Lane 3	control region (A) (from Figure 5.2)
Lane 4	SRH2 f3
Lane 5	SRH2 f10
Lane 6	SRH2 f20
Lane 7	25 bp DNA ladder

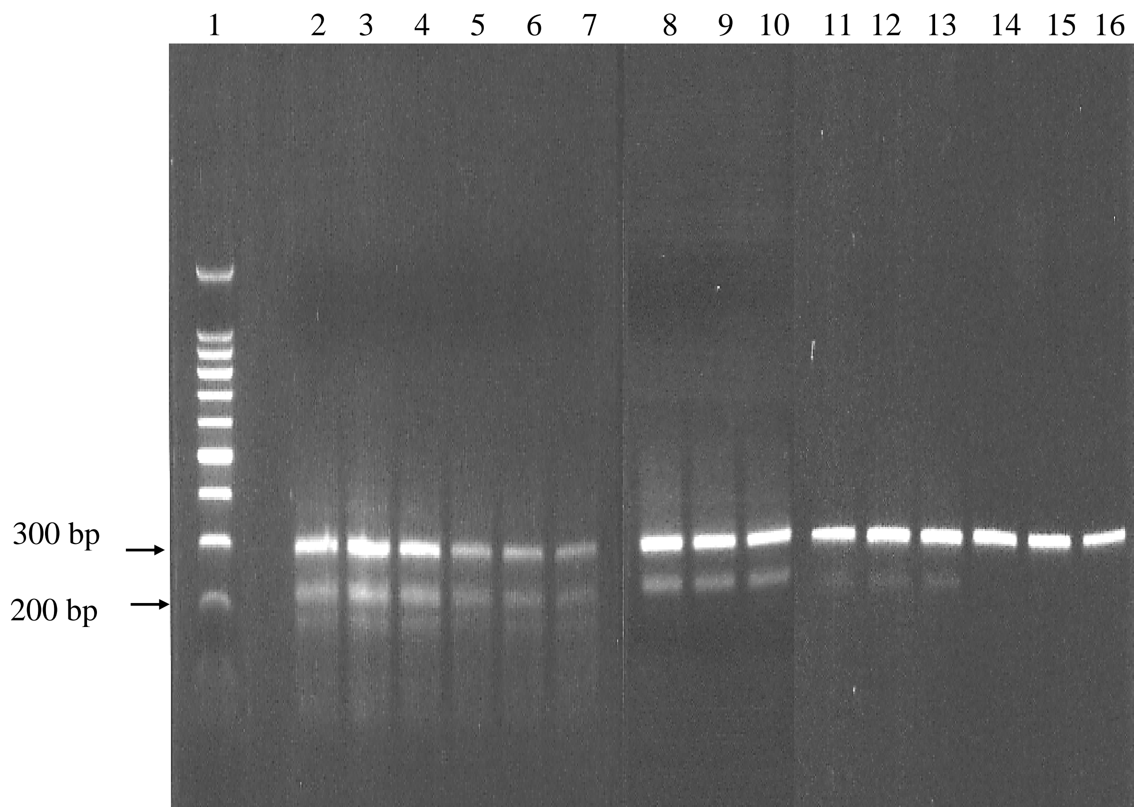


Figure 5.10 RT-PCR amplified product of RNA preparations from SRH2 f3, f10 and f20 analysed using 2% agarose gel electrophoresis showing additional bands in the 200 bp region. No 200 bp band was observed for CCCVd. Amplification was done twice using primer set I based on protocols by Hodgson *et al*. (1998).

Lane 1	100 bp DNA ladder
Lane 2-4	SRH2 f3 (circular region of 2D-PAGE, figure 5.1)
Lane 5-7	SRH2 f3 (1D-PAGE, figure 5.2)
Lane 8-10	SRH2 f10 (circular region of 2D-PAGE, figure 5.1)
Lane 11-13	SRH2 f20 (1D-PAGE, figure 5.2)
Lane 14-16	CCCVd (circular region of 2D-PAGE, figure 5.1)

5.4 DISCUSSION

RT-PCR of nucleic acid eluted from the circular region of the 2D-PAGE of an asymptomatic (SRH2) oil palm using CCCVd-specific primers produced an amplicon 300 bp in size that corresponded to the size of the amplified product from the circular region of 2D-PAGE fractionated CCCVd-infected coconut leaf. Amplicons of similar size were also observed in all three fronds of SRH 2, which represents different stages of the disease; early (f20), middle (f10) and late (f3). Moreover, additional bands were observed in the 200 bp region when analysed by 2 % agarose gel electrophoresis indicating that additional variants of CCCVd may be present in nucleic acid extracts from all three fronds of SRH2.

Amplification was only successful when nucleic acids were partially purified using 1D or 2D-PAGE. PEG extracted nucleic acids used as starting material for RT-PCR amplification failed to produce any amplified products indicating that the PEG extracted nucleic might contain impurities that inhibit the synthesis of cDNA. On the other hand, RT-PCR amplification using guanidine extracted nucleic acids only showed an amplified product in the CCCVd-infected coconut sample. The starting leaf material used for the guanidine extraction was 0.02g, which may not be enough for extracting sufficient CCCVd-like RNAs from the oil palm samples as the concentration of these RNAs have been shown to be low in Chapter 3 and 4.

The low concentration probably explains why the RT-PCR products from the oil palm sample were not generally detected with EtBr staining but detectable by silver staining. Hence, the RT-PCR products of the first cycle had to be eluted after fractionation by 5 % PAGE and re-amplified by PCR using the same primer set. This second product was sufficient to be detected by EtBr staining and obtained with both primers sets. This result indicates that RT-PCR could not be used for diagnosis of the

CCCVd-like RNAs in oil palm. However, RT-PCR using the primer sets described in this chapter could be useful as an alternative diagnostic method for CCCVd-like RNAs from oil palms provided the template is free from impurities and the nucleic acids are concentrated to have sufficient amount of template for the RT reaction.

The detection of additional amplified products (Section 5.3.5) indicates that there could be more than one variant of the CCCVd-like RNA in the oil palm sample, which agrees with the observations in Chapter 3 and 4. These bands were not observed in the CCCVd sample.

The absence of amplification from samples collected from zones outside the expected viroid region indicates that the primers are specific for amplification of CCCVd and CCCVd-like RNAs from the oil palm. This confirms previous observation (Chapter 4) that the CCCVd-like RNAs in the oil palm have high sequence similarity with CCCVd such that they can be amplified using CCCVd-specific primers.

Primer sets I and V could be useful for RT-PCR amplification for cloning and sequencing as these primer sets allow the amplification of full length circular viroid molecules. Hadidi and Yang (1990) and Yang *et al*. (1992) have reported that using primers that anneal to the central conserved region of the circular viroid with 3’ ends facing away from each other, full length cDNA copies of a number of viroids can be amplified.

The RT-PCR experiments show that the RNA molecule in the oil palm sample has high sequence similarity with CCCVd, which enabled the CCCVd-specific primers to amplify it even though amplification had to be done more than once. However, these CCCVd-like RNAs need to be sequenced to determine their similarity to CCCVd. This is described in Chapter 6.

CHAPTER 6

CLONING AND SEQUENCING OF CCCVd-RELATED RT-PCR PRODUCTS

6.1 INTRODUCTION

Viroids were first characterized by direct RNA sequencing (Gross *et al.*, 1978), which was a time consuming approach that required a large amount of RNA. With the introduction of cDNA-based methodologies, direct sequencing has been restricted to attaining a portion of the RNA sequence for the synthesis of one or more specific primers (Visvader and Symons, 1985; Koltunow and Rezaian, 1988**;** Hernandez and Flores, 1992).

Visvader and Symons (1985) and Puchta and Sanger (1989) used viroid-specific DNA primers for the complete first and second strand cDNA synthesis but this method required prior knowledge of at least a portion of the RNA sequence of the viroid. An RT-PCR approach based on the use of random hexamers (Lakshman *et al.*, 1992) allowed unknown target sequences to be determined but this method required a large amount of template as well as the presence of single restriction sites in the cDNA to be cloned. Navarro *et al.*, (1998) developed a cloning method based on random-PCR approaches (Froussard, 1992; Don *et al.*, 1993) which only required very low amount of template (Navarro *et al.*, 1996) and no prior knowledge of the viroid sequence.

Sequence similarity searches are used to identify which family the viroid belongs to or whether it is a new species or a variant of a viroid. The main search program used to determine sequence similarity is the BLAST. Several data bases are

available for viroid nucleic acid sequences including GenBank (MD, USA) and EMBL (Cambridge, UK).

This chapter describes the cloning and sequencing of RT-PCR amplified products obtained from an asymptomatic oil palm sample (SRH2) using CCCVd specific primers.

6.2 MATERIALS AND METHODS

6.2.1 Materials

The CCCVd-like RNAs used for cloning were obtained by RT-PCR experiments of the RNA extracted from fronds 3, 10 and 20 of the asymptomatic oil palm (SRH 2) (Chapter 5).

6.2.2 Methods

6.2.2a Synthesis of cDNA from CCCVd-like RNA by RT-PCR and cloning of the PCR products

cDNA was transcribed from gel purified CCCVd-like RNA with AMV reverse transcriptase (Section 2.2.5a). Amplicons were synthesized according to Hodgson *et al.* (1998) and Rodriguez (1993) (Section 2.2.5). They were analysed by 2 % agarose gel electrophoresis and PCR products similar in size to the PCR product expected for CCCVd were excised and extracted from the gel fragment (Section 2.6.6a)

Cloning of PCR products was done using the QIAGEN PCR Cloning kit (QIAGEN Australia Pty Ltd) as described in Section 2.2.6. Amplicons were ligated into the pDrive cloning vector (Section 2.6.6b) which was then transformed into competent

cells by electroporation (Section 2.2.6c). Plasmids were prepared from the transformed bacteria as described in Section 2.2.7. The plasmids were analysed to check the insert size (Section 2.2.8) and clones with the expected insert size were selected for sequencing.

6.2.2b Sequence analysis

Selected clones were sequenced, by AGRF as described in Section 2.2.9b. Sequences were edited with the CHROMAS program. Comparisons with sequences in GenBank were done using BLAST in the NCBI website (Section 2.2.9c). Sequence alignment was done with Clustal W (Chenna *et al.*, 2003) and the GENEDOC program (Nicholas *et al.*, 1997). Secondary structure was predicted by Mfold version 3.1 (Zuker, 2003).

6.3 RESULTS

6.3.1 Selection of recombinant clones

The RT-PCR amplified products (Chapter 5) were cloned into the *Eco*RI site of the pDrive cloning vector and 20 recombinant bacterial colonies were selected. Inserts ranged from 150 bp to 300 bp in size (Figure 6.1) but the majority (16 clones) were about 300 bp in length.

6.3.2 Sizes of cloned inserts

Of the 20 clones that were sequenced, twelve contained inserts of 297 nts (OP_{297}), four contained inserts of 293 nts (OP_{293}), two contained inserts of 232 nts (OP_{232}), one contained an insert of 165 nt (OP_{165}) and one contained an insert of 270 nt (OP_{270}). All showed high sequence similarity with 296 nt variant of CCCVd ($CCCVd_{296}$).

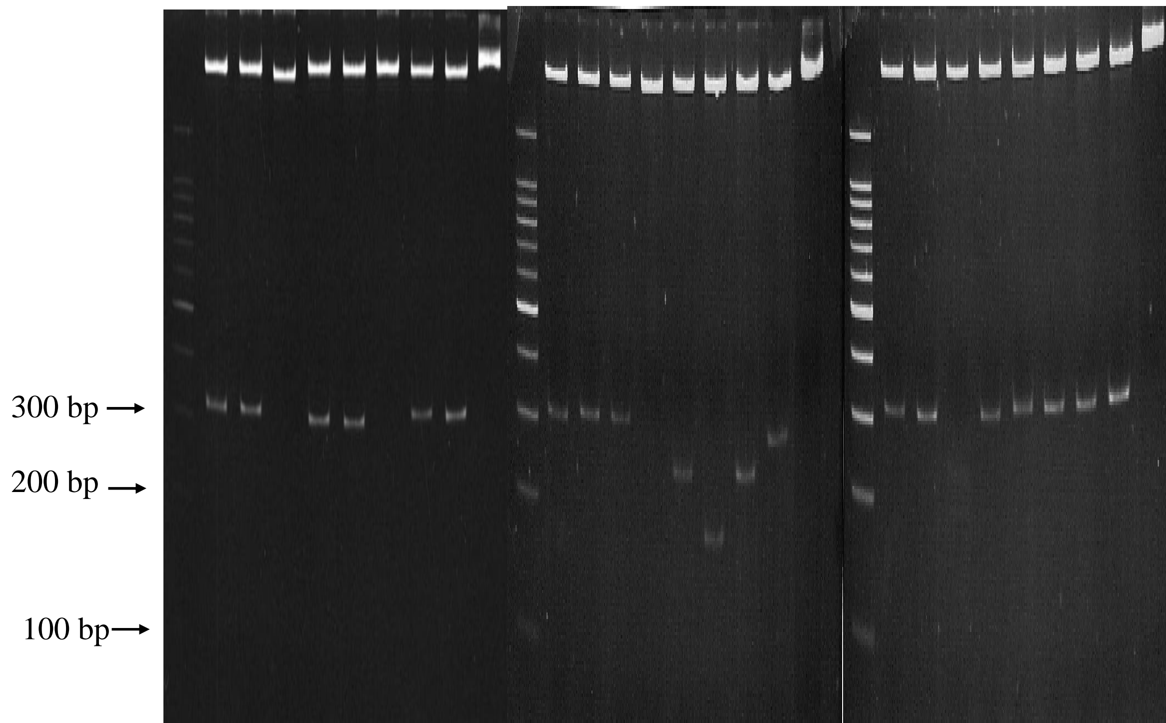


Figure 6.1 Identification of clones with inserts by digestion of plasmid with EcoRI. 20 clones contained inserts ranging from 150 bp to 300 bp in size.

6.3.3 Analysis of primary sequences

Of the 12 OP_{297} clones, four were identical and were defined as the consensus sequence for this variant (Appendix H). Alignment of the consensus sequence of OP_{297} with $CCCVd_{296}$ showed that they had 98 % sequence similarity (Figure 6.2). OP_{297} had an insertion and four single base substitutions compared to $CCCVd_{296}$ (Figure 6.2). The determination of the secondary structure at 37 ^{o}C with a minimum computed folding energy of approximately -138.2 kcal/mole showed that the duplication of the right hand terminal domain (TR) in $CCCVd_{296}$ (Figure 6.3b) was also present in OP_{297} (Figure 6.3c). Compared with $CCCVd_{296}$, OP_{297} had an insertion of G at position 124 in the TR domain and the four single base substitutions were at positions 31 (C→U) of the pathogenicity (P) domain, 75 (G→A) of the central conserved region (CCR), 105 (A→U) and 175 (A→C) in the TR of $CCCVd_{296}$. The remaining eight clones had one to five additional base substitutions in their sequence compared with the consensus sequence and these were distributed across all five domains of the secondary structure (Figure 6.3c)

The consensus sequence of OP_{293} (Appendix H) was derived from two identical clones of the four clones sequenced. It differed from OP_{297} only in that it had four deletions at positions 20 (-A), 243 (-A), 281 (-G), 282 (-U) and two base substitutions at positions 113 (G→U) and 171 (G→C) of OP_{297} (Figure 6.2). It therefore had 97 % sequence similarity with $CCCVd_{296}$. As shown in Figure 6.4a, three deletions and a base substitution occurred in the P domain of the molecule. Another deletion occurred in the C domain. Three other base substitutions occurred at TR domain of the molecule. The other two clones had three and five additional base substitutions respectively, which occurred in the variable (V), TR and P domains (Figure 6.4a).

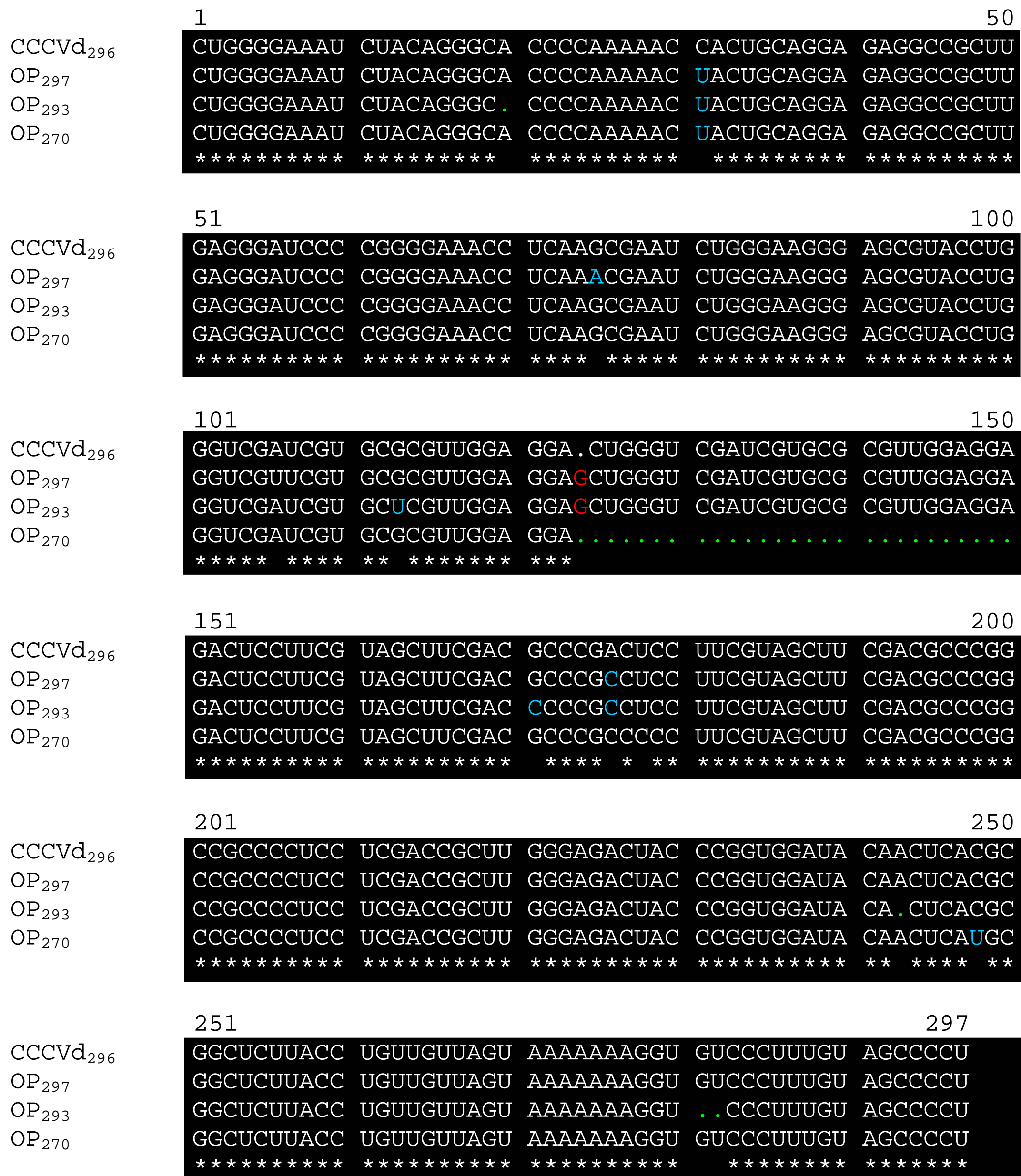
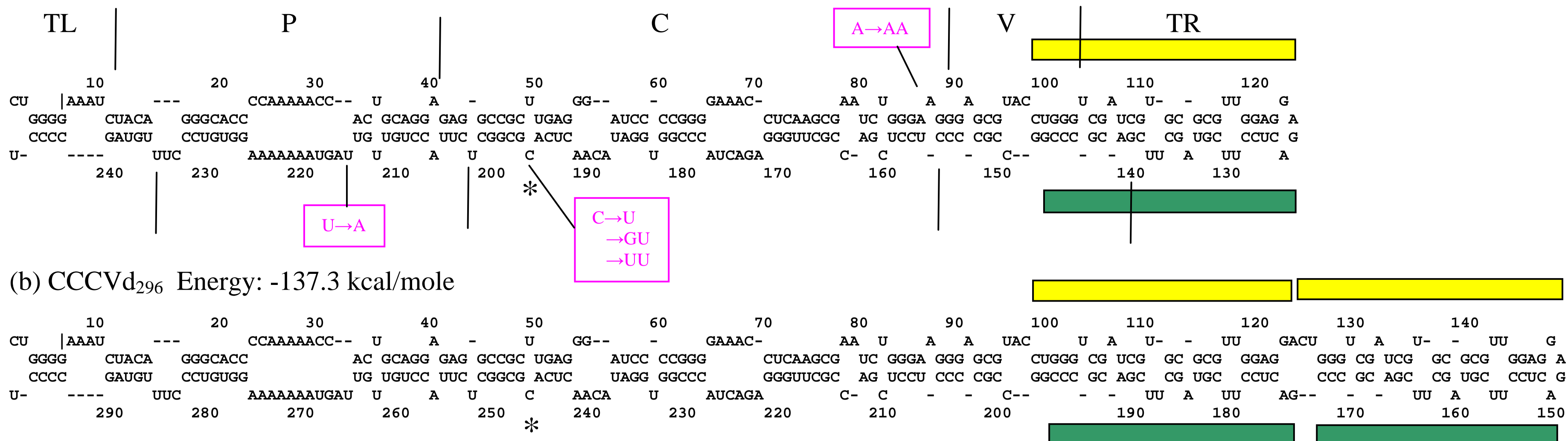
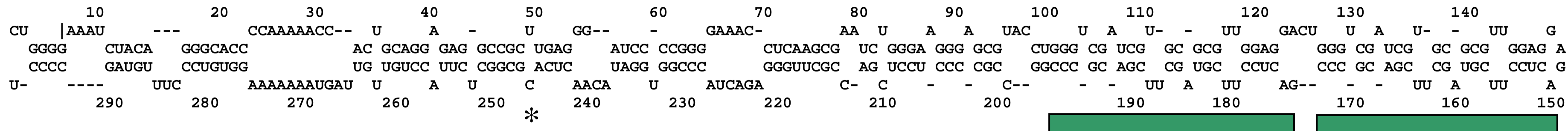


Figure 6.2 Alignment of consensus sequences of OP_{297}, OP_{293} and OP_{270} (Appendix G) with the sequence of $CCCVd_{296}$ and using Clustal W. Letters in blue indicate substitution; red indicates an insertion; a green dot indicates deletion. "*" indicate nucleotides which are identical in all sequences in the alignment.

Figure 6.3 Secondary structures with a minimum computed folding energy at 37 ^{o}C of (a) $CCCVd_{246}$, (b) $CCCVd_{296}$ and (c) consensus OP_{297}. The sites where mutations occurred compared with $CCCVd_{296}$ are indicated. Those found in OP_{297}, OP_{293} and OP_{270} are indicated in blue, whereas those specific to one variant are shown in black. Additional mutation sites found in single clones of OP_{297} are indicated in orange (Appendix G). Duplicated sequences are indicated with the same colour. White dots in the duplicated sequences indicate imperfect duplication due to base substitutions. The base substitutions indicated in pink in $CCCVd_{246}$ are the mutation points of the 'brooming' mutant as reported by Rodriguez and Randles (1993).


(a) CCCVd246 Energy: -112.5 kcal/mole
TL
P
C
V
TR
A→AA
10 20 30 40 50 60 70 80 90 100 110 120
CU |AAAU --- CCAAAAACC-- U A - U GG-- - GAAAC- AA U A A UAC U A U- - UU G
GGGG CUACA GGGCACC AC GCAGG GAG GCCGC UGAG AUCC CCGGG CUCAAGCG UC GGGA GGG GCG CUGGG CG UCG GC GCG GGAG A
CCCC GAUGU CCUGUGG UG UGUCC UUC CGGCG ACUC UAGG GGCCC GGGUUCGC AG UCCU CCC CGC GGCCC GC AGC CG UGC CCUC G
U- ---- UUC AAAAAAAUGAU U A U C AACA U AUCAGA C- C - - C-- - - UU A UU A
240 230 220 210 200 190 180 170 160 150 140 130
U→A
C→U
→GU
→UU


(b) CCCVd296 Energy: -137.3 kcal/mole
10 20 30 40 50 60 70 80 90 100 110 120 130 140
CU |AAAU --- CCAAAAACC-- U A - U GG-- - GAAAC- AA U A A UAC U A U- - UU GACU U A U- - UU G
GGGG CUACA GGGCACC AC GCAGG GAG GCCGC UGAG AUCC CCGGG CUCAAGCG UC GGGA GGG GCG CUGGG CG UCG GC GCG GGAG GGG CG UCG GC GCG GGAG A
CCCC GAUGU CCUGUGG UG UGUCC UUC CGGCG ACUC UAGG GGCCC GGGUUCGC AG UCCU CCC CGC GGCCC GC AGC CG UGC CCUC CCC GC AGC CG UGC CCUC G
U- ---- UUC AAAAAAAUGAU U A U C AACA U AUCAGA C- C - - C-- - - UU A UU AG-- - - UU A UU A
290 280 270 260 250 240 230 220 210 200 190 180 170 160 150

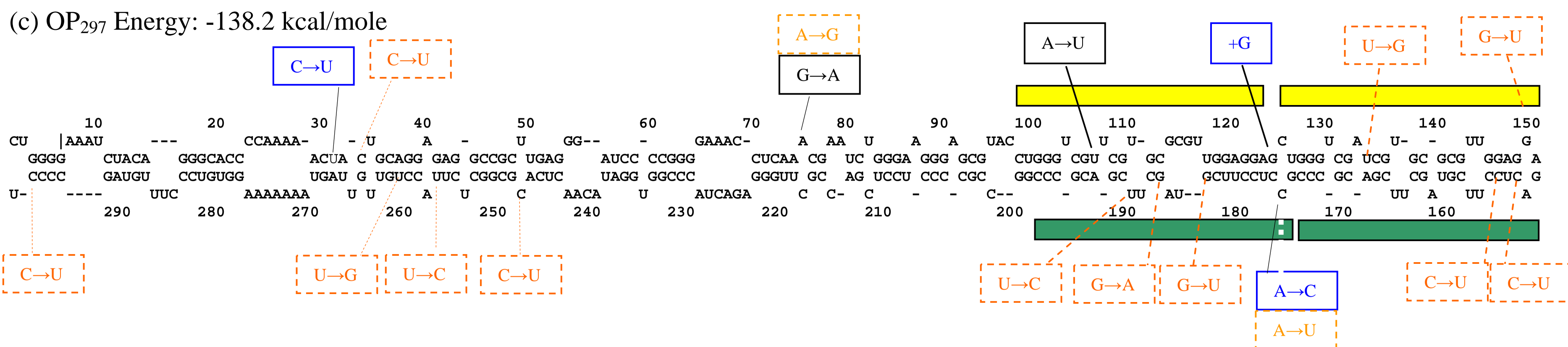

(c) OP297 Energy: -138.2 kcal/mole
C→U
C→U
A→G
G→A
A→U
+G
U→G
G→U
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
CU |AAAU --- CCAAAA- - U A - U GG-- - GAAAC- A AA U A A UAC U U U- GCGU C U A U- - UU G
GGGG CUACA GGGCACC ACUA C GCAGG GAG GCCGC UGAG AUCC CCGGG CUCAA CG UC GGGA GGG GCG CUGGG CGU CG GC UGGAGGAG UGGG CG UCG GC GCG GGAG A
CCCC GAUGU CCUGUGG UGAU G UGUCC UUC CGGCG ACUC UAGG GGCCC GGGUU GC AG UCCU CCC CGC GGCCC GCA GC CG GCUUCCUC GCCC GC AGC CG UGC CCUC G
U- ---- UUC AAAAAAA U U A U C AACA U AUCAGA C C- C - - C-- - - UU AU-- C - - UU A UU A
290 280 270 260 250 240 230 220 210 200 190 180 170 160
C→U
U→G
U→C
C→U
U→C
G→A
G→U
A→C
A→U
C→U
C→U

Figure 6.4 Secondary structures with a minimum computed folding energy at 37 ^{o}C of (a) consensus OP_{293} and (b) OP_{270}. The sites where mutations occurred compared with $CCCVd_{296}$ are indicated. Those found in OP_{297}, OP_{293} and OP_{270} are indicated in blue, whereas those specific to one variant are shown in black. Additional mutation sites found in single clones of OP_{293} are indicated in orange (Appendix G). Duplicated sequences are indicated with the same colour. White dots in the duplicated sequences indicate imperfect duplication due to base substitutions.

a) OP_{293} Energy: -134.4 kcal/mole

```
        10              20          30          40          50           60          70          80          90         100         110         120         130         140
CU    |AAAU      ---    CCCAAAA    - U       A    -       U     GG-       -      GAAAC-          AA  U    A    A   UAC       U  A   U-    -  UU      AGCU         UC   GC   UU    G
  GGGG     CUACA    GGGCC          ACUA C GCAGG GAG GCCGC UGAG    AUCC CCGGG         CUCAAGCG  UC GGGA GGG GCG   CUGGG CG UCG  GCU CG  GGAGG    GGGUCGA  GU  GCG  GGAG A
  CCCC     GAUGU    CCUGG          UGAU G UGUCC UUC CGGCG ACUC    UAGG GGCCC         GGGUUCGC  AG UCCU CCC CGC   GGCCC GC AGC  CGA GC  CCUCC    CCCAGCU  CG  UGC  CCUC G
U-     ----    UUC      AAAAAAA    U U     A    U      C    ACA       U      AUCAGA          C-  C    -    -   C--       -  -  UU  U  UU     GCC-         U-   A-   UU    A
  290             280        270         260         250         240          230         220         210          200          190         180         170         160        150
```

-A

C→U

-GU

U→C

-A

A→U

A→U

U→G

G→U

+G

G→U

G→A

A→C

G→C

C→G

b) OP_{270} Energy: -119.0 kcal/mole

```
        10              20            30          40          50           60          70          80          90         100         110         120
CU    |AAAU      ---      CCAAAA-    - U       A    -       U     GG--      -      GAAAC-          AA  U    A    A   UAC       U  A   .-UGC    UU    G
  GGGG     CUACA    GGGCACC        ACUA C GCAGG GAG GCCGC UGAG    AUCC CCGGG         CUCAAGCG  UC GGGA GGG GCG   CUGGG CG UCG       GCG  GGAG A
  CCCC     GAUGU    CCUGUGG        UGAU G UGUCC UUC CGGCG ACUC    UAGG GGCCC         GGGUUCGC  AG UCCU CCC CGC   GGCCC GC AGC       UGC  CCUC G
U-     ----    UUC         AAAAAAA    U U     A    U      U     AACA      U      AUCAGA          C-  C    -    -   C--       -  -  / \---    UU    A
.              260       250        240         230         220          210         200         190          180          170       U    A   130
                                                                                                                                       U    -
                                                                                                                                        CG
                                                                                                                                        GC
                                                                                                                                        AU
                                                                                                                                       U  U
                                                                                                                                         GC
                                                                                                                                         CG
                                                                                                                                         UA
                                                                                                                                        U  C
                                                                                                                                        C  G
                                                                                                                                        C  C
                                                                                                                                        C  C
                                                                                                                                        C  C
                                                                                                                                         CG
```

160

140

150

C→U

C→U

U→C

A→C

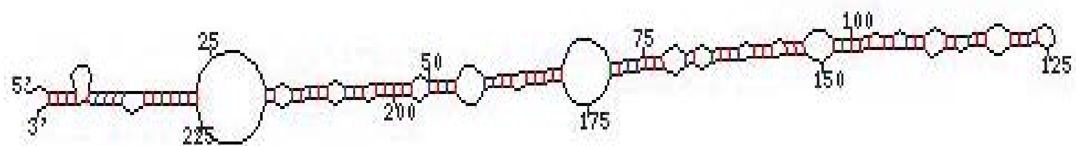
The base substitutions at 75 (G→A) and 105 (A→U) of OP_{297} were not observed in OP_{293}. However, the insertion of G at position 124 and the base substitutions at 31 (C→U) and 175 (A→C) of OP_{297} were present in OP_{293}. It also had the duplication of the TR domain observed in $CCCVd_{296}$ and also in OP_{297}.

Alignment of OP_{270} with $CCCVd_{296}$ showed 90 % sequence similarity and also that it lacked 26 nt from position 124 to 149 of $CCCVd_{296}$ together with four single base substitutions (Figure 6.2, Appendix H). Analysis of the secondary structure of OP_{270} showed no duplication of the top strand TR domain (Figure 6.4b-indicated by yellow bar), which compared with OP_{297} and $OP_{293,}$ represented a deletion of 27 nt (figure 6.2). However, the lower strand TR was duplicated (Figure 6.4b-indicated by green bar) except that the two base substitutions at positions 149 (A→C) and 151 (U→C) made this an imperfect duplication. In addition, there were two more single base substitutions at positions 31 (C→U) in the P domain and 221 (C→U) in C domain of the OP_{270} molecule.

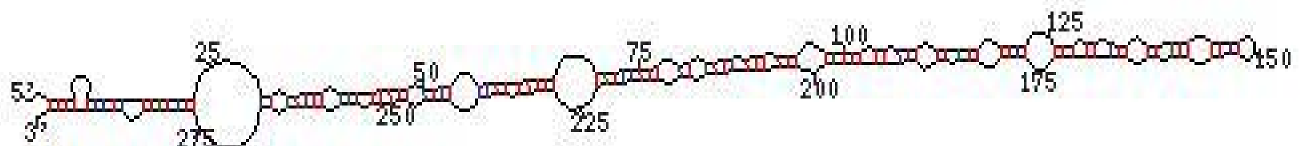
The changes in the nucleotide sequences observed in the oil palm variants resulted in changes in the secondary structure of these variants compared to the secondary structure of $CCCVd_{296}$. The base substitutions observed in OP_{297} seems to have added additional loops to the secondary structure of OP_{297} (Figure 6.5c) compared with $CCCVd_{296}$ (Figure 6.5b) as indicated by the boxed region of the secondary structure. An addition of G at position 124 appeared to have induced a G-C base pairing thus reducing the size of the loop, which now consists only of a C loop. The secondary structure of OP_{293} showed minor changes in the TR compared with $CCCVd_{296}$ (Figure 6.5d) but OP_{270} assumed a branched conformation at TR to accommodate the duplication of the lower TR domain alone (Figure 6.5e).

Figure 6.5 Secondary structures with a minimum computed folding energy at 37 ^{o}C of (a) $CCCVd_{246}$, (b) $CCCVd_{296}$, (c) consensus OP_{297}, (d) consensus OP_{293} and (e) OP_{270}. Boxed regions indicate the changes in the secondary structures of the oil palm variants compared to $CCCVd_{296}$. OP_{270} showed a branched conformation due to the changes in the sequence of the molecule.

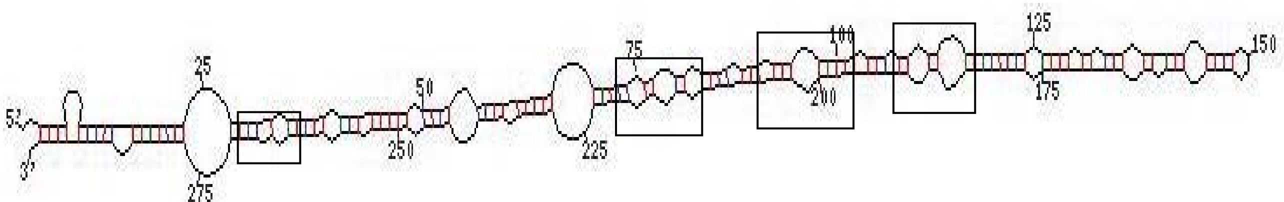
a) $CCCVd_{246}$



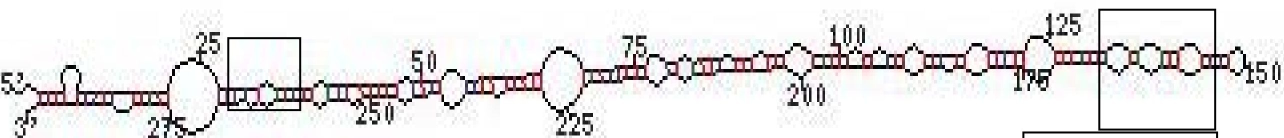
b) $CCCVd_{296}$



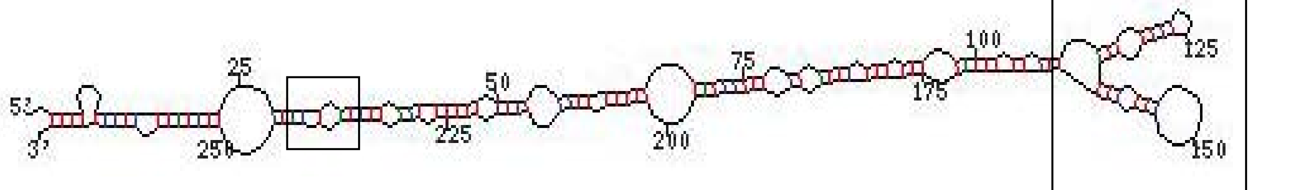
c) OP_{297}



d) OP_{293}



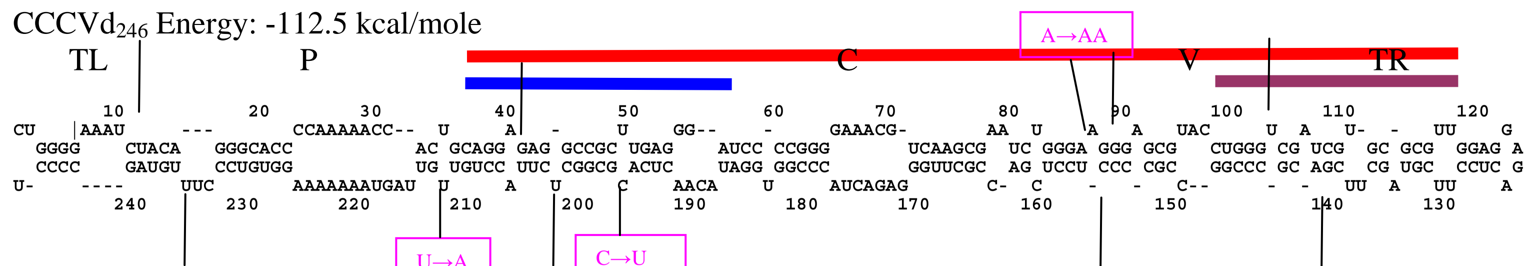
e) OP_{270}

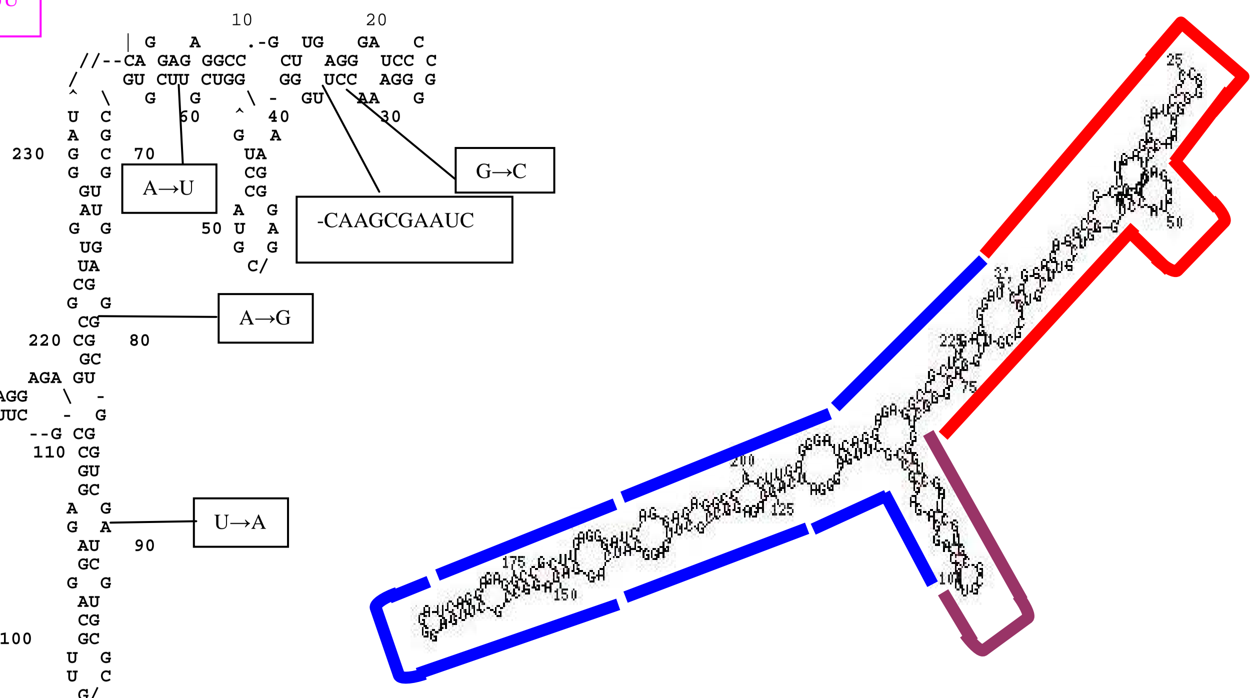


Alignment of OP_{232} sequence with $CCCVd_{246}$ showed that it contains partial sequence of $CCCVd_{246}$ of 89 nt in length from position 36 to 124 which includes a part of the P domain and the entire C, V and TR domain with a deletion of 6 nt from position 76 to 81 (Figure 6.6, Appendix H). The secondary structure of OP_{232} (Figure 6.6) with a minimum computed folding energy of approximately -91.1 kcal/mole showed that position 125 to 145 of OP_{232} is a 20 nt duplication of position 98 to 118 of the $CCCVd_{246}$. The rest of the sequence of the variant contains a 22 nt sequence from position 36 to 57 of $CCCVd_{246}$, which is duplicated six times. Apart from these major differences, there were also three single base substitutions at positions 70 (G→C) of C domain, 106 (A→U) of V domain and 123 (A→G) of TR of $CCCVd_{246}$ (Figure 6.6).

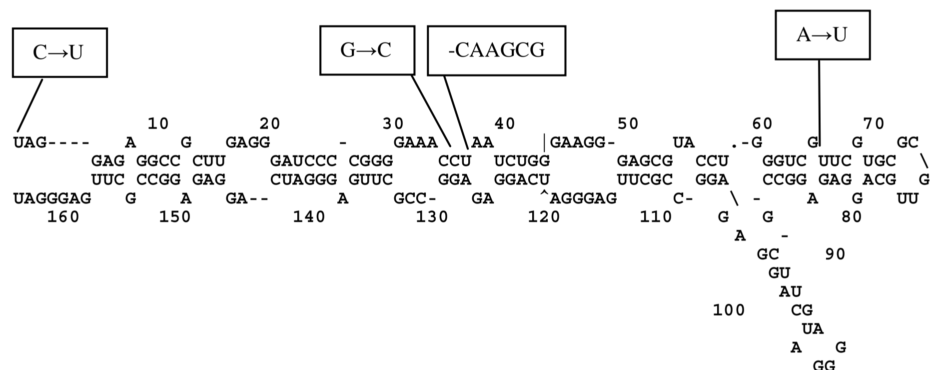
Comparison of the OP_{165} sequence with $CCCVd_{246}$ and analysis of the secondary structure of OP_{165} showed that it also contains a partial sequence of $CCCVd_{246}$, however the length is only 83 nt (position 36 to 118 of $CCCVd_{246}$), a deletion of 6 nt compared to OP_{232} (Figure 6.7, Appendix H). However, there were only 2 nt deletion from position 76 and 77 compared to the 6 nt deletion in the same region of OP_{232}. OP_{165} also lacks the 20 nt duplication (position 98 to 118 of $CCCVd_{246}$), which can be found in OP_{232}. In addition, the duplication of the 22 nt sequence from position 36 to 57 of $CCCVd_{246}$ in OP_{232} also occurs in this variant but only repeated four times. Three single base substitutions were also observed in this variant, which occurred at positions 36 (C→U) of P domain, 70 (G→C) of C domain and 106 (A→U) of V domain of $CCCVd_{246}$ (Figure 6.7).

Figure 6.6 Secondary structures with a minimum computed folding energy at 37 ^{o}C of $CCCVd_{246}$ and OP_{232}. The sites where mutations occurred compared with $CCCVd_{246}$ are indicated. Duplicated sequences are indicated with the same colour. The base substitutions indicated in pink in $CCCVd_{246}$ are the mutation points of the 'brooming' mutant as reported by Rodriguez and Randles (1993).


CCCVd246 Energy: -112.5 kcal/mole
TL
P
C
V
TR
A→AA
U→A
C→U
→GU
→UU


OP232 Energy: -91.1 kcal/mole
A→U
G→C
-CAAGCGAAUC
A→G
U→A

OP_{165} Energy: -56.1 kcal/mole




```
               10        20        30        40         50          60         70
UAG----     A    G    GAGG       -    GAAA \ AA     |GAAGG-     UA    .-G     G    G    GC
       GAG GGCC CUU     GAUCCC CGGG     CCU  UCUGG       GAGCG  CCU    GGUC UUC UGC  \
       UUC CCGG GAG     CUAGGG GUUC     GGA  GGACU       UUCGC  GGA    CCGG GAG ACG  G
UAGGGAG   G    A    GA--      A    GCC-    GA    ^AGGGAG     C-    \ -    A   G    UU
   160        150        140        130        120        110     G   G      80
                                                                    A   -
                                                                      GC    90
                                                                       GU
                                                                        AU
                                                               100       CG
                                                                          UA
                                                                         A   G
                                                                           GG
```

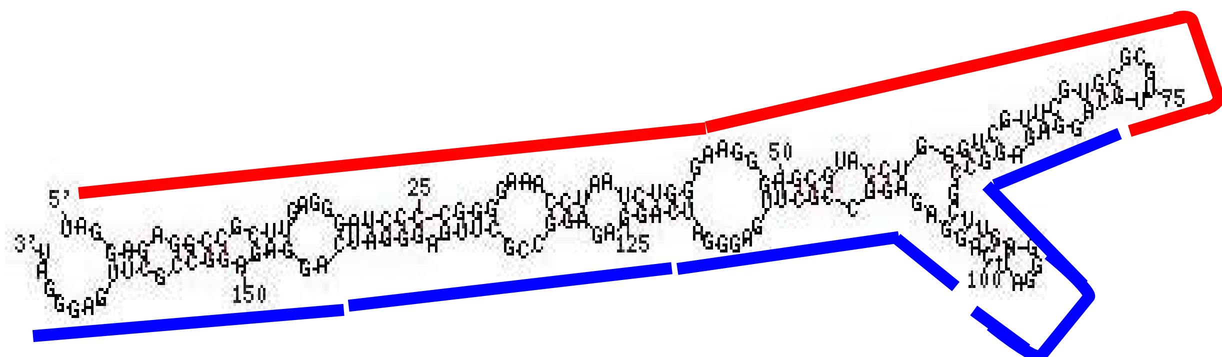


Figure 6.7 Secondary structures with a minimum computed folding energy at 37 °C of OP_{165}. The sites where mutations occurred compared with $CCCVd_{246}$ are indicated. Duplicated sequences are indicated with the same colour.

6.4 DISCUSSION

Cloning and sequencing of the RT-PCR product of an asymptomatic oil palm (SRH2) identified five clones of 297, 293, 270, 232 and 165 nts in length. OP_{297} and OP_{293} were obtained from all three fronds sampled and OP_{270}, OP_{232} and OP_{165} was attained from frond 10. OP_{297}, OP_{293} and OP_{270} showed high sequence similarity (greater than 90 %) with $CCCVd_{296}$, but were not identical to CCCVd. OP_{232} and OP_{165} had some sequence similarity with $CCCVd_{246}$. Because an arbitrary level of 90 % sequence similarity is accepted as separating viroid species from variants (Flores *et al.*, 2005b), OP_{297}, OP_{293} and OP_{270} can be considered as variants of CCCVd. No variants of the 'fast' $CCCVd_{246}$ form were obtained in this study.

Although more than one variant was recovered from a single oil palm, the majority of clones (71 %) were of the 297 nt variant suggesting that this was dominant in the nucleic acid samples of the three fronds. This agrees with a previous report by Hanold and Randles (1991) that the CCCVd-like RNA from an OS affected oil palm in the Solomon Islands was about 296 nt in size, and suggested that the 'slow' viroid form may be dominant in oil palm. In addition, there were minor and apparently sporadic variations among the individual variants, which supports previous reports that viroids propagate in their hosts as populations of closely related sequence variants (quasi-species), with one or more predominating in the population (Flores *et al.*, 2005b).

Compared with $CCCVd_{296}$, 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 TR, producing a predicted branched secondary structure. Absence of duplications of either the upper or lower strand of the TR domain has been previously reported in CEVd (Szychowski *et al.*, 2005). Changes in sequence have been reported to affect the

stability of the structure that could affect the replication and symptom expression (Owens *et al.*, 1995).

The absence of symptoms in the oil palm from which these variants were isolated raises the possibility that these oil palm variants could be a mild mutant of CCCVd. Changes as little as one to three nucleotides in the pathogenic domain of pospiviroids have been shown to affect infectivity (Wassenegger *et al.*, 1996) as well as symptom severity (Gross *et al.*, 1981) by affecting the virulence modulating (VM) region (Schnolzer *et al.*, 1985). Compared with $CCCVd_{296}$, OP_{297}, OP_{293} and OP_{270} substituted (C→U) at position 31 in the P domain and (A→C) at position 175 in TR. These changes, especially the base substitution at position 31 in the P domain could have affected their ability to induce symptoms. Therefore, it is important that clones of these molecules should be tested for pathogenicity by inoculation into oil palm seedlings in future studies.

Mutations have been found to regulate replication and symptom expression of PSTVd variants (Owens *et al.*, 1995; Qi and Ding, 2002). The mutations observed in the oil palm variants described in this study may have affected the replication of these variants resulting in them reaching a low concentration as described in previous chapters (Chapters 3,4,5). The low concentration of these variants may be responsible for the lack of obvious symptoms such as orange spotting and stunting in this four year old palm. Further studies are needed to evaluate the relationship between mutation and viroid concentration.

The base changes in the oil palm variants described here are unique and not similar to any mutations previously reported in CCCVd (Haseloff *et al.*, 1982; Rodriguez and Randles, 1993). This indicates that mutants of CCCVd occur in the field, and these may give rise to a range of disease phenotypes. It has already been found that

mutation in the P and C domain of CCCVd is associated with a severe form of coconut cadang-cadang disease known as 'brooming' (Rodriguez and Randles, 1993).

The small variants such as OP_{232} and OP_{165} have not been described for CCCVd. However, the ability of these variants to induce disease is questionable as these variants lack the entire bottom strand sequence and almost all the pathogenicity domain. Moreover, these smaller variants could be either a replication intermediate or a by product of RT-PCR amplification as the 22 nt repeat fragment, which is prominent in both these variants, partly consists of the sequence of the reverse primer (17 nts) of primer set I. These variants were only present when amplified using primer set I. Further study with other primer sets is needed to verify whether these small variants are the artifacts of RT-PCR amplification.

CHAPTER 7

DETECTION OF siRNA's IN CCCVd INFECTED COCONUT PALM

7.1 INTRODUCTION

Post-transcriptional gene silencing (PTGS), also known as RNA silencing, is a mechanism that regulates gene expression in eukaryotes and results in the sequence-specific degradation of single-stranded RNAs (ssRNAs) from genetic elements of internal or foreign origin (Baulcombe, 2002). PTGS is triggered by double-stranded RNA (dsRNA), which is subsequently processed into 21-25 nt fragments called small interfering RNAs (siRNAs) by an RNase III-like enzyme (Dicer). The siRNAs guide a second RNase (RNA induced silencing complex [RISC]) for degrading their associated ssRNA (Hammond *et al*., 2000). Because siRNAs homologous with and complementary to the targeted ssRNA have been detected in all systems exhibiting PTGS, the siRNAs are regarded as markers for this phenomenon.

In plants, PTGS has been reported for cytoplasmic ssRNAs from endogenous nuclear genes, transgenes and RNA and DNA viruses (Vance and Vaucheret, 2001). Recently, PSTVd-specific siRNAs have been detected in plants infected by this pathogen, suggesting that PSTVd induces PTGS (Itaya *et al*., 2001; Papaefthimiou *et al*., 2001). siRNAs of similar length were also detected in plants infected by members of the Avsunviroidae, *Peach latent mosaic viroid* (PLMVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Martinez de Alba *et al*., 2002) and *Avocado sunblotch viroid* (ASBVd) (Markarian *et al*., 2004).

An inverse correlation has been found in chloroplastic viroids between the accumulation levels of mature viroid forms and their corresponding siRNAs. The *in*

vivo PLMVd and CChMVd concentrations were low but their siRNAs were easily detectable, whereas in tissues where the *in vivo* concentration of ASBVd was very high, the corresponding siRNAs were either undectectable (Martinez de Alba *et al*., 2002) or they accumulated to low levels (Markarian *et al*., 2004). This inverse correlation between viroid accumulation levels and the presence and/or absence of their siRNAs is consistent with the involvement of the latter in a PTGS defence response of the host that would attenuate the detrimental effect of viroids by lowering their *in vivo* titre (Martinez de Alba *et al*., 2002).

This chapter reports an investigation of PTGS in CCCVd-infected coconut samples from the Philippines.

7.2 MATERIAL AND METHODS

7.2.1 Material

Fresh leaf samples of 11 CCCVd-infected coconut samples from the Philippines as listed in Table 7.1 were used in this study. Leaf samples from a healthy coconut seedling raised from an Australian seed nut and maintained in the glasshouse at Waite Campus, University of Adelaide were used as negative control.

Table 7.1 Coconut leaf samples used in this study

Sample	Characteristic	Source
HCoCo	- healthy coconut seedling	Coconut collection, Waite Glasshouse
E1, E2, E3	- early stage coconut cadang-cadang disease leaflets from the Philippines	J.W. Randles
M1, M2	- middle stage coconut cadang-cadang disease leaflets from the Philippines	J.W. Randles
L1, L2, L3	- late stage coconut cadang-cadang disease leaflets from the Philippines	J.W. Randles
B1, B2, B3	- coconut cadang-cadang disease leaflets with brooming symptoms from the Philippines	J.W. Randles

7.2.2 Methods

7.2.2a Total nucleic acid extraction

Nucleic acids were extracted from fresh leaf materials (Section 7.2.1) using the method described in Section 2.2.1c.

7.2.2b PAGE

Analysis of nucleic acids was done by PAGE as described in Section 2.2.2a. Nucleic acids were fractionated on a 15 % denaturing (8M Urea) PAGE buffered in 1X TBE (Appendix C). Electrophoresis was at 30 mA for 2 hr. Nucleic acids were electroblotted onto a nylon membrane (Zeta Probe®, BioRad) as described in Section 2.2.4b.

7.2.2c Hybridization assay

A full-length cRNA probe was transcribed by T7 RNA polymerase from a plasmid vector containing the $CCCVd_{246}$ clone and labelled with ^{32}P (Section 2.1.5). The probe was heated at 100 ^{o}C for 2 min and added to the pre-hybridization solution at about 10^6 cpm/ml and used for the hybridization assay.

Electroblotted membranes (Zeta Probe®, BioRad) were hybridised according to the method of Hanold and Randles (1991) with some modifications as described in Section 2.2.4c.

7.3 RESULTS

Hybridization of nucleic acids extracted from the CCCVd-infected coconut leaf samples with the cRNA probe showed two bands of approximately 20 nt and 25 nt in size (Figure 7.1) after a high stringency wash. These small RNAs were present in nucleic acid extracts from leaf samples of all three stages of coconut cadang-cadang disease. Similar results were also obtained in leaf samples exhibiting the severe 'brooming' symptoms of cadang-cadang.

The hybridization signals obtained with these small RNAs were weaker than for CCCVd RNA. In addition, the hybridization signal intensity varied between palms at the same stage of cadang-cadang disease. The small RNAs of two palms in the late stage (Figure 7.1- lane 7-9) and all three palms with brooming symptoms (Figure 7.1- lane 10-12) showed the strongest hybridization signals compared with the small RNAs from the early (Figure 7.1- lane 2-4) and middle stage (Figure 7.1- lane 5-6). No small RNA was detected in the nucleic acid extract healthy coconut (Figure 7.1- lane 1)

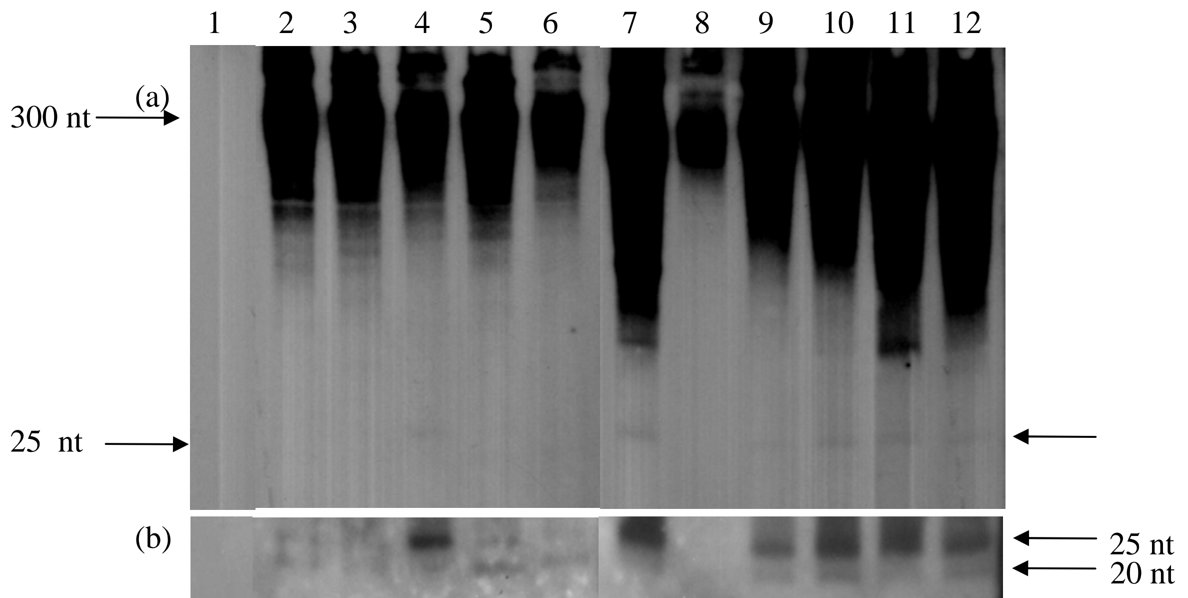


Figure 7.1 Northern blots of total nucleic acids extracted from CCCVd infected coconut leaves from different stages of cadang-cadang disease fractionated on a 15 % denaturing PAGE showing the presence of small RNAs of 20-25 nt long (indicated by arrows) after a high stringency wash. Autoradiography was done for (a) 12 hr or (b) for 72 hr at -70 ^{o}C. Panel (b) shows the small RNA region only.

Lane 1 healthy coconut leaf sample

Lane 2-4 extracts from leaf samples of palms at the early stage of coconut cadang-cadang disease

Lane 5-6 extracts from leaf samples of palms at the middle stage of coconut cadang-cadang disease

Lane 7-9 extracts from leaf samples of palms at the late stage of coconut cadang-cadang disease

Lane 10-12 extracts from leaf samples of palms with brooming symptoms

7.4 DISCUSSION

CCCVd-infected coconut leaf collected in the Philippines and extracted within 3 days of harvesting contained two small RNAs approximately 20 nt and 25 nt in size. A high stringency wash of the Northern blots failed to remove the hybridization signal suggesting that these small RNAs had sequences closely similar to CCCVd. Thus, these small RNAs satisfy the criteria for identification as siRNAs and suggest that PTGS is induced by CCCVd in coconut palm.

The siRNAs were present at low concentrations compared to CCCVd. They were present in all three stages of the cadang-cadang disease but the amount varied between stages and also between samples at same stage. They were absent or present in low concentrations at early and middle stages when the concentration of CCCVd was high. The concentrations of the siRNAs were higher at the late stage and samples with brooming symptoms but the CCCVd concentration was also high in those samples as indicated by the strong hybridization signals.

The result of this experiment is not sufficient to draw conclusions on the possible role of these siRNAs in PTGS but it suggests that there are no regulating molecular activities in field grown coconut palms.

CHAPTER 8

STUDIES OF RELATIONSHIP BETWEEN OIL PALM ORANGE SPOTTING AND PHYTOPLASMAS

8.1 INTRODUCTION

Plant infecting non-culturable mollicutes, known as phytoplasmas, were first discovered in plants in 1967 (Doi *et al*., 1967). Before their discovery, diseases caused by phytoplasmas were thought to be caused by viruses but no viruses could be consistently visualized in diseased tissues or isolated from infected plants (Lee and Davis, 1992). Transmission electron microscopy identified bacteria without cell walls that inhabited phloem sieve elements, thus indicating that phytoplasmas were the causal agent (Lee and Davis, 1992). To date, phytoplasmas have been associated with diseases in several hundred plant species (McCoy *et al*., 1989). Typical symptoms are yellowing, deformation of leaves and abnormalities such as sterile flowers, phyllody and proliferation (McCoy *et al*., 1989).

Traditionally, the identification and classification of phytoplasmas was based primarily on biological properties such as the symptoms induced in infected plants, plant host range and relationships with insect vectors (Lee *et al*., 2000). Recent advances in molecular-based biotechnology have made it possible to gain new knowledge about phytoplasmas and to develop systems for their accurate identification and classification (Lee *et al*., 2000). Molecular-based probes, such as mono- and polyclonal antibodies, and cloned phytoplasma DNA fragments developed in the 1980s have been used to detect various phytoplasmas associated with plants and insects and to

study their genetic interrelationships (Kirkpatrick *et al*., 1987; Ahrens and Seemüller, 1992; Lee *et al.*, 2000).

Phylogenetic analyses of the 16S rRNA gene of phytoplasmas and ribosomal protein (rp) gene sequences placed phytoplasmas in the class Mollicutes (Lim and Sears, 1989). Specific oligonucleotide probes for the phytoplasma 16S rRNA gene improved the sensitivity of tests to detect phytoplasmas when used in place of cloned chromosomal DNA probes (Kirkpatrick *et al*., 1987). This suggested that the phytoplasma 16S gene could be useful for identification and differentiation of phytoplasmas. Subsequently, universal oligonucleotide primers based on conserved 16S rRNA gene sequences (Deng and Hiruki, 1991) were designed and used in PCR assays (Ahrens and Seemüller 1992; Lee *et al*., 1993; Namba *et al*., 1993) that allowed the detection of a broad array of phytoplasmas associated with plants and insect vectors.

The detection of a phytoplasma in lethal yellowing affected coconut (Harrison *et al.,* 1992) showed that phytoplasmas occur in palms and they have recently been shown to be associated with, and are the probable cause of a number of lethal diseases of coconut palm (Tsai and Harrison, 2003). Hence, this chapter describes experiments to test the possibility that a phytoplasma is associated with oil palm orange spotting disorder.

8.2 MATERIAL AND METHODS

8.2.1 Materials

8.2.1a Leaf samples

The leaf samples used in this study are listed in Table 8.1. Altogether, seven symptomatic and three asymptomatic oil palms from Malaysia were used in this study. Additional palm samples maintained at the Waite Glasshouse were also tested for the presence of phytoplasma-like DNA.

Australian grapevine yellows phytoplasma (AGYp) and Coconut lethal yellowing phytoplasma (LYp) DNAs used as a positive control in this study were provided by Dr. Nuredin Habili from Waite Diagnostics, School of Agriculture and Wine, University of Adelaide.

8.2.2 Methods

8.2.2a DNA extraction

DNA was extracted from leaf samples using the CTAB extraction procedure as described in Section 2.2.1f.

8.2.2b PCR assay

Four sets of universal primers were used to amplify the 16S rRNA operon (Table 8.2, Appendix G). LY specific primer sets were also used in this study. Nested PCR was used as described in Section 2.2.5b. Amplified products were analysed by 1.5 % agarose gel and visualized by EtBr staining and UV fluorescence.

Table 8.1 Oil palm and other palm species used in this experiment.

Oil palm samples from Malaysia		
Sample	Characteristic	Source
SRH 1 f20	- Asymptomatic oil palm	Selangor River Estate, Malaysia
SRH 2 f20	- Asymptomatic oil palm	Selangor River Estate, Malaysia
SRH 3 f20	- Asymptomatic oil palm	Selangor River Estate, Malaysia
SRD 1 f20	- Symptomatic (orange spotted) oil palm	Selangor River Estate, Malaysia
SRD 2 f20	- Symptomatic (orange spotted) oil palm	Selangor River Estate, Malaysia
SRD 3 f20	- Symptomatic (orange spotted) oil palm	Selangor River Estate, Malaysia
CEP 1 f20	- Symptomatic (orange spotted) oil palm	CEP Renggam Estate, Malaysia
CEP 2 f20	- Symptomatic (orange spotted) oil palm	CEP Renggam Estate, Malaysia
CEP 3 f20	- Symptomatic (orange spotted) oil palm	CEP Renggam Estate, Malaysia
CEP 4 f20	- Symptomatic (orange spotted) oil palm	CEP Renggam Estate, Malaysia
Other palm samples from Waite glasshouse		
Sample	Characteristic	Source
HOP	- asymptomatic oil palm seedling (RHS)	J.W. Randles
OP40-	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	J.W. Randles
OP43-	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	J.W. Randles
OP29-	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	J.W. Randles
OP12+	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	J.W. Randles
OP14+	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	J.W. Randles
GCP	- golden cane palm	J.W. Randles
CP	- cane palm	J.W. Randles
COCO	- coconut inoculated with viroid-like RNA from nucleic extract of coconut palm from the Solomon Islands	J.W. Randles
Hcoco	- healthy coconut	J.W. Randles

Table 8.2 Properties of the primers used for the detection of phytoplasma in the oil palms

Primer set	Sequence	Polarity	Reaction	PCR product length
P1	5'-d(AAG AGT TTG ATC CTG GCT CAG GAT T)-3'	+ve	Nested PCR	1200 bp
P7	5'-d(CGT CCT TCA TCG GCT CTT)-3'	-ve		
R16F2n	5'-d(GAA ACG ACT GCT AAG ACT GG)-3'	+ve		
R16R2	5'-d(TGA CGG GCG GTG TGA CAA ACC CCG)-3'	-ve		
LY16SF	5'-d(CAT GCA AGT CGA ACG GAA ATC)-3'	+ve	PCR	1400 bp
LY16SR	5'-d(GCT TAC GCA GTT AGG CTG TC)-3'	-ve		

P1 - Deng and Hiruki (1991)

P7 - Smart *et al*. (1996)

R16F2n - Lee *et al*. (1993)

R16R2 - Lee *et al*. (1993)

LY16Sf - Harrison *et al.* (2002)

LY16Sr - Harrison *et al.* (2002)

8.2.2c Restriction Fragment Length Polymorphism (RFLP)

10 µl of PCR product was digested with 5 U of Mse I (Invitrogen, Australia) in the appropriate buffer and incubated at 37 ^{o}C for 2 hr. The digested fragments were separated by 8 % non-denaturing PAGE buffered in 1X TBE and electrophoresed at 75 mA/350 V for 1 hr. Gel was stained with silver as described in Section 2.2.2d. AGYp was used as a standard.

8.3 RESULTS

8.3.1 PCR analysis using primers P1/P7 and R16F2n/R16R2

A preliminary analysis of DNA extracted from the oil palm samples by nested PCR using the P1/P7 and R16F2n/R16R2 primer sets showed PCR products of either approximately 800 bp or 1.2 kbp (Figure 8.1) in both symptomatic and asymptomatic palms; however, the intensity of the bands varied. All three asymptomatic oil palms contained a phytoplasma-like DNA while only four of the seven symptomatic palms were positive. One asymptomatic palm (SRH3 f20) and two symptomatic palms (CEP 3 f20 and CEP 4 f20) were selected for further analysis as these three samples showed the strongest band intensity.

PCR analysis of DNAs extracted from other palm samples from the Waite glasshouse together with the selected oil palm samples from Malaysia showed two bands of approximately 800 bp and 1.2 kbp in size in all samples but OP40-, which only produced the 1.2 kbp band (Figure 8.2). CEP3 f20, OP 29-, GCP, CP and COCO did not show any bands.

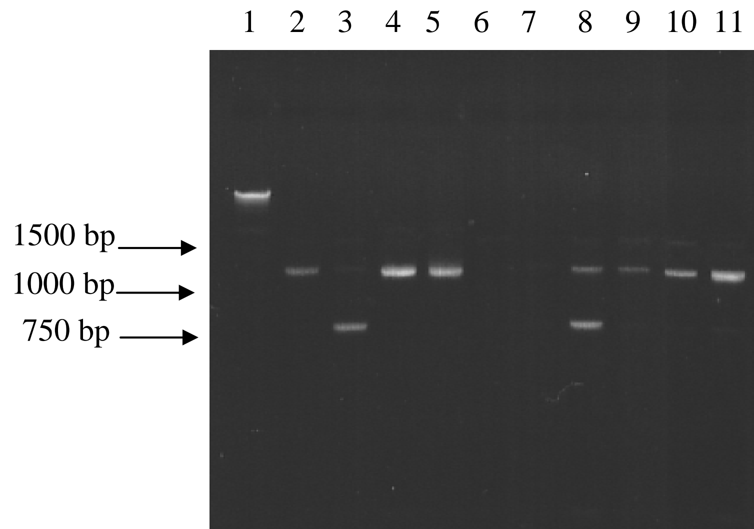


Figure 8.1 Nested PCR analysis of DNA extracted from oil palm leaf samples from Malaysia using the P1/P7 and R16F2n/R16R2 primer sets

Lane 1	1 kb DNA marker
Lane 2	SRH1 f20
Lane 3	SRH2 f20
Lane 4	SRH3 f20
Lane 5	SRD1 f20
Lane 6	SRD2 f20
Lane 7	SRD3 f20
Lane 8	CEP1 f20
Lane 9	CEP2 f20
Lane 10	CEP3 f20
Lane 11	CEP4 f20

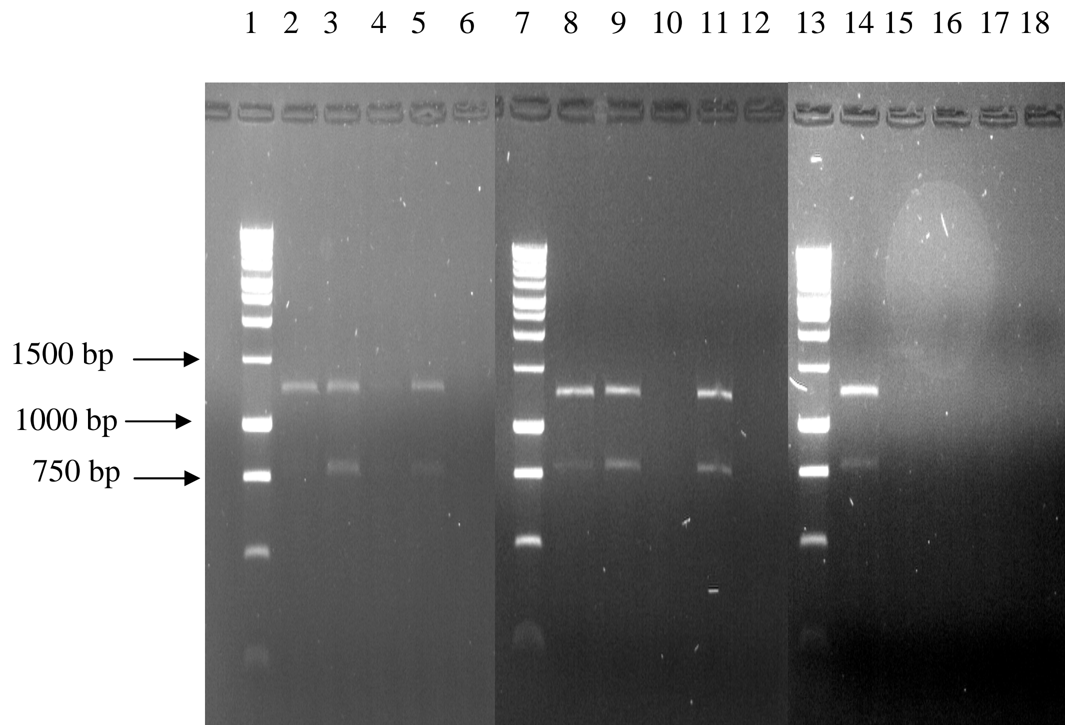


Figure 8.2 Nested PCR analysis of DNA extracted from leaf samples of oil palms and other palm samples using the P1/P7 and R16F2n/R16R2 primer sets

Lane 1, 7, 13	1 kb DNA marker
Lane 2	OP 40-
Lane 3	SRH3 f20
Lane 4	CEP3 f20
Lane 5	CEP4 f20
Lane 6, 12,	SDDW
Lane 8	OP12+
Lane 9	OP14+
Lane 10	OP29-
Lane 11	OP43-
Lane 14	HOP
Lane 15	GCP
Lane 16	CP
Lane 17	COCO
Lane 18	Hcoco

Nested-PCR analysis of AGYp and LYp DNAs using the combination of P1/P7 and R16F2n/R16R2 primers sets produced a band approximately 1.2 kbp, which corresponded with those from the oil palm samples but the 800 bp band was not observed (Figure 8.3).

8.3.2 RFLP analysis and comparison with AGYp

The oil palm samples containing phytoplasma-like DNA could be separated into four groups based on RFLP analysis with Mse I (Figure 8.4). OP40- and CEP4 f20 were placed in separate group, each with a unique RFLP pattern. SRH3 f20 and HOP with similar RFLP pattern, were placed into one group while the rest of the samples were grouped into one as they had similar RFLP patterns. However, none of the RFLP patterns of the oil palms were similar to AGYp.

8.3.3 PCR analysis using Coconut lethal yellowing phytoplasma (LYp) specific primers

PCR analysis of the lethal yellowing phytoplasma DNA with LY-specific primers, LY16SF and LY16SR, produced a band of approximately 1.5 kbp, which was the expected PCR product size using this primer set (Figure 8.5). However, no bands were observed in the oil palm samples using these LY specific primers (Section 8.3.1).

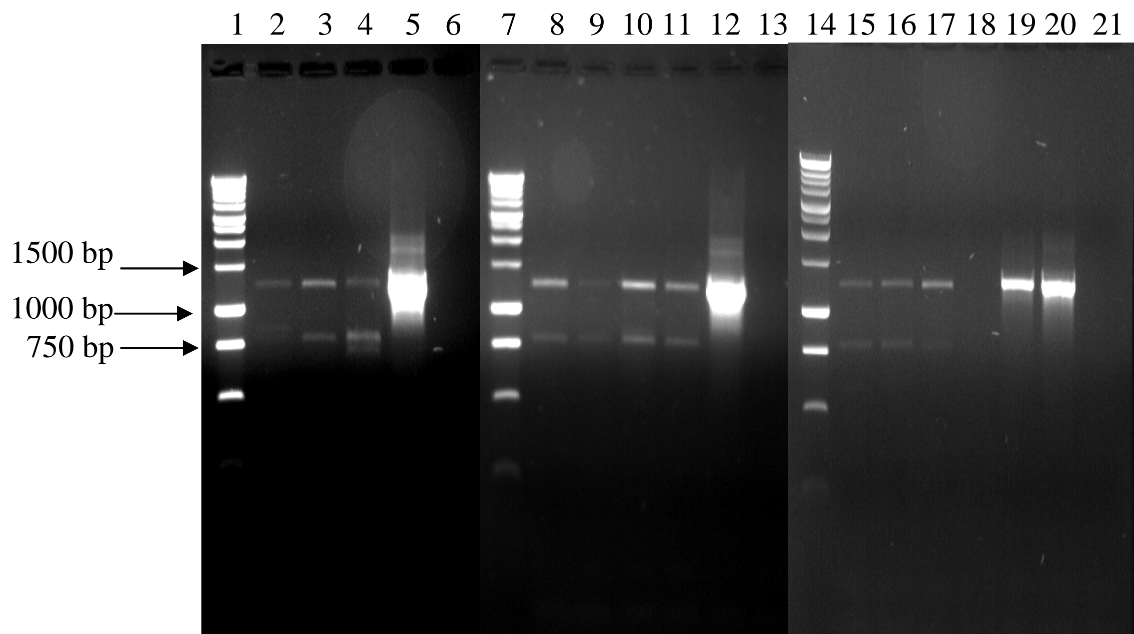


Figure 8.3 Nested PCR analysis of AGYp, LYp and DNA extracted from leaf samples of oil palm and other palm samples using the P1/P7 and R16F2n/R16R2 primer sets

Lane 1, 7, 14	1 kb DNA marker
Lane 2	OP 40-
Lane 3, 15	SRH3 f20
Lane 4, 16	CEP4 f20
Lane 5, 12, 20	AGYp
Lane 6, 13, 21	SDDW
Lane 8	OP12+
Lane 9	OP14+
Lane 10	OP43-
Lane 11, 17	HOP
Lane 18	COCO
Lane 19	LYp

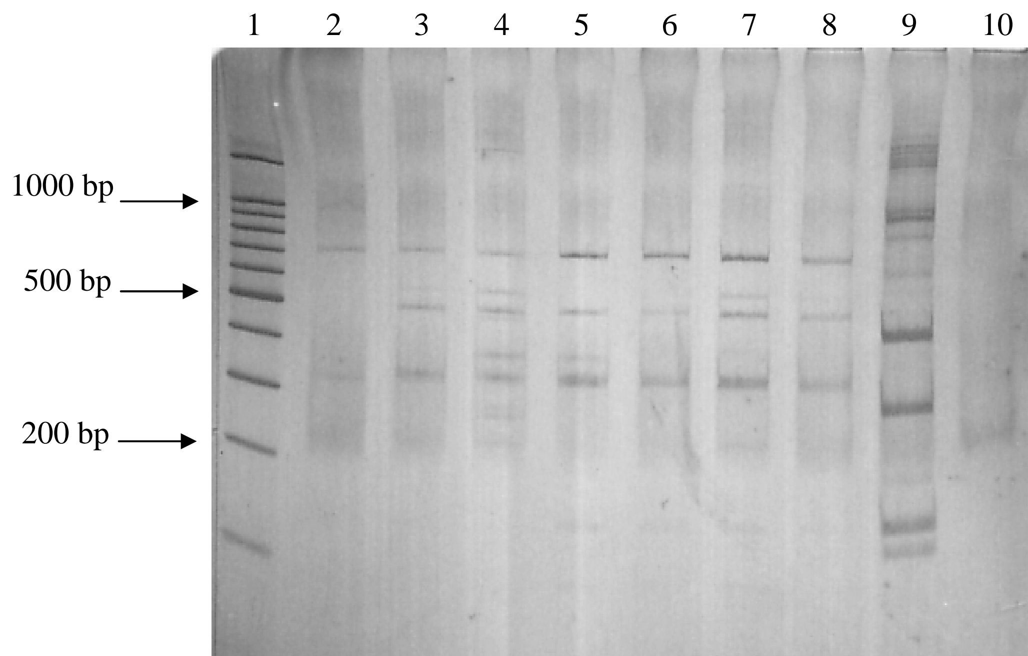


Figure 8.4 Comparison of RFLP analysis with Mse I of AGYp DNA with phytoplasma-like DNA from oil palms.

Lane 1	100 bp DNA marker
Lane 2	OP 40-
Lane 3	SRH3 f20
Lane 4	CEP4 f20
Lane 5	OP12+
Lane 6	OP14+
Lane 7	HOP
Lane 8	OP43-
Lane 9	AGYp
Lane 10	SDDW

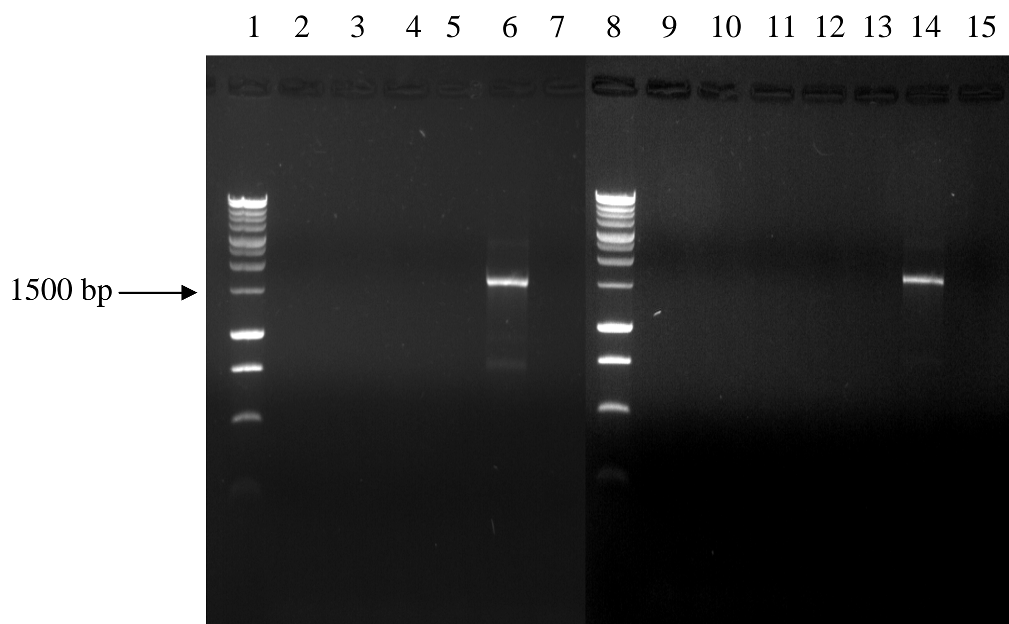


Figure 8.5 PCR analysis of DNA extracted from leaf samples of oil palm and other palm samples using the LY16SF and LY16SR primer set.

Lane 1, 8,	1 kb DNA marker
Lane 2	SRH3 f20
Lane 3	CEP3 f20
Lane 4	CEP4 f20
Lane 5	HOP
Lane 6, 14	LYp
Lane 7, 15	SDDW
Lane 9	OP 40-
Lane 10	OP43-
Lane 11	OP12+
Lane 12	OP14+
Lane 13	COCO

8.4 DISCUSSION

Analysis of DNA extracted from both symptomatic and asymptomatic oil palms from Malaysia by nested PCR using universal primer sets to amplify the 16S rRNA operon showed the presence of phytoplasma-like DNA in both types of samples. Furthermore, DNA extracted from several oil palm seedlings maintained at the Waite glasshouse also contained phytoplasma-like DNA.

RFLP analysis of the phytoplasma positive palms indicates that there could be four different phytoplasmas present in the oil palm samples. However, none of them were related to AGYp. The phytoplasma-like DNA in the oil palm sample was also not related to LYp as PCR analysis with LY specific primers did not produce any amplicon.

In these experiments, phytoplasma-like DNA was only detected in DNA extracted from the oil palm leaf samples brought from Malaysia and from seedlings growing in the glasshouse but not in the other palm species maintained in the glasshouse. This result suggests that this phytoplasma-like DNA is specific to oil palms but that it is not closely related to LYp. The possibility remains that the nucleic acid extracts from the oil palms may contain DNA which can be primed with the phytoplasma universal primer sets to produce an amplified product of similar size to that of a phytoplasma. Further investigation by cloning and sequencing of these phytoplasma-like DNAs from the oil palms could resolve this issue.

The results of these experiments could not relate the phytoplasma-like DNA found in the oil palms specifically with orange spotting disorder of oil palms, thus no further work was done to characterize this molecule.

CHAPTER 9

GENERAL DISCUSSION

Results described in this thesis show that oil palms from commercial plantations in Malaysia contain nucleic acids similar to *Coconut cadang-cadang viroid* (CCCVd). These nucleic acids were detected both in oil palms with the orange spotting disorder and in asymptomatic palms by Northern blot hybridization of single-dimensional and two-dimensional polyacrylamide gels with ^{32}P-labelled full length $CCCVd_{246}$ cRNA probe (Chapter 3). Based on their mobility in 5 % non-denaturing PAGE, these nucleic acids were in the size range of 250-300 nt. 2D-PAGE showed that they had the electrophoretic mobility expected for a circular molecule. Hybridization assays done under high stringency conditions showed that there was strong sequence similarity with $CCCVd_{246}$. Although their identity as RNA was not confirmed in this thesis by direct tests of ribonuclease sensitivity, the criteria that they were small, circular and complementary to the CCCVd cRNA probe provided evidence that they were viroid RNAs and they were thus described as CCCVd-like RNAs.

These CCCVd-like RNAs seemed to be present at low concentration in the oil palms tested compared with CCCVd in coconut. Weak hybridization signals were obtained in the oil palm samples even when large amounts of nucleic acid extract (leaf weight equivalent of 20-100 g) were loaded onto the gel (Chapter 3). Low efficiency of extraction from the oil palm samples could have contributed to this but even with the PEG extraction procedure (Hanold, 1993; Hanold and Randles, 1991), which has been found to concentrate CCCVd in both coconut and oil palms (Randles *et al*., 1980), detection of these CCCVd-like RNAs required long exposures of Northern blots.

Hybridization assays have been used for routine viroid detection (Hanold, 1993; 1998) but the weak signals obtained in the hybridization assays here with oil palm indicate that this technique may have been at the limit of its sensitivity.

The 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 (Chapter 4). RPA showed that 90 % of the symptomatic and 50 % of the asymptomatic oil palms from Malaysia contained RNAs which partially protected the ^{32}P-labelled full length $CCCVd_{246}$ cRNA probe. The RPA pattern closely resembled that of CCCVd suggesting that there were very few mismatches between the CCCVd-like RNAs and $CCCVd_{246}$. Typically, there were additional protected fragments in the oil palm samples. RPA showed that more sequence variation was evident in younger than the older fronds. As the younger fronds represent a later stage of disease, it appears that sequence variation may increase as the plant ages. Generally, the RPA signal was weak in the oil palm samples supporting earlier observations (Chapter 3) that the CCCVd-like RNAs may be present in low concentrations in the oil palm nucleic acid extracts. The fact that the RPA patterns of the CCCVd-like RNAs from the oil palms were similar to CCCVd from coconut palms show that the weak signal is not due to sequence variability.

RT-PCR has been reported to detect viroids in very small amounts of total nucleic acid from infected tissues (Hadidi and Yang, 1990). In this thesis, RT-PCR amplification using CCCVd-specific primers (Chapter 5) confirmed the presence of CCCVd-like RNAs in an asymptomatic oil palm (SRH2). Amplification was only successful when nucleic acids were partially purified using 1D or 2D-PAGE. RT-PCR of RNAs eluted from the circular viroid region of 2D-PAGE of the asymptomatic palm produced amplicons approximately 200 to 300 bp in size, but these were detectable by

silver staining only. The successful amplification of CCCVd-like RNAs from the oil palm using CCCVd-specific primers indicates that these RNAs have high sequence similarity with CCCVd. However, the need for re-amplification of the first round RT-PCR products for detection by EtBr staining concurs with the earlier conclusion that the concentration of the CCCVd-like RNAs is low.

In summary, then, the very low concentration of the CCCVd-like RNAs compared with CCCVd illustrates the need for a sensitive diagnostic method. PAGE and hybridization assays have been consistent and reliable techniques for CCCVd detection (Hanold and Randles, 1991) but were not definitive for the CCCVd-like RNAs in oil palm. RPA was found to be as sensitive as Northern hybridization for detecting the CCCVd-like RNAs in the oil palm samples, and an added advantage was that it showed whether variations were present in the CCCVd-like RNA population (Lopez-Galindez *et al*., 1988; Kurath and Palukaitis, 1989b). RPA is also much less labor intensive than cloning and sequencing of nucleic acid samples (Palukaitis *et al*., 1994). This technique could be exploited for a large-scale survey for CCCVd variants in commercial oil palm plantations. The use of non-radioactive probes should be evaluated, as this is a more feasible option for large-scale studies than radioactive probes.

The low concentration of the CCCVd-like RNAs meant that further characterisation was limited to one palm (SRH2). Insufficient leaf material was brought from Malaysia to attempt isolation and sequencing of RNAs from symptomatic palms as it was found that samples larger than 100 g were required for each extraction.

Twenty cloned sequences were obtained from SRH2 (Chapter 6). They fell into five size classes comprising 297 nt (OP_{297}), 293 nt (OP_{293}), 270 nt (OP_{270}), 232 nt (OP_{232}) and 165 nt (OP_{165}). They were obtained from three fronds of SRH2, frond 3 (3rd

youngest), 10 and 20. OP_{297} and OP_{293} were obtained from all three fronds while OP_{270}, OP_{232} and OP_{165} were obtained from frond 10. A comparison of the consensus sequences of OP_{297}, OP_{293}, and of OP_{270} with known viroid sequences showed that OP_{297}, OP_{293} and OP_{270} had 98 %, 97 % and 90 % sequence similarity with $CCCVd_{296}$ respectively. No variants of the 'fast' $CCCVd_{246}$ form were obtained.

CCCVd comprises two monomeric 'fast' ($CCCVd_{246}$ and $CCCVd_{247}$) and 'slow' ($CCCVd_{296}$ and $CCCVd_{297}$) electrophoretic forms as well as dimeric forms of each monomer (Randles, 1985). $CCCVd_{247}$ differs from $CCCVd_{246}$, and $CCCVd_{297}$ from $CCCVd_{296}$, by the insertion of a cytosine at position nt 197 (numbering scheme for $CCCVd_{246}$). The slow RNAs are directly derived from the corresponding 'fast' forms by a partial duplication of the right-hand terminal (TR) domain of the molecule (Haseloff *et al*., 1982).

Of the 20 clones that were sequenced, twelve contained inserts of OP_{297}, four contained inserts of OP_{293}, two contained inserts of OP_{232}, one contained an insert of 165 nt OP_{165} and one contained an insert of OP_{270}. Four of the 12 OP_{297} clones were identical and were therefore defined as the consensus sequence for this variant. The consensus sequence of OP_{293} was derived from two identical clones from four clones sequenced. The remaining clones of OP_{297} and OP_{293} had additional base substitutions in their sequence compared with the consensus sequence and these were distributed across all five domains of the secondary structure. The possibility that the base changes were generated by PCR errors, including PCR-derived recombination (Fernandez-Delmond *et al*., 2004) has been considered but the consistent base changes observed across all variants suggests that these are not random errors introduced by the *Taq* polymerase used for PCR amplication of these variants.

Compared with $CCCVd_{296}$, the consensus sequence of OP_{297} 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 a deletion of a 26 nt repeat at the TR, producing a predicted branched secondary structure. Common to all three large variants were the substitution (C→U) at nt 31 in the pathogenicity (P) domain and (A→C) at nt 175 in the TR of $CCCVd_{296}$. Because an arbitrary level of 90 % sequence similarity and distinct biological properties are accepted as separating viroid species from variants (Flores *et al.*, 2005b), OP_{297}, OP_{293}, and OP_{270} can be considered as variants of CCCVd.

The occurrence of these variants agrees with the RPA analysis (Chapter 4), which showed that the CCCVd-like RNAs in this palm had several mismatches when compared with CCCVd.

The presence of sequences equivalent to OP_{232} and OP_{165} has never been reported for CCCVd. These variants contain a partial sequence of $CCCVd_{246}$ 89 nt in length from position 36 to 124, which includes a part of the P domain and the entire central (C), variable (V) and TR domain. These variants also contain a 22 nt sequence from position 36 to 57 of $CCCVd_{246}$ which is repeated six and four times in OP_{232} and OP_{165} respectively. The ability of these variants to replicate independently of OP_{297} or OP_{293} is questionable as these variants lack the entire bottom strand sequence and almost all the P domain. Although they may be intermediates of replication, they could also be by-products of the RT-PCR amplification as the 22 nt repeat fragment, which is prominent in both these variants, contains 17 nt of the sequence of reverse primer set I. Moreover, these variants were only detected when primer set I was used for RT-PCR. Use of other primer sets might help determine whether these small variants are artifacts of RT-PCR amplification.

Although more than one variant was recovered from a single oil palm, the majority of clones (71 %) were of the 297 nt variant suggesting that this was dominant in the nucleic acid sample of the three fronds. In addition, there were minor and apparently sporadic single base variations among the individual variants. As mentioned above, the possibility remains that some of these were introduced by RT-PCR. However, it may also be a further example of viroids propagating in their hosts as populations of closely related sequence variants (quasi-species), with one or more predominating in the population (Flores *et al*., 2005b).

The lack of RNA similar to the 'fast' $CCCVd_{246}$ form in this study agrees with a previous report by Hanold and Randles (1991) that the CCCVd-like RNA from an OS affected oil palm in the Solomon Islands was about 296 nt in size. The evidence presented here that the majority of clones were of OP_{297} supports the observation that the 'slow' CCCVd form may be dominant in oil palm.

The base changes in the oil palm viroids described here differ from mutations previously reported in CCCVd (Haseloff *et al*., 1982; Rodriguez and Randles, 1993). This indicates that mutants of CCCVd occur in the field, some of which may give rise to a range of disease phenotypes. It has already been found that mutation in the P and C domain of CCCVd is associated with a severe form of coconut cadang-cadang disease known as 'brooming' (Rodriguez and Randles, 1993). This study shows that mutations occur in other sites, and their relationship to other disease phenotypes should be investigated in future work.

The detection of CCCVd-like RNAs in oil palms with the OS disorder (Hanold and Randles, 1991) has led to the suggestion that OS is an infectious disease caused by a viroid allied to CCCVd. The results reported here provide insufficient evidence that the CCCVd-like RNA described here is the causal agent of OS in oil palm as

identification of a putative pathogen alone is not sufficient to establish it as the cause of a disease or disorder.

In this study, the CCCVd-like RNAs have been shown to be associated with OS by RPA (Chapter 4). The RPA pattern of CCCVd-like RNAs from symptomatic palms was very similar to that of CCCVd from coconut (Section 4.3.4), the causal agent of the lethal coconut cadang-cadang disease. Moreover, the symptoms in oil palm inoculated with CCCVd (Imperial *et al.*, 1985) were similar to those in coconut, where spotting and decline of leaflets had been observed (Randles, 1975; Randles *et al.*, 1980).

Further isolation and sequencing of the CCCVd variants from both symptomatic and asymptomatic palms will be necessary for understanding their role in symptom expression. Tests of infectivity and pathogenicity by inoculating the clones obtained here (Tabler and Sanger, 1984; Matousek *et al.*, 2004) will be necessary to establish whether these CCCVd variants can infect and induce orange spotting in oil palms.

Inoculation of the range of clones of the oil palm variants may indicate whether mutations affect symptoms. Mutations have been found to regulate replication and symptom expression of PSTVd variants (Owens *et al.*, 1995; Qi and Ding, 2002). Changes in one to three nucleotides in the pathogenic domain of pospiviroids have been shown to affect infectivity (Wassenegger *et al.*, 1996) as well as symptom severity (Gross *et al.*, 1981) by affecting the virulence modulating (VM) region (Schnolzer *et al.*, 1985). Compared with $CCCVd_{296}$, OP_{297}, OP_{293} and OP_{270} substituted (C→U) at position 31 in the P domain and (A→C) at position 175 in the TR. These changes, especially the base substitution at position 31 in the P domain may be sufficient to affect their ability to induce symptoms.

The nature and severity of symptoms in a viroid infected plant is a reflection of the presence or predominance of a particular sequence variant within the viroid

population (Singh *et al.*, 2003). The absence of symptoms in the palm from which the described sequences were isolated raises the possibility that these variants could be mild variants of CCCVd. A field survey of oil palms in a trial plot in the Solomon Islands showed that CCCVd-like RNAs was detected in five out of 10 asymptomatic trees sampled (Hanold and Randles, 1991) suggesting that mild variants of CCCVd may have occurred in oil palms. ASBVd infection in avocados is an example where a tree infected with a variant of ASBVd remained symptomless while maintaining a stable viroid population (Semancik and Szychowski, 1994).

On the other hand, the palm may still be in the pre-symptomatic stage and may require more time for symptom development. Generally, viroids have been detected prior to symptom development (Singh *et al.*, 2003) and CCCVd has also been detected in leaves of cadang-cadang affected coconut palms before the leaf symptoms appeared (Randles *et al.*, 1998). Furthermore, viroid symptom expression has been found to be influenced by host and environmental factors (Semancik, 2003).

This thesis also reports the presence of phytoplasma-like DNA in both symptomatic and asymptomatic oil palms from Malaysia (Chapter 8). Nested PCR with universal primer sets amplified the 16S rRNA operon. This assay also detected the DNA in samples of oil palm maintained in the Waite glasshouse. Therefore, no evidence has been provided here to associate a phytoplasma with the orange spotting disorder. Due to time constraints, no attempts were made to sequence the DNA.

The detection of siRNAs in CCCVd-infected coconut palms is reported in this thesis (Chapter 7). siRNAs are regarded as the marker for the occurrence of post-transcriptional gene silencing (PTGS) in plants infected by viroids. An inverse correlation has been found between the accumulation levels of mature viroid forms and their corresponding siRNAs (Martinez de Alba *et al.*, 2002; Markarian *et al.*, 2004).

However, the results described in this thesis showed that there was a weak hybridization signal for the siRNA and there was no indication that PTGS had regulated the accumulation of CCCVd.

In conclusion, the identification of CCCVd variants in a commercially grown oil palm shows that a viroid closely related to the lethal CCCVd is present in oil palm plantations in a region outside the Philippines, the country where CCCVd is thought to be contained. The observation that they occur at a low concentration suggests that CCCVd variants may exist below the threshold of detection in some plant reservoirs. Such subliminal infections may provide a source of viroid sequences, which could mutate into more pathogenic variants, with the potential to cause an economically devastating disease. CCCVd detection methods for quarantine also need to be revised to prevent the movement of these variants. Further work should include a study of the range of variants of CCCVd in oil palm, their effects on symptom development and commercial yield, and their geographical distribution.

APPENDIX A

LEAF MATERIALS USED IN THIS STUDY

1. Oil palm leaf samples from Malaysia

Sample	Characteristic	Seed source	Site
SRH 1 f3, f10, f20	- Asymptomatic - 4 year old palm - tree bearing small fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRH 2 f3, f10, f20	- Asymptomatic - 4 year old palm - tree bearing small fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRH 3 f3, f10, f20	- Asymptomatic - 6 year old palm - tree bearing small fruit bunch	*Dura* x *Dura* (Nigerian germplasm)	Selangor River Estate, Sime Darby Plantations
SRH 4 f20	- Asymptomatic - 6 year old palm - no fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRH 5 f20	- Asymptomatic - 6 year old palm - tree bearing large fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRH 6 f20	- Asymptomatic - 14 year old palm - tree bearing large fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRH 7 f20	- Asymptomatic - 14 year old palm - tree bearing large fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRH 8 f20	- Asymptomatic - 14 year old palm - tree bearing large fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations

- f3, f10, f20 denotes frond no. 3, 10 and 20, which indicates late, mid and early stages of disease respectively.

Sample	Characteristic	Seed source	Site
SRD 1 f3, f10, f20	- Symptomatic - 4 year old palm - 89% of fronds with orange spotting - no orange spotting in f3 - tree bearing small fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRD 2 f3, f10, f20	- Symptomatic - 4 year old palm - 84% of fronds with orange spotting - no orange spotting in f3 - tree bearing small fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRD 3 f3, f10, f20	- Symptomatic - 6 year old palm - 83% of fronds with orange spotting - no orange spotting in f3 - tree bearing small fruit bunch	*Dura* x *Dura* (Nigerian germplasm)	Selangor River Estate, Sime Darby Plantations
SRD 4 f20	- Symptomatic - 6 year old palm - tree bearing small fruit bunch	*Dura* x *pisifera* (Golden Hope commercial)	Selangor River Estate, Sime Darby Plantations
SRD 5 f20	- Symptomatic - 6 year old palm - orange spots were less frequent and palm was stunted - tree bearing no fruit bunch - suspected with crown disease	*Dura* x *Dura* (Nigerian germplasm)	Selangor River Estate, Sime Darby Plantations
SRD 6 f12	- Symptomatic - 3 year old palm - no fruit bunch - stunted compared to adjacent asymptomatic palms	EBOR material	Selangor River Estate, Sime Darby Plantations

- f3, f10, f20 denotes frond no. 3, 10 and 20, which indicates late, mid and early stages of disease respectively.

Sample	Characteristic	Seed source	Site
CEP 1 f3, f10, f20	- Symptomatic - 22 year old palm - 49% of fronds with orange spotting - no orange spotting in f3 - no fruit bunch	*Dura* x *pisifera* (Chemara)	CEP Renggam Estate, Sime Darby Plantations
CEP 2 f3, f10, f20	- Symptomatic - 22 year old palm - 69% of fronds with orange spotting - no orange spotting in f3 - no fruit bunch	*Dura* x *pisifera* (Chemara)	CEP Renggam Estate, Sime Darby Plantations
CEP 3 f3, f10, f20	- Symptomatic - 22 year old palm - 78% of fronds with orange spotting - no orange spotting in f3 - no fruit bunch	*Dura* x *pisifera* (Chemara)	CEP Renggam Estate, Sime Darby Plantations
CEP 4 f3, f10, f20	- Symptomatic - 22 year old palm - 77% of fronds with orange spotting - no orange spotting in f3 - no fruit bunch	*Dura* x *pisifera* (Chemara)	CEP Renggam Estate, Sime Darby Plantations

- f3, f10, f20 denotes frond no. 3, 10 and 20, which indicates late, mid and early stages of disease respectively.

2. Oil palm samples from Waite glasshouse

Sample	Characteristic	Source
HOP	- asymptomatic oil palm seedling (RHS)	J.W. Randles
OP40-	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	- as indicated -
OP43-	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	- as indicated -
OP29-	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	- as indicated -
OP29+	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	- as indicated -
OP12+	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	- as indicated -
OP14+	- oil palm inoculated with nucleic extract of OS palm from the Solomon Islands	- as indicated -
Co28	- healthy oil palm seedling	- as indicated -
E21	- oil palm inoculated with cloned CCCVd	- as indicated -
T31A	- oil palm inoculated with cloned CCCVd	- as indicated -

3. Coconut leaf materials

Sample	Characteristic	Source
CCCVd	- middle stage coconut cadang-cadang disease leaflets from the Philippines	D. Hanold
HCoCo	- healthy coconut seedling	Coconut collection, Waite Glasshouse
E1, E2, E3	- early stage coconut cadang-cadang disease leaflets from the Philippines	J.W. Randles
M1, M2	- middle stage coconut cadang-cadang disease leaflets from the Philippines	J.W. Randles
L1, L2, L3	- late stage coconut cadang-cadang disease leaflets from the Philippines	J.W. Randles
B1, B2, B3	- coconut cadang-cadang disease leaflets with brooming symptoms from the Philippines	J.W. Randles
H1	- 4 year old coconut palm from Sri Lanka - leaf spots in frond 3 - not bearing nuts	J.W. Randles
H2	- 4 year old palm from Sri Lanka - not bearing nuts	J.W. Randles
LSD	- 16 year old palm from Sri Lanka (B/E 4039) - lethal scorch decline - bearing nuts	J.W. Randles
CRD	- 16 year old palm from Sri Lanka (B/E CRD1) - coconut rapid decline	J.W. Randles

APPENDIX B

BIOCHEMICALS AND MISCELLANEOUS CHEMICALS

BIOCHEMICAL	SOURCE
Ampicillin	Roche Mannheim, Germany
AMV-RT	Promega, USA
dATP	Promega, USA
dCTP	Promega, USA
dGTP	Promega, USA
dTTP	Promega, USA
BamH I	Promega, USA
DNA step ladder, 25 bp	Promega, USA
DNA ladder, 100 bp	Promega, USA
DNA ladder, 1 kb	Promega, USA
EcoR I	Promega, USA
Herring testis DNA	Sigma, USA
Mse I	Invitrogen, Australia
^{32}P-dUTP	Geneworks, Australia
pDrive cloning vector	Qiagen, Australia
Proteinase K	Amresco, USA
PCR Master mix	Promega, USA
rATP	Promega, USA
rCTP	Promega, USA
rGTP	Promega, USA
rUTP	Promega, USA
RNase A	Roche Mannheim, Germany
RNase T1	Roche Mannheim, Germany
RNasin® Ribonuclease inhibitor	Promega, USA
RQ1 DNase (RNAse-free)	Promega, USA
Sac I	Promega, USA
Sal I	Promega, USA
tRNA (Wheat germ)	Sigma, USA
T7 RNA polymerase	Promega, USA

CHEMICAL	SOURCE
Acetic acid, glacial	BDH Chemical, England
Acrylamide	Sigma, USA
Agarose 3:1 high resolution blend	Amresco, USA
Ammonium acetate	BDH Chemical, Australia
Ammonium persulfate	Sigma, USA
Boric acid	BDH Chemical, Australia
Bromophenol blue	BDH Chemical, England
Butan-1-ol	BDH Chemical, Australia
Calcium chloride	BDH Chemical, Australia
Cellulose, medium fibrous (CF11)	Whatman Laboratory, England
Chloroform	BDH Chemical, Australia
CTAB	Ajax chemicals Ltd., Australia
DTT	Promega, USA
EDTA, disodium salt	BDH Chemical, Australia
Ethanol	BDH Chemical, Australia
EtBr	Amresco, USA
Ficoll®400	Pharmacia, Sweden
Formaldehyde, 40 % (w/v)	May & Baker (M&B), Australia
Formamide	Sigma, USA
Glycerol	BDH Chemical, Australia
D-Glucose	BDH Chemical, Australia
Hydrochloric acid	Ajax chemicals Ltd., Australia
8-Hydroxy-quinoline	Sigma, USA
IPTG	Amresco, USA
Iso-Amyl alcohol	Ajax chemicals Ltd., Australia
Iso-propyl alcohol (Propan-2-ol)	BDH Chemical, Australia
N-Lauroyl-sacosine (sarkosyl)	Sigma, USA
Lithium chloride	Sigma, USA
Luria Agar	Sigma, USA
Luria Broth	Sigma, USA
Magnesium chloride	BDH Chemical, Australia

CHEMICAL	SOURCE
N, N'-Methylene-bis-acrylamide	Sigma, USA
α-Monothioglycerol	Sigma, USA
Phenol	Ajax chemicals Ltd., Australia
PEG 8000	Sigma, USA
PIPES	Sigma, USA
PVP 40	Sigma, USA
PVPP	Sigma, USA
Silver nitrate	Merck, Australia
Sodium borohydride	Sigma, USA
Sodium acetate	BDH Chemical, Australia
tri-Sodium citrate	BDH Chemical, Australia
Sodium chloride	BDH Chemical, Australia
Sodium metabisulphite	Ajax chemicals Ltd., Australia
Sodium dihydrogen orthophosphate dehydrate	BDH Chemical, England
di-Sodium hydrogen orthophosphate	BDH Chemical, England
Sodium hydroxide	Amresco, USA
Sodium sulphite anhydrous	BDH Chemical, Australia
SDS	BDH Chemical, England
TEMED	Sigma, USA
Trizma base	Sigma, USA
Tryptone peptone (Pancreatic digest of Casein)	Becton Dickinson, USA
Urea	BDH Chemical, Australia
X-gal	Amresco, USA
Xylene cyanol F.F.	Ajax chemicals Ltd., Australia
Yeast extract	Oxoid Ltd., England

APPENDIX C

POLYACRYLAMIDE AND AGAROSE GELS, BACTERIAL MEDIA, BUFFERS AND SOLVENTS

1. Acrylamide solutions

Analytical gels

30 % Acryl : Bis (39:1) 29.25 g acrylamide and 0.75 g bisacrylamide were dissolved in SDDW and the volume was adjusted to 100 ml. The solution was sterilised by filteration (0.4 µm) and stored at room temperature in the dark.

40 % Acryl : Bis (39:1) 39 g acrylamide and 1 g bisacrylamide were dissolved in SDDW and the volume was adjusted to 100 ml. The solution was filter sterilised and stored as above.

40 % Acryl : Bis (32.3:1) 38.8 g acrylamide and 1.2 g bisacrylamide were dissolved in SDDW and the volume was adjusted to 100 ml. The solution was filter sterilised and stored as above.

2. Polyacrylamide gels

Components	5 % Non-Denaturing 1X TAE	5 % 8M Denaturing 0.25X TBE	5 % 8M Denaturing 1X TBE	15 % 8M Denaturing 1X TBE	20 % Non-denaturing 1X TAE
Urea	-	16.8 g	25.2 g	16.8 g	-
Buffer - 10 X TAE - 10 X TBE	3 ml	- 0.87 ml	- 5.25 ml	- 3.5 ml	3 ml -
30% Acryl : Bis (39:1) 40% Acryl : Bis (39:1) 40% Acryl : Bis (32.3:1)	5 ml - -	5.8 ml - -	8.7 ml - -	- 13.1 ml -	- - 15 ml
10% APS	450 µl	500 µl	750 µl	500 µl	450 µl
TEMED	45µl	30 µl	45 µl	30 µl	45µl
SDDW	21.5 ml	14.1 ml	17.25 ml	4.4 ml	11.5 ml
Total gel volume	30 ml	35 ml	52.5 ml	35 ml	30 ml

3. Analytical agarose gels

Agarose gel	1.5-2.0 g agarose was dissolved by heating in a microwave oven with 100 ml 1X TAE/1X TBE/0.5X TBE, cooled to 60 ^{o}C. EtBr was added to 0.5 μg/ml.

4. Bacterial media

LB medium (1 L)	25 g of Luria broth (Sigma) was dissolved in 1 L of DDW. The solution was sterilised by autoclaving for 20 min at 121 ^{o}C/15 psi on liquid cycle.
SOC medium (100 ml)	2 g tryptone, 0.5 g yeast extract, 1 ml of 1M NaCl and 250 µl of 1M KCl was mixed with 97 ml DDW and shaken until the solutes dissolved. Adjusted to pH 7.0 with 1N NaOH and sterilise by autoclaving for 20 min at 121 ^{o}C/15 psi on liquid cycle. The solution was cooled to less than 60 ^{o}C and 1 ml of 1M $MgCl_2$ and 1 ml of filter sterilized (0.22 µm) 2M glucose were added. The volume was adjusted to 100 ml with SDDW.

5. Bacterial agar

LB agar (1 L)	40 g of Luria Agar (Sigma) was suspended in 1 L of DDW. The solution was sterilised by autoclaving for 20 min at 121 ^{o}C/15 psi on liquid cycle. It was cooled ca. 50 ^{o}C and ampicillin was added to 25 mg/ml. Approximately about 20 ml of the melted agar solution was poured into 90 mm Petri dishes in a lamina-flow and allowed to set. Plates were inverted and stored at 4 ^{o}C for 1 month. For blue/white colony selection, 80 mg/ml X-gal (20 µl/plate) and 1 M IPTG (4 µl per plate) were mixed and spread over the surface of LB-ampicillin plates and allowed to absorb for 30 min at 37 ^{o}C prior to use.

6. Glycerol cultures

0.85 ml of overnight culture was added to 0.15 ml of sterile glycerol. The mixture was vortexed and transferred to a cryotube (NUNC, USA). The culture was freezed in liquid nitrogen before storage at -70 ^{o}C.

7. Buffers

10X TBE: 0.9 M Tris, 0.8 M HBO_3, 20 mM Na_2EDTA, pH 8.0

10X TAE: 0.4 M Tris, 0.3 M NaOAc, 9 mM Na_2EDTA, adjusted to pH 7.2 with acetic acid

20X SSPE: 0.3 M Na_2HPO_4, 2.4 M NaCl, 0.3 M Na_3-citrate, 40 mM Na_2EDTA pH 8.0

20X SSC: 3 M NaCl, 0.3 M Na_3-citrate, adjusted to pH 7.2 with NaOH

10X STE: 0.5 M Tris, 10 mM Na_2EDTA, 1 M NaCl, adjusted to pH 7.2 with HCl

1X TE: 10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA pH 8.0

8. Gel loading buffers

10X Non-denaturing (10ml): 0.25 % (w/w) bromophenol blue, 0.25 % (w/w) xylene cyanol, 50 % glycerol, 10 mM Na_2EDTA pH 8.0. Sterilised by autoclaving for 20 min at 121 ^{o}C/15 psi on liquid cycle.

10X Denaturing (10ml): 0.25 % (w/w) bromophenol blue, 0.25 % (w/w) xylene cyanol, 10 mM Na_2EDTA pH 8.0 and add formamide to 10 ml

9. Blotting solution

Capillary blotting: 0.01 N NaOH / 3 M NaCl or 0.02 N NaOH

Electroblotting: 25 mM $NaH_2PO_4.2H_20$, adjusted to pH 6.5 with NaOH

10. Pre-hybridization /Hybridization solution

Hybridization solution	50 % Formamide, 5X SSPE, 0.1 % Ficoll®400, 0.1 % PVP, 1 % SDS, 100 µg/ml denatured Herring testis DNA

11. Organic reagents

CA solution	24 volumes of chloroform were mixed with 1 volume of iso-amyl alcohol and stored in a light protected bottle at 4 °C.
Ethanol	Absolute and 70 % (v/v) ethanol stored at room temperature. 75 % (v/v) ethanol stored at -20 °C.
PCA solution	One volume of CA solution was mixed with 1 volume of water saturated phenol and stored in a light protected bottle at 4 °C.
Water saturated Phenol	500 g phenol was melted in a 65 °C waterbath and had 8-hydroxyquinoline added to 0.1 % (w/w) and 55 ml of SDDW was added and stirred overnight to mix the phases. The saturated phenol was stored in a light sensitive bottle at 4 °C.

APPENDIX D

COMPONENTS OF KITS USED IN THIS THESIS

Riboprobe® in vitro transcription system (Promega, USA)

Components	Final concentration (20µl volume)
Linearised plasmid (0.2 µg/µl)	1 µg
Transcription optimized 5X buffer - 40 mM Tris-HCL pH 7.9 - 6 mM $MgCl_2$ - 2 mM spermidine - 10 mM NaCl	1X Transcription buffer - 8 mM Tris-HCL pH 7.9 - 1.2 mM $MgCl_2$ - 40 µM spermidine - 2 mM NaCl
100 mM DDT	10 mM
Recombinant RNasin®Ribonuclease Inhibitor (20 u/µl)	20 u
* rNTP's (rATP, rCTP, rGTP), 2.5 mM each	0.5 mM each rATP, rCTP, rGTP
T7 RNA polymerase (20 u/µl)	20 u
α-^{32}P-UTP (270 µCi; 3000 Ci/µMole)	3 µCi

* 2.4 µl of 100 µM cold UTP was added to the reaction mix for RPA experiments.

Reverse transcription system (Promega, USA)

Components	Final concentration (20µl volume)
25 mM $MgCl_2$	5 mM
10 X Reverse Transcription buffer - 100 mM Tris-HCl (pH 9.0 at 25°C) - 500 mM KCl - 1% Triton®X-100	1X Transcription buffer - 10 mM Tris-HCl (pH 9.0 at 25°C) - 50 mM KCl - 0.1% Triton®X-100
10 mM dNTP mixture	1 mM each dNTP
Recombinant RNasin®Ribonuclease Inhibitor (40 u/µl)	20 u
AMV Reverse Transcriptase (24 u/µl)	24 u
Primer, 10 µM	0.5 µM
RNA sample	2 µl
Nuclease free water	To a final volume of 20 µl

PCR master mix (Promega, USA)

2X, master mix — 50 units/ml *Taq* DNA polymerase, 400 µM each, dATP, dCTP, dGTP, dTTP, 3 mM $MgCl_2$. 1X master mix was utilized for PCR reaction.

PCR cloning kit (Qiagen, Australia)

Components	Final concentration (6µl reaction)	Final concentration (8µl reaction)
pDrive cloning vector (25ng/µl)	1 µl (25 ng)	1 µl 25 ng
PCR product (50 ng/µl)	2 µl (100 ng)	3 µl (150 ng)
2X ligation master mix	3 µl (1X)	4 µl (1X)

APPENDIX E

CCCVd PROBE

A) pGEM-T Easy plasmid vector (Promega, USA)

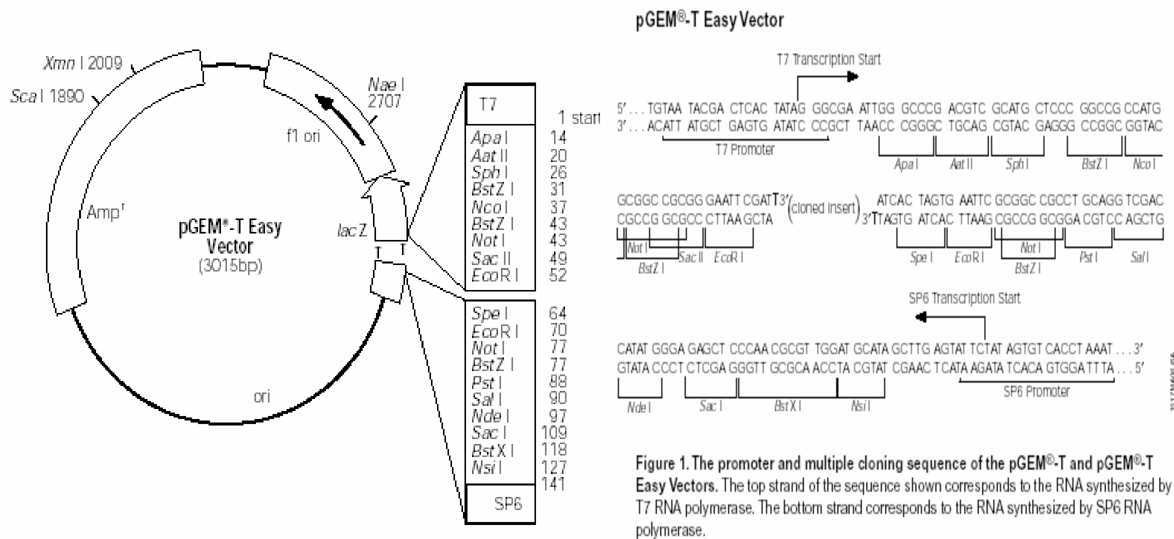


Figure 1. The promoter and multiple cloning sequence of the pGEM®-T and pGEM®-T Easy Vectors. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

B) Sequence of $CCCVd_{246}$ inserted in the multiple cloning site of the above vector

clamp *EcoRI* *

5'-GGCGCGGCGAATTCCCCCGGGGATCCCTCAAGCGGCCTCTCCTGCAGTAGTTTTTGG
3'-CCGCGCCGCTTAAGGGGGCCCCTAGGGAGTTCGCCGGAGAGGACGTCATCAAAAACC

GGTGCCCTGTAGATTTCCCCAGAGGGGCTACAAAGGGACACCTTTTTTTACTAACAACA
CCACGGGACATCTAAAGGGGTCTCCCCGATGTTTCCCTGTGGAAAAAAATGATTGTTGT

GGTAAGAGCCGCGTGAGTTGTATCCACCGGGTAGTCTCCCAAGCGGTCGAGGAGGGGCG
CCATTCTCGGCGCACTCAACATAGGTGGCCCATCAGAGGGTTCGCCAGCTCCTCCCCGC

GCCGGGCGTCTAAGCTACGAAGGAGTCTCCTCCAACGCGCACGATCGACCCAGGTACGC
CGGCCCGCAGATTCGATGCTTCCTCAGAGGAGGTTGCGCGTGCTAGCTGGGTCCATGCG

TCCCTTCCCAGATTCGCTTGAGGTTTGTCGACGCCGCGCC-3'
AGGGAAGGGTCTAAGCGAACTCCAAACAGCTGCGGCGCGG-5'

*Sal1** clamp

- Alphabets in black- complementary sense sequence of $CCCVd_{246}$
- Alphabets in red – sequence of $CCCVd_{246}$ (sequence starts at nt 65 of $CCCVd_{246}$)

*- restriction sites

APPENDIX F

STAINING SOLUTIONS

1. Silver staining

Silver nitrate solution (0.2 %)	0.3 g of silver nitrate was dissolved in 150 ml DDW
Developing solution	375 mM NaOH, 0.15 % (v/v) CH_2O and 3 mM $NaBH_4$

2. Ethidium bromide

Ethidium bromide solution	Ethidium bromide was dissolved in DDW to 10 mg/ml and stored at room temperature in a light protected bottle. The solution was diluted to 1 ppm for agarose and 0.5 ppm for polyacrylamide gels.

APPENDIX G

PRIMERS USED IN THIS THESIS

RT-PCR primers (Geneworks, Australia)

PC (30 mer) 5'-d(CGA ATC TGG GAA GGG AGC GTA CCT GGG TCG)-3'

RT (31 mer) 5'-d(AGG TTT CCC CGG GGA TCC CTC AAG CGG CCT C)-3'

GV1 (25 mer) 5'-d(ACG TCA AGC GAA TCT GGG AAG GGA G)-3'

GVR1 (23 mer) 5'-d(GAT CCC TCA AGC GGC CTC TCC TG)-3'

GV2 (19 mer) 5'-d(ATC TAC AGG GCA CCC CAA A)-3'

GVR2 (20 mer) 5'-d(AAG AGC CGC GTG AGT TGT AT)-3'

GV3 (25 mer) 5'-d(GTA CCT GGG TCG ATC GTG CGC GTT G)-3'

GVR3 (25 mer) 5'-d(CCC AGA TTC GCT TGA CGT TTC CCC G)-3'

GV4 (18 mer) 5'-d(ACT CAC GCG GCT CTT ACC)-3'

GVR4 (20 mer) 5'-d(TGT ATC CAC CGG GTA GTC TC)-3'

Sequencing primers (Geneworks, Australia)

M13 forward (17 mer) 5'-d(GTA AAA CGA CGG CCA GT)-3'

M13 reverse (16 mer) 5'-d(AAC AGC TAT GAC CAT G)-3'

SP6 promoter (19 mer) 5'-d(CAT TTA GGT GAC ACT ATA G)-3'

T7 promoter (19 mer) 5'-d(GTA ATA CGA CTC ACT ATA G)-3'

PCR primers for phytoplasma detection (Geneworks, Australia)

P1 (25 mer) 5'-d(AAG AGT TTG ATC CTG GCT CAG GAT T)-3'

P7 (18 mer) 5'-d(CGT CCT TCA TCG GCT CTT)-3'

R16F2n (20 mer) 5'-d(GAA ACG ACT GCT AAG ACT GG)-3'

R16R2 (24 mer) 5'-d(TGA CGG GCG GTG TGA CAA ACC CCG)-3'

LY16Sf (21 mer) 5'-d(CAT GCA AGT CGA ACG GAA ATC)-3'

LY16Sr (20 mer) 5'-d(GCT TAC GCA GTT AGG CTG TC)-3'

APPENDIX H

SEQUENCES OF CLONES OF OIL PALM CCCVd VARIANTS

A) OP_{297}

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CCCVd296    CUGGGGAAAUCUACAGGGCACCCCAAAAACCACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV1         CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV9         CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV17        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV18        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV3         CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GVP8        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV2         CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GVP1        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GVP2        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV16        CUGGGGAAAUCUACAGGGCACCCCAAAAACUAUUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GVP7        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV15        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
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CCCVd296    CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCGCGUUGGA 120
GV1         CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 120
GV9         CGGGGAAACCUCAAACGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 120
GV17        CGGGGAAACCUCAAACGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 120
GV18        CGGGGAAACCUCAAACGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 120
GV3         CGGGGAAACCUCAAACGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 120
GVP8        CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
GV2         CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
GVP1        CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
GVP2        CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
GV16        CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
GVP7        CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
GV15        CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 116
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CCCVd296    GGA-CUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGACUCC 179
GV1         GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 180
GV9         GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 180
GV17        GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 180
GV18        GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 180
GV3         GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 180
GVP8        GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 176
GV2         GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGUCUCC 176
GVP1        GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGAUUCCUUCGUAGCUUCGACGCCCGCCUCC 176
GVP2        GGAGCUGGGUCGAGCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 176
GV16        GGAGCUGGGUCGAUCGUGCGCGUUGGAGUAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 176
GVP7        GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUUCUUCGUAGCUUCGACGCCCGCCUCC 176
GV15        GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC 176
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CCCVd296    UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 239
GV1         UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 240
GV9         UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 240
GV17        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 240
GV18        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 240
GV3         UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 240
GVP8        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
GV2         UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
GVP1        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
GVP2        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
GV16        UUCGUAACUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
GVP7        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
GV15        UUCUUAGCCUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 236
            *** ** * ***************************************************

CCCVd296    CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 296
GV1         CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 297
GV9         CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 297
GV17        CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 297
GV18        CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 297
GV3         CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 297
GVP8        CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 293
GV2         CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 293
GVP1        CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 293
GVP2        CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCUU 293
GV16        CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 293
GVP7        CAACUCAUGCGGCUCUUACCGGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 293
GV15        CAACUCAUGCGGCUCUCACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 293
            ******* ******** *** ********************************** *
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"*" indicate nucleotides which are identical in all sequences in the alignment.

- Letters in blue indicate substitution; red letter indicate an insertion; '----' indicate nucleotides not read because primers (set I) did not include this region.

- Sequences highlighted in yellow indicate identical clones which were used to define the consensus sequence of this variant

B) OP_{293}

```
CCCVd296   CUGGGGAAAUCUACAGGGCACCCCAAAAACCACUGCAGGAGAGGCCGCUUGAGGGAUCCC  60
GV10       CUGGGGAAAUCUACAGGGC.CCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC  59
GV12       CUGGGGAAAUCUACAGGGC.CCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC  59
GVP4       CUGGGGAAAUCUACAGGGC.CCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC  59
GVP5       CUGGGGAAAUCUACAGGGC.CCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC  59
           ******************* ********** *****************************

CCCVd296   CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCGCGUUGGA  120
GV10       CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCUCGUUGGA  119
GV12       CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCUCGUUGGA  119
GVP4       CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUUCCUGGGUCGAUCGUGCUCGUUGGA  115
GVP5       CGGGGAAACCU----CGAAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA  115
           ***********    ******************** ********* ****** *******

CCCVd296   GGA-CUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGACUCC  179
GV10       GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACCCCCGCCUCC  179
GV12       GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACCCCCGCCUCC  179
GVP4       GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACCCCCGCCUCC  175
GVP5       GGAGCUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGCCUCC  175
           *** ********************************************** **** ****

CCCVd296   UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA  239
GV10       UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA  239
GV12       UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA  239
GVP4       UUCGUAGCUUCGACUCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA  235
GVP5       UUCGUAGCUUCGACACCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA  235
           ************** *********************************************

CCCVd296   CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU  296
GV10       CA.CUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGU..CCCUUUGUAGCCCCU  293
GV12       CA.CUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGU..CCCUUUGUAGCCCCU  293
GVP4       CA.CUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGU..CCCUUUGUAGCCCCU  289
GVP5       CA.CUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGU..CCCCUUGUAGCCCCU  289
           ** *************************************  *** ***********
```

"*" indicate nucleotides which are identical in all sequences in the alignment.

- Letters in blue indicate substitution; red letter indicate an insertion; '....' indicates deletions; '----' indicate nucleotides not read because primers (set I) did not include this region.

- Sequences highlighted in yellow indicates identical clones which were used to define the consensus sequence of this variant

C) OP_{270}

```
CCCVd296   CUGGGGAAAUCUACAGGGCACCCCAAAAACCACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV8        CUGGGGAAAUCUACAGGGCACCCCAAAAACUACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
           ****************************** *****************************

CCCVd296   CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCGCGUUGGA 120
GV8        CGGGGAAACCUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCGCGUUGGA 120
           ************************************************************

CCCVd296   GGA.CUGGGUCGAUCGUGCGCGUUGGAGGAGACUCCUUCGUAGCUUCGACGCCCGACUCC 179
GV8        GGA..........................GACUCCUUCGUAGCUUCGACGCCCGCCCC 153
           ***                          ************************* *  **

CCCVd296   UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 239
GV8        UUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACCCGGUGGAUA 213
           ************************************************************

CCCVd296   CAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 296
GV8        CAACUCAUGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUAGCCCCU 270
           ******* *************************************************
```

"*" indicate nucleotides which are identical in all sequences in the alignment.

- Letters in blue indicate substitution; '....' indicates deletions.

D) OP_{232} and OP_{165}

```
CCCVd246   CUGGGGAAAUCUACAGGGCACCCCAAAAACCACUGCAGGAGAGGCCGCUUGAGGGAUCCC 60
GV5        ...................................CAGGAGAGGCCGCUUGAGGGAUCCC 25
GV7        ...................................CAGGAGAGGCCGCUUGAGGGAUCCC 25
GV6        ...................................UAGGAGAGGCCGCUUGAGGGAUCCC 25
                                               ************************

CCCVd246   CGGGGAAACGUCAAGCGAAUCUGGGAAGGGAGCGUACCUGGGUCGAUCGUGCGCGUUGGA 120
GV5        CGGGGAAACCU----......UGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 75
GV7        CGGGGAAACCU----......UGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUGGA 75
GV6        CGGGGAAACCU----..AAUCUGGGAAGGGAGCGUACCUGGGUCGUUCGUGCGCGUUG.. 77
           ********* *          *********************** ************

CCCVd246   GGAGACUCCUUCGUAGCUUCGACGCCCGGCCGCCCCUCCUCGACCGCUUGGGAGACUACC 180
GV5        GGGGCUGGGUCGAUCGUGCGCGUUGCAGGAGAGGCCGCUUGAGGGAUCAGGAGAGGCCGC 135
GV7        GGGGCUGGGUCGAUCGUGCGCGUUGCAGGAGAGGCCGCUUGAGGGAUCAGGAGAGGCCGC 135
GV6        .........................CAGGAGAGGCCGCUUGAGGGAUCAGGAGAGGCCGC 112

CCCVd246   CGGUGGAUACAACUCACGCGGCUCUUACCUGUUGUUAGUAAAAAAAGGUGUCCCUUUGUA 240
GV5        UUGAGGGAUCAGGAGAGGCCGCUUGAGGGAUCAGGAGAGGCCGCUUGAGGGAUCAGGAGA 195
GV7        UUGAGGGAUCAGGAGAGGCCGCUUGAGGGAUCAGGAGAGGCCGCUUGAGGGAUCAGGAGA 195
GV6        UUGAGGGAUCAGGAGAGGCCGCUUGAGGGAUCAGGAGAGGCCGCUUGAGGGAU....... 165

CCCVd246   GCCCCU.............................. 246
GV5        GGCCGCUUGAGGGAUCAGGAGAGGCCGCUUGAGGGAU 232
GV7        GGCCGCUUGAGGGAUCAGGAGAGGCCGCUUGAGGGAU 232
GV6        .....................................
```

"*" indicate nucleotides which are identical in all sequences in the alignment.

- Letters in blue indicate substitution; pink letters indicate the 21 nt repeat found in OP_{232}; green letters indicate the 22 nt repeat found in both OP_{232} and OP_{165}; '....' indicate deletions; '----' indicates nucleotides not read because primers (set I) did not include this region.

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