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CHARACTERIZATION OF dsRNA SPECIES IN TOBACCO EXHIBITING NON-GEMINIVIRUS LEAF CURL SYMPTOMS

JENNIFER CALVERT-EVERS

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science.

Johannesburg 1996

ABSTRACT

Tobacco in Southern Africa has been found to exhibit three forms of leaf curl (class I, II and III) based on symptomology. Class I tobacco leaf curl is a disorder of unknown etiology, appearing as bent or twisted leaves exhibiting frilly enations (leafy outgrowths) along the midrib and secondary veins of severely stunted tobacco plants. The disease was in the past thought to be attributed to a complex of virus strains, including a geminivirus transmitted by the whitefly, *Bemisia tabaci*. However all attempts to purify virus or virus-like particles from symptomatic leaves have been unsuccessful. Research carried out more recently has revealed the consistent detection of at least ten double-stranded RNA species (*c.*1.1 to 4.8 kilobase pairs) in leaf extracts of tobacco plants exhibiting class I leaf curl symptoms. Also of unknown etiology, though reported to be associated with two dsRNA species (*c.* 4.2 and 4.6 kilobase pairs), is the silverleaf syndrome of squash *(Cucurbita pepo)* which is induced by the feeding of nymphs of the whitefly, *Bemisia tabaci*, Biotype B. In both disorders, the non-endogenous nature of the detected dsRNA species has led to the speculation that the causal agent may be a virus or a viruslike agent, either carried by or in the whitefly. The aims of this research were to further characterize the dsRNA species in class I tobacco, to ascertain if these dsRNAs are associated only with class I leaf curl etiology and if there is a relationship between suspected whitefly-induced tobacco leaf curl and silverleaf etiology. This study confirmed the presence and double-stranded nature of the RNA species present in partially purified preparations of class I tobacco leaf curl and whitefly. In contrast, no dsRNA species were observed in class II and class III tobacco leaf curl or silverleaf squash extracts. Although dsRNA species were detected in two cultivars of healthy squash, these were thought to be either cellular dsRNA of unknown origin or dsRNA of a cryptic virus or latent viruslike agent. Using an oligo(dT) trapping method, it was established that the dsRNA species isolated from the class I tobacco leaf curl do not have a poly(A)$^+$ tail, and thus are not

considered to be inactivated mRNAs possibly associated with modified proteins induced by whitefly feeding. Results obtained from hybridization studies, using specific labelled dsRNA bands isolated from tobacco class I leaf curl extracts as probes, showed n/ detectable levels of sequence homology between class I leaf curl and the two dsRNA species (c.4.2- and 4.6-kbp) reported to be associated with silverleaf whitefly and whitefly-induced squash silverleaf. Although two class I dsRNA probes (3.6- and 3.3-kbp) hybridized weakly to class II and class III tobacco leaf curl extracts, it was suspected that a TMV-contamination of both probe and plant material have been responsible. With the exception of dsRNA-3 .Kbp probe cross-reacting with the dsRNA-2.4kbp band, the dsRNA bands did not hybridize with each other, nor with healthy tobacco, healthy squash, squash silverleaf or whitefly extracts. It would appear therefore that the dsRNA molecules are unique sequences of a multipartite genome. The striking similarities between class I tobacco leaf curl symptoms and dsRNA pattern with plant-infecting reoviruses warrant further investigation.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted in partial fulfilment of the requirements for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Jennifer Calvert-Evers

this 22ND day of FEBRUARY 1996.

DEDICATION

For my parents, for believing in me; my son, Matthew, for his understanding and patience during this year of study; and my husband, Maurice, without whose constant encouragement and support this work would not have been possible.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to :

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LIST OF ABBREVIATIONS

AMV	avian myeloblastosis virus
bp	base pair/s
c.	about
°C	degrees Celcius
cm	centimetres
cv	cultivar
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminence
EDTA	ethylenediamine tetra-acetic acid
et al.	and others
FDV	fiji disease virus
Fig./s	figure/s
g	gram/s
h	hour/s
kbp(s)	kilobase pair(s)
LEV	*Lolium* enation virus
LMT	low melting temperature
MRDV	maize rough dwarf virus
M	molar
µg	microgram/s
µl	microlitres
mg	milligrams
min	minute/s
ml	millilitre/s
mm	millimetre/s

mM	millimolar
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MW	molecular weight
ng	nanogram/s
nm	nanometre
RDV	rice dwarf virus
RF(s)	replicative form(s)
RBSDV	rice black-streaked dwarf virus
RGDV	rice gall dwarf virus
RNA	ribonucleic acid
RRSV	rice ragged stunt virus
rpm	revolutions per minute
RLCD	rugose leaf curl disease
sec	second/s
ss	single-stranded
SSC	Sodium chloride-sodium citrate
STE	Sodium chloride-Tris-EDTA
TBE	Tris-boric-EDTA
TE	Tris-EDTA
TLC	tobacco leaf curl
TLCD	tobacco leaf curl disease
TLCV	tobacco leaf curl virus
TMV	tobacco mosaic virus
TRNA	total ribonucleic acid
v/v	volume per volume
WTV	wound tumor virus
w/v	weight per volume

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CHAPTER 1: INTRODUCTION

1.1 TOBACCO LEAF CURL DISEASE

1.1.1 A Brief History

Leaf curl was first noted in 1912 by Peters and Schwartz in the Netherlands East Indies, however according to Lucas (Hill, 1968), a disease tentatively identified as leaf curl was reported from South Africa by Lounsbury in 1902. Later, two independent workers, Story (1931) in Tanganika and Thung (1932) in Java, almost simultaneously announced that the disease was induced by the tobacco leaf curl virus (TLCV) transmitted by the whitefly, *Bemisia tabaci* (Lucas, 1975). The viral particle structure and composition were eventually characterized in Japan by Osaki and Inouye in 1978 (Osaki and Inouye, 1981), but their findings have never been confirmed by other workers.

1.1.2 Distribution

Tobacco leaf curl occurs mainly in tropical and sub-tropical countries but has been reported in temperate regions such as Japan and parts of Europe and USA (CMI/AAB, 1981). It was, at one time, considered one of the most destructive tobacco diseases in East Africa, Zimbabwe (then Rhodesia) and the Transvaal (Lucas, 1975). Although less prevalent today as a result of effective control measures, leaf curl still occurs in many parts of the world, especially in Venezuela (Gooding, 1991) and India (Valand and Muniyappa, 1992), causing substantial yield losses.

1.1.3 Leaf Curl Symptoms

Numerous workers have classified the different forms of leaf curl based on the description of symptoms displayed by the infected tobacco plant (Pal and Tandon, 1937; Wolf *et al.*, 1949; Osaki and Inouye, 1981; and Valand *et al.* 1992). Five forms of South African leaf curl were originally recognized by McClean in 1940: severe leaf curl, mild strains A, B and C, and latent leaf curl. These forms differed from each other in their ability to induce enations and/or vein thickenings and in the severity and intensity of the symptoms they produced in tobacco. The classification used in this study is based on work carried out by Paximadis (1995), who reclassified leaf curl symptoms by placing them into three classes according to the range of symptoms they displayed and possible causal agent (s).

1.1.3.1 Class I (*N.tabacum* cv TL 33)

This class exhibits the most characteristic features of severe leaf curl, viz. twisted, deformed and stunted leaves (Fig.1.1) which have a thick, leathery texture and develop prominent leafy or "cup-like" outgrowths (enations) along the veins on the lower surface (Fig.1.2); vein thickenings and puckering on the ventral surface (Fig.1.3). Plant height is reduced with the uppermost leaves of the plant being more severely affected (Fig.1.4).

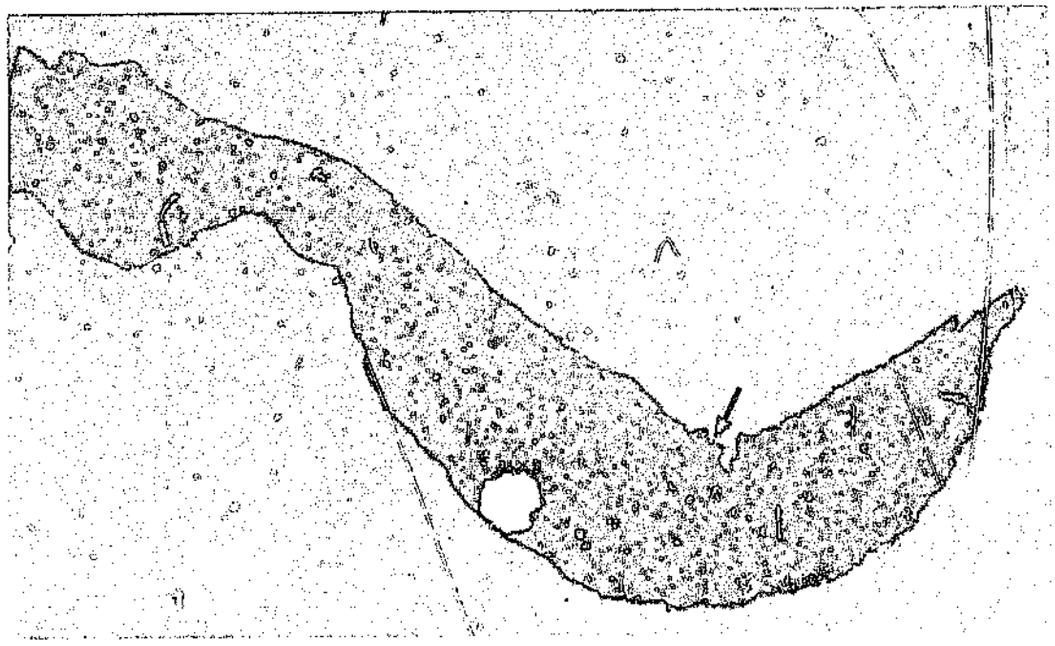


Fig.1.1 *N. tabacum* cv TL 33 exhibiting typical class I leaf curl symptoms *viz.* downward curling of the margins of twisted, deformed and stunted leaves and the development of leafy enations (arrow) along the veins on the lower surface.

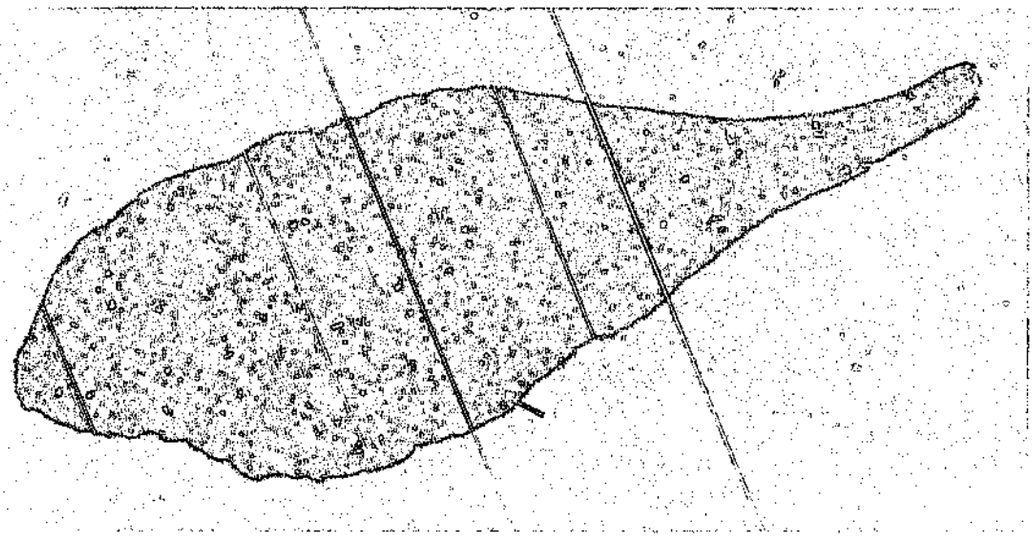


Fig. 1.2 "Cup-like" leafy outgrowths (enations) on the midrib vein and dark vein thickening (arrow) on the under surface of a tobacco leaf collected from *N.tabacum* cv TL 33.

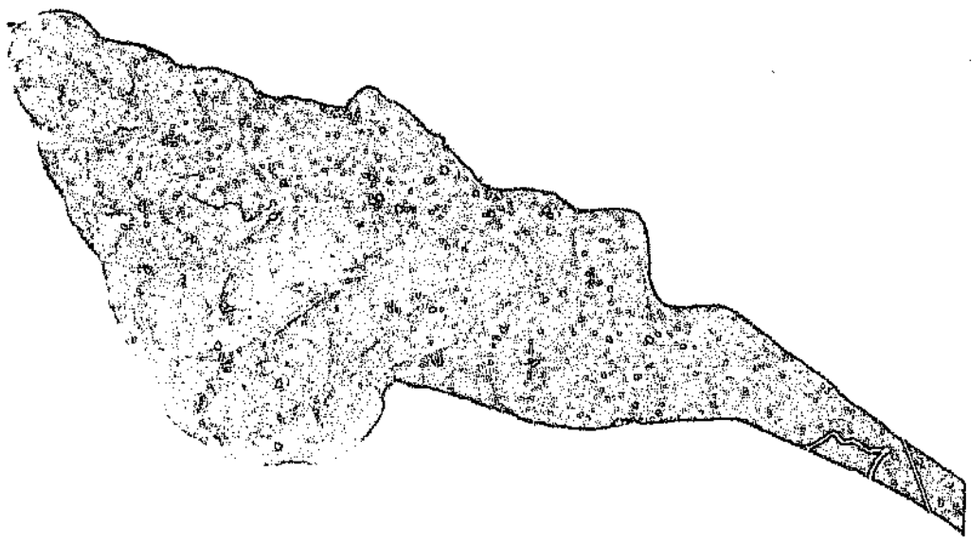


Fig.1.3 Ventral surface of *N.tabacum* cv TL 33 showing dark green vein thickening and puckering of the leaf margin.

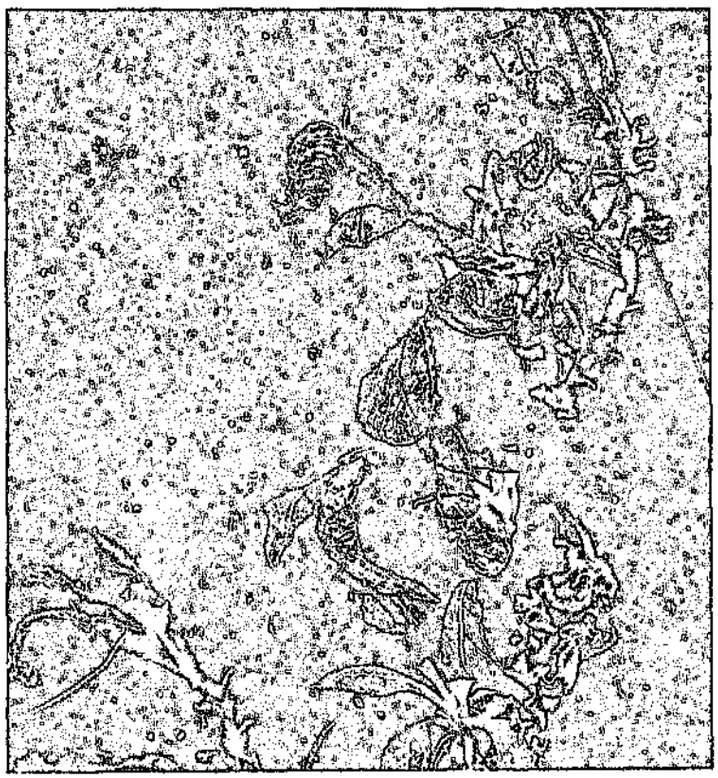


Fig. 1.4 Infected *N.tabacum* cv TL 33 plant. Uppermost leaves appearing more severely deformed and twisted.

1.1.3.2 Class II (*N.tabacum* cv Mammoth 10)

The margins of leaves of plants exhibiting class II symptoms roll upward with the tips occasionally curling or twisting. Prominent enations appear on the midribs and secondary veins, giving the leaf a "velvet-like" appearance. Leaves are also slightly puckered on their dorsal surface (Fig.1.5).

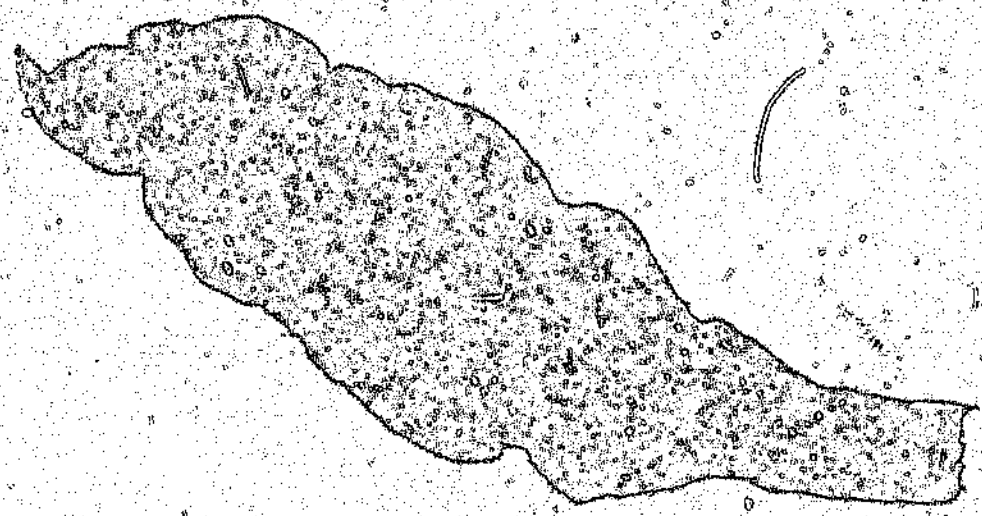


Fig.1.5 *N.tabacum* cv mammoth 10 exhibiting typical class II symptoms *viz.* the upward curling of the margins of the leaf and numerous leafy enations along the midrib and secondary veins .

1.1.3.3 Class III (*N.tabacum* cv HG)

Class III plants vary in their general appearance. Leaves differ in the severity of their symptoms from small thickenings on the veins and mild puckering (Fig.1.6) to the downward curl of the entire leaf, with irregular leaf margins.

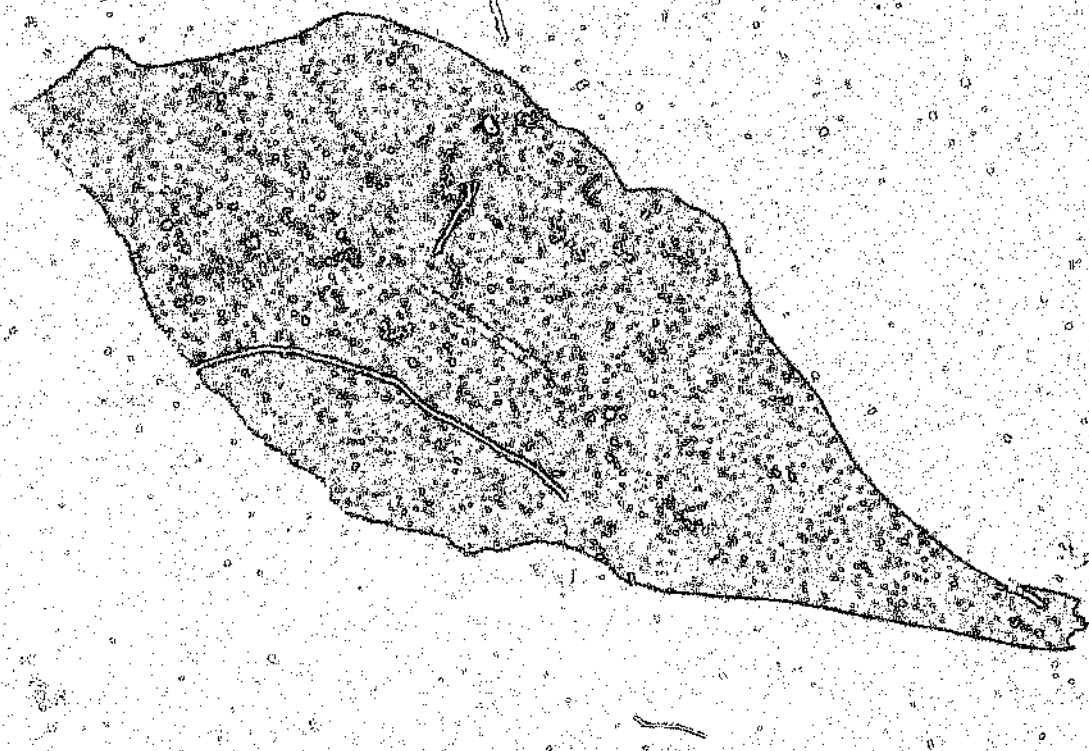


Fig.1.6 *N.tabacum* cv HG exhibiting mild class III leaf curl symptoms : slightly wrinkled and puckered leaf margins and absence of enations along the midrib and secondary veins.

1.1.4 Leaf Curl Etiology

Although it has been reported that a virus was involved in the etiology of leaf curl symptoms, there has been only one successful virus extraction to date (Osaki and Inouye, 1961). Geminate particles (c. 18 x 30 nm in size) consisting of two incomplete T=1 icosahedral structures, with one shared subunit, were detected in partially purified preparations of TLCD-infected tobacco plants. Electron microscopy of the virus nucleic acid revealed small circular molecules, suggesting that the virus particles, like those of some other geminiviruses, contain circular DNA molecules. Despite numerous attempts by many different workers in South Africa to extract and characterize virus particles from tobacco class I leaf curl plants, these, too, were unsuccessful (Thatcher, 1976; Forster, 1990; Duyver, 1990; and Paximadis, 1995). This led to the initial conclusion that the causal agent (s), must be present in low concentrations or are unstable *in vitro*. However, research carried out by Paximadis (1995) showed that not all forms of leaf curl are, in fact, caused by viruses. Class I leaf curl disease was found not to be associated with a geminivirus, and although a potyvirus (potato virus Y strain) was detected in a large number of tobacco plants exhibiting class I leaf curl, it was shown to be a latent infection not contributing to class I leaf curl symptoms. The consistent presence, however, of at least ten dsRNA bands from class I leaf extracts raised the question of whether these dsRNA species were in some way associated with class I leaf curl symptomatology. Class II leaf curl was found to be associated with neither a geminivirus nor potyvirus infection. It appeared that class II leaf curl symptoms were transmitted through seed, and may be associated with a seed-transmitted agent or a genetic disorder of tobacco, once described by McClean as "curly-leaf", an inherited peculiarity of tobacco (McClean, 1940). Class III leaf curl was positively associated with a whitefly-transmitted geminivirus infection. Symptom variation observed in this class may be attributed to

the presence of more than one geminivirus strain, but this is still to be unequivocally demonstrated.

1.2. WHITEFLY AS A DISEASE-INDUCING AGENT

It is well established that the whitefly *Bemisia tabaci* (Gennadius) transmits a number of bacterial, fungal and viral plant diseases, affecting a wide range of ecomonically important crops (Costa, 1976; Bharathan *et al.*, 1990; Jiménez *et al.*, 1995). It is also the only known whitefly vector of the geminivirus which is responsible for class III tobacco leaf curl ((Brown, *et al.*, 1992; Paximadis, 1995). Although it has not been unequivocally established that a virus is not responsible for class I leaf curl etiology, an investigation of the role of whitefly as a disease- or symptom-inducing agent is warranted, especially since biotype B of the genus *B.tabaci*, has been reported to have the ability to produce a "silverleaf" syndrome of squash plants after exposure to the whitefly feeding (Brown *et al.*, 1992) and since two sizes of dsRNA (*c.*4.6- and 4.2- kbp) were consistently observed in leaf extracts of whitefly-infested, symptomatic tobacco plants (Bharathan *et al.*, 1990). Squash silverleaf (SSL) symptomatology includes an initial veinal chlorosis of leaves, followed by the progressive silvering of the interveneal tissue which appears only in new leaves that develop following whitefly infestation. When the whitefly insects are removed, affected leaves retain their silvering, but new growth returns to normal. This lead to the speculation that SSL was caused by a translocated toxicogenic factor associated with whitefly feeding (Yokomi *et al.*, 1990).

The purported finding of dsRNAs (*c.* 4.6- and 4.2- kbp) and increased RNA-dependent RNA polymerase activity in leaf extracts from infected silverleaf squash plants led Bharathan *et al.* (1990) to suggest that the causal agent was a virus or viruslike agent, either carried or transmitted by the whitefly since no dsRNA species were found in asymptomatic leaves of squash plants. Yokomi *et al.* (1990) confirmed the presence of these dsRNA species from nymph-infested leaves of SSL-affected plants. Identical size dsRNA bands were detected from both nymph and adult *B.tabaci.* The relationship between the whitefly and SSL suggested that a toxicogenic factor associated with nymphal feeding may be causing SSL. The role of the dsRNA in SSL etiology is not yet understood. More recently, Jiménez *et al.* (1995) presented evidence that whitefly-induced SSL is a host-specific response to the silverleaf whitefly. SSL symptomatology was accompanied by an alteration in the production of proteins. Two new intercellular fluid proteins were induced in silverleaf tissue and the constitutive expression of another protein was suppressed. It has, therefore, been suggested that the induction of these intercellular fluid proteins are in response to insect attack; however, the possibility of a translocated whitefly-borne toxin or virus cannot, at present, be precluded. While it is well recognized that whitefly-mediated squash silverleaf is induced by the feeding of nymphs of the silverleaf whitefly, the mechanism involved in symptom expression is unknown. Yokomi *et al.* (1995) tested several plant biochemical regulators to determine whether they could mitigate expression of squash silverleaf in *C.pepo.* Application of a gibberellic acid biosynthesis inhibitor, such as chlormequat chloride, induced leaf silvering symptoms similar to those induced by the silverleaf whitefly in squash plants. Although phenotypically similar, plant bioregulator-induced and whitefly-induced leaf silvering were accompanied by different physiological responses. Chlormequat chloride did not produce detectable dsRNAs or changes in intercellular fluid proteins, whereas whitefly-mediated squash silverleaf

extracts were found to possess two induced intercellular leaf proteins. Although the dsRNA species, previously reported by both Yokomi (1990) and Bharathan (1990, 1992), were detected in silverleaf whitefly extracts, no dsRNAs were detected in whitefly-induced silvered tissue free of nymph or adult whitefly. The finding that bioregulators could induce leaf silvering without dsRNAs led these investigators to speculate that leaf silvering may be the result of hormonally-mediated alterations in the plant's physiology. In addition, hormonal inhibition was thought to be a general component of whitefly phytotoxicity since silverleaf whitefly also induces discolouration disorders in plants other than squash.

1.3 DOUBLE-STRANDED RNA IN PLANTS

1.3.1 dsRNA in Healthy Plants

Healthy plants grown from seed are generally free of detectable amounts of endogenous high molecular weight dsRNA. However, the possible presence of cryptic viruses or latent viruslike agents in apparently healthy plants must be considered as these cause mild or no visible symptoms. They are transmitted efficiently in pollen and seed but are not transmitted mechanically or by vectors (Jordan *et al.*, 1983). Although they occur in very low concentrations in infected plants, cryptic viruses have molecular characteristics that might be expected of disease-producing viruses. The genome of cryptic viruses consists of 2-5 dsRNA segments which have been translated *in vitro*. (Matthews, 1991). Other than unclassified cryptic viruses, the only other kind of dsRNA which may be encountered in healthy plants is cellular dsRNA of unknown origin. These are nonviral and are usually present in specific cultivars or plant species but not

detected in others (Jordan *et al.*, 1983; Valverde *et al.*, 1986). The presence of cellular dsRNA in healthy plants can led to misinterpretations of results, which emphasizes the importance of appropriate healthy controls (same plant or cultivar species) to show that endogenous dsRNA, which has been found in tissues of certain plant species not infected with RNA viruses, is not present in tissues from healthy plants. The functional role and significance of dsRNA species in healthy plants is largely unknown, though it has been proposed that a regulatory system may be involved. There have been numerous reports concerning the biological control of activities such as the down-regulation and expression of different genes by naturally occurring anti-sense RNAs (Simmons, 1988; van der Krol *et al.*, 1988). Through Watson and Crick base-pairing, anti-sense RNAs bind to their complementary target mRNAs, thus controlling their function and expression. The consequences of anti-sense RNA binding may involve the inhibition of ribosome binding, resulting in a message not being translated efficiently. Such biological controls may be exerted at many levels, having both direct and long-range effects. Although in most documented cases, the anti-sense RNA exerts negative control, it is plausible that anti-sense binding could increase target function and/or expression.

1.3.2 dsRNA in Plant Viral Disease

The most common kind of dsRNA detected in infected plant cells is the replicative form (RF) of single-stranded RNA viruses which compose approximately 90% of all known plant viruses. This form of dsRNA is the intermediate product produced during replication and is consistently detected when a plant is infected with an ssRNA virus, regardless of the host. Replicative intermediate (RI) forms consisting of dsRNA that are

partially single-stranded may also be produced, however these are more susceptible to RNase degradation (Valverde, 1990). Replicative dsRNA has also been identified for satellites associated with RNA plant viruses and may even occur in greater concentrations than the virus-specific RNA itself (Schneider *et al.*, 1977). The only other characterized dsRNA form found in infected plant tissue belongs to the genera *Fijivirus* and *Phytoreovirus* of the family *Reoviridae* (section 1.3.3).

1.3.3 Plant Reoviruses

1.3.3.1 Introduction

Members of the *Reoviridae* family are distributed throughout living organisms. Among their natural hosts are vertebrates, insects and plants. They differ widely in the virulence that they exhibit in these hosts. The first discovered mammalian reovirus literally is, as the name signifies, a "respiratory enteric orphan", meaning a virus unassociated with disease. However, more recently discovered members of this family, the rotavirus, was found to cause infantile gastroenteritis, one of the most important causes of death in infants under the age of two year in less developed countries (Steele, 1990). In the early 1960s, Gomatos and co-workers showed that the nucleic acid in reovirus particles is dsRNA, thus providing the first demonstration of the occurrence of dsRNA in nature (Francki *et al.*, 1983). Although dsRNA was being investigated as the replicative form of viral genomes consisting of single-stranded RNA, this discovery identified the source of the dsRNA. Equally important was the finding that the reovirus genome consists of 10 or 12 discrete segments, each with its specific sequence content and each transcribed into its own messenger RNA. Since then, a number of viruses were shown to possess

a multisegmented dsRNA genome and have been classified as members of the *Reoviridae* family . The genera and host ranges are listed in table 1.1 (Joklik, 1983).

Table 1.1 Host ranges of members of the family *Reoviridae*

Genus	Host Range
Orthoreovirus	Vertebrates
Orbivirus	Vertebrates, insects
Rotavirus	Mammals
Cypovirus	Insects
Phytoreovirus	Plants, insects
Fijivirus	Plants, insects
Presently unassigned viruses	Insects, fish, oysters

1.3.3.2 Members of the Plant *Reoviridae*

Plant-infecting reoviruses contain genomes consisting of either 10 or 12 distinct segments of dsRNA and are transmitted by either leafhopper or planthopper vectors. These two criteria form the primary basis for dividing this group of viruses into two genera, *Phytoreoviruses* and *Fijivirus*. The genome of Phytoreoviruses consists of 12 dsRNA segments, which are transmitted by leafhoppers (Cicadellidae, Jassoidea) and infect a variety of hosts including dicot- and monocotyledons. Fijiviruses, however, have a genome consisting of 10 dsRNA segments, are plant hopper-transmitted (Delphacidae, Fulguroidea) and infect only monocotyledons (Gramineae), in which they induce neoplastic growths that have been referred to as vein swellings, galls, or enations by various authors. Table 1.2 lists some of the the members of the *Reoviridae* that infect plants (Francki *et al.*, 1983).

Table 1.2 Members of the plant reoviruses*

Virus	Host (s)	Vector(s)
Genus *Phytoreovirus*		Leafhoppers
1. Wound Tumor (WTV)	*Trifolium incarnatum*	*Agellia constricta*
	Melilotus alba	*A. quadripunctata*
	M.officinalis	*Agalliopsis novella*
2. Rice Dwarf (RDV)	*Oryza sativa*	*Nephotettix cincticeps*
	Echinoclos crus-galli	*N. apicalis*
3. Rice gall Dwarf (RGDV)	*Oryza sativa*	*Nephotettix nigropictus*
		N. cincticeps
Genus *Fijivirus*		Planthoppers
1. Fiji disease (FDV)	*Saccharum officinarum*	*Perkinsiella saccharicida*
		P. vastatrix
		P.vitiensis
2. Maize rough dwarf (MRDV)	*Zea mays*	*Laodelphax striatellus*
	Digitaria sanguinalis	*Delphacodes propinqua*
3. *Lolium* enation (LEV)	*Lolium* spp.	*Javesella pellucida*
Unclassified		
1. Rice ragged stunt (RRSV)	*Oryza* spp.	*Nilaparvata lugens*
2. Italian ryegrose leaf curl disease (RLCD)	*Trifolium pratense*	*Austroagallia torrida*

* Only members of the plant reoviruses pertinent to the text are listed.
(Adapted from The *Reoviridae*. Francki, R.I.B and Boccardo, G. 1983).

1.3.3.3 Properties of Plant Reoviruses

Several of the leafhopper- and planthopper-borne diseases, now known to be caused by viruses that belong to *Reoviridae*, were studied for many years without their etiological agents being recognized. It was only after the development of suitable purification techniques that the viruses were isolated and characterized. The first two plant reoviruses to be purified and studied were wound tumor virus (WTV) in America (1954) and rice dwarf virus (RDV) in Japan (1959). Subsequent studies have shown that several other plant viruses, such as maize rough dwarf virus (MRDV) and Fiji disease virus (FDV), possess reovirus-like particles and dsRNA (Francki *et al*, 1983). However, research progress on the plant reoviruses has been generally slow, mainly due to technical difficulties associated with studies of these viruses, as they usually only replicate in vascular or neoplastic tissue and they cannot be transmitted by mechanical inoculation. In addition, their transmission is dependent on insect vectors, in which they multiply. Although the viruses multiply in their vectors, no neoplastic growths like those in plants are produced. However, cytopathological effects similar to those induced in plants cells have been observed in organs of various hosts. All plant reoviruses have a similar appearance in thin section of infected cells. Reovirus particles are large, about 70 nm in diameter, and characteristic enough to be detected and identified in infected cells by electron microscopy. They have double shells of icosahedril symmetry, with densely staining inner cores due to the presence of dsRNA. Except for FDV, some of the 70-nm virus particles may be found enclosed in long tubules of unknown origin and function. However, the majority of these particles are usually scattered in the cytoplasm or aggregated into crystalline inclusions. Although slightly smaller particles (*c.* 60 nm) have been reported, Ikegami and co-workers have shown that certain purification treatments cause particles

to lose their outer protein shells (Ikegami *et al.*, 1974). This is especially noticable with Fiji disease virus (FDV), in which all intact particles (IPs) about 75 nm in diameter, after extraction and sedimentation, are converted to cores or subviral particles (SVPs), about 55 nm in diameter (Hatta *et al.*, 1977). Consequently, most purified preparations of fijiviruses studied consist mainly or entirely of SVPs. In developing virus-purification procedures, the difficulty of obtaining sufficient virus has been the most serious limitation. Virus particles are restricted to the neoplastic tissues of plants infected by most of the plant *Reoviridae*, hence obtaining large quantities of plant tissues containing virus as starting material for purification is usually difficult. This is particularly true of fijiviruses, resulting in this genus not being investigated as extensively as the phytoreoviruses, whose genomic biological properties include the lack of affinity for ribosomes and mRNA activity, as would be expected of dsRNA. A unique feature of this group of plant viruses is that each double-stranded genome has been transcribed *in vitro* to give a mRNA that produced a single protein product which hydridized selectively to one genome segment. The resolution of these mRNA transcripts into discrete species has helped establish the relationship between the viral genome segments and the transcripts which they encode. Such information may be useful in developing an understanding of gene expression in the vectors and plant hosts (Nuss *et al.*, 1981).

1.3.3.4 Classification Problems

A number of plant viruses with isometric particles and multisegmented dsRNA genomes have been assigned to the family *Reoviridae* according to the accepted criteria, however, there are some newly described viruses, whose viruslike particle structure and dsRNA genome suggest that they belong to the *Reoviridae*, but are difficult to

accommodate within the existing genera (Table 1.3). According to the accepted criteria the taxonomic position of such viruses is not clear. An example of a virus which does not fit into any of the currently recognized genera is rice ragged stunt virus (RRSV). It infects rice plants, producing symptoms similar to those of Graminae infected with fijiviruses and is vectored by the planthopper *Nilaparvata lugens*. However, the genome of the virus contains only 8 dsRNA segments, thus precluding its assignment to the genus *Fijivirus* or to any other currently accepted genera.

Table 1.3 Biological, Structural and Cytopathological Properties of Plant Reoviridae

	Genus	
Properties	Phytoreovirus	Fijivirus
Hosts		
Vertebrate	-	-
Insects	+	+
Plants	+	+
Particle Structure		
Diameter (nm)	73	67-70
Intact particles	59	55
Core or subviral particles	+	+
Outer shell		
Spikes A	-	+
B	-	+
Number		
RNA segments	12	10
Viral Proteins	7	6(?)
Cytopathology		
Viroplasms	+	+
Tubules	+	+
Matrix pattern	-	-

(Adapted : Francki, R.i.B and Boccardo, G. 1983. *In* : The Reoviridae)

Since notable differences in biological characteristics exist within each genus, efforts to understand plant reovirus biology in molecular terms has relied on molecular cloning, sequences analysis and in vitro expression studies. Already, complete nucleotide sequences have been determined for genomic segments belonging to several members of the genus *Phytoreovirus* (Anzola *et al.*, 1989; Nakashima *et al.*, 1990 and Suzuki *et al.*, 1990). Marzachi *et al.* (1991) have succeeded in cloning and partially characterizing the MRDV genome, a member of the genus *Fijivirus*. The information obtained from such sequence analyses can, when compared with an appropriate database, provide insight into evolutionary relationships which may help clarify current classification problems.

1.3.4 Diagnosis of Viral or Viruslike Diseases

Since healthy plants do not contain readily detectable amounts of endogenous segments of high molecular weight dsRNA (> 0.1 x 10^6), diagnostic tests based on the detection and identification of specific dsRNA species have been useful in the analysis of previously unrecognized virus or viruslike infections (Dodds *et al.*, 1984; Falk *et al*, 1984, Haber, 1992). In many instances, the analysis and characterization of these dsRNA species have helped to determine the etiology of virus strains (Dale *et al.*, 1986; Smith *et al.*, 1991; Lukács, 1994). Different groups of plant viruses have characteristic dsRNA patterns or profiles. The uniqueness of a profile is based on the numbers and molecular weights of the dsRNA segments. A virus whose genome consists of one ssRNA should produce one major dsRNA of approximately twice the molecular weight of the ssRNA. In practice, however, other minor or less prominent dsRNAs of lower molecular weight are detected. Some of

these dsRNAs are the RFs of subgenomic RNAs derived from the genomic RNA, while the origin of others is not known (Valverde *et al.*, 1988). Evaluation of results should be made in consultation with literature regarding described dsRNA profiles for members of different viral groups. In addition, findings should be confirmed by means of other detection techniques such as serological detection using specific monoclonal or polyclonal antisera and dot immunoassay (Aramburu *et al.*, 1991).

CHAPTER 2: AIMS AND OBJECTIVES

The general objective of the project is to investigate the nature of the dsRNA species isolated from tobacco class I leaf curl and their possible involvement in the etiology of class I leaf curl symptoms. The successful characterization of these dsRNA species may contribute to an understanding of the complex interactions that give rise to tobacco leaf curl syndrome.

To achieve the primary aim, the following specific objectives were proposed:

1. To optimize dsRNA extraction procedures for experimentation ;

2. To confirm the double-stranded nature and pattern of the RNA species and to ascertain whether the dsRNAs have a poly(A) * tail. This information may be useful in determining whether the dsRNA species may be modified messenger RNA (such as a physiological response of the plant induced by the feeding action of whitefly or a toxin associated with whitefly feeding) or viral dsRNAs.

3a. To determine whether tobacco leaf curl and squash silverleaf symptoms, both suspected whitefly-induced etiologies, are related by using selected dsRNA species extracted from class I leaf curl, in cross-hybridization studies with silverleaf squash and whitefly nucleic acid extracts ;

3b. To determine whether the dsRNAs are non-homologous separate species by cross-hybridizing them with each other ;

3c. To determine whether these dsRNA species are exclusively associated with the etiological agent of class I leaf curl by hybridization with RNA extracts from tobacco exhibiting class II and class III symptoms.



CHAPTER 3: MATERIALS AND METHODS

3.1 PLANT MATERIAL

DsRNA and total RNA extractions were carried out on the following :

(a) fresh plant material, from infected tobacco plants exhibiting class I (*N. tabacum* cv. TL 33) or class II (*N.tabacum* cv. mammoth 10) symptoms, which were originally collected from tobacco fields in the Eastern Transvaal and Brits area, or supplied by the Tobacco and Cotton Research Institute near Rustenburg. Class III (*N. tabacum* cv.HG) plants were collected in Zimbabwe. Leaf material was collected from these infected plants and stored frozen at -20°C until extraction at a later date.

(b) healthy tobacco (*N.tabacum* cv. TL 33) and squash plants (*Cucurbita pepo* cv. Long Green Bush and Rolet), grown from seed and maintained in growth chambers under controlled conditions at a mean temperature of 25°C ± 2°C. The seedlings were watered to saturation three times a week and provided with nutrients on a weekly basis.

(c) asymptomatic tobacco (*N.tabacum* cv.TL 33) grown from seeds collected from plants severely affected with class I tobacco leaf cu.' Same growth conditions as healthy tobacco and squash seedlings.

(d) squash plants exhibiting silverleaf symptoms (figs. 3.1 and 3.2) induced by whitefly feeding. Healthy squash plants (*C.pepo* cv. Long Green Bush), grown from seed, were introduced to colonies of whitefly (*B.tabaci*), maintained in screen cages in growth chambers at a mean temperature of ±25°C.

Leaves developing silverleaf symptoms (figs.3.1 and 3.2) were harvested, usually after 3 weeks' exposure to whitefly feeding, and pupal cases, if present, were removed prior to extraction.

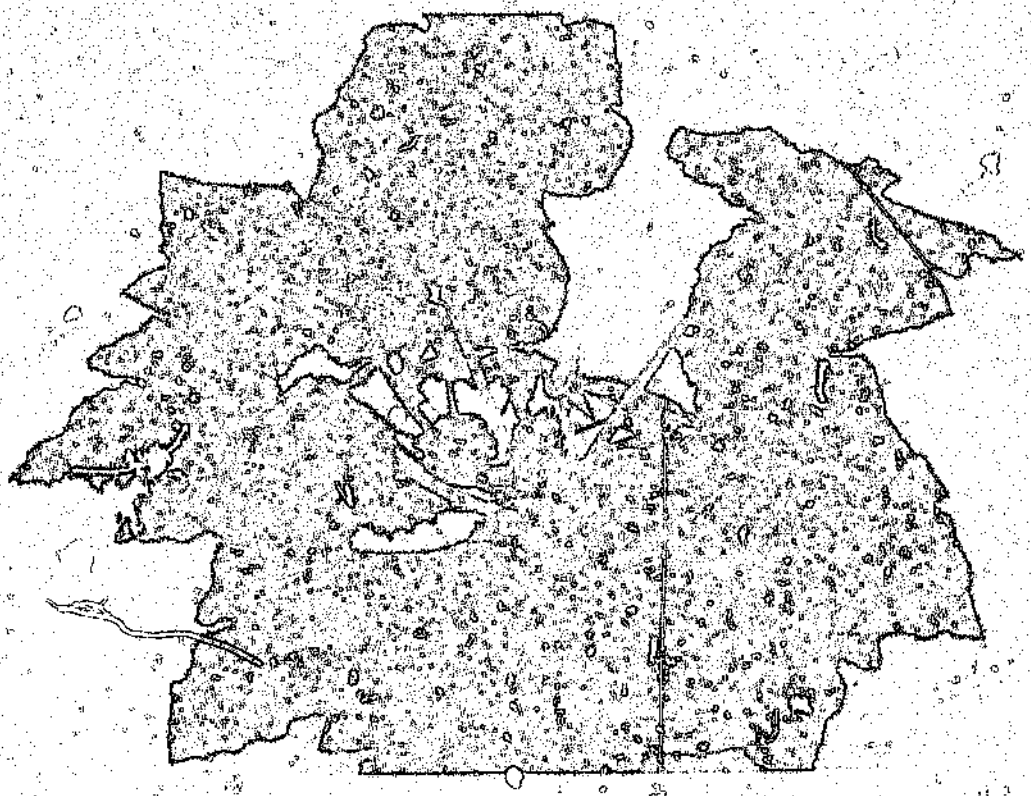


Fig.3.1 Squash plant (*Cucurbita pepo cv. Table Queen*) showing the different stages of silverleaf development.



Fig.3.2 Squash silverleaf induced by whitefly feeding, showing veinal chlorosis and progressive silvering of leaf tissue

3.2 WHITEFLY COLONIES

Colonies of whitefly were maintained in screen cages in a growth chamber (mean temperature 25°C) on a variety of host plants, including squash (*C.pepo* cv. Long Green Bush) and tobacco (*N.tabacum* cv. TL 33). The colony of whitefly was obtained from local populations infesting poinsettias. Judy Brown (Department of Plant and Biological Sciences,

University of Tuscon, Arizona), biotyped the whitefly colony and confirmed that it was analogous to silverleaf whiteflies that have been described as *B.tabaci* biotype B (Jiménez *et al.*, 1995). Except for non-viral silverleaf on squash, no other symptoms were noted on plants on which these whitefly colonies were mainained. For double-stranded and total RNA extractions approximately 100 whiteflies were aspirated into a glass tube, anaesthetized with a small volume of chloroform and homogenized with extraction buffer. The method for the isolation and purification of whitefly dsRNA and total RNA is the same as that for plant tissue, which differentially elutes dsRNA at a particular concentration of ethanol-free buffer.

3.3 DsRNA EXTRACTION

3.3.1. Introduction

dsRNA extractions, previously described by Morris and Dodds (1979), were carried out on tobacco plants exhibiting the three different leaf curl class symptoms, squash plants showing silvering of leaves and on whitefly cultures. DsRNA extractions were also performed on asymptomatic tobacco grown from seeds collected from class I leaf curl plants, and on healthy tobacco and healthy squash plants as controls. Unless otherwise stated, attempts were not made to remove other nucleic acids (DNA, single-stranded RNA) which did not interfere with dsRNA visualization. A list of suppliers and the reagents used in the extraction are listed in Appendix 6.1 and 6.2 respectively. A slightly modified method was used for large-scale extraction and purification of dsRNA. The extraction procedure involves the homogenization of plant tissues in the presence of an extractant designed to denature proteins and release undegraded nucleic acids. The extraction buffer, at an elevated pH (6.8)

and moderate (0.2M) salt content to reduce ribonuclease activity, contains an additive, bentonite, which reduces nuclease activity and a detergent, sodium dodecyl sulfate (SDS), to ensure denaturation of proteins and the release of the nucleic acid. In addition, mercaptoethanol prevents the oxidation and browning of plant tissue extracts, while the use of phenol/chloroform, as the organic protein denaturant component, ensures good separation of the phases after centrifugation and provides more complete removal of proteins from the aqueous phase. Purification of the nucleic acids is based on the differential binding of single- and double-stranded RNA and DNA nucleic acids to cellulose in the presence of various concentrations of ethanol. DNA and ssRNA fractions elute in buffer containing 15-18% ethanol, while dsRNA remains bound. The bound dsRNA is then eluted in a small volume of ethanol-free buffer.

3.3.2 Small Scale dsRNA Extraction

Plant material (0.5 g), usually symptomatic leaves, was ground to a fine powder in liquid nitrogen using a mortar and pestle. The frozen powder was extracted with 1.3 ml of double-strength STE buffer, 3% SDS, 25 mg/ml Bentonite, and 0.5% β-mercaptoethanol. The extract was shaken for 20 min, at room temperature, with an equal volume of STE-saturated phenol and chloroform: isoamyl alcohol (24:1). The resulting emulsion was centrifuged at 4 500 rpm for 15 min at 4°C in a Beckman J2-21 centrifuge, using a Beckman JA-14 rotor. Cellulose columns were prepared by equilibrating 2.5 g of Whatman CF-11 cellulose powder with 50 ml of STE:ethanol (16.5% v/v). 1 ml fractions of the well mixed slurry were transferred to sterile microfuge tubes and centrifuged at 4°C for 5 min. Unless otherwise stated, all centrifugations were carried out for 5 min at 4°C in a Hägar microfuge. The supernatant from each cellulose column was discarded and approximately 1.1ml fractions of the phenol-extracted aqueous phase transferred to the cellulose columns. The final

ethanol concentration was adjusted to 16.5% (v/v), and, after a thorough mixing, the columns were allowed to stand at room temperature for 15-20 min. The cellulose, with bound dsRNA, was then washed with several volumes of STE:ethanol (16.5%) to remove unbound impurities. After the final wash, the cellulose columns were resuspended in a small volume (100μl) of 1 x STE to elute the DNA and ssRNA fractions. The dsRNA was finally eluted in 500 μl 1 x STE, concentrated by adding 0.1 vol. 3M sodium acetate, pH 5.5 and 2.5 vol. 100% ethanol and incubating the solution at -20°C for 4 h or longer to precipitate the dsRNA. The samples were centrifuged for 10 min, the ethanol discarded and the pellets dried under vacuum using a Savant Speedyvac Concentrator or incubated at 37°C for 5-10 min. The pellets were resuspended in 5 μl TE buffer and stored at 4°C. Enzymatic treatment of the final sample was not necessary as dsRNA obtained by means of this procedure is usually free from detectable host DNA and RNA (Valverde, 1990).

3.3.3 Large Scale dsRNA Extractions

Whatman CF-11 powder (2.5 g) was mixed in 30 ml STE: ethanol (16.5%) in a large plastic centrifuge bottle. The bottle was centrifuged for 15-20 min at 8 500 rpm and the supernatant discarded. Fresh or frozen plant material (7-10 g) was ground in liquid nitrogen using a sterile mortar and pestle. The powder was transferred to a large plastic centrifuge bottle to which was added :14 ml 1 x STE (2 x STE may also be used); 2 ml 10% SDS; 18 ml water-p; 1 x STE-saturated phenol and 16 mg fractionated bentonite. The bottle was shaken for 20-30 min and then centri" ged for 15 min at 8 500 rpm. The top aqueous phase was transferred to a clear centrifuge tube and the volume adjusted to 20 ml with 1 x STE. Twenty-one μl 100 % ethanol per 100μl aqueous solution was added to obtain a final concentration of 16.5% ethanol. The contents of the tube were

mixed well and the solution was transferred to the large plastic centrifuge bottles containing the cellulose column. The mixture was shaken thoroughly and allowed to stand at room temperature for 20 min. All future steps were carried out at room temperature unless indicated. The bottle was centrifuged at 8 500 rpm for 15 min. The supernatant was discarded and 30ml 1 x STE:ethanol (16.5%) was added to the cellulose. The bottle was well shaken for 5-10 min and then centrifuged at 8 500 rpm for 15 mins. This step was repeated twice more. After the final wash the dsRNA was eluted by adding 3 x 10 ml aliquots of 1 x STE buffer. The supernatant was collected after each wash and precipitated by adding 2.5 volumes 100% ethanol and 0.1 volume 3 M sodium acetate, pH 5.5. The solution was stored overnight at -20°C. After incubation, the tube was centrifuged for 30 min at 8 500 rpm and the pellet resuspended in 500μl 70% ethanol. The tube was then centrifuged for 15 min at 8 500 rpm and supernatant discarded. The pellet was allowed to air-dry and finally resuspended in 500μl TE buffer. The resuspended pellet was stored at 4°C.

3.4 TOTAL RNA EXTRACTION

3.4.1 Introduction

To obtain good preparations of total RNA, it was necessary to minimize the activity of RNases by taking the following precautions. All glassware was treated by baking at 180°C for 8 hours or more; plasticware and electrophoretic trays were soaked overnight in 1M NaOH and rinsed several times with sterile water treated with 0.1% diethyl pyrocarbonate (DEPC). All solutions and reagents were treated with 0.1% DEPC for at least 12 hours at 37°C and then autoclaved for 15 min. at 15 lb/sq.

Disposable gloves were worn during the preparation of reagents and during all manipulations involving RNA.

3.4.2 Method

The procedure is a modification of the method described by Sambrook *et al.* (1989) for the extraction of RNA using guanidine HCl and organic solvents. The same method was used for both plant and whitefly total RNA extractions. Solutions and reagents are listed in Appendix 6.3. Plant material (0.2 g) was ground in a sterile eppendorf in 1ml guanidine HCl homogenization buffer using a sterile pestle. Fifty μl of 10% sodium lauroyl sarcosinate was added and the tube centrifuged at 10 000 rpm for 5 min. at 4°C. Unless otherwise stated, all centrifugations were carried out in a Häger microfuge. The top aqueous phase was transferred to a fresh eppendorf tube and an equal volume of chloroform:isoamyl alcohol ($CHCl_3$:IAA)-saturated phenol was added. The tube was shaken well and centrifuged at 10 000 rpm for 5 mins at 4°C. The top aqueous layer was transferred to a fresh eppendorf tube and an equal volume of 8M lithium chloride was added. The mixture was vortexed vigorously and allowed to stand for at least 2 hours at -20°C. After incubation, the tube was centrifuged at 10 000 rpm for 15 min. at 4°C. The supernatant was transferred to a fresh eppendorf and ethanol precipitated by adding 0.1 volume 3M sodium acetate, pH 5.5 and 2.5 volume 100% ethanol. The pellet was resuspended in 500 μl DEPC-treated water and the 8M lithium chloride step repeated. One tenth volume 3M sodium acetate, pH 5.5 and 2.5 volume 100% ethanol were added to the supernatant, vortexed as above and incubated overnight at -20°C. After incubation, the tubes were centrifuged at 10 000 rpm for 15 min at 4°C and the supernatant discarded. The pellet was washed in 500μl 70% DEPC-treated ethanol and the tubes centrifuged at 10 000 rpm for 15 min. at 4°C. The

supernatant was removed and the pellets dried at room temperature. The pellets were resuspended in DEPC-treated water or DEPC-treated TE buffer and stored at -70°C.

3.5 AGAROSE GEL ELECTROPHORESIS

Initial detection and analysis of dsRNA purified from plant material was achieved by the electrophoretic separation of the species in agarose horizontal gels (0.5mm thick, 10cm long and 8cm wide). A 1% agarose gel was prepared by adding 0.4 g agarose to 40ml 1 x TBE buffer. Agarose in solution was heated in a microwave oven to melt the agar. The solution was allowed to cool before adding 1 μl ethidium bromide (10mg/ml) and then poured into the electrophoretic tray. The comb was positioned and the gel allowed to solidify. Once set, the comb was removed and the gel placed in the electrophoretic tank. TBE buffer was added to each tank and allowed to cover the gel by approximately 1 mm. Ethidium bromide (1μl per 30ml TBE) was then added to the TBE buffer in the electrophoretic tanks. Tracking dye (3μl dye per 6μl sample) was added to each sample and approximately 20-30μl of sample was loaded into the wells. Electrophoresis was conducted at 90V at room temperature for 1½-2 hr or until adequate band resolution was achieved. Once electrophoresis was complete the gel was placed on a ultraviolet (UV) transilluminator (302 nm) for examination of the fluorescing nucleic acid bands and photographed on Polaroid Type 665 film. Reagents are described in Appendix 6.4. The molecular weights of the dsRNA species were estimated from gel mobility of the species, relative to *Hind* III- and *Pst* I-cut λ DNA molecular weight markers (Appendix 6.5).

3.6 RECOVERY OF dsRNA FROM LMT GELS

3.6.1 Introduction

The method for the recovery and isolation of selected dsRNA bands is a modified phenol-based method described by Falson (1992). Residual phenol and agarose are centrifugally separated from the dsRNA solution in the presence of salt following phenol extraction and prior to ethanol precipitation. Reagents and solutions are described in Appendix 6.6.

3.6.2 Method

Tobacco class I extraction samples were run in a 0.9% LMT agarose gel, containing 3.025μl of ethidium bromide (10 mg/ml) per ml TBE buffer, at 90 V for 1½ - 2 h at room temperature. Following electrophoresis the dsRNA bands of interest were located by exposing the gel to UV light (302 nm) on the transilluminator. The bands were excised in the smallest possible volume using a sharp scalpel. Each sliced segment was transferred to a sterile eppendorf tube and the volume estimated from its tared weight. Two volumes of TE buffer were added and the tube placed in a 65°C waterbath for 5 to 10 min. Once the agarose was fully melted, 1 volume of TE-saturated phenol was added and the tube thoroughly mixed by inversion. The tube was then reheated to 65°C for a few min. and centrifuged in a Häger microfuge for 3 min at 10 000 rpm. The aqueous phase was transferred to a new sterile eppendorf tube containing 1 volume TE-saturated phenol. The tube was mixed by inversion, centrifuged and the aqueous phase transferred to a fresh eppendorf tube containing 1 volume TE-saturated isoamyl alcohol. The tube was mixed by inversion, centrifuged at 10 000 rpm for 3 min. The

supernatant was discarded and the isoamyl alcohol step repeated twice more. After the final wash, the bottom phase was transferred to a fresh sterile eppendorf tube containing 0.1 volume of 4 M lithium chloride. The tube was mixed by inversion and placed on ice for 2 min. The tube was then spun as above for 3 min. The supernatant was transferred to a new tube, leaving a transparent pellet behind. Since most of the phenol and agarose have been eliminated from the sample, 1μl glycogen of a 20mg/ml solution, was added to improve the final yield of recovered dsRNA. Finally, the sample was precipitated by adding 2.5 volumes of cold 100% ethanol, mixed by inversion and left for 30 min at -70°C. The tube was spun as above for 10 min and the pellet washed in 500μl cold 70% ethanol. The dried pellet was resuspended in 5-10μl TE buffer and stored at 4°C.

3.7 RIBONUCLEASE A DIGESTION

3.7.1 Introduction

A recognized feature of dsRNA is its resistance to digestion by pancreatic ribonuclease A (RNase A). The double-stranded nature of the RNA species was confirmed by digestion of the sample with DNase-free ribonuclease A in the presence of water or a high salt (0.45M) solution based on a method described by Falk and Duffus (1984). The preparation of reagents and solutions are described in Appendix 6.7.

3.7.2 Method

Double-stranded RNA samples, suspended in either 500 μl of 3 x SSC or 500 μl sterile H_2O, were incubated at room temperature for 15 min with

DNase-free RNase A (1μg/ml). Samples were adjusted to contain 5μg/ml of Proteinase K and 0.05% SDS, then incubated for an additional 10 min to digest RNase A. Undigested dsRNAs were precipitated in 2.5 volumes 100% ethanol and incubated overnight at -20°C or for 1 hour at -70°C. After incubation, the samples were centrifuged for 15 min and the supernatants discarded. The pellets were washed four times in 500μl 70% ethanol and finally suspended in 5μl TE buffer. RNase stability of the RNA species was evaluated by electrophoresis in a 1% agarose gel.

3.8 OLIGO(dT) TRAPPING OF POLY (A)$^+$RNA

3.8.1 Introduction

To ascertain whether the dsRNA species contain a poly (A)$^+$ tail, an oligo(dT) trapping method (Amersham) was used to specifically bind any poly(A)$^+$ RNA present in the extraction sample. Hybond-mAP (messenger affinity paper) is a diazonium-activated paper, substituted covalently by polyuridylic acid to give a high affinity for poly(A) sequences. Any mRNA, present in the sample, possessing a 3'-poly(A) tail, will bind specifically to the Hybond-mAP via hydrogen bonds. Poly(A)$^-$ RNA is removed from the sample by a series of salt washes. Following a 70% ethanol wash, mRNA is released in a salt-free solution by heating the Hybond-mAP/RNA complex in distilled water to disrupt the hydrogen bonds. Subsequent gel electrophoresis of the sample allows the identification of the isolated mRNA species. The protocol described is capable of isolating 8-12μg poly(A)$^+$ RNA per cm^2 Hybond-mAP from volumes up to 200μl. Extreme care was taken to eliminate contamination by exogenous ribonuclease. Therefore any glassware, plastic tips, buffers or reagents used were treated with DEPC to inactivate any RNase present. The preparation of reagents and solutions are given in Appendix 6.8.

3.8.2 Method

Three pieces of Hybond-mAP were cut into squares, approximately 1cm^2 in size, pre-wet with 0.5M NaCl (~20µl/cm^2) and allowed to air dry completely on clean, sterile filter paper. Two control tubes were prepared by adding 5 µl (~1µg) of mRNA (positive control) and 5 µl (~1µg) of λ- DNA (negative control) to two sterile eppendorf tubes containing 10µl sterile DEPC-treated water. The controls, together with the sample tube containing 15µl (~1µg) of dsRNA, were heated for 5 min at 65°C to denature the RNA. The tubes were immediately placed on ice and NaCl added to each tube to give a final concentration of 0.5M. The three pieces of dried Hybond-mAP were placed on parafilm™ (American Can Company) and the contents of each tube slowly applied to the Hybond affinity paper (ideally ~ 20µl/cm^2). The papers were allowed to dry for at least 5 min. Each paper was transferred to a sterile eppendorf tube and washed twice in at least 5 ml 0.5M NaCl for 5 mins. Fresh 0.5M NaCl was used for each wash. After the final wash, the Hybond-mAP was placed in at least 5 ml 70% (v/v) ethanol and gently shaken for 2 min. The Hybond-mAP was transferred to filter paper and allowed to air dry completely before being placed in a sterile eppendorf tube. The minimum amount of distilled water required to cover the Hybond-mAP was added to each tube. The tubes were then placed in a 70°C waterbath for 5 min to release the poly(A)$^+$ RNA bound to the affinity paper. The tubes were vortexed briefly and then centrifuged with the Hybond-mAP wedged at the top of the tube. The Hybond-mAP papers were removed, leaving the poly(A)$^+$ RNA in a salt-free solution. Gel electrophoresis was carried out to visualize and identify the RNA species.

3.9 HYBRIDIZATION STUDIES

3.9.1 Introduction

Selected dsRNA bands, extracted and purified from a LMT agarose gel, were labelled using an enhanced chemiluminer e (ECL) nucleic acid labelling technique (Amersham). The reagents used in this kit are protected by the patent holder and therefore no details concerning the chemical composition and concentrations of these reagents can be given. The system involves directly labelling denatured, single-stranded probe RNA with the enzyme horseradish peroxidase. The addition of gluteraldehyde causes the formation of chemical cross-links so that the probe becomes covalently labelled with the enzyme. Once labelled, the probe is used in hybridization with target RNA immobilized on a membrane. After hybridization, the filters are washed to remove unlabelled probe. Probe/RNA hybrids are detected by the addition of hydrogen peroxide, which acts as a substrate for peroxidase. Reduction of hydrogen peroxide by the enzyme is coupled to a light-producing reaction by another detection reagent, containing luminol. Luminol, upon oxidation, produces a blue light which can then be captured on autoradiography film.

3.9.2 Preparation of labelled probes

Selected class I dsRNA bands, measuring c. 4.4-, 3.6-, 3.3- and 2.4-kbp in size (fig.4.1), were excised separately from a 0.9% LMT agarose gel and purified as previously described in section 3.6. Each dsRNA band was labelled separately. The dsRNA to be labelled was diluted to a concentration of 10 ng/μl us g the sterile water supplied with the ECL kit.

The dsRNA sample (10 μl) was denatured by heating the sample for 5 min in a boiling water bath and then immediately snap-cooling in an ice-ethanol slurry for 5 min. After briefly centrifuging to collect the contents at the bottom of the tube, 10 μl of DNA labelling reagent was added and the tube mixed gently but thoroughly. Equal volumes (10 μl) of glutaraldehyde solution was then added, the tube mixed thoroughly and centrifuged briefly. Finally the tube was incubated for 10 min at 37°C. If not used immediately, the tube was held on ice for a short period (10-15 min).

3.9.3 Northern Blot

DsRNA and total RNA extractions carried out on tobacco exhibiting class I leaf curl symptoms, classes II and III, asymptomatic tobacco grown from seed collected from class I leaf curl, healthy squash, silverleaf squash and whitefly were electrophoresed on a 1% non-denaturing agarose gel. After electrophoresis, the gel was soaked in at least 300 ml 50mM NaOH (denaturing buffer) for 30 min at room temperature with gentle shaking. The gel was then neutralized in 300 ml 0.025M sodium phosphate, pH 6.5 (transfer buffer) for an additional 30 min at room temperature with gentle shaking. Reagents and buffers are described in Appendix 6.9. A piece of Whatman 3MM filter paper was positioned on top of a support tray placed in a glass baking dish. The dish was filled with transfer buffer until the level of the buffer almost reached the top of the support tray. The buffer was allowed to saturate the filter paper and any bubbles were removed by rolling a glass pipette across the filter paper. The gel was then placed (RNA-side down) on top of the saturated filter paper and the right-hand corner of the gel cut off to orient the gel. A piece of nylon membrane, cut the same size as the gel, was soaked briefly in transfer buffer and, using a pair of forceps, positioned on top of the gel. Using a

sharp scalpel blade, the right-hand corner of the membrane was cut to match the orientation of the gel. Air bubbles were smoothed out using a glass pipette. Two pieces of Whatman 3MM filter paper were cut to size, soaked briefly in transfer buffer and placed on top of the nylon membrane. Again, any air bubbles between the membrane and the filter papers were removed. The edges of the gel were surrounded by Saran wrap to prevent liquid from flowing directly from the reservoir to the paper towels stacked on top of the gel. A stack of paper towels (5-8cm high) was placed on the filter papers, and a glass plate and a 500-g weight were added to the stack in order to weigh it down (Fig. 3.3).

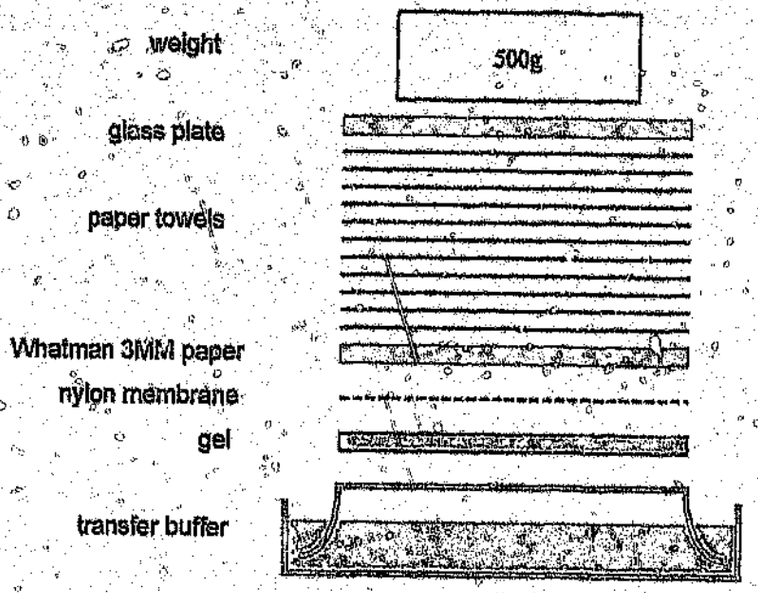


Fig. 3.3 Capillary transfer of RNA from an agarose gel to a nylon membrane.

Transfer was allowed to proceed for 16-18 h. Following transfer, the paper towels were removed and the nylon membrane rinsed briefly in transfer buffer to remove any pieces of agarose that may have stuck to the membrane. The membrane was placed on sterile filter paper and allowed to air dry completely at room temperature. On air dry the RNA was fixed to the membrane by exposing the membrane to UV light (302 nm) on a transilluminator for 5 min. The membrane was then placed between two pieces of filter paper, wrapped in Saran wrap and stored at 4°C. To assess the efficiency of transfer of RNA, the gel was stained for 45 min in a solution of ethidium bromide (0.5µl/ml) and examined under UV light.

3.9.4 Dot Blots

A separate dot blot was prepared for each labelled dsRNA probe. A small volume of dsRNA (~ 3 µl equivalent to 50 ng), extracted from each plant species and whitefly, was denatured by heating in a rapidly boiling waterbath for 5 min. The sample was immediately snap-cooled in an ice-ethanol slurry for 5 min and microfuged briefly to collect the contents at the bottom of the tube. The denatured sample, approximately 1µl at a time, was slowly applied to the Hybond-N membrane. The spot was allowed to dry between each application. The membrane was air dried completely before fixing under UV light (RNA-side down) for 5 min. A total RNA blot, using total RNA extracted from each plant species and whitefly, was prepared in the same manner. Each blot was placed between two pieces of 3MM Whatman filter paper, wrapped in clingwrap and stored at room temperature until hybridization with a labelled probe.

3.9.5 Hybridization and Stringency Washes

Each dot blot was placed in a separate sterile plastic container and prehybridized in hybridization buffer for 1 h at 42°C with gentle agitation. Following prehybridization, a specific labelled RNA probe (section 3.9.2) was added to the hybridization buffer and incubation, at 42°C with gentle agitation, was continued overnight. After incubation, the blot was transferred to approximately 40 ml primary wash buffer (2-5ml/cm^2 of membrane), preheated to 55°C. Two 10 min washes at 55°C were carried out with gentle shaking. Fresh primary buffer was used for each wash. The blot was then placed in a clean container filled with approximately 40 ml of secondary buffer (2-5ml/cm^2 of membrane). The blot was washed for 5 min with gentle agitation at room temperature. A second wash was carried out using fresh secondary buffer. If necessary, stringency was controlled by altering the SSC concentration of the primary wash buffer; 1 x SSC is low stringency, 0.1 SSC is high stringency. Preparation of hybridization and wash buffers are described in Appendix 6.10.

3.9.6 Signal Generation and Detection

Following the final wash in secondary wash buffer, the blot was transferred, RNA-side uppermost, to a clean glass container. Equal volumes of detection reagent 1 and detection reagent 2 (0.125ml/cm^2 of membrane) were added to the membrane. Detection reagent 1 decays to hydrogen peroxide, the substrate for peroxidase. Reduction of hydrogen peroxide by the enzyme is coupled to the light producing reaction by detection reagent 2. This contains luminol, which on oxidation produces blue light. After incubation for 1 min at room temperature, excess

detection reagent was drained off and the blot placed in a plastic sleeve. Once the air pockets were removed, the plastic sleeve was heat-sealed and the blot placed, RNA-side up, in a film cassette. The following steps were carried out in the darkroom, using red-safe lights. A sheet of ECL blue-light sensitive autoradiography film (Hyperfilm™-ECL) was placed on top of the blot and the cassette firmly closed. Exposure time ranged from 1-4 h at room temperature.

3.9.7 Film Development

The following procedure was carried out in the dark room, using red safe lights. The exposed film was removed from the cassette and immediately placed in a tray containing approximately 500 ml film developer. The film was agitated gently for 2-5 minutes. The film was then rinsed briefly in water and transferred to a tray containing 500 ml fixative. The film was agitated gently for approximately 2 min., rinsed briefly in water and allowed to air dry.

CHAPTER 4: RESULTS

4.1 NUCLEIC ACID ANALYSIS

4.1.1 Detection of Double-stranded RNA Species

The CF-11 chromatography fractionation of tobacco leaves exhibiting class I leaf curl yielded 12 visible double-stranded RNA species (fig.4.1).

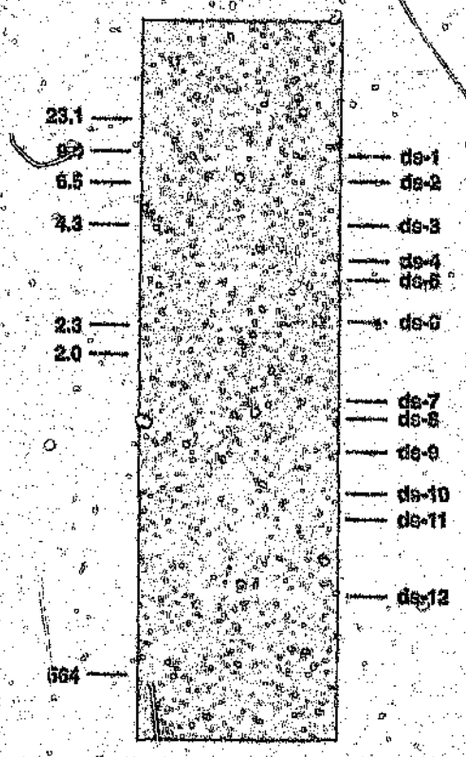


Fig.4.1 Electrophoretic profile of dsRNA fractionation from class I tobacco leaf curl run in a 1% agarose gel containing 0.025µl ethidium bromide per ml TBE buffer and photographed under ultraviolet radiation. Relative positions (in kilobase pairs) of λ DNA *Hind* III-digested molecular weight markers are indicated on the left. Migration of 12 dsRNA species, labelled ds-1-12, are indicated on the right.

The dsRNA preparation was virtually free of DNA and ssRNA contamination provided that the columns were not overloaded in excess of the binding capacity and at least two 1 x STE:ethanol (16.5%) washes were carried out. One cycle of CF-11 chromatography was usually enough to fractionate dsRNA efficiently, however when necessary, a second cycle was applied under the same conditions to recover additional dsRNA. Twelve dsRNA bands, ranging from *c.* 0.8 - 7.6kbp in size, were detected after gel electrophoresis. Sizing (in kilobase pairs) of each dsRNA band was determined by comparing the relative mobility of the band with that of known co-electrophoresed *Hind* III-and *Pst* I-cut λ DNA molecular weight markers (fig.4.2).

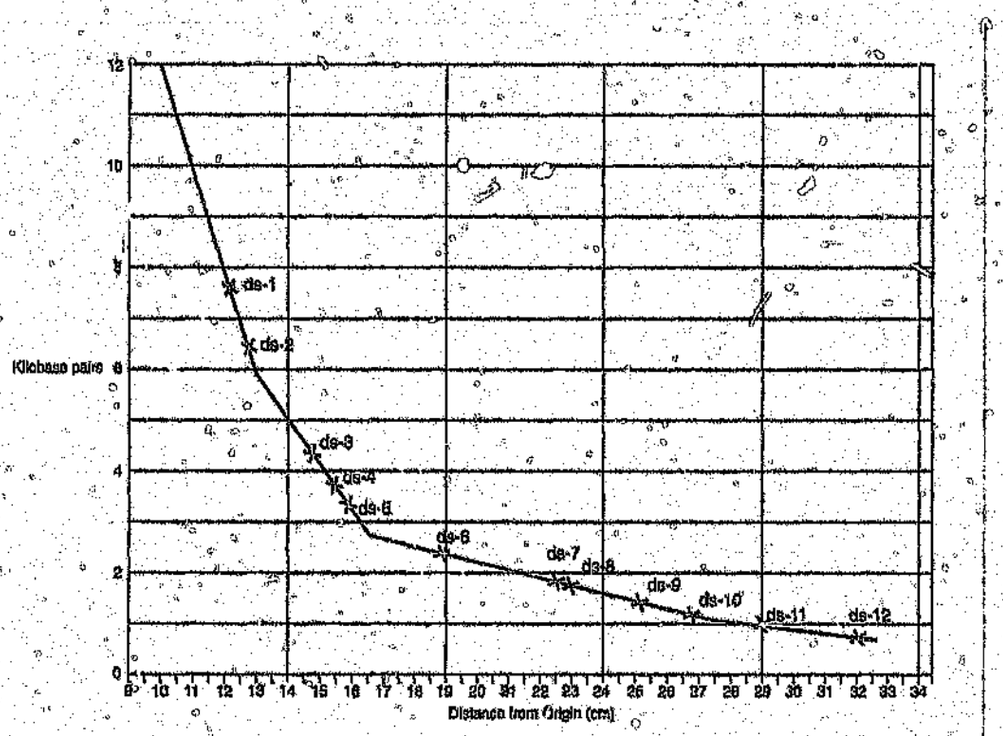


Fig.4.2 Relative sizing (in kilobase pairs) of class I tobacco leaf curl dsRNAs as interpolated from electrophoretic migration of *Hind* III- and *Pst* I-cut λ DNA molecular weight markers.

The relative molecular weights of at least 10 dsRNA band compared favourably with those reported by Paximadis (1995), however, two additional bands, *c.* 5.16 x 10^6 and 4.40 x 10^6, were observed (table 4.1).

Table 4.1 Comparison between the relative molecular weights of class I tobacco dsRNA bands obtained in this study and those reported by Paximadis (1995).

MOLECULAR WEIGHT ESTIMATE OF dsRNA BANDS					
Present Study			*Paximadis (1995)*		
Band	Basepairs (bp)	M_r	Band	Basepairs (bp)	M_r
ds-1	7 600	5.16 x 10^6		Not observed	
ds-2	6 500	4.40 x 10^6		Not observed	
ds-3	4 400	2.99 x 10^6	ds-1	4 800	3.26 x 10^6
ds-4	3 600	2.44 x 10^6	ds-2	3 900	2.65 x 10^6
ds-5	3 300	2.34 x 10^6	ds-3	3 500	2.38 x 10^6
ds-6	2 400	1.63 x 10^6	ds-4	2 600	1.76 x 10^6
ds-7	1 800	1.22 x 10^6	ds-5	1 800	1.22 x 10^6
ds-8	1 700	1.15 x 10^6	ds-6	1 700	1.15 x 10^6
ds-9	1 400	9.52 x 10^6	ds-7	1 500	1.02 x 10^6
ds-10	1 200	8.16 x 10^5	ds-8	1 480	1.0 x 10^6
ds-11	1 000	6.80 x 10^5	ds-9	1 400	9.5 x 10^5
ds-12	800	5.44 x 10^5	ds-10	1 100	7.4 x 10^5

The results of the dsRNA extractions carried out on all three classes of tobacco leaf curl, three cultivars of healthy squash, squash silverleaf and whitefly are shown in fig. 4.3.

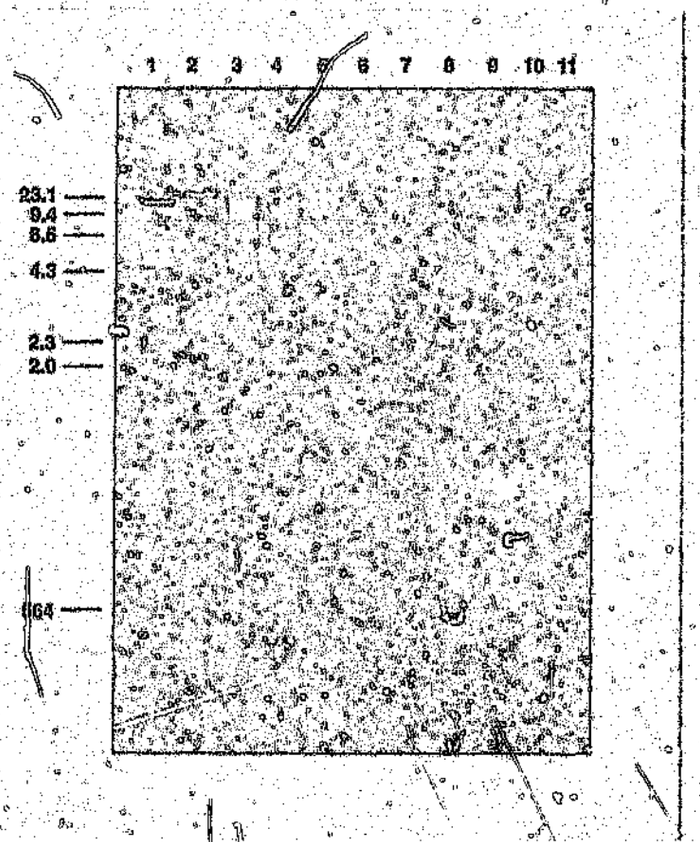


Fig.4.3 Detection of dsRNA species by agarose gel electrophoresis. Lane 1, *Pst* I molecular weight marker; lane 2, *Hind* III molecular weight marker; lane 3, class I tobacco leaf curl; lane 4, class II tobacco leaf curl; lane 5, class III tobacco leaf curl; lane 6, asymptomatic tobacco grown from seed collected from tobacco showing class I leaf curl symptoms; lane 7, healthy squash *(C.pepo cv. Table Queen)*; lane 8, healthy squash *(C.pepo cv. Long Green Bush)*; lane 9, healthy squash *(C.pepo cv. Ronel)*; lane 10, silverleaf squash; lane 11, whitefly *(B. tabaci)*. Samples were electrophoresed in 1% agarose gel containing 0.025μl ethidium bromide per ml TBE buffer and photographed under ultraviolet radiation. Relative positions (in kilobase pairs) of λ DNA *Hind* III-digested molecular weight markers are indicated on the left.

The dsRNA bands detected in class I tobacco leaf curl (lane 3) were absent in leaves from class II (lane 4); class III (lane 5) and asymptomatic tobacco grown from seed collected from class I leaf curl tobacco plants (lane 6). The 23.1-kbp fragment observed in most of the dsRNA extracts (lanes 3-8 and lanes 10) was shown to be genomic DNA as demonstrated by its sensitivity to DNase treatment (data not shown). The dsRNAs isolated from two of the three healthy, asymptomatic cultivars of squash, *C.pepo* cv. *Table Queen*, cv. *Long Green Bush* and cv. *Ronel* (lanes 7-9, respectively) were distinctly different. Besides the large 23.1-kbp band (which later proved to be genomic DNA), a second smaller band estimated to be *c.*9.6 kbp was observed in lane 7, representing *C.pepo* cv. *Table Queen*. With the exception of the 23.1-kbp genomic DNA band, a faint single dsRNA band was detected in *C.pepo* cv. *Long Green Bush* (lane 8). The size of this band could not be accurately measured as it lies outside the range of the molecular weight markers. No dsRNA bands were observed in extracts from *C.pepo* cv. *Ronel* (lane 9). The two dsRNA bands, (*c.*4.6- and 4.4-kbp) previously reported to be associated with squash silverleaf (lane 10) and silverleaf whitefly (lane 11), were not detected. A faint, single dsRNA band (*c.*8.8-kbp), however, was isolated from the whitefly extraction (lane 11). Ribonuclease A sensitivity tests were carried out on the two squash cultivars (cv. *Table Queen* and cv. *Long Green Bush)* and whitefly extracts to confirm the double-stranded nature of the RNA bands (Fig.4.6).

4.1.2 Total RNA Extractions

The results obtained from plant and whitefly total RNA extractions are shown in figure 4.4. The consistent detection of a single broad band, representing a high molecular weight species (estimated relative MW

23-kbp, as indicated by H*ind* III molecular weight marker), suggests that it may represent the host genomic nucleic acids of each plant species. An additional band, the same size (*c.* 8.8-kbp) as that observed in the dsRNA extraction, may be seen in lane 11, containing the TRNA sample extracted from whitefly.

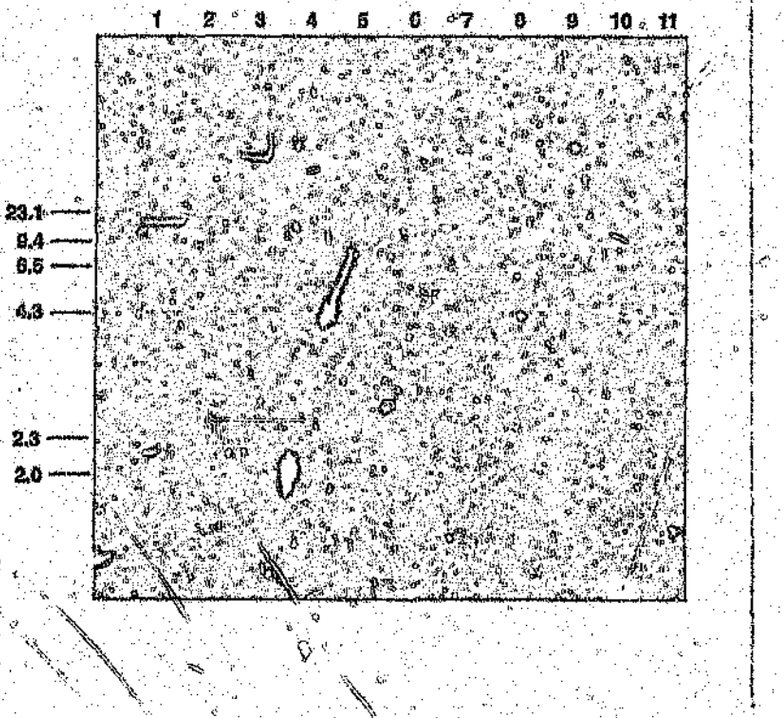


Fig.4.4 Agarose gel electrophoresis resulting from total RNA extractions. Lane 1, P*st* I molecular weight marker; lane 2, H*ind* III molecular weight marker; lane 3, class I tobacco leaf curl; lane 4, class II tobacco leaf curl; lane 5, class III tobacco leaf curl; lane 6, asymptomatic tobacco grown from seed collected from tobacco showing class I leaf curl symptoms; lane 7, healthy squash *(C.pepo cv. Table Queen)*; lane 8, healthy squash *(C.pepo cv. Long Green Bush)*; lane 9, healthy squash *(C.pepo cv. Ronel)*; lane 10, silverleaf squash; lane 11, whitefly (*B. tabaci*). Samples were electrophoresed in 1% agarose gel containing 0.025μl ethidium bromide per ml TBE buffer and photographed under ultraviolet radiation. Relative positions (in kilobase pairs) of λ DNA H*ind* III-digested molecular weight markers are indicated on the left.

4.2 RIBONUCLEASE A DIGESTION

RNase sensitivity of the dsRNA species extracted from class I tobacco leaf curl, healthy squash (cv. *Table Queen* and *Long Green Bush*), squash silverleaf (SSL) and whitefly (WF), were tested at high salt (3 x SSC) and no salt (water only) concentrations. The results of class I leaf curl dsRNA sensitivity to RNase A hydrolysis are seen in fig.4.5. The dsRNA preparation was resistant to RNase A digestion in a high salt solution (0.45M NaCl) after 15 min incubation (lanes 2 and 5), but was very susceptible to degradation when incubated in water only (lanes 3 and 4), thus confirming double-stranded nature of class I leaf curl RNA species. Lambda DNA *Hinc* II-cut molecular weight markers are represented in lanes 1 and 6.

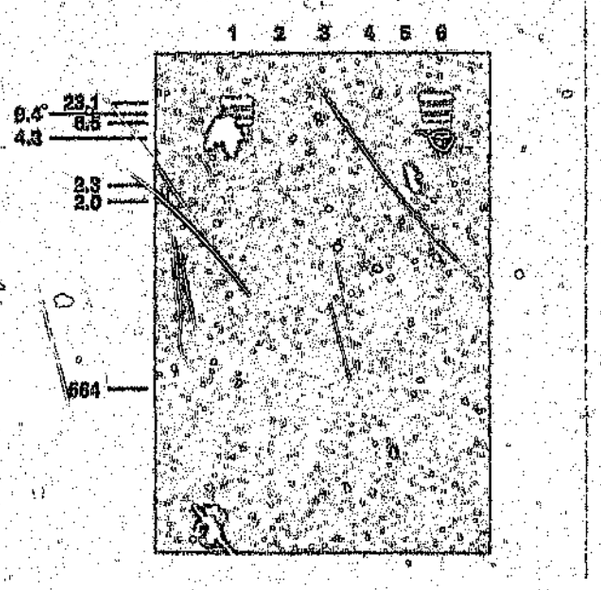


Fig.4.5 Ribonuclease (RNase A) treatment of dsRNA species associated with class I leaf curl. Lane 1and 6, λ DNA *Hind* III-cut molecular weight marker; lane 2 and 5, class I tobacco leaf curl dsRNA species after 15 min incubation in 0.45M NaCl containing 1µg/ml RNase A; lane 3 and 4, class I dsRNA species after incubation in water and 1µg/ml RNase A. Relative positions (in kilobase pairs) of λ DNA *Hind* III-digested molecular weight markers are indicated on the left.

Fig.4.6 demonstrates the double-stranded nature of the RNA species associated with healthy squash, SSL and WF. In a high salt solution (lanes 2-5) the RNA species isolated from each plant species and whitefly are resistant to RNase A digestion, whereas in water (lanes 6-9), the RNA species of all extracts are hydrolysed.

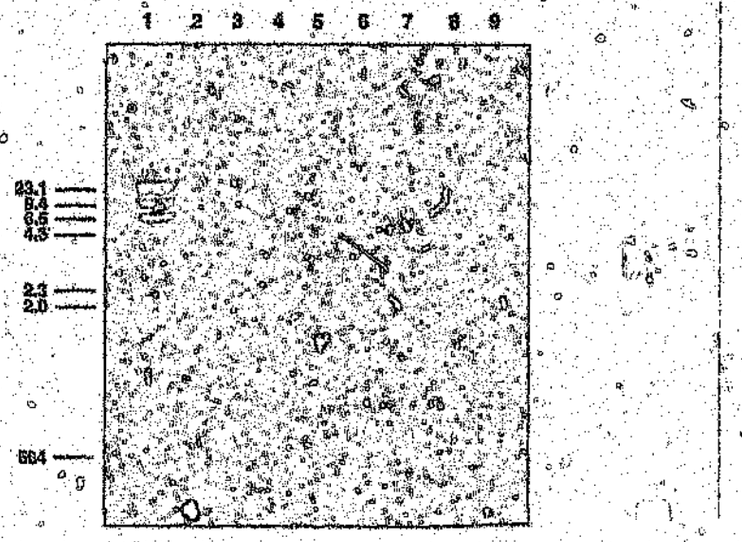


Fig.4.6 Ribonuclease (RNase A) treatment of dsRNA species associated with healthy squash, squash silverleaf and whitefly. Lane 1, λ DNA Hind III-cut molecular weight marker; lanes 2-5, dsRNA species after 15 min incubation in 0.46M NaCl containing 1µg/ml RNase A; lane 2, healthy squash (cv. *Table Queen*); lane 3, healthy squash (cv. *Long Green Bush*); lane 4, squash silverleaf; lane 5, whitefly. Lanes 6-9, dsRNA species after incubation in water containing 1µg/ml RNase A; lane 6, healthy squash (cv. *Table Queen*); lane 7, healthy squash (cv. *Long Green Bush*); lane 8, squash silverleaf; lane 9, whitefly. Relative positions (in kilobase pairs) of λ DNA *Hind* III-digested molecular weight markers are indicated on the left.

4.3 OLIGO(dT) TRAPPING OF POLY(A)+ RNA

The results of the oligo(dT) trapping of poly(A)$^+$RNA are shown in fig 4.7. A known positive mRNA control (1μg) is represented in lane 1(before binding to messenger affinity paper- mAP) and lane 2 (after binding and elution). No loss of mRNA was detected. Class I dsRNA species are represented in lane 3 (before binding) and lane 4 (after binding and elution). No dsRNA bands were detected after binding to mAP indicating that the class I RNA species do not have a poly (A)$^+$ tail. A known negative control, λ DNA (1μg), is represented in lane 5 (before binding) and after binding (lane 6). As expected, no band was detected in lane 6.

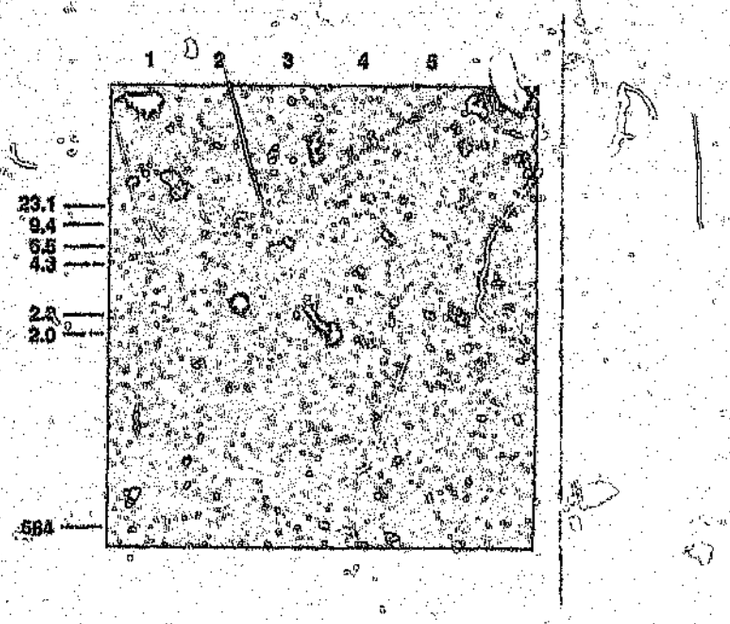


Fig.4.7 Gel electrophoresis following the oligo(dT) trapping of poly(A)$^+$RNA. Lane 1, known positive control containing 1μg of mRNA before binding to Hybond messenger affinity paper (mAP); lane 2, known positive mRNAcontrol (1μg) after binding to mAP; lane 3, class I dsRNA extraction sample before binding; lane 4, class I dsRNA extraction sample after binding to mAP; lane 5, known negative control, λ DNA (1μg) before binding to mAP; lane 6, known negative control, λ DNA, after binding. Relative positions (in kilobase pairs) of λ DNA *Hind* III-digested molecular weight markers are indicated on the left.

4.4 HYBRIDIZATION STUDIES

4.4.1 Northern Blot

DsRNA preparations extracted from all plant species and adult whitefly were electrophoresed on a non-denaturing 1% agarose gel (fig.4.8A). Transfer of these RNAs was made to Hybond-N membrane as described in Materials and Methods. A preliminary study was first carried out using labelled dsRNA prepared from pooled class I dsRNA bands as probe. Although the labelled *Hind* III DNA probe hybridized effectively to the target DNA marker control (lane 2, fig.4.8B), the labelled RNA probe failed to hybridize with the target RNA on the blot. Similarly, hybridization studies carried out on total RNA preparations extracted from all plant species and whitefly, using labelled pooled class I dsRNA bands as a probe, were unsuccessful (data not shown). Since the efficiency of hybridization is largely related to the degree of denaturation of RNA samples, it is possible that either the target RNA (which was denatured by an alkali), or the labelled RNA probe (which was denatured by heating), partially renatured during subsequent steps, thereby reducing hybridization efficiency. Since transfer of RNA from the gel to the nylon membrane continued overnight, it is also possible that the RNA species were degraded and lost during this long process. Perhaps a shorter transfer time (eg. using a vacuum transfer unit) may improve the efficiency of transfer, resulting in better hybridization between target RNA and labelled probe. Since Northern blotting was unsuccessful, dot-blots were attempted. Preliminary experiments were carried out using control mRNA and class I dsRNA samples (fig. 4.9A and 4.9B). Both labelled probes hybridized to their respective RNA targets, demonstrating that (i) the RNA species were adequately denatured by heating, and (ii) the labelled denatured RNA probe hybridized efficiently to its complementary target sequence; and (iii) the labelling kit was functional.

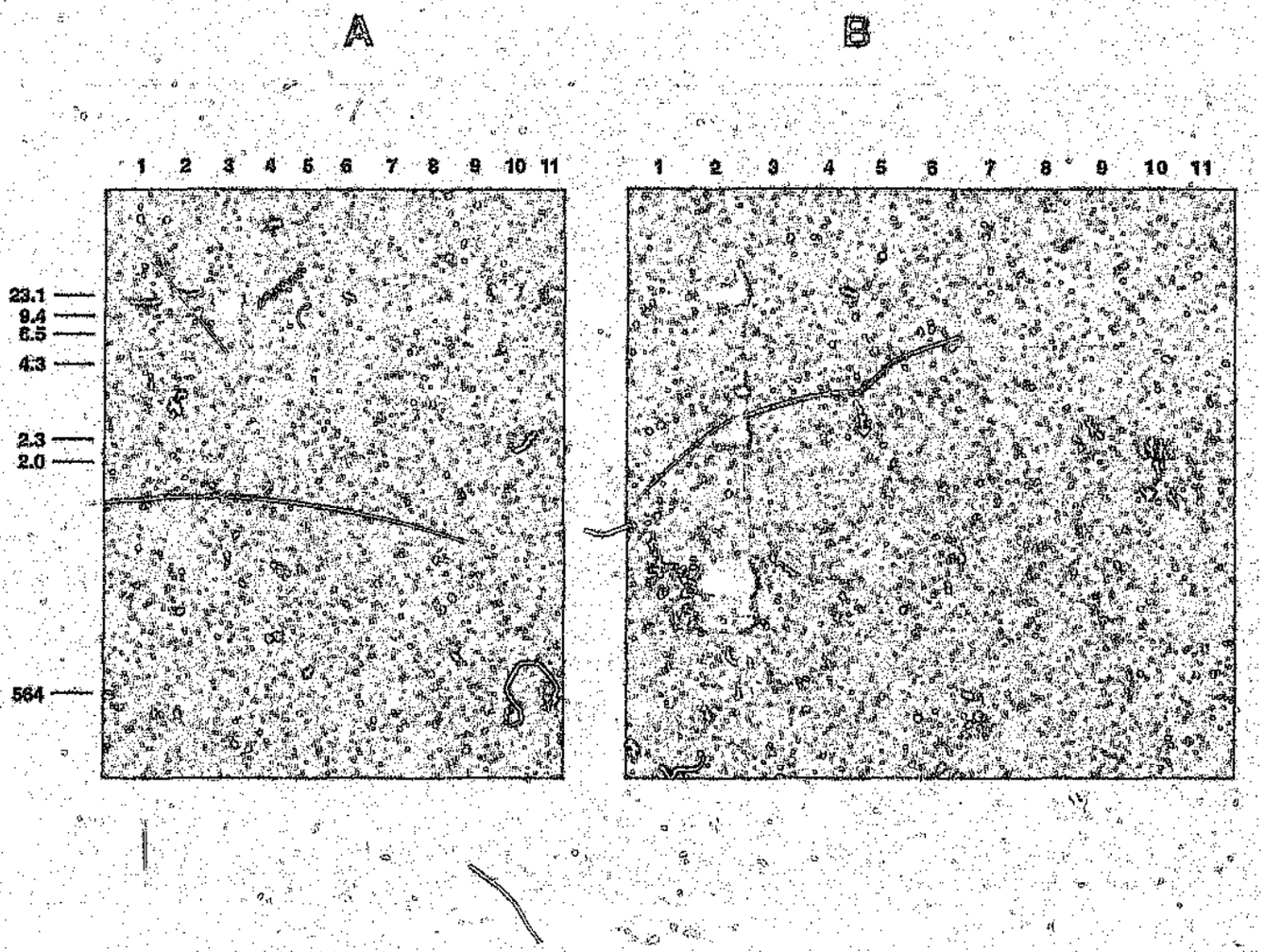


Fig.4.8 Hybridization of RNA extracted from class I leaf curl to dsRNA preparations. A, Double-stranded RNA preparations from all plant species and adult whitefly in 1% agarose gel stained with ethidium bromide. B, Northern blotting.Nucleic acids were transferred by capillary elution to a nylon membrane and the blot was hybridized to RNA probe prepared from all 10 visible dsRNA species from class I tobacco leaf curl. Lane 1, *Pst* I molecular weight marker; lane 2, *Hind* III molecular weight marker; lane 3, class I tobacco leaf curl; lane 4, class II tobacco leaf curl; lane 5, class III tobacco leaf curl; lane 6, asymptomatic tobacco grown from seeds collected from class I leaf curl; lane 7, healthy squash *(C.pepo cv. Table Queen)*; lane 8, healthy squash *(C.pepo cv. Long Green Bush)*; lane 9, healthy squash *(C.pepo cv. Ronel)*; lane 10, silverleaf squash; lane 11, whitefly *(Bemesia tabaci)*. Relative positions (in kilobase pairs) of λ DNA *Hind* III-digested molecular weight markers are indicated on the left.

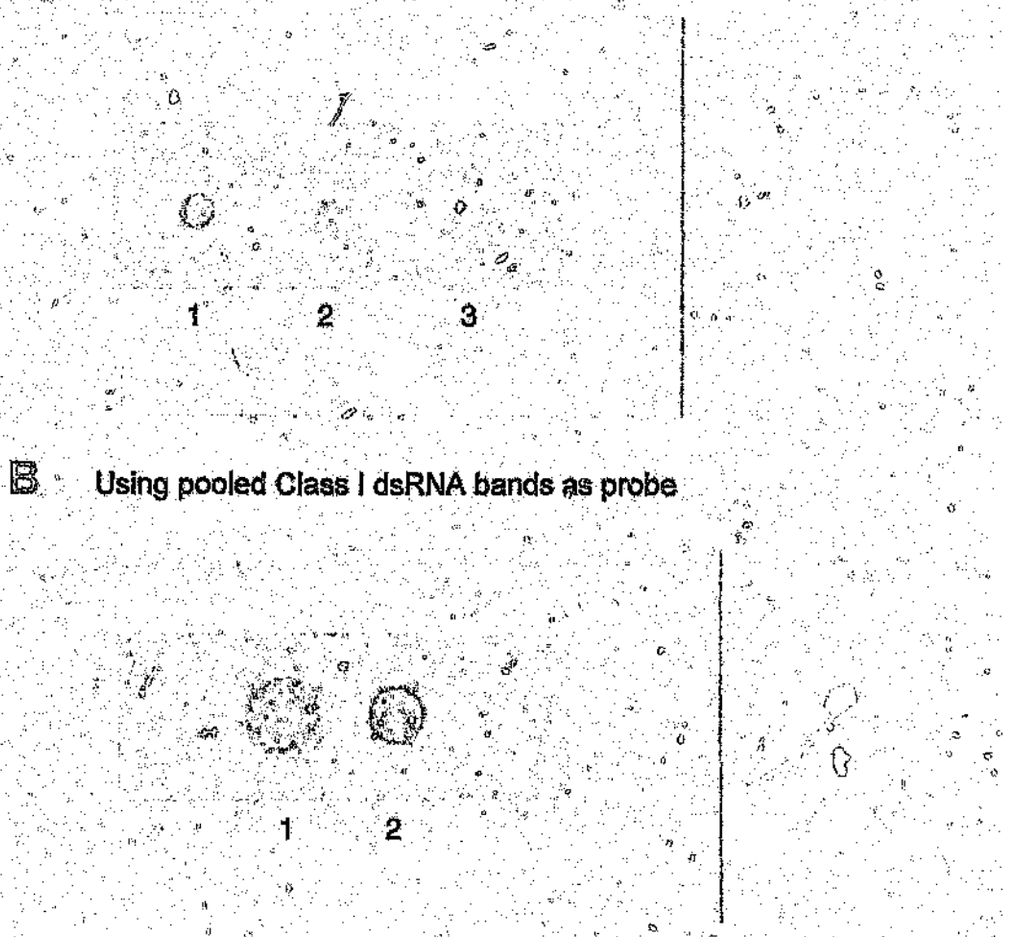


Fig.4.9 Dot blot hybridization of mRNA and dsRNA. A. Labelled mRNA as probe. Spot 1, mRNA 50 ng; spot 2, mRNA 5 ng; spot 3, mRNA 0.5 ng. B. Labelled dsRNA as probe. Spot 1, dsRNA 150 ng; spot 2, dsRNA 75 ng.

4.4.2 Dot Blot Hybridizations

Results of dot-blot hybridization studies, in which dsRNAs from each plant isolate and whitefly were probed with four separately ECL-labelled dsRNA bands (4.4-, 3.6-, 3.3- and 2.4-kbp) from class I leaf curl, are shown in fig. 4.10.

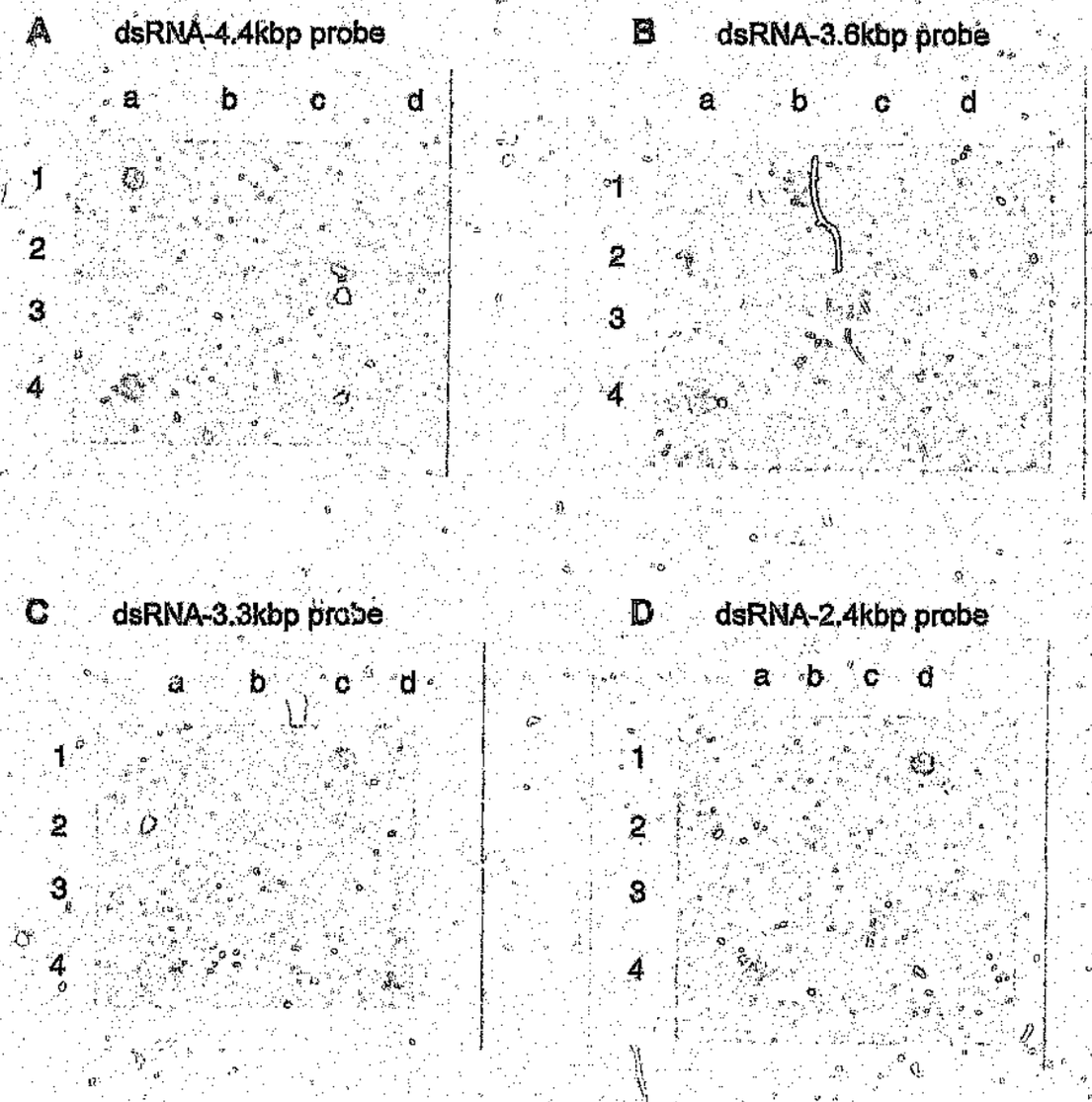


Fig 4.10 Detection of class I dsRNA homology by dot hybridization using four separately ECL-labelled dsRNA bands as probes. A. dot blot hybridization with labelled dsRNA 4.4-kbp probe. B. Dot blot hybridization with labelled dsRNA 3.6-kbp probe. C. Dot blot hybridization with labelled dsRNA 3.3-kbp probe. D. Dot blot hybridization with labelled dsRNA 2.4-kbp probe. Spot 1a, dsRNA 4.4-kbp band; spot 1b, dsRNA 3.6-kbp band; spot 1c, dsRNA 3.3-kbp band; spot 1d, dsRNA 2.4-kbp band; spot 2a, pooled lower dsRNA bands; spot 2b, class II tobacco leaf curl; spot 2c, class III tobacco leaf curl; spot 2d, healthy squash (*cv Long Green Bush*); spot 3a, healthy squash (*cv Ronel*); spot 3b, squash silverleaf; spot 3c, whitefly; spot 4a, TRNA extracted from class I leaf curl (positive control); spot 4b,TRNA extracted from healthy tobacco grown from seed (negative control).

Hybridization studies with the four dsRNA class I probes showed that each specific labelled dsRNA probe hybridized strongly to its respective complementary target RNA (Figs.4.10A, spot 1a; 4.10B, spot 1b, 4.10C, spot 1c; 4.10D, spot 1d). As expected, none of the probes hybridized with preparations from healthy tobacco plants (Fig.4.10A-D; spots 4b). This establishes that the dsRNA probes were uncontaminated by any host-specific nucleic acids and that the dsRNAs are not a natural component of healthy tobacco. DsRNA 4.4-kbp and 2.4-kbp probes did not hybridize to class II and III tobacco, however both dsRNA 3.6-kbp and 3.3-kbp probes hybridized weakly to tobacco class II leaf curl dsRNA (fig.4.10B and 4.10C, spot 2b) and to tobacco class III leaf curl dsRNA (fig.4.10B and 4.10C, spot 2c). These isolates still tested positive when the dot-blot assays were repeated under high stringency conditions (data not shown). In contrast, the two faint spots (fig.4.10A, spot 2b and 2c) were not observed following the same high stringency conditions and are thus considered negative. With the exception of dsRNA-3.6kbp probe (fig.4.10B, spot 1d) which hybridized weakly with the 2.4-kbp band, the other three labelled probes did not hybridize with each other. In addition, all four dsRNA probes failed to detect sequence homology with pooled lower class I dsRNA bands (fig.4.10A-D, spot 2a), indicating the uniqueness of the nucleotide sequences of the RNA molecules. The two healthy squash cultivars, *Long Green Bush* (Fig.4.10A-D, spot 2d) and *Ronel* (fig.4.10A-D, spot 3a), squash silverleaf (fig.4.10A-D, spot 3b) and whitefly (Fig.4.10A-D,spot 3c) did not cross-react with any probes.

CHAPTER 5: DISCUSSION

The primary objective of this project was to characterize the dsRNA species detected in extracts from tobacco plants exhibiting class I leaf curl symptoms and to establish whether they play a role in the etiology of leaf curl symptomology. Furthermore the possibility that the dsRNA species in class I tobacco show homology with the dsRNAs in whitefly and squash silverleaf symptomology was also investigated.

5.1 CHARACTERIZATION OF dsRNA SPECIES

Results presented from this reseach confirm the consistent presence and double-stranded nature of at least 10 visible RNA species isolated from class I tobacco leaf curl plant material. Although the relative molecular weights of these 10 dsRNA fragments (Table 4.1) correlate well with those obtained by Paximadis (1995), two additional dsRNA bands (MW *c.* 5.1 and 4.4 x 10^6) were observed. It was later discovered that the three classes of tobacco plants used in this study, had become inadvertently infected with tobacco mosaic virus (TMV) although the symptoms were initially masked. Although several papers have been published concerning the number of dsRNA species associated with TMV (Palukaitis *et al.*, 1983), these dsRNA species were not always consistently obtained. When the dsRNAs were purified by CF-11 cellulose chromatography, a doublet was often observed at the position of ds-1 (MW *c.* 5.16 x 10^6) and ds-2 (MW *c.* 4.40 x 10^6) (table 4.1). Thus, the additional bands observed in tobacco class I leaf curl extracts may be attributed to this TMV-dsRNA doublet. According to Zelcer *et al.* (1981), TMV-infected tobacco plants contain, in addition to the viral replicative form of 4 x 10^6 MW, three smaller dsRNA species with apparent

molecular weights of 2.25, 1.1, and 0.23 x 10^6. Consequently the top two dsRNA bands, suspected to be contaminating TMV bands, were ignored. In addition, only dsRNA bands which correlated in size with those originally isolated by Paximadis (1995), were excised from LMT agarose gel and used for further testing. Despite these precautions, it would appear that a TMV-dsRNA species, having an apparent molecular weight of approximately 2.25 x 10^6 MW, may have co-migrated with the class I dsRNA doublet (2.44 and 2.34 x 10^6 MW), since hybridization studies using these two class I dsRNA species as labelled probes (3.6 and 3.3 kbp) showed sequence homology with tobacco class II and III leaf curl. It seems probable that the TMV-dsRNA species hybridized with its complementary target RNA in TMV-infected class II and III tobacco leaf curl plants. Although these molecular weight dsRNAs were not visible as clear bands on agarose gels (lanes 4 and 5, fig.4.3), it is possible that they are were present in low concentrations which could be detected by sensitive hybridization methods (fig.4.10B-C, spots 2b and 2c). In order to clarify the question of the 3.3- and 3.6-kbp weak hybridization to class II and III tobacco, TMV-free tobacco would need to be tested.

Electrophoretic analysis of the dsRNA species (Fig. 4.3) shows the presence of dsRNA in infected class I leaf curl but not healthy tobacco plants, suggesting the non-endogenous nature of the detected dsRNA species. In addition, asymptomatic plants grown from seed collected from plants exhibiting severe class I leaf curl, do not contain dsRNA (lane 6, fig. 4.3), indicating that the etiological agent is not seed transmitted. Similarly, the 12 dsRNAs could not be detected in tobacco plants exhibiting class II and class III symptoms indicating that the etiology or mechanism involved in symptom expression is not the same for all three classes of leaf curl. In fact, it has been demonstrated that class III symptoms are due to a geminivirus and class II is suspected (but not proven) to be a genetic disorder (Paximadis, 1995). Furthermore, the dot-

blot results demonstrate that class I dsRNA are unique sequences, suggesting that these RNA molecules comprise a multipartite genome. Although dsRNA-3.6kbp probe weakly hybridized with class I dsRNA band 2.4kbp (fig. 4.10B, spot 1d), all other hybridizations between bands were not detected. The reason for the 3.6- and 2.4-kbp weak cross-hybridization is not known, however, the possibility that there is a small stretch of sequence homology between the two class I dsRNA genome fragments (3.6- and 2.4-kbp) cannot be ruled out.

Results of oligo(dT) trapping for RNA possessing a poly(A)$^+$ tail show that the dsRNA species isolated from class I tobacco leaf curl are not polyadenylated. Reoviruses have also been found not to possess a poly(A)$^+$ tail. Although most eukaryotic mRNAs have a 3' poly(A) sequences, not all plant viruses possess such a sequence (Bol *et al.*, 1975). Since *in vitro* transcription and translation of dsRNAs of several members of the genus *Phytoreovirus* have been successful (section 1.3.3.1), it is possible that each of the class I dsRNA segments is unique, acting as template for a single mRNA molecule that, in turn, directs the synthesis of a single viral polypeptide. McCrae *et al.* (1978), in an attempt to assign cognate RNA and polypeptide species, turned to an alternate supply of reovirus messenger RNA, namely, the ds genome RNA segments themselves. Unlike ssRNA species, the dsRNA segments migrate as very tight bands, enabling all 10 segments of reovirus dsRNA to be isolated in virtually pure form, thus providing a means of determining the nature of the polypeptide encoded by each of the 10 segments of reovirus dsRNA. Perhaps such as technique may be employed in further studies to identify virus-specific polypeptides encoded by each class I dsRNA segment in tobacco.

The results of hybridization studies between the class I dsRNA-4.4 probe and dsRNA extracts from squash silverleaf and whitefly were of special interest for two reasons. Firstly, both Bharathan *et al.* (1990) and Yokomi *et al.* (1990) reported that there is an unequivocal relationship between the presence of two dsRNAs (4.2 and 4.6-kbp) in adult whitefly and the whitefly-mediated silverleaf syndrome. Since Bharathan hypothesized that the dsRNA may be the replicative form of a virus or viruslike agent causing the silverleaf syndrome, the detection of sequence homology between the class I dsRNA-4.4kbp probe and either squash silverleaf or whitefly, may shed some light on the etiology of tobacco class I leaf curl symptomology. The second reason is that this particular dsRNA band lies within the cryptic virus RNA size range. However, in this study, no detectable levels of sequence homology were observed between the class I 4.4-kbp probe and these two extracts (fig. 4.10A spot 3b; spot 3c). It is significant to note that Bharathan and co-workers made no effort to remove whitefly eggs and nymphs from leaf and plant material prior to dsRNA extraction and it is therefore likely that the two dsRNA species detected in squash by Bharathan were derived from the silverleaf whitefly.

High molecular weight dsRNAs were also unexpectedly detected in extracts from two of the three asymptomatic squash cultivars, cv. *Table Queen* (MW >15.7 x 10^6 and *c.*6.5 x 10^6) and *Long Green Bush* (MW >15.7 x 10^6) intended as squash controls. The top band (MW >15.7 x 10^6), detected in both cultivars, was identified as genomic DNA as demonstrated by its sensitivity to DNAse I (data not shown). Cv. *Table Queen* was subsequently excluded from the study as the lower dsRNA band (MW *c.*6.5 x 10^6) could easily be confused with dsRNA of viral origin and thus lead to erronous conclusions. This dsRNA species may be cellular dsRNA of unknown origin, specific to a particular cultivar or plant species, or may indicate the presence of a cryptic virus or latent

viruslike agent. The detection of these unexpected dsRNAs emphasizes the need to evaluate healthy, symptomless seed-grown controls and to select only those cultivars which lack dsRNA species.

5.2. ETIOLOGY OF CLASS I TOBACCO LEAF CURL

Although no evidence has been presented that conclusively demonstrates the etiology of class I tobacco leaf curl disease, two hypotheses have emerged. Support for the latter (5.2.2) is presented.

5.2.1 WHITEFLY-MEDIATED SYMPTOMOLOGY

The first hypothesis is based on the ability of whitefly to induce physiological and cytological disorders, suggesting that class I tobacco leaf curl may be due to hormonally mediated alterations in the plant's physiology induced by whitefly feeding or by a virus or viruslike agent carried by the whitefly. This hypothesis was initially proposed based on literature reports (McClean, 1940) that tobacco leaf curl was the result of a whitefly-transmitted geminivirus infection. Whitefly-mediated squash silverleaf was originally thought to be associated with two distinctive dsRNA species (*c.* 4.2- and 4.6-kbp) and increased RNA-dependent RNA polymerase activity (Bharathan *et al.* 1990 and 1992). Yokomi *et al.*(1995), however, correlated the induction of leaf silvering with the feeding action of silverleaf whitefly, suggesting rather that a toxicogenic factor was involved. These investigators produced further evidence by showing that plant biochemical regulators such as chlormequat chloride (a gibberellic acid biosynthesis inhibitor) induced leaf silvering symptoms similar to those induced by silverleaf whitefly on *Cucurbita pepo* L.

without any dsRNAs being detected. These results suggest that plant bioregulator silvering and whitefly-induced leaf silvering result from similar hormone-mediated alterations in growth and development and symptom expression is not necessarily a direct result of a virus or viruslike agent. However, results from this study do not support the hormone-mediated theory as the two dsRNA species, isolated from silverleaf-associated whitefly, were not detected in the whitefly tested in this study. Only a single *c.* 8.8-kbp dsRNA was noted (lane 11, fig. 4.3) and no dsRNAs have been detected in squash silverleaf material. Furthermore dsRNAs have now been shown not to be linked to physiological disorders (Jiménez *et al.*, 1995).

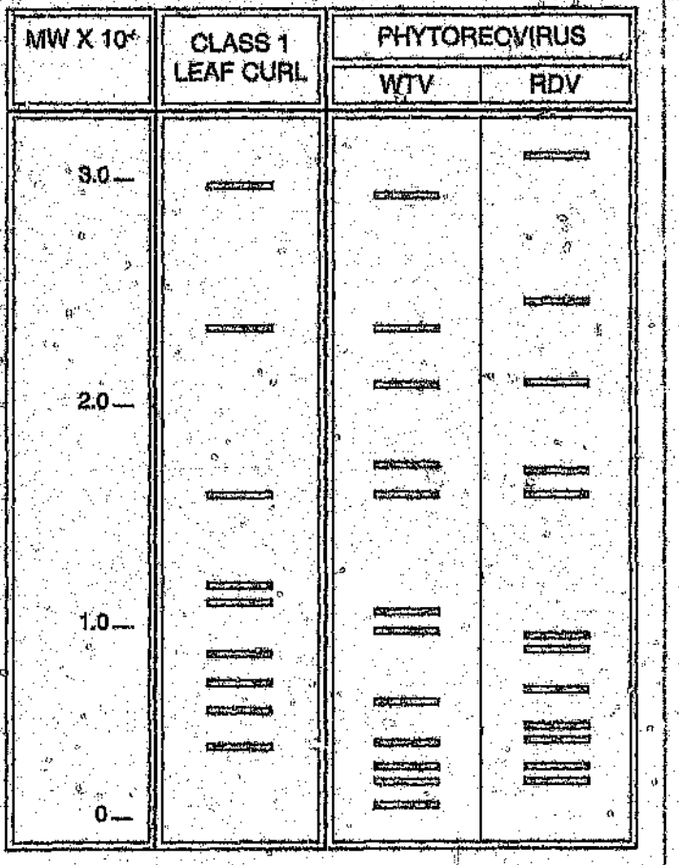
5.2.2 VIRAL ETIOLOGY

The second hypothesis is based on the detection of the dsRNA species often associated with disorders of unknown, though suspected viral etiology, and whose detection has often been used as evidence for viral etiology when viral particles could not be purified (Dale *et al.*, 1986). This approach is based on the premise that plants, not infected with RNA viruses or virus-like agents, do not contain readily detectable amounts of endogenous segments of high-molecular-weight (>0.1 x 10^6) dsRNA (Ikegami *et al.* 1979; Dodds *et al.* 1984) and that RNA viruses replicate via a double stranded intermediate. Accepting this premise, it is suggested that the dsRNA species, isolated from class I tobacco leaf curl, may be viral in origin, possibly the replicative dsRNA of a single-stranded (ss) RNA virus, the genome of a dsRNA virus, or some plasmid-like molecule of unknown etiology. The possible presence of a cryptic virus being responsible for the dsRNA species detected can be eliminated since cryptic viruses are present in usually symptomless plants in low

concentrations and do not consist of 10-12 dsRNA segments. Dodds *et al.* (1984) report that most of the cryptic viruses studied and characterized have a dsRNA genome of 2-5 segments, with molecular weights ranging from 0.5-2.6 x 10^6. In addition, cryptic viruses are seed-transmitted and no dsRNA species were detected in asymptomatic tobacco plants grown from seed derived from severely affected class I tobacco leaf curl plants (fig.4.3). Failure of tobacco seedlings, grown from class I leaf curl seeds, to produce class I leaf curl symptoms also eliminates the possibility that class I leaf curl is an inherited, genetic disorder of tobacco.

It could be postulated that the dsRNA species isolated from class I tobacco leaf curl is the replicative form (RF) of ssRNA viruses since this form is the most common kind of dsRNA detected in infected plant cells. Numerous flexuous particles resembling potyviruses (potato virus Y) in morphology and size were detected in ultrastructural studies carried out on thin sections prepared from enations of leaves from severely infected tobacco plants (Duyver, 1990 and Paximadis, 1995). However, these infections were thought to be latent, not contributing to class I leaf curl etiology, since transmission studies, involving potyvirus purified from tobacco exhibiting class I leaf curl symptoms, failed to induce similar symptoms in inoculated indicator plants (Paximadis, 1995). In addition, potyviruses do not induce visible dsRNA species. Furthermore, serological studies carried out by Paximadis (1995) demonstrated that class I leaf curl tobacco plants failed to cross-react with a variety of geminivirus antisera. Although other infective viral particles were not observed, this may have been the result of inadequate sampling, uneven distribution or restriction to certain tissues, or being detectable only during certain stages of infection.

A striking resemblance of tobacco leaf curl disease with several leaf and planthopper-borne diseases, known to be caused by the plant- infecting family, *Reoviridae*, is noted. Class I tobacco leaf curl plants possess many features which are characteristic for some plant-infecting reoviruses, such as the development of enations and vein thickenings on the lower leaf surfaces, the detection of at least 10-12 dsRNA segments in partially purified preparations, and transmission from infected to healthy plants by an insect vector. All previous attempts were to purify a geminivirus or potyvirus from class I tobacco leaf curl extracts, and reovirus-like particles corresponding to the structure and size (~ 55 nm) of the genus, *Fijivirus* subviral particle (SVP) or core, may easily have been overlooked. Another reason which may account for the failure to detect a reovirus is that reoviruses, fijiviruses in particular, are sensitive to a variety of physical and chemical agents (which were used in geminivirus and potyvirus purification), and therefore care ought to be taken not to expose virus particles to these reagents during purification and isolation processes. While possession of at least 10 dsRNA segments was the initial reason for suggesting that class I leaf curl may be associated with plant-infecting reoviruses, it was the electrophoretic banding patterns that highlighted the remarkable similarity (fig. 5.1). Plant- and leafhoppers are endemic to areas in the Eastern Transvaal and Zimbabwe. Since the tobacco plants used in this study were originally collected from these areas, it is highly probable that the plants have been in contact with these insect vectors, and it is possible that transmission of a reovirus to tobacco may be responsible for class I leaf curl .



(Adapted : Francki, R.I.B and Boccardo, G. 1983. *In*: The *Reoviridae*)

Fig.5.1 Diagramatic representation of the relative sizes of class I tobacco leaf curl and two members of the genus *Phytoreovirus*

5.3 SUMMARY

Although no evidence has been presented that unequivocally demonstrates the etiology of class I tobacco leaf curl, evidence from this research supports the hypothesis that a plant-infecting reovirus may be involved in the etiology of class I tobacco leaf curl disease. Distinctive features common to both class I leaf curl and certain plants infected by a reovirus are :

* development of symptoms such as neoplastic tissues, vein thickenings and stunting;
* detection of 10-12 dsRNA segments in plant extracts;
* similar dsRNA banding pattern;
* dsRNA segments do not possess a poly(A)$^+$ tail;
* each dsRNA segment hybridized selectively to one genome fragment.

Two additional features, not carried in this study, but reported by Paximadis (1995) are :

* transmission dependent on insect vectors;
* not seed-transmitted

These observations emphasize the need for further research in this area as there appears to be a number of plant diseases that, at one time or another, have been claimed to be caused by, or associated with reolike viruses but the etiology of which remains obscure.

6. APPENDICES

6.1 SUPPLIERS OF REAGENTS

Acetic acid, glacial	Holpro
Agarose	Promega
Agarose (LMT)	FMC Bioproducts
β-mercaptoethanol	Calbiochem
Boric acid	Saarchem
Bromophenol blue	Merck
Cellulose CF-11 powder	Whatman
Chloroform	ASE
Diethylpyrocarbonate (DEPC)	Sigma
Dithiothreitol	BM
EDTA	Saarchem
Ethanol	BDH
Ethidium bromide	BM
Formaldehyde	BDH
Glycerol	ASE
Hydrochloric acid	BDH
Isoamyl alcohol	Merck
Lithium chloride	Saarchem
Phenol	Biochemika (Fluka)
Sodium acetate	BDH
Sodium bicarbonate (anhydrous)	Saarchem
Sodium citrate	Saarchem
Sodium chloride	Associated Chemical Enterprises

Sodium dodecyl sulphate	Biorad
Soulum hydroxide	Holpro/BDH
Tris	BM
Tris-HCl	BDH

ENZYMES	
Hind III	BM
Pst I	BM
Proteinase K	BM
RNase I	BM

6.2 REAGENTS USED FOR EXTRACTION OF dsRNA

6.2.1 STE Extraction Buffer (0.1M NaCl, 0.05M tris, 1mM EDTA)

A 10 x stock solution was prepared by adding 61g Tris base, 58g NaCl and 3.7g EDTA to 800ml distilled water. The pH was adjusted to 6.8 using concentrated HCl and then made up to 1 litre with distilled water.

6.2.2 STE-Ethanol (16.5%)

100ml of 10x STE extraction buffer was added to 174ml 100% ethanol and made up to 1 litre with distilled water.

6.2.3 TE Buffer (1mM tris-HCl, 0.1mM EDTA)

A 10x stock solution was prepared by adding 12.1g Tris HCl and 3.7g EDTA to 800ml distilled water. The pH was adjusted to 8.0 with concentrated HCl and then diluted to 1 litre with distilled water.

6.3 REAGENTS USED FOR EXTRACTION OF TOTAL RNA

All manipulations involving DEPC were carried out in the fume cupboard. Gloves were worn when handling such solutions and DEPC-contaminated waste was regarded as extremely toxic and disposed of accordingly. All stock solutions prepared below were made up in DEPC-treated water, incubated overnight at 37°C and then autoclaved for 20 min. at 15lb/sq. Working solutions were prepared from stock solutions and made up to volume using DEPC-treated water, previously incubated and autoclaved.

6.3.1 0.1% DEPC Water

A 0.1 % solution was prepared by adding 1 ml DEPC to 999 ml sterile water. The solution was incubated overnight at 37°C and then autoclaved for 20 min. at 15 lb/sq.

6.3.2 Guanidine HCl Homogenization Buffer
(8M guanidine HCl in 10mM DTT)

18 ml of 8M guanidine HCl was added to 2 ml of a 0.1M DTT stock solution prepared in DEPC-treated water.

6.3.3 1 M Dithiothreitol (DTT) Stock Solution

3.09 g of DTT was dissolved in 20 ml of 0.01M sodium acetate (pH 5.2). The solution was filter-sterilized and dispensed in 1ml aliquots and stored at -20°C.

6.3.4 Chloroform:Isoamyl Alcohol-Saturated Phenol

To prepare 50 ml ($CHCl_3$:IAA)-saturated Phenol, 48 ml chloroform was added to 2 ml IAA. 50 ml phenol was then added to the solution and mixed well. The solution was transferred to a dark bottle and stored at 4°C.

6.3.5 8M Lithium Chloride

3.39 g lithium chloride was added to 10 ml DEPC-treated water, incubated at 37°C overnight and then autoclaved for 15-20 min at 15 lb/sq.

6.4. AGAROSE GEL ELECTROPHORESIS

6.4.1 Tris Borate EDTA (TBE) Buffer

(0.089M tris, 0.089M boric acid, 0.002M EDTA)

A 10 x TBE stock solution was prepared by adding 108g of Tris base, 55g of boric acid and 40 ml 0.5M EDTA (pH 8) to 800 ml sterile distilled water. The pH was adjusted to 8.3 with acetic acid and the solution made up to 1 litre with sterile distilled water. To prepare 1 x TBE buffer, 100 ml of stock solution was added to 900 ml sterile distilled water.

6.4.2 Tracking Dye

2% bromophenol blue was dissolved in 40% RNase-free sucrose prepared in sterile TE buffer.

6.5 MOLECULAR WEIGHT MARKERS

6.5.1 Preparation of MW Markers

To prepare lamba-cut molecular weight markers, 4 µl of the selected restriction enzyme was added to 15 µl λ-DNA, 2 µl of the appropriate marker buffer and 2µl of filtered, autoclaved SABAX water in a sterile eppendorf tube. The tube was incubated for 3 h at 37°C with thorough mixing every 30 min. After incubation, 20µl of filtered, autoclaved SABAX water was added to the tube which was then either kept at 4°C for immediate use or stored at -20°C.

6.5.2 Lambda-cut *Hind* III MW Marker (Boehringer Mannheim)

Fragment lengths (base pairs): 23 130; 9 416; 6 557; 4 361; 2 322; 2 027; 564; 125

6.5.3 *Pst* I Molecular Weight Marker (Boehringer Mannheim)

Fragment lengths (base pairs): 11 497; 5 977; 4 747; 4 507; 2 838; 2 560; 2 459; 2 443; 2 140; 1 986; 1 700; 1 159; 1 093; 805; 514.

6.6 RECOVERY OF dsRNA FROM LMT GELS

6.6.1 Preparation of A 0.9% LMT Gel

The protocol for the preparation for a 0.9% gel is essentially the same as already described in section 3.5, except 0.45g of low melting temperature agarose (SeaPlaque) was added to 50 ml 1x TBE buffer. The agarose was heated in a microwave oven and once cool, 1 μl ethidium bromide (10mg/ml) was added and the gel poured into the electrophoretic tray and allowed to solidify.

6.6.2 4M Lithium Chloride

A 4M lithium chloride solution was prepared by adding 1.69 g of lithium chloride to 10 ml sterile distilled water.

6.7 REAGENTS USED FOR RIBONUCLEASE A DIGESTION

6.7.1 SSC Buffer (0.15M NaCl, 0.015M sodium citrate, pH 7.0)

A 20 x stock solution of SSC was prepared by dissolving 175.4 g of NaCl and 88.2 g sodium citrate in 800 ml sterile distilled water. The pH was adjusted to 7.0 with a few drops of 10N HCl and the volume made up to 1 litre with sterile distilled water. 1.5ml of this stock solution was added to 8.5 ml sterile distilled water to prepare a 3 x SSC solution.

6.7.2 DNase-free RNase A

A stock solution of 10 mg/ml was prepared by dissolving pancreatic RNase (RNase A) in 10 mM Tris HCl (pH 7.5) and 15mM NaCl. The solution was heated to 100°C for 15 min and then allowed to cool slowly to room temperature. The stock solution was dispensed into 1 ml aliquots and stored at -20°C.

6.7.3 Proteinase K

A stock solution of 200µg/ml was prepared by filter-sterilizing 13.6 µl of Proteinase K (14.7mg/ml) in 1 ml sterile distilled water. 12.5 µl of the stock solution was added to 500 µl of sample to obtain a final concentration of 5µg/ml.

6.7.4 0.05% Sodium Dodecyl Sulphate (SDS)

A stock solution of 0.5% SDS was prepared by adding 0.5 g SDS to 100 ml sterile distilled water. To prepare a 0.05% solution, 1 ml of stock was added to 9 ml sterile distilled water.

6.8 REAGENTS USED FOR OLIGO(dT) TRAPPING

6.8.1 Preparation of Glassware and Plastic tips

All glassware was autoclaved and baked overnight at 180°C; plastic tips, electrophoretic trays and eppendorfs were soaked overnight in 1M NaOH and rinsed several times with autoclaved, sterile DEPC-treated water. Plastic tips and eppendorfs were then autoclaved for 20 min at 15lb/sq.

6.8.2 0.5M NaCl

A 2M NaCl stock solution was prepared by adding 11.6 g of NaCl to 100 ml sterile 0.1% DEPC-treated water. The stock solution was then autoclaved for 20 min at 15 lb/sq. before preparing working solutions. To prepare a 0.5M NaCl solution, 10 ml of stock solution was added to 30 ml sterile DEPC-treated water. To adjust the samples to contain a final concentration of 0.5M NaCl, 5 µl of 2 M NaCl was added to 15 µl of sample.

6.9 BUFFERS USED FOR NORTHERN BLOTS

6.9.1 Denaturing Buffer (50mM NaOH)

A stock solution of 500mM NaOH was prepared by adding 10 g NaOH to 500 ml DEPC-treated water. To prepare a working solution of 50mM NaOH, 50 ml of stock solution was added to 450 ml DEPC-treated water.

6.9.2 Transfer Buffer (0.025M sodium phosphate, pH 6.5)

A stock solution of 0.25M sodium phosphate was prepared by adding 13 g to 400 ml DEPC-treated water and the pH adjusted to 6.5 with HCl. Volume was then made up to 500 ml with DEPC-treated water. To prepare a working solution of 0.025M, 50 ml of stock solution was added to 450 ml DEPC-treated water.

6.10 REAGENTS AND BUFFERS FOR HYBRIDIZATION

6.10.1 Hybridization Buffer (Supplied by Amersham)

Sodium chloride was added to the hybridization buffer (0.125ml/cm^2) to obtain a final concentration of 0.5M. The blocking agent was then added to a final concentration of 5% (w/v). The buffer was immediately thoroughly mixed for 1 h at room temperature on a magnetic stirrer, then heated to 42°C for 0.5-1 h with occassional mixing.

6.10.2 Low Stringency Primary Wash Buffer (0.4% SDS, 1 x SSC)

Low stringency primary wash buffer was prepared by adding 4 g SDS in 800 ml DEPC-treated water and 50 ml of a 20 x SSC stock solution to 800 ml DEPC-treated water. The buffer was made up to 1 litre and kept at 4°C.

6.10.3 High stringency Primary Wash Buffer (0.4% SDS, 0.1 x SSC)

High stringency primary wash buffer was prepared by adding 4 g SDS in 800 ml DEPC-treated water and 5 ml of a 20 x SSC stock solution to 800 ml DEPC-treated water. The buffer was made up to 1 litre and kept at 4°C.

6.10.4 Secondary Wash Buffer (0.5 x SSC)

Secondary wash buffer was prepared by adding 25 ml of 20 x SSC stock solution to 975 ml DEPC-treated water. The buffer was stored at 4°C.

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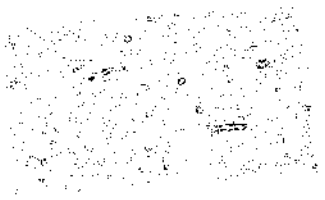

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