THE ETIOLOGY OF SUGARCANE STRIATE MOSAIC DISEASE

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SUMMARY

Sugarcane striate mosaic disease (ScSMD) is a sugarcane disease of unknown etiology that has virus-like symptoms. It occurs in the Burdekin River district of North Queensland. A characteristic symptom of ScSMD is the development of fine chlorotic striations on the lamina and stem. Other effects of the disease in the field are poor germination of cuttings and ratoons, shortening of internodes and stunting and yellowing of whole leaf blades. Sugarcane fields affected by ScSMD are ploughed out prematurely.

In this thesis, glasshouse trials showed that cuttings from ScSMD affected sugarcane had delayed germination and the germinated sugarcane plants from ScSMD affected cuttings showed reduced growth rate and yield when compared with the cuttings from healthy sugarcane plants. For example, the ScSMD affected sugarcane cuttings germinated 1-2 weeks later than the cutting from healthy sugarcane. The newly germinated shoots from the ScSMD affected cutting were thin, short and showing striation. The ScSMD sugarcane had 2-3 less leaves than healthy sugarcane plants. The height of the diseased plants was shorter than those of the healthy sugarcane plants after germination. The ScSMD affected sugarcane were thinner and shorter than that of the healthy sugarcane. There was no difference in the length of the first and the second oldest leaves, but above these the ScSMD affected sugarcane had an average 20 cm shorter leaves than healthy sugarcane.

There were disease associated morphological and structural changes in the cells of the ScSMD affected sugarcane leaf. The chloroplasts in ScSMD affected leaf were more spherical than in those in the healthy leaf. The grana of the ScSMD affected chloroplasts were not different in shape when compared with those in the healthy sugarcane. However, the thylakoids in the spherical chloroplasts were deformed and undulating in appearance. There were also some electron dense materials between the deformed thylakoids. Numerous widened plasmodesmata could be detected in cell walls of the ScSMD affected sugarcane. Some empty membrane bound vesicles were present close to the deformed plasmodesmata or the deformed chloroplasts. The vesicles varied in size.

dsRNAs were isolated by the standard CF11 cellulose chromatography method from symptomatic leaves of ScSMD affected sugarcane. The ScSMD associated dsRNAs

contained a major component ca. 9 kbp in size. Minor smaller subgenomic dsRNAs (about 6.0, 2.6 and 2.5 kbp) of the 9 kbp dsRNA were also observed. The ScSMD associated dsRNAs were isolated from ScSMD affected sugarcane collected in several sites in Queensland but not from the healthy sugarcane.

An improved dsRNA extraction method was developed using microgranular cellulose in a batch procedure. The microgranular cellulose had higher binding capacity than the CF11 cellulose. The yield was at least 10 fold higher than for the CF11 cellulose method.

The dsRNA preparation was treated with RNase and DNase to eliminate the host DNA and RNA in a buffer containing 0.3 M sodium salt. It was fractionated by agarose. The purified dsRNAs was fractionated by polyacrylamide gel electrophoresis, and extracted, melted and used for making cDNA.

The random PCR method was adapted to make a cDNA library from dsRNA. This process was successfully produced cDNA library from 10-100 ng of the dsRNA. The cDNAs were cloned into pGEM-7zf(+). All clones obtained hybridised to the ScSMD associated dsRNA.

Five of the clones were sequenced and found to represent three segments that together comprised about 2.55 kb (28 %) of the dsRNA sequence. A sequence similarity search of the three ScSMD segments revealed that they had similarity to 18 viruses in 6 genera. Five of these genera comprised viruses with helical symmetry (*Capillo-*, *Carla-*, *Potex-*, *Poty-* and *Trichovirus* genera) whereas the sixth was the icosahedral genus *Tymovirus*. The greatest similarity was found between these virus sequences and ScSMD segment 3 and the least similarity was with ScSMD segment 2. The greatest overall similarity was found between the three ScSMD segments and apple stem pitting virus (ASPV), with slightly less similarity to blueberry scorch and potato virus M carlaviruses. The three ScSMD specific segments had 52 to 68 % nucleotide sequence and 52 % amino acid sequence similarity with the proposed replicase open reading frame of ASPV, a putative carlavirus with a 9.3 kb genome.

A ScSMD associated virus particle, sugarcane striate mosaic virus (ScSMV), was purified and identified by hybridisation with probes prepared from the ScSMD specific clones. The virus particle was a slightly flexible filament, 900- 1,000x 15 nm in size. A purified preparation of ScSMV had a single protein component ca. 51 kDa in size which was assumed

to represent the capsid protein. The nucleic acid isolated from the ScSMV particles was ssRNA with a polyadenylic acid sequence at the 3' end and ca. 9 kb in size. The ScSMV particles most closely resembled those of the longer carlaviruses such as ASPV, BlSV and PVM, and the capsid protein was also close in size to these than that of the shorter carlaviruses.

A diagnostic method was developed using dot blot hybridisation or RT-PCR assay. Dot hybridisation assay using a ^{32}P labelled probe transcribed from cloned inserts 640 or 612 bp in size was specific for detection of ScSMD. Three primers (SD100, SD500 and SD900) were designed for RT-PCR to detect ScSMD by amplification of a segment in ScSMD segment 2 and a set (SD100 and SD900) detected ScSMV in total nucleic acid extracts of ScSMD affected sugarcane leaves.

It was observed that some of the ScSMD affected sugarcane had phytoplasma-like symptoms such as white leaf striations, severe stunting and phytoplasma-like vesicles in the phloem cells. PCR assay was used to determine whether phytoplasmas were associated with ScSMD. PCR was done with three pairs of phytoplasma 16S rRNA specific universal primers. Phytoplasmas were detected in some plants using all three sets of primers but they were found in both ScSMD affected and healthy sugarcane. It was concluded that they were not specifically associated with ScSMD, and their effect on sugarcane needs to be further investigated.

In conclusion, this thesis describes the use of molecular nucleic acid methods to associate a ssRNA plant virus, ScSMV, with ScSMD. Diagnostic methods for future studies have been described. The possible interaction of the ScSMV with other potential pathogens in sugarcane plants has been suggested.

STATEMENT

This thesis contains no material which has been previously presented for any other degree in any university and to the best of my knowledge contains no material previously published or written by another person, except where due reference is 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.

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ABBREVIATIONS

A	Absorbance
amp	Ampicillin
AMV	Alfalfa mosaic virus
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APS	Ammonium persulfate
ASPV	Apple stem pitting virus
BAA	N, N'-Methylene-bis-acrylamide
BAC	N, N'-Bisacrylylcystamine
BlSV	Blueberry scorch virus
BME	2-Mercaptoethanol
bp	Base pair
BSA	Bovine serum albumin
CF11	Fibrous medium cellulose
CMV	Cucumber mosaic virus
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-thimidine-5'-triphosphate
dNTP	Mixture of deoxynucleoside-triphosphates in equimolar amounts
DEPC	Diethyl pyrocarbonate
DIW	De-ionized water
DNA	Deoxyribonucleic acid
DNase-rf	RNase free deoxyribonuclease
DTT	Dithiothreitol
ds-	Double stranded
EDTA	Ethylenediamine *tetra* acetic acid
ELISA	Enzyme-linked immnosorbent assay
EtBr	Ethidium bromide
E-OH	Ethanol
g	Gram
g	Gravity
IPA	*iso*-Propyl alcohol
IPTG	*iso*-Propyl-β-D-thiogalactopyranoside
kbp	Kilo base pair

kDa	Kilo dalton
L	Litre
M	Molar
µ-	Micro-
m-	Milli-
MOPS	3-N-[Morpholino]propane sulfonic acid
M-OH	Methanol
M-MLV-RT	Moloney Murine leukemia virus reverse transcriptase
MTG	α-Monothioglycerol
MWt	Molecular weight
n	Nano-
NaAc	Sodium acetate
ORF	Open reading frame
PCI	Phenol: chloroform: *iso*-amyl alcohol (ratio of 25: 24: 1)
PCR	Polymerse chain reaction
PEG	Polyethylene glycol
PMV	Pea mosaic virus
ppm	Parts per million
PSbMV	Pea seed born mosaic virus
PVM	Potato virus M
PVP	Polyvinylpyrollidone
RH	Random hexamer
rPCR	Random PCR
rpm	Revolutions per minute
RNase-df	DNase free-ribonuclease
ScSMD	Sugarcane striate mosaic disease
SDIW	Sterilised de-ionised water
SDS	Sodium dodecyl sulphate
ss-	Single stranded
TEMED	N, N, N'-N'-Tetramethylethylenediamine
TLC	Thin-layer chromatography
TMV	Tobacco mosaic virus
Tris	Tris(hydroxymethyl) aminomethane
UN	Universal nucleotides
UV	Ultra violet
V	Voltage
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 Sugarcane industry in Australia

The commercial sources of sugar are sugarcane and sugar-beet. Sugarcane was first introduced into Australia in 1788 from New Guinea to access its potential for commercial production. 64% of Australian raw sugar is produced from sugarcane by 6,400 cane growers in a 2,100 kilometre long zone on the north-east coast of Australia between Mossman in Queensland and Grafton in New South Wales (Fig. 1.1). The total area of land allocated to cane growing is 365,000 hectares. The sugarcane industry is expanding at about 5% a year. Over 90% of the crop is grown in Queensland and 80% of production is exported (Croft and Smith, 1996).

1.2 Sugarcane plant

Sugarcane is a large grass of the genus *Saccharum* within the family *Gramineae*. The genus *Saccharum* has been selected and used as a source of sugar since the days of primitive man, but the origins of sugarcane continue to be debated. The term '*Saccharum* complex' has been used to describe a closely related interbreeding group believed to be progenitors of modern sugarcane (Daniels and Roach, 1987).

Cultivated sugarcanes are thought to have originated in New Guinea and other islands of the Malayan Archipelago. The other original sugarcane forms (*Saccharum beberi, S. edule, S. officinarum, S. robustum, S. sinense and S. spontaneum*) were imbued with many desirable characters including sweetness, lack of fibre, thick stalks and vigour. It is believed that *Saccharum officinarum* L. mostly grown in Australia may have been selected by man for its sweetness and low fibre (Alexander, 1973).

Interspecific hybridisation of *S. officinarum* ('noble' canes) with the wild *S. spontaneum* L. and *S. robustum* Brandes and Jeswiet ex Grassl., and subsequent backcrossing gave significant yield increases, a process known as 'nobilisation'. In this process the female parent, *S. officinarum*, contributes its somatic chromosome number to the progeny, whilst the

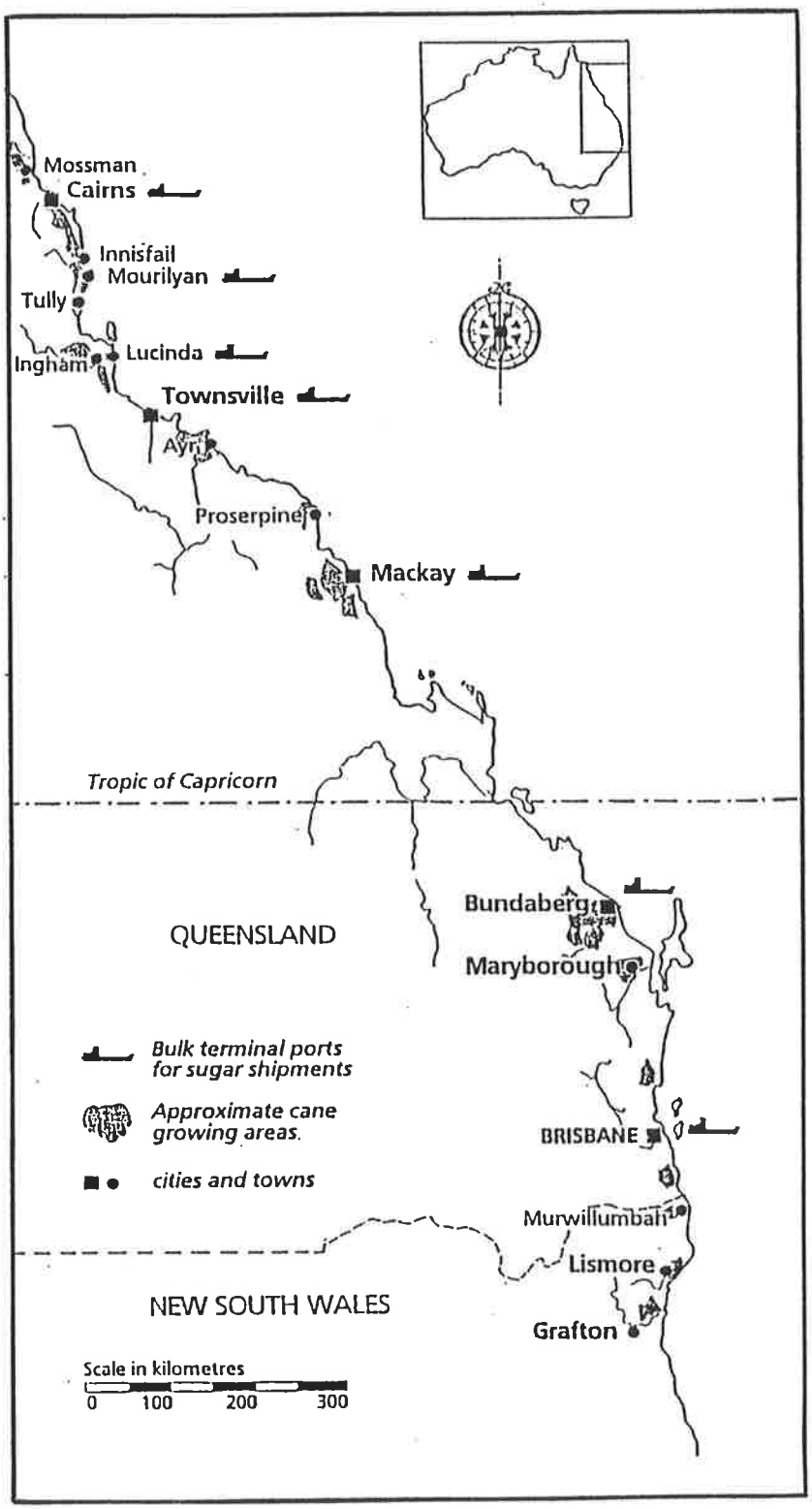


Figure 1.1 Map of sugarane growing areas in Australia (Anonymous, 1985).

male parent contributes vigour and disease resistance in a normally reduced chromosome complement. Commercial sugarcane cultivars are complex hybrids arising from this original 'nobilisation' and have high aneuploid chromosome numbers due to chromosomal increases accompanying certain crosses and backcrosses (Daniel and Roach, 1987).

Propagation of sugarcane is generally by asexual planting with setts, which are cut from cane with one or more lateral buds. A primary shoot will develop from the lateral buds and second shoots will develop from the primary shoot. Sugarcane is harvested after 12-16 months in Australia. The stalks of cane are able to regrow, a process known as ratooning (Alexander, 1973).

There are many different viral, bacterial and fungal diseases of sugarcane as well as diseases with unknown causal agents. They are listed in Tables 1.1 and 1.2.

1.3 Sugarcane striate mosaic disease

Sugarcane striate mosaic disease (ScSMD) is a severe disease of unknown etiology and first reported (Hughes, 1961) in the sugarcane (*Saccharum* spp.) hybrid clones, Pindar and Q56 in the lower Burdekin district of North Queensland (near Ayr, Queensland). Its distribution is limited but, due to premature plough-out of fields with the disease, complete crop loss occurs in the affected areas (B. Croft, pers. comm., 1996). Spread is slow but the mode of natural spread is unknown. Evidence that the disease recurs on the same farms and that soil treatment with nematicidal and fungicidal fumigants reduces incidence (Anon., 1969) suggested that it is soil-borne. It is also transmitted by vegetative propagation. Cuttings from ScSMD affected sugarcane plants could not be cured and the only means of control is the use of resistant cultivars (Hughes, 1961).

The symptom of ScSMD is characteristic striations on the leaf lamina. The youngest two leaves are usually symptomless. Although the pathology of ScSMD suggests a viral etiology (Hughes, 1961), no virus has been found to be associated with the disease. A preliminary report that three double-stranded (ds) RNAs could be isolated from affected sugarcane, suggested that a virus may be associated with ScSMD (Anon., 1985).

Table 1.1 Some major bacterial, fungal and phytoplasma diseases of sugarcane.

GROUP	DISEASE	PATHOGEN
Bacteria	Bacterial mottle	*Pectobacterium carotovorum*
	Gumming	*Xanthomonas campestris pv. vasculorum*
	Leaf scald	*Xanthomonas albilineans*
	Mottle stripe	*Pseudomonas rubrisubalbicans*
	Ratoon stunting	*Clavibacter xyli* spp. *xyli*
	Red stripe	*Pseudomonas rubrilineans*
Fungi	Banded sclerotial	*Pellicularia sasakii*
	Root rot	*Marasmius sacchari*
	Black rot	*Ceratocystis sp.*
	Brown rot	*Corticium sp.*
	Brown strip	*Cochliobolus stenopilus*
	Rust	*Puccinia melanocephala*
	Downy mildew	*Peronosclerospora sacchari*
	Dry top rot	*Sorosphaera vascularum*
	Eye spot	*Bipolaris sacchari* and *Drechslera sacchari*
	Pineapple	*Ceratocystis paradoxa*
	Red rot	*Glomerella tucumanensis*
	Sclerophthora	*Sclerophthora macnospora*
	Smut disease	*Ustilago scitaminea*
	Wilt	*Fusarium moniliform* and *Cephalosporium sacchari*
Phytoplasma	Grassy shoot	Nk*
	White leaf	Nk

* Nk; not known

Table 1.2 Sugarcane disease caused by viruses and by unknown agents

PATHOGENE	GENUS	Name (Acronym)	Genome
VIRUS	*Badnavirus*	Sugarcane bacilliform virus (SCBV)	dsDNA
	Closterovirus	Sugarcane mild mosaic virus (SCMMV)	ssRNA
	Fijivirus	Fiji disease virus (FDV)	dsRNA
	Potyvirus	Sugarcane mosaic virus (SCMV)	ssRNA
Unknown	Unknown	Chlorotic streak (SCCS)	unknown
		Ramu stunt disease (RSD)	"
		Striate mosaic disease (ScSMD)	"
		Yellow leaf syndrome (YLS)	"

1.4 Diagnostic methods for an unknown virus disease

A disorder on a plant could be associated with several causes, such as effects of the environment, malnutrition, herbicides, the plant genotype, or it may be infected with internal or external pathogens. A possible pathogen is implicated if the disorder can be still seen on the plant when no natural or environmental factors are involved. Extracellular pathogens such as nematodes, fungi and bacteria that either grow outside their host cells, propagate in the intercellular spaces or xylem, or produce intracellular haustoria, are generally detected routinely by existing procedures of microscopy, isolation, and/or culture. Intracellular pathogens such as membrane bound parasites that may be entirely located in the vascular tissue (vascular wilt fungi, fungal endophytes, fastidious phloem-infecting bacteria, flagellate protozoa or phytoplasmas) and those which are not membrane bound and which parasitise the symplast (viruses and viroids) are identified by more complex procedures (Randles *et al.*, 1996).

Diagnostic methods are either biological or non-biological. Biological diagnostic methods rely on transmission and amplification of intracellular pathogens in sensitive alternative hosts by using the biological properties of pathogens. The sensitivity of the tests is usually determined by the minimum number of pathogen propagules required to initiate infection. The minimum number of tobacco mosaic virus particles needed is probably in the range of 10-450 (Matthews, 1991). However, non-biological diagnosis methods depend on recognition of specific components of pathogens or pathogen groups. Electron microscopy, immunology, component isolation, nucleic acid probing and PCR amplification are applications of component analysis. These methods provide information on the presence of an organism (Randles *et al.*, 1996).

Recently developed diagnostic methods for plant viruses are listed in Table 1.3. Some of the more important methods are briefly explained below.

1.4.1 Light microscopy

Inclusions and aggregated particles produced by viruses, and host constituents may be identified with specific staining techniques, and followed by light microscopy. Examples of stains are azure A or B, acridine orange, methyl green-pyronin, a combination of

Table 1.3 Methods of diagnosis of plant viruses

METHOD	TEST	REFERENCES
Serological	ELISA	Clark & Adams,1977
	Enzyme-linked fluorescent assay	Neurath & Strick, 1981
	Dot immunobinding assay	Converse & Martin,1990
	Western blotting	Kumar *et al.*, 1985
	Single antibody dot immunoassay	Graddon & Randles, 1986
	Serological electron microscopy	Breyel & Casper, 1988
	Monoclonal antibody	Halk *et al.*, 1984
Nucleic acid	Double-stranded RNA analysis	Valverde *et al.*, 1990
	PCR	Randles *et al.*, 1996
	Hybridisation	
	Radiolabeled probes	
	Nucleic acid probes	Randles *et al.*, 1996
	Nicktranslated cDNA	Candresse *et al.*, 1988
	RNA probes	Melton *et al.*, 1984
	Non-radiolabelled probes	Hopp *et al.*, 1991

calcomine orange and Luxol brillant green® BL, and toluidine blue. Table 1.4 lists different colours by various stains between bodies induced by viruses and plant constituents. Overman (1992), and Christie and Edwardson (1986) have done field surveys of virus or phytoplasma diseases in plants by this technique.

1.4.2 Electron microscopy to study viral effects on plants

Electron microscopy (EM) is a useful method to study cytopathological effects in plants infected by viruses and to detect them.

1.4.2.a Tissue preparation

Preparation of plant tissues for EM includes fixation , dehydration, embedding, thin sectioning and staining. Fixation chemicals for plant materials usually are aldehydes (formaldehyde or glutaraldehyde). Post fixation is done with osmium tetra oxide. Ethanol or acetone have been used to dehydrate tissues before embedding. The most common embedding media are Epoxy resin and LR white resin. Different mixtures or resins can be used for different samples. Different compositions of Epoxy resin are listed in Table 1.5 (Hayat, 1989).

1.4.2.b Negative staining method

Many stains are available for electron microscopy and double staining of thin sections is often done with uranyl acetate and lead nitrate. For partially purified samples, negative staining is still often used because of its simplicity.

A negative stain should give high contrast and high resolution, while supporting the particles against flattening. It also should not affect the specimen, and should be easy to handle, reliable, and protect the specimen from beam damage. Most commonly used negative stains and their characteristics are summarised on Table 1.6.

1.4.2.c Cytopathological effects on tissues infected by plant viruses

The main cytopathological effects of virus infection are changes in cell components such as nuclei, mitochondria, chloroplasts and cell walls.

Table 1.4 Staining reactions of plant cell constituents (Christie and Edwardson, 1986, and Matthews, 1991).

CONSTITUENTS	STAINS			
	Azure A	O-G	R-G	P-B
Cell Wall	Colourless	Yellowish Green	Nr	Nr
Chromatin	Blue	Green	Nr	Nr
Cytoplasm	Colourless	Yellowish Green	Nr	Nr
Inclusions of TMV	Nr	Green	Red	Red
Inclusions of TEV	Nr	Green	Dark Red	Reddish Pink
Inclusion of CYMV	Nr	Olive Green	Red	Light Pink
Microcrystals	Colourless	Green	Nr	Nr
Nucleolus	Red-violet	Green	Slate	Violet
Nucleoplasm	Colourless	Orange	Aqua	Blue
P-protein (phloem)	Colourless	Green	Nr	Nr

O-G; Calcomine orange-"luxol" brilliant(1:4), R-G; congo rubin-methyl green(1:10),

P-B; phloxine-methylene blue

Nr; Not-reported

Table 1.5 Composition of Epoxy resin to embed samples for transmission electron microscopy (Hayat, 1989).

EPOXY RESIN		MIXTURE		
MEDIUM	COMPONENT	STANDARD	SOFT	HARD
Spurr's	ERL 4206	10.0	10.0	10.0
	DER 736	6.0	8.0	4.0
	NSA	26.0	26.0	26.0
	DMAE (S-1)	0.4	0.4	0.4
	Total (in g)	42.6	44.4	40.4
Araldite	Araldite	29.0		
	DDSA	24.0		
	DMP 30	0.5		
	Total	53.5		
Epon	Epon 812	16		
	DDSA	8		
	NMA	8.7		
	DMP-30	0.4		
	Total	33.1		

ERL 4206: Vinylcyclohexane dioxide, DER 736: Diglycidyl ether of polypropylene glycol, NSA: Nonenyl sussinic anhydride, DMAE (S-1): Dimethyl aminoethanol, DDSA: Dodecyl succinic anhydride, DMP 30 (DY 064): 2,4,6 tridimethylamino methyl phenol, NMA: Methylnadic anhydride.

Table 1.6 Commonly used stains in transmission electron microscopy and their characteristics (Milne, 1993).

STAINS	Prefered pH	Damage to Samples	Contrast	Resolution	Stability
Phosphotungstate	3.0-5.0	No	Low	Low	Unstable
	7.0	Yes (on membranes)	High	Low	Stable
Sodium silicotungstate	9.0	Yes (to unfixed viruses)	Variable with particle structure	High (for antibody related work)	Stable
Uranyl acetate	5.5	No (except unfixed rhabdoviruses)	High	High	Stable
Uranyl formate	5.5	No	High	High	Unstable
Ammonium molybdate	5.5	No	Low	High	Stable

Nuclei of infected cells may disintegrate, and some small isometric viruses (southern bean mosaic virus and tomato bushy stunt virus) and their coat protein may be found in or next to the nuclei as well as in the cytoplasm (Matthews, 1991). Mitochondria in plant cells infected by viruses are bounded by membranes (Hatta *et al.*, 1971), or aggregated in the cell. Chloroplasts may become rounded and clumped together (Matthews, 1991), and some peripheral vesicles and changes have been also found in and near the chloroplasts in cells infected by turnip yellow mosaic virus (Hatta and Matthews, 1976).

The cell walls are physical support and barrier structures. The plasmalemma bounds the cytoplasm which is a distinct biochemical and physiological compartment containing a substantial proportion of the total enzymes of the cell (Martelli and Castellano, 1971). Three main abnormalities in walls have been found; wall- thickening, cell wall protrusion involving the plasmodesmata and deposition of electron dense material between the cell wall and the plasma membrane (Bailey and Davidson, 1979).

Three types of inclusion bodies have been described; crystalline, cytoplasmic and pinwheel inclusions. Crystalline inclusions may be induced when virus particles accumulate in infected cells in sufficient numbers and exist under suitable conditions to produce three-dimensional crystalline arrays. Cytoplasmic inclusion bodies are induced under some conditions such as water deficit (Milne, 1967) and high temperature (Ushiyama, 1971). Pinwheel inclusion bodies are found in plants infected with potyviruses (Hiebert and McDonald, 1973).

1.4.3 Purification of putative causal agents

Pure virus preparations are essential for chemical, physical, biochemical studies, and other biological investigations. Hence, during the past few decades techniques for purification of plant viruses has been developed (Francki, 1972)

Extraction buffer and ionic environment - The pH is a very important factor governing the solubility of proteins and hence of viruses. The charge on virus particles varies with the pH of the solution. The majority of viruses have isoelectric points on the acid side of neutrality and hence require neutral or slightly alkaline conditions to remain in solution (Brakke, 1967). However, the pH must not be too high because at low hydrogen ion

concentrations the bonding between viral coat protein and nucleic acid becomes weaker. Viruses also have different ionic environment and some ions, such as magnesium and calcium, have stabilising effects (Francki, 1972).

Additives to protect viruses- When tissues are homogenised to release virus, plant constituents may inactivate viruses by forming insoluble precipitates, degrading them or by inactivating. Compounds most commonly encountered are tannins, polyphenols and enzymes. Phenolic compounds inactivate virus by forming complexes with them. Formation of these complexes can be reversed by raising pH, by dilution or by adding substances such as chemicals-caffeine, nicotine or albumin, chelating agents-sodium dithiocarbamate or EDTA, or reducing agents-ascorbic acid, cysteine, sodium sulphite or thioglycollic acid. Some synthetic polymers such as PVP and PEG are known to complex with polyphenols and thereby minimize the phenolase activity. To prevent enzyme activity, bentonite can be added, but care must to be taken that it does not adsorb the virus (Randles, 1993).

Precipitation and purification - Separation of particles from plant debris can be carried out with muslin cloth and differential centrifugation. Further purification can be done for many viruses by density gradient centrifugation with sucrose, caesium sulfate or Nycodenz (Randles *et al.*, 1987). After density gradient centrifugation, fractions which contain virus particles are dialyzed in buffer and concentrated by high speed centrifugation (Ralph and Bergquist, 1967).

1.4.4 Extraction of nucleic acids

Total nucleic acid extracts of the leaf of almost all plants show the same pattern when fractionated under non denaturing conditions by polyacrylamide gel electrophoresis. DNA has the lowest electrophoretic mobility, with the 25S, 23S, 18S, 16S, and 5S ribosomal RNAs, and 4S tRNA separating ahead of the DNA. In principle, plant virus RNAs, would be detectable under the appropriate conditions as additional species against the background of host plant nucleic acids.

Because of the relatively low amounts of virus-associated nucleic acids usually present in tissue from plants with a disease of unknown etiology, methods must be developed for concentrating the putative virus (and its nucleic acid) relative to host nucleic acids, and

reducing the level of other compounds which may interfere with detection of the target nucleic acid.

Plant tissue - Early symptomatic tissue has the highest probability of containing a viral pathogen, and it should be trimmed of any non symptomatic part. Young tissue would be the most likely to support the replication of a putative virus if symptomatic tissue is not available. Control tissue should come from a number of disease-free plants.

Extraction method - Cell and organelle breakage should be maximized in the extraction procedure, ranging from blending or juice extraction from soft tissues, to mechanical pulverisation or blending in liquid nitrogen for highly fibrous tissue.

Extraction medium - The ionic strength of extraction buffers can affect the stability of nucleic acids. Double-stranded DNA begins to denature to single strandedDNA at cation concentrations below 100 nM, and RNA also has less base pairing and less secondary structure (Marmur and Doty, 1961). Both molecules become more susceptible to nucleases under these conditions.

Clarification - Some chemicals such as bentonite, polyvinyl sulfate, dextran sulfate and macaloid have been used to remove cell components or proteins. However, these agents reduce the amount of extracted nucleic acid because they adsorb nucleic acids. High pH, for example at pH 9 or above, can inhibit RNase activity but RNA can be hydrolysed at high pHs (Sambrook *et al.*, 1989). To separate nucleic acids from protein and cell debris, phenol, chloroform or a mixture of chloroform and iso-amylalcohol is used. The extracted nucleic acid can then be concentrated with PEG, by chromatography or ultra-centrifugation.

Precipitation - Ethanol (2.5 volumes) or isopropanol (0.8 volumes) and 0.3% CTAB in the presence of 0.1 M NaCl can also be used. The latter two precipitants minimize co-precipitation of polysaccharides with the nucleic acids (Randles, 1993).

1.4.5 Fractionation of nucleic acids

Electrophoresis or chromatography can be used to fractionate nucleic acids. Gel electrophoresis is the most commonly used method because of its high resolution, simplicity and convenience. DNA and RNA molecules can be fractionated and analysed by agarose and polyacrylamide gel electrophoresis.

Non-denaturing gels can be employed for separation of double stranded nucleic acids, and denaturing gels should be used to analyse ssRNA whose mobility varies on non-denaturing gels due to variations in secondary structure (Ogden and Adams, 1987). Denaturants that have been used in electrophoresis are formaldehyde, methylmercuric hydroxide, sodium hydroxide, urea or glyoxal (Sambrook *et al.*, 1989). Glyoxylation of nucleic acids is done before electrophoresis (Murant *et al.*, 1981). Single-stranded DNA can be also fractionated by agarose or polyacrylamide gel with inclusion of a denaturing reagent. There are several systems for ssRNA, but the size of fragment to be resolved and the experimental protocol should be considered before choosing a system because the result can be affected by interactions between gel and denaturants. Sodium hydroxide is most useful for ssDNA analysis and glyoxal denaturation electrophoresis for nucleic acids results in separation of bands according to their molecular weight. Methylmercuric hydroxide (Baily and Davidson, 1979) and formaldehyde gels (Lebrah *et al.*, 1977) can be used for accurate sizing of nucleic acids with molecular markers.

1.4.6 dsRNA in diagnosis

dsRNA isolated from virus infected plants could be the genome of a dsRNA virus or the replicative dsRNA of a ssRNA virus (Hamilton *et al.*, 1981). The replicative form of dsRNA of ssRNA viruses involves the synthesis of a complementary negative-stranded template RNA in the form of a partially double-stranded replicative intermediate (RI). In addition, when replicative nucleic acids are isolated from infected cells, genome length double-stranded RNA molecules termed replicative form (RF) can also be identified (Garnier *et al.*, 1980). dsRNAs of several viruses had been studied and some of the dsRNAs can be used as molecular weight markers (Dodds, 1993; Valverde *et al.*, 1986; Table 1.7).

For confirmation of its virus origin, the isolated dsRNA should be further characterised because the use of dsRNAs in diagnosis is complicated by the fact that uninoculated healthy plants contain a series of dsRNA species. For example, virus-free French bean (*Phaseolus vulgaris cv.* Black Turtle Soup) contains high molecular weight dsRNA but other bean varieties contained no detectable dsRNA.

Table 1.7 Relative migration during electrophoresis of dsRNAs of citrus tristeza virus (CTV), tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), tobacco necrosis virus (TEV) in 6.0 % polyacrylamide gels, with current molecular weight (Valverde *et al.*, 1986)

Viral dsRNA segment	Distance (cm) migrated	Molecular weight ($x10^6$)	Size (kbp)*
CTV-1	0.40	13.30	19.5
TEV	Nr***	6.5	9.5
TMV-1**	0.80	4.30	6.3
TMV-2	1.39	2.10	3.1
CMV-1	1.40	2.00	2.9
CMV-2	1.45	1.90	2.8
CMV-3	1.90	1.30	1.9
TMV-3	2.40	0.95	1.4
CMV-4	3.75	0.55	0.8
TMV-4	5.00	0.42	0.6

*; Has been calculated assuming that molecular weight of 1 kbp dsRNA is $6.8x\ 10^5$ Da.

**; Numbers refer to genomic or subgenomic forms of dsRNA detected with virus infected plant.

***; Not reported.

Nevertheless, analysis of dsRNA can be applied to detect viruses. For example, antibody to dsRNA can trap or locate molecules for subsequent observation by electron microscopy of sap extracts or tissue sections (Stollar *et al.*, 1978). Hybridisation methods may also be applied (Boedtker, 1959).

1.4.7 PCR assay

The importance of PCR lies in its ability to amplify a specific DNA or cDNA transcript *in vitro* from trace amounts of a complex template. It is possible to amplify the sequences from a specific template in the size range of 50-10,000 bp in length, more than a million fold in a few hours (Hadidi *et al.*, 1995).

PCR assay is an *in vitro* method for enzymatically synthesising defined sequences of target DNA. The PCR normally uses two oligo nucleotide primers that hybridise to opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalysed by a thermostable enzyme which is isolated from microorganisms which are living in a high temperature condition, such as *Thermus aquaticus* or *T. thermophilus*. The PCR involves a series of cycles of denaturation of the target template, annealing of the primers to the target and extension of the primers by the thermostable DNA polymerase. The factors considered in PCR are selection of primers, the thermostable enzyme, and concentration of primers, dNTPs and $MgCl_2$. The temperature of the three steps - denaturation, annealing and extension and the length of the steps are important for a successful PCR assay.

The availability of nucleotide sequences of many plant pathogens has made it possible to develop the PCR assay for the detection and diagnosis of several viruses and other pathogens. Because of its great sensitivity, the PCR provides a good alternative to other diagnostic methods and can speed diagnosis, reduce the sample size required, and often eliminates the need for radioactive probes. Detection of viruses, viroids, bacteria, phytoplasma, fungi, and nematodes by PCR has impacted on diagnostic practices (Hadidi *et al.*, 1995).

1.5 Classification of plant viruses

The generally accepted classification system for viruses by ICTV (International Committee on Taxonomy of Viruses) is the classical monothetic hierarchical system applied by Linnaeus to plants and animals (Murphy *et al.*, 1995). The name of a virus is listed by the vernacular name, the genus and family. There have been many errors and complications in virus taxonomy. The reason for these problems arose from the inadequate characterisation and description of viruses, and inadequate reviews of data by international speciality groups.

There are two main concerns involved in attempting to classify viruses. First, related viruses are placed in genera or families. The more stable properties of the viruses, such as amount and kind of nucleic acid, particle morphology, and genome strategy, are most useful in the identification of viruses. The second concern is to be able to distinguish between related viruses and give some assessment of degrees of relationship within a group or family of viruses. For this purpose more variant properties are useful. These include amino acid composition of the coat protein, and symptoms and host range of viruses. Some properties, such as amino acid sequence and serological specificity may be useful both for defining groups and for distinguishing viruses within groups.

1.5.1 Structure of particles

Structure of virus particles is important in the group classification of viruses into genera because the ICTV classification is based largely on the morphology of particles.

With isometric viruses, particle morphology has not been as generally useful as for the rod-shaped particles. This is mainly because many isometric particles lie in the same size range (25-30 nm in diameter) and are of similar appearance unless preparations and photographs of high quality are obtained (Hatta and Francki, 1984). Where detailed knowledge of symmetry and arrangement of subunits has been obtained by X-ray analysis of high-resolution electron microscopy, these properties give an important basis for grouping isometric viruses. The dimension and structure of the rod-shaped virus particles is important because the recent classification of the rod-shaped viruses is based on the particle properties (Murphy *et al.*, 1995).

1.5.2 Viral coat protein

Structural proteins make up most of a virus particle and coat protein molecular weight can be used to allocate viruses to provisional taxonomic groups (Lane, 1992). The properties of viral proteins, and in particular the amino acid sequences, are of prime importance in virus classification at all levels, for indicating evolutionary relationships between families and groups of viruses (Matthew, 1991). In particular, amino acid homologies have been used to estimate degrees of relationship within the genera.

1.5.3 Nucleic acids

The organisation and strategy of the viral genome is of prime importance for the placing of viruses into families and genera, or for the establishment of a new family and genus (Murphy *et al.*, 1995). Nucleic acid hybridisation and physical structure of the genomic nucleic acid, and a map of restriction endonuclease sites are important. Moreover, as the complete nucleotide sequences of more and more viruses become known, virus genotypes will become increasingly important for classification.

1.5.4 Serological and biological properties

Serological relationships are important to distinguish between genera because it is independent of the dimension of the particles. This characteristic was one of the most important single criteria for placing viruses in related groups (Shukla *et al.*, 1989). Biological properties such as host range, symptoms in particular host plants and method of transmission can be considered in classification. Macroscopic symptom differences will often reveal the existence of a different strain of a virus if there is no other criterion. However, these characteristics can be ambiguous and lead to confusion of identification.

1.5.5 Classification of rod-shaped viruses

According to the ICTV, when an "unknown" is first studied in a laboratory, its initial characterisation may involve only standardised protocols. One key to simplifying and

Table 1.8 Characteristics of genera of rod-shaped viruses (Murphy *et al.*, 1995).

FAMILY	*GENUS*	Size of particle (nm)	Shape of particle	Type of nucleic acid	Size of nucleic acid (kb)	Size of coat protein (kDa)
Potyviridae	*Potyvirus*	11-15x 650-900	flexuous rod	ssRNA	8.5-10	30-47
	Rymovirus	690-720x 11-15	"	"	8.5-10	"
	Bymovirus	250-300 and 500-600	"	"	4.5 and 7.9	"
Nc	*Tobamovirus*	300x 18	stiff rod	"	6.4	17-18
Nc	*Tobravirus*	180-215 or 46-115x 21.3-23.1	"	"	6.8, 4.5 or 1.8	22-24
Nc	*Hordeivirus*	110-150x 20	"	"	3.8 and 2.8, or 3.3 and 3.2	22
Nc	*Furovirus*	250-300 or 92-160x 20	"	"	5.9-7.1. 3.5-4.3 or 2.1-2.4	19.7-23
Nc	*Closterovirus*	1200-2200x 12	very flexuous rod	"	15.5-20	27-28
Nc	*Capillovirus*	640x 12	"	"	6.5	36
Nc	*Trichovirus*	640-800x 12	"	"	6.3-7.6	22-27
Nc	*Carlavirus*	610-700x 12-15	slightly flexuous rod	"	7.4-8.5	31-49
Nc	*Potexvrius*	470-580x 13	very flexuous rod	"	5.8-6.9	18-27

Nc; Not classified

Nr; Not reported

rationalising such study is to set useful techniques into a proper sequence based upon taxonomic characteristics because of the recent rapid accumulation of complete nucleotide sequence information for many viruses in a range of different families and groups. In virus classification, the most important key is the sequence of the virus nucleic acid, the structure, and the type of genome and serological relationships. Some important rod shaped viruses are listed in Table 1.8 with their specific characteristics.

1.6 Scope of this thesis

This thesis reports an investigation of the etiology of ScSMD by biological and microscopic studies, and by nucleic acid studies. Studies of ScSMD affected sugarcane are followed by the detection, isolation, cloning and partial sequencing of a disease specific dsRNA, and the tentative classification of the putative viral agent from the partial sequence. This thesis also reports the properties of the ScSMD associated virion. Because routine methods of cloning the dsRNA were unsuccessful, this thesis reports an improved protocol for the purification of dsRNA, the use of randomly primed PCR (rPCR) (Froussard, 1992) to produce a 'library' of cDNA representing the dsRNA sequence, the selection of disease specific inserts for sequencing, and the tentative classification of the agent of ScSMD as a rod-shaped virus in the genus *Carlavirus*.

This thesis also reports on the development of diagnostic probes for nucleic acid hybridisation assay and of diagnostic primers for RT-PCR to detect the ScSMD associated virus, ScSMV.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plants and soil

Table 2.1 lists sources of sugarcane material used in this work. Seeds, cuttings and leaf samples of sugarcane plants were consigned by express mail. Midribs of leaves (samples 7-11) were stripped and stored at -20 °C. Seedlings (samples 1-3) and cane cuttings (samples 4-6) were raised in a glasshouse at 25-30 °C and fertilised with complete nutrients every 2 week. Other plants (Table 2.2) used in this study were grown in sterilised soil with fertilisation every two week.

2.1.2 Biochemicals and miscellaneous chemicals

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

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

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

2.1.4 Primers

Primers for PCR were designed and selected from known sequences. Stability and primability of the selected primers were checked by using Oligo™ and Amplify programs. The best selected primers synthesised by Integrated DNA Technologies, Inc. (USA). Primers for sequencing (M13 and SP6) and random hexamers were purchased from Promega (USA). All primers used in this study are listed in Appendix D.

Table 2.1 Sources of sugarcane samples used in this study

Sample	source	cv.	site (property)	
1	Healthy seed	(Q96)	BSES*, Meringa Sugar Experiment Station	
2		(Q117)		
3		(Q124)		
4	Healthy cane	(Q96)	BSES, Ayr	
5		(Q117)		
6	ScSMD affected cane	(Q96)		
7	ScSMD affected leaf and cane	(Q96)	Burdekin River, Ayr	(Marshall)
8		(Q96)		(Michelin)
9		(Q117)		(Fava)
10		(Q117)		(Pellizari)
11		(Q117)		(Ballao)

*: Bureau of Sugar Experiment Stations, Queensland.

Table 2.2 Sources of indicator plants other than sugarcane used in this work.

Family	Species	Common name	Source
Chenopodiaceae	*Chenopodium amaranticolor*		WARI
	Chenopodium quinoa		"
Cucurbitaceae	*Cucumis sativus*	Cucumber	Yates
Fabaceae	*Phaseolus vulgaris* cv. Hawkesbury wonder	Broad bean	WARI
	Pisum sativum cv. Green feast	Pea	"
Graminaceae	*Oryza sativa* cv. Amaroo	Long rice	"
	Oryza sativa cv. Pelde	Medium rice	"
Poaceae	Zea mays	Sweet corn	Yate
Solanaceae	*Lycopersicon esculentum*	Tomato	WARI
	Nicotiana clevelandii		"
	Nicotiana glutinosa		"
	Nicotiana tabacum cv. White Burley	Tobacco	"
	Nicotiana tabacum cv. Xanthi n. c.		"

WARI; Waite Agricultural Research Institute.

Yates; Arthur Yates & Co. Ltd., NSW, Australia.

2.2. METHODS

2.2.1. Growth studies

Growth measurement of ScSMD-affected and healthy sugarcane plants were done on glass-house grown plants. Cuttings of the diseased and healthy sugarcane were planted in a pot with sterilised soil. Development of the primary shoots was recorded when the first leaf had emerged from the surface of the soil. Height was measured from the bottom node to the top node. Width of leaves was measured at the widest part. Length of leaves was measured from the base to the tip.

2.2.2 Microscopy

2.2.2.a Light microscopy

Light microscopy was done according to the method of Christie and Edwardson (1986) with some modifications.

For epidermal tissue, the leaf surface was lifted and peeled with sharp-pointed tweezers and immediately stained (Appendix C) for 5-10 min. The stained tissue was cut into small pieces on a glass slide, and the excess was removed with several changes of 95% (v/v) E-OH (5-10 sec/change). The stained tissue was either examined with a light microscope, or stored in 100% (v/v) 2-methoxyethanol at 4 °C for future use.

For cross sections, tissues were cut into thin sections with a razor blade and soaked in 2-methoxyethanol for 30 min to remove chlorophyll and other pigments. The decolourised tissue was stained and examined, as above.

2.2.2.b Scanning electron microscopy

Environmental scanning electron microscopy (ESEM, ElectroScan Corp., USA) was used to examine structural changes in tissue.

Fresh leaf tissues of ScSMD affected and healthy sugarcane plants were used. The leaf was cut into pieces (2 cm) and transferred to a sample chamber in the ESEM. The surface structure and thickness were measured for both healthy and ScSMD-affected sugarcane leaf,

and quantitative differences were observed at 20 kV and the temperature was kept just over the dew point in the chamber of the ESEM.

2.2.2.c Transmission electron microscopy

Sugarcane plants were kept in a glasshouse at 25-30 °C. They were covered with black fabric for 36 hr before being used for electron microscopic work to eliminate starch from chloroplasts. Three treatments were used to look for either a disease causing agent, inclusion bodies or cytopathological changes in tissues.

The embedding procedure was carried out according to the method of Hatta and Francki (1978). For low temperature treatment, sugarcane plants were kept in a cold room (4 ± 2 °C) for 24 hr. For high temperature treatment, plants were kept at 60 °C for 5 min and 24 °C (Ushiyama and Matthews, 1970).

Stems, roots, mid leaves and leaves of ScSMD-affected and healthy sugarcane plants were cut into pieces (ca. 0.5x 1 cm) and fixed in 60 mM phosphate buffer (pH 7.5) containing 4% (v/v) paraformaldehyde and 5% (v/v) glutaraldehyde for 15-16 hr at 4 °C. The fixed tissues were rinsed three times with washing solution [60 mM phosphate buffer, pH 7.0 and 10% (w/v) sucrose] for 9 hr. The rinsed tissues were cut into small pieces (ca. 1-2x 5 mm) with a razor blade.

At this point, tissues for RNase treatment were incubated in high salt buffer (2x SSC; Appendix B) or in low salt buffer (0.1x SSC) twice for 6 hr at room temperature. The washed tissues were treated with pancreatic RNase A-df 100 µg/ml (type IIIA, Sigma) 16 hr at 25 °C in either the high salt or in the low salt buffer solution and rinsed in Millonig's phosphate buffer, pH 7.0 (Hatta and Francki, 1978) for 9 hr. Rinsed tissues were refixed in 1% (w/v) osmium tetraoxide in Millonig's phosphate buffer for 3 hr at room temperature and rinsed in the same buffer three times for 9 hr at room temperature. Dehydration was done with a series of different concentrations of acetone, followed by 100 % acetone. The dehydrated samples were embedded in Epoxy resin according to Hayat (1989) and is described in Section 4.2.2.b.

2.2.2.d Staining of samples for TEM

For TEM, 400 mesh copper grids were coated with formvar and carbon (Hayat, 1989). At first, separate 30 µl droplets of a stain solution and water were placed on Parafilm®. The carbon coated grids were made hydrophilic with a spark tester *in vacuo* (Edwards, England). The hydrophilic grids were placed on the droplets of the samples for 10-30 sec. The grids were picked up and the excess was removed by touching the edge of the grids with pieces of filter paper. They were then stained on droplets of a staining solution (Appendix E) for 10 sec and the excess was removed, as above. The grids were examined by a Phillips EM 400 electron microscope at 100 kV.

2.2.3 Total nucleic acid extraction

2.2.3.a Crush method

This extraction method was carried out according to the method of Hodgson (1996). Midribs were removed from sugarcane leaves and the leaves were than cut into small pieces and about 0.5 g placed in a thick walled plastic bag (7x 13 cm). If fresh tissue was to be processed immediately it was kept on ice or in a refrigerator at 4 °C. Alternatively it was stored in a sealed bag at -20 °C, for later processing.

For extraction, 1.5 ml of 0.5x NETM [1x is 0.5 M NaCl, 100 mM NaAc, pH 4.6, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, pH 8.0 and 0.25% (v/v) BME] was added. Leaf samples were crushed with low impact hits from a hammer with a slightly rounded plastic head. The crushed tissue was pushed into a corner of the bag, and then squeezed to separate the extraction sap into the other corner. 800 µl of the solution was collected into a microcentrifuge tube containing 1/20 volume of 20% (w/v) SDS. The mixture was shaken gently at room temperature for 30 min. 350 µl of water saturated phenol and 350 µl of the water saturated chloroform and *iso*-amylalcohol solution (Appendix B) were added (Sambrook *et al.*, 1989). The solution was mixed vigorously then the phases were separated by centrifugation for 10 min at 10,000 *g* at room temperature. The upper aqueous phase was carefully removed to a microcentrifuge tube containing 1 volume of IPA. The solution was mixed gently and incubated at -20 °C for at least 3 hr. The precipitated nucleic acid was pelleted by centrifugation for 15 min x 10,000 *g* at room temperature. The supernatant was carefully discarded and 400

µl of 70 % (v/v) E-OH was added and washed by gentle agitation for 4-12 hr. The pellet was dried after the supernatant was discarded. 35-50 µl of SDIW was added to the pellet and it was dissolved for 12 hr at 4 °C or for 3 hr at room temperature.

2.2.3.b CTAB extraction

Isolation of TNA with CTAB from sugarcane leaves was done by the method of Doyle and Doyle (1990).

One gram of fresh leaf tissue was powdered in liquid nitrogen in a chilled mortar and pestle. The powdered tissue was mixed gently with a preheated aliquot of 7.5 ml of CTAB extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) BME, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0] in a 50 ml centrifuge tube. The solution was incubated at 60 °C for 30 min with occasional gentle swirling. The solution was extracted once with CI solution. The aqueous phase was collected after centrifugation (1600 *g*) in a swinging bucket rotor (SORVALL®). Nucleic acids were precipitated with IPA, dried and dissolved, as above.

2.2.3.c RNA extraction

Total nucleic acid (TNA) was extracted from ScSMD affected and healthy plants, and other plant tissues infected with viruses by modifications of the method of Randles *et al.* (1986).

Leaf tissue was cut into small pieces, ground in a pestle and mortar with liquid nitrogen, and mixed with extraction buffer [100 mM Tris, pH 7.4, 10 mM EDTA, 1% (w/v) sarkosyl and 1% (v/v) MTG] at 3 ml/g of leaf. The extract was transferred to a 10 ml centrifuge tube and extracted with 1 volume of PCI solution (Appendix B). The liquid phase was collected after centrifugation (10,000 *g* at 4 °C) and extracted with 1 volume of CI solution (Appendix B). The liquid phase was collected again and precipitated with 0.1 volume of 3 M NaAc and 0.8 volumes of IPA. The mix was incubated at -20 °C for 4-12 hr and the precipitated TNA was collected by centrifugation (10,000 *g* at 4 °C for 20 min). The IPA solution was poured off and the pellet was washed with 75% E-OH and dried *in vacuo*. The TNA pellet was dissolved in 50 µl of TE (pH 7.4) and analysed on 1.2% agarose gel. The gel was stained and photographed.

2.2.3.d Quantitation of DNA and RNA

Spectrophotometry and EtBr staining methods were used to estimate concentration of nucleic acids in a solution, and these were carried out by the method of Sambrook *et al.* (1989).

A spectrophotometer (Beckman, USA) was used to calculate concentrations of DNA or total nucleic acid in a solution by measuring the OD at 260 nm and 280 nm. The ratio between OD 260 and 280 was calculated to determine purity of the nucleic acids.

EtBr fluorescent quantitation was used to calculate small amounts of nucleic acids by dotting 1 µl samples on a EtBr containing gel with a series of same amount of a nucleic acid standard. The dots were left until they were absorbed. The gel was photographed and the intensity compared with the intensity of the series of standard dots to determine concentrations.

2.2.4 Isolation and purification of dsRNA

2.2.4.a Extraction of TNA

One hundred gram lots of lamina tissue without midribs and with clear striations were chopped into small pieces (ca. 5 $mm^{2)}$, placed in liquid nitrogen, ground briefly in a pestle and mortar, then pulverised by blending in a VirTis® homogeniser in liquid nitrogen. Ten volumes of extraction buffer [0.1 M NaCl, 0.05 M Tris-HCl, pH 8, 1% (w/v) sarkosyl, 10 mM EDTA and 1% (v/v) MTG) was added and the mixture was stirred vigorously for 20 min at room temperature. One volume of phenol: chloroform (1:1) was added and stirring continued for 30 min. After centrifugation at 10 000 *g* for 20 min, the supernatant was recovered by precipitation with 0.8 volumes of IPA. The nucleic acid pellet was washed with 70% (v/v) E-OH and dried. It was then dissolved in 1.3x STE (STE is 0.1 M NaCl, 10 mM Tris-HCl, pH 8, 10 mM EDTA) for 3-5 hr at room temperature or at 4 °C overnight. Undissolved material was removed by low speed centrifugation.

2.2.4.b dsRNA isolation by microgranular cellulose method

To bind to microgranular cellulose (Macherey Nagel, Duren; MN 300), the cellulose was added in the ratio of 10 mg per 5 g leaf equivalent of the TNA extract. Ethanol was added to 16.5% (v/v) with initial vigorous shaking then with mixing for 30 min or more at room

temperature to bind dsRNAs. The cellulose fraction was collected by centrifugation at 10,000 *g* for 30 sec, washed 8x with the washing buffer (16.5% ethanol and 1x STE), then dried *in vacuo*. The dsRNA was eluted with STE (100-200 µl per 10 mg of cellulose) by mixing at room temperature for 30 min. The supernatant was collected after centrifugation and the dsRNA precipitated with IPA as above in the presence of 0.3 M NaAc. The dsRNA pellet was washed with 70% ethanol, dried and dissolved in TE (pH 8) (Appendix B). It was then treated with 5 units of ribonuclease free DNase I (RQ1, Promega) according to the manufacturers recommendation for 30 min. EDTA was added to 20 mM, and it was extracted with phenol: chloroform and precipitated with IPA and NaAc, as above.

2.2.4.c dsRNA isolation by the standard CF11 method

The extracted TNA (Section 2.2.4.a) was mixed with CF11 cellulose (2 g/30 g of leaf tissue equivalent) for 30 min. The solution was adjusted dropwise to 19% (v/v) E-OH with slow dropping, stirred and mixed for a further 30 min. The solution was poured into a column and washed with 30 volumes of 1x STE containing 18% ethanol. A commercial column (BioRad) or a syringe(3 or 20 ml) column was used. A syringe column was made of a disposable syringe and two layers of Miracloth® fitted to the bottom of the syringe. Nucleic acid bound to the washed column was equilibrated with 5 ml of STE/4 g CF11 and eluted with 1x STE (20 ml/g of CF11). The eluate containing the dsRNA was mixed with fresh CF11 (0.2 g/6 g of 1st cycle of CF11 cellulose) for 30 min and the E-OH concentration was adjusted, as above. Columns were washed with 1x STE containing 17% ethanol (250 ml/0.1 g of CF11). The dsRNA was eluted with 1x STE (3 ml/0.1 g of CF11) and precipitated with IPA.

2.2.4.d dsRNA purification by gel fractionation

All the following steps were done under RNase free conditions (Sambrook *et al.*, 1989). The dsRNA isolated with the microgranular cellulose was run on 0.9% agarose gel and stained with EtBr. The gel was photographed.

dsRNA in the agarose gel was collected using a RNaid^{TM} kit (BIO 101) according to the manufacturer's manual. The bands were cut out with a razor blade and they were cut into small pieces (ca. 2 mm^2). The diced gel was transferred to a microcentrifuge tube and weighed.

Three volumes of RNA binding salt was added and mixed. The mix was incubated at 50 °C for 10 min to dissolve the agarose. An approximate amount of dsRNA was estimated and RNAMATRIX™ (1 µl/µg) was added. The solution was well mixed for 5 min and centrifuged for 1 min in a microcentrifuge at maximum speed (ca. 12,000x *g*). The matrix bound to dsRNA was washed twice with RNAWASH solution and dried. It was resuspended in 50 µl of DEPC treated SDIW. The eluted dsRNA was collected by centrifugation and precipitated with IPA and NaAc. This recovered dsRNA was used for the following step.

Polyacrylamide gel fractionation of dsRNA was done according to a modification of the method of Dulieu and Bar-Joseph (1989). A 6.5% polyacrylamide gel (Mini Protean, BioRad) containing the dissolvable cross linker (BAC) was prepared and pre-run at 100 V for 30 min to remove monomers and to equilibrate the pH of the gel. The dsRNA recovered from agarose gel was loaded and further run at 100 V for 3 hr. The polyacrylamide gel was stained with EtBr and the dsRNA band was excised and transferred into a microcentrifuge tube. One volume of BME and 1 volume of DEPC treated SDIW was added. The solute was vortexed and incubated at 50 °C for 10 min until the gel was dissolved. The volume was adjusted to 500 µl with 10x STE until the concentration of STE reached 1.5x. The pre-warmed (37 °C) E-OH was added to 33% (v/v) and vortexed. Microgranular cellulose (20 mg) was added to the solution and shaken for 30 min. The cellulose was collected by centrifugation at maximum speed in a microcentrifuge for 30 sec. The solution was discarded carefully and the microgranular cellulose was washed at least 5 times with 10 volumes of washing solution [1x STE and 20% (v/v) E-OH)]. The washed pellet was dried *in vacuo* and eluted with 100 µl of DEPC treated SDIW. The dsRNA was precipitated with 2 volume of E-OH and 0.3 M NaAc at -20 °C for 12 hr. The precipitated dsRNA was collected by centrifugation at 4 °C.

2.2.5 Gel electrophoresis

2.2.5.a Polyacrylamide gel

Analysis of nucleic acids were done on polyacrylamide gels buffered in TAE or TBE (Appendix B). The gels were pre-run for 30 min at 100 V and samples were loaded into wells. Electrophoresis was in 80x 65x 1.5 mm minigels (BioRad) at 100 V for 2.5 hr. The gel was removed and stained with silver (Section 2.2.5.c).

Analysis of protein was done on SDS-PAGE using the method of Sambrook *et al.* (1989) and the discontinuous system (Laemmli, 1970). The gel was run at 5 V/cm until the tracking dye had reached the beginning of the resolving gel. The voltage was increased to 10 V/cm and the gel was run until the tracking dye (bromophenol blue) reached the bottom of the resolving gel. The gel was stained with Coomassie brilliant blue or with silver nitrate (Section 2.2.6.c).

2.2.5.b Agarose gel

Analytical or preparative agarose gels were either 0.9 % or 1.2 % buffered with TAE (Sambrook *et al.*, 1989). Bands were visualised by ethidium bromide and fluorescent imaging in UV light at 254 nm.

Formaldehyde denaturing gels were used for analyses of ssRNA, according to the method of Sambrook *et al.* (1989). Agarose [final concentration 1% (w/v)] was dissolved in DEPC treated SDIW, cooled to 60 °C and mixed with pre-warmed 0.1 M MOPS, pH 7.0, 40 mM NaAc, 5 mM EDTA and 2.2 M HCHO. The gel was pre-run at 5 V/cm for 5 min. 4.5 µl of sample, 2.0 µl of electrophoresis buffer, 3.5 µl of formaldehyde and 10 µl of deionised formamide (Appendix B) were mixed. They were incubated at 65 °C for 15 min and cooled on ice. The samples were loaded on a pre-run agarose gel with an RNA MWt marker. The gel was run at 4 V/cm for 3-4 hr until the tracking dye had migrated 8 cm. The gel was rinsed with water for 10 min to remove formaldehyde and stained with EtBr solution containing 0.1 M ammonium acetate for 40 min or stained with toluidine blue solution (Section 2.2.5.c).

2.2.5.c Staining of gels after electrophoresis

Gels were removed from the electrophoresis apparatus to a container and one of the following staining procedures was carried out.

EtBr solution (Appendix C) was added at 1-2 ppm. After 15 min-4 hr incubation, the gel was photographed under UV light (Sambrook *et al.*, 1989).

The toluidine blue solution (Appendix C) was added to the EtBr stained gels and incubated 6-12 hr and destained with several changes of 1% (v/v) acetic acid until the marker band was visible. The gel was photographed and/or further stained with silver.

To stain proteins in the SDS-PAGE gels, five volumes of the Coomassie brilliant staining solution (Appendix C) was added and incubated at room temperature for 4- 12 hr. The gel was destained by several changes of destaining solution [CH_3OH: H_2O (1:1, v/v)] until the bands were clearly visible. The gel was photographed and stored in water containing 20 % (v/v) glycerol in a sealed plastic bag (Sambrook *et al.*, 1989).

Polyacrylamide gels stained with other stains or removed from the plates were fixed [10% (v/v) ethanol and 5% (v/v) acetic acid) for 10 min] and washed [10% (v/v) ethanol and 0.5% (v/v) acetic acid]. The gel were briefly rinsed with 200 ml of DIW and the silver staining solution (Appendix C) was added and incubated with gentle shaking for 1 hr. After incubation, the silver solution was removed and the excess was washed 4 times every 2 min in DIW. After the final wash, 200 ml of developing solution [375 mM NaOH, 0.15% (v/v) CH_2O and 3 mM $NaBH_4$] was added. The colour was developed until the marker was visible and the developing solution was removed and the gel was rinsed twice with DIW. 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).

2.2.6 cDNA synthesis

2.2.6.a cDNA synthesis and amplification by rPCR

cDNA synthesis was primed with a random hexanucleotide (RH) linked to the 3' end of a 'universal oligonucleotide' (UN) which had an *Eco*R 1 site and an additional 5' sequence suitable for priming a PCR. This approach was a modification of the method of Froussard (1992). dsRNA (5 µl, ca. 50 ng) was mixed with 1 µg of UN-RH primer (Appendix D) and incubated at 100 °C for 10 min, cooled to room temperature, and the reverse transcription reaction set up in a final volume of 50 µl {50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 40 units RNasin, 2 mM dNTPs and either 16 units of AMV or 200 units of M-MLV reverse transcriptase}. Following incubation at 43 °C for 60 min the mixture was boiled for 5 min and chilled on ice.

The second strand was synthesised by Klenow fragment DNA polymerase I (ca. 10 units) in a final volume of 100 µl (50 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 1 mM dNTPs and 0.1 mM DTT). Incubation was at 37 °C for 1 hr.

This reaction yielded dsDNA originating at random priming sites within the sequence of the dsRNA, but representative of the whole sequence of the dsRNA. Fragments smaller than 200 bp, including primer and dNTPs, were removed with a Sephacryl® 400 (Pharmacia) column. All fragments had the same termini to allow them all to be amplified by a single PCR (Froussard, 1992).

PCR amplification of cDNA was done in a 50 µl reaction volume containing 5 µl of a second strand cDNA reaction solution, and PCR reaction buffer [50 mM KCl, 10 mM Tris-HCl pH 9.2 at 25 °C, 1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 500 µM dNTPs, 0.1 µM UN primer and 1 unit of *Taq* DNA polymerase (Promega)]. Following denaturation at 94 °C for 2 min, the mixture was subjected to 30 cycles of amplification; 94 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min; followed by a final extension step of 72 °C for 5 min. The size range of the PCR products was determined by analytical agarose gel electrophoresis. The products were digested with *Eco*R I, and small fragments again removed by Sephacryl® chromatography as above. The resulting PCR products were cloned into dephosphorylated pGEM-7zf(+) and transformed into *E. coli* (Sambrook *et al.*, 1989)

2.2.6.b A standard method for synthesis of cDNA

A standard cDNA synthesis was done according to the method of Sambrook *et al.* (1989).

First strand cDNA was made with AMV-RT. At first, the purified dsRNA from gel was denatured by boiling with 12 mM methyl mercuric hydroxide in the presence of random hexamer primer at 100 °C. The reaction mixture was 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 µl of 2.5 mM dNTPs and 40 units of RNasin® in a final volume of 25 µl. The mix was warmed to 37 °C, and prewarmed 4 mM sodium pyrophosphate and 15 units of AMV-RT were added to the mixture. The mixture was incubated at 42 °C for 1.5 hr. The reaction was stopped by adding 75µl of 50 mM EDTA.

Second strand DNA was synthesised using the RNase H method. 23 units of *E. coli* DNA polymerase I, 0.8 units of *E. coli* RNase H and 10 µl of second strand reaction buffer (500 mM Tris-HCl, pH 7.6, 1 M KCl, 50 mM $MgCl_2$ and 50 mM DTT) were added to 20 µl of

the first strand reaction. The mixture was incubated at 14 °C for 2-3 hr and enzymes were denatured at 70 °C for 10 min.

The repair synthesis was done with T4 DNA polymerase. The second strand reaction mixture was cooled on ice and 2 units of T4 DNA polymerase were added and incubated at 37 °C for 20 min. The reaction was stopped by adding 10 µl of 200 mM EDTA. The reaction mixture was extracted with PCI and precipitated with E-OH and NaAC.

2.2.6.c Synthesis of ^{32}P-labelled ss-cDNA probes by random priming

The concentration of the isolated dsRNA or dsDNA (10 µl) was calculated and aliquots of 50-100 ng were transferred to tubes. The target nucleic acids were denatured by boiling for 10 min and cooled on ice. In a 50 µl reaction mixture, one unit of Klenow fragment DNA polymerase I for dsDNA, or 200 units of M-MLV-RT for dsRNA, and the reaction buffer (final concentration of 50 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$, 1 mM DTT, 50 µM dNTPs and 50 µCi -^{32}P-dCTP) were added, and incubated at 37 °C for 2-4 hr. The reaction was stopped by adding EDTA to 20 mM and fractionated into 12 aliquots of 200 µl with a Sephadex® G-50 column according to Sambrook *et al.* (1989). The radioactivity of probes was determined with a liquid scintillation system (LS 5000 TD, Beckman®). The probe was diluted to 1.5-3x 10^6 cpm per ml for hybridisation assays.

2.2.7 Molecular cloning

2.2.7.a Ligation of ds-cDNA to a plasmid vector

The amplified ds-cDNA by rPCR (Section 2.2.6.a; Froussard, 1992) was digested with *Eco*R I and small fragments were removed with a Sephacryl® 400 spin column. 100 ng of *Eco*RI digested and dephosphorylated plasmid [pGEM-7zf(+)] (Sambrook *et al.*, 1989) were mixed and ligated with bacteriophage T4 DNA ligase buffer in a solution containing 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP. The mixture was incubated at 16 °C for 4-12 hr. An aliquot of this ligation mix was used for transformation.

2.2.7.b Preparation of competent cells

Competent cells were prepared according to the method of Sambrook *et al.* (1989). To produce competent bacterial cells, cells stored in glycerol at -70 °C were plated on an LB agar plate and incubated at 37 °C overnight. A single colony was selected and inoculated into 2 ml of LB. It was incubated in a shaking incubator at 37 °C overnight. The following morning, 100 ml of SOB medium was inoculated with 1 ml of overnight grown cells and incubated at 37 °C until the OD_{600} of the SOB medium (Appendix B) was in a range of 0.5~0.9.

The cells were placed on ice for 15 min and transferred to pre-cooled 50 ml Corning centrifuge tubes (Corning Incorporated, New York) and centrifuged at 1000 *g* at 4 °C for 15 min. The supernatant was poured off and traces of media were removed by draining. The cells were gently resuspended in 13 ml of RF1 buffer [0.1 M RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$ and 15% (v/v) of glycerol, pH 5.8 with (0.2 M CH_3.COOH)] and placed on ice for 30 min. The cells were centrifuged as above and the supernatant was poured off. 4 ml of RF2 [10 mM RbCl, 75 mM CaCl2, 10 mM MOPS and 15% (v/v) glycerol, pH 6.8 (with NaOH)] was added. They were resuspended gently and placed on ice for a further 15 min. The competent cells were dispensed as 200 µl aliquots into pre-cooled sterile microcentrifuge tubes. The aliquoted cells were frozen in liquid nitrogen and stored at -70 °C until used.

2.2.7.c Transformation by heat shock

Transformation was done according to the method of Sambrook *et al.* (1989) with some modifications. The competent cells were thawed on ice and mixed gently by swirling. 100 µl of thawed competent cells were transferred to prechilled 2 ml Eppendorf® tubes and BME was added to 25 mM. The solutes were mixed by swirling 5 times every 2 min. The cells were incubated on ice for a further 10 min, and 1-50 µl of the ligation mix was added and mixed. The mixed solution was placed on ice for 30 min and the cells were treated with heat at 42 °C for 45-50 sec. The heat treated cells were cooled on ice for 2 min and 0.9 ml of pre-warmed SOC (37 °C) was added. The mixture was incubated at 37 °C for 1 hr with shaking and 50-200 µl of solution was plated on an ampicillin, IPTG and X-gal containing LB plate (Appendix B). The plates were incubated for 12-16 hr at 37 °C and transformed white colonies were picked and used for plasmid preparation.

2.2.8 Preparation of cloned plasmids

2.2.8.a Mini-preparation

Extraction of plasmid DNA was done by the alkaline lysis method (Sambrook *et al.*, 1989).

The selected white colonies were transferred into 5 ml LB or DYT medium containing 60 µg/ml of ampicillin. The culture was incubated overnight at 37 °C with shaking. The cells was harvested from 1.5 ml aliquot of each culture by centrifugation at 10,000 *g* for 1 min at room temperature. The supernatant was poured off and the bacterial pellet was resuspended by vortexing in 200 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA and 20 µg/ml of RNase A). 300 µl of freshly prepared solution II [0.2 N NaOH and 1% (w/v) SDS] was added and the solution was mixed by inversion before placing on ice for 5 min. The solution was neutralised by adding 300 µl of ice-cold solution III (3 M NaAc, pH 4.8). The contents of the tube were mixed by inversion and then incubated on ice for 5 min. Cellular debris was removed by centrifugation at 10,000 *g* for 10 min at room temperature. The supernatant was transferred to a clean tube and extracted with an equal volume of PCI solution. The supernatant was transferred to a fresh tube and precipitated with 2.5 volumes of E-OH. After 30 min incubation, the plasmid was pelleted and washed with 70% (v/v) E-OH. The pellet was vacuum dried and resuspended in 20 µl of TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA).

2.2.8.b Midipreparation

Extraction of plasmid DNA were done by the alkaline lysis method (Sambrook *et al.*, 1989) with some modifications.

The selected clones were transferred into 100 ml of LB or DYT medium containing 60 µg/ml of ampicillin. The cultures was incubated overnight at 37 °C with shaking. The cells were harvested from 50 ml of each culture by centrifugation at 12,000 rpm for 5 min at 4 °C. The supernatant was poured off and the bacterial pellet was resuspended by vortexing in 3 ml of ice-cold Solution I. A 6 ml of freshly prepared solution II was added and the solution was mixed by inversions and incubated at room temperature for 5 min and placed on ice for 5 min.

The solution was neutralised by adding 4.5 ml of ice-cold solution III. The contents of the tube were mixed by inversion and then incubated on ice for 5 min. Cellular debris was removed by centrifugation at 10,000 *g* for 10 min at room temperature The supernatant was transferred to a clean tube and extracted with an equal volumeume of PCI solution. The supernatant was transferred to a fresh tube and precipitated with 0.8 volumes of IPA. After incubation at -20 ^{o}C, the plasmid was pelleted by centrifugation and washed with 70% (v/v) E-OH. The pellet was vacuum dried and resuspended in 20 µl of TE (pH 8.0). This plasmid DNA was further treated with RNAse A to remove bacterial RNA and proteins were removed as above. The amount of collected plasmid was checked by the spectrophotometric method. An aliquot was digested with *Eco*RI and analysed on an agarose gel. The fragments were purified to make probes with a gel extraction kit (Section 2.2.11.b).

2.2.9 Analysis of inserts in the recombinant plasmids

2.2.9.a Selection of ScSMD specific clones.

Transformed bacterial colonies were selected using the X-Gal system (Sambrook *et al.*, 1989). Minipreps of plasmids (Section 2.2.8.a) from over 60 colonies (Sambrook *et al.*, 1989) were restricted with *Eco*R I and analysed on agarose gels to determine sizes of inserts. Southern blots of the gels were probed with cDNA prepared by random priming of the PCR product (Sambrook *et al.*, 1989). The specificity of the clones was also tested by probing dot blots of healthy and diseased sugarcane total nucleic acid extracts, and the purified dsRNA, with cDNA synthesised by random priming (Section 2.2.6.c) of the clones to be tested.

2.2.9.b dsDNA Purification from agarose gel

dsDNA was extracted with a QIAGEN gel extraction kits (QIAEX II; QIAGEN GmbH, Germany) according to the manufacturer's recommendation (1995).

Bands were cut out, sliced and weighed. 3 volumes of QX1 was added to 1 volume of gel. QIAX II was resuspended by vortexing for 30 sec. QIAX II (30 µl/10 µg of DNA but at least 10 µl) was added. The solution was incubated at 50 °C for 10 min with mixing every 2 min to keep QIAEX II in suspension. The solution was centrifuged at 10,000 *g*

for 30 sec and the supernatant was carefully removed. The pellet was washed twice with 500 µl of QX1 then three times with 500 ml of PE buffer. The pellet was air dried for 20 min and the bound DNA was eluted with 100 µl of TE (pH 8.0).

2.2.10 Molecular hybridisation

Blotting and hybridisation were done according to Sambrook *et al.* (1987) with some modifications.

2.2.10.a Dot blot

For RNA blotting, extracted nucleic acid samples were dissolved in TE (pH 8.0) and incubated at 65 °C for 5 min in three volumes of RNA blotting solution [500 µl of formamide, 162 µl of 37% (v/v) formaldehyde and 100 µl of 1 M MOPS, pH 7.0]. For DNA blotting, samples were heated to 95 °C then chilled on ice. One volume of 20x SSC was added and 3 µl of samples were spotted on to a sheet of nylon membrane (Zeta-probe®, Bio-Rad). After application of the samples, they were UV cross linked (125 kJoule) with a UV Cross Linker (Bio-Rad).

2.2.10.b Capillary blotting

The stained gels were washed with SDIW to remove excess EtBr and 20 mM NaOH solution was added. In a container, an absorbing pad was layered and pre wetted with 20 mM NaOH 3 sheets of Whatman 3MM filterpaper and bubbles were removed with a glass rod. The washed gel was layered at the top of the filter paper and bubbles were removed. Nylon membrane cut in the gel size was layered at the top of the gel and bubbles were removed, as above. A sheet of plastic transparency cut to the gel size was layered on top of the nylron membrane to mask the gel. Three sheets of pre-wet Whatman 3MM filter paper were layered and bubbles were removed, as above. A 3 cm thick layer of paper tissue was placed on top and a weight of 500-1,000 g was applieed. The nucleic acids were transferred for 4-12 hr and the nucleic acids blotted to the nylon membrane were UV cross linked (150 kJoule) as above.

2.2.10.c Hybridisation assay

Dot or capillary blotted membranes (Zeta probe®, BioRad) were pre-hybridised for 4 or 16 hr at 42 °C in prehybridisation buffer[5x SSC, 0.02% (w/v) BSA fraction V, Ficoll®, 0.02% (w/v) PVP, 0.5% (w/v) SDS, 100 µg/ml of denatured herring sperm DNA and 50% (w/v) deionised formamide].

The cDNA probes (Section 2.2.6.d) were heated at 80 °C for 5 min in 50% formamide and added to the hybridisation solution (same as the prehybridization solution but containing ^{32}P labelled probe at about 1.5-3x 10^6 cpm/ml). The membrane was hybridised at 42 °C for at least 18 hr (Sambrook *et al.*, 1989). The filter was then washed twice at room temperature for 15 min with 2x SSC and 0.1 % SDS for low stringency. The washed membrane was exposed to an X-ray film (Kodak AR) for 4-12 hr. The membrane was washed twice again in a solution containing 0.1x SSC and 0.1% SDS at 67 °C two times every 20 min for high strigency and the membrane was autoradiographed, as above.

2.2.11 Sequence analysis

Selected clones were sequenced by automatic cycle sequencing (Applied Biosystems, USA) and compiled with the SeqEd™ program (Applied Biosystems). Comparisons between sequenced nucleotides and sequence data bases were done with Fasta, Blast and MPsrch programs in the internet site of DISC Homology Search (http//www.dna.affrc.go.jp/htdocs/homology/homology.html) (Goto, 1982 and Lipman, 1985). The protein motifs were searched according to Prestige (1996) in the Net. The alignment was done with Clustal W program (Version 4.1; Thompson *et al.*, 1994)

2.2.12 PCR assay

2.2.12.a RNA PCR

The purified TNA of healthy and ScSMD -affected sugarcane was dissolved in SDIW. cDNA was synthesised with M-MLV RT and a set of primers. A solution (20 µl) containing 1 µg of each primers and 2 µg of TNA was boiled for 10 min with a PCR machine and cooled on ice. The primers were annealed to the template at room temperature for 30 min.

The reaction mixture contained 25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 1.5 mM MgCl, 5 mM DTT, 200 units of M-MLV-RT, 0.3 M BME, 10 mM dNTPs, 40 units of RNasin®, and the primer annealed template in a volume of 50 µl, and incubated at 42 °C for 2 hr (Sambrook *et al.*, 1989).

5 µl of the cDNA reaction mixture was added to 50 µl of a PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 9.2 at 25 °C, 1.25 µM of a set of primers and 0.5 units of *Taq* DNA polymerase. After PCR the products were analysed on a 1.2 % agarose gel.

2.2.12.b PCR for detection of phytoplasma

Three sets of primers [AFW and ARV(Ahrens and Seemuller, 1992), R2 and F2 (Schaff *et al.*, 1992) and SN10601 and SN910502 (Namba *et al.*, 1992)] (Appendix D) were used to examine the relationship between ScSMD and the presence of phytoplasma in sygarcane.

The following reagents were mixed in the order given; SDIW, 10x PCR buffer (100 mM Tris-HCl, pH 8.3 and 500 mM KCl), 10 mM dNTPs, 2.25 µM of each forward and reverse primer and 0.2 units of *Tth* DNA polymerase. To a volume of 5 µl of CTAB extracted TNA was added. The tube was transferred to a PCR machine (Perkin Elmer Cetus, USA) and subjected to a single denaturing step and 35 cycles of denaturing, annealing, extension , and final extension step to optimise the PCR conditions for each set of primer.

2.2.13 Studies of virus particle compositon

2.2.13.a RNA extraction from viral particles

The purified virus was digested with RNase (50 ng/ml) and DNase (50 ng/ml) for 30 min at 37 °C. Proteinase K (20 µg/ml) was added to a buffer (50 mM Tris-HCl, pH 7.4 and 1% SDS) and incubated at 37 °C for 4 hr. This sample was analysed on an agarose gel or extracted with PCI and precipitated. The collected samples were used for cDNA synthesis, gel analysis or hybridisation assay.

2.2.13.b Checking the 3' end of virus RNA with $d(T)_{15}$ primer

Proteinase K (100 µg/ml) was added to an aliquot of partially purified virus and the mixture was incubated at 45 °C (Sambrook *et al.*). The mixture was extracted with one volume of PCI and once with CI solution. The aqueous solution was recovered and ssRNA was precipitated with NaAc and E-OH and dissolved in TE. The RNA of pea seed borne mosaic potyvirus was extracted the same method as above for use as a control.

The extracted RNA (5 µl) and 5 µl of $d(T)_{15}$ primer (0.5 µM) were mixed and denatured by boiling. A solution containing [1x M-MLV RT reaction buffer (375 mM KCl, 250 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$ and 50 mM DTT), 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 0.5 mM dCTP, 25 µCi of (^{32}P)-dCTP and 200 units of M-MLV-RT) was added. The solution was incubated at 42 °C for 1.5 hr and the incubated solution was fractionated by a Sephadex®-G50 column to removed unbound dNTPs, according to Sambrook *et al.* (1989). The nucleic acid fraction was analysed on an 1.2% agarose gel, photographed and transferred to a nylon membrane. The transblotted nylon membrane was exposed to an X-ray film.

CHAPTER 3

BIOLOGICAL STUDIES ON ScSMD AFFECTED SUGARCANE

3.1 INTRODUCTION

ScSMD is a disease of unknown etiology with virus like symptoms. This disease has only been found in the lower Burdekin River district of northern Queensland (Hughes, 1961). The disease occurs on both sides of the Burdekin River in the main sugarcane growing area and is often associated with areas of poor growth, such as excessively sandy or shallow soils, or poorly irrigated areas. Some sugarcane hybrids, such as Pindar, Q57, Q63 and Q96, are known to be susceptible varieties (Anon., 1972).

Some features of ScSMD had been described. The characteristic symptom of ScSMD on sugarcane is the presence of short, fine, light green striations on the leaf lamina. ScSMD is transmissible by the pin-prick method. Spread is slow and ScSMD recurs on the same farms. Soil treatment with nematicidal and fungicidal fumigants also reduces incidence, suggesting it is soil borne. It has been reported that cuttings from ScSMD affected sugarcane plants could not be cured by heat treatment of cuttings (Anon., 1969). There is a need for a further study of ScSMD to determine its cause, to evaluate its economic impact and to establish control strategies.

Biological assays for a plant disease of unknown etiology are time consuming. Nevertheless, these studies are very important for improving the understanding of the mode of transmission and spread, and symptomatology is particularly important to assist in diagnosis of a disease before isolation and characterisation of an agent is achieved. However, dependence on disease symptoms for identification and classification can lead to much confusion because many factors can have marked effects on the disease produced by an agent such as a virus (Matthews, 1991). There are two main types of symptoms of virus infection, local and systemic. Local symptoms develop near the site of entry. They can be identified as chlorotic, ring spot or necrotic lesions according to the colour and shape. There are various systemic

symptoms: reduction in size, mosaic patterns, yellowing, and necrosis on the leaf or on the fruit.

The experimental transmission of a virus from infected to healthy tissue is a procedure fundamental to the study of a virus disease. Plant viruses require a sub-lethal wound because they are unable to enter the cuticle of the their host plant unaided. In the laboratory a sublethal wound is usually accomplished by mechanical transmission, which involves grinding the diseased leaf material, and rubbing with a mild abrasive (carborundum) which damages the cuticle and epidermis of the plant. This method is used in the laboratory to isolate viruses from diseased field plants, to transmit them to test hosts, to sub-culture viruses, to study virus symptoms in a range of host species, and to assay for virus infectivity.

In this chapter, the symptoms of ScSMD-affected sugarcanes are described. The effects of ScSMD on the growth rate and germination of sugarcane cuttings were studied under glasshouse conditions. The transmissibility of ScSMD through mechanical inoculation was also studied.

3.2 MATERIALS AND METHODS

3.2.1 Plants

All plants used were raised in a glasshouse kept at 27± 3 °C and fertilised every two week. Seedlings and cuttings of sugarcane hybrids (Q96, Q114 and Q117; Table 2.1) and other species of indicator plants (Table 2.2) were used at the three to four leaf stage.

Symptom development was observed in a glasshouse at an average day length of 14 hr at 27± 3 °C. Also, naturally infected sugarcane samples collected from the Burdekin district were examined for ScSMD symptoms

3.2.2 Inoculation of ScSMD

Pin-pricking mechanical inoculation and a grafting-like inoculation method were used. The symptoms and effects on the inoculated plants were observed in the glasshouse every week for 6 months after inoculation.

To prepare inoculumn, young ScSMD affected leaves were chopped into small pieces and ground in a pre-chilled pestle and mortar with either 2 vol of 100 mM phosphate buffer, pH 7.0 or 50 mM borate buffer, pH 7.5. Inocula of other viruses (AMV, CMV and TMV) were prepared in the same way and inoculated at the same time using the same methods as used for the ScSMD affected plant material. These inocula were applied to the plant leaves, which had been dusted with carborundum. The leaves were rubbed with a finger or punctured with a bundle of 8 pins tied with thread. The grafting-like procedure was carried out by replacing two buds on a healthy sugarcane with two buds of ScSMD-affected sugarcane and sealing the site with parafilm®. The replaced buds were left until they died.

3.2.3 Studies on ScSMD-affected sugarcane plants

Sugarcane cuttings from ScSMD-affected and healthy sugarcane as controls were planted in pots (25x 35 cm), placed in a glasshouse, irrigated every second day and fertilised every two week. Germination was recorded when the shoot emerged through the surface of the soil. Growth rates of diseased and healthy sugarcanes were measured every week. The height was measured from the bottom node to the top node. Overall height was measured from the bottom to the longest tip of the sugarcane. The width of leaves was measured at the widest part. The length of every leaf was measured from the bottom to the tip after it had emerged completely.

3.3 RESULTS

3.3.1 Symptoms of ScSMD affected sugarcane

Symptoms were short chlorotic striations, yellowing and stunting. The best conditions for production of symptoms were at 25-30 °C in a glasshouse.

Characteristic chlorotic striations and yellowing were observed on the leaves of ScSMD affected sugarcane. The striations normally did not appear on the two youngest leaves. Yellowing could be observed on the tertiary stems (Fig. 3.1A).

Some leaf sheaths of ScSMD affected sugarcane plants also showed chlorotic striations. This symptom could not only be observed on leaves without chlorotic striations, but also on the primary stems that germinated from cuttings of ScSMD affected sugarcane plants. More pronounced chlorotic striations on the leaf sheath were found on older plants (Fig. 3.1C).

The stems of ScSMD affected plants showed mosaic, shortening of the internode length and less thickening of the diameter. The mosaic symptoms could be found on stems of affected plants that were collected from the field but not on glasshouse grown plants (Fig. 3.1D). The internodes of affected sugarcane plants were shorter and the diameter of the stems was thinner than for healthy sugarcane (Fig. 3.1E).

The regrown shoots from the third ratoons had clear chlorotic striations. The striations could be observed also on the leaf sheath and the leaf was showing yellowing.

3.3.2 Growth rate

Under glasshouse conditions, ScSMD reduced the growth rate of sugarcane. This reduced growth was associated with delayed germination, stunted height, and reduced number and length of leaves.

ScSMD affected cuttings germinated an average of 1 week later than healthy sugarcane cuttings. The emerged shoots from ScSMD cuttings were weak and some had the characteristic striation on the sheath of the first leaves (Fig. 3.1F). The leaves that emerged from shoots from ScSMD affected cuttings were weaker and thinner, and sometimes had the

Fig 3.1 Symptoms of sugarcane striate mosaic disease;

A - Leaves of sugarcane from ScSMD affected sugarcane (ScSMD) compared with healthy (H).

B - Symptoms of ScSMD affected leaves (ScSMD) from early to late stages. (Age of leaf increases from left to right.)

B - Chlorotic striations on ScSMD affected leaf sheath.

C - Striations associated with ScSMD on canes which were collected from the field.

D - Internodes of the diseased sugarcane compared with healthy one, showing reduced internode length and diameter of stems.

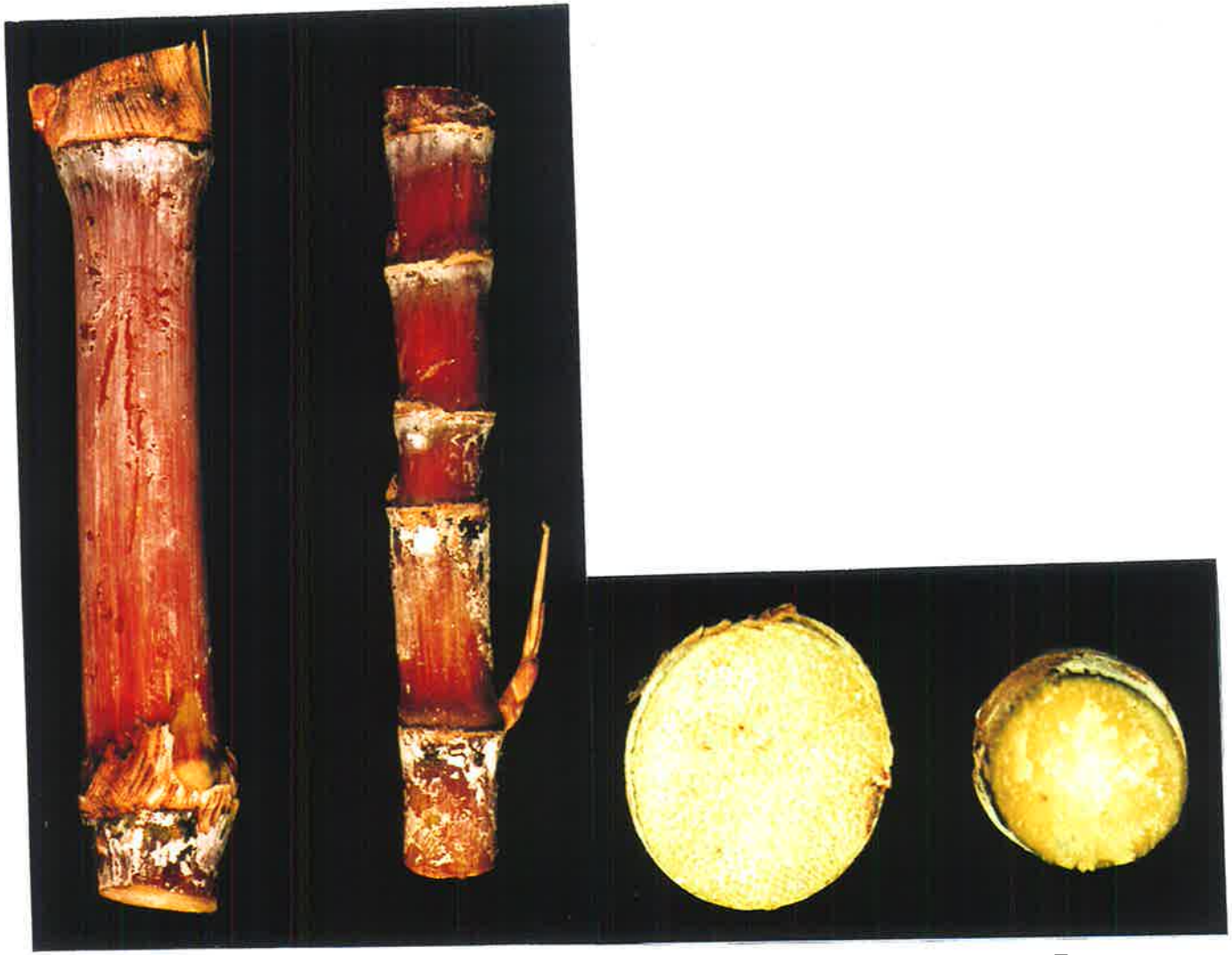
E - Shoots from diseased (ScSMD) compared with healthy cuttings (H).

(A)

(B)

(C)

(D)



H D H D

(E)

H D

(F)

chlorotic striation on the lamina with yellowing. Sometimes the shoots died shortly after germination.

Germinated shoots of ScSMD affected sugarcanes had fewer and shorter leaves, but the width of leaves was similar to that of controls. The number of completely emerged leaves of ScSMD affected sugarcane were 3±1 fewer than the healthy sugarcane plants at the same growing stage. The length of ScSMD affected sugarcane leaves was not significantly different from that of healthy sugarcane for the first and the second leaves from the bottom. However, the length of leaves from the third to the ninth from the bottom was less than that of healthy sugarcane plants. Leaves above the ninth from the bottom were similar in length to those of the healthy sugarcane (Fig. 3.2). ScSMD affected sugarcane leaves had a similar width to that of healthy sugarcane leaves, except for the third and fourth leaves (Fig. 3.3).

Up to two weeks after germination, there was no significant difference in leaf number between ScSMD affected and healthy. The total number of leaves of germinated ScSMD affected sugarcane plants was then 2-4 fewer than for healthy sugarcane (Fig. 3.4).

The growth of ScSMD affected sugarcane was reduced. The overall heights of ScSMD affected sugarcanes were not significantly different from healthy up to 5 weeks after germination. The ScSMD affected sugarcanes then grew at about 20 cm/week while the healthy canes at about 50 cm/week after germination (Fig. 3.5A). The same pattern was observed for the overall height comparison (Fig 3.5B). The stems of ScSMD affected sugarcane plants were also shorter and thinner (Fig. 3.1E).

3.3.3 Inoculation

No visible symptoms were produced on any of the possible viral indicator plants or susceptible sugarcane hybrids after inoculation with the ScSMD by the pin-pricking or the applied grafting methods.

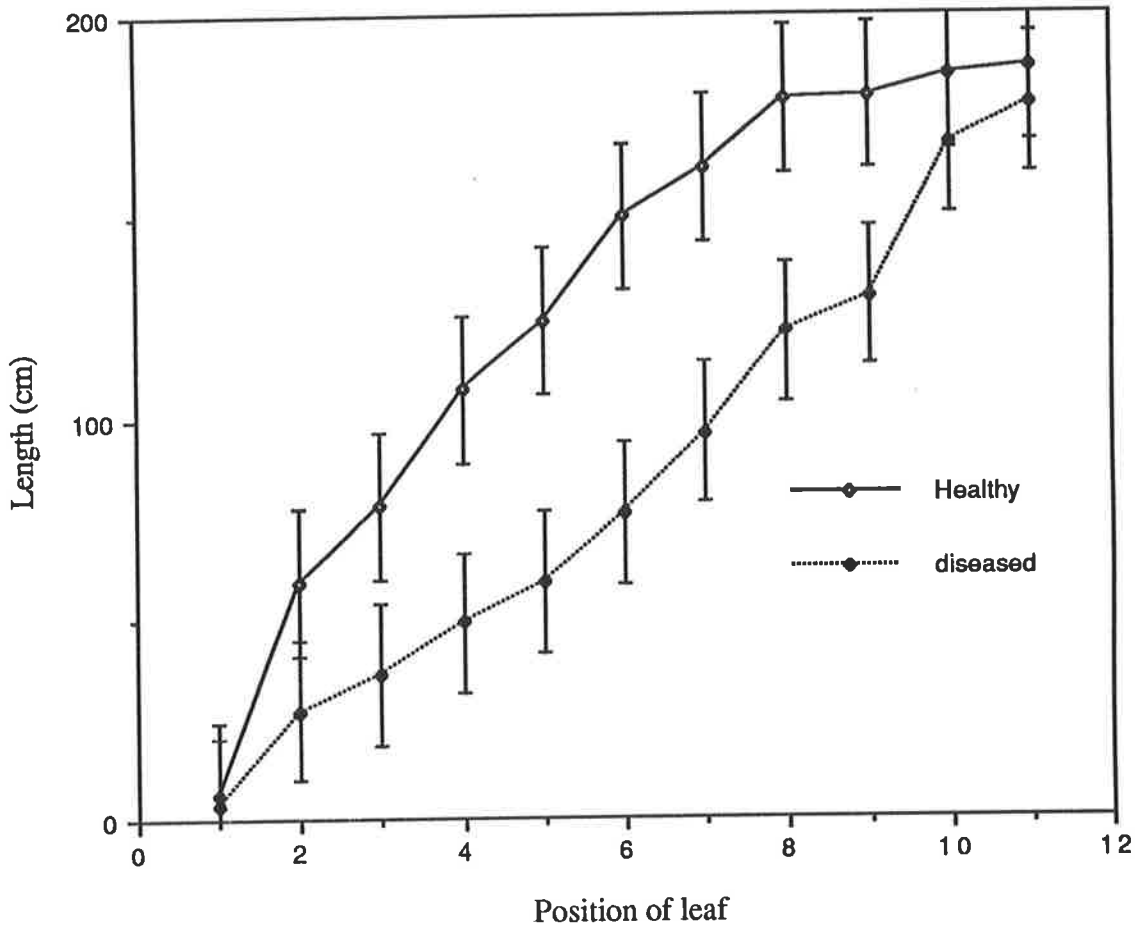


Figure 3.2 Mean length of leaves at nodes 1 to 11 of ScSMD affected and healthy sugarcane. Canes were selected which had the same number of nodes. (n=9, mean± standard error)

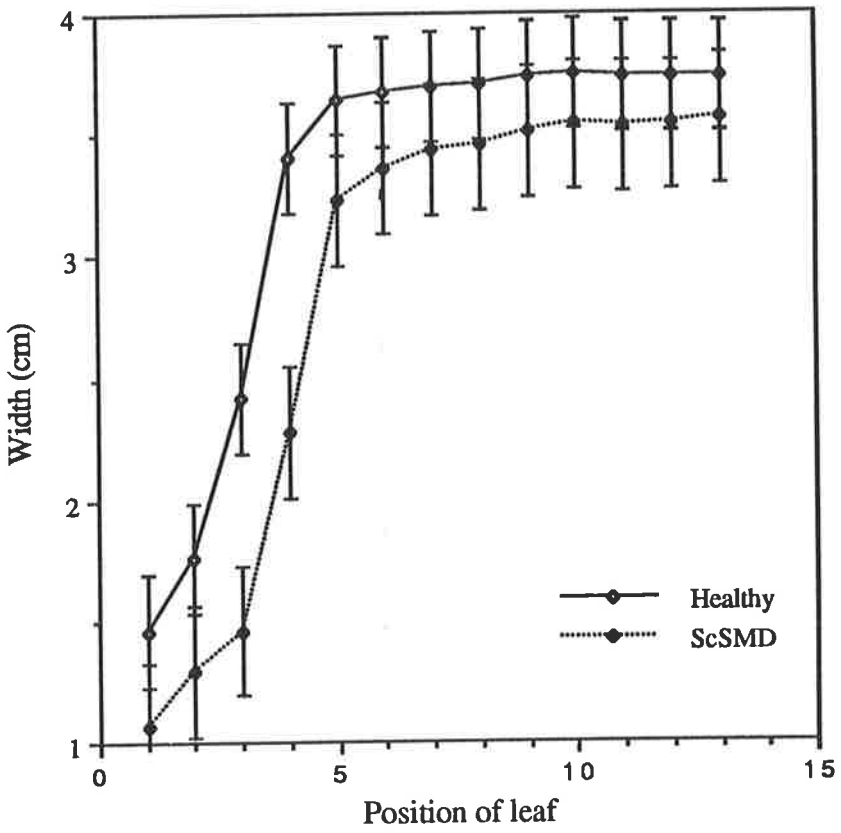


Figure 3.3 Mean maximum width of sugarcane leaves at nodes 1 to 13 of ScSMD affected and healthy canes. Canes were selected which had the same number of nodes. (n=9, mean= standard error)

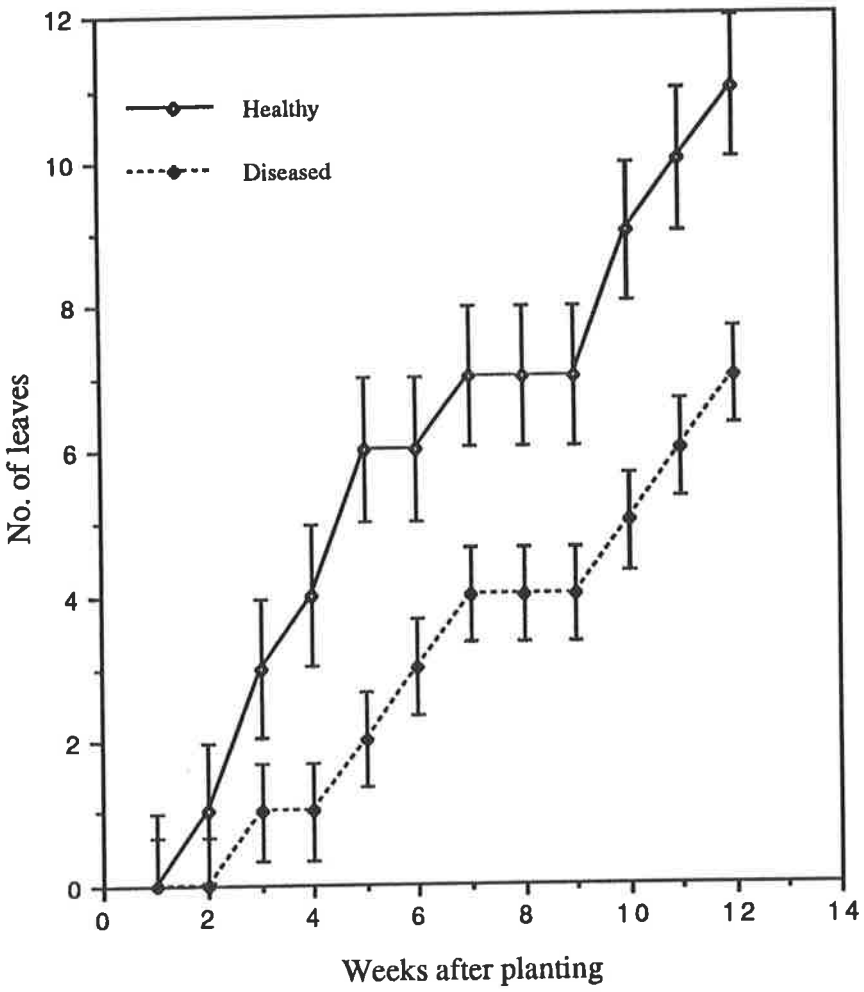


Figure 3.4 Mean number of leaves on canes from ScSMD affected and healthy at 1 to 12 weeks after planting. (n=9, mean± standard error)

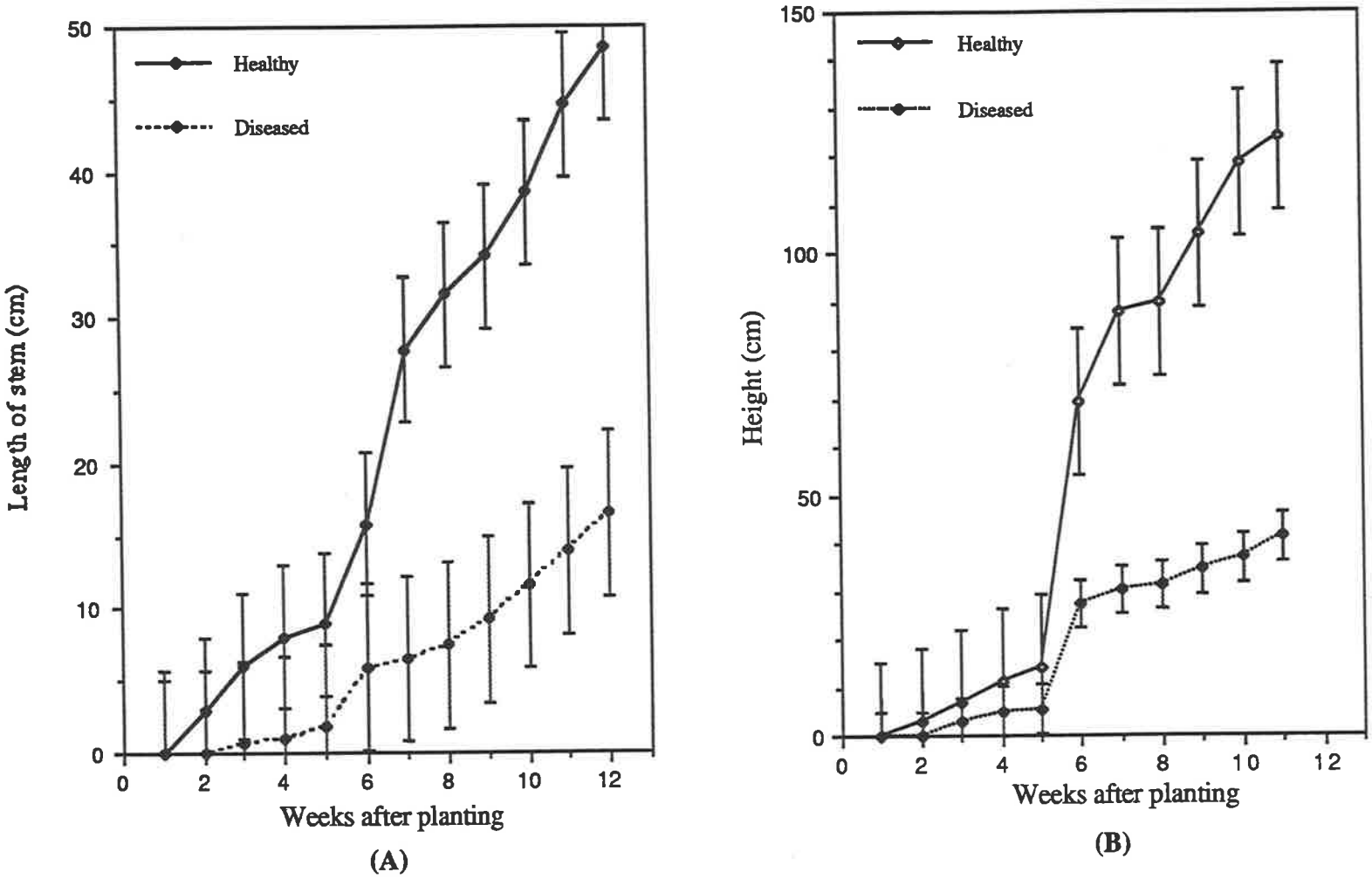


Figure 3.5 **(A) Mean increase in stem length of ScSMD and healthy sugarcane at 1 to 12 weeks after planting. (n=9, mean± standard error)**
(B) Mean increase in overall height of ScSMD affected and healthy sugarcane at 1 to 11 weeks after planting.

3.4 DISCUSSION

The chlorotic striations associated with ScSMD were observed on the leaf sheath as well as on the lamina of the first and second leaves. Stunting was also observed in ScSMD affected sugarcane plants as the disease progressed. Clearer symptoms could be found on older growth stage leaves than at younger stages. The early stages of growth, especially of shoots from cuttings had no ScSMD symptoms. The best time to observe symptoms would thus be after three months germination or from the third ratoon stage.

The germination of cuttings from the diseased plants was delayed and numbers of emerged leaves were fewer than for healthy plants at the same stage after germination. The germinated shoots of ScSMD affected sugarcane, especially shoots grown from older ratoons, were thinner than those of controls. Also, the width of leaves tended to be narrower than in healthy sugarcane.

Inoculation with ScSMD failed to produce any visible symptoms on disease susceptible sugarcane hybrids or on viral indicator plant species. The transmission may thus be vector dependent or require a helper virus to infect the host sugarcane. The search for vectors and the mode of transmission should be continued when an appropriate diagnosis method is available.

CHAPTER 4

CYTOPATHOLOGY OF ScSMD AFFECTED SUGARCANE

4.1 INTRODUCTION

Viruses are economically important only when they cause some significant deviation from normal in the growth of a plant. There are two main types of effects, cytopathological and histological. Microscopes have been used to study those effects in virus infected plant tissues.

Light microscopy is used for detecting inclusion or aggregated bodies of virus particles in epidermal cells or thin sections of virus-infected plants with different stains (Christie and Edwardson, 1986). However, this technique is limited to observing larger inclusions, bodies and aggregates of virus particles, and changes in organelles, such as chloroplasts and nuclei. Light microscopy is still important for studying cytopathological abnormalities, particularly because it can scan much greater areas of a thin section than electron microscopy. Light microscopy may also assist in the interpretation of electron microscopic observations (Matthews, 1991).

Transmission (TEM) and scanning (SEM) electron microscopes have been used to study cytopathological effects on virus infected tissues. Improvements have taken place in procedures for the fixing, staining, and sectioning of plant tissues over the past 30 years. The wide availability of high resolution TEM has led to an understanding of cytopathological effects on virus infected cells. SEM is of less value in the study of virus diseases compared with TEM, but it is useful for some viruses (Hatta and Francki, 1976). However, it also has to be considered that small differences in preparation techniques can have a major effect on the organelles and virus-induced structures (Matthews, 1991). In SEM, a recent advanced technique using fresh untreated tissues in an environmental scanning electron microscope (ElectroScan Corp., USA), eliminates the disadvantages of sample preparation, including the time taken and sample preparation effects.

The macroscopic symptoms induced by viruses frequently reflect histological changes in the virus infected plant cells. The histological changes are necrosis, hypoplasia, and hyperplasia. Necrosis is the death of tissue, organs or the whole plant, and it often occurs in combination with other histological changes (Shepardson *et al.*, 1980). Hypoplasia can be found in the yellow area on leaves with mosaic symptoms , the lamina of which is thinner, or the mesophyll cells are less differentiated with fewer chloroplasts (Matthews, 1991). Hyperplasia is enlargement of cells near the veins and undifferentiated cell division (Matthews, 1991).

In the work reported this chapter, cytopathological effects of ScSMD on sugarcane plants were studied. Causal agents or like bodies were sought, and differences between the organelles in the cells of ScSMD affected and healthy sugarcane leaf tissue have been studied.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The sample sizes of sugarcane tissue and plants used in this work are listed in Tables 2.1 and 2.2.

The copper grids used for the thin sections were coated with 2% formvar solution (v/v in chloroform) according to Hayat (1989). The coated grids were checked by light microscopy.

4.2.2 Methods

4.2.2a Microscopy

Light microscopy was carried out according to the method of Christie and Edwardson (1986) with some modification as described in Section 2.2.2.a. The SEM procedure was as described in Section 2.2.2.b. TEM was performed according to the method of Hatta and Francki (1978), and is described in Section 2.2.2.c.

4.2.2.b Embedding sugarcane tissue in Spurr's medium

The samples (see 2.2.2.c) prepared for thin sectioning were dehydrated and embedded according to the following standard schedule and embedded in Spurr's medium (Table 1.5).

SOLVENTS and RESIN		TIME
5 % Acetone in water		20 min
25 % "		30 min
50 % "		40 min
75 % "		"
90 % "		"
95 % "		1 hr
100 % "		2x 1 hr
100 % Acetone: Spurr's medium	[3: 1 (v/v)]	1 hr
	(1: 1)	"
	(1: 3)	"
	"	"
Spurr's medium		2x 1 hr

After the last incubation with Spurr's medium, fresh medium was added and vacuum infiltrated three times *in vacuo* for 3 hr. Samples were transferred to a resin mould and hardened at 65 °C for 8-12 hr until the resin turned yellow (Hayat, 1987). The hardened resin was then cooled slowly to room temperature. The embedded samples were cut with a diamond knife (PROBING & STRUCTURE, QLD) into 50-120 nm (silver or gold) sections by an ultramicrotome (2120 Ultrotome®, LKB, Bromma).

4.2.2.c Staining samples for TEM

Thin sections were stained with lead citrate and/or uranyl acetate (Appendix C). One group of thin sections was transferred to a formvar coated 200 mesh copper grid. Uranyl acetate staining was done according to Hayat (1989) and has been described in Section 2.2.2.d.

Lead citrate staining was carried out with the following modifications. To create a CO_2 free atmosphere, pellets of NaOH were dropped into a petri dish containing water droplets and the dish was covered for 10 min. Droplets of lead citrate solution were then placed into the petri dish. The grid with thin sections mounted was placed onto the lead citrate droplet and incubated for 2 min covered with lid. The grid was then washed with CO_2 free water, and dried or stained again with uranyl acetate. Stained samples were examined with a Phillips 400 electron microscope at 100 kV.

Staining of the grids with sap of ScSMD affected sugarcane was done as described in Section 2.2.2.d.

4.3 RESULTS

4.3.1 Light microscopy

Sugarcane tissue was examined by light microscopy using standard transverse sections prepared by hand with razor blades. It was difficult to examine peeled epidermal strips of sugarcane because the surface of the sugarcane was covered with a thick waxy layer which interfered with observation. No inclusion bodies or like structures were observed in ScSMD affected sugarcane tissues.

4.3.2 Scanning electron microscopy

The leaf surface of ScSMD affected sugarcane had a smoother texture than that of healthy plants. There were also fewer spikes on the affected sugarcane leaves (Fig. 4.1B) than on the healthy leaves (Fig. 4.1A). The surfaces of the affected leaves were covered with a thicker layer of waxy material than healthy leaves (Fig. 4.1B & D). Older diseased leaves were smoother than on younger leaves. The lower surface of affected leaves was more heavily covered than the upper. These surface differences were found on yellowed leaves with chlorotic striations and on diseased older leaves on stems of the third ratoon stage in the glasshouse.

Figure 4.1 Scanning electron micrographs of sugarcane leaves.

A - Upper epidermis of healthy sugarcane.

B - Upper epidermis of ScSMD-affected sugarcane.

C - A stomate on the upper epidermis of healthy leaf.

D - Stomata on the upper epidermis of sugarcane leaf affected by ScSMD.

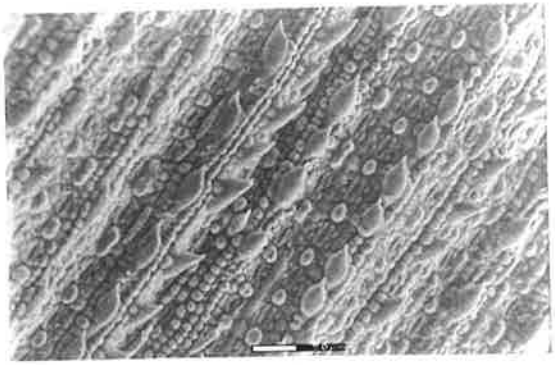
E - Outer surface of ScSMD affected leaf sheath.

F - Inner surface of ScSMD affected leaf sheath.

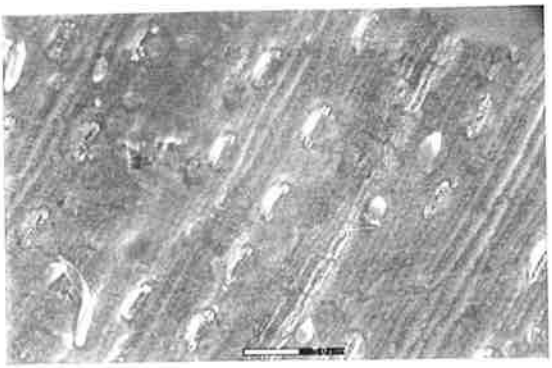
G - A cross section of healthy lamina.

H - A cross section of ScSMD affected lamina.

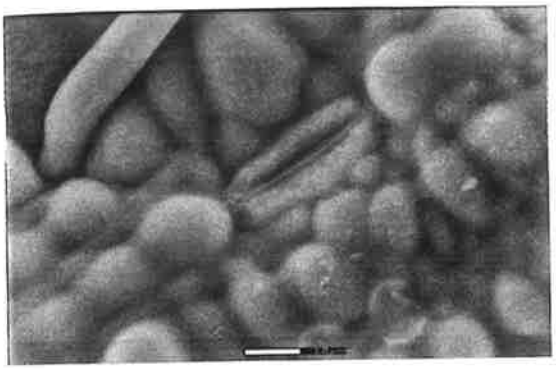
see corrigendum



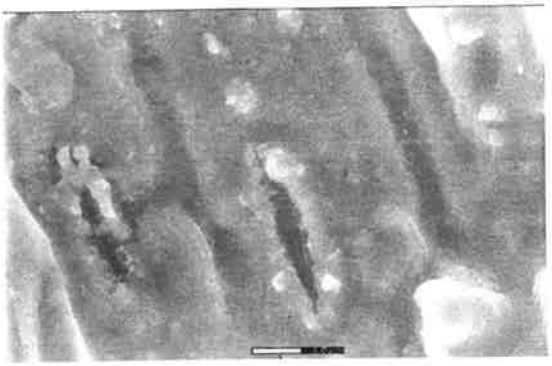
(A)



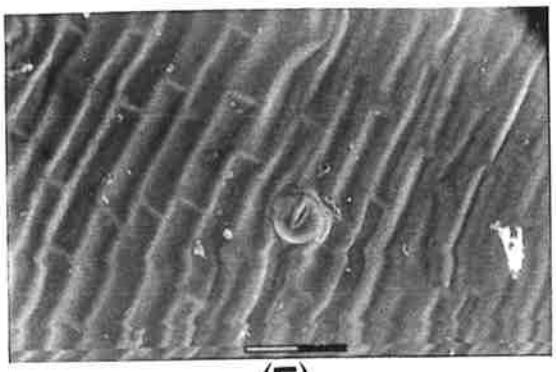
(B)



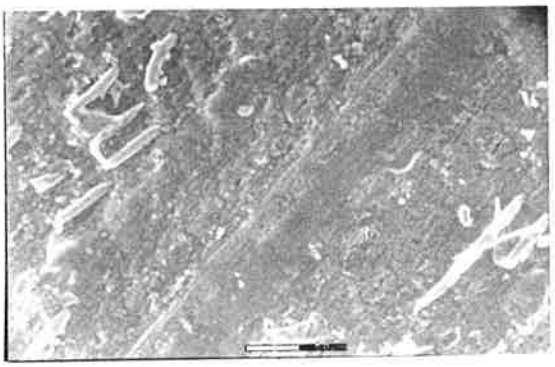
(C)



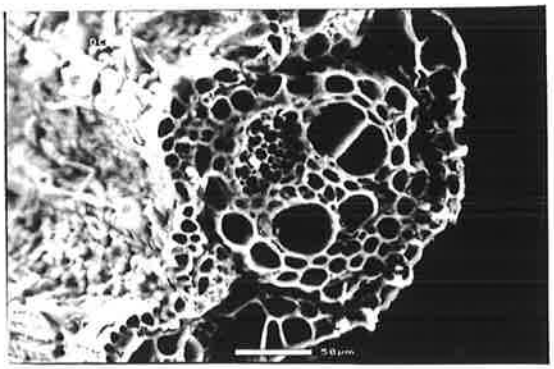
(D)



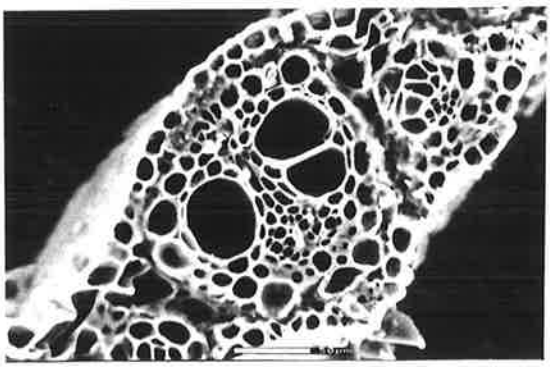
(E)



(F)



(G)



(H)

There was no difference in cell size between ScSMD affected and healthy sugarcane leaves (Fig. 4.1G & H). There was also no texture difference on the surface of the leaf sheaths (Fig. 4.1 E and F).

4.3.3 Structural changes in organelles of ScSMD affected cells

Differences were observed between diseased and healthy sugarcane in chloroplasts, plasmodesmata and mitochondria. There were empty vesicles in the disease affected cells.

In the chloroplasts in ScSMD affected cells, the thylakoid membrane was deformed. The affected thylakoid was wavy whereas thylakoids in chloroplasts of healthy leaves were straight. There were also electron dense material between the membranes (Fig 4.2C).

Various sizes of single walled empty vesicles were found in the cytoplasm of disease affected cells, especially next to the deformed chloroplasts (Fig. 4.2B). The vesicles in the cytoplasm of the ScSMD affected sugarcane had one layer of membrane and the size varied.

More mitochondria could be seen in the thin sections of ScSMD affected sugarcane cells than in those of healthy cells (Fig. 4.2D).

More and thicker plasmodesmata were observed in the affected sugarcane cells than in healthy cells, and some electron dense particles were observed in the plasmodesmata (Fig. 4.2E).

4.3.4 Negative staining of the sap of ScSMD affected sugarcane leaves

No putative particles were found in sap from ScSMD affected sugarcane leaves. However, the grids to which sugarcane sap had been applied were hard to observe due to the presence of other organelles of sugarcane cells.

4.4 DISCUSSION

In this chapter, light microscopy failed to detect any differences between healthy and ScSMD affected sugarcane and provided no evidence of the likely cause of ScSMD. If the causal agent is a virus, it may either not aggregate or not produce inclusion bodies in the cells.

Figure 4.2 Transmission electron micrographs of thin sections of sugarcane leaf tissue.

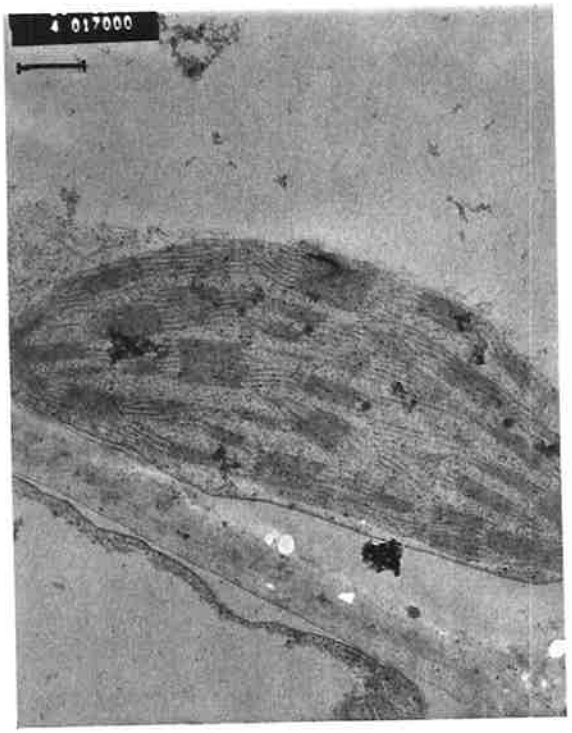
A - Chloroplast in the cell of healthy sugarcane leaf.

B & C - Chloroplast in the cells of ScSMD affected sugarcane leaf with small vesicles.

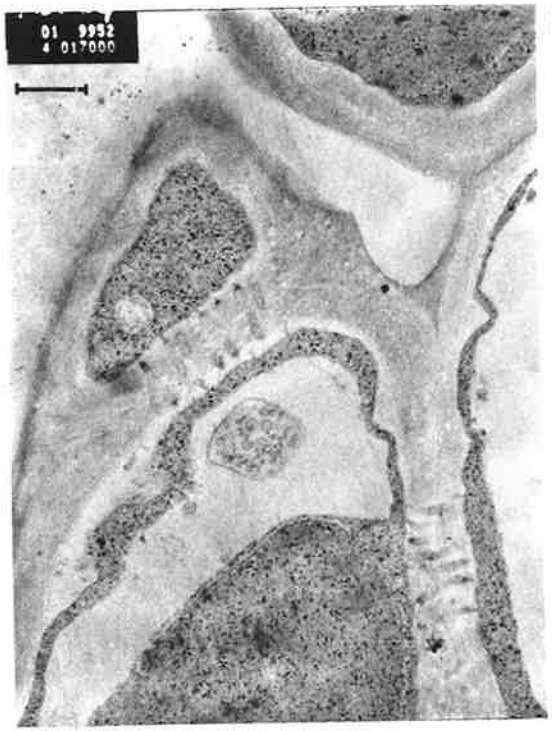
D - Mitochondria in the cell of ScSMD affected sugarcane.

E - Plasmodesmata in the cell wall of ScSMD affected sugarcane.

see corrigendum

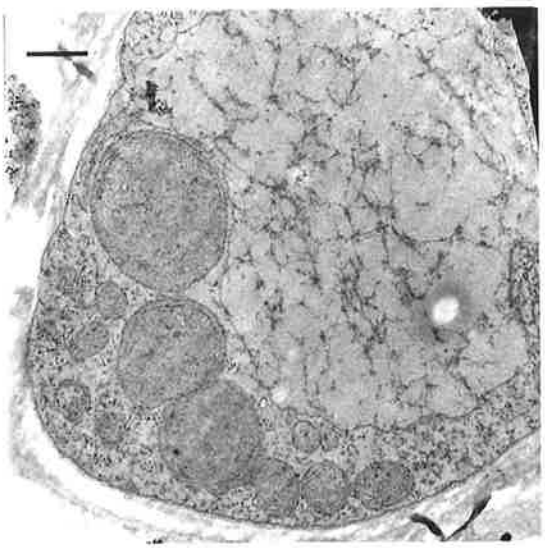


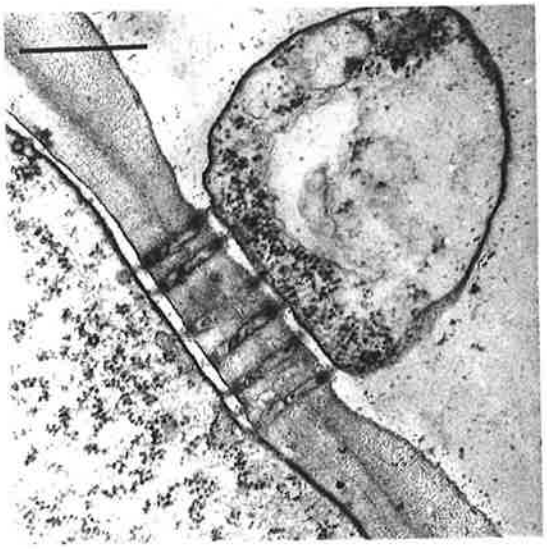
(A)

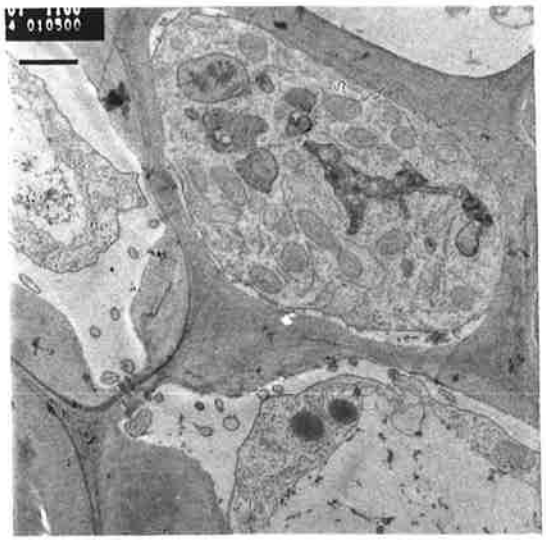


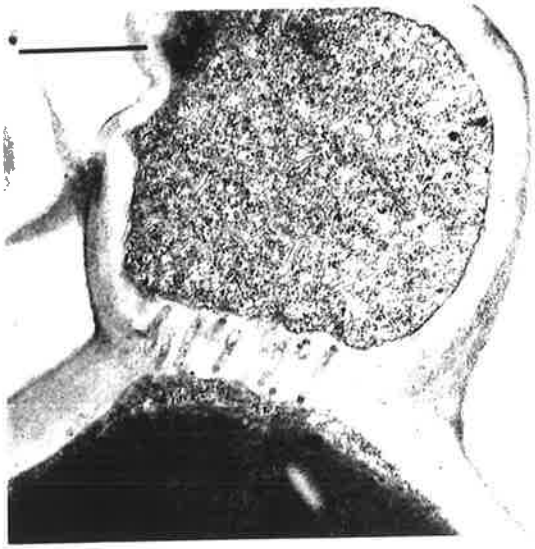
(B)

(C)









(D)

(E)

The surface structure of ScSMD affected sugarcane leaf was different from that of healthy sugarcane. The surfaces of leaves were smoother in texture. They were covered with an extra amount of wax layer and had fewer spikes on the surface. These symptoms were observed on leaves after the third ratoon stage. These structural changes became more severe at the older growth stages and after the leaves were already yellow. This structural difference was limited to the leaves.

Also associated with the disease were changes in the membrane structure of chloroplasts, the number of mitochondria and the diameter of the plasmodesmata, which have a role in the movement of nutrients and viruses between cells. No putative causal agent or virus-like particle was detected in thin sections by TEM. Similarly, negative staining of sap showed no detectable causal agent.

In conclusion, microscope studies showed that the ScSMD causal agent affected sugarcane organelles and tissue but no possible causal agent was found. Changes to organelles similar to these seen in the ScSMD affected sugarcane have been reported previously for numerous plant infectious viruses (Matthews, 1991). Therefore a virus may be involved in ScSMD. Methods other than electron microscopy may be required to detect and identify the causal agent of ScSMD and to study its etiology.

CHAPTER 5

ANALYSIS OF NUCLEIC ACID EXTRACTS FROM ScSMD AFFECTED SUGARCANE AND FRACTIONATION OF dsRNA

5.1 INTRODUCTION

The symptoms of ScSMD on sugarcane plants are virus-like with chlorotic striations, stunting and a reduced growth rate (Chapter 3). The cytopathological effects of ScSMD are also virus-like such as deformation of chloroplasts and reduction of number of mitochondria, and changes in plasmodesmata (Chapter 4). These symptoms and cytopathological effects strongly support the contention that ScSMD has a viral etiology. However, no causal agents were detected in the sap of ScSMD affected sugarcane leaf or in tissue sections by TEM.

Virus-infected plant tissue contains the viral genome in several forms: genomic, subgenomic and replicative. To determine whether these forms are in the tissue or not, it is necessary to isolate, fractionate and detect them.

Many viruses infecting plants have an RNA genome, which can be either single or double stranded (Murphy *et al.*, 1995; Appendix E). Viral genomes may also be segmented, and the number and size of these segments is diagnostic for the genus or family of viruses. Replication of each of these occurs through double-stranded RNA (dsRNA) forms. These are relatively stable, and their length is the same as that of the genomic segments that they are replicating. They are thus a valuable indicator of the presence of an RNA virus and its likely taxonomic position. Purification of dsRNA from RNA virus infected plants by the CF11 cellulose method was initially developed by Franklin (1966) and it has been well described by Dodds (1993) and his colleagues (Valverde *et al.*, 1986).

This chapter describes the composition of nucleic acids in healthy and ScSMD affected sugarcane tissues, and isolation of ScSMD associated dsRNAs by the standard CF11 cellulose method. The chapter also discuses the development of an improved dsRNA isolation

procedure called the microgranular cellulose method, from nucleic acid extracts of sugarcane tissues, and a purification procedure for dsRNA.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Sugarcane leaf material was from the glasshouse or collected in the field (Table 2.1). dsRNA molecular markers were prepared by the CF11 cellulose method (Dodds, 1993; Section 2.2.5.a) from *Nicotiana glutinosa* infected with AMV or CMV, *N. tabacum* cv. Xanthi infected with TMV and *Vicia faba* infected with PMV.

5.2.2 Methods

5.2.2.1 Total nucleic acid extraction

Total nucleic acid (TNA) was extracted from ScSMD affected and healthy plants, and other plant tissues infected with viruses (Section 5.2.1) by modifications of the method of Randles *et al.* (1986). The method is described in Section 2.2.3.

5.2.2.2 dsRNA extraction

dsRNA extraction by the microgranular cellulose method was modified from the method of Dulieu and Bar-Joseph (1989). The method is described in Section 2.2.5.a and summarised in Table 5.1. The standard CF11 cellulose method was performed according to the method of Dodds (1992) and is described in Section 2.2.5.a.

5.2.2.3 Gel fractionation of the ScSMD associated 9 kbp dsRNA

ScSMD associated dsRNA (Table 5.1) isolated by the microgranular cellulose method was used for gel fractionation and the procedure is described in Section 2.2.5.b.

Table 5.1. Microgranular cellulose batch procedure for dsRNA isolation from leaf

- Powder frozen tissue by blending in liquid nitrogen
- Add 4 volumes (w/v) of STE (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl and 10 mM EDTA) containing 1 % SDS and 1 % MTG
- Stir mixture at room temperature for 30 min
- Add 0.5 volume of phenol-chloroform (1:1) and emulsify for 30 min
- Centrifuge (10,000 *g*, 10 min), collect the aqueous phase after centrifugation and precipitate nucleic acids with 0.8 volumes of *iso*-propyl alcohol.
- Collect the nucleic acid pellet, dissolve in 1.3x STE (at 0.8 ml/3 g of leaf extracted) and clarify by centrifugation (10,000 *g*, 20 min).
- Add microgranular cellulose to the supernatant (at 10 mg/3 g of leaf extracted) and mix for 30 min
- Add ethanol drop wise to 20 % with constant vigorous stirring and mix for 30 min
- Collect the cellulose by centrifugation (10,000 *g*, 2 min)
- Wash with STE containing 16.5 % ethanol (5 cycles of centrifugation for 0.5 min alternating with discarding and replacement of the supernatant)
- Drain and air-dry the cellulose pellet
- Elute with STE (100-200 µl/10 mg) and remove cellulose by centrifugation (10,000 *g*, 5 min)
- Digest with RNase free DNase (RQ1, Promega, in the recommended buffer, 100 units/ml, 37 °C, 60 min)
- Extract with 1 volume of phenol-chloroform and precipitate with *iso*-propyl alcohol, as above
- Analyse by agarose gel electrophoresis

5.3 RESULTS

5.3.1 Analysis of TNA by gel electrophoresis

Among the methods described in Section 2.2.3, the best extraction method for TNA from sugarcane tissue was the RNA extraction method. Analysis of TNA extracts by agarose gel electrophoresis of leaf of sugarcane, and of *Nicotiana* spp. infected with AMV, CMV and TMV had gene patterns of characteristic non-degraded ssRNA. No difference in band patterns was detectable between healthy and ScSMD affected sugarcane on agarose gels using EtBr staining. However, the genomic ssRNAs of viruses could be detected from TNA of plants infected with AMV, CMV and TMV in these gels (Fig. 5.1).

corrigendum

When TNAs from healthy and ScSMD affected leaf samples were analysed by PAGE using silver staining, there was no detectable difference between the band patterns (Fig. 5.2). The relatively high concentration of chromosomal DNA and rRNA of sugarcane may have obscured disease associated nucleic acids. An attempt was therefore made to isolate a dsRNA fraction from the diseased sugarcane.

5.3.2 Extraction of dsRNA

The standard CF11 cellulose method yielded small amounts of dsRNA, and other possible host nucleic acids from leaves of both field and glasshouse grown sugarcane with symptoms of ScSMD. Similar preparations from healthy sugarcane contained negligible amounts of nucleic acid. All nucleic acid extracts from diseased leaves showed a sharp band on silver stained polyacrylamide gels with an apparent molecular size of ca. 9 kbp (using dsRNA size markers from AMV, CMV, TMV and PMV), together with a variable number of lower molecular weight bands. No such bands were isolated from sugarcane seedlings or from healthy sugarcane collected in the field outside the region of occurrence of ScSMD (samples 1 and 4, Fig. 5.3). Leaves from diseased plants grown in the glasshouse had higher yields of the 9 kbp dsRNA than diseased leaves collected from the field (Fig. 5.3).

The band was resistant to DNase and to RNase in high salt concentration, but was digested with RNase at low salt, as expected for dsRNA (Fig. 5.4).

Figure 5.1 Analysis by 1.2% agarose gel electrophoresis of total nucleic acid (TNA) extracted with the RNA extraction procedure from plant leaves. The gel was stained with EtBr.

1 - Healthy sugarcane

2 - ScSMD affected sugarcane

3 & 4 - *Nicotiana tabacum* cv. Xanthi infected with TMV

5 & 6 - *N. glutinosa* infected with AMV

7 & 8- *N. glutinosa* infected with CMV

9 & 10- Healthy *N. glutinosa.*

see corrigendum

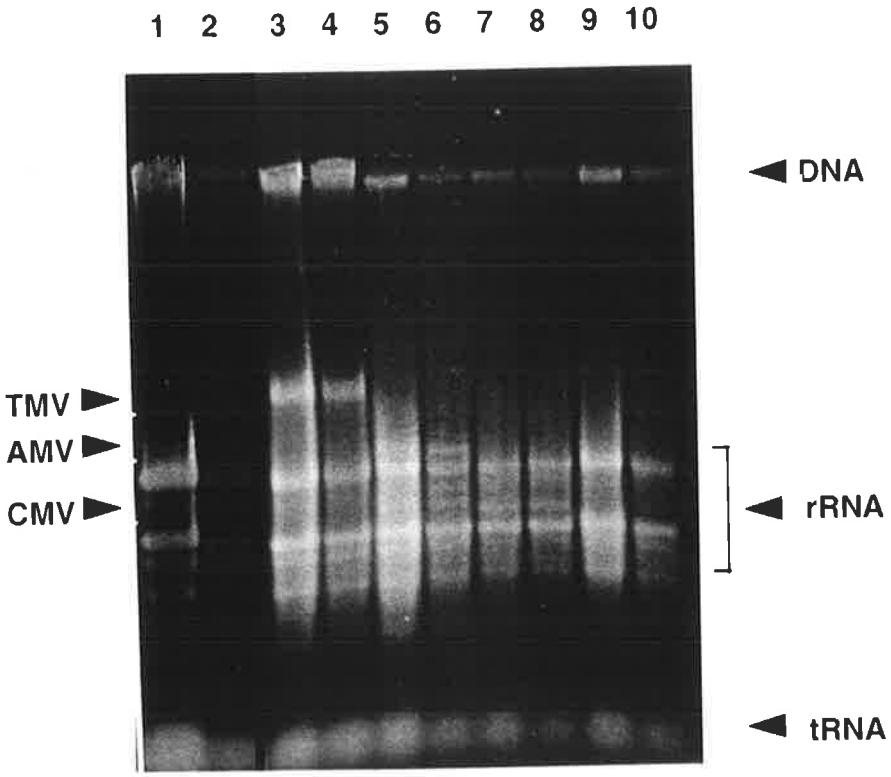

1 2 3 4 5 6 7 8 9 10
DNA
TMV
AMV
CMV
rRNA
tRNA

Figure 5.2 Analysis by 3.3% polyacrylamide gel electrophoresis of TNA extracted with the RNA extraction procedure from sugarcane leaves. The gel was stained with silver.

rRNAs; ribosomal RNAs

H - healthy sugarcane

D - ScSMD affected sugarcane.

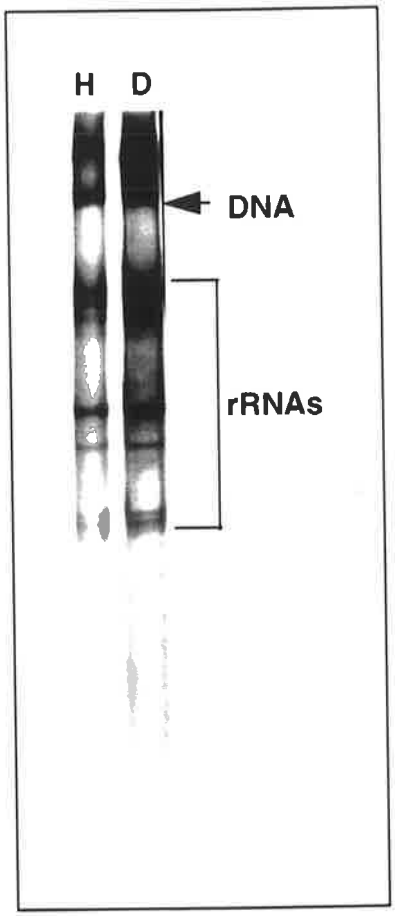

H
D
DNA
rRNAs

Figure 5.3 dsRNA preparations by the standard CF11 cellulose method. Nucleic acid preparations were analysed on a non denaturing 6.5% polyacrylamide gel and stained with silver. The dsRNA size marker (0.88-3.25 kbp) is dsRNA of AMV.

1 & 4 - Healthy sugarcane.

6 - ScSMD symptomatic sugarcane grown in the glasshouse.

7 to 11 - ScSMD symptomatic sugarcane collected from the field (Table 2.1).

6t - TNA of sample 6 prepared by the RNA extraction procedure.

see corrigendum

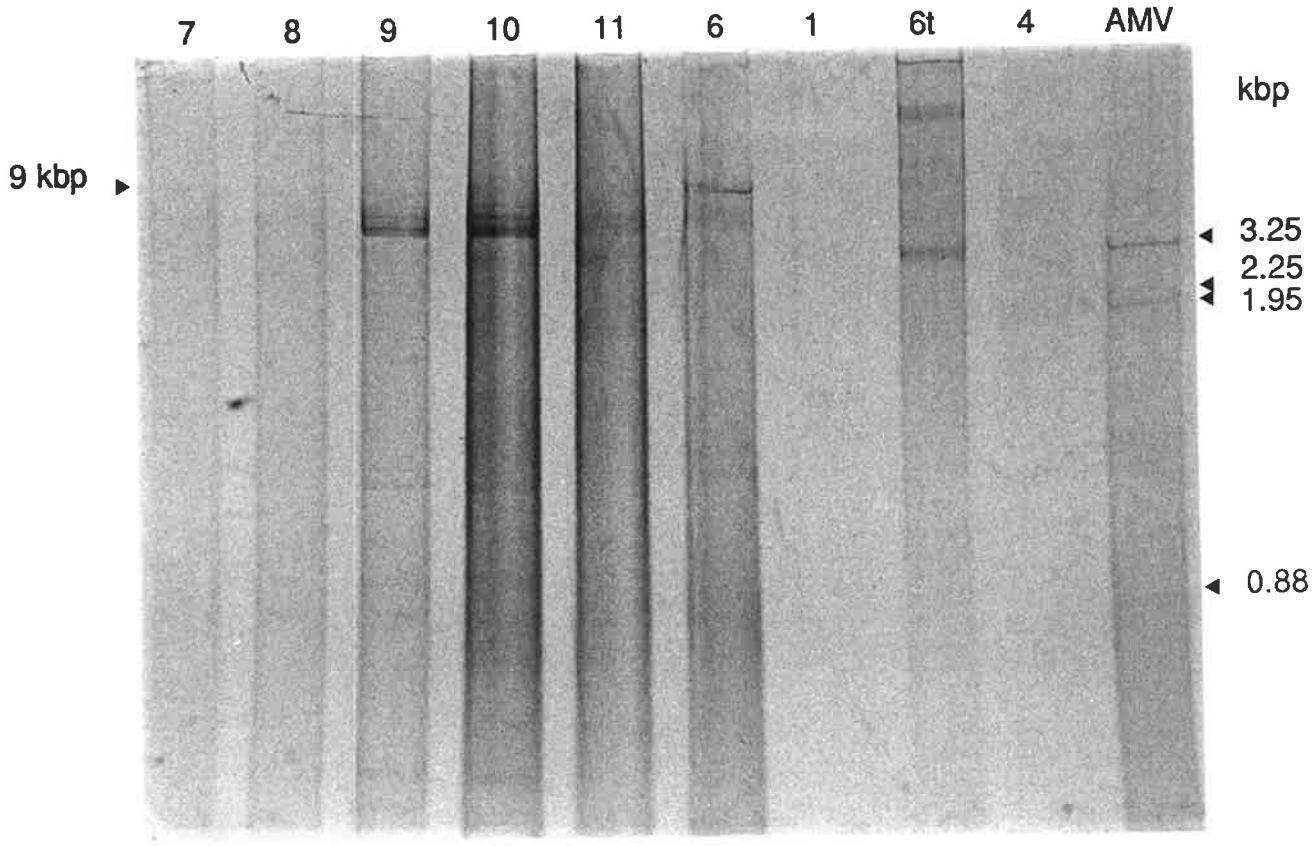

7
8
9
10
11
6
1
6t
4
AMV
kbp
9 kbp
3.25
2.25
1.95
0.88

Figure 5.4 Incubation of ScSMD associated dsRNA with nucleases at 37 °C for 1 hr, followed by analysis by 0.9% agarose gel electrophoresis. The gel was stained with EtBr.

1 - dsRNA preparation from ScSMD affected sugarcane digested with DNase (0.1 unit/µl) in a buffer containing 10 mM Tris-HCl, pH 7.0 and 10 mM $MgCl_2$.

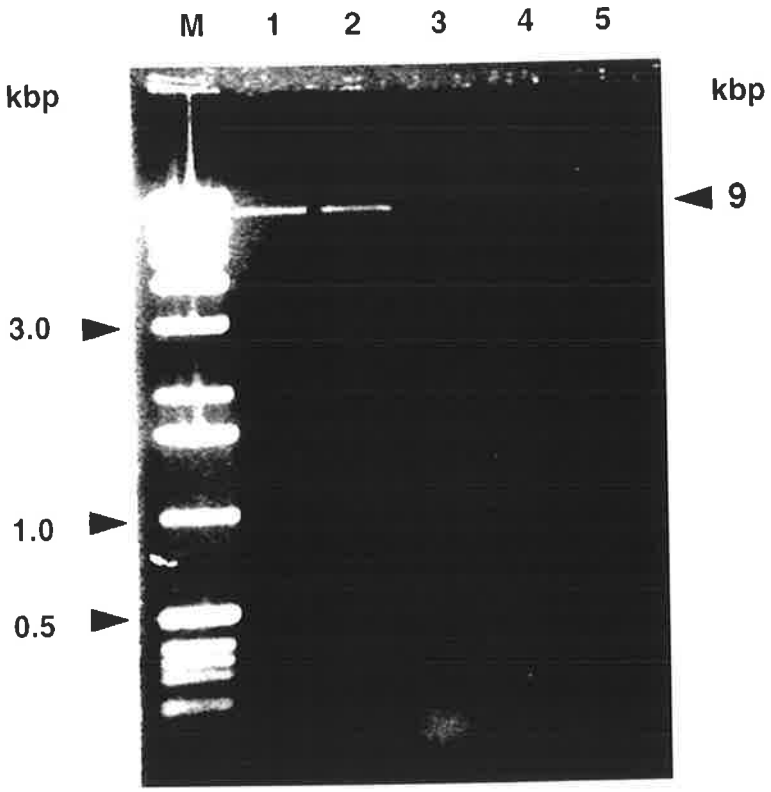
2 - dsRNA preparation from ScSMD affected sugarcane digested with RNase (100 ng/µl) in a buffer containing 10 mM Tris-HCl, pH 7.5 and 0.3 M NaCl.

3 - dsRNA preparation from ScSMD affected sugarcane digested with RNase (100 ng/µl) in a buffer containing 10 mM Tris-HCl, pH 7.5.

4 - dsRNA preparation from healthy sugarcane digested with DNase as in lane 1.

5 - dsRNA preparation from healthy sugarcane digested with RNase as in lane 2.

M - 1 kbp DNA ladder was used as a marker.


M
1
2
3
4
5
kbp
kbp
9
3.0
1.0
0.5

It was found that the use of microgranular cellulose (Table 5.1) for concentrating dsRNA increased yields by about 10-fold (Fig. 5.5). The preparations from the microgranular cellulose method also contained ssRNA and dsDNA, presumably of host origin. These nucleic acids were partially removed by one cycle of CF11 chromatography (Section 2.2.4.c). The microgranular batch method was thus shown to be suitable for preparing dsRNA from sugarcane leaf tissue for further studies.

The method routinely adopted to isolate dsRNA from sugarcane tissue was to extract dsRNA with the microgranular cellulose method from the initial large volume of plant extracts and then to reduce contaminating host ssRNA and dsDNA by CF11 cellulose chromatography.

5.3.3 Specific association of dsRNA with ScSMD

It was found that the 9 kbp dsRNA band was only present in diseased plants (for example, see Fig. 5.3). Therefore, it could be used both as a molecular marker for the disease, and as evidence that ScSMD is a disease of viral etiology. The dsRNA was therefore purified and cloned.

5.3.4 Electrophoretic patterns of dsRNA isolated from ScSMD affected plants

Four dsRNA species(9, 6, 2.6 and 2.5 kbp) were detected in microgranular cellulose preparations but 9 kbp band was the most prominent (Fig. 5.6A).

The smaller dsRNAs (6, 2.6 and 2.5) hybridised to a probe made from the 9 kbp. There were also some bands below 1 kbp in size that were not visible by staining with EtBr, but which also hybridised with the probe (Fig. 5.6B).

see corrigendum

5.3.5 Purification of ScSMD associated dsRNAs by gel fractionation

ScSMD associated dsRNAs were recovered from agarose gel with the RNaid™ kit. The 9 kbp dsRNA when recovered also contained a small amount of the 6 kbp dsRNA. The dsRNAs were fractionated by BAC cross-linked PAGE (Fig. 5.7A) and the 9 kbp dsRNA band

Figure 5.5 Comparison of yields of dsRNA from 50 g of ScSMD affected sugarcane leaf tissue.

1 - 1 kbp DNA ladder.

2 - Prepared by the standard CF11 method (first cycle of column chromatography only, Section 2.2.4.c).

3 - Prepared by the microgranular cellulose method.

4 - dsRNA size marker (0.88-3.25 kbp) is alfalfa mosaic virus dsRNA.

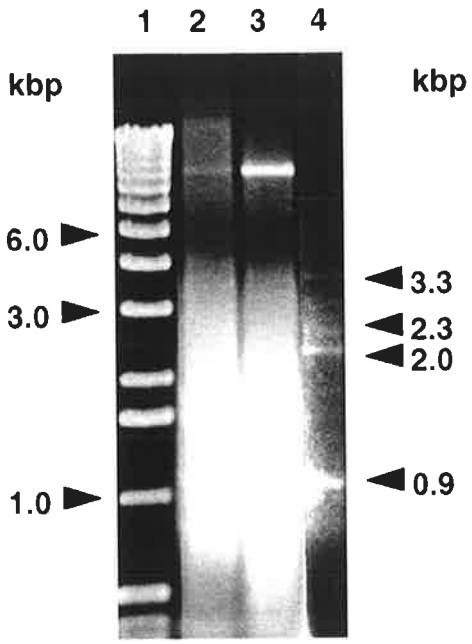
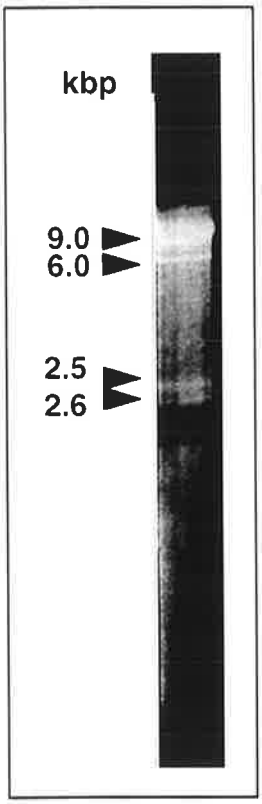

1
2
3
4
kbp
kbp
6.0
3.0
1.0
3.3
2.3
2.0
0.9

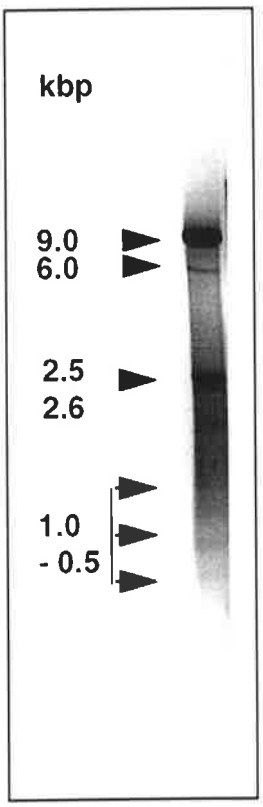
Figure 5.6 (A) - dsRNA prepared by the microgranular cellulose batch method treated with DNase and RNase in a buffer containing 0.3 M NaCl as described in Fig. 5.4, and analysed on a 0.9% agarose gel.

(B) - The dsRNA in gel (A) was transblotted and hybridised with a probe prepared from the 9 kbp dsRNA and autoradiographed.

see corrigendum



(A)



(B)

was recovered successfully from the polyacrylamide gel using BME to solubilise the gel and microgranular cellulose binding to recover the dsRNA (Fig. 5.7B).

5.4 DISCUSSION

Factors in the extraction of TNAs from sugarcane leaf tissues were temperature and time. The RNA extraction method (Section 2.2.3.c) was found most satisfactory and it was necessary to powder samples in liquid nitrogen and thaw in the buffer.

No differences in the TNA patterns were found between the healthy and ScSMD affected sugarcane when fractionated by either AGE or PAGE. dsDNA and ssRNA originating from sugarcane plants were present in high concentrations, so it would be difficult to find different band patterns if the concentration of the disease associated nucleic acid in the extracts was relatively low. It was therefore found necessary to fractionate the TNA extracts further.

Using the CF11 cellulose method, ScSMD associated 9 kbp dsRNA was isolated from field and glass house grown sugarcane. Although the CF11 method yielded dsRNA from *Nicotiana* spp., at least 50 g of ScSMD infected plant material had to be used to detect ScSMD-associated dsRNA by silver staining and the yield was only ca. 5 ng. Therefore, an improved method was required.

To increase the yield of dsRNA microgranular cellulose, a higher binding capacity cellulose was substituted for CF11 cellulose. This method gave about a 10 fold higher yield than the standard CF11 method from sugarcane tissue, and was easier and faster. However, the products of the microgranular method contained considerable amounts of host dsDNA and ssRNA in the high salt buffer. These host nucleic acids could be removed by treatment with DNase and RNase.

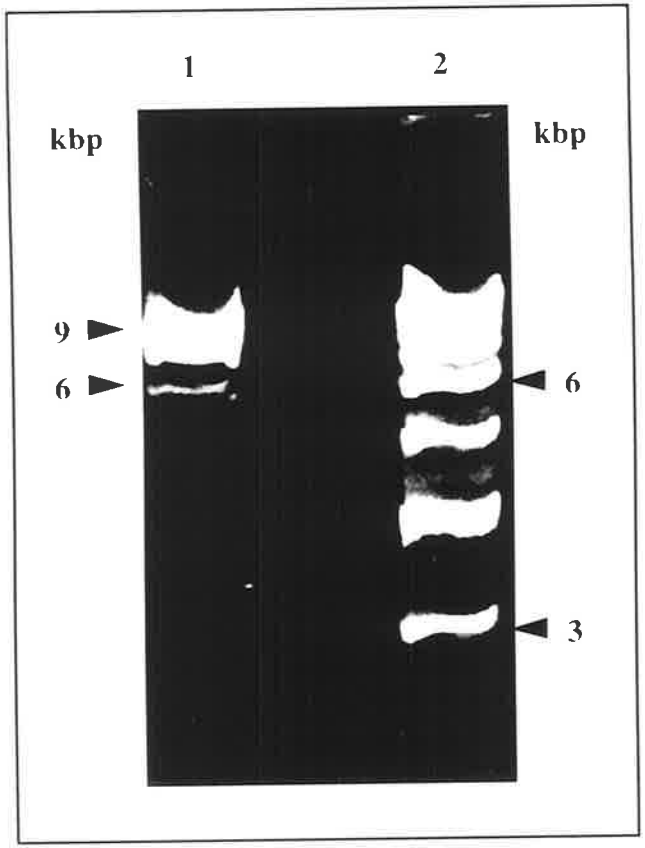
The technique routinely adopted in this work to extract dsRNA from ScSMD affected sugarcane leaf was to combine both of the above methods. First, microgranular cellulose was used to collect large amounts of dsRNA rapidly from the initial plant extract. The amount of other contaminating nucleic acids was then reduced by one cycle of CF11 cellulose column chromatography. Isolated dsRNAs were fractionated by two cycles of gel electrophoresis and used for cloning.

Figure 5.7 Analysis by gel electrophoresis of ScSMD associated dsRNA. Gels were stained with EtBr.

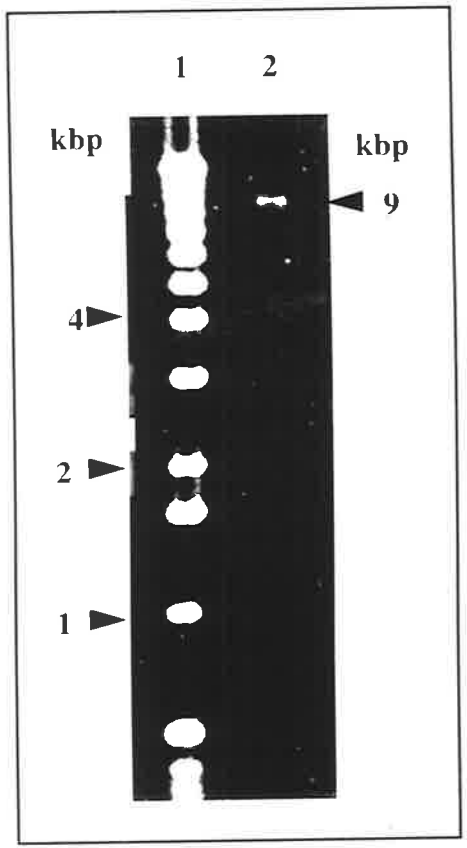
(A) - The ScSMD associated dsRNA extracted from the gel shown in Fig. 5.6A by an RNA extraction kit (RNaid^{TM} kit) and analysed by 6.5% polyacrylamide gel electrophoresis to separate the ScSMD associated 6 kbp dsRNA from the 9 kbp dsRNA.

(B) - The 9 kbp dsRNA from gel A and analysed by 0.9% agarose gel electrophoresis.

see corrigendum



(A)



(B)

Hybridisation between the ScSMD associated 9 kbp and small dsRNAs showed that the ScSMD associated dsRNAs had sequence homology, and suggests that the 6, 2.6 and 2.5 kbp are subgenomic forms fo the 9 kbp dsRNA. The electrophoretic pattern of the other disease associated 6, 2.6 and 2.5 kbp dsRNAs differed between the glasshouse grown and field collected sugarcane. If they are subgenomic, this variation may only reflects rate of replication (Fig. 5.6A).

The isolation of the 9 kbp dsRNA suggests that a virus with a ssRNA genome of 9 kb may be the causal agent of ScSMD.

CHAPTER 6

CLONING AND SEQUENCING OF ScSMD ASSOCIATED dsRNAs

6.1 INTRODUCTION

A direct procedure for identifying a virus is to obtain the sequence of its dsRNA and to compare it with other sequences in genome data bases. To sequence from a dsRNA, the accepted method is to synthesise the first strand of cDNA from target templates with a primer and reverse transcriptase, followed by the second strand synthesis.

The primers for the first strand cDNA can be a specific sequence if the sequences of the target are known, or a random primer if the target sequences are unknown. Many approaches have been made to generate cDNA libraries from a target, and these have been developed to preserve as much of the original sequence as possible. To improve cloning efficiency, the copied ds-cDNA can be manipulated to have restriction sites in common with the vector. The inserts ligated into vectors are transformed into bacterial cells. The transformed cells may be used for further analysis, such as subcloning, preparation of specific probes or sequencing of the inserted fragment of the template.

Several procedures have been used for the selection of cloned target sequences from transformed bacterial colonies. Each of the transformed cell lines within a library can be screened for homology with nucleic acid sequences, for expression of an antigen (antibody recognition). Selection using only a single approach is rarely proof that a clone has been identified correctly, because even the most stringent criterion for screening may select false positives. Therefore, a combination of selection schemes is often needed and further characterisation is usually essential for correction. Such analyses must rely on information that supplements that originally used for screening (Klimmel, 1987). The most convenient method of characterisation is often just a re-identification of some of the same properties used to select the clone originally.

The most commonly used method for sequencing is the enzymatic method (dideoxy chain termination sequencing) because it is rapid and a large number of samples can be processed (Sambrook *et al.*, 1989).

Sequence similarity searches are used to predict the possible function of an unknown sequence, to look for relationships to previously published sequences, or to identify potential phylogenetic relationships. Three main sequence similarity searching programs are FastA, BLAST and MPsrch. The FastA program is suitable for a sequence similarity search, BLAST for a basic local alignment search and MPsrch for database searching. Several databases are available, such as databases for nucleic acid- DDBJ (Mishima, Japan), EMBL (Cambridge, UK), and GenBank (MD, USA); databases for protein- GenPept, MIPS (Martinsried, Germany), PIR (Noda, Japan) and Swiss-Prot(Cambridge, UK) (Gotoh, 1982).

6.2 MATERIALS AND METHODS

6.2.1 Materials

All primers used in these experiments are listed in Appendix D. All dsRNAs used in the standard cloning method were extracted by the standard CF11 cellulose method (Section 2.2.4.c). The further purified ScSMD associated dsRNAs (Section 5.3.5) were used in cloning.

6.2.2 Methods

see corrigendum

6.2.2.a Synthesis of cDNA from ScSMD associated 9 kbp dsRNA with rPCR and cloning of the rPCR products

First strand cDNA was made with M-MLV-RT. The 20 µl sample of purified dsRNA (Section 5.3.5) and 0.5 µg of UN-RH primer was mixed, and denatured by boiling with 0.35 µl of 1M BME. cDNA synthesised and amplified according to Froussard (1992) as described in Section 2.2.6.a. The rPCR products were analysed on 1.2% agarose gels. The gel was stained with EtBr and photographed to record and the gel was transblotted to hybridise (Section 2.2.10) with probes made from ScSMD associated dsRNAs (Section 2.2.6.c).

For cloning, the rPCR products were digested with *Eco*RI. The digested *Eco*RI arms were removed by using a Sephacryl® 400 spin column (2.2.6.a). The *Eco*RI digested cDNAs were ligated with pGEM-7zf(+) (Section 2.2.7.a) and transformed into *E. coli* by the heat shock method (Sambrook *et al.*, 1989; Section 2.2.7.c). Plasmids were prepared from transformed bacteria as described in Section 2.2.8. The plasmids were further analysed for check the insert size as well as their specificity to ScSMD. ScSMD specific clones were selected as described in Section 2.2.9.a.

6.2.2.b Cross-hybridisation of clones

Three plasmid preparations containing ScSMD specific clones were selected according to their size from 50 plasmid preparations with ScSMD specific inserts. The plasmids were digested with *Eco*RI (Section 2.2.2.9.b), and analysed on preparative agarose gels. The inserts were recovered from the gels with a QIAGEN gel extraction kit (Section 2.2.9.b). The recovered inserts were ^{32}P-labelled by random priming method (Section 2.2.6.c). The labelled probes were used for hybridisation to check relationships among ScSMD specific clones (Section 6.2.2.a).

6.2.2.c Sequence analysis

Five ScSMD specific clones were selected and sequenced. The method for sequencing is described in Appendix F. The sequences were edited with SeqEd™ software (Applied Biosystems, Inc., USA). The search for possible motifs in the ScSMD sequences was carried out by Signal Scan version 4.0 (Prestrige, 1996) using the Australian National Genomic Information Service (ANGIS).

Comparisons with genome data bases were carried out with the Mpsrch™ (Oxford Molecular Ltd., UK) and the FastA in the DISC homology search programs in the World Wide Web (*http//www.dna.affrc.go.jp/htdocs/homology/homology.html/*; Gotoh, 1982; Lipman & Pearson, 1985).

Alignments between ScSMD segments and other viruses were done using the ANGIS with Clustal W (Version 1.4; Thomas *et al.*, 1994).

6.3 RESULTS

6.3.1 Amplification of dsRNA by rPCR

The rPCR method described by Froussard (1992) successfully amplified cDNA from 50-100 ng of the purified dsRNA. Optimum conditions for rPCR were found to be a UN-RH primer concentration of 2.5 μM and an annealing temperature of 60 °C. There was main species of cDNA that were about 350 and 600 bp, but there was no detectable amount of PCR products from controls (Fig. 6.1A).

Most of the cDNAs amplified by rPCR hybridised with the probes made from ScSMD associated 9 kbp dsRNA whereas all the controls were negative (Fig. 6.1B).

6.3.2 Cloning of rPCR products

cDNAs amplified with rPCR (Section 6.3.1) were cloned into the *Eco*RI site of pGEM-7zf(+), and about 50 recombinant colonies were identified. Inserted fragments ranged from 0.22 to about 1 kbp in size (Fig. 6.2A). The clones from the 9 kbp dsRNA and the 6 kbp dsRNA contained inserts ranging from 0.3 to 1.5 kbp in size, but both of the smaller dsRNA (2.5 and 2.6 kbp) were below 300 bp. 24 recombinant colonies were selected for further analysis.

All of the inserts in the selected colonies were shown to be homologous to the dsRNA by probing of the Southern blots with a dsRNA specific probe (Fig. 6.2B).

6.3.3 Selection of clones for sequencing

Table 6.1 shows characteristics of the clones. Three of the clones (DA2, DA10 and DA14) were selected because they were large and differed from each other. DA20 was selected because of it was the largest. DA4 was chosen because it was distinct from the others.

Figure 6.1 (A) Analysis by 1.2 % agarose gel electrophoresis of rPCR products from ScSMD associated 9 kbp dsRNA (DA), 6 kbp dsRNA (MA) and 2.5 and 2.6 kbp dsRNA (SA), and stained with EtBr. 1 kbp DNA ladder was used as a standard marker. (B) Southern blot hybridisation of rPCR products from the ScSMD associated dsRNAs (DA, MA and SA) and controls with a probe made from ScSMD associated 9 kbp dsRNA by random priming.

1 - DA as a template.

2 - MA as a template.

3 - S as a template.

Controls for PCR - 4 - Without templates.

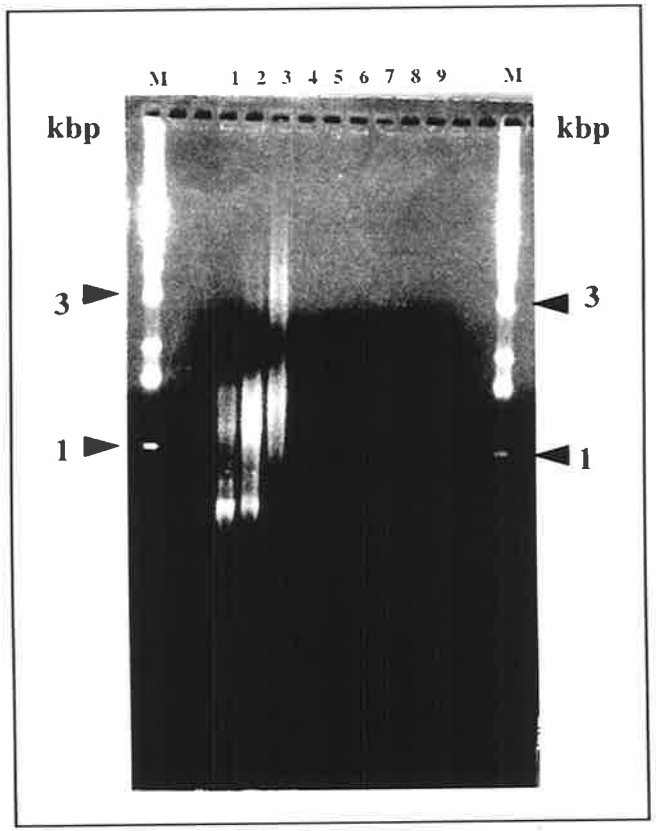
5 - Without primers.

6 - Without primers and templates

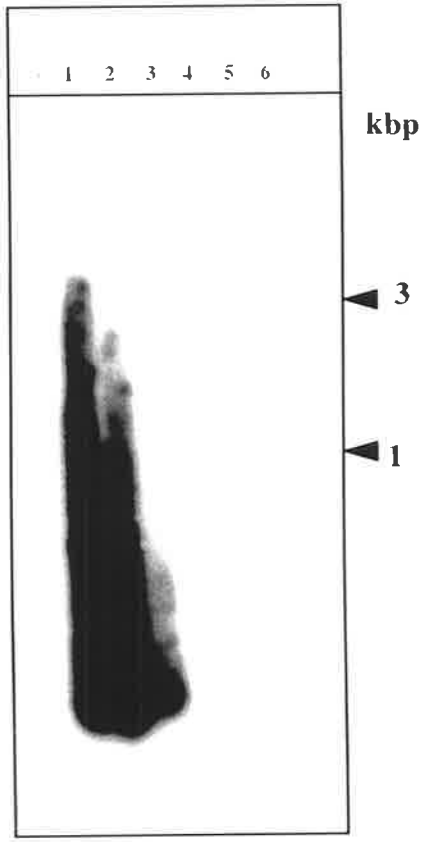
7 - TNA of healthy sugarcane extracted by the RNA extraction method was used as a template.

8 - TNA of ScSMD affected sugarcane extracted by the RNA extraction method was used as a template.

9 - TNA of *Nicotiana glutinosa* infected with AMV was used as a template.



(A)



(B)

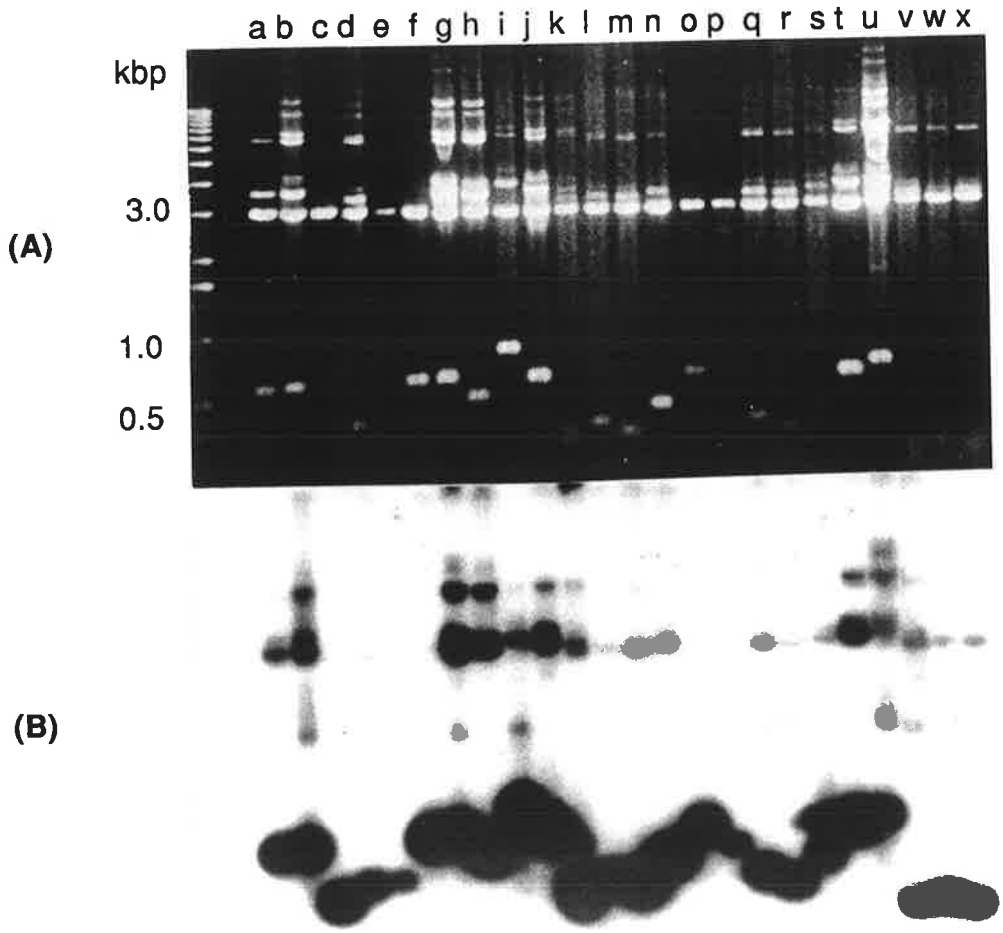
Figure 6.2 (A) - Plasmids were prepared from the ScSMD specific clones to check the size of the inserts. The prepared plasmids were digested with *Eco*RI and analysed by 1.2% agarose gel electrophoresis. The gel was stained with ethidium bromide

Lanes a to j - Inserts of cDNA prepared from the 9 kbp dsRNA of ScSMD.

Lanes k to u - Inserts of cDNA prepared from the 6 kbp dsRNA of ScSMD.

Lanes v to x - Inserts of cDNA prepared from the 2.5 and 2.6 kbp dsRNA of ScSMD.

(B) A southern blot of the gel (A) probed with cDNA prepared from ScSMD 9 kbp dsRNA. The size marker is in lane 1. Clones a, b, c, d and e were selected for sequencing.


a b c d e f g h i j k l m n o p q r s t u v w x
kbp
3.0
1.0
0.5
(A)
(B)

see corrigendum

Table 6.1 Cross hybridisation of ScSMD specific clones with randomly labelled with ^{32}P-dCTP of three clone fragments and ScSMD associated 9 kbp dsRNA.

CLONES		PROBES			
NAME	SIZE (bp)	DA2	DA7	DA14	dsRNA
DA2 (a)*	650	+++	-	-	+++
DA4 (b)	650	-	-	-	+++
DA7 (c)	300	+	+++	+	+++
DA8 (d)	370	+++	-	-	+++
DA9 (e)	420	-	-	-	+++
DA10 (f)	700	+	+++	+	+++
DA14 (g)	700	-	-	+++	+++
DA19 (h)	600	+	+++	+	+++
DA20 (i)	1,000	+++	+++	+	+++
DA22 (j)	700	-	-	++	+++
MA1 (k)	570	+++	-	+	+++
MA2 (l)	350	+	-	-	+++
MA3 (m)	300	+	+++	+	+++
MA5 (n)	440	++	+++	+	+++
MA6 (o)	700	+	+++	++	+++
MA8 (p)	500	++	-	-	+++
MA11 (q)	350	-	-	-	+++
MA12 (r)	300	++	-	-	+++
MA14 (s)	400	-	-	+	+++
MA16 (t)	600	+++	+++	++	+++
MA17 (u)	700	-	-	+++	+++
SA1 (v)	200	+	++	++	+++
SA4 (w)	230	+	++	++	+++
SA6 (x)	200	+	++	++	+++

*; Numbers in Figure 6.2

**; Strength of hybridisation on the autoradiograph, +++: strong, ++: medium, +: weak, -: not hybridised

6.3.4 Sequencing

The selected five clones, which represented inserts of 0.9 (DA20), 0.67 (DA14), 0.63 (DA10), 0.6 (DA2) and 0.63 (DA4) kbp in size, were sequenced. When the sequences were aligned, the three crosshybridising clones (DA2, DA10 and DA20) were found to have overlapping sequences representing a segment of 1.25 kbp. The other two clones showed no sequence homology with any of the other inserts, and were defined as unique segments of 0.63 (DA4) and 0.67 (DA14) kbp respectively (Fig. 6.3)

6.3.5 Comparison of segments with sequences in the genome data base

A comparison of the sequences with the genome data base identified nucleotide sequence similarities between the ScSMD segments and a number of rod-shaped viruses in the genera *Potex-*, *Capillo-*, *Tricho-*, *Carla-* and *Potyvirus*, as well as one virus in the icosahedral virus genus *Tymovirus* (Table 6.2). ScSMD segment 3 had significant sequence similarity to viruses in all 6 genera, segment 1 had significant similarity to viruses in the genera *Tricho-* and *Carlavirus*, whereas ScSMD segment 2 had significant similarity to only three viruses in the genus *Carlavirus*. The genome sense orientation of the segments was determined from this comparison. The strongest overall similarity was observed at both the nucleotide and amino acid sequence level with species within the genus *Carlavirus*, and of this the greatest similarity was found within the sequence of the putative replicase gene (ORF1) of apple stem pitting virus (ASPV; Table 6.2) (Jelkman, 1994).

Figure 6.4 shows the sites on the ASPV genome map that matched the ScSMD segments 1, 2 and 3. Figure 6.5 shows the best fit alignment of the three ScSMD segments within the ORF1 of ASPV. Marked variation in the degree of similarity between the sequences is evident. For example, fragment 3 showed the highest homology with ASPV, whereas segment 2 showed the lowest (Table 2). In segment 1, part was similar (shaded region) whereas both the 5' and 3' ends were dissimilar (Fig. 6.4). Moreover, the motifs for replicase binding (TATA box) and transcription initiation (CAAT box) noted in the ScSMD sequence did not match with those of the ASPV sequence.

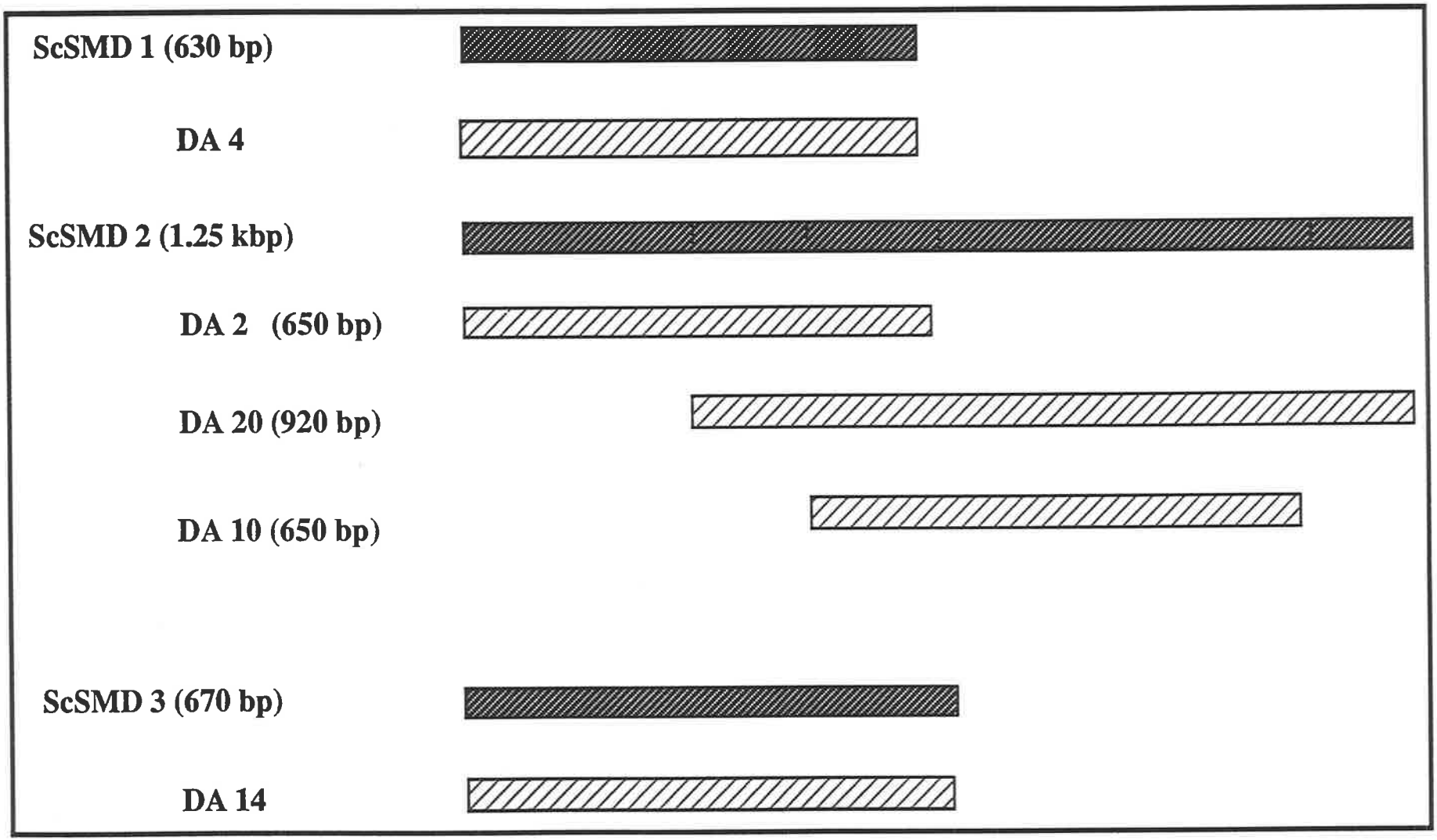


Figure 6.3 The sequenced ScSMD segments (ScSMD1, 2 and 3), and their respective clones (DA2, 4,10, 14 and 20) with their sizes.

Table 6.2. Percentage sequence similarity of ScSMD clones versus carlaviruses and other related viruses.

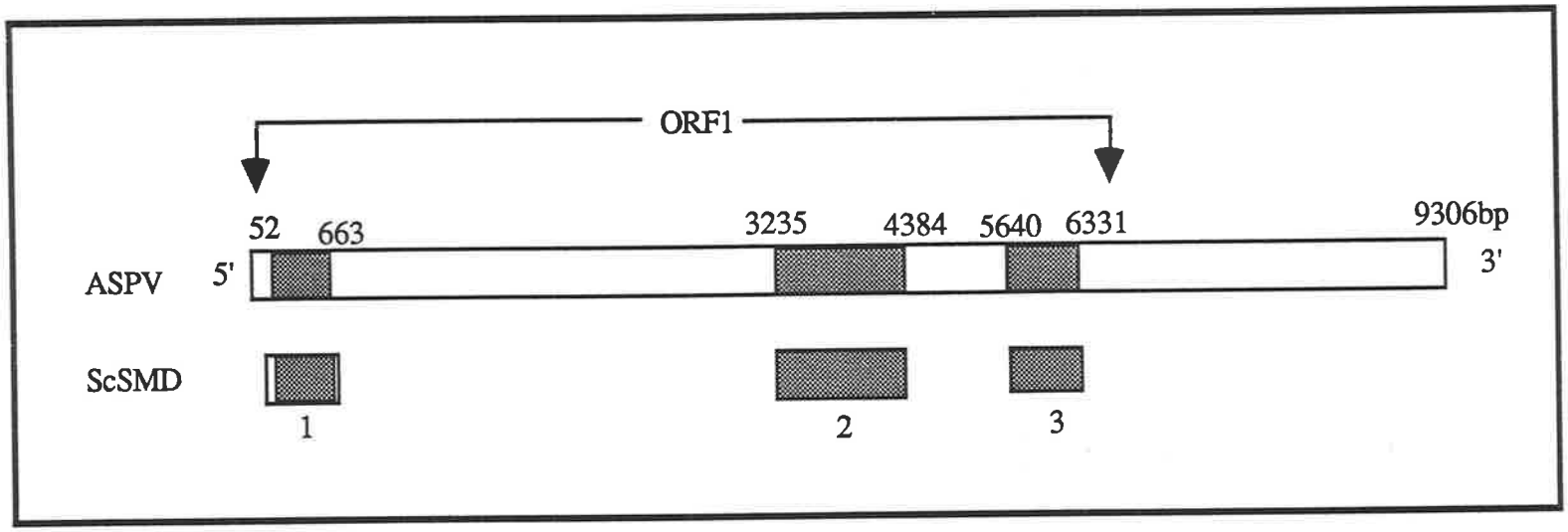
Virus	ScSMD clones					
Genus	1		2		3	
Species	nn*	aa**	nn	aa	nn	aa
Potex virus						
WClMV	-	5.8	-	-	53.7	9.4
BaMV	-	-	-	-	53.3	-
PapMV	-	-	-	-	52.0	-
Capillovirus						
ASGV	-	6.9	-	-	59.6	26.4
CCA	-	-	-	-	64.7	-
CTLV	-	-	-	-	58.8	-
Trichovirus						
ACLSV	56.5	9.3	-	-	60.1	37.5
GVB	58.9	9.9	-	-	56.3	26.6
PVT	-	-	-	-	61.8	22.7
Carlavirus						
ASPV	57.9	14.4	51.6	5.9	67.6	52.1
BlSV	-	13.5	-	6.5	67.7	52.4
GLV	-	-	-	-	65.8	-
GarMV	-	-	-	-	64.9	49.1
PVM	59.9	14.1	-	3.2	63.7	47.7
PVS	-	-	-	-	68.1	52.1
ShVX	-	-	-	-	63.0	10.7
Potyvirus						
PRSV	-	-	-	-	52.6	-
Tymovirus						
OYMV	-	-	-	-	52.6	-

* : nucleotide, ** : amino acid, and - : homology below 40 % for nucleotide and below 3.0 % for amino acid. Acronym and Genebank number of compared

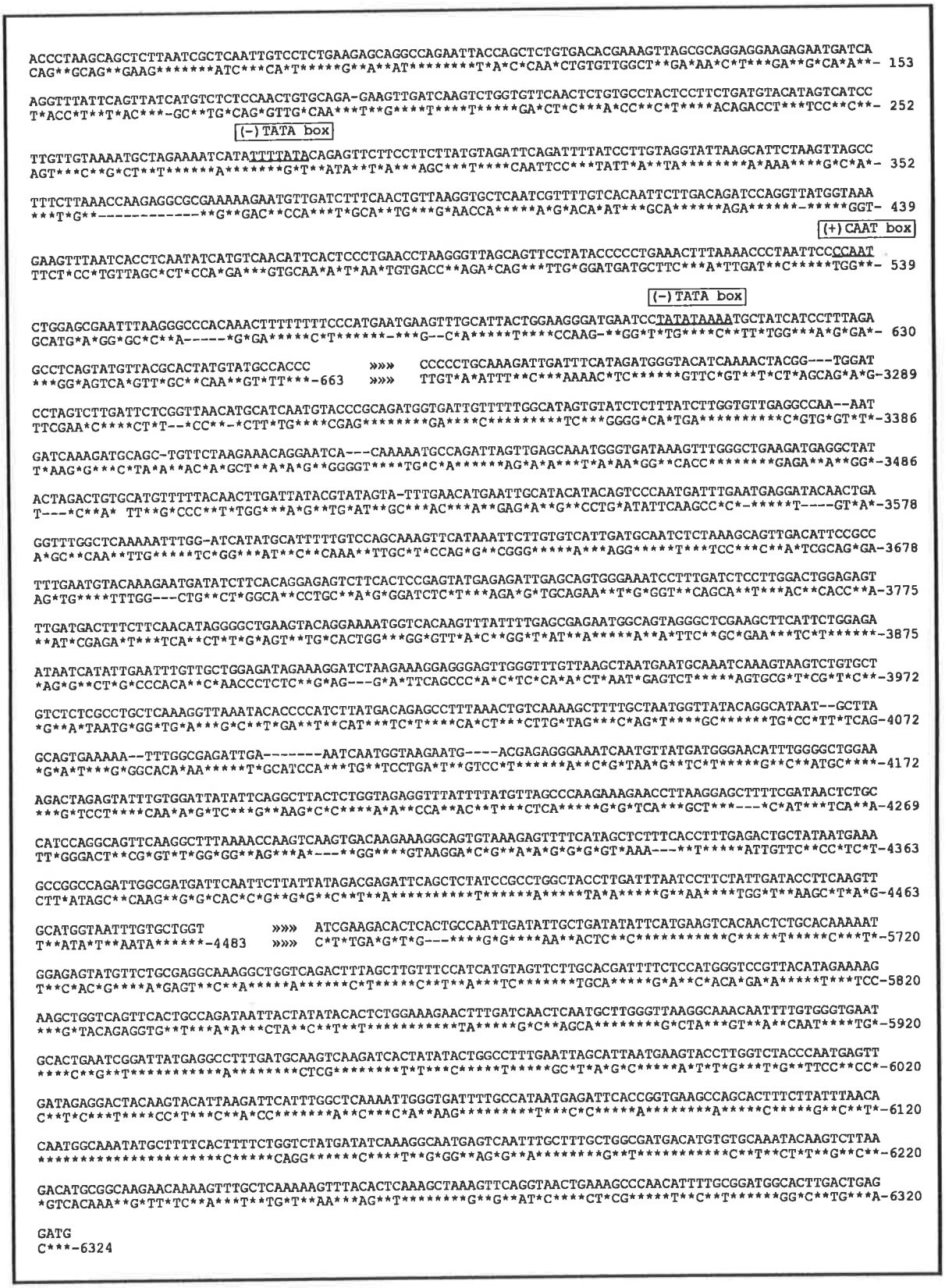
viruses ; ACLSV : apple chlorotic leaf spot virus (D14996), ASGV : apple stem grooving virus (D14995), ASPV : apple stem pitting virus (D21829), BaMV : bamboo mosaic virus (D26017), BSV : blueberry scotch virus (L25658), CCA : cherry capillovirus A (X82547), CTLV : citrus tatter leaf virus (D14455), GarMV : garlic mosaic virus (D11161), GLV : garlic latent virus (Z68502), GVB : grape vine virus B (X75448), OYMV : ononis yellow mosaic virus (04375), PapMV : papaya mosaic virus (D13957), PVM : potato virus M (D14449), PVS : potato virus S (D00461), PVT : potato virus T (D10172), ShVX : shallot virus X (M97264) and WClMV : white clover mosaic virus (D13957).

Figure 6.4 (A) - Diagram showing regions of homology between segments 1, 2 and 3 of ScSMD and the genome of ASPV (Jelkman, 1994). Regions with high homology are shaded and those with negligible homology are unshaded.

B - Alignment between sequences of ScSMD segments 1, 2 and 3 depicted in Fig 6.4 (top row), and ASPV (bottom row). Identical nucleotides are denoted by the symbol '*' and '-' is inserted to adjusted the spacing of the alignment. Discontinuities are indicated with arrows at ASPV nucleotides 663, 4483 and 6324.



(A)



(B)

These similarities strongly support the conclusion that the sequence obtained from ScSMD affected sugarcane is from an associated ScSMD virus (ScSMV), that this virus is likely to be rodshaped, and with currently available data that it is most closely related to some members of the genus *Carlavirus*.

6.4 DISCUSSION

It was found in the first attempt at cloning that pure dsRNA was required. Contaminants were efficiently removed by using both DNase and RNase treatment in high salt conditions before gel fractionation, as well as omitting all size markers from the gel fractionation procedures.

Heat denaturation of dsRNA prior to reverse transcription was used instead of methyl mercuric hydroxide because the latter gave inconsistent results. The highest yields of cDNA were obtained after heat denaturation in the presence of random hexanucleotide primers followed by slow cooling. rPCR successfully amplified cDNA from a small amount of pure dsRNA. The annealing step in the PCR was done at 60 ^{o}C instead of 55 ^{o}C (Froussard, 1992) to maximise specificity. PCR products were reduced in size when a large excess of the UN-RH primer was used. The amplified ScSMD associated cDNAs were cloned and selected clones were sequenced.

The nucleotide sequences obtained from the dsRNA represented 2.55 kb of the estimated 9 kb of the ScSMD specific RNA. A homology search showed that the three sequenced segments had some identity with several species within the genus *Carlavirus*. Overall, the greatest similarity was with the ASPV ORF1 that is the putative replicase region (Jelkman, 1994). Less but significant similarity was observed with other genera representing both rod-shaped and icosahedral viruses. The sequence similarity to one of the tymoviruses is probably due to the known similarity between the replicases of potexviruses and tymoviruses (Koenig *et al.*, 1995). Other motifs, such as the coat protein gene of the viruses in these genera, are not similar, so that nucleotide sequences homology between these ORFs would not be expected.

These results indicate that the agent of ScSMD is probably a rodshaped virus in the genus *Carlavirus*. Because the size of the ScSMD associated dsRNA is longer than for most

carlaviruses, except ASPV, blue berry scorch virus and potato virus M, the virus particle would be expected to be longer than the mean length discribed for this genus (Brunt, 1995).

CHAPTER 7

PURIFICATION AND CHARACTERISATION OF VIRUS PARTICLES ASSOCIATED WITH ScSMD SPECIFIC dsRNA

7.1 INTRODUCTION

To study structures and other basic properties of a virus, it is essential to be able to obtain a disease associated particle. Plant viruses vary over a 10,000 fold range in the amount present in their hosts (Matthews, 1991). They also differ in their stability to various physical conditions and chemical agents and enzymes that may be encountered during isolation. For these reasons, different procedures are available to purify viruses from their hosts.

During purification of viruses, it is often necessary to design an extraction medium that will preserve the virus particles in an intact and unaggregated state during isolation and storage, but which will disrupt or absorb host organelles and proteins. Important factors to consider in an extraction medium are pH, concentration of divalent cations, reducing agents and protection against phenolic compounds. Abilities to remove plant proteins and ribosomes, and to release virus agents from host components are also required (Francki, 1971).

When virus particles have been isolated, electron microscopy can be used to study their structure. This technique depends on differences in electron scattering of different parts of the specimen. Various techniques for preparation of specimens have been developed and applied to study the structure of viruses because virus particles themselves have little contrast. These include shadowing, positive staining and negative staining. Shadowing obscures surface details and positive staining can cause alteration or disintegration of the particles. Negative staining is the most widely used procedure (Hayat, 1989) because it requires only short treatment and shows good contrast on surfaces. Characteristics of commonly used negative stains are described in Section 1.4.2.b.

7.2 MATERIALS AND METHODS

7.2.1 Materials

ScSMD affected and healthy plant materials were used for the virus purification procedure. The healthy sugarcane plants were raised from seed (Table 2.1), and they were tested with ScSMD specific probes made by the random priming (Section 2.2.10) to ensure that they did not contain ScSMD associated dsRNA (Section 2.2.6.c).

see corrigendum

7.2.2 Methods

7.2.2.a Virus purification

An attempt was made to purify a virus from ScSMD affected sugarcane leaves by a modification of the method of Randles *et al.* (1989).

All steps in the procedure were carried out at 4 °C. One kg of healthy and ScSMD affected leaves were harvested and the midribs were removed. The leaves were chopped into small pieces (ca. 1 cm^2) and blended in a pre-chilled commercial blender (WARING, USA) with 5 volumes of extraction buffer (0.1 M phosphate buffer, pH 7.0, 10 mM EDTA and 1% MTG). The slurry was filtered through 2 layers of muslin cloth. Triton X-100 (5%) was added to clarify the extracts and they were incubated with stirring for 30 min. To precipitate possible virus particles, PEG 6000 was added to a final concentration of 8% (w/v) and incubated with stirring for 40 min. The solution was centrifuged at 10,000 *g* for 10 min. The supernatant was discarded and the pellet was resuspended overnight in 0.2 volume of 10 mM Tris-HCl (pH 7.4) in relation to the original volume of plant extract. Insoluble material was removed by centrifugation at 10,000 *g* for 10 min and the pellet was discarded. The supernatant was layered on top of a 20% sucrose cushion (6 ml) in a 60 Ti centrifuge tube (capacity: 38.5 ml, Beckman) and centrifuged at 45,000 rpm for 2 hr. The pellet was resuspended in 23 ml of 10 mM Tris-HCl, pH 7.2, for 6 hr to overnight. Insoluble material was removed by centrifugation, as above. 7.5 g of Cs_2SO_4 was added and the solution was centrifuged in an SW 65 Ti (4.4 ml, Beckman) rotor at 45,000 rpm for 18 hr. Bands were visualised by light scattering and collected with a syringe using an 18 gauge needle and the rest

of the Cs_2SO_4 gradient was fractionated by recovering 0.5 ml steps from the top. They were then dialysed against 10 mM Tris-HCl, pH 7.4, for 16 hr. The dialysate was centrifuged at 50,000 rpm for 2 hr in a TLA 100.3 rotor (Beckman) and the pellet resuspended in storage buffer [10 mM Tris-HCl, pH 7.4 and 0.02% (w/v) NaN_3]. This was used for electron microscopy or 1 volume of glycerol was added for storage.

A sample from every step of the virus purification procedure were dotted on a nylon membrane and hybridised to probes made from a ScSMD specific clone (DA4, Section 2.2.10) see corrigendum to monitor the purification procedure. The fractions that hybridised to the ScSMD specific probes were used for TEM (Section 2.2.2.d) and examined with a Phillips CM 100 electron microscope. The fractions were also pelleted by centrifugation and used for SDS-PAGE.

7.2.2.b Size measurement of detected virus-like particles

Virus-like particles were photographed and the electron micrographs were scanned into an image analyser (Adobe Photoshop™) with a photoscanner (Relysis™). The size of TMV particles used as an internal size marker, and virus-like particles were measured with a computer program (Kontron™).

7.2.2.c SDS-PAGE

A SDS-PAGE discontinuous polyacrylamide gel electrophoresis system (Laemmli, 1970) was used. The gel electrophoresis was carried out according to the procedure of Sambrook *et al.* (1989) and is described in Section 2.2.5.a.

Final pellets of the fraction that contained virus-like particles (Section 7.2.2.a) were dissolved in loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and loaded into wells in a gel with an adjacent prestained protein marker (BioRad). The gel was run at 5 V/cm until the tracking dye had reached the top of the resolving gel. The voltage was increased to 10V/cm and the gel was run until the tracking dye had reached the bottom of the resolving gel. The gel was stained with Coomassie brilliant blue or silver (Section 2.2.5.c).

7.2.2.d Nucleic acid fractionation

Nucleic acid was isolated (Section 2.2.13.a) from the fraction that contained virus-like particles. The samples were analysed by formaldehyde gel electrophoresis and hybridised. The procedure is described in Section 2.2.5.b.

7.2.2.e 3' terminus

Nucleic acid was isolated from ScSMD associated virus-like particles and used for making cDNA with a primer $\{d(T)_{15}\}$ to check for the presence of poly A at the 3' end. The procedure is described in Section 2.2.12.b.

7.3 RESULTS

7.3.1 Virus purification

The dot blot of the pellet sample of ScSMD affected plants from the sucrose cushion step bound the 9 kbp dsRNA specific probe. A sample from the band (1.27 g/cm^3) in the Cs_2SO_4 density gradient also hybridised to the probe. Samples from other fractions of the ScSMD affected sugarcane and the plant extracts of healthy sugarcane did not hybridise to the probe (Fig. 7.1).

In samples prepared from the samples that hybridised to the dsRNA specific probe, filament-shaped virus-like particles were observed (Fig. 7.2). Because the particles were associated with ScSMD and their nucleic acid was homologous with the disease-associated dsRNA, it was assumed that they were the viral agent of ScSMD. They were therefore named sugarcane striate mosaic virus (ScSMV) particles.

7.3.2 Size of ScSMV

Under the TEM, the TMV particles which were used as an internal standard had a mean length 300 nm and a mean diameter of 18 nm (Fig. 7.2B). The diameter of ScSMV was a uniform 15 nm but the lengths of ScSMV particles ranged from 400 to 3,400 nm (Fig. 7.2).

Figure 7.1 Dot blot hybridisation with a probe prepared from clone DA14. ScSMD associated dsRNA was dotted as a positive control.

Bottom row is from ScSMD affected sugarcane.

Top row is from healthy sugarcane.

1 - High speed pellet after 20% sucrose cushion centrifugation.

2 - High speed pellet from the band isolated after $CsSO_4$ gradient centrifugation.

3 - Pellet after PEG precipitation.

4 - Supernatant after PEG precipitation.

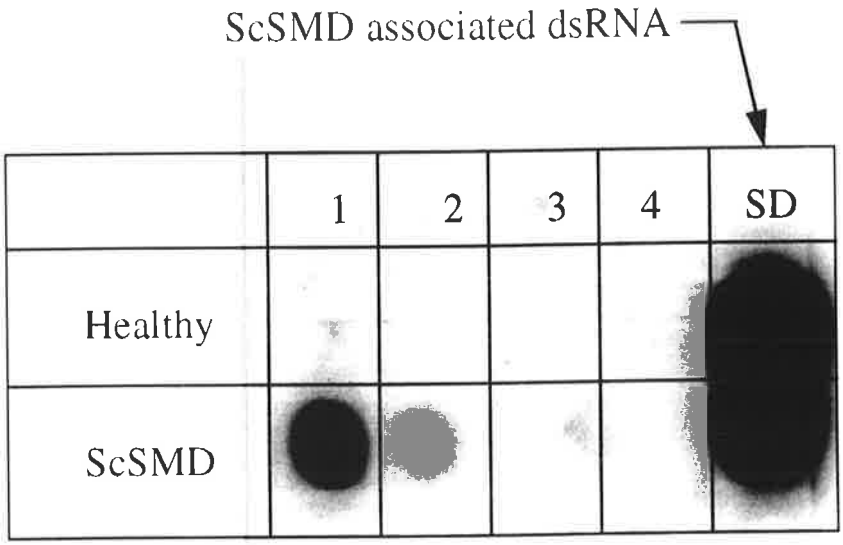

ScSMD associated dsRNA
1
2
3
4
SD
Healthy
ScSMD

Figure 7.2 Electron micrograph of isolated virus particles from ScSMD affected sugarcane leaf tissue. The bar represents 500 nm.

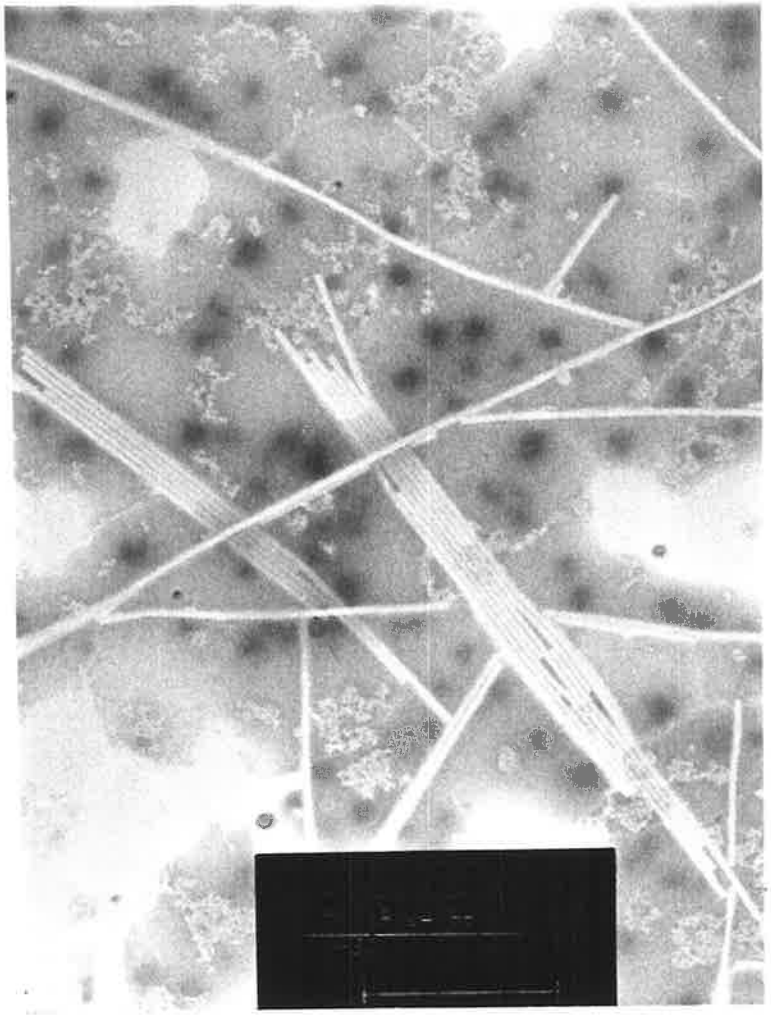
(A) ScSMD associated virus particles from the high speed pellet of the band of the Cs_2SO_4 gradient centrifugation.

(B) The sample (A) mixed with purified TMV.

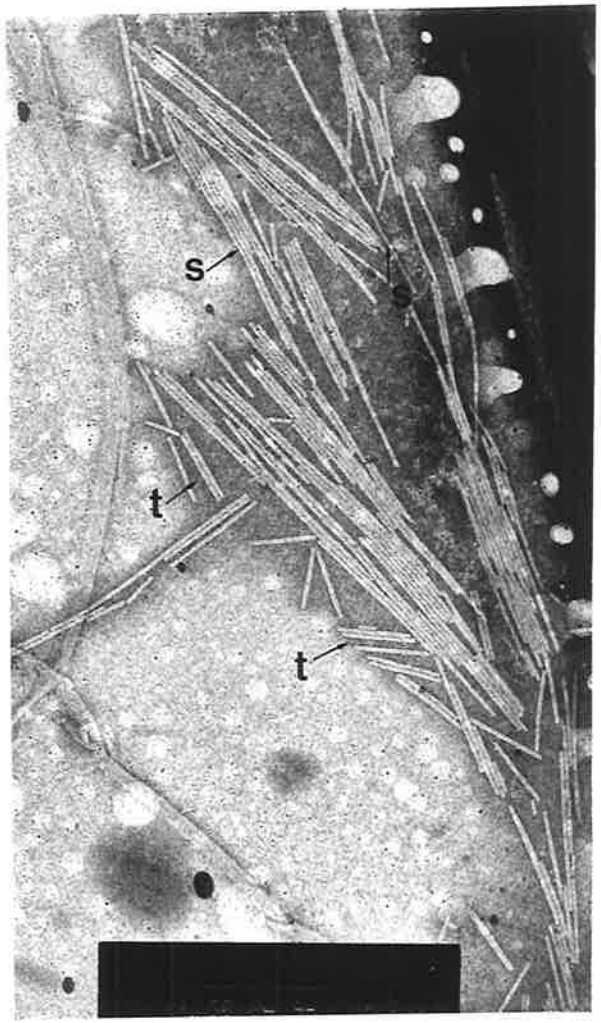
T - Tobacco mosaic virus.

S - Sugarcane striate mosaic virus.

see corrigendum



(A)



(B)

The length distribution was bimodal and the main two components were 1,000± 100 nm and 2,000± 100 nm in length (Fig. 7.3).

7.3.3 Size of the capsid protein of ScSMV

On the SDS-PAGE, a major protein band was observed which is assumed to be the capsid protein of ScSMV. The size of this capsid protein was ca. 51 kDa. No proteins were detected in the comparable fraction of healthy sugarcane (Fig. 7.4).

7.3.4 Nucleic acid of ScSMV

The nucleic acid isolated from ScSMV was a ssRNA because it was not digested with DNase, but was digested by RNase in a buffer containing 300 mM NaCl. The size of the RNA was ca. 9 kb. The method using proteinase K treatment without phenol extraction showed some degradation but more degradation of the RNA occurred after a phenol extraction step (Fig. 7.5).

7.3.5 3' terminus of ScSMD RNA

When $d(T)_{15}$ primers were used for cDNA synthesis, extension of the primers occurred and the size of the products was in range of 0.3-3 kbp (Fig. 7.6). ScSMV thus assumed to have poly A at the 3' terminus.

7.3.6 An isometric virus particle

Icosahedral virus-like particles (30 nm in diameter) were observed from the $CsSO_4$ gradient fraction (ca. 1.22 g/cm^3) of one of the ScSMD affected sugarcane plants (Fig. 7.7). The nucleic acid of the particles did not hybridise to the probes made from a ScSMV specific clone DA14.

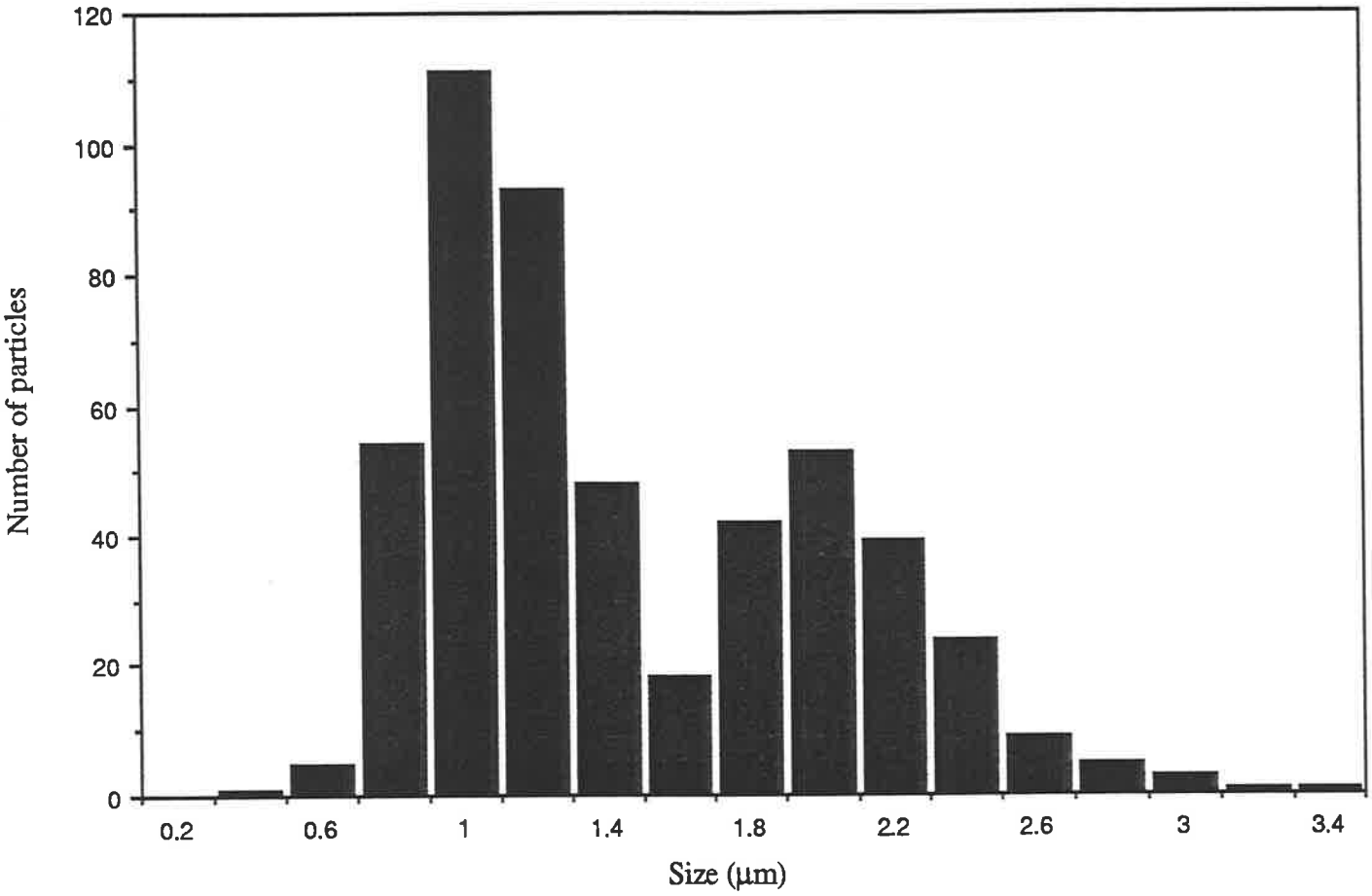


Figure 7.3 Particle length distribution of ScSMV particles.

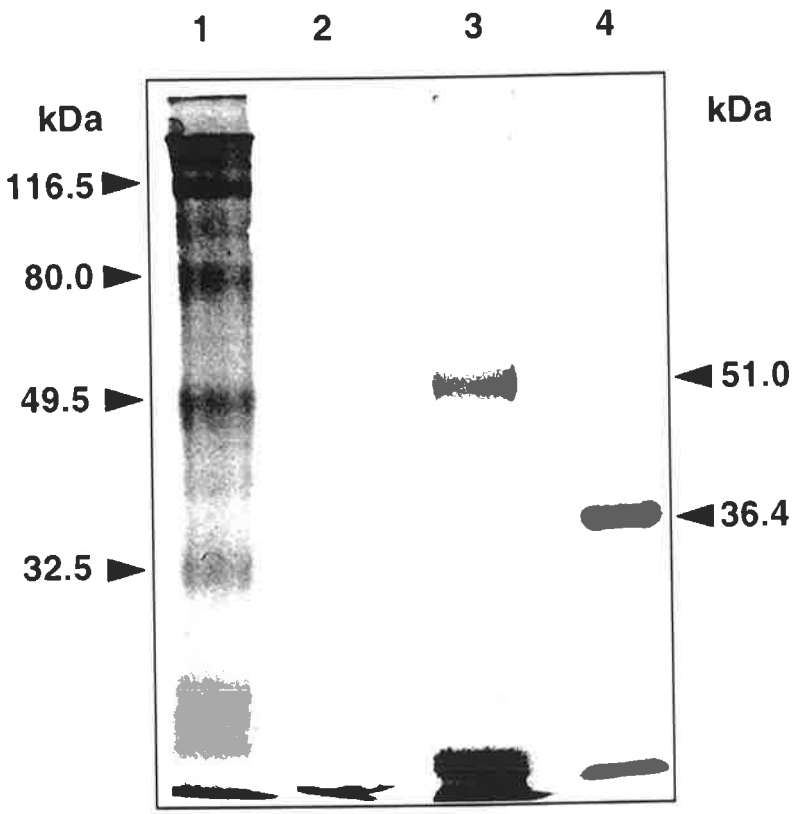
Figure 7.4 Samples prepared from the high speed pellets of ScSMD affected and healthy plants from the Cs_2SO_4 gradient fraction and analysed by discontinuous SDS-PAGE (Laemmli, 1970). The gel was stained with silver.

Lane 1 - standard protein size marker.

2 - High speed pellet of healthy sugarcane from the same Cs_2SO_4 gradient fraction as the diseased plant (Lane 3).

3 - High speed pellet from the Cs_2SO_4 gradient fraction in which ScSMV particles were observed.

4 - Purified PSbMV.



17K

Figure 7.5 Northern blot hybridisation of RNA extracted from ScSMV fractionated by Cs_2SO_4 gradient centrifugation and probed with a ScSMD specific probe prepared from clone DA14. A RNA size marker (Promega) was used to calculate the size.

Lane 1 - Nucleic acid of ScSMV digested with RNase A (100 ng/µl) in 50 mM Tris-HCl, pH 7.0 and 0.3 M NaCl at 37 °C for 30 min.

2 - RNA prepared by proteinase K treatment in 50 mM Tris-HCl, pH 7.4 and 1% SDS and precipitation with E-OH.

3 - RNA prepared by proteinase K treatment as in lane 2 followed by phenol-SDS extraction and precipitation with E-OH.

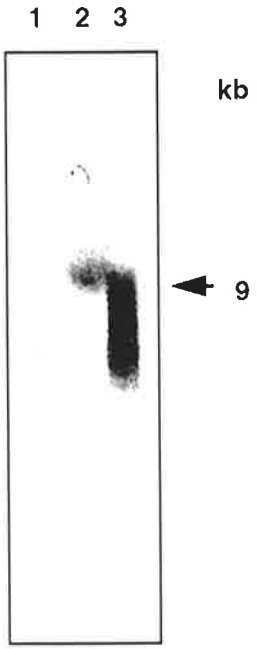

1 2 3
kb
9

Figure 7.6 ^{32}P-labelled cDNA products primed with oligo d(T)$_{15}$ and analysed by 1.2% agarose gel electrophoresis and followed by blotting. The blotted membrane was autoradiographed.

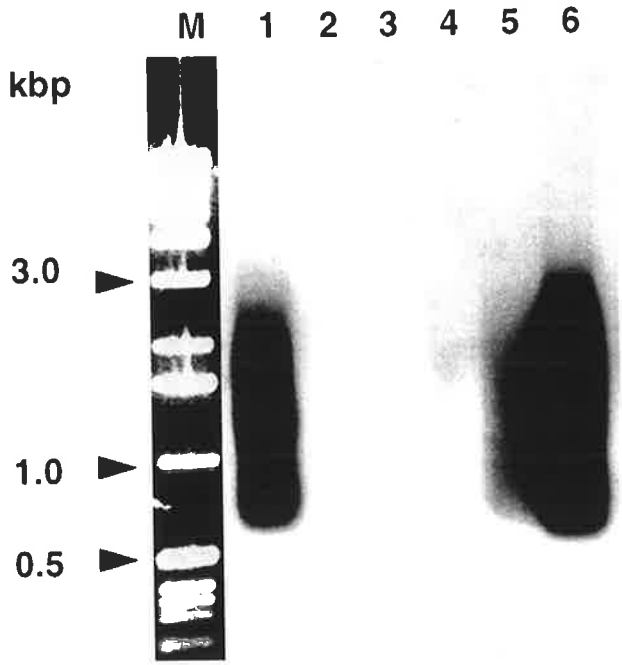
Lane 1 - 1 kbp DNA ladder as marker.

2 - ssRNA of PSbMV used as a template with a poly A 3' terminus.

3 - Nucleic acid extracted from the pellet of healthy sugarcane after the sucrose cushion centrifugation.

4 to 6 - ScSMV RNA.

see corrigendum


M
1
2
3
4
5
6
kbp
3.0
1.0
0.5

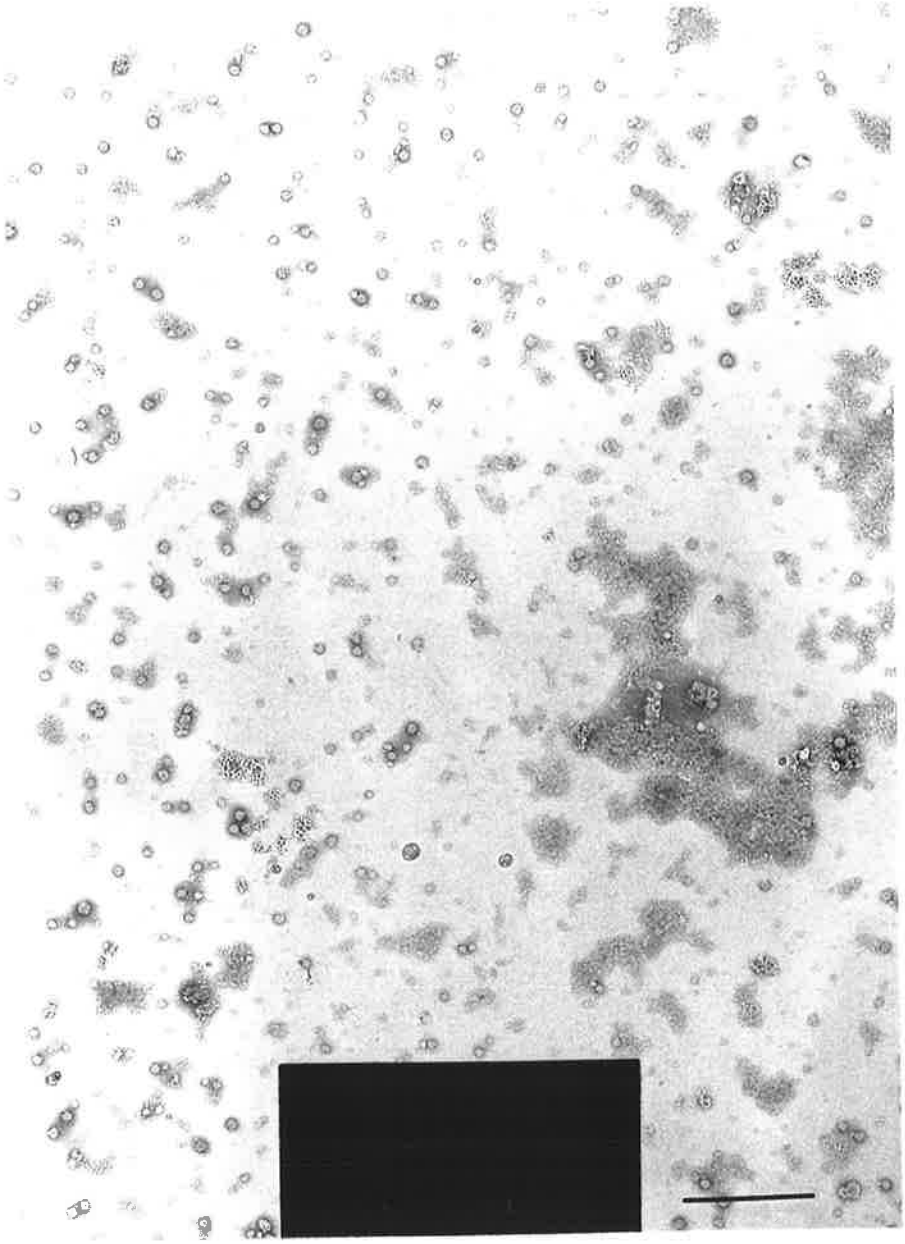


Figure 7.7 Electron micrograph of spherical virus-like particles (30 nm in diameter) isolated from leaf tissue of one ScSMD affected sugarcane leaf tissue. The particles were negatively stained with phosphotungstic acid. The bar represents 500 nm.

7.4 DISCUSSION

During the purification of ScSMD related virus particles, it is important to note that the pellets had to be resuspended with stirring overnight before undissolved materials were removed by low speed centrifugation. This would probably be due to the isolated virus particles being rod-shaped and aggregating tightly during high speed centrifugation.

The size of the virus particles was 400-3,400 nm in length and 15 nm in diameter. The ScSMV particles showed a bimodal distribution. The ratio between the size of its RNA and the particle length was in the range between 10-13 (nucleotide/nm) in filamentous viruses, for example 11 for CLV, 11 for ASPV, 12 for BlSV and 13 for PVM (Table 7.1). The unit size of the 9 kb ScSMV would therefore be the 900-1,100 nm particle class and the 1,900-2,100 nm would be the dimer.

The 900-1100 nm length class and the putative capsid protein (51 kDa) was larger than for most of the carlaviruses and the potyviruses (Table 7.1). However, the structure of filament-shaped ScSMV particles with little flexibility is similar to the carlaviruses. The genome size of ScSMV, 9 kb, is similar to that of the proposed carlaviruses ASPV and BlSV which are longer viruses within the *Carlavirus* genus (Table 7.1). The sequences of these viruses also had highest sequence similarity with ScSMV segments.

From these results, the ScSMD associated ScSMV particles may be a virus in the genus *Carlavirus*. The ScSMV particles resemble thoise of members of the larger group of the carlaviruses.

Table 7.1 Characteristics of ScSMV compared with some members of the *Carlavirus* and *Potyvirus* genera.

Genera	Species	Particle structure	Modal length (nm)	RNA size (kb)	Coat protein size (kDa)
	ScSMV	Slightly flexuous filament	900-1,000	9.0	51
Carlavirus	Carnation latent virus	Slightly flexuous filament	610-700	7.4-7.7	34
	Potato virus M	"	650	8.5	34
	Blueberry scorch virus*	"	675-720	8.9	37
	Apple stem pitting virus**	Very flexuous filament	800	9.3	48
Capillovirus	Apple stem grooving virus	"	640	6.5	27
Trichovirus	Apple chlorotic leaf spot virus	"	640-800	6.3-7.6	22-27
Potexvirus	Potato virus T	"	470-580	6.3	18-27
Potyvirus	Potato virus Y	Flexuous filament	650-900	8.5-10	37.4
Closterovirus	Beet yellows virus	Very flexuous filament	1250	15.5	23-28

*; Martin and Bristow, 1988.

**; Koganezawa and Yanase, 1990.

CHAPTER 8

DIAGNOSIS OF ScSMD BY HYBRIDISATION AND RT-PCR

8.1 INTRODUCTION

There are two main types of diagnostic methods for plant viruses, biological and non-biological. Biological methods are an essential part of diagnosis, but they are expensive in time, labour and facilities. Improved methods are required to recognise plant diseases or pathogens. Non-biological diagnostic methods developed recently depend on the recognition of components specific to the group of viruses. Electron microscopy, immunology, component isolation, nucleic acid hybridisation and PCR amplification are applications of component analysis (Randles *et al.*, 1996). Nucleic acid hybridisation and PCR in particular they have made a major impact on the diagnosis of viruses (Hadidi *et al.*, 1995).

The basis of all molecular hybridisation is the interaction by base pairing between the bases, adenine and thymidine/uridine, and cytosine and guanine. The base paired nucleic acids can be separated by various physical and chemical procedures. The separated strands then can be reinstated into the double-stranded nucleic acid (termed hybridisation) enabling the various factors controlling the stability of the duplex to be examined. Many factors are involved in this process (Hull, 1993).

The simplest format is dot blot hybridisation, in which the target sample is spotted onto the membrane. Results of dot blot hybridisation do not give any information on the size or number of species of the target nucleic acid. Such information can be gained by electrophoresing the nucleic acid in a gel and then transferring on to a membrane by capillary blotting (Southern or Northern blot) (Hull, 1993).

Dot or capillary blot hybridisation can be used to identify specific nucleic acid sequences in preparations ranging from crude extracts to the purified RNA or DNA of interest. For quantitation of viral DNA, it appears that no additional sample preparation is

necessary, but for viral RNA various interfering compounds need to be removed by phenol extraction before testing (Maule *et al.*, 1983).

PCR assay provides a good alternative to other diagnostic methods in plant viruses and can speed diagnosis because of its great sensitivity. 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). Before PCR can be used for detection of RNA viruses, viral RNA is first reverse transcribed to cDNA (RT-PCR). This technique has been used successfully to detect very small amounts of viral nucleic acids, and is more sensitive than molecular hybridisation or ELISA for the diagnosis of many plant viruses (Hadidi *et al.*, 1993).

8.3 MATERIALS AND METHODS

8.3.1 Materials

The sugarcane plants used in these experiments are listed in Tables 2.1 and 2.2. All chemicals used in these experiments are described in Appendices A, B and C. Primers used for the RT-PCR assay to detect ScSMD associated sequences are listed in Appendix D.

8.3.2 Methods

8.3.2.a Dot blotting

Nucleic acid samples extracted by the crush method (Section 2.2.3.a) were dissolved in TE (pH 8.0). Dot blotting of the samples was carried out according to the method of Sambrook *et al.* (1989) as described in Section 2.2.10.a.

8.3.2.b Capillary blotting

TNA from sugarcane tissues was extracted by the crush method (Section 2.2.3.a) and analysed by formaldehyde agarose gel electrophoresis (Section 2.2.5.b).

Capillary blotting of the gel was carried out according to the method of Sambrook *et al.* (1987) with some modifications. The procedure is described in Section 2.2.10.b.

8.2.3.c Hybridisation assay

see corrigendum

ScSMD clones (DA2, 4, and 10) were transcribed by DNA polymerase I with random primers and labelled with ^{32}P (Section 2.2.6.c). The transcribed probes were heated at 80 °C for 5 min in 50% formamide and added to the prehybridisation solution at about 1.5-3x 10^6 cpm/ml, and used for the hybridisation assay.

Dot or capillary blotted membranes (Zeta probe®) were hybridised according to the method of Sambrook *et al.* (1989) with some modifications and the procedure is described in Section 2.2.10.c.

8.2.3.d Design of primers for RT-PCR

The sequence of clone DA20 was used for designing the primers for ScSMD detection because of its size (920 nucleotides) and least homology to other viruses (Table 6.2).

The sequence of DA20 was analysed in the Oligo™ program. Several forward and reverse primers were designed in the size range of 20-25 nucleotides. They were checked for possible hairpin structures and stability and the most stable with least self priming were selected for further analysis.

The selected primers were checked by the Amplify™ program. The known sequences of DA20, ASPV, BlSV and PVM were used as templates in the computer analysis. Three primers (1 forward and 2 reverse) which had the highest primability and stability were selected for the RT-PCR. They were SD900 and SD500 for reverse, and SD100 for forward. The expected sizes of RT-PCR products in the computer analysis were 818 or 458 bp (Fig. 8.1).

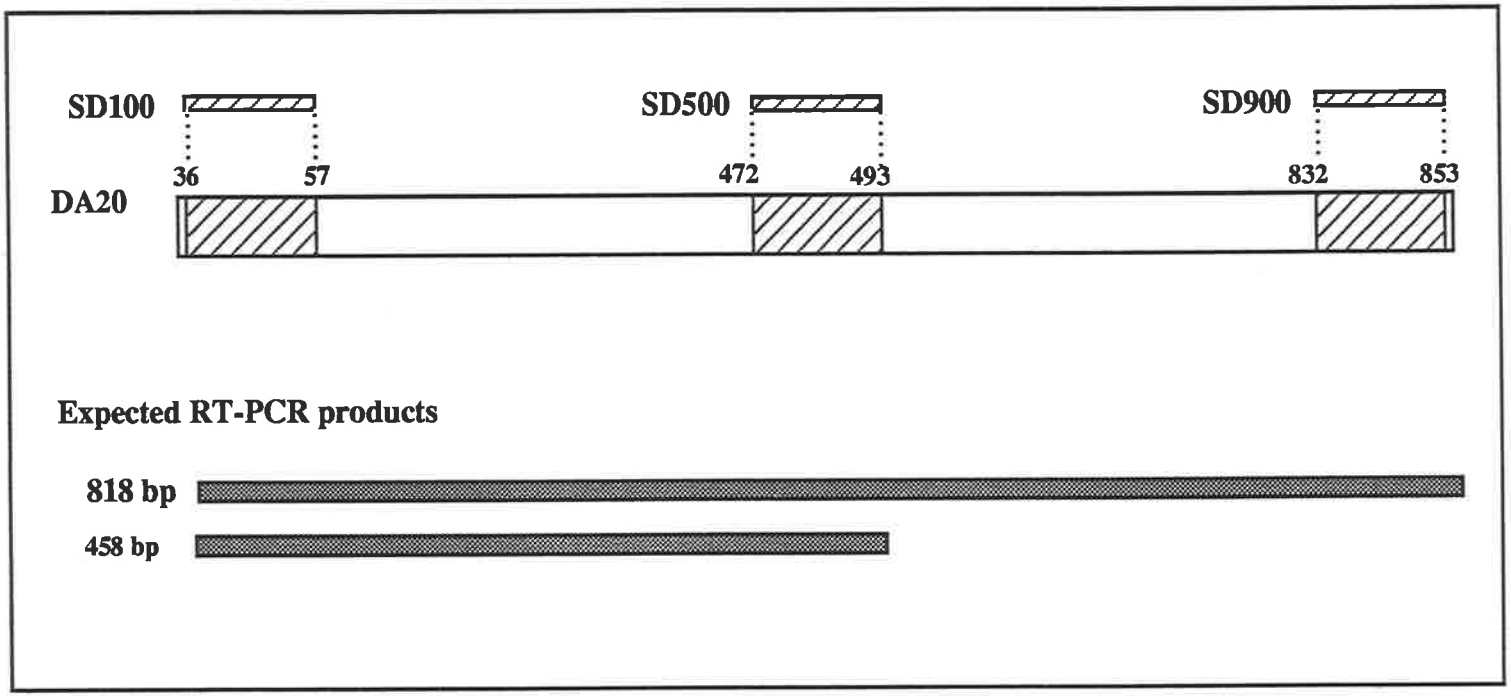


Figure 8.1 Sites of the primers which were designed and selected for RT-PCR detection of ScSMD in clone DA20. The expected RT-PCT product is 818 bp using primers SD100 and SD900, and 458 bp using SD100 and SD500.

8.2.3.e RT-PCR assay

The RT-PCR assay was carried out using primers SD100, SD500 and SD900 according to the method described in Section 2.2.12.a. .

Several different RT-PCR programs were carried out to find the optimum conditions for amplification of ScSMV. The products were analysed on a 1.2% agarose gel. The gels were then transblotted and hybridised with ScSMD specific probes made from clone DA10 (Section 2.2.6.c) because it didn't contain sequences of the primers SD100 and SD900 (see Fig. 6.3).

8.3 RESULTS

8.3.1 Hybridisation with ScSMD diagnostic probes

All probes transcribed from the clones (DA2, DA4 and DA14) hybridised to the ScSMD associated dsRNAs and TNA. The probes made from DA14 were chosen because they hybridised most strongly to total nucleic acids of the ScSMD-affected sugarcane leaves. The probe also hybridised weakly with dot blots of one of the field collected healthy plants but not to dot blots from healthy sugarcane plants grown from seeds (Fig. 8.2).

Results from the Northern blots of the TNA showed that there was a ca. 9 kb band in the sample of the ScSMD affected leaf, but not in samples of healthy sugarcane plants raised from seeds (Fig. 8.3).

8.3.2 RT-PCR assay for ScSMD diagnosis

The optimum conditions for the PCR with the primer set SD900 and SD100 were initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min , with a final extension at 72 °C for 7 min. The annealing temperature could be varied over the range of 45 to 57 °C. There was 800 bp of PCR products using DA20 (lane 1 in Fig. 8.4) but not from PCR controls (lanes from 2 to 7 in Fig. 8.4).

The optimum conditions for the primer set SD500 and SD100 was initial denaturation at 94 °C for 1 min, 30 cycles of 94 °C for 1 min, 50 °C for 40 sec and 72 °C for

Figure 8.2 (A) Results of dot blot hybridisation assay with a ScSMD specific probe prepared from clone DA14.

(B) Sample code and lay out:

DA14 plasmid used dotted as a positive control.

Q96 and Q117 - Sugarcane hybrids.

T - Total nucleic acid.

D - microgranular cellulose prepared dsRNA.

d - ScSMD affected sugarcane plants.

SH - Healthy sugarcane raised from seed in a glass house.

FH - Healthy sugarcane collected from the field.

G - Glasshouse grown sugarcane.

BA - ScSMD symptomatic sugarcane collected from Ballao.

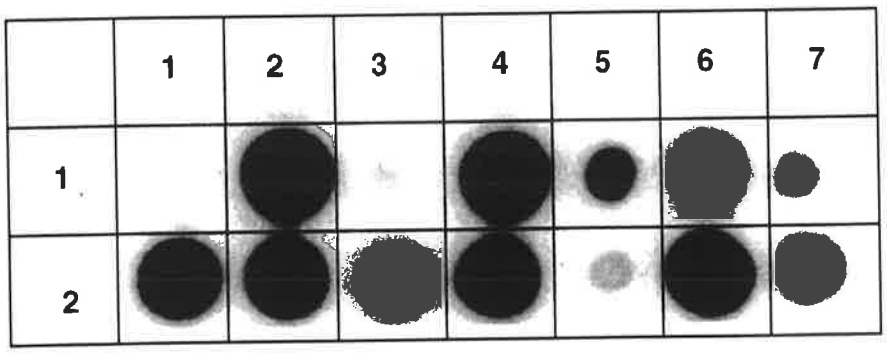
MA - ScSMD symptomatic sugarcane collected from Marshall.

MI - ScSMD symptomatic sugarcane collected from Michelin.

FA - ScSMD symptomatic sugarcane collected from Fava.

PE - ScSMD symptomatic sugarcane collected from Pellizari

(A)



(B)

	1	2	3	4	5	6	7
1	SH-T	dD-G	FH-T	dD-G	dT-G	dD-BA	dT-BA
2	dD-MA	dD-MI	dD-FA	dD-PE	dT-PE	DA14	DA14

Figure 8.3 Northern blot analysis of TNA from ScSMD affected and healthy sugarcane plants hybridised with ScSMD specific probes transcribed from the clone DA14.

1 - TNA of field collected ScSMD affected sugarcane from Ballao.

2 - TNA of the glasshouse grown ScSMD affected sugarcane.

3 - TNA of the glasshouse grown healthy sugarcane raised from seeds.


1
2
3
kb
9.0

1 min, and final extension at 72 °C for 7 min (lane 8 in Fig. 8.4). There was 500 bp of PCR products using DA20 (lane 8 in Fig. 8.4) but not from PCR controls (lanes from 9 to 14 in Fig. 8.4).

Using TNA from the diseased plants only one sample of RT-PCR products using the primers SD100 and SD900 from TNA showed a virus specific band by staining of the agarose gel (Fig. 8.5A).

To find out whether there were any PCR products which could not be detected with EtBr staining, the gel was transblotted and hybridised with the probe transcribed from DA10. Several samples of the diseased plants (Fig. 8.5B) had a small amount of RT-PCR products. There was also a small amount of the products in one of the field collected healthy plants which weakly hybridised to the ScSMD specific probe (Fig. 8.2; lane 13 in Fig. 8.5B), but not from healthy plants raised from seed.

When 1 µl of the first RT-PCR reaction of samples was used as a template for a second PCR reaction, a 800 bp product could be seen on an EtBr stained agarose gel from TNA of all ScSMD affected but not from healthy sugarcanes (Fig. 8.6A) . All products of the 2nd PCR reaction hybridised to the DA10 probes (Fig. 8.6B).

When the primer set SD100 and SD500 was used for RT-PCR, there was no detectable amount of PCR products by staining with EtBr as well as by hybridisation with the ScSMV specific probes.

8.4 DISCUSSION

An essential tool for the study of a virus disease is to find a specific and rapid diagnostic method. This chapter describes dot and Northern blot hybridisation, and RT-PCR to detect ScSMD.

Probes which were prepared from clones and used in the dot hybridisation method were specific for detecting ScSMV sequences in the TNA of ScSMD-affected sugarcane. One field collected sugarcane without ScSMD symptoms showed weak hybridisation with the ScSMD specific probe (DA14). This suggests that this assay may be suitable for detecting latent or pre-symptomatic infection. Northern blot hybridisation of

Figure 8.4 Analysis of RT-PCR products by 1.2% agarose gel electrophoresis. The gel was stained with EtBr.

Lane 1 - Clone DA20 was used as a template with primers SD900 and SD100.

2 - As in lane 1 except that TNA of *E. coli* was used as a template.

3 - As in lane 1 except that TNA of healthy sugarcane was used.

4 - As in lane 1 except that plasmid pGEM-7zf(+) was used as a template.

5 - As in lane 1 but without template.

6 - As in lane 1 but without primer.

7 - Without templates and primers.

8 to 14 As in lane 1-7 except that the primers SD500 and SD100 were used.

M - 1 kbp DNA ladder as a standard molecular weight marker.

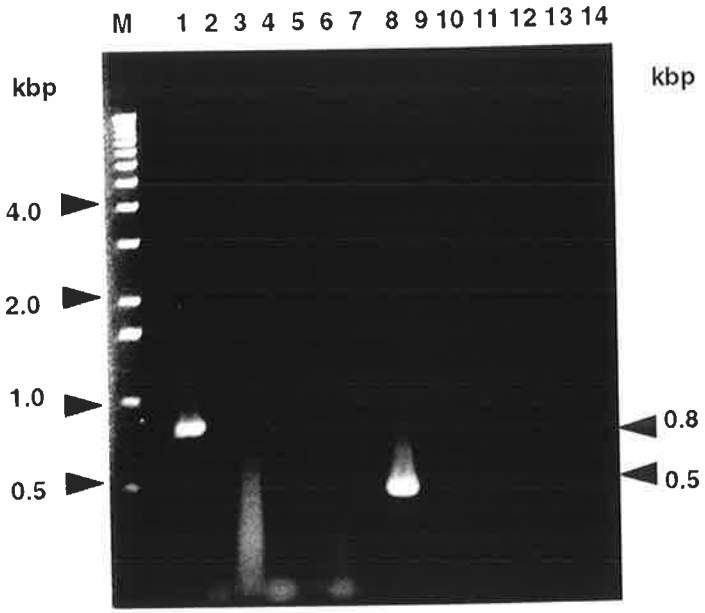

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
kbp
4.0
2.0
1.0
0.5
kbp
0.8
0.5

Figure 8.5 (A) Analysis of RT-PCR products using primers SD900 and SD100. The products were analysed by 1.2 % agarose gel electrophoresis and the gel was stained with EtBr.

Lane 1 - Clone DA20 used as a template for positive control.

Lane 2 & 15 - RT-PCR of TNA of healthy sugarcane raised from seed.

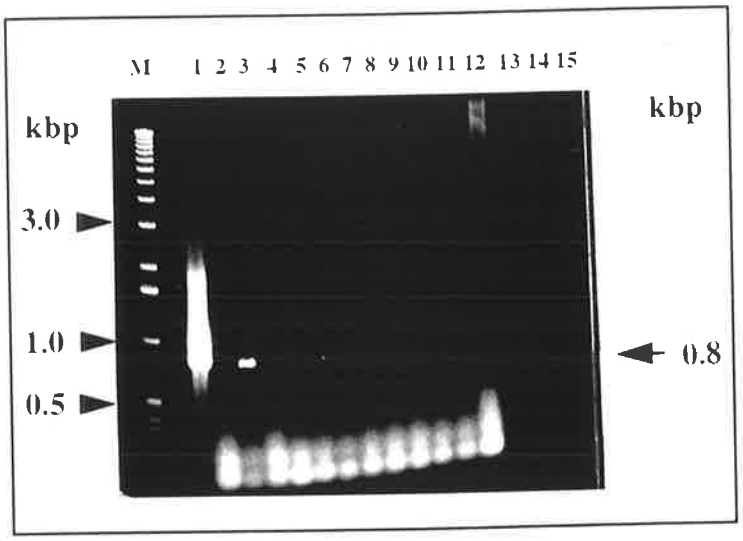
Lane 3 to 13 - RT-PCR of TNA of ScSMD symptomatic sugarcane plants.

Lane 14 - RT-PCR of TNA of field collected healthy sugarcane plant.

Lane M - 1 kbp DNA ladder used as a molecular weight marker.

(B) A southern blot hybridisation of the gel in A with a probe prepared from clone DA17.

(A)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kbp
3.0
1.0
0.5
kbp
0.8

(B)

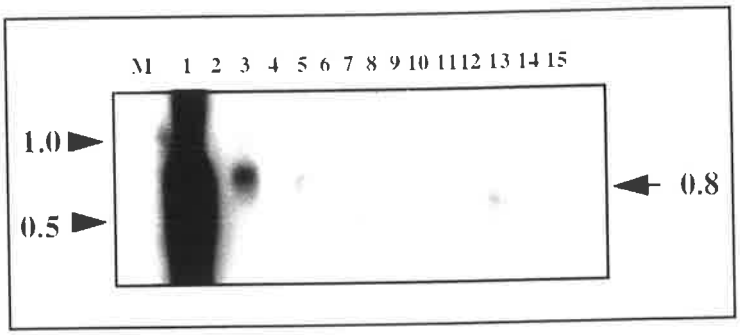

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1.0
0.5
0.8

Figure 8.6 (A) The products of a second PCR using 1 µl of the first RT-PCR reaction products as a template (see Fig. 8.5). Analysis in 1.2 % agarose gel electrophoresis with EtBr staining.

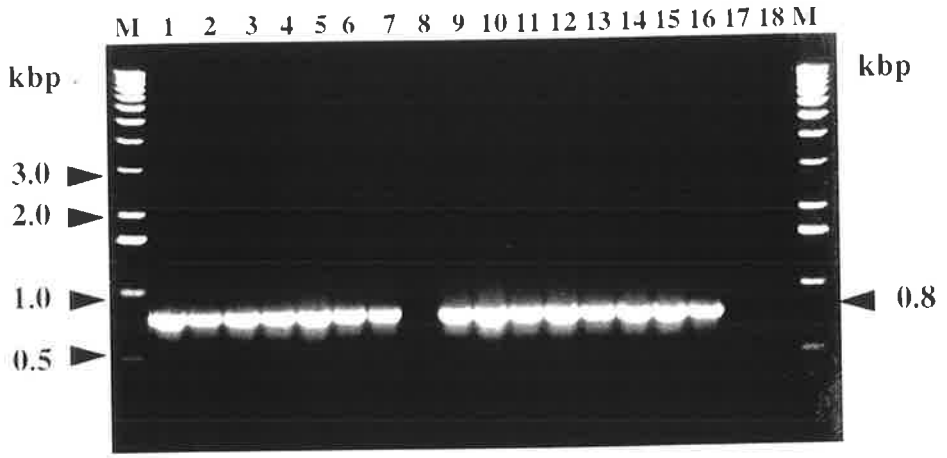
lanes 1 to 7 & 9 to 16 - PCR of RT-PCR reaction product from ScSMD affected sugarcane plants

Lanes 8 & 18 - PCR of RT-PCR reaction products from healthy sugarcane plants raised from seeds.

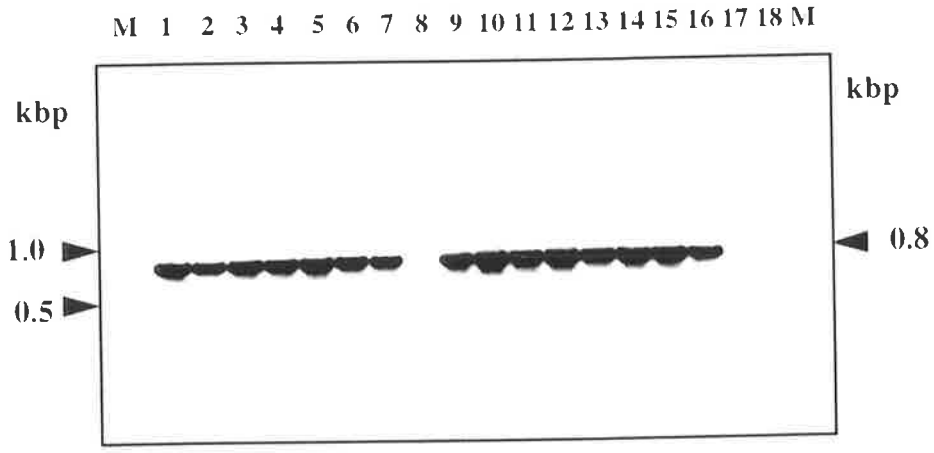
Lane 17- PCR of RT-PCR reaction product from field collected healthy sugarcane.

Lane M - 1 kbp DNA ladder used as a standard molecular weight marker.

(B) A southern blot of the gel in A hybridised with a probe prepared from clone DA17.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
kbp
3.0
2.0
1.0
0.5
kbp
0.8

(A)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
kbp
1.0
0.5
kbp
0.8

(B)

TNA from ScSMD affected sugarcane detected a band of about 9 kb which can be assumed to be the ScSMV genomic RNA. These methods need to be further developed for use in the field.

Three primers were used for RT-PCR assays to detect the ScSMD associated sequences. Using DA20 as a template , PCR conditions for the two set of primers were optimised and the PCR products were 800 bp (with DA900 and DA100) and 500 bp (DA500 and DA100).

Using the primers DA900 and DA100 in the RT-PCR, PCR products were detected from TNA of ScSMD affected plants. Using DA500 and DA100, PCR products were not detected from TNA of the diseased plants.

The RT-PCR products were not generally detected with EtBr staining probably because the amount of the product was very low for many samples. Detection of RT-PCR products by hybridisation was not much more sensitive than by staining. However, by using the first cycle of the RT-PCR products as a template in a second PCR reaction, all of the samples for diseased plants were strongly positive.

CHAPTER 9

STUDIES OF RELATIONSHIP BETWEEN ScSMD AND PHYTOPLASMAS

9.1 INTRODUCTION

Diseases caused by phytoplasmas were thought for many years to be caused by viruses, but transmission electron microscopy identified the presence of cell wall-less bacteria in the phloem, thus indicating that phytoplasmas were the causal agent. Plant non-culturable mollicutes, commonly referred to as phytoplasmas, were first discovered in plants in 1967 (Doi *et al.*, 1967). Many plant species have been reported to have phytoplasma-associated diseases. Typical symptoms are yellowing, deformation of leaves and abnormalities such as sterile flowers, phyllody and proliferation (McCoy *et al.*, 1989).

Until recently, the detection of phytoplasmas was mainly based on electron microscopy and a fluorescence technique with UV light microscopy using the DNA fluorochrome 4'-6-diamidino-2-phenylindole (DAPI). Neither method allows differentiation between phytoplasmas. Moreover, their sensitivity depends on the number of phytoplasma cells in the tissue. Thus, electron microscopy can only be used successfully for detection in hosts with a relatively high concentration of phytoplasmas. The DAPI technique is considerably more sensitive but is of limited value when the phytoplasma population is very low especially in woody plants (Ahrens and Seemüller, 1992). Progress has been made toward specific detection of phytoplasmas by both serological methods and DNA/DNA hybridisation assay. However, it is not known whether these are more sensitive than the DAPI method.

Molecular phylogenetic analyses of the 16S rRNA gene of phytoplasmas have been reported (Lim and Sears, 1989) and recent studies on the 16S rRNA gene sequence of many phytoplasmas suggest that these phytoplasmas are most closely related to *Anaeroplasma* and *Acholeoplasma*, but are evolutionary distinct from animal mollicutes. Specific oligonucleotide probes for the phytoplasma 16S rRNA gene improve the sensitivity of tests to detect phytoplasmas when used in place of cloned chromosomal DNA probes (Kirkpatrick *et al.*,

1990). This suggests that the phytoplasma 16S gene can be useful for identification and differentiation of phytoplasmas.

The introduction of PCR (Saiki *et al.*, 1988) has increased the sensitivity of diagnosis of plant pathogens (Hadidi *et al.*, 1995). Deng and Hiruki (1991) reported that the 16S rRNA genes of phytoplasmas could be amplified by using specific primers. Ahrens and Seemüller (1992), Lee *et al.* (1993) and Namba *et al.* (1993) established a diagnostic PCR assay using a set of phytoplasma specific primers which will amplify the phytoplasma 16S rRNA gene.

This chapter discusses a study of the relationship between ScSMD and phytoplasmas. It was done because two phytoplasma diseases on sugarcane have been reported (Raychaudhuri and Mitra, 1993; Table 1.1).

9.2 MATERIALS AND METHODS

9.2.1 Primers

PCR primers for the detection of phytoplasmas were chosen from reported primers. Sequences of 16S rRNA of phytoplasmas from *Oenothera hookeri*, the evening primrose, and 15S rDNA sequences of *Mycoplasma capricolumn* (AC X00921), *M. hyopneumoniae* (Y00149), *M. synoviae* (X52082), and the *Mycoplasma* strain PG50 (M10588), *Spiroplasma citri* (M23942), *Nicotiana tabacum* (V00165) and *Pisum sativum* (M30826) were extracted from the GenBank using ANGIS. The extracted sequences were used as templates in the Amplify program to check the primability and specificity of the reported primers. Their references and the expected sizes of the PCR products are described in Table 9.1.

9.2.2 Methods

9.2.2.a TEM

TEM was carried out according to the method of Hayat (1987). The procedure is described in Section 2.2.2.c.

Table 9.1 The expected sizes of PCR products for selected phytoplasma specific primers.

Primers (names in the references)		Expected PCR products	
Forward	Reverse	(nucleotides in 16S rRNA) gene map	References
ASFWR (F2)	ASREV (F2)	558 bp (759-1,316)	Ahrens and Seemüller, 1992
LFWR (R16F2)	LREV (R16R2)	1,245 bp (152-1,397)	Lee *et al.*, 1993
NBFWR (SN910601)	NBREV (SN910602)	1,348 bp (1-1,348)	Namba *et al.*, 1993

9.2.2.b PCR assay

TNA extracted by the crush method (Section 2.2.4.a) was dissolved in TE and the concentration and the purity was checked with a spectrophotometer (Section 2.2.4.c, Sambrook *et al.*, 1989). The concentration of TNA was adjusted to 200 ng/µl and 5 µl of this TNA solution was used for the PCR assay.

The PCR assay was done according to the method of Lee *et al.* (1993) with some modifications and is described in Section 2.2.12.b. PCR products were analysed on agarose gels and stained with EtBr. The gels were photographed.

9.3 RESULTS

9.3.1 Phytoplasma-like symptoms and particles in the ScSMD affected sugarcane plants.

There were some phytoplasma like symptoms such as white leaf and proliferation symptoms on some plants maintained in the glasshouse (Fig. 9.1A). The white leaf streak continued from the bottom to the tip of the leaf blade. Sugarcane with the white leaf symptom showed a progressive leaf tip die back and eventually the growing point of the sugarcane died (Fig. 9.1A). Multiple stems then grew from the base of the dead sugarcane shoot, giving a proliferation symptom (Fig 9.1B). Most of the germinated shoots were also stunted in glass house studies, but the white leaf symptoms could not be found until the third ratoon stage.

During the cytopathological studies of ScSMD by TEM, phytoplasma-like cells were observed in some of the young leaves of plants at the late growth stage which had the white streak symptom. The particles were present in the phloem cells and had a single membrane. The size of the circular shaped particles was from 0.3-0.5 µm (Fig 9.2). However, there were no similar detectable particles in the healthy sugarcane.

Figure 9.1 Symptoms of phytoplasma-like disease on ScSMD affected sugarcane plants

(A) - Different types of white stripe on lamina of young leaves in the third ratoon stage.

(B) - Proliferation after death of shoots from the third ratoon.

(A)

(B)

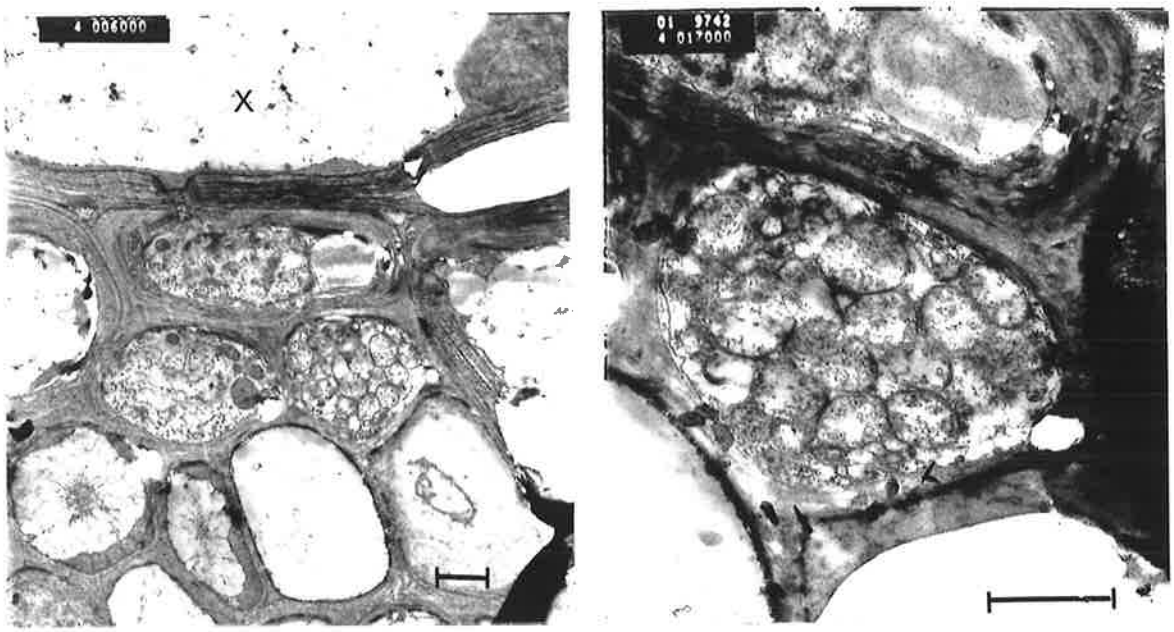


Figure 9.2 Transmission electron micrograph of phytoplasma like particles in the phloem of a ScSMD affected sugarcane. The bar represents 50 nm.

(A) - Overview of cells in a vascular bundle.

(B) - Magnification of (A) showing phytoplasma-like cells in a phloem-associated cell.

see corrigendum

9.3.2 PCR assay to detect phytoplasmas

All the chosen primers amplified PCR products of the expected size from some of the TNA extracts of the ScSMD affected and healthy sugarcane plants in the field. However, PCR products were not produced from samples of healthy sugarcane raised from seeds or from some of the ScSMD affected sugarcane (Table 9.2).

The most consistent results were obtained from PCR assay with the primer set, ASFWR and ASREV. The optimum conditions for the all primer sets were;

LFWR and LREV -an initial denaturing temperature of 94 °C for 1 min and 35 cycles of 94 °C for 40 sec, 60 °C for 1 min and 70 °C for 1 min, and the final extension at 70 °C for 4 min. The size of PCR products was 1.2 kbp (Fig. 9.3).

NBFWR and NBREV -an initial denaturing temperature of 94 °C for 1 min and 35 cycles of 94 °C for 40 sec, 55 °C for 1 min and 70 °C for 1 min, and the final extension at 70 °C for 4 min. The size of PCR products was 1.3 kbp (Fig. 9.4).

ASFWR and ASREV -an initial denaturing temperature of 94 °C for 1 min and 35 cycles of 94 °C for 40 sec, 57 °C for 1 min and 70 °C for 40 sec, and the final extension at 70 °C for 3 min. The size of PCR products was 0.5 kbp (Fig. 9.5)

9.4 DISCUSSION

Phytoplasma-related symptoms and some phytoplasma-like particles were present in some of the ScSMD affected sugarcane plants. The phytoplasma-like particles were about 500 nm in diameter and were observed in the phloem cells of some ScSMD affected sugarcane plants, but not in those from healthy sugarcane grown from seed. The phytoplasma-like particles had a single layer membrane which is a typical feature of mycoplasma-like organisms in plants (Ghosh *et al.*, 1988).

The PCR assay is fast and sensitive and many phytoplasma specific primers are available. Therefore the PCR assay was used to detect phytoplasmas and to study the relationship between phytoplasmas and ScSMD.

Table 9.2 Relationship between ScSMD and phytoplasmas as shown by hybridisation with the ScSMD specific probes (DA14) and PCR using phytoplasma specific primers respectively.

Sugarcane plants	Samples on Figures 9.3 and 9.4	DA14*	PCR**
ScSMD affected plants	3	+	+
"	4	+	+
"	5	+	+
" with white stripe	6	+	+
" with proliferation	7	+	+
" with white stripe	8	+	+
ScSMD affected plants	9	+	-
"	10	+	-
Sugarcane raised from seeds	11	-	-
"	12	-	-
Healthy plant collected from the field	13	-	-
"	14	-	-
"	15	-	+
"	16	-	+
"	17	-	+
"	18	-	+
"	19	-	+
"	20	-	+
"	21	-	+
"	22	-	+

*; +, Hybridised with probes made from clone DA14; - did not hybridise.

**; +, PCR products detected by using phytoplasma specific primers; -, PCR products not detected.

Figure 9.3 Analysis of PCR products with the phytoplasma specific primers (LFWR and LREV) by 1.2% agarose gel electrophoresis. The gel was stained with EtBr. Arrow indicates 1.2 kbp dsDNA.

1 - TNA from *Catharanthus roseus* infected with sweet potato little leaf phytoplasma.

2 - TNA from symptomless *C. roseus*.

3-10 - TNA from ScSMD affected sugarcane which tested positive with the disease specific probes made from DA14 (Section 2.2.6.c).

11 & 12 - TNA from healthy sugarcane raised from seeds.

13-15 - TNA from healthy sugarcane which did not hybridise with ScSMD specific probes made from clone DA14 (Section 2.2.6.c).

M - 1 kbp DNA ladder used as a standard molecular weight marker.

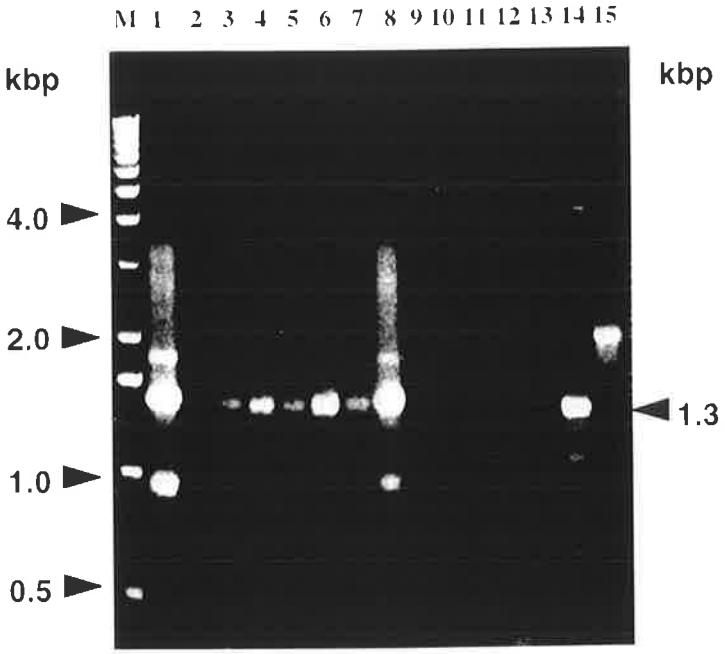

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kbp
4.0
2.0
1.0
0.5
kbp
1.3

Figure 9.4 Analysis of PCR products using primers NBFWR and NBREV by 1.2% agarose gel electrophoresis. The gel was stained with EtBr. Arrow indicates 1.3 kbp dsDNA.

Lane 1 - TNA from *Catharanthus roseus* infected with sweet potato little leaf phytoplasma.

Lane 2 - TNA from healthy *C. roseus*.

Lanes 3-8 TNA from ScSMD affected sugarcane which tested positive with the disease specific probes made from DA14 (Section 2.2.6.c).

Lanes 15-20 - TNA from healthy sugarcane which did not hybridise with ScSMD specific probes made from clone DA14 (Section 2.2.6.c).

Lane 21 - TNA from healthy sugarcane raised from seeds.

M - 1 kbp DNA ladder was used as a standard molecular weight marker.

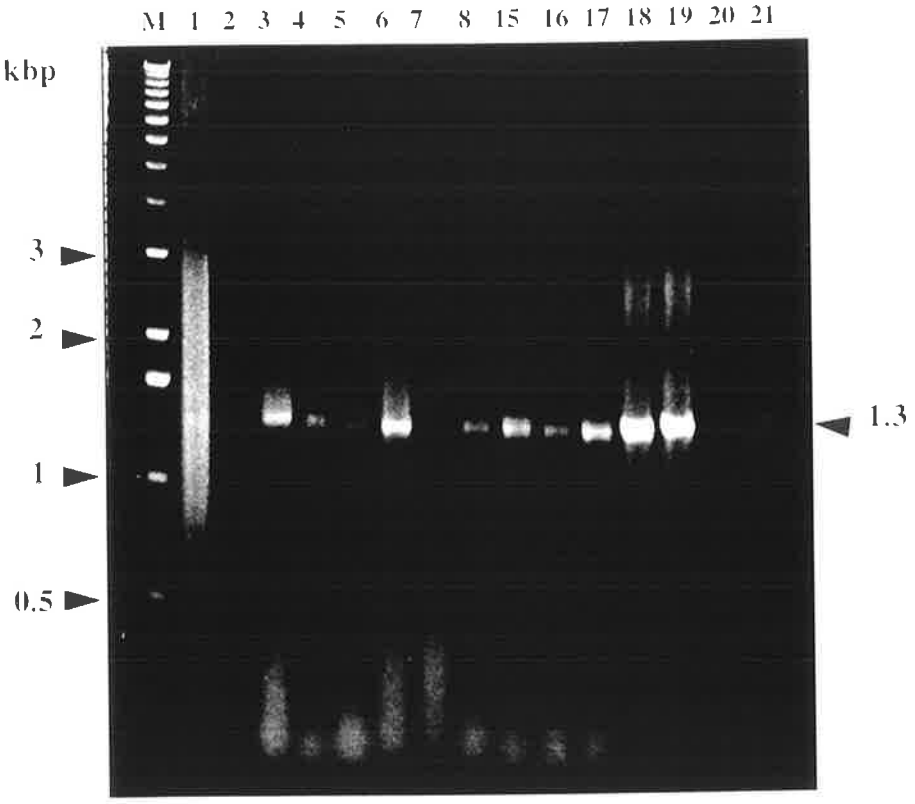

M 1 2 3 4 5 6 7 8 15 16 17 18 19 20 21
kbp
3
2
1
0.5
1.3

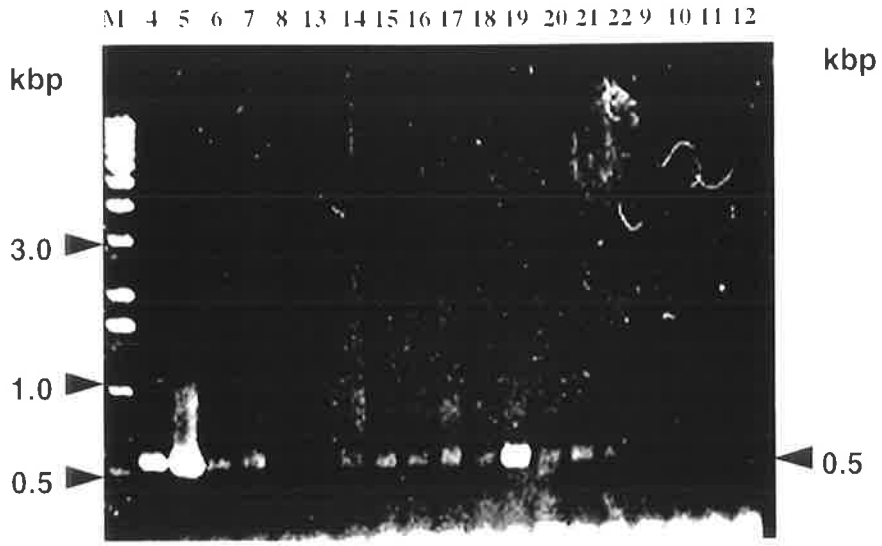
Figure 9.5 Analysis of PCR products using primers ASFWR and ASREV by agarose gel electrophoresis. The gel was stained with EtBr. Arrow indicates 0.5 kbp dsDNA.

Lanes 4 to 10 - TNA from ScSMD affected sugarcane plants.

Lanes 13 to 22 - TNA from ScSMD free sugarcane plants which did not hybridise with ScSMD specific probes made from clone DA14 (Section 2.2.6.c)

Lanes 11 & 12 - TNA from sugarcane seeds.

M - 1 kbp DNA ladder was used as a standard molecular weight marker.


M 4 5 6 7 8 13 14 15 16 17 18 19 20 21 22 9 10 11 12
kbp
3.0
1.0
0.5
kbp
0.5

Although the phytoplasmas were detected in glasshouse and field grown sugarcane plants, there was no evidence that the phytoplasmas were related specifically to ScSMD because some of the ScSMD symptomatic plants were negative for phytoplasma by PCR. There were also detectable amounts of phytoplasma specific PCR products from field collected healthy sugarcane plants which did not react with the ScSMV specific probes.

There was some variation in the PCR products from sugarcane samples. Some samples gave a product with one set of primers but not with others and the amount of the PCR products differed from sample to sample. This suggests that the phytoplasmas may vary in different plants.

It is concluded that the phytoplasmas observed were not the cause of ScSMD but that they may interact with ScSMV to produce a more severe disease of sugarcane. The importance of these phytoplasmas should be further investigated.

CHAPTER 10

GENERAL DISCUSSION

The characteristic symptom of ScSMD on sugarcane was previously reported to be chlorotic striations on the lamina (Hughes, 1961). In this thesis, chlorotic striations are reported on the other parts of sugarcane as well, such as the leaf sheaths and the stems when plants were grown in a glasshouse. Stunting was also observed on ScSMD affected sugarcane plants, and growth was retarded. The distribution of symptoms was such that clear chlorotic striations could be found on the older leaves, whereas the younger leaves had no symptoms. The best stage to search for symptoms of ScSMD in the field may be late growth, or on regrowth in the older ratoon stages.

Several further effects of ScSMD were observed on sugarcane. The germination of ScSMD-affected sugarcane cuttings was delayed by one week so that the number of leaves was subsequently less than for healthy plants at the same time after germination. The germinated shoots of ScSMD affected cuttings were also thinner and shorter than those of healthy cuttings.

Inoculations of ScSMD to susceptible sugarcane hybrids and virus indicator species with sap extracts from the diseased plants failed to produce any visible symptoms. Transmission in the field may thus be vector dependent. When an appropriate diagnostic method is available, the search for the vector or other transmission method should be pursued in order to clarify the epidemiology of ScSMD.

The surfaces of diseased leaves were smoother in texture than those of healthy leaves. This smooth texture could be found in affected leaves that were showing yellowing and striations. This is probably because the surface of the diseased leaves was covered with a thicker layer of wax, especially around the stomata, and because fewer spikes were present on the surface of leaves.

The thylakoid membrane structure of chloroplasts and the number of mitochondria were altered in the ScSMD affected sugarcane leaf cells. Effects on these organelles have been reported for numerous plant infecting viruses (Matthews, 1991). The

plasmodesmata in the cell wall of ScSMD affected leaves apparently also differed from that in healthy leaves.

Although ScSMD affected sugarcane showed virus-like symptoms and cytopathological effects, no putative causal agent or virus-like particle could be detected in thin sections of ScSMD affected sugarcane tissue by transmission electron microscopy. Despite an extensive search, negative staining of sap of ScSMD affected sugarcane also failed to detect any putative causal agent. Other procedures were required to find evidence that an intracellular pathogen was associated with ScSMD.

Total nucleic acid extraction methods were developed to compare the nucleic acid compositions of diseased and healthy sugarcane. The best extraction of RNA was achieved with the RNA extraction buffer. The TNAs were analysed by electrophoresis, but there was no difference in the composition between the healthy and ScSMD affected sugarcane. Further experiments therefore adopted a different approach.

More than 90% of plant viruses have an RNA genome (Murphy *et al.*, 1995; Appendix F). If a plant is infected by a virus with an RNA genome, the plant tissue is likely to contain dsRNAs. Such dsRNAs could be either genomic or replicative forms. The dsRNAs will also be segmented if the RNAs of plant viruses are segmented, and the number and size of segments could be diagnostic for species and groups of viruses (Dodds, 1993).

An attempt was therefore made to extract dsRNA using the standard CF11 cellulose chromatography method. ScSMD associated dsRNAs were found in the leaves of both field collected and glass house grown sugarcane. All ScSMD affected sugarcane plants contained a 9 kbp dsRNA, which was the largest, but the band pattern differed between the glasshouse grown and field collected sugarcane in showing smaller dsRNAs in different amounts. Dodds (1993) also found differences in dsRNA band patterns from plants infected with a virus in a glasshouse and plants naturally infected with the same virus .

The smaller dsRNAs (6, 2.6, 2.5 and between 0.5 to 1.0 kbp) were hybridised to a probe reverse transcribed from the 9 kbp dsRNA. Thus, the small dsRNAs can be concluded to represent subgenomic forms of the 9 kb genome of the disease causal agent.

The standard CF11 cellulose method was satisfactory for isolating dsRNA from AMV, CMV, PMV and TMV infected tissues. However, this method produced a very

low yield of dsRNA from ScSMD-affected tissue that led to difficulties for further molecular biological studies of ScSMD associated dsRNA. An increased yield of dsRNA was needed.

A batch method using a microgranular cellulose was developed which increased the yield of dsRNA from ScSMD affected sugarcane tissues probably because of the higher binding capacity of this cellulose. The new batch method gave at least a 10 fold higher yield of dsRNA than the CF11 method, and was also easier and faster to use.

The products of the microgranular cellulose batch method contained considerable amounts of plant dsDNA and ssRNA was less amount than the CF11 cellulose method. It was necessary to treat them with DNase and RNase to eliminate these nucleic acids.

The technique finally adopted for routine extraction of dsRNA from ScSMD affected sugarcane leaf was to use the microgranular cellulose to rapidly collect large amounts of dsRNA from a large volume of plant extract. The collected dsRNA was further isolated by a cycle of CF11 cellulose column chromatography to reduce the amounts of host nucleic acids. This method proved to be generally suitable for extracting dsRNA from other plant tissues with low concentrations of dsRNA, such as *Vicia fava* infected with PMV.

For cloning of the dsRNA, it was found that further purification was required to remove contaminating host DNA and RNA. These were successfully removed by a combination of two cycles of gel fractionation and nuclease treatments. The dsRNA isolated by the microgranular cellulose batch method was first treated with DNase then with RNase A in high salt to remove host nucleic acids. The nuclease treated dsRNA was then fractionated on 0.9 % agarose gel and recovered. PAGE was then used because the band between the 9 kbp and 6 kbp dsRNA was not clearly separated on the agarose gel. Dissolvable disulphide cross-linkage was also used in the PAGE to increase recovery from the gel because the amount of dsRNA remaining was very low.

An efficient method for cloning from the small amounts of dsRNA was developed using the rPCR method. It was found that the standard method of cloning with the random hexamer primer method produced only small sized cDNAs from the dsRNA templates. cDNAs were also reduced in size when a large excess of the UN-RH primer was used. To obtain a longer cDNA by reverse transcriptase, it was found to be desirable to use a

small amount of primer. However, this produced only a small amount of cDNA, so a method to amplify it was required.

Random PCR (rPCR), developed by Froussard (1992), was used. The cDNAs amplified by rPCR were cloned and all of them were ScSMD-associated sequences. ScSMD specific clones were selected and sequenced. Thus rPCR can be used to make a cDNA library when only a small amount of target nucleic acid is available.

The sequenced clones were combined into three segments, which together represented 2.55 kbp of the estimated 9 kbp size of the ScSMD associated dsRNA. A homology search showed that the sequenced ScSMD segments had some identity with several species within six plant virus genera; the *Tymovirus, Potexvirus, Capillovirus, Carlavirus, Potyvirus* and *Closterovirus* genera. Overall, the greatest similarity was with the ASPV ORF1 which is the putative replicase region (Jelkman, 1994). Less but significant similarity was observed with other genera representing rod-shaped and icosahedral viruses. The sequence similarity to one of the tymoviruses is probably due to the known similarity between the replicases of the other three genera of rod-shaped viruses and tymoviruses (Koenig *et al.*, 1995).

Nucleic acid which was isolated from the pellet of the sucrose cushion centrifugation hybridised with the ScSMD specific probe. It was shown to be a ssRNA by its sensitivity to RNase in a high salt buffer and its resistance to DNase.

It was ca. 9 kb in size which is similar to that reported for viruses in the genera *Potex-, Capillo-, Tricho-* and *Carlavirus,* which all have an ORF 1 of 4- 6 kbp, a 3' triple gene block and a poly A 3' terminus . The size most closely resemble to that of some carlaviruses.

A filament-shaped virus particle 15 nm in diameter was isolated which co-purified with the ScSMD associated RNA. Its length was between 400 and 3500 nm. Two modal lengths were recognised, 900-1,100 nm and 1,900-2,100 nm. The 900-1,100 nm particles could represent monomers, and the longer particles dimers. The monomer length is longer than that described for most of the carlaviruses (600-700 nm; Koenig *et al.*, 1995) but it is close to that of the group of longer carlaviruses, such as PVM, ASPV and BlSV.

Because the length of the RNA of carlaviruses is related to the length of their

particles, the 9 kbp RNA is from the 1,000 nm particle. The 6 and 2.26 kb RNAs would have particles of 600 and 250 nm if encapsidated, but because those could not be detected, these dsRNAs are assumed to be replicative forms of subgenomic,ScSMV RNA. Hybridisation with 9 kbp dsRNA supports this contention.

It is concluded the ScSMV associated with ScSMD is most closely related to carlaviruses because of the similarity of the length of its genome, to the three larger carlaviruses, ASPV, BlSV and PVM. It can therefore be proposed that the genus carlavirus may be divided into two sub-groups based on length and the sequences of the viruses and that ScSMV appears to fit into the larger subgroup.

The identification of a virus associated with ScSMD has demonstrated the viral etiology of ScSMD and has led to the development of a diagnostic method for the disease. Two diagnostic methods for ScSMD have been developed in this thesis, dot blot hybridisation and RT-PCR.

Hybridisation probes prepared from the ScSMD specific clones distinguished between the TNAs of ScSMD affected and healthy sugarcane when used in dot blot hybridisation assays. Some field collected sugarcane not showing ScSMD symptoms nevertheless showed hybridisation with ScSMD-specific probes which was possibly due to presymptomatic infection.

The RT-PCR assay was tried because it has been shown to be more sensitive than other diagnostic methods for some plant viruses (Hadidi *et al.*, 1993). To carry out RT-PCR, two sets of primers were derived from ScSMD associated sequences. The set of SD900 (FWD) and SD100 (REV) primers was able to amplify a 800 bp segment when the ScSMD associated dsRNA and TNA of ScSMD affected sugarcane was used. However, the amount of the 800 bp product was generally too small to be detectable on agarose gels with ethidium bromide staining. To detect the small amount of RT-PCR product, analysed gels had to be transblotted to a nylon membrane and to be hybridised with ScSMD specific probes. Alternatively, the first cycle of PCR products could be used for another cycle of PCR. This RT-PCR assay needs to be further developed so that it can be applied in the field for detection of ScSMV.

Some symptoms on sugarcane were observed to be similar to those induced by

phytoplasmas, such as proliferation and white-streak on leaves. Phytoplasma-like particles about 500 nm in diameter with a single membrane were also observed in the ScSMD-affected tissue by TEM in phloem cells in the ScSMD affected tissue. A PCR assay was employed to study the relationship between the phytoplasmas and ScSMD using phytoplasma specific universal primers.

Phytoplasmas were detected by PCR, but no relationship was observed between ScSMD and the presence of PCR products of the size expected to be produced by phytoplasma in the TNAs of the ScSMD-affected and healthy sugarcane plants. Most ScSMD symptomatic sugarcane plants were positive but not all affected plants showed detectable phytoplasma specific PCR products. There were also detectable amounts of PCR products from field collected healthy sugarcane that did not react with ScSMD specific probes or by RT-PCR assay for the detection of ScSMV.

In the future, the sequence of the full genome of ScSMV could be obtained using oligo d(T)n primers or internal primers in the genomic region. Sequencing of the full length of the ScSMV RNA will assist classification of ScSMV and development of a diagnostic method for field application. Mode of transmission should also be addressed to find the mechanism of spread of ScSMD in the field. This will be possible when a sensitive RT-PCR assay is available. This work would assist in the development of control methods.

In the current study, an icosahedral virus like particle was also observed, but the nucleic acid extracted from a preparation of these did not hybridise to a ScSMV specific probe. The relationship of this virus-like particle to sugarcane diseases requires further study.

The phytoplasma that was detected with phytoplasma specific primers has to be classified and its possible role as a disease causal agent needs to be further investigated, because it has possible implications for ScSMD in the later growth stage of sugarcane.

In conclusion, this thesis describes the use of molecular nucleic acid methods to associate a ssRNA virus with ScSMD. A diagnostic method for further studies has also been described. The possible interaction of ScSMV with other potential pathogens has been demonstrated, and further directions in research on this poorly understood disease are suggested.

APPENDICES

1. Appendix A - Biochemicals and miscellaneous chemicals
2. Appendix B - Polyacrylamide and agarose gels, bacterial media, buffers and solvents
3. Appendix C - Staining solution
4. Appendix D - Primers
5. Appendix E - Fluorescent dye primer cycle sequencing
6. Appendix F - Table of characteristics of plant viruses
7. Appendix G - Published work

APPENDIX A

BIOCHEMICALS AND MISCELLANEOUS CHEMICALS

BIOCHEMICAL	SOURCE
Ampicillin	Boehringer Mannheim, Germany
AMV-RT	Promega, USA
dATP	Promega, USA
dCTP	Promega, USA
dGTP	Promega, USA
dTTP	Promega, USA
DNA ladder, 123	GIBCO BRL, USA
DNA ladder, 1kb	GIBCO BRL, USA
EcoRI	Promega, USA
Klenow enzyme (DNA polymerase I, large fragment)	Promega, USA
Lysozyme	Sigma, USA
M-MuLV-RT	GIBCO BRL, USA
^{32}P-dCTP	Bresatec, Australia
Proteinase K	Boehringer Mannheim, Germany
RNA MWt marker	Promega, USA
RNase A (type IIIA)	Sigma, USA
RNAsin® Ribonuclease inhibitor	Promega, USA
RQ1 DNase (RNase-free)	Promega, USA
pGEM®-7zf(+)	Promega, USA
Salmon sperm DNA	Sigma, USA
Taq DNA polymerase	Promega, USA Pharmacia Biotech, Sweden
Tth DNA polymerase	Promega, USA

CHEMICAL	SOURCE
Acetic acid, glacial	BDH Chemical, England
Acridine orange	Aldrich Chem. Co., USA
Acrylamide	Bio-Rad Laboratories, USA
Agar, purified	Oxoid Ltd., England
Agarose (Type II: medium EEO)	Sigma, USA
Ammonium persulfate	BioRad, USA
Azure A	George T. Gurr Ltd., England
BAC	BioRad Laboratories, USA
Bacto-trypton	Oxoid Ltd., England
Bacto-yeast extract	Oxoid Ltd., England
Boric acid	BDH Chemical, England
BSA	Sigma, USA
X-gal	Promega, USA
Bromphenol blue	BDH Chemical, England
Calcium chloride	BDH Chemical, England
Calcomine orange 2RS	Aldrich Chem. Co., USA
Cellulose, medium fibrous (CF11)	Sigma, USA
Cellulose powder MN 300 (microgranular cellulose)	Machery, Nagel & Co., Germany
Caesium sulphate	BDH Chemical, England
CTAB	MERCK, Germany
Dextran sulphate	Pharmacia Biotech, Sweden
DEPC	Sigma, USA
DTT	Sigma, USA
Epoxy resin	Probing & Structure co., Australia
EtBr	Sigma, USA
E-OH	BDH Chemical, England
EDTA	BDH Chemical, England
Ficoll® 400	Pharmacia Biotech, Sweden
Formaldehyde	Ajax chemicals Ltd., Australia
Formamide	BDH chemical, England
Glutaraldehyde EM	BDH, England
Glycerol	BDH Chemical, England
D-Glucose	BDH Chemical, England
IPTG	Boehringer Mannheim, Germany
IPA	BDH Chemical, England
N-Lauroyl-sacosine (sarkosyl)	Sigma, USA
Lead citrate	BDH Chemical, England

CHEMICAL	SOURCE
Luxol® brilliant green Br	Aldrich Chem. Co., USA
Manganeous chloride	BDH Chemical, England
Magnesium chloride	BDH Chemical, England
BME	Sigma, USA
2-Methoxyethanol	BDH Chemical, USA
Methylmercuric hyroxide	SERVA, USA
Mineral oil, light	Sigma, USA
MTG	Sigma, USA
N, N'-Methylene-bis-acrylamide	Bio-Rad, USA
MOPS	Sigma, USA
Phenol	BDH Chemical, England
PEG 6000	Sigma, USA
PVP 10	Pharmacia Biotech, Sweden
PVP 40	Pharmacia Biotech, Sweden
Potassium acetate	BDH Chemical, England
Resin, analytical grade mixed bed [AG® 501-X8(D)]	Bio-Rad Laboratories, USA
Rubidium chloride	BDH Chemical, England
Sephacryl® S-400	Pharmacia Biotech, Sweden
Sephadex® G-50, fine	Pharmacia Biotech, Sweden
Silver nitrate	BDH Chemicals, England
Sodium borohydride	Sigma, USA
Sodium acetate	BDH Chemical, England
tri-Sodium citrate	BDH Chemical, England
Sodium chloride	BDH Chemical, England
Sodium hydroxide	BDH Chemical, England
Sucrose	BDH Chemical, England
SDS	BDH, England
TEMED	Sigma, USA
Toluidine blue	Aldrich Chem. Co., USA
Triton X-100	Sigma, USA
Trizma base	Sigma, USA
dodeca-Tungstosilicic acid	BDH Chemical, England
Xylene cyanol F.F.	Ajax chemicals Ltd., Australia

APPENDIX B

POLYACRYLAMIDE AND AGAROSE GELS, BACTERIAL MEDIA, BUFFERS AND SOLVENTS

1. Acrylamide solutions

For soluble gels 20% solution- 4.8 g of acrylamide and 0.2 g of BAC were dissolved in separate beakers. Two solutions were mixed and adjusted to 25 ml and filtered with a syringe filter (0.4 µm). This solution was stored in a light protected bottle at room temperature and used within 24 hr.

For analytical gels 30% solution - 29 g of acrylamide and 1 g of BAA were dissolved in SDIW and the volume was adjusted to 100 ml. The polyacrylamide solution was sterilised with a filter, as above. The sterilised solution was stored at 4 °C in a light protected bottle and used for 6 months.

40% solution-39 g of acrylamide and 1 g of BAA were dissolved in SDIW and the volume was adjusted to 100 ml. The solution was filter sterilised and stored, as above.

2. Polyacrylamide gels

6.5% soluble polyacrylamide gel	20 ml solution
20% acrylamide mix	6.5
10x TBE	2.0
SDIW	11.04
10% APS	0.06
TEMED	0.4

3.5% polyacrylamide gel for analysis	10 ml solution
30% acrylamide mix	1.75
10x TBE or TAE	1.0
SDIW	7.18
10% APS	0.07
TEMED	0.004

6.5% polyacrylamide gel for analysis	10 ml solution
40% acrylamide mix	1.63
10xTBE or TAE	1.0
SDIW	7.3
10% APS	0.07
TEMED	0.004

12 (15)% polyacrylamide gel for SDS-PAGE

	Resolving gel(10 ml)	Stacking gel (5 ml)
30% acrylamide mix	4.0 (5.0) ml	0.83 ml
1.5 M Tris (pH 8.8)	2.5 (2.5)	0.63
SDIW	3.3 (2.3)	3.4
10% SDS	0.1 (0.1)	0.05
10% APS	0.1 (0.1)	0.05
TEMED	0.004 (0.0004)	0.005

3. Agarose gel 0.7~2.0 g of agarose was dissolved by heating with a microwave oven in 100 ml of TAE buffer (40 mM Tris-acetate, pH 8.3, 1 mM EDTA).

4. Bacterial media

LB medium (1 L) To 950 ml of deionized water, add :

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 1 or 10 N NaOH. Adjust the volume of the solution to 1 litre with deionized water. Sterile by autoclaving for 20 min at 15 lb./sq. in. on liquid cycle with a Autoclave.

SOB medium (1 L) To 950 ml of deionized water, add :

bacto-tryptone	20 g
bacto-yeast extract	5.0 g
NaCl	0.5 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 1 N NaOH. Adjust the volume of the solution to 1 litre with deionized water. Sterilise by autoclaving for 20 min at 15 lb./sq. in. on liquid cycle. Cool to room temperature and just before use add 10 ml of filter sterilised of 1 M $MgCl_2$ and 10 ml of filter sterilised 1 M $MgSO_4$.

SOC medium (1 L) SOC medium is SOB medium, containing 35% (w/v) glucose. After SOB medium has been autoclaved, allow it to cool to below of 60 °C and then add 1 ml of 2 M filter-sterilised glucose solution prior to use.

5. Storage of bacterial culture containing glycerol

To 0.85 ml of overnight bacterial culture, add 0.15 ml of sterile glycerol. Vortex the mixture to ensure that the glycerol is evenly

dispersed and transfer to a cryotube (NUNC, USA). Freeze the culture in liquid nitrogen and then transfer the tube to a -70 °C freezer for long term storage.

6. Solvents

CI solution	24 volumes of chloroform were mixed with 1 volume of *iso*-amyl alcohol and stored in a light protected bottle.
ETHANOL	Absolute and 75 % (v/v) ethanol stored at -20 °C.
FORMAMIDE	Formamide was mixed with a mixed bed resin (AG 501-X8) (10 g/100 ml) to deionize and filtered with two layers of Whatmann filter paper No. 1. It was stored at -20 °C.
PCI solution	One volume of CI solution was mixed with 1 volume of a saturate phenol and stored in a light protected bottle at 4 °C.
Phenol	For TNA preparation, 8-hydroxyquinoline [0.1 % (w/w)] was added to solid phenol and 0.1 M Tris-HCl (pH 8.0) saturated and equibliated with DEPC treated TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). For RNA preparation, phenol was prepared same as above except that saturation was done with SDIW.
Iso-propyl alcohol	Absolute *iso*-propyl alcohol was stored at -20 °C

7. BUFFERS

SSC	150 mM NaCl and 0.15 M $Na_3C_6H_5O_7.2H_2O$)
STE	100 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA
TAE	40 mM Tris-CH_3COOH, pH 8.3 and 1 mM EDTA
TBE	25 mM Tris-borate, pH 8.0 and 1 mM EDTA
TE	10 mM Tris-HCl, pH 7.4 or 8.0 and 1 mM EDTA

Formaldehyde gel-running buffer

20 mM MOPS, pH 7.0, 8 mM sodium acetate and 1 mM EDTA

APPENDIX C

STAINING SOLUTION

I. Staining solutions used in light microscopy

*i> **Azure A solution*** - 0.1% (w/v) Azure A was made in 2-methoxyethanol as a stock solution. A working Azure A solution was made by mixing 0.1% Azure A and 0.2 M Na_2HPO_4 in a ration of 9:1 before use.

*ii> **O-G solution*** - 1% (w/v) Calcomine orange 2RS and 1% (w/v) Luxol Brilliant Green BL were made in 2-methoxyethanol and filtered with a Whatmann No. 1 filter paper and stored in a light protected bottle. Before use, water and the orange dye were mixed in a ratio of 1:1 and adjusted with the green dye content according to the leaf tissue.

*iii> **Acridine orange*** - 0.01% (w/v) acridine orange was made in 0.1 M potassium phosphate buffer, pH 6.0.

II. Staining solutions used in electron microscopy

*i> **Lead citrate*** - 33 g of $Pb(NO_3)_2$ and a 1.76 g of $Na_3C_6H_5O_6.2H_2O$ were dissolved in CO_2 free water. The solution was mixed vigorously for 1 min. After 30 min, 8 ml of 1 M NaOH (free of Na_2CO_3) was added and the volume of the solution was adjusted to 50 ml. The solution was stored in a glass stoppered brown bottle at room temp. This solution could be used for 1 month.

*ii> **1% Phosphotungstic acid*** - 1 g of $H_3PO_4.12WO_3+xH_2O$ was dissolved in 100 ml of SDIW and the pH of the solution adjusted with 1 N NaOH to pH 6.8.

*iii> **Saturated uranyl acetate*** - $UO_2(CH_3{\cdot}COO)_2{\cdot}2H_2O$ was dissolved with 1 ml of 70% (v/v) M-OH in a microcentrifuge tube. The solution was vortexed vigorously for a few minutes and undissolved salt was removed with a quick centrifugation (ca. 10,000 x *g* for 30 sec). The supernatant was transferred to a centrifuge tubes and rapped with aluminium foil. It was stored at 4 ^{o}C and used for a week.

III. Staining solution for gel electrophoresis

i> Ethidium bromide- Ethidium bromide was dissolve in SDIW to 10 mg/ml and stored in a light protected bottle at room temperature. The solution was diluted just before use to either 1 ppm for agarose gels or 5 ppm for polyacrylamide gels and for formaldehyde denaturing agarose gel.

ii> 0.25% Coomassie brilliant blue- 0.25 g of Coomassie brilliant blue R250 was dissolved in 90 ml of 45% M-OH and 10% of gracial acetic acid. The undissolved material was removed by filtration with Whatmann filter paper No. 1 and stored in a light protected bottle.

iii> Silver nitrate solution- 0.3 g of silver nitrate was dissolved in 150 ml of DIW containing 0.5 ml of 40% formaldehyde solution. The solution was used immediately.

iv> Toluidine blue-w 1 g of toluidine blue was dissolved in 10 ml of 5% of glacial acetic acid and store in a light protected bottle until used.

APPENDIX D

PRIMERS USED IN THIS THESIS

Random hexamer (Promega, USA)

RH (6mer)	5' - d(NNN NNN) - 3'

Sequencing primers (Promega, USA)

T7 Promoter (20mer)	5'- d(TAA TAC GAC TCA CTA TAG GG) - 3'
SP6 Promoter (19mer)	5'- d(GAT TTA GGT GAC ACT ATA G) - 3'

rPCR primers (Integrated DNA Technologies, Inc., USA)

UN-RH (26mer)	5' - d(GCC GGA GCT CTG CAG AAT TC-N NNN NN) - 3'
UN (20mer)	5' - d(GCC GGA GCT CTG CAG AAT TC) - 3'

RT-PCR primers (Integrated DNA Technologies, Inc., USA)

SD 900 (22mer)	5' - d(CAC TGC TAA GCA TTA TGC CTG T) - 3'
SD 540 (22mer)	5' - d(ATG GAA GAC TCT CCT GTG AAG A) - 3'
SD 100 (22mer)	5' - d(CAA AAC TAC AGT GGA TCC TAG T) - 3'

PCR Primers to detect phytoplasma (Integrated DNA Technologies, Inc., USA)

ASFWD (20mer)	5' - d(ACG AAA GCG TGG GGA GCA AA) - 3'
ASREV (20mer)	5' - d(GAA GTC GAG TTG CAG ACT TC) - 3'
LFWD (20mer)	5' - d(ACG ACT GCT GCT AAG ACT GG) - 3'
LREV (25mer)	5' - d(TGA CGG GCG GTG TGT ACA AAC CCC G) - 3'
NBFWD (21mer)	5' - d(TTA GGA CTC GGT CCT AGT TTG) - 3'
NBREV (21mer)	5' - d(CCA CTT ATG CAA GAG CCC CAA) - 3'

APPENDIX E

FLUORESCENT DYE PRIMER CYCLE SEQUENCING

1 Preparation of dsDNA template

The plasmid containing the template DNAs were prepared as in Section 2.2.10.b and further purified by PEG preparation (Applied Biosystems User Bulletin 18, 1991). The precipitated pellet was dissolved in 1 ml of SDIW. 0.25 ml of 4 M NaCl and 1.25 ml of sterile 13 % PEG 8,000 were then added. After thorough mixing, the solution was incubated on ice for 20 min and the plasmid DNA was pelleted by centrifugation at 10,000 *g* for 15 min at 4 °C. The supernatant was removed carefully and the pellet was rinsed with 2.0 ml of 70 % E-OH. The pellet was dried under vacuum for 3 min, resuspended in 10 ml of SDIW and stored at -20 °C.

2 Cycle sequencing reaction

Cycle sequencing of the dsDNA was done using the *Taq* dye primer cycle sequencing kit manufactured by Applied Biosystems Inc. (USA) and the recommended protocol (ABI dye primer cycle sequencing/Part No. 901482/Rev. B).

The following reagents were aliquoted into four 0.5 ml tubes:

REAGENT	A (µl)	C (µl)	G (µl)	T (µl)
d/ddNTP mix	1	1	2	2
Dye primer (0.4 pM/µl)	1	1	2	2
5x cycle seq. buffer (400 mM Tris-HCl, pH 8.9 at 24 °C, 100 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$)	1	1	2	2
DNA Template (200-250 ng/µl)	1	1	2	2
Diluted *Taq* DNA polymerase	1	1	2	2
Total Volume (µl)	1	1	2	2

The d/ddNTP mixtures contained the following:

A: 1.5 mM ddATP, 62.5 μM dATP, 250 μM dCTP, 375 μM C^7dGTP and 250 μM dTTP

C: 0.75 mM ddCTP, 250 μM dATP, 62.5 μM dCTP, 375 μM C^7dGTP and 250 μM dTTP

G: 0.125 mM ddGTP, 250 μM dATP, 250 μM dCTP, 94 μM C^7dGTP and 250 μM dTTP.

T: 1.25 mM ddTTP, 250 μM dATP, 250 μM dCTP, 375 μM C^7dGTP and 62.5 μM dTTP

The diluted *Taq* was prepared by mixing 0.5 μl of AmpliTaq® DNA polymerase (8 units/ml), 1.0 μl of 5x cycle sequencing buffer and 5.5 μl of deionized water.

The four reaction tubes above were overlaid with 30 μl mineral oil and placed in a thermal cycler (PTC-100™ Programmable Thermal Controller, MJ Research, Inc). The cycling was started as follows: 95 °C for 30 sec, 55 °C for 30 sec and 70 °C for 1 min for 15 cycles. Then it was continued for another 15 cycles at 95 °C for 30 sec and 70 °C for 1 min. At the completion of the run, the temperature was rapidly dropped to 4 °C. For best results, the total time required for 30 cycles should be 1 hr and 45± 5 min.

The extension reaction mixtures from the four tubes were pipetted out and combined into a 1.5 ml tube containing 80 μl 95 % ethanol with 1.5 μl 3 M NaAc, pH 5.3. The tube was placed on ice for 10-15 min and centrifuged at 10.000 *g* for 15-30 min. The supernatant was carefully discarded. The pellet was rinsed with 250 μl of 70 % ethanol, dried *in vacuo* for 1-3 min and resuspended in 6 μl of deionized formamide (Sambrook *et al.*, 1989) with 8.3 mM EDTA, pH 8.0. Insoluble materials were removed by brief centrifugation. The DNAs in the supernatant were denatured by heating at 90 °C for 2 min and loaded immediately on a pre-electrophoresed 6 % PAG with 8 M urea assembled in a ABI DNA sequencer, Model 373A (Operated by Dr N. Shirley of Dept. of Plant Science, WARI).

APPENDIX F

TABLE OF CHARACTERISTICS OF PLANT INFECTING VIRUSES (Murphy *et al.*, 1995).

FAMILY	*GENUS*	Size of particle (nm)	Shape of particle	Type of nucleic acid	Size of nucleic acid	Size of coat protein (kDa)
Geminiviridae	Subgroup I	18x 30	two incomplete icosahedral	circular ssDNA	2.5-2-8 kb	28-34
	Subgroup II	"	"	"	2.7-3.0 kb	"
	Subgroup III	"	"	"	2.5-2.8 kb	"
Nc	*Badnavirus*	60-900x 30	bacilliform	dsDNA	7.5-8.0 kbp	35-37
Nc	*Caulimovirus*	50	isometric	"	"	57
Reoviridae	*Fijivirus*	65-70	double shelled spherical	dsRNA	1.5-4.35 kbp	64-139
	Phytoreovirus	"	spherical	"	0.45-4.45 kbp	45-160
	Oryzavirus	57-65	spherical with spikes	"	3.75-27 kbp	32-125
Partitiviridae	*Alphacryptovirus*	30	isometric	dsRNA	1.7 and 2.0 kbp	55
	Betacryptovirus	38	"	"	2.1 and 2.25 kbp	Nr
Rhabdoviridae	*Cytorhabdovirus*	100-430x 45-100	bacilliform	ssRNA	11-15 kb	47-62
	Nucleorhabdovirus	"	"	"	"	"
Bunyaviridae	*Tospovirus*	90-100	spherical	ssRNA	11-20 kb	29
Nc	*Tenuivirus*	3- 10x ?	various filament	ssRNA	2.0- 10 kb	Nr

Sequiviridae	*Sequivirus*	30	isometric	"	9-12 kb	23, 26 and 32
	Waikavirus	"	"	"	11 kb	"
Comoviridae	*Comovirus*	28-30	icosahedral	"	3.5-7.2 kb	22-43
	Fabavirus	"	"	"	4.5-6.3kb	"
	Nepovirus	"	"	"	3.9-8.4 kb	55-60
Potyviridae	*Potyvirus*	11-15x 650-900	flexuous helical	"	8.5-10 kb	30-47
	Rymovirus	690-720x 11-15	"	"	8.5-10 kp	"
	Bymovirus	250-300 and 500-600	"	"	4.5 and 7.9 kb	"
Nc	*Sobemovirus*	30	icosahedral	"	4.2 kb	30
Nc	*Luteovirus*	25-30	icosahedral	"	5.6-5.8 kb	22
Nc	*Enamovirus*	25-28	"	"	2.7, 4.5 and 7.9 kb	21 and 54
Nc	*Umbravirus*	52	vacuole like	"	4.5	Nr
Tombusviridae	*Tombusvirus*	30	icosahedral	"	4-4.7	19-22
	Carmovirus	"	"	"	4.0	"
Nc	*Necrovirus*	28	"	"	3.8	29-30
Nc	*Dianthovirus*	32-35	"	"	1.5 and 3.9	37
Nc	*Machlomovirus*	30	"	"	4.4	25.1
Nc	*Tobamovirus*	300x 18	helical		6.4	17-18
Nc	*Tobravirus*	180-215 or 46-115x 21.3-23.1	helical		6.8, 4.5 or 1.8	22-24
Nc	*Hordeivirus*	110-150x 20	helical		3.8 and 2.8, or 3.3 and 3.2	22

Nc	*Furovirus*	250-300 or 92-160x 20	helical		5.9-7.1. 3.5-4.3 or 2.1-2.4	19.7-23
Bromoviridae	*Alfamovirus*			"	0.88, 2.5, 3.6 and 4.3	20-26
	Ilarvirus	26-35	spherical and isosahedral	"	0.85, 2.2, 2.7 and 2.9	"
	Bromovirus	"	"	"	0.87, 2.1, 2.8 and 3.2	"
	Cucumovirus	"	"	"	1.0, 2.19, 3.0 and 3.4	"
Nc	*Ideaovirus*	33	isometric	"	1, 2.2 and 5.4 kb	30
Nc	*Closterovirus*	1200-2200x 12	helical symmetry (very flexuous)	"	15.5-20	27-28
Nc	*Capillovirus*	640x 12	flexous filament	"	6.5	36
Nc	*Trichovirus*	640-800x 12	very flexuous filament	"	6.3-7.6	22-27
Nc	*Tymovirus*	30	icosahedral	"	6.3	20
Nc	*Carlavirus*	610-700x 12-15	helical symmetry (little flexuous)	"	7.4-8.5	31-36
Nc	*Potexvrius*	470-580x 13	flexuous helical	"	5.8-6.9	18-27
Nc	*Marafivirus*	28-32	icosahedral	"	2.0-2.4	27

Nc; Not classified

Nr; Not reported

APPENDIX G

Published work

1. Choi, Y.-G. and J. W. Randles (1995). Studies on the etiology of sugarcane striate mosaic disease. *Proceedings of the 10th Biennial Australasian Plant Pathology Society Conference* -August 1995, Lincoln, New Zealand. p 84

2. Choi, Y.G. and J. W. Randles (1996). Double-stranded RNA extraction with cellulose powder from plant tissue. In *Molecular Tools For Plant Pathogen Diagnosis*. Ed., R. A. J. Hodgson. Adelaide : Department of Crop protection, University of Adelaide.

3. Choi, Y.G. and J.W. Randles (1996). Sequences cloned from sugarcane striate mosaic disease affected plants indicate possible virus identity. *Proceeding of the 10th International Congress of Virology*, August 1996, Jerusalem, Israel. PW44-43.

4. Choi, Y.G. and J.W. Randles (1996). Microgranular cellulose improves dsRNA recovery from plant nucleic acid extracts. *BioTechniques*. (submitted).

The tenth Conference of the Australasian Plant Pathology Society Inc. held at Lincoln University, Christchurch, New Zealand from 28–30 August 1995

161. Studies on the etiology of sugarcane striate mosaic disease

Choi, Y.G., and Randles, J.W.
Department of Crop Protection, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, SA 5064, Australia

Sugarcane striate mosaic disease is a disease of unknown etilolgy of sugarcane in norther Queensland. Diseased plants show foliar symptoms, and both in the field and in glasshouse trials diseased plants are severely stunted. No potential causal agent has been detected by light or electron microscopy, but structural changes in tissue have been found to be associated with the disease. Analysis of nucleic acid components by gel electrophoresis showed that there were no detectable qualitative or quantitative effects of the disease on the composition of total nucleic acids of sugarcane leaf. A method for isolating and assaying dsRNA was developed and used to show that diseased sugarcane contains a unique dsRNA ca. 9kbp in size. Cloning of this component is in progress to determine whether it is related to host nucleic acids, or represents the replicative form of a possible virus agent of the disease.

Xth INTERNATIONAL CONGRESS OF VIROLOGY
JERUSALEM, ISRAEL, 11 - 16 AUGUST, 1996

PW44-43

Sequences cloned from sugarcane striate mosaic disease affected plants indicate possible virus identity

Choi, Y. G., and Randles, J. W.
Department of Crop Protection, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond. SA 5064, Australia

Sugarcane striate mosaic disease(ScSMD) is a disease of unknown etiology of limited distribution in northern Queensland. A disease associated dsRNA(ca. 9kbp) has been isolated by cellulose chromatography. Reverse transcription of dsRNA with a random hexamer linked to a "universal primer" produced a cDNA library. This library was then amplified by PCR primed with the "universal primer"(*P. Froussard, 1992, Nucleic Acid. Res. 20, 2900*). The PCR products were cloned and selection was done by hybridisation to the dsRNA. Four disease specific clones were sequenced . A data base search revealed that the dsRNA had the greatest sequence homology with the carlaviruses in the putative replicase gene. This is the first evidence of a carla-like virus infecting sugarcane.

Further work is in progress to isolate a putative virus particle and to develop a PCR based diagnostic method for ScSMD.

THE UNIVERSITY OF ADELAIDE
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Molecular Tools For Plant Pathogen Diagnosis

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Edited by Richard Hodgson

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RH

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Double-Stranded RNA Extraction From Plant Tissue With Cellulose Powder

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BACKGROUND AND COMMENTS

EXPERIMENT 03

In plants the usual from of RNA is as a single stranded (ssRNA) molecule. However, in virus infections double-stranded RNA (dsRNA) is often a characteristic feature. These dsRNAs are a feature viruses which contain either ssRNA or dsRNA genomes. They represent the replicative form of viruses.

The genome of viruses vary in their nucleic acid type, their size and number of segment, and these properties are often diagnostic for a virus species, group and family. Also, there are often subgenomic segments of the virus genome. Characteristic dsRNAs corresponding to virus genomic and subgenomic RNAs are detectable in infected plants. The most abundant dsRNAs usually correspond to the complete genomic nucleic acid.

Columns of fibrous cellulose powder (CF11) are commonly used for isolating dsRNA from plant tissue by chromatography using an ethanol and salt solvent in the column. In this report, the advantages of using thin layer chromatography grade (TLC) cellulose powder instead of using CF11 powder are described.

The principle of this method is to utilise the differential binding affinity of dsRNA, ssRNA and DNA for the cellulose powder. This differential binding is dependent upon the ethanol and salt concentration, such that elution occurs for DNA and ssRNA at ~20% ethanol while dsRNA elutes with ~15% ethanol. All nucleic acid is bound at 30% ethanol and none is bound at 5% ethanol. Thus, the chromosomal DNA and ssRNA of plant tissue can be effectively removed by washing with a salt buffer containing 15.5-17.5% ethanol. Another useful feature of dsRNAs is that they are resistant to some RNAse enzymes under high salt conditions (0.1-0.3M NaCl). The ssRNA is susceptible.

With the CF11 method, commercial columns can be used for the larger initial extraction but syringe columns are preferred in the second extraction step.

Comparison between CF11 and TLC extraction of dsRNA

	Method of Extraction	
	CF11	TLC
Yield	LOW	HIGH
Time	LONG (2 DAYS)	HALF DAY
ssRNA contamination	NO	YES
dsDNA contamination	VERY LOW	LOW
Usage	Good for analytical test, particularly on polyacrylamide gel	Good for preparation of samples over 2.0×10^6 in molecular weight

AIMS

1. Isolate from plant tissue the replicative form dsRNA of a ssRNA virus.
2. Preparation of disease associated pure dsRNAs for further studies.

REAGENTS

10X STE: (buffer for dsRNA extraction from common plant tissue): 1M NaCl, 100mM Tris-HCl pH 8.0 and 10mM EDTA

1X TESSa: (buffer for dsRNA extraction when polyphenol oxidase activity is expected in the tissue): 100mM NaCl, 50mM Tris-HCl pH 8.0, 10mM EDTA, 1% w/v sarkosyl and 1% v/v thioglycollic acid (optional)

Wash buffer: 17% ethanol, 1xSTE

Elution buffer: 5% ethanol, 1xSTE

Syringe column: 3, 5 or 10ml disposable syringe with a luer lock tip. Cut 2 sheets of miracloth® to fit into the bottom of the syringe and put some clear plastic tube over the luer tip

CF11 and TLC grade cellulose powder: Check the uniformity of powder because some batches can contain large granules which will bind protein when ethanol is added and the coagulated protein is hard to wash away (source; Sigma or Whatman)

PCA mix: Phenol/Chloroform/iso-amylAlcohol (25:24:1) washed with SW (store at 4°C).

CA mix: Chloroform/iso-amylAlcohol (24:1) washed with SW (store at 4°C).

Ethanol-KOH solution: (for removing RNAse from equipment): 90% v/v ethanol and 10% w/v KOH

DNAse-RF (RNase free): RQ1® (Promega) or RNase free DNase from other companies

Isopropanol: propan-2-ol (isopropyl alcohol)

SW: Sterile DEPC-treated distilled water

CF11 METHOD

❶ TISSUE PREPARATION

1. Remove the mid rib or thick veins from tough plant leaves (these will be hard to crush in the liquid nitrogen) and cut the tissue into small pieces. However, removing midribs is not necessary if liquid nitrogen is not used and tissue is processed directly in extraction solution. Keep tissue cool until used.
2. Put the sample immediately into liquid nitrogen if frozen tissue is used.
3. Crush and powder the tissue in a mortar with liquid nitrogen. Pour in buffer (10ml/1g) immediately and transfer into an appropriate screw cap tube resistant to phenol and chloroform.

❷ EXTRACTION OF TOTAL NUCLEIC ACID

1. Add 1 volume of PCA solution to the tissue slurry and mix thoroughly.
2. Incubate the mixture for 30min at room temperature with constant shaking
3. Transfer to a centrifuge tube and centrifuge at 10,000g x 20 min
4. Carefully transfer the supernatant into a fresh container and add 1 volume of CA solution. Mix well at room temperature for 10 min and centrifuge as above
5. Transfer the supernatant to a sterilised beaker or a centrifuge tube. Precipitate with 0.9 volumes of isopropanol or use directly for the **TLC cellulose method**

❸ dsRNA EXTRACTION (1st column)

1. MIXING - To the extract from ❷ part 5. add 200mg of CF11 for each 5g of original leaf tissue and mix well before adding ethanol
2. BINDING - Add ethanol to the mixture dropwise with constant stirring (It is very important to add ethanol drop by drop because, otherwise if the ethanol is added too quickly the dsRNA will not bind but protein and other nucleic acid will bind to the cellulose powder and a nucleic acid/protein complex will be precipitated)
3. Incubate the solution for 30 min at room temperature while stirring
4. Pour the slurry into a large column
5. WASHING - Wash the column with at least 500ml of Wash buffer/2g of CF11 powder (larger volumes are better)
6. ELUTING - At first, equilibrate with 1ml of 1xSTE per 1g of CF11, and then elute with 10ml of 1xSTE per 1g of CF11 (Force out all remaining solution out of the column)

❹ 2nd COLUMN

1. MIXING - To the eluted solution of 1st column add CF11 (200mg/to 5g equivalent of tissue)
2. BINDING- To this slurry stir in ethanol to 17% v/v and mix for 20min
3. COLUMN - Make a column with a syringe (~0.3g:3ml syringe, 0.3~1g:10ml and 1g:20ml or 30ml syringe) then pour in the slurry
4. WASHING - Rinse the column with Washing buffer (at least 100ml/0.1g of CF-11)
5. ELUTING - Equilibrate with 1ml of 1xSTE/0.2g of CF-11, and then elute with 9ml of 1xSTE.
6. PRECIPITATION - Precipitate by adding 1/10 volume of 3M Na acetate and 0.9 volume of isopropanol, and incubate for 4hr or overnight at -20°C

TLC METHOD

❶ TISSUE PREPARATION ❷ EXTRACTION OF NUCLEIC ACID METHOD

1. This procedure is the same as CF11 method to the end of step ❷ part 5. above
2. Precipitate the nucleic acid with 0.9 volume of isopropanol and incubate at -20°C for at least 2 hr or this sample can be stored at -20°C for future processing
3. Wash the pellet with 70% ethanol, pellet by centrifugation at 10,000g for 20 min. and air dry
4. Dissolve the dried pellet in 1.3 x STE with mixing for 3-5 hr at room temperature or 12 to 18hr at 4°C

❸ dsRNA EXTRACTION

1. Add TLC cellulose (10mg/extract from 5g tissue)
2. BINDING - While mixing the slurry add ethanol slowly to a final concentration of 17% then mix for a further 30 min
3. WASHING - Centrifuge at 10,000g x 1 min and pour off the supernatant
4. Wash at least eight times with 1 volume of Washing buffer by centrifugation as above.
5. After the last wash centrifuge to pellet the nucleic acid and dry, but do not overdry
6. ELUTION - Add 200µl of 1xSTE/10mg of TLC and incubate at room temperature for 30 min
7. Centrifuge the solution at 10,000g x 2 min
8. PRECIPITATION - Transfer the supernatant to a fresh centrifuge tube and add 0.1 vol of 3M Na acetate and 0.9 vol of isopropanol
9. Incubate at -20°C for 4hr or overnight, then centrifuge at 12,000g x 15 min and wash the pellet with 70% ethanol before drying the pellet

❹ DNAse-RF TREATMENT

1. ENZYME TREATMENT - Dissolve the pellet with 1X TE and treat with DNAse-RF.
2. EXTRACTION - with 1 volume of PCA then 1 volume CA
3. PRECIPITATION - Precipitate with 1/10 vol 3m Na acetate and 0.9 vol of isopropanol
4. GEL ANALYSIS - Analyse on polyacrylamide gel (5-10%) or agarose gel (0.5 ~ 1.2%), and stain with silver or ethidium bromide (polyacrylamide gel is preferred for analytical purpose).

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MICROGRANULAR CELLULOSE IMPROVES dsRNA ISOLATION FROM PLANT NUCLEIC ACID EXTRACTS

Yoon Gi Choi and John W Randles

Double-stranded RNA (dsRNA) is an important non-specific indicator of the presence of RNA viruses in bacteria, fungi and plants (1, 2). The dsRNA usually represents either the replicative form or the genome of an RNA virus, or it may be an intermediate in the replication of viroids or satellite RNAs. Its size(s) can provide specific information on the likely identity of a virus-like agent infecting a host, and successful isolation and purification of the dsRNA allows it to be cloned for use as a specific diagnostic molecular probe without the need for purifying the virus (3). Sequence analysis of clones provides information on the likely taxonomic position of a virus-like agent associated with the dsRNA (4) and can lead to identification of the virus.

The preferred method for dsRNA isolation uses differential absorption of the dsRNA fraction from nucleic acid extracts to chemically unmodified fibrous cellulose powder (eg. CF11, Whatman, Maidstone, England) in the presence of sodium chloride and at a specific ethanol concentration. This is followed by washing, elution in ethanol-free buffer, concentration and analysis by gel electrophoresis (1). During an investigation of a sugarcane disease of unknown etiology (sugarcane striate mosaic disease, ScSMD) in which a disease-associated dsRNA band of about 9 kbp was identified by the routine CF11 procedure, we found that the band intensity in analytical agarose and polyacrylamide electrophoretograms was low and variable. For further study, large amounts of infected leaf were required and dsRNA had to be isolated from large volumes of nucleic acid extract. We report here an improved method for dsRNA isolation which routinely increased yields in the sugarcane system, and suggest that it may be applicable to other systems in which the CF11 procedure is unsatisfactory.

Microgranular cellulose has been used previously to recover dsRNA from dissolved polyacrylamide gel (5). We therefore tested a more finely divided form of cellulose as a replacement for CF11 cellulose. We used a thin-layer chromatography grade of microgranular cellulose (MN 300 cellulose powder; Macherey-Nagel GmbH & Co. KG, Düren D-5160, Germany). Direct substitution of the MN 300 cellulose powder for CF11 cellulose in a modification of the column purification method of Dodds (1) was not successful because of extremely low flow rates through the chromatography column. However, when a batch method was used (Table 1), the yield of 9 kbp dsRNA was consistently at least 10-fold higher than for the CF11 column method (Figure 1). The MN 300 powder also recovered dsRNA from *Nicotiana tabacum* infected with tobacco mosaic virus and *N. glutinosa* infected with either alfalfa mosaic virus (Figure 1, lane 4) or

cucumber mosaic virus at a higher yield than the CF11 column method. A DNase digestion step was included routinely in this procedure to remove host DNA which tended to co-migrate with the 9 kbp dsRNA.

Single-stranded RNA contaminants were obtained with both cellulose materials and these interfered with the detection of dsRNA smaller than 4 kbp (Figure 1, lanes 2 & 3). These could be removed either by predigestion of the sample with ribonuclease A (Sigma Chemical Co., St. Louis)(50 ng/ml in 0.3 M NaCl, 0.1 M Tris-HCl, pH 7.0, at 37 °C for 0.5-1 hr) or by an absorption-elution cycle on a CF11 cellulose column (1). RNase A treatment allowed minor subgenomic dsRNAs of 6, 2.6 and 2.5 kbp in size (which are not visible in Figure 1) to be seen only in the MN300 cellulose prepared samples when they were fractionated on agarose gels and stained with ethidium bromide.

The yields of 9 kbp dsRNA from the two methods were estimated after the absorption-elution cycle on a CF11 cellulose column (as above). A dilution series of each preparation and a marker dsDNA were dotted onto and absorbed into agarose gel, and their staining intensity with ethidium bromide compared (6). From 50 g of sugarcane leaf, the CF11 cellulose method yielded 1-5 ng of 9 kbp dsRNA whereas the MN300 microgranular cellulose method yielded 50-100 ng.

Further purification of the dsRNA for randomly primed reverse transcription PCR and cloning was done by eluting it after the agarose gel electrophoresis step (Table 1), treating it with RNase in high salt as above, fractionating it by polyacrylamide gel electrophoresis and eluting it from the gel. Hybridization analysis showed that the PCR products and clones (up to 1.2 kbp in size) were specific to the dsRNA (unpublished result).

We conclude that this procedure is particularly useful for the efficient binding and recovery of dsRNA from the initial large volumes commonly obtained when extracting nucleic acids from plants, and when the content of dsRNA relative to cellular nucleic acids is expected to be low. In our hands, the greatly increased yields of ScSMD-associated dsRNA obtained by this method have made it possible to further purify, clone and sequence virus specific sequences and thus determine the etiology of this disease (unpublished results). We believe that this modification of a routine method may have general application to other systems in which dsRNA analysis is essential.

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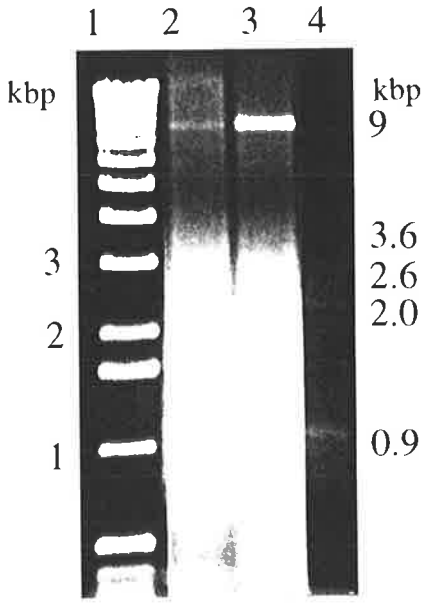
Figure 1. **A typical agarose gel electrophoretic analysis of dsRNA extracted with microgranular cellulose.** dsRNA obtained from 50 g of ScSMD affected sugarcane leaf either by a single cycle of CF11 cellulose column chromatography (lane 2) or by the microgranular cellulose batch method (lane 3) and analysed by 0.9 % agarose gel electrophoresis with ethidium bromide staining. DNA molecular weight markers (GibcoBRL, Gaithersburg; 1 kbp ladder) are shown in lane 1, and dsRNA isolated by the microgranular cellulose method from alfalfa mosaic virus infected *Nicotiana glutinosa* and treated with RNase (see text) is shown in lane 4.

Table 1. Microgranular cellulose batch procedure for dsRNA isolation from leaf

- Powder frozen tissue by blending in liquid nitrogen
- Add 4 volumes (w/v) of STE (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl and 10 mM EDTA) containing 1 % SDS and 1 % monothioglycerol
- Stir mixture at room temperature for 30 min
- Add 0.5 volume of phenol-chloroform (1:1) and emulsify for 30 min
- Centrifuge (10,000 *g*, 10 min), collect the aqueous phase after centrifugation and precipitate nucleic acids with 0.8 volumes of *iso*-propyl alcohol
- Collect the nucleic acid pellet, dissolve in 1.3x STE (at 0.8 ml/3 g of leaf extracted) and clarify by centrifugation (10,000 *g*, 20 min)
- Add microgranular cellulose to the supernatant (at 10 mg/3 g of leaf extracted) and mix for 30 min
- Add ethanol drop wise to 20 % with constant vigorous stirring and mix for 30 min
- Collect the cellulose by centrifugation (10,000 *g*, 2 min)
- Wash with STE containing 16.5 % ethanol (5 cycles of centrifugation for 0.5 min alternating with discarding and replacement of the supernatant)
- Drain and air-dry the cellulose pellet
- Elute with STE (100-200 µl/10 mg of cellulose) and remove cellulose by centrifugation (10,000 *g*, 5 min)
- Digest with RNase free DNase (RQ1, Promega Corporation, Madison) in the recommended buffer (100 units/ml, 37 °C, 60 min)
- Extract with 1 volume of phenol-chloroform and precipitate with *iso*-propyl alcohol, as above
- Analyse by agarose gel electrophoresis


1
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CORRIGENDUM

Fig. 4.1 Sizes of the scale bars are 50 µm in A, B, E, F, G and H, and 10 µm in C and D.

Fig. 4.2 The size of the scale bars is 500 nm.

Section 5.3.1 Line 4 should be written as "The lower nucleic acid content of lane 2 compared with lane 1 (Fig. 5.1) was noted but no qualitative differences were observed between diseased and healthy sugarcane."

Fig. 5.1 The size of TMV RNA is 6.3x 10^6, AMV RNA1 is 3.2x 10^6, and CMV RNA3 is 2.1x 10^6.

Fig. 5.3 Add following sentence to the end of the figure legend. "Promega RNA mol. wt. markers were used (Appendix A). In addition, tobacco mosaic virus dsRNA (6.3 kbp) and potyvirus dsRNA(9.4 kbp) were used to estimate the size of the ScSMD associated dsRNA.

Fig. 5.6 Label of "2.6" and "2.5" should be reversed in the figure.

Fig. 5.7 The following information should be added in the legend,
"(A) lane 1- Purified ScSMD associated dsRNA from the gel (A) in fig. 5.6.
lane 2- DNA 1 kbp marker.
(B) lane 1- DNA 1 kbp marker
lane 2- Purified 9 kbp dsRNA from the gel A.

Section 5.3.4 Add sentence to the section as follows "The probe made from the 9 kbp dsRNA hybridised to it self but not to TNA of healthy sugarcane."

Section 6.2.2 Change section heading to "Synthesis of cDNA from purified ScSMD associated dsRNAs with rPCR and cloning of the rPCR products."

Table 6.1 Change table heading to "Relationships among the ScSMD specific clones."

Section 7.2.1 "All plants were glasshouse grown." should be added at the end of the section.

Section 7.2.2.a "DA4" should read "DA14" in line 7 of ~~the second parpagraph.~~ page 59

Fig. 7.2 "Magnification of (A) is 42,000x, and (B) is 17,000x" should be added.

Fig. 7.6 Add the following information to the figure legend.
lane 4 - 0.01 ng of ssRNA purified from ScSMV was used as a template.
lane 5 - 1.00 ng of ssRNA purified from ScSMV was used as a template.
lane 6 - 100 ng of ssRNA purified from ScSMV was used as a template.

Section 8.2.3.c Add following sentence to the beginning of the section "ScSMD clones (DA2, 10,14 and 17) were selected because they had different sequence similarities to ScSMV RNA and specific to ScSMD. The selected clones were transcribed by DNA polymerase I with random primers and labeled with ^{32}P."

Fig. 9.2 The bars represent "500" nm.