CHARACTERISATION OF MINOR RNAs ASSOCIATED WITH PLANTS INFECTED WITH CUCUMBER MOSAIC VIRUS

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by

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TABLE OF CONTENTS

Chapter 3 Identification and isolation of new ds-RNA fragments in Cucumber Mosaic Virus infected plants

Chapter 4 Characterisation of new ds-RNA fragments in Cucumber Mosaic Virus infected plants

"Characterisation of minor RNAs associated with plants infected with cucumber mosaic virus "

ABSTRACT

The main aim of this study was to characterise the minor double stranded RNAs (dsRNA) and single stranded RNAs (ssRNA) which are consistently associated with plants infected with Q strain of cucumber mosaic virus (Q-CMV). *Cucumovirus* is one of the four genera of the family Bromoviridae and includes three species, cucumber mosaic virus (CMV), tomato aspermy virus (TAV) and peanut stunt virus (PSV). Strains of CMV are divided into two subgroups (I and II) on the basis of nucleotide sequence homology. Viruses from the Bromoviridae contain a tripartite RNA genome which encodes four proteins. Proteins 1a and 2a, translated from RNAs 1 and 2 respectively, are components of the viral RNA polymerase. RNA 3 encodes 3a and coat protein (CP) and CP is translated through a subgenomic mRNA, RNA 4. It was shown recently that RNA 2 of CMV strains (in both subgroups) encode an additional 2b protein which is translated from RNA 4A, a subgenomic RNA of RNA 2 (Ding et al., 1994; Shi et al, 1997).

Polyacrylamide gel electrophoresis of double-stranded RNAs (dsRNA) extracted from Q-CMV infected plants indicated the presence of three new dsRNA species of about 2.4, 1.2 and 0.9 kb (designated dsB1, dsB2 and dsB3), in addition to the dsRNA forms of the known major viral RNA species. Northern analysis showed that these new dsRNAs are of viral origin and that they have sequence homology with Q-CMV RNA2. Moreover, their consistent presence in dsRNA profiles of Q-CMV and some other strains implied that they may be biologically important.

A prerequisite for investigation of these ds-RNAs, which are present in low amounts, was to find an efficient method for their isolation. For this purpose, different isolation

procedures were examined. The isolation of the minor ds-RNAs was most efficiently achieved using the Bis-acrylylcystamine (BAC) method of Dulieu and Bar-Joseph (1989) with some modifications. Dissolving the acrylamide and BAC at less than 50°C, keeping the mixture of dsRNA and acrylamide at 50°C during the isolation process and increasing the washing times, were found to be efficient modifications allowing elimination of undissolved acrylamide gel pieces in the eluted dsRNA samples.

The isolated dsRNA fragments were used to make a cDNA library using the random-primer PCR method which has already been used for cDNA synthesis of single stranded RNAs. For each dsRNA fragment, overlapping cDNA clones were generated and sequenced. RACE-PCR was also employed to amplify 5'-terminal sequences. The results revealed that dsB1, dsB2 and most likely dsB3 have both 3' and 5' termini identical to those of the Q-CMV RNA2. Furthermore, due to the deletion of some sequences, they are smaller than full length RNA2. Therefore, although full length sequences have yet to be obtained, the data so far suggest their similarity with an internal deletion class of defective (D) RNAs.

Subsequently, single stranded RNA profiles of Q-CMV infected plants were studied for the possible presence of additional RNAs. This study showed that, in addition to the known genomic RNAs of Q-CMV, there were three new RNAs (designated TB1, TB2 and TB3) which were not encapsidated. Northern analysis showed that these RNAs were only related to RNA2 and not to RNAs 3 or 4. These minor ss-RNAs were isolated from total RNAs of Q-CMV infected cucumber and used for amplification using the RT-PCR and RACE-PCR methods. Cloning and sequencing of the amplified regions indicated that the 3' and 5' terminal sequences of TB1, TB2 and TB3 were identical to those of the Q-CMV RNA2. However, the amplification of internal regions using a range of primer pairs was not successful. Even though amplification of full-length RNA 2 (positive control) with the same primer pairs succeeded. This implied the possible lack of hybridisation sites for the primers on the minor ss-RNAs. Taken together these results implied that TB1, TB2 and TB3 possibly represent partially

deleted molecules, lacking different numbers of nucleotides in the central region of RNA2. The results so far indicate a close affinity between the minor ds- and ss-RNAs, and it is possible that some or all of the ds-RNA fragments may be the replicative forms of the ss-RNA species. Sequencing of more cDNA clones of the minor ds-and ss-RNAs is required to give a more precise picture.

In addition to the subjects described above, the genetic stability and relative fitness of CMV-qt hybrid virus (which was previously constructed by exchange of the 2b coding sequence between Q-CMV and V-TAV; Ding et al., 1996) were investigated by serial passages and mixed infections. CMV-qt was serially passaged in *Nicotiana glutinosa* plants. No sequence variation was found within the 2b coding region of the progeny virus after ten passages, and the progeny virus remained as virulent to *N. glutinosa* plants as the original CMV-qt, indicating that the hybrid virus is genetically stable. In mixed inoculation experiments, relative fitness of the hybrid virus was studied in competition with its parental viruses. The results showed that in mixed infection of CMV-qt hybrid virus and V-TAV, only CMV-qt hybrid virus was detectable in the systemic leaves of *N. glutinosa* plants, while in mixed inoculation of Q-CMV and CMV-qt, hybrid virus failed to invade *N. glutinosa* plants systemically. These results suggest that the hybrid virus is relatively fitter than V-TAV but not than Q-CMV.

Furthermore, to investigate the important factor(s) involved in the competition of the hybrid virus and its parental viruses, their movement rates and accumulation levels of the RNAs were compared. These experiments showed that there were no detectable differences between the movement rate of Q-CMV, V-TAV and the hybrid virus in the (non inoculated) systemic leaves (measured at 12 hours intervals). However, mixed inoculation of protoplasts with Q-CMV+CMV-qt and V-TAV+CMV-qt revealed a higher accumulation level of the hybrid virus RNAs than the TAV RNAs, but a lower level of the hybrid virus RNAs than the Q-CMV RNAs. These results suggest that in competition between the hybrid virus and its parental viruses, the replication level of each virus is the key factor.

STATEMENT

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

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Chapter 1

General Introduction

1.1 Introduction

Viruses are one of the smallest forms of life and consist mainly of protein and nucleic acid. The viral nucleic acid can be DNA or RNA, either single stranded (ss) or double-stranded (ds). Viruses are obligate parasites that, upon infection of a host cell, are able to adapt the cell's biological machinery for the synthesis of viral nucleoprotein.

The majority of plant viruses have ss-RNA genomes and, due to their small size, they have limited genetic information and encode primarily some specific functions required for virus replication. However, because of severe evolutionary constraints they have developed efficient strategies, e.g. subgenomic RNA synthesis, utilizing read through proteins, frame shifting, polyprotein processing, overlapping genes and synthesis of sub-viral RNAs such as satellite and defective interfering RNAs. Indeed, by these mechanisms viruses have increased the efficiency of their replication, genome expression and packaging strategies, as well as generating genetic diversity which enables them to survive and increase their flexibility to adopt to intra or extracellular changes.

Among the plant viruses with single stranded RNA, cucumber mosaic virus (CMV), is of economic importance and therefore has been studied extensively. An important aspect of the research program describes in this thesis is to determine the roles of various minor RNA species of CMV. This information is necessary as a contribution to our understanding of basic aspects of CMV replication and its disease induction.

The investigations described in this thesis were focused on the structural elucidation of new RNAs which have been observed in ss- and ds-RNA profiles of Q strain of CMV. As an introduction, in this chapter a review of literature will be made as background information for the interpretation and discussion of the experimental results which are described in chapters 3 to 6 hereafter.

1.2 Classification of cucumoviruses

The Cucumovirus Genus comprises three major species, cucumber mosaic (CMV), tomato aspermy (TAV) and peanut stunt (PSV) viruses (Harrison et al., 1971).

All these viruses share similarities in particle morphology, vector transmission and genome organization (See reviews by Kaper and Waterworth, 1981; Francki et al., 1985, Edwardson and Christie 1991; Palukatis *et al.*, 1992). However, on the basis of several criteria, including nucleic acid hybridisation, serological relationships and peptide mapping of the viral coat protein those CMV strains investigated to date fall into two subgroups (see review by Palukaitis *et al.*, 1992).

1.3 Biological aspects of cucumoviruses

The cucumoviruses, and CMV in particular, have been found in most countries of the world and are very important plant pathogens with the largest host range of any virus (see reviews by Kaper and Waterworth, 1981; Francki, 1985; Wood, 1991; Palukaitis *et al.*, 1992). Tomato aspermy virus (TAV) was described from diseased tomato plants by Blencowe and Caldwell (1949) and infects over 100 species in 27 families (Hollings and Stone, 1971). This virus is best known in connection with chrysanthemum diseases throughout the world (Kaper and Waterworth, 1981) and is less able to infect cucumber and other cucurbits.

The most common symptom of CMV or TAV infection is mosaic; although symptoms depend on factors such as host species, the age of plant, the strain of virus and the environmental conditions. Their symptoms cover the whole range from the fernleaf, ring spot, seedless fruits and death to symptomless infection (Kaper and Waterworth, 1981; Palukaitis *et al.*, 1992).

CMV and TAV are naturally transmitted mainly by a large number of aphid species in a non-persistent manner (Edwardson and Christie, 1991). However, these two viruses are readily mechanically transmissible among herbaceous species (Kaper and Waterworth,

1981). CMV and TAV are also transmitted in the seed of several species and by some dodder species (Francki *et al.*, 1979; Holling and Stone, 1971; Edwardson and Christie, 1991). Infected chrysanthemum cuttings is reported as an important means of TAV spread (Kaper and Waterworth, 1981).

1.4 Virion properties

CMV and TAV, like other cucumoviruses, are icosahedral particles with a diameter range from 24 to 42 nanometer (nm) and a central hole which is penetrable by negative stain (Tolin, 1977; Francki *et al.*, 1979; Edwardson and Christie, 1991). The nucleoproteins consist of 18.25% RNA and hexamer-pentamer clustering of 180 identical protein subunits with T=3 surface lattice symmetry (Finch *et al.*, 1967; Hollings and Stone, 1971; Kaper and Waterworth, 1981).

The sensitivity of CMV and TAV particles to low concentrations of sodium dodecyl sulphate (SDS) (Kaper, 1973; Habili and Francki, 1974b) or high concentrations of neutral chloride salts (Kaper *et al.*, 1965; Francki *et al.*, 1966), indicates that the stability of their particles highly depends on RNA-protein interactions (Kaper, 1975). Moreover, TAV is stabilized by Mg2+, while CMV precipitates in the presence of the cation. CMV is stabilized but TAV is degraded, by EDTA (Palukaitis *et al.*, 1992).

The loose packing of protein subunits in the capsid, which leaves holes in the centres of each hexamer and pentamer cluster may explain the ability of ribonuclease to penetrate intact capsids and degrade encapsidated RNA (Palukaitis *et al.*, 1992).

When the molecular weights of coat protein subunits and individual RNA species are taken into account, it can be concluded that the whole genome of CMV is encapsidated, not in a single particle, but in three different particles (Peden and Symons, 1973).

Different procedures have been used to purify the viral particle of cucumoviruses (Kaper and Waterworth, 1981). Development of a method for purification of a cucumovirus

obviously depends on the virus and strain under study as well as on other factors (Kaper and Waterworth, 1981). Commonly used methods for purification of CMV(Lot *et al.*, 1972; Peden and Symons, 1973), are basically modifications of that described by Scott (1963). Tobacco and cucumber are preferential plant species for propagation of most CMV strains (Kaper and Waterworth, 1981).

1.5 Interaction between cucumoviruses in plants

The linkage between protein and RNA in CMV particles can be dissociated easily into the RNA and protein components and these can subsequently be reassembled into biologically active virus particles (Palukaitis *et al.*, 1992). It has been shown that heterologous RNAs such as those of tobacco mosaic virus (TMV) and turnip yellow mosaic virus (TYMV) can be encapsidated by cucumovirus coat proteins (Chen and Francki, 1990; Kaper and Geelen, 1971). Cucumoviral RNAs, also be encapsidated in heterologous coat proteins such as that of alfalfa mosaic virus (AlMV) (Candelier-Harvey, 1993). The lack of specificity in encapsidation of heterologous RNAs by cucumovirus coat proteins may have important epidemiological impacts in nature (Palukaitis *et al.*, 1992). Mechanisms of RNA encapsidation are not well understood, however it has been shown that RNA structure may play an important role in selective protein-RNA recognition (Turner *et al.*, 1988).

1.6. Genome organisation and functions

Cucumoviruses have a single stranded plus sense RNA genome, consisting of three RNA species, designated RNAs 1, 2 and 3 (Fig. 1.1) (Kaper and Waterworth, 1981). In addition, the viral particles encapsidate a fourth RNA (RNA 4), that is derived from the 3'-terminal region of RNA 3. Moreover, some cucumovirus strains contain subgenomic RNAs 4A, 5 or 6. RNAs 1, 2 and 3 are necessary for infection (Peden and Symons, 1973; Lot *et al.*, 1974).

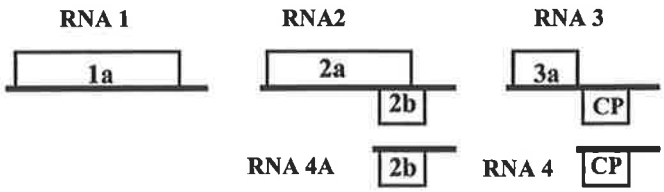

Fig. 1.1 Genome organisation of Q-CMV

All three genomic RNAs contain a conserved untranslated 3'-terminal region of about 300 nucleotides with an adenosine at the end and their 5' termini contain a 7-methylguanosine cap (Symons, 1975). In a reaction similar to the aminoacylation of tRNA the 180 nt of the 3'-terminal can be folded into a three-dimensional tRNA-like structure which accepts tyrosine (Wilson and Symons, 1981; Rizzo and Palukaitis, 1989).

1.6.1 RNA 1

In CMV, RNA 1 contains only one major open reading frame (ORF), encoding a polypeptide of about 990 amino acids, designated 1a protein. There is significant homology between RNA 1 of cucumoviruses at amino acid and nucleotide levels. There is significant homology (85%) between Fny- and Q-CMV strains, at the 1a protein level. Moreover, the nucleotide sequence comparison of RNA 1 between Fny-CMV and Q-

CMV shows 71.4% homology, whereas the homology between Fny-CMV and two other subgroup I strains, O-CMV and Y-CMV, is 92-96% (Palukaitis *et al.*, 1992).

1.6.2 RNA 2

RNA 2s of cucumoviruses have more than one initiation codon. In Fny-CMV and V-TAV, RNA 2s contain one long ORF after the first initiation codon while in RNA 2 of Q-CMV there is a 26-nucleotide ORF after the first initiation codon. A large ORF appears after the second initiation codon which encodes a polypeptide ranging from 829 to 858 amino acids (aa), designated the 2a protein, within RNA 2 of these three cucumoviruses.

It has been shown recently (Ding *et al.*, 1994) that the RNA 2 of all cucumoviruses sequenced to date contain an additional conserved ORF called 2b. The sequence surrounding the AUG codon (an A at position -3 and a G at position +4) agrees with the optimal sequence context of Kozak (1984). The translation product of this ORF 2b has been detected in Q-CMV-infected plants (subgroup II, Ding *et al.*, 1994) and more recently in V-TAV and WAII-CMV, a subgroup I strain (Shi et al., 1997).

The three species of cucumoviruses share an equal homology (46%) at the level of their RNA 2s which on average is lower than their homology at the RNA1 level.

The distribution of homologous regions within the coding area of Q-CMV and Fny-CMV RNA 2s is quite different from that found in the RNA 1. In the RNA 2 the homology is maximal for the central part between amino acids 216 and 757 (71%), higher than for the other two regions, N- (1-223) and C-terminal (750-857), which show 36 and 23.5% identity, respectively (Moriones *et al.*, 1991). In all cucumovirus 2a proteins, a GDD motif for polymerase activity is present in the C-terminal half (Moriones *et al.*, 1991).

1.6.3 RNA 3

RNA 3 contains two ORFs, one encoding a putative movement protein, designated 3a, and the other encoding CP. Comparisons of cucumovirus RNA 3 sequences have revealed a considerable nucleotide sequence divergence between any two of the RNA 3s,

which is higher than that observed between RNA 1s of cucumoviruses, but lower than that between the RNA 2s. The sequence homology at the amino acid level between strains in the two subgroups was high for both ORFs (3a and CP) on RNA3, but was somewhat higher in the 3a protein than the CP (Palukaitis *et al.*, 1992).

1.6.4 RNA 4

RNA 4 is a subgenomic RNA produced from the 3'-terminal of RNA 3. In all CMV strains, RNA 4 encodes a CP (Schwinghamer and Symons, 1975; Davies and Symons, 1988) of 218 aa, while in TAV strains (B, P, V, and C) it encodes a CP of 217 aa, 219 aa, 219 aa and 230 aa, respectively.

The level of either aa or nt sequence identity between CMV and TAV is much lower than that between CMV subgroups. The nt sequence of the CP ORF is the most conserved within subgroups, but not between subgroups or between CMV and TAV. The N-terminal region contains a cluster of basic amino acids (Palukaitis *et al.*, 1992), which is implicated in protein/RNA interactions in the virion (Harrison, 1984), whereas no functional reason is known for the homology at the C-terminus (Palukaitis *et al.*, 1992).

In RNA 4 , the 5' untranslated region is highly conserved either within and between the CMV subgroups. The overall nt sequence homology of RNA 4 is 93.6% within subgroup II strains, while within subgroup I it is only 69% .

1.6.5 RNA 4A

RNA 4A was first reported in 1973 as a minor RNA which encapsidated in Q-CMV (Peden and Symons, 1973). However, its functional role as a subgenomic RNA was not understood until 1994 (Ding *et al.*, 1994). RNA 4A is 682 nucleotides long and is identical in sequence to the 3'-terminal portion of the 2a gene. This RNA is generated from the 3'-terminus of RNA 2 and expresses a 2b protein encoded by RNA 2. RNA 4A is capped at the 5' end (Ding *et al.*, 1994), and is present in subgroup II strains (Palukaitis *et al.*, 1992), as well as subgroup I strains (Shi *et al.*, 1997). The complete

sequence of Q-CMV RNA 4A is 682 nt, identical to the 3'-terminal 682 nt of Q-CMV RNA 2 (Ding *et al.*, 1994), and encodes a small ORF, called ORF 2b, of 100 amino acids which overlaps, with the C-terminal portion of the 2a gene in RNA 2. RNA 4A functions as mRNA and its translation product, the 2b protein has been detected in plants infected by Q-CMV (Ding *et al.*, 1994), V-TAV and WAII-CMV (Shi *et al.*, 1997).

Sequence analysis has revealed that nucleotide and predicted aa sequences of 2b proteins are conserved among different cucumoviruses with more than 30% sequence homology in most cases (Ding et al., 1994).

1.6.6 RNA 5 and RNA 6

In addition to RNA 4A, CMV strains contain two minor RNA species, designated RNA 5 and RNA 6 in order of decreasing molecular weight (Palukatis *et al.*, 1992; Symons, 1978). Recently it has been demonstrated that RNA 5 in Q-CMV is a mixed population without any coding ability derived from the conserved 3'-terminal of the genomic RNAs 2 and 3 (Blanchard *et al.*, 1996). RNA 5 is not found in subgroup I strains of CMV, while RNA 6 is present in both CMV subgroups (Palukaitis *et al.*, 1992). RNA 6 of Q-CMV was shown to be a mixture of plant RNAs contaminated with fragments of the genomic CMV RNAs but why these RNAs are coencapsidated is not clear (Palukaitis *et al.*, 1992).

1.7 Untranslated regions

In the cucumoviruses, the 5' untranslated regions of genomic RNA 1s and 2s have similar lengths. The 3' untranslated regions of all cucumovirus RNA 2s are about 100 nt longer than those of the other RNAs. These extra nucleotides encode part of the 2b gene (Ding et al., 1994).

In cucumovirus RNAs, the 3' untranslated regions are less conserved between viruses but highly conserved within each virus. The sequence homology of the 5' untranslated

region between cucumoviruses is higher than that of the 3' untranslated regions between the same viruses. Cucumoviruses have a tRNA-like structure at the 3' terminus of each genomic or subgenomic RNA. The role of this structure has been demonstrated in TAV to be recognition by and interaction with aminoacyl tRNA synthetase (Joshi and Haenni, 1986). Moreover, it has been shown that the tRNA-like structure is essential for the replication of CMV RNAs (Boccard and Baulcombe, 1993).

Secondary structure of the 3' untranslated region is an important feature of cucumoviruses. In CMV, the 3'-terminal of all three RNAs are similar in secondary structure and sequence (Palukaitis *et al.*, 1992), indicating that the pseudoknot structures present in the 3'-terminal sequences have a functional role (Palukaitis *et al.*, 1992). The importance of the RNA pseudoknot structure has been shown in some other viruses. In TMV, this structure in the 3' untranslated region is essential for the regulation of protein translation (Pleij, 1995) and might be involved in recombination events (Pleij *et al.*, 1987) while in BMV, it is involved in viral replication (Duggal *et al.*, 1994).

1.8 RNA functions

Individual CMV RNAs or combinations of them are associated with different functions. A variety of functions were shown to be controlled by RNA1: seed transmission in *Phaseolus vulgaris* (Hampton and Francki, 1992), local infection of squash and tobacco by NL-CMV (Lakshman and Gonsalves, 1985), CMV transmission and symptom severity (Zitter and Gonsalves, 1991; Roossinck and Palukaitis, 1990; Roossinck 1991). In addition, RNA 2 was found to be associated with CMV systemic infection of cowpea (Edwards *et al.*, 1983; Hanada and Tochihara, 1980; Marchoux *et al.*, 1974) and maize (Rao and Francki, 1982) and the necrosis induction phenotype (Sleat *et al.*, 1994). Furthermore, CMV RNA 3 was found to be involved in hypersensitivity response of arabidopsis (Takahashi *et al.*, 1994), serological specificity (Mossop and Francki, 1977; Hanada and Tochihara, 1980; Marchoux *et al.*, 1974), aphid transmissibility (Mossop

and Francki, 1977; Zitter and Gonsalves, 1991) and local infection of some plants (Rao and Francki, 1982).

A combination of CMV RNAs 2 and 3 have been found to determine the induction of primary symptoms in cowpea (Marchous et al., 1974), the ability of systemic infection in *Lactuca saligna* (Edwards *et al.*, 1983), local infection of some plants (Marchoux *et al.*, 1974; Rao and Francki, 1982) and yellow mosaic symptoms in several CMV host species (Rao and Francki, 1982). Symptom control in some other hosts, the ability to support the systemic movement and accumulation of CMV-satRNA are determined primarily by RNAs 1 and 2 (Moriones *et al.*, 1994).

In pseudorecombinant studies between TAV and CMV, Rao and Francki (1981) showed that their RNA 1 and RNA 2 could not be exchanged, although their RNA 3 could be. Thus, the proteins encoded by RNAs 1 and 2 may interact in concert (Palukaitis *et al.*, 1992).

1.9 Functions of genome-encoded proteins

Q-CMV RNA 1 directs the synthesis of a single polypeptide of Mr 95K (Rezaian *et al.*, 1985) and RNA 2 directs a single polypeptide of Mr 110K (Rezaian *et al.*, 1984). RNA 3 directs the synthesis of a 35K protein (Davies and Symons, 1988). The 24.5K coat protein cistron in RNA3 is translated through the subgenomic RNA 4 (Schwinghamer and Symons, 1975; Davies and Symons, 1988). A polypeptide of Mr 15K is also reported as the translational product of RNA 4A (Ding et al., 1994).

1.9.1 Proteins 1a and 2a

Pseudorecombination studies using protoplasts infected with RNAs 1 and 2 of CMV showed that these two RNAs are capable of producing a replicase (Nitta *et al.*, 1988, Wood, 1991). In addition, further evidence suggest the involvement of 1a and 2a proteins in RNA replication. This evidence consists of amino acid sequence similarities with other viral RNA polymerases (Ishikawa *et al.*, 1986; French *et al.*, 1986; Nassuth

and Bol, 1983; Rizzo and Palukaitis, 1988; Wood, 1991), the presence of 1a and 2a proteins in an active isolated replicase from CMV-infected plants (Hayes and Buck, 1990), and the inhibition of RNA dependent RNA polymerase (RdRp) activity by RNA 1 and 2 antibodies (Hayes *et al.*, 1994).

Further studies revealed that the 1a and 2a proteins may play some roles in cell-to-cell and systemic movement of the virus via either independent functions or a complex of both proteins (Gal-On *et al.*, 1994; Carr *et al.*, 1994).

1.9.2 Protein 2b

Mutational analysis revealed that the 2b gene contributes to long distance movement and systemic symptom expression (Ding *et al.*, 1995b). Further studies showed that the two conserved regions of the 2b protein (KSPSE and the C-terminal 16 aa) are both important for long distance virus movement in cucumber and tobacco plants. In addition, the C-terminal 16 codons are essential for symptom expression in cucumber. These results indicate that the 2b gene encodes a host-specific long-distance CMV movement function and is an important virulence determinant of the virus (Ding *et al.*,1995b).

1.9.3 3a Protein

Several lines of evidence have implied that 3a protein is involved in the movement of CMV. Suzuki *et al.* (1991) showed that a deletion mutant of 3a protein was not able to induce systemic infection in host plants, whereas it was able to replicate in protoplasts. Protein 3a was then seen in a CMV infected cell wall fraction (Burman *et al.*, 1994) and was subsequently revealed that it is associated with the modification of plasmodesmata (Vaquero *et al.*,1994). Further mutational analysis confirmed the function of 3a protein as the short distance movement protein of CMV (Ding *et al.*, 1995; Kaplan *et al.*, 1995). Furthermore, transgenic CMV 3a plants were able to complement the cell-to-cell and long-distance movement of 3a protein mutants which were not able to perform such functions (Kaplan *et al.*, 1995; Cooper *et al.*, 1996).

In addition, it has been shown that the CMV 3a protein contains RNA binding activity similar to that of the TMV 30K movement protein which is a further evidence for involvement of the 3a in the movement process (Li and Palukaitis, 1996).

1.9.4 Coat protein

Coat protein (CP) is a multi-functional protein in cucumoviruses. Besides being the structural protein of virus particles, CMV CP has also been shown to be able to complement the defective long distance movement of TAV in cucumber plants (Taliansky and Garcia-Arenal, 1995).

Furthermore, it has been demonstrated that CMV CP is involved in symptom expression. An amino acid residue at position 129 in CMV CP had been shown to be a symptom determinator (Shintaku *et al.*, 1992; Suzuki *et al.*, 1995). It has been suggested that the symptom expression is affected by a particular local secondary structure surrounding amino acid 129 rather than the nucleotide acid sequence itself or a particular amino acid per se (Shintaku *et al.*, 1992). However, in some other cases, it has been shown that the induction of symptoms is controlled by a combination of the virus coat protein gene and two host recessive genes (Takahashi and Ehara, 1993).

It has also been reported that the CP is involved in the recognition of CMV by aphid vectors which is necessary for virus transmission (Chen and Francki, 1990; Palukaitis *et al.*, 1992). Perry *et al.* (1994) used chimeric RNA 3s which contain sequences from the efficiently aphid-transmitted strain Fny-CMV and the very poorly aphid-transmissible M-CMV, and revealed that two amino acid regions in the coat protein, positions 129 and 162, were crucial for aphid transmission.

1.10 Subviral RNAs associated with cucumoviruses

1.10.1 Satellite RNA

In addition to the genomic and subgenomic RNAs, CMV particles often contain a satellite RNA which was designated CARNA 5 by Kaper and Waterworth (1977). These molecules are not necessary for CMV infection, however they require CMV for both replication and encapsidation (Roossinck *et al.*, 1992). CMV and TAV, but not PSV, support the replication of CMV satellite RNAs (Palukaitis *et al.*, 1992). Over 28 isolates of satellite RNAs of CMV have been sequenced (Roossinck *et al.*, 1992). They contain between 332 and 342 nt. The sequence homology between CMV satellite RNAs ranges from 70% to 99%. However, no homology between CMV satellite RNAs and CMV genomic RNAs, plant RNAs or genomic DNA has been detected (Palukaitis *et al.*, 1992).

An important feature of satellite RNAs is their ability to modify the symptoms induced by helper viruses. Some symptom modifications are associated with the replication of satellite RNAs. In most cases, satellite RNAs attenuate CMV symptoms and reduce the amount of infectious virus in plant tissues (McGarvey *et al.*, 1994). However, a few satellite RNAs can induce new disease symptoms when combined with an appropriate CMV strain and plant host (Jorda *et al.*, 1992; Kaper *et al.*, 1990).

The effects of these molecules on the host depends on the particular combination of satellite, helper virus and host (Kaper and Collmer, 1988). Some strains increase the accumulation of satellite RNAs whereas others decrease it (Kaper *et al.*, 1995). In another words, satellite RNA reduces the accumulation of one strain while not reducing the accumulation of another strain (Gal-On *et al.*, 1995). It has been shown that some satellite RNAs anneal to RNA 3 and RNA 4, and some others anneal to RNA 1 and RNA 2 as well as to RNA 3 and RNA 4 (Fraile *et al.*, 1993). In the latter case they probably form an unusual knot-like structure or base-pairing (Rezaian and Symons, 1986), by which the satellite RNA could regulate CMV RNA replication. A recent finding that

satellite RNAs competed with CMV RNAs for RdRp (Wu and Kaper, 1995) provides experimental evidence that these RNAs may regulate CMV RNA replication.

1.10.2 Defective and defective interfering RNAs

Defective (D) or defective interfering (DI) RNAs are subgenomic deletion and/or rearrangement mutants of the viral genome (Roux *et al.*, 1991; Holland, 1990). These molecules retain the essential cis-acting elements that mediate RNA packaging and replication (Levis *et al.*, 1986, Pattnaik *et al.*, 1992, Weiss *et al.*, 1989). However, deletion of essential coding regions makes them dependent upon the parental helper genome for provision of *trans*-acting factors required for replication and packaging (Roux *et al.,* 1991). These particles have been found in almost every animal virus group (Holland, 1990). DI RNAs have also been found in several plant RNA virus groups. The DI RNAs that have been found in association with plant viruses can be divided into two groups. In one type, DI RNAs have single internal deletions. This type includes clover yellow mosaic potexvirus (CYMV) (White *et al.*, 1991), tomato spotted wilt tospovirus (TSWV) (Resende *et al.*, 1991) and in three furoviruses: soil-borne wheat mosaic virus (SBWMV) (Chen *et al.*, 1994), beet necrotic yellow vein virus (Bouzoubaa *et al.*, 1991), and peanut clump virus (Manohar *et al.*, 1993). The other type of DI RNAs is more complex and consists of a mosaic of the parental viral genome.Viruses in this group include tomato bushy stunt tombusvirus (TBSV) (Hillman *et al.*, 1987), cymbidium ringspot (CymRSV) (Burgyan *et al.,* 1989), and cucumber necrosis tombusviruses (CNV) (Finnen and Rochon, 1993), as well as turnip crinkle virus carmovirus (TCV) (Li *et al.*, 1989). The presence of these molecules in viral infections can have various effects on the symptoms produced by the helper virus, ranging from symptom attenuation (e.g., CymRSV on *Nicotiana clevelandii*) (Burgyan *et al.*, 1991), to enhancement (Li *et al.*, 1989) and to no effect on symptoms (e.g., cymv on broad bean) (White *et al.*, 1991).

In the family of Bromoviridae, defective RNAs have been found to be associated with two strains of broad bean mottle bromovirus (BBMV) (Romero *et al.*, 1993). The BBMV DI RNAs were derived from RNA2 by small in-frame deletions in the 2a open reading frame. The presence of these DI RNAs in the viral infection in some hosts reduced the accumulation of BBMV RNA2. Furthermore, the DI RNAs enhanced the symptoms produced by the helper virus in pea plants, and DI RNAs were not encapsidated in either pea or bean plants. Also artificial DI RNAs have been produced in brome mosaic bromovirus (Marsh *et al.*, 1991). Similar to BBMV DI RNAs, the DI RNAs of brome mosaic virus were also derived from RNA2.

Recently, two defective RNAs were characterised for the first time in the Cucumovirus genus (Graves and Roossinck, 1995). These two defective RNAs of Fny-CMV, designated 3α and 3ß, were obtained after serial passages of Fny-CMV in a tobacco host. The defective RNAs are derived from RNA 3 by single, in-frame deletions in the ORF 3a. Defective RNA 3α which is smaller than D RNA 3ß RNA resides totally within the region deleted from 3β and is maintained at a very low level, while the 3ß reaches levels equal to those of the genomic RNAs. These data indicate that the 3α could be an intermediate in the production of the 3ß. These two defective RNAs had no apparent effect upon the level of helper virus replication or symptom production. In vitro translation of defective RNA 3ß produced a 20 K truncated 3a protein implying the possibility of its role as a mRNA. It appears that other CMV strains are able to support a defective RNA once it is formed. The mechanism by which the CMV defective RNAs arise is still unknown however the polymerase "copy choice" model which is also suggested for the formation of the DI RNAs of BBMV is the generally accepted process.

1.11 Unresolved RNA molecules in the profile of cucumoviruses

1.11.1 ss-RNA

On the basis of current information about the genome organisation of cucumoviruses, it is known that they have three genomic RNAs (1, 2 and 3) and three subgenomic RNAs (4,

4a, and 5). However, there are some additional RNAs (Ding *et al.*, 1995a) the identities of which are not clear. Similar bands are present in two other cucumoviruses, V-TAV and a subgroup I strain of CMV (Bu-Jun Shi, 1997).

1.11.2 ds-RNA

Viral ss-RNAs that are packaged in the viral particles and obtained from purified virions are usually only those which are required for infection (genomic RNA(s)). However, double-stranded RNAs that are extracted from whole plant tissue represent all RNAs involved in replication and expression of the viral genome.

Most plant viruses have a genome of single-stranded RNA of positive sense, which replicates within infected cells through a double-stranded replicative form (RF). In addition to these full length double-stranded RNAs, distinct dsRNA bands smaller than expected RF molecules have frequently been detected in extracts from different hosts infected by single stranded RNA viruses (Morris, 1983; Dodds *et al.*, 1984; German *et* al., 1992). Indeed, the number and size of these segments are often diagnostic for a virus or a group or family of viruses (Francki *et al.*, 1991; Matthews, 1991). Furthermore, each of these segments may play an important role or roles in the life cycle of viruses, i.e. as a template for replication or as mRNAs which may act in protein synthesis (German *et al.*, 1992). The dsRNAs representing known genomic and subgenomic ssRNAs and additional virus-specific dsRNAs of even more uncertain origin are not present in equal amounts, and the most abundant ones seem to be those that correspond to the genomic ssRNAs (Dodds, 1993). Characterisation of these minor dsRNAs for a specific virus to elucidate their structure as well as their functional role(s) provides valuable information for diagnosis of the virus and investigation of the life cycle (Dodds, 1993).

ds-RNA analysis has been used for differentiation of CMV isolates which share some important biological properties (Wang *et al.*, 1988; Rizos *et al.*, 1992; Pares *et al.*, 1992; Ryu and Park,1995; Dodds *et al.*, 1985). In these studies, in addition to the genomic RNAs, a few minor bands were also visible on ethidium bromide or silver stained gels

(Pares *et al.*, 1992; Wang et. al., 1988). These minor dsRNA bands are mainly distributed between RNAs 2 and 3, as well as between RNAs 3 and 4 (Wang *et al.*, 1988; Pares *et al.*, 1992; Dodds, 1993). The identity of these minor ds-RNA fragments is not clear.

Chapter Two

General Materials and Methods

2.1 Materials

In order to avoid unnecessary repetition and also make it easy to find the details of materials, the common materials which were used in this thesis are described below. Others more specific are described in the relevant chapter.. All chemicals were at least analytical grade in standard. Solutions were prepared under sterile conditions with ultra-pure water and autoclaved where appropriate.

2.1.1 General reagents

The following were obtained from Sigma Chemical (USA): Kanamycin, bovine serum albumin (BSA), dithiothreitol (DTT), ethidium bromide, 3-(N-morpholine) propanesulfonic acid (MOPS), Tris base, spermidine, salmon sperm DNA, Polyvinylpyrrolidone (PVP).

The following were obtained from BDH (Australia): Sodium chloride (NaCl), sodium hydroxide (NaOH), magnesium chloride ($MgCl_2$), Polyethylene glycol (PEG) 6000 and 8000, sodium acetate (NaOAc), ammonium acetate (NH_4Ac), ethylenediaminetetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS), urea, sucrose, glucose, ethanol, iso-propyl alcohol, iso-amyl alcohol, methanol, glycerol, chloroform, bromophenol blue, hydrochloric acid (HCl), glacial acetic acid, Phenol.

Sources of other important reagents were as follows :

Ammonium persulfate (APS), tetramethethylethenediamine (TEMED) from Bio-Rad (Tokyo)

Ampicillin, tRNA, *E. coli* and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) from Boehringer Mannheim (Germany).

Agarose, isopropyl-b-thiogalactopyranoside (IPTG) from Promega (USA).

Nitrocellulose membrane (BA83 0.2mm pore diameter) from Schleicher and Schuell (Germany).

Bacto-agar, bacto-tryptone and yeast extract from Difco Laboratories (USA).

2.1.2 Enzymes

Calf intestinal alkaline phosphatase (CIP) and RNase T_1 from Boehringer Mannheim (Germany).

Klenow fragment of *E. coli* DNA polymerase I (Klenow) from Bresatec (Australia).

Lysozyme from Sigma

Pancreatic RNase A, proteinase K from Sigma (USA).

Restriction enzymes from Bresatec (Australia), Promega (USA), Boehringer Mannheim (Germany) and New England Biolabs (USA).

Taq Thermostable DNA polymerase from Bresatec or Biotech International Ltd.

T4 DNA ligase from Bresatec

Vent DNA polymerase from New England Biolabs (USA).

2.1.3 Sample loading buffer

Urea loading buffer (3xULB): 2 M urea, 50 mM Tris-HCl, 10 mM EDTA, 20% ($^{W}/_{V}$) sucrose, 0.06% ($^{W}/_{V}$) xylene cyanol, 0.06% ($^{W}/_{V}$) bromophenol blue.

Formamide loading buffer (2XFLB): 95% formamide, 10mM EDTA pH 8.0, 0.1% ($^{W}/_{V}$) xylene cyanol, 0.1% ($^{W}/_{V}$) bromophenol blue.

Sucrose loading buffer (SLB): 20% sucrose, 0.01% bromophenol blue, 0.01% xylene cyanol, 10mM Tris-HCl pH 8.0, 1mM EDTA.

2.1.4 Common solutions and growth media

Denhardt's solution: 0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidine, 0.1%(w/v) BSA.

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 11 mM Na_2HPO_4, pH 7.4.

SSC: 150mM NaCl, 15mM sodium citrate.

TAE (Electrophoresis running buffer): 40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, pH 8.2.

TBE (Electrophoresis running buffer): 89 mM Tris-borate, 2 mM EDTA, pH 8.3.

TE: 10mM Tris-HCl, 0.1mM EDTA, pH 8.0.

TEN: 100mM NaCl, 1mM EDTA, 10mM Tris HCl, pH 7.5.

LB (growth medium): 1% (w:v) bacto-tryptone, 0.5% (w:v) yeast extract, 1% (w:v) NaCl, pH 7.0.

2YT (growth medium): 1.6% (w:v) bacto-tryptone, 1% (w:v) yeast extract, 0.5% (w:v) NaCl, pH 7.0.

GET buffer: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0.

STE buffer: 50 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl, pH 8.0.

Tris buffer saline (TBS): 20 mM Tris-HCl, 500 mM NaCl, pH 7.5.

AP colour development buffer: 100 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 9.5.

All buffers were sterilised by autoclaving or where necessary by filtration through a Sartorius ™ Minisart NML 0.2μm filter.

2.1.5 Kits

DNA sequencing kits using α-^{35}S-dATP and modified T7 DNA polymerase (Sequenase® version 2.0) (Sequenase) from US Biochemicals (USA).

Geneclean® II and Mermaid® kits from Bio 101 (USA).

Ni-NTA Spin column from QIAGEN (Germany).

2.1.6 Molecular weight markers

SPP-1 bacteriophage DNA restricted with *Eco* RI and plasmid pU19 DNA digested with *Hpa* II from Bresatec (Australia).

2.1.7 Synthetic oligodeoxyribonucleotides

Synthetic oligodeoxyribonucleotides (primers) were prepared on an Applied Biosystems Model 380B DNA synthesiser either by Neil Shirely or by Jing Li. All primers were unphosphorylated and purified from premature termination products by Mono Q column, high pressure liquid chromatography (HPLC).

2.1.8 Nucleotides and radionucleotides

Deoxynucleotide triphosphates (dNTPs; Pharmacia)

Dideoxynucleotide triphosphates (ddNTPs; Pharmacia)

Ribonucleotide triphosphates (NTPs; Pharmacia)

α-^{32}P-dATP (10 mCi/ml; Bresatec)

γ-^{32}P-ATP (10 mCi/ml; Bresatec)

α-^{32}P-UTP (10 mCi/ml; Bresatec)

α-^{35}S-dATP (12.5 mCi/ml; Bresatec)

2.1.9 Bacterial strains and plasmid vectors

Escherichia coli strain DH5a : Stratagene (USA) was used for all routine cloning work in this thesis.

Routine cloning was carried out using the vector pBluescript (SK+/-) Stratagene, (USA), a phagemid carrying ampicillin resistance and with promoter sequences for T3 and T7 RNA polymerases flanking the polylinker. Other vectors were used as indicated in the text of this thesis.

2.2 Methods

Methods were carried out according to standard procedures (*e.g.* Sambrook *et al.*, 1989) or using manufacturers' specifications except where indicated. Routine methods used throughout this thesis are recorded here with listings of suppliers and solution components where appropriate. Specific methods are listed in each chapter as necessary.

2.2.1 Virus sources

Three viruses were chosen for this study. The V strain of tomato aspermy virus (V-TAV) was originally isolated from chrysanthemum sp. in Victoria, Australia (Habili and Francki, 1974a). Two strains from each of the two subgroups (Owen and Palukaitis, 1988) of cucumber mosaic virus (CMV): Q strain (Q-CMV), a subgroup II strain (Owen and Palukaitis, 1988), was originally isolated from capsicum sp. in Queensland, Australia (Francki, 1964); WAII strain (WAII-CMV), a subgroup I strain (Wahyuni *et al.*, 1992), was previously described as T strain and originally isolated from capsicum sp. in Western Australia (Hatta and Francki, 1979).

2.2.2 Storage of viruses

Dried *Nicotiana glutinosa* leaves infected with the viruses listed above were available in the Symons laboratory. All dried leaf inocula were stored at 4°C in sterile glass vials containing fused $CaCl_2$ as the dehydrating agent. Contact between leaf material and $CaCl_2$ was prevented by a sterile cotton wool plug.

2.2.3 Propagation of viruses .

A small quantity of the dried leaf material was ground to a paste in a few drops of 50 mM phosphate buffer pH 7.2 [72:28 (v/v) 50 mM Na_2HPO_4:NaH_2PO_4]. The mortar and pestle were autoclaved, kept at 4°C and rinsed in distilled water before use. The extract was then rubbed by finger or pestle onto 2 to 3 weeks old *Chenopodium quinoa* leaves

using sterile carborundum powder (500 mesh) as the abrasive. The plants used had been heavily watered the night before and kept in the dark for 24 hours prior to inoculation. Inoculated plants were grown in a glasshouse kept at a 15 hour day length at 230 lumens/m^2 and 20-22°C. Five to seven days after inoculation, individual local lesions were isolated and used as the inoculum for infecting *C. quinoa*, and the process repeated three times. Finally a single lesion was inoculated onto *N. glutinosa*. Virus infection was confirmed using the northern blot hybridisation as described below. Infected leaf material was harvested, and was either stored or used to infect large numbers of *N. glutinosa* or other hosts for virus propagation.

2.2.4 Purification of viral particles

The propagation host for Q-CMV was *Cucumis sativus* cv. Green Gem and *N. glutinosa* for WAII-CMV and V-TAV. The infected leaves were harvested 10 to 15 days after inoculation. Q-CMV and WAII-CMV were purified according to the method of Lot *et al.* (1972) as modified by Peden and Symons (1973). Briefly, to each gram of leaf material, 1 ml of the extraction buffer containing 0.5 M sodium citrate, 5 mM ethylenediaminetetraacetic acid (EDTA) (pH9.0) and 0.5% thioglycollic acid (BDH) and 1 ml of chloroform were added. The mixture was homogenised by a blender and centrifuged at 12,000 g for 10 minutes at 4°C. To every 1 ml of the aqueous phase, 3.3 ml of the buffer containing 5 mM sodium borate, 0.5 mM EDTA (pH 9) and 40% (w/v) polyethylene glycol (PEG, mol. wt 6000) was added and the mixture was stirred slowly for about 30 minutes at 4°C. Viral particles were precipitated by centrifugation at 12,000 g for 10 minutes at 4°C. The pellets were resuspended in 3-4 ml of the suspension buffer containing 5 mM sodium borate, 0.5 mM EDTA (pH 9) and 2% (w/v) Triton X-100 per gram of the infected leaf materials and centrifuged again at 12,000 g for 10 minutes at 4°C. The viral particles were further purified by three cycles of low (12,000 g, 10 minutes) and high speed (100,500 g, 2 hours) centrifugations at 4°C. The virus pellets

were resuspended in the suspension buffer without Triton X-100 and stored at 4°C or at -80°C in the presence of 50% glycerol.

TAV was purified essentially as described by Peden and Symons (1973) except that 1% thioglycollic acid was added to the extraction buffer and the virus was resuspended in 0.02M phosphate buffer pH 7.6 [87:13 (v/v) 20 mM Na_2HPO_4:NaH_2PO_4] (Habili and Francki, 1974a).

The purified virions were examined for morphology under electron microscope or used for the isolation of virion RNAs.

2.2.5 Isolation of RNAs

Virion RNAs were isolated from purified virus as described by Peden and Symons (1973). One ml of viral particle suspension containing 0.5% SDS, 0.3 M sodium acetate, pH 5.2 and 10 mM EDTA (pH7.0) was extracted with an equal volume of 50 mM Tris-HCl (pH8.0)-saturated phenol (stored at 4°C). The aqueous phase was reextracted with an equal volume of the phenol and the viral RNAs precipitated in the presence of 2.5 volumes of ethanol by centrifugation at 12,000 g for 30 minutes at 4°C. The precipitated RNAs were dissolved in 0.3 M sodium acetate (pH 5.2) and 1 mM EDTA (pH 7.0) and ethanol reprecipitated again. The RNA pellets were twice washed with 70% ethanol, dried *in vacuo* and suspended in 0.1 mM EDTA (pH 7.0) and stored at -20°C.

Total RNAs were isolated from plants essentially as described by Verwoerd *et al.* (1989) except that cold extraction buffer and a single chloroform extraction were used. Fresh leaves, as little as 100 mg, were collected in 2 ml Eppendorf tubes, frozen by liquid nitrogen and ground with a steel bar. The fine powder was homogenised with 400 µl extraction buffer (stored at 4°C) containing 0.1 M LiCl, 100 mM Tris-HCl (pH8.0), 10 mM EDTA and 1% SDS and 400 µl phenol by vortexing for 1 minute, 400 µl chloroform was then added and the mixture was vortexed for 1 minute. After centrifugation in an

Eppendorf microcentrifuge at 14000 rpm for 10 minutes at room temperature, the aqueous phase was removed and mixed with an equal volume of 4M LiCl and left at -20°C overnight. The RNAs were collected by centrifugation at 14,000 rpm for 15 minutes at 4°C and redissolved in 400 µl water containing 0.3 M sodium acetate (pH 5.2), and reprecipitated in the presence of 2.5 volume of ethanol by centrifugation at 14,000 rpm for 15 minutes at 4°C. The RNA pellets were twice washed with cold 70% ethanol, dried *in vacuo* and suspended in 0.1 mM EDTA (pH 7.0) and stored at -20°C.

2.2.6 Isolation of DNA from agarose gels

DNA bands of interest were excised from the gels under long wavelength UV light and DNA purified using the Geneclean Kit of BIO 101 as described below. Each excised gel slice was placed in a 1.5 ml of Eppendorf tube and 3 volumes of 6 M NaI added. After incubation at 55°C for 5 minutes and the gel was completely melted, 5 µl of GLASSMILK was added. The mixture was incubated at room temperature for 5 minutes. The silica matrix with the bound DNA was precipitated by a brief spin of 5 seconds at 14000rpm (Eppendorf) and washed twice with NEW WASH. The DNA was eluted from the GLASSMILK in 10-15 µl water by incubating at 55°C for 5 minutes.

2.2.7 Phenol:chloroform extraction and ethanol precipitation of DNA

DNA solutions were vortexed thoroughly with one volume of phenol:chloroform (containing one volume of redistilled phenol (BDH, Australia) equilibrated in 50 mM Tris-HCl pH 8.0, and one volume of chloroform) and centrifuged for 10 minutes at room temperature (full speed in an Eppendorf micro-centrifuge for small quantities, or in a Sorvall HB4 rotor at 10,000 rpm for larger solutions). The aqueous phase was recovered and the extraction repeated as necessary.

DNA was routinely precipitated from solutions with ethanol. Briefly, 0.1 volume of 3M sodium acetate (pH 4.6) was added followed by 2.5 volumes of ice-cold ethanol. The

solutions was incubated on ice for 15 minutes, followed by centrifugation at high speed at room temperature for 15 minutes in an Eppendorf micro-centrifuge for small volumes, or in a Sorvall HB4 rotor at 10,000 rpm for 15 minutes at 4°C for larger volumes. Pellets were washed in 70% ethanol prior to drying *in vacuo* or on the bench at room temperature.

2.2.8 Restriction digestion of DNA

The DNA solution was placed in a 1.5 ml Eppendorf tube and mixed with the appropriate restriction endonuclease and buffer recommended by manufacturer and incubated at the appropriate temperature for the required period of time. Where appropriate, the restricted DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP, Promega) as described below. Digested DNA was added with CIAP (0.01unit/pmol ends) and incubated at 37°C for 1 hour in the presence of 1x CIAP buffer (50 mM Tris-HCl, pH9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine). The digested and/or dephosphorylated DNA was purified by agarose gel electrophoresis as described in 2.2.6 and 2.2.7.

2.2.9 First-strand cDNA synthesis

RNA was denatured in the presence of 50 ng of specific first-strand oligonucleotide primer in TE buffer by heating to 80°C for 2 min, followed by cooling at room temparature for 5 min. The annealed RNA-primer mixture was then subjected to reverse transcription under the following buffer conditions; 10 mM Tris-HCl pH 8.5, 50mM KCl, 5 mM $MgCl_2$, 10mM DDT, 1mM each dNTP, 1 U/µl RNasin (Promega,USA), and 8U of AMV reverse transcriptase (Promega) in a final volume of 20 µl. The rection was incubated initially at 37°C for 5, and then continiued at 50°C for a further 30 min, then stopped by heating at 80°C for 5 min. cDNAs were purified by phenol:chloroform extraction and ethanol precipitation (2.2.7).

2.2.10 Polymerase chain reaction (PCR)

Conditions for PCR varied depending on the DNA polymerase used to calalyse the reaction. Reactions using *Taq* polymerase were set up in 0.5 ml microfuge tubes as follows: 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100) supplied with the enzyme, 1.5-3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 1μl of reverse transcription mixture and 1U of *Taq* DNA polymerase. A layer of sterile oil (20 μl, Sigma) was added on top of the reaction mixtures. Amplification was carried out in an MJ minicycller (MJ Research Inc., USA, model PTC 150) using the following protocol: 92°C for 1 min, 40°C for 1min and 72°C for the first 10 cycles, followed by 92°C for 1 min, 62°C for 1 min and 72°C for 2 min for 25 cycles and a final elongation step of 5 min at 72°C.

PCR reaction products were separated by electrophoresis on 1.2 or 2% agarose gels.

2.2.11 End-filling using Klenow enzyme

End-filling of double-stranded DNA fragments with 3' recessed ends for cloning or radioactive labelling using the large fragment of *E. coli* DNA polymerase I (Klenow fragment) was performed in a reaction containing 50 mM NaCl, 6 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, and 100 mM each dNTP. Individual dNTPs were substituted with radioactively labelled species where appropriate. The reaction was incubated at 37°C for 15 minutes, then terminated by incubation at 70°C for 10 minutes. DNAs were purified by phenol:chloroform extraction and ethanol precipitation (Section 2.2.3).

2.2.12 Ligation of cDNA into plasmid vectors

For dephosphorylated vectors, approximately 20 ng of the linearised DNA was mixed with the insert DNA at a molar ratio of approximately 1:3 (vector:insert) in the presence of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and either 0.25 units (blunt ended insert) or 0.05 units (sticky ended insert) of T4 DNA ligase (Bresatec),

in a reaction volume of 10 µl. Reactions were incubated at 25°C for at least 1 hour or at 4°C overnight. Non-dephosphorylated vectors were treated in the same manner as dephosphorylated vectors, except the amount of linearised vector DNA used was decreased to 5 ng.

2.2.13 Transformation of *E. coli* with plasmid recombinants

•Preparing competent cells: The desired *E. coli* strain was streaked on LB plates overnight and a single colony picked and grown overnight at 37°C in LB medium. The overnight culture was diluted 100 times with LB and grown at 37°C to log phase (A_{600} 0.4 - 0.6). After chilling on ice, the cells were sedimented by centrifugation at 4,000 g at 4 °C for 10 minutes and resuspended in ice-cold 0.1 M $CaCl_2$ (1 ml $CaCl_2$ per 100 ml culture). The resuspended cells were then incubated on ice for at least 1 hour prior to use.

•Transformation with calcium chloride and heat shock

To prepare competent cells, a single colony of the *E.coli* host strain was inoculated into 5 ml of L-broth (where appropriate the L-broth was supplemented with an antibiotic) and the culture incubated overnight at 37°C with continuous shaking. The overnight culture was then diluted 100 fold into 50 ml of L-broth (plus antibiotic) and the incubation continued at 37°C, with shaking, until the culture reached an absorbance (l600) of 0.45-0.55. The cells were then pelleted by centrifugation in a SS-34 rotor at 4,000 rpm for 5 minutes at 4°C, resuspended in 10 ml of ice cold 0.1 M $CaCl_2$ and left on ice for at least 30 minutes. The centrifugation step was then repeated and the cells resuspended in 1 ml of ice cold 0.1 M $CaCl_2$. One hundered microlitres of this cell suspension was mixed with 2-5µl of the DNA ligation reaction mix (Section 2.2.9) and left on ice for 40 minutes. The cells were heat shocked at 42°C for 90 sec, 900µl of L-broth was added and the cells were incubated at 37°C for 45 minutes. Three-hundred microlitres of culture were then plated onto solid media containing appropriate antibiotic. The agar plates were routinely incubated at 37°C overnight.

• **Transformation with electroporation**

Transformation of bacteria with plasmids was also performed by electroporation, using the *E. coli* strain DH5a and the Gene-Pulser (Bio-Rad, USA). The essentials of the procedure used to prepare electrocompetent cells was described in the Gene Pulser manual. Ten ml of an overnight culture of *E. coli* strain DH5a was grown overnight, and used to inoculate 1 L of LB broth. The 1 L culture was grown in a 2 L non-baffled flask to an optical density (λ600) of 0.9, and the flask was then chilled on ice for 15 to 30 minutes. The culture was transferred to 200 ml pots, and the cells pelleted in a GSA rotor at 3,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the cells gently resuspended in 0.5 L of ice-cold 10 % glycerol solution. The cells were pelleted as above, the supernatant discarded and resuspended in 20 ml of ice-cold 10 % glycerol solution. Cells were transferred to 30 ml tubes and pelleted in a HB4 rotor at 4,000 rpm for 15 minutes at 4°C and resuspended in 2.0 ml of ice-cold, 10 % glycerol solution. The electrocompetent cells were transferred to 1.5 ml Eppendorf tubes in aliquots of 200 ml, snap frozen in liquid nitrogen, and stored at -80°C.

Transformation of electrocompetent cells with plasmid was performed according to the recommendations supplied with the Gene-Pulser. Electrocompetent cells (40 µl) were combined with 1.0 ml milliQ H_2O containing 5 ng of plasmid DNA or 60 ng of ligated DNA. The mixture was transferred to an ice-cold, disposable electroporation cell (0.1 cm electrode gap, supplied with the Gene-Pulser), and subjected to electroporation using a Gene-Pulser, set at 1.8 kV, 125 mFD and 200 W. Immediately following electroporation, the cells were mixed with 1.0 ml LB media without antibiotic, and grown at 37°C in a 1.5 ml Eppendorf tube for one hour on a shaker. Two-hundred microlitres of culture were then plated onto solid media containing antibiotic, and grown at 37°C overnight.

•**Selection of clones**: Bacterial colonies or white colonies where the blue/white colour selection was available were selected for minipreparation of plasmid DNA. Restriction

enzyme digestion analysis was used to further confirm the presence or absence of the inserted DNA fragment.

2.2.14 Growth of bacteria

Cultures of *Escherichia coli* bacteria were grown overnight at 37°C using solid or liquid media. Solid media was composed of LB broth and 1.5 % bacteriological agar. Liquid cultures were set up by inoculating LB or 2YT broth from single bacterial colonies or from a scraping of a frozen culture from a glycerol solution. Incubation was then completed on a rotator or shaker. Where appropriate, the growth media contained the antibiotics ampicillin or kanomycin at concentrations of 100 mg/ml and 50 mg/ml, respectively.

2.2.15 Isolation of plasmid DNA

2.2.15.1 Minipreparation of plasmid DNA: A single colony was picked into 2 ml of LB containing ampicillin (100μg/ml). After growing overnight at 37°C, the bacterial cells were pelleted. After the supernatant was removed, the pellet was resuspended in 200 μl of STET [0.1 M NaCl, 10 mM Tris-HCl (pH8.0), 1 mM EDTA (pH8.0), 5% Triton X-100] containing 0.1 mg/ml lysozyme (Sigma) and the suspension placed in 95°C heating block for 45 seconds, and centrifuged at 14000rpm (Eppendorf) at 4°C for 30 minutes. The "gloopy" pellet was removed with a toothpick and the supernatant was mixed with 200 μl ice-cold isopropanol. After 5 minutes on ice, the mixture was re-centrifuged at 14000rpm (Eppendorf) at room temperature for 10 minutes. The supernatant was carefully removed and the DNA pellet was washed with 70% ethanol and resuspended in 30 μl H_2O.

2.2.15.2 Large scale preparation of plasmid DNA: 500 ml of an overnight culture of the relevant clone was centrifuged at 4000rpm at 4°C for 15 minutes in a Sorvall GS3 rotor. The bacterial pellet was resuspended in 100 ml of ice-cold STE [0.1

M NaCl, 10 mM Tris-HCl (pH8.0), 1 mM EDTA (pH8.0)] and the suspension centrifuged at 4000rpm at 4°C for 15 minutes. The pellet was resuspended in 18 ml GTE [25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM glucose] and the suspension then mixed with 2 ml of lysozyme [10 mg/ml in 10 mM Tris-HCl (pH 8.0)], 40 ml of freshly prepared 1% (w/v) SDS, 0.2 M NaOH. The mixture was gently inverted several times and incubated at room temperature for 10 minutes. The mixture was neutralised by the addition of 20 ml of 3 M potassium acetate followed by incubation on ice for 10 minutes. Cellular debris and insoluble potassium-SDS were sedimented by centrifugation at 4000rpm at 4°C for 15 minutes (Sorvall GS3 rotor) and the supernatant was carefully filtered through four layers of cheesecloth into a 250-ml centrifuge bottle and mixed with 0.6 volume of isopropanol and the mixture stored at room temperature for 10 minutes. The mixture was centrifuged as before. The pellet was resuspended in 4 ml of TE [10 mM Tris-HCl (pH8.0), 1 mM EDTA (pH8.0)] and 4 ml 5 M LiCl/50 mM Tris-HCl (pH8.0) added. The mixture was kept on ice for 15-30 minutes. The supernatant was cleared by centrifugation at 3000rpm (Sorvall SS-34 rotor) at 4°C for 5 minutes and plasmid DNA precipitated by adding 2.5 volume of ethanol and 0.1 volume of 3M sodium acetate. The pellet was resuspended in 5 ml TE and incubated at 37°C for 1 hour in the presence of 50 μg/ml DNase-free RNase A (Promega). The suspension was phenol:chloroform (1:1 v:v) extracted twice and the nucleic acid recovered from the aqueous phase by ethanol precipitation using 0.3 M sodium acetate (pH 5.2) and centrifugation at 10000rpm (Sorvall HB-4 rotor) at 4°C for 40 minutes.

The nucleic acid pellet was resuspended in 1-2 ml of TE and the suspension then filtered through a minipore filter (0.2 μm pore size; Schleicher&Schuell) before being loaded onto a Sepharose 6 HPLC column. The column was washed with the HPLC buffer [0.1 M sodium acetate (pH7.0), 0.05% SDS (v/v), 20% ethanol)]. Fractions containing plasmid DNA were pooled and the plasmid DNA was recovered by ethanol precipitation as in 2.2.5.

2.2.16 Agarose gel electrophoresis

Agarose minigels were prepared from 0.7-2.0% ($^{W}/_{V}$) solutions of SeaKem GTG agarose (FMC, USA) in 1xTBE. Ten millilitres of the molten agarose solution was poured onto a 7.5 x 5.0 cm glass microscope slide after positioning of an appropriate well comb. One half volume of urea loading buffer (3xULB) was added to DNA samples before loading of the wells. Preparations of phage SPP-1 DNA digested with *Eco* RI, or pUC19 DNA digested with *Hpa* II (Bresatec, Australia), were used as medium and low range molecular weight markers respectively. Gels were electrophoresed in 1xTBE running buffer at 80-120 mA. DNA was visualised by staining gels with ethidium bromide (10 mg/ml ($^{W}/_{V}$) in water). Gels were destained in water before photography under short wavelength UV light.

2.2.17 Polyacrylamide (sequencing) gel electrophoresis

Denaturing polyacrylamide gels were prepared from 50 ml solutions containing 6% ($^{W}/_{V}$) acrylamide, 0.3% ($^{W}/_{V}$) bisacrylamide, 7 M urea and 1xTBE. Polymerisation was initiated by the addition of 400 ml freshly prepared 10% ($^{W}/_{V}$) ammonium persulfate and 40 ml of TEMED. The polymerising solution was poured into gels of 20 x 40 x 0.04 cm, with well formation by shark's tooth combs. Gels were allowed to set for at least 60 minutes, then pre-electrophoresed at 50 W until gel temperature was approximately 50°C. Gels were electrophoresed at 50°C at constant power after loading and denaturing of samples in formamide loading buffer (2xFLB).

2.2.18 DNA sequencing

Dideoxy chain termination sequencing methods (Sanger *et al.*, 1977; 1980) was used to determine DNA sequence. DNA sequencing was performed with Sequenase Version 2.0 T7 DNA polymerase using DNA sequencing kits (USB). Plasmid DNAs purified on a minipreparation or large-scale as described (Section 2.2.12) were denatured by the

addition of 5 µl of 1 M NaOH containing 1 mM EDTA to 20 µl of plasmid DNA. The denatured DNAs were purified from solution components by passage through micro-spin columns containing Sepharose CL-6B (Pharmacia), followed by elution in 25 µl TE. Seven µl of the purified denatured plasmid DNA was used in each sequencing reaction.

2.2.19 *In vitro* transcription of plasmid clones by RNA Polymerase:

2.2.19.1 Preparation of radioactive RNA probes

Appropriate linearised DNAs (1-2 µg) were mixed with 40 mM Tris-HCl pH7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 unit/µl RNasin, 0.5 mM each of dATP, dCTP and dGTP, 12 µM UTP, 50-100 µCi α-^{32}P-UTP, and 20-40 units T3, T7 or SP6 RNA polymerase (Promega) as appropriate, and the mixture incubated at 37°C for 1 hour. The DNA template was destroyed by addition of 1 unit of RNase-free DNase (Promega) and incubation at 37°C for 15 minutes. The transcripts were extracted with phenol:chloroform (1:1 v:v), precipitated in 2.5 M ammonium acetate/2.5 volume of ethanol and the pellet finally resuspended in 100 µl TE.

2.2.20 Northern blot hybridisation

The method was adapted from Ding *et al* (1995b).

Gel preparation: 2.16 g of agarose was boiled in 156.6 ml water and cooled to 60°C. 5.4 ml of 37% formaldehyde and 18 ml of 10x MOPS buffer [0.23 M MOPS (3-(N-Morpholino) propanesulfonic acid; Sigma) (pH7.0), 0.01 M EDTA and 0.05 M NaAc] were then added before the gel was poured into a gel box (14x21cm). The running buffer was 1x MOPS.

Sample preparation: 5.8 µl RNA (0.1-0.5 µg for purified RNA and 2-10 µg for total plant RNA), 2.5 µl 10x MOPS, 4.4 µl formaldehyde and 12.5 µl formamide were mixed

and the mixture incubated at 65°C for 15 minutes. Five µl formamide loading buffer (FLB: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was then added before loading on an agarose gel. The gel was run at 6V/cm until the bromophenol blue reached the bottom 1/4 of the gel.

Northern blotting: The RNA transfer sandwich consisted of the following layers (from bottom to top): 2 sheets of Whatman 3MM prewetted with HETS buffer (CINNA/Biotecx Laboratories), the gel (upside down), the nylon membrane (Hybond$^+$, Amersham) and the dry paper towels. After the transfer was completed in at least 4 hours, the sandwich was disassembled. RNA was fixed onto the membrane by UV light (GS Genelinker, BIORAD) and photographed under short-wave length UV light. Prehybridisation was done at 65°C for at least 4 hours in a bottle containing 10 ml of solution consisting of 5 ml deionized formamide, 2.5 ml 20x SSC [3 M NaCl, 0.3 M sodium citrate (pH7.0)], 1 ml 50x Denhardts [1% (w/v) Ficoll 400 (Pharmacia), 1% (w/v) polyvinylpyrrolidone (Sigma), 1% (w/v) BSA, 0.5% SDS and 20 µg/µl sheared and denatured salmon sperm DNA (BDH)], 1 ml 10% SDS, 0.2 ml phosphate buffer (pH 6.8) [49:51 (v:v) 50 mM Na_2HPO_4:NaH_2PO_4], 1 mg each of denatured salmon sperm DNA and *E. coli* tRNA (BDH). The labelled RNA probe was then added and the hybridisation continued at the same temperature overnight. The membrane was then washed twice in 2x SSC/0.1% SDS at 55°C for 15 minutes and once in 0.2x SSC/0.1% at 75°C for 45 minutes and then autoradiographed.

2.2.21 preparation of ^{32}P-labelled cDNA 5' probe

The only DNA probe was excised from pQCD2 (Ding et al., 1995a) by digestion with *PstI/EcoRI* (Section 2.2.8) and after separation of the fragment on agarose (Section 2.2.16) it was purified with Gene Clean kit as described in Section 2.2.6. ^{32}P-labelled DNA probe was prepared by the random priming method of Feinberg and Vogelstein, (1983) and purified by the method of Collins et al., (1996). DNA templates (20 ng) were

denatured in the presence of 200 pmoles of random-sequence 9-mer oligonucleotide primer in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by heating to 95°C for 2 minutes, followed by cooling on ice for 5 minutes. The labelling reaction was carried out overnight at room temparature or for 2 hours at 37°C in a 25 µl reaction volume in 1x labelling buffer (2.5xlabelling buffer was 0.5 M HEPES (4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid), pH 6.6, 0.125 M Tris-HCl, pH 8.0, 12.5 mM $MgCl_2$, 12.5 mM DTT, 1.0 mg/ml BSA), 30 µCi ^{32}P-dCTP, 20 µM each dATP, dGTP and dTTP and 1 U *E. coli* DNA polymerase I (Klenow fragment).

Unincorporated dNTPs were removed by passage through Biogel P-10 (BIORAD) spin columns prepared as follows: 1.0 ml syringes, with plungers removed, were plugged at the bottom with sterile glass wool and filled to the top with Biogel P-10 equilibrated in TEN buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1.0 mM EDTA) and centrifuged at 3000 g for 4 minutes to remove excess TEN buffer. Probe reaction mixtures were diluted to 200 µl with TEN buffer and passed through the column by centrifugation at 3000 g for 4 minutes.

2.2.22 Southern hybridisation analysis

PCR products were, separated by electrophoresis on a 1.2 % agarose gel. The gel was denatured for 15 min in 100 ml denaturing solution (1.5 M NaCl, 0.5M NaOH) and rinsed in 10xSSC (see section 2.1.4) for 2 min. DNA was transferred from the gels to Hybond N^+ membranes (Amersham) by Southern transfer (Southern, 1975) for 4 hours to overnight using 10xSSC as transfer buffer. DNA was fixed to the membranes by contact with a pad of Whatman 3MM paper soaked with 0.4 M NaOH for 20 min and the membranes subsequently neutralised by rinsing the membranes in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA) for 10 min.

The memranes were kept in 2xSSC at 4°C or were transferred to a hybridisation bottle on a fine plastic mesh, then prehybridised overnight at 65°C in 10 ml of hybridisation solution containing, 3 ml 5xHSB solution (3 M NaCl, 100 mM PIPES (1, 4-Piperazine diethanesulfonic acid), 25 mM Na_2EDTA, pH 6.8), 2 ml 50xDenhardt's solution, 2%(w/v) Ficoll, 400 (Pharmacia), 2% (w/v) PVP (Sigma), 2% (w/v) BSA fraction V (Sigma), 3 ml 25% (w/v) dextran sulphate, 2 ml MilliQ H_2O and 200 µl salmon sperm DNA (10 mg/ml) sheared and denatured by heating at 95°C for 3 minutes prior to addition. Hybridisation analysis was performed overnight at 65°C using ^{32}P-dCTP-labelled cDNA probes prepared by random priming from RNA 2 cDNA clones. After incubation the hybridisation solution was discarded and the filter washed as follows: 2x SSC, 0.1% SDS at 65°C for 15 minutes; 1x SSC, 0.1% SDS at 65°C for 15 minutes; 0.5x SSC, 0.1 %SDS at 65°C for 15 minutes and a final wash 0.2x SSC, 0.1 %SDS at 65°C for 20 minutes. The filters were blotted dry and exposed for a few hours to X-ray film (Fuji RX).

Chapter 3

Identification and Isolation of New ds-RNA Fragments in Cucumber Mosaic Virus Infected plants

3.1 Introduction

Cucumber mosaic virus (CMV) is the type species of the genus Cucumovirus. It has a single-stranded RNA genome of messenger sense divided into three RNA molecules (RNAs1, 2 and 3). In addition to the genomic RNAs, CMV has three subgenomic RNAs (RNAs 4, 4a and 5) which in total encode five proteins (1a, 2a, 3a, 2b and coat protein) (Palukatis et al., 1992; Ding et al., 1994). Recently, two defective(D) RNAs were characterised and added to the profile of the CMV RNAs (Graves et al., 1995).

The replication mechanism of CMV is probably very similar to that of other plant RNA viruses and performs through a double-stranded replicative form (RF) (Palukatis et al., 1992). In addition to the full length RF molecules, distinct double stranded RNA (dsRNA) fragments smaller than the expected RF molecules have been detected in cellular extracts of various hosts infected with single-stranded RNA viruses (Dodds et al., 1984; German et al., 1992). These dsRNA fragments can be an important feature of viruses since, on the one hand, they can be used as a diagnostic tool or as a differentiation criteria between viral isolates (Francki et al., 1991; Matthews, 1991) and, on the other hand, each of these segments may be functionally active and play important roles in the life cycle of viruses (German et al., 1992).

Fractionated dsRNA profiles of different CMV strains used for differentiation of CMV isolates revealed that, in addition to the known genomic and subgenomic RNAs, a few minor bands were also visible on ethidium bromide or silver stained polyacrylamide gels (Dodds et al., 1985; Wang et al., 1988; Pares et al., 1992; Rizos et al., 1992; Ryu and Park, 1995). These minor ds-RNA fragments are mainly distributed between RNAs 2 and 3 and between RNAs 3 and 4. However, the question of whether these minor dsRNAs are virus specific or related to the host (Wang et al., 1988; Pares et al., 1992) is still to be resolved. Since these dsRNA bands have been observed consistently in

different strains of CMV, regardless of host species and method of extraction, they may be important in the life cycle of the virus.

The aims of this chapter are (i) to determine if the minor dsRNAs are of CMV origin or host origin and, if they are CMV origin, to try to understand their relation to CMV genomic RNAs; and (ii) to find an efficient method for isolation of these dsRNAs, which are present in low concentrations.

3.2 Materials and methods

3.2.1 Extraction and purification of total double-stranded RNA

Extraction of total dsRNA was performed as described by Smith et al. (1991) with some modifications (Fig. 3.1). Q-CMV infected cucumber leaves (30 grams) harvested twelve days post-inoculation and frozen in liquid nitrogen were pulverised into a fine powder in a pre-cooled mortar and pestle. The ground tissue was transferred to a 1000 ml beaker and mixed with 40 ml 2 x STE (1 x STE is 100 mM NaCl, 50 mM Tris-HCl pH 7.0, 1mM EDTA), 6 ml 10% SDS and 50 ml Tris-HCl saturated phenol (pH 8.0). The resulting slurry was stirred for 30 minutes at room temperature and centrifuged at 10,000 rpm for 15 minutes at 4°C in a Sorvall GSA rotor. The aqueous phase was transferred to a new beaker and an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added. The mixture was stirred for 30 minutes at room temperature, and the centrifugation step repeated. The final aqueous phase was collected, diluted with 2 x STE to a total volume of 60 ml, and then adjusted to 16% (v/v) ethanol before the addition of 5 g Whatman CF-ll cellulose. The ds-RNA was allowed to bind to the CF-11 while being stirred for 2 hours at room temperature. The cellulose was repeatedly washed with 50 ml 2 x STE containing 16% (v/v) ethanol and centrifuged at 10,000 rpm for 10 minutes at 4°C in a GSA rotor. Washing was continued until the cellulose appeared white, and it was then suspended in 50 ml 2 x STE containing 16% (v/v) ethanol. The cellulose was transferred to a sterile 50 ml syringe and allowed to settle. It was subsequently washed at room temperature with 350 ml 1 x STE containing 16%

(v/v) ethanol to remove single stranded RNA (ss-RNA). ds-RNA was eluted from the column with 36 ml of 1 x STE and precipitated from solution by the addition of 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol. The precipitated nucleic acids were pelleted by centrifugation in 10 ml tubes at 10,000 rpm at 4°C for 30 minutes in an SS-34 rotor and the supernatant was discarded. The ds-RNA pellets were washed with 70% ethanol at -20°C and centrifuged as before. The nucleic acid pellets were then air dried for 5 minutes at room temperature and resuspended in 300 µl water.

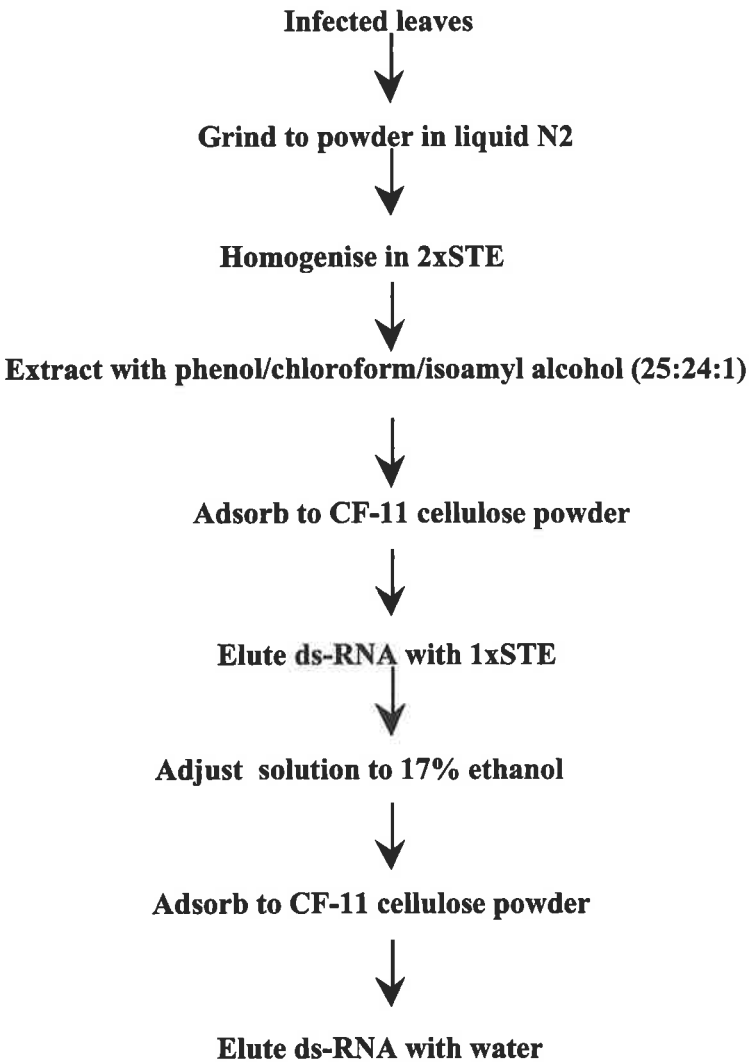

Fig. 3.1 ds-RNA extraction method from CMV infected plant

3.2.2 DNase and RNase digestion of dsRNA

The purified dsRNA was digested with RNase-free DNase as described by Dodds (1993). Four µl of the purified dsRNA was diluted with water (1:5) and digested at 37°C for 1 hour with 1.0 unit of RNase-free DNase (Promega) in a 25 µl reaction volume containing 0.1 volume of 0.05 M $MgCl_2$. The reaction mixture was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) and precipitated by mixing with ethanol. After one 70% ethanol wash and an air drying step, the pellet was resuspended in 4 µl water, in readiness for RNase digestion.

The DNase treated dsRNA was diluted with water (1:5) and digested with 500 ng/ml of pancreatic RNase A in 2x SSC (high-salt buffer) at 37°C for 10 minutes. After phenol/chloroform extraction and ethanol precipitation the pellet was resuspended in 4 µl water and the digested dsRNA was electrophoresed on a 6% polyacrylamide gel. dsRNA bands were then visualised with silver staining as describe below in Section 3.2.3.2.

3.2.3 Analysis of dsRNA

3.2.3.1 Electrophoresis conditions

Polyacrylamide minigels (9 x 7.5 x 0.1cm) were prepared from 10 ml solutions containing 6% polyacrylamide (Ultrapure Accugel 19:1 sequencing grade) in 1xTAE, 70 µl of freshly prepared 10% (w/v) ammonium persulphate and 7.5 µl TEMED (N, N, N', N'-Tetra- methylethylenediamine). Gels were allowed to polymerise for 30 minutes before pre-electrophoresis for 20 minutes at 100 V. Nucleic acid samples were applied to the gels in 5 µl aliquots containing an equal volume of formamide loading buffer (FLB; Section 2.1.3). Electrophoresis was carried out for 3 hours at 100V in a BIO-RAD Mini Protein Cell II with 1xTAE as running buffer and dsRNA bands were visualised with silver staining.

3.2.3.2 Silver staining procedures

Silver staining was performed according to the method described by Bassam et al. (1991). After electrophoresis, gels were fixed in 10% acetic acid for 10 minutes and washed 3 times in distilled water (2 minutes each). The washed gels were impregnated with silver solution (1.5g/L $AgNO_3$, 0.056% formaldehyde) for 20 minutes and quickly rinsed (5-20 seconds) with distilled water. The gels were then put in developer solution (30g/L Na_2CO_3, 0.056% formaldehyde, 400 µg/L sodium thiosulphate) until optimal image contrast was obtained. Image development was stopped by adding fixative (7.5% acetic acid, at 4°C) for periods ranging from 30 seconds to 5 minutes, and gels were washed with water.

3.2.4 Comparison of four methods for isolation of ds-RNA fragments

Further investigation of the minor dsRNAs required their isolation. However, with regard to their low concentrations, the efficiency of isolation method was very important. For this purpose four methods were compared and at the conclusion of each procedure a sample was run in a 6% bis-acrylamide gel and stained with ethidium bromide to evaluate the efficiency of recovery.

a. *Recovery of dsRNA from agarose*

Agarose minigels were prepared from a 1% (w/v) solution of agarose (SeaKem GTG:Genetic technology Grade 9 FMC, USA) in 1xTBE. dsRNA samples were mixed with a 0.5 x volume of RNA loading buffer and, after electrophoresis, gels were stained with ethidium bromide and the desired dsRNA bands were excised. The agarose slices were dissolved by adding a 0.5 x volume (w/v) of TBE modifier and 4.5 volumes of NaI (Gene Clean Kit). dsRNA fragments were recovered by the addition of CF-11 cellulose as described in Section 2.1.4.

b. ***Purification of dsRNA by electroelution***

After electrophoresis of dsRNA and ethidium bromide staining as described in Section 3.2.3, individual bands of interest were excised from gels and cut into small pieces. The dsRNAs were electroeluted from the gel pieces using two types of apparatus: BIOTRAP electroeluter (Schleicher & Schuell Inc.) and the Model 422 Electroeluter (Bio-Rad) according to the manufacturers' protocols. In these methods electroeluted dsRNAs (from bis-acrylamide gel pieces) were concentrated by ethanol precipitation as described in Section 2.2.7.

c. Isolation of dsRNA from bis-acrylamide cross-linked gel using an embedding method

dsRNA samples were run in a 6% bis-acrylamide cross linked gel in 1xTAE buffer for 3.5 hours followed by staining with ethidium bromide. The target dsRNA segments were subsequently excised and embedded in 1.8% TAE agarose minigels. Gels were electrophoresed in 1xTAE running buffer at 100 V for 30-60 minutes; the dsRNA bands then migrated from the acrylamide pieces into the agarose gel. The bands were excised from the agarose and processed as in Section 2.1.5.

d. **Isolation** ***of dsRNAs from BAC-acrylamide cross-linked gels***

- ***Electrophoresis conditions***

Electrophoresis conditions were essentially as described by Dulieu and Bar-Joseph (1989). A stock solution containing 20% acrylamide and 0.8% BAC (N, N'-bisacrylylcystamine) was made according to Ghaffari et al. (1988). Acrylamide and BAC were added simultaneously to a small volume of water and dissolved by heating (care was taken that the temperature remained below 50°C). The solution was degassed by placing under vacuum for 20 minutes before being passed through a 0.45μm

nitrocellulose filter (Millipore) to remove insoluble materials. Gels were prepared using a solution of 6.5% acrylamide in TAE buffer containing 2.1% TEMED and 0.015% ammonium persulphate, and poured into a 9 x 7.5 x 0.1 cm BIO-RAD slab gel apparatus. Gels were pre-run for 1 hour at 100V to remove excess reagents. The dsRNAs were then dissolved in distilled water and samples of 10–50 µl were loaded on the gel. Electrophoresis was carried out at 100V for 210 minutes and gels were stained with ethidium bromide. The desired bands were then excised under UV light and processed immediately.

- ***Isolation of dsRNAs from BAC-acrylamide cross-linked gels***

To the excised dsRNA segments, 2-mercaptoethanol and distilled water were added at a ratio of 1:2:2 (v/v/w) following the method of Ghaffari et al. (1988). The samples were then mixed and heated for 5 minutes at 50°C (Fig.3.2). After the gel slices had dissolved, the volume was adjusted to 500 µl with 2 x STE buffer; the STE buffer was added drop-wise while vortexing to avoid precipitation of partially dissolved acrylamide. The concentration of the solution was subsequently adjusted to 33% ethanol, before the addition of 10 mg of microgranular cellulose CC-41. The mixture was then shaken for 30 minutes to keep the CC-41 in suspension, and the CC-41 adsorbed dsRNA separated from the dissolved acrylamide by a short centrifugation step (1 minute at maximum speed). Finally, the pellet was washed in a solution containing 1 xSTE, 33% ethanol and 10% 2-mercaptoethanol, by shaking at room temperature for 15 minutes, followed by three washes with a solution of 1xSTE and 20% ethanol. After removal of residual ethanol by vacuum drying, the dsRNA was eluted from the cellulose by resuspending the pellet in 100 µl water while shaking for 5 minutes at room temperature. After a short centrifugation the supernatant containing the dsRNAs was transferred to a new tube and precipitated with 2.5 volumes ethanol.

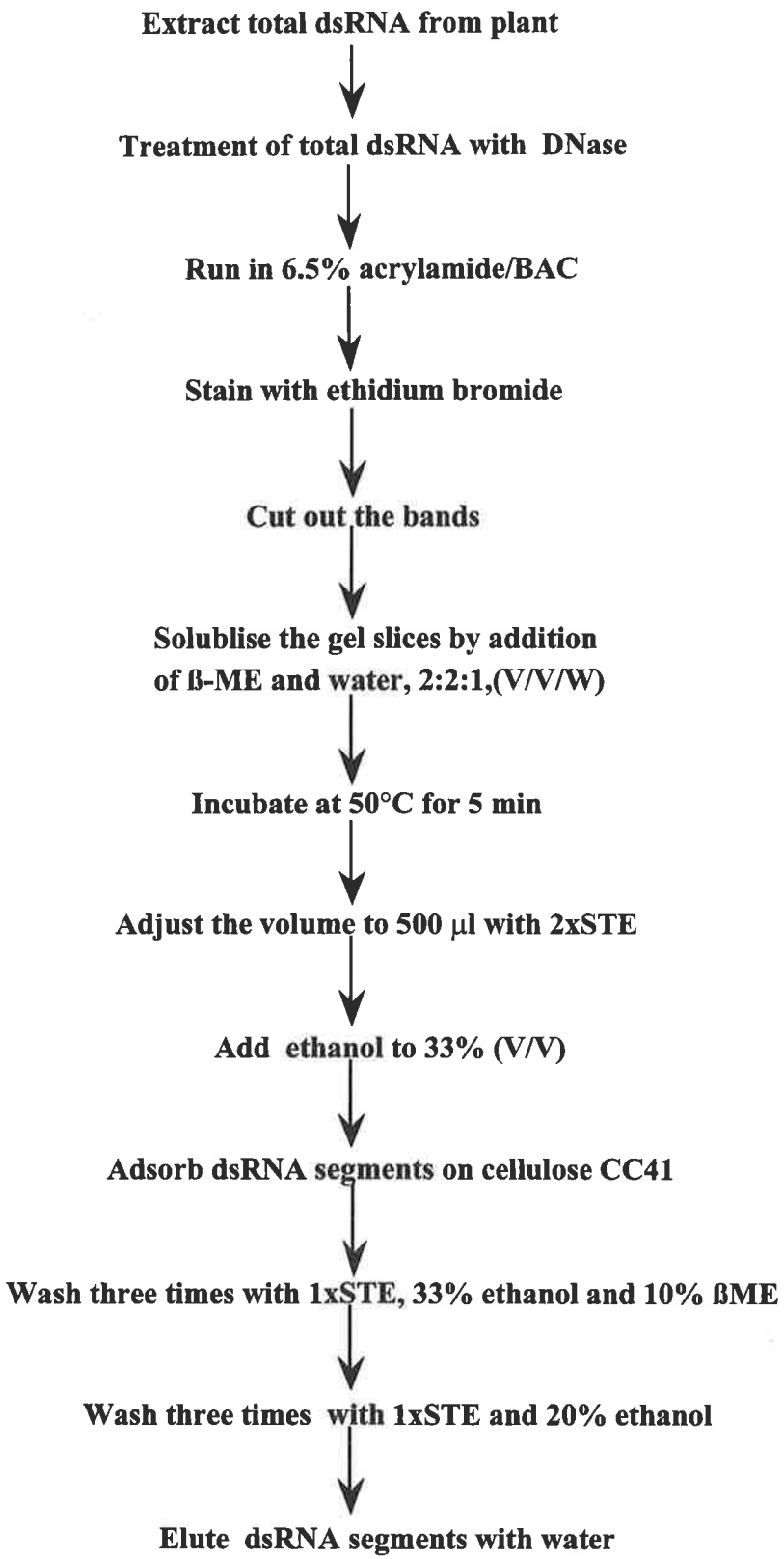

Fig.3.2 Isolation procedure of the minor dsRNAs from polyacrylamide gel with bisacrylylcystamine (BAC) crosslinker

3.2.5 Northern hybridisation analysis

Northern analysis was carried out following electrophoresis of ds RNA samples in 6% polyacrylamide gels in TAE buffer as in Section 2.2.20. The gels were subsequently rinsed in 0.5xTBE buffer and transferred to positively-charged Hybond N^+ membranes (Amersham) in a Mini trans-blot electrophoretic transfer cell (BIO-RAD), with cooling, in 0.5xTBE buffer at 300 mA for 1 hour. The membranes were soaked in 50 mM NaOH and 10 mM NaCl for 5 minutes and rinsed twice in 2xSSC for 5 minutes. The RNA was then fixed to the membrane by cross linking in the Gene Linker UV chamber (BIO-RAD), using the CL program. ^{32}P-labelled riboprobes were prepared from cDNA clones (Section 2.2.19) by in vitro transcription.

3.3 Results

3.3.1 Three minor dsRNA species from CMV-infected plants

Total dsRNAs were initially extracted from infected cucumber plants by the method of Smith et al. (1991). However, since the extracted dsRNAs were consistently found in the present work to be contaminated by additional nucleic acids, two modifications were made. (1) A second round of CF-11 chromatography was added, as recommended by Dodds (1993) to reduce the levels of contaminating nucleic acids. (2) A DNase treatment step was added which also had been reported to intensify the ds-RNA bands after electrophoresis (Ryu et al., 1995) and found to be efficient in removing contaminating DNAs (Dodds, 1993).

Silver stained polyacrylamide gels of CMV dsRNA samples revealed five prominent bands (Fig. 3.3). These bands correspond to RNAs 1 and 2, which under the conditions described (Section 3.2.3.1), migrated as one band, and RNAs 3, 4, 4A and 5. In

addition, three faint bands were consistently observed in dsRNA samples extracted from CMV infected plants (Fig. 3.3 lane 2), while there were no similar bands in dsRNA samples extracted from healthy plants (Fig. 3.3 lane 1). The additional bands include one between RNAs 2 and 3, another between RNAs 3 and 4 and a third running just below RNA 4. In this thesis these fragments are referred to as dsB1, dsB2 and dsB3, respectively.

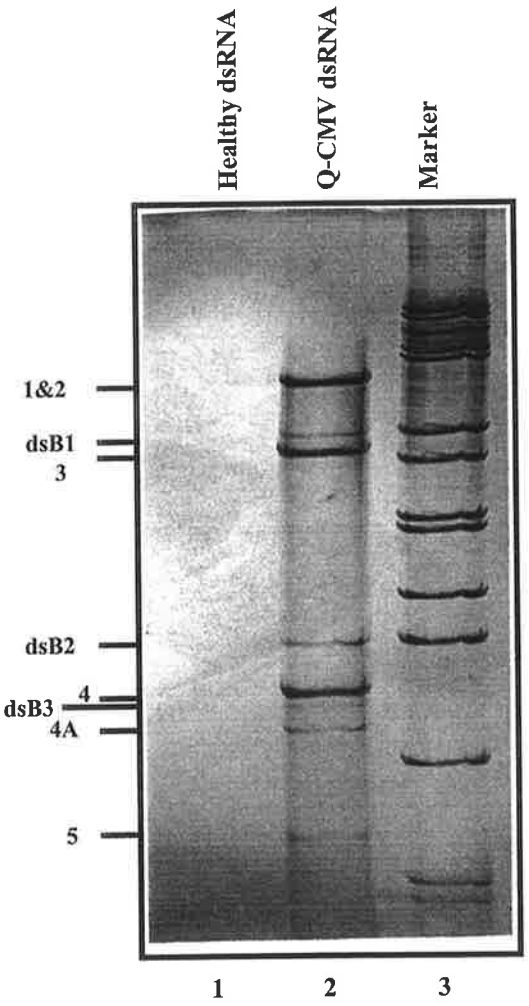

Fig. 3.3 ds RNA species isolated from cucumber plants infected with Q-CMV. 6% polyacrylamide gel electrophoresis pattern of total dsRNA extracted from healthy and Q-CMV infected cucumber plants. Three minor dsRNAs (dsB1, dsB2 & dsB3) can be seen in lane 2, in addition to the known CMV RNAs (1, 2, 3, 4, 4A and 5). No dsRNA was present in healthy cucumber (lane 1).

The double-stranded nature of these three species was confirmed by the fact that they were not digested by DNase I and RNase A (Section 3.2.2); if an RNA molecule is double-stranded, it is resistant to RNAse A in high-salt buffer because no single-stranded regions are accessible to the enzyme as a result of the stable base pairing.

3.3.2 Gel isolation of the minor dsRNAs

Due to the low concentrations of the desired dsRNA species, an efficient method was required for their isolation. Four different isolation methods were compared.

***a. Isolation from agarose*:** In the purification method from agarose, all three ds-RNA fragments were isolated successfully by CC-41 cellulose after agarose gel electrophoresis (Section 3.2.4 a). However the electrophoretic mobilities of the isolated ds-RNA molecules were sometimes different from those expected on the basis of their sizes. This problem was much less severe when the concentration of total ds-RNA used for isolation was increased. Thus it can be concluded that, while size is not a limiting factor, dsRNA concentration has an important impact on the efficiency of this method. Fig. 3.4 (lanes 1 and 2) shows dsB1 and dsB2 RNAs isolated from 50 grams Q-CMV infected cucumber tissue.

b. Isolation using the electroelution method: Analysis by electrophoresis in a 6% polyacrylamide gel of the dsRNA species purified by the electroelution method showed that dsB2 and dsB3 segments were successfully isolated by this method. However the recovery of the dsB1 fragment was not efficient (data not shown). These results imply that the electroelution method is better for shorter molecules.

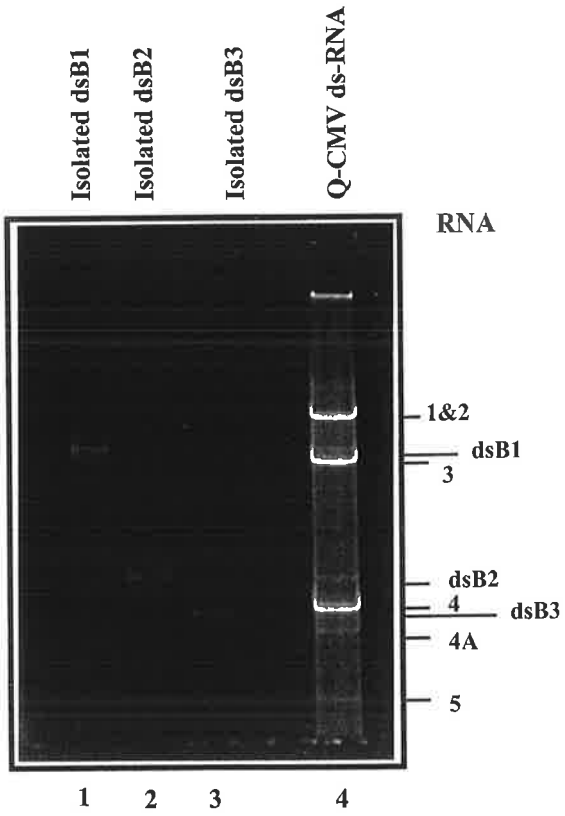

Fig.3.4 Analysis of the gel purified minor dsRNAs by polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis pattern of isolated minor ds-RNAs separated on a 6% polyacrylamide bis-cross linked gel for 3 h in TAE buffer and stained with ethidium bromide. Lanes 1-3, dsB1, dsB2 and dsB3 (respectively), isolated from total dsRNA using agarose gel method. Lane 4, Q-CMV total dsRNAs.

c. *Isolation using the embedding method*

In this method, initial difficulties encountered in the recovery of dsRNAs from acrylamide gels were overcome by transferring nucleic acids from acrylamide gel pieces to agarose gels. The method then resulted in the successful isolation of all three additional dsRNA bands (Fig. 3.5); however, sometimes after polyacrylamide gel electrophoresis the isolated dsRNA bands were not sharp.

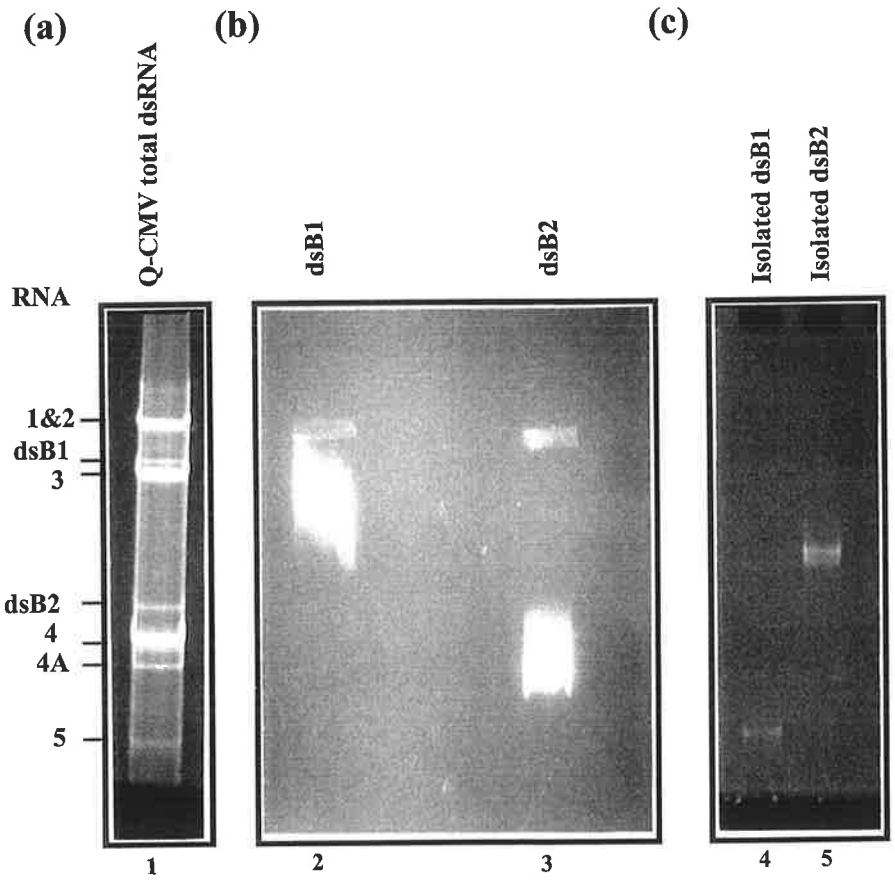

Fig. 3.5 Isolation of minor ds-RNAs using the embedding method

(a) Q-CMV total ds-RNAs were run in a 6% polyacrylamide gel and then the minor ds-RNA bands were excised from the gel. **(b)** The excised polyacrylamide gel pieces were then embedded in a 2% agarose gel and after electrophoresis, the dsRNAs were recovered from the agarose as in Section 3.2.4 c. (**c**) Electrophoresis pattern of the recovered dsB1 and dsB2 on a 6% polyacrylamide gel.

d. *Isolation using the BAC cross-linker method*

In order to use the BAC crosslinker in polyacrylamide gels, the method described by Dulieu and Bar-Joseph (1989) was used. However, after final elution in water and concentration by ethanol precipitation, some pieces of acrylamide were consistently coprecipitated with the desired dsRNAs, and poor recovery of dsRNA segments resulted. To overcome this problem several modifications were made.

(a) Acrylamide and BAC were dissolved together under lower temperature conditions (less than 50°C)

(b) After dissolution of acrylamide gel pieces, the mixture was kept at 50°C for all subsequent steps.

(c) After addition of cellulose CC-41 to the mixture of dissolved acrylamide and ds-RNAs and adsorption of the ds-RNAs, the pellet (which was obtained by a centrifugation) was washed at least three times with 1xSTE, 33% ethanol and 10% 2-mercaptoethanol.

As a result of these modifications, relatively good yields and clean dsRNAs were recovered for all three ds-RNAs (Fig. 3.6).

The results showed that the isolation of minor ds-RNA bands was most efficiently achieved using the BAC method of Dulieu and Bar-Joseph (1989) with some modifications. This approach was therefore used in subsequent experiments.

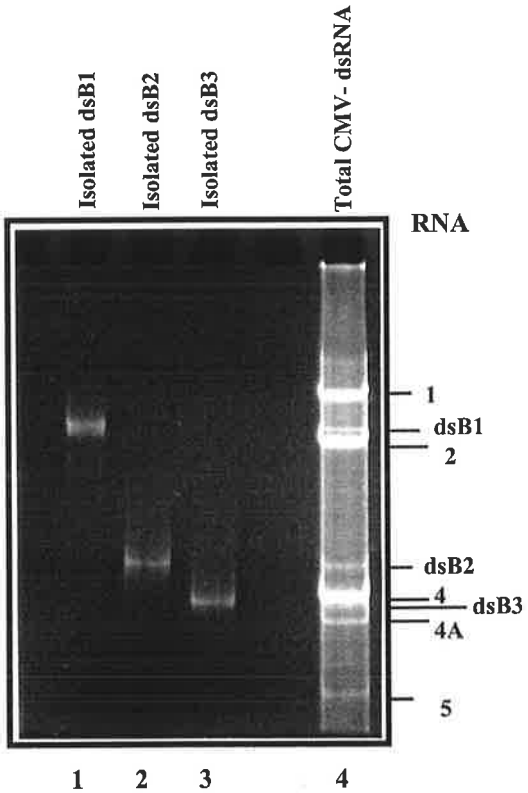

Fig.3.6 Analysis of the gel purified minor dsRNAs by polyacrylamide gel electrophoresis. Patterns of the minor ds-RNAs on a 6% BAC-polyacrylamide gel after electrophoresis for 3.5 h in TAE buffer and staining with ethidium bromide. Lanes 1-3, purified minor ds-RNAs (dsB1, dsB2 & dsB3 respectively) using the BAC method. Lane 4, total Q-CMV dsRNAs.

3.3.3 dsB1, dsB2 and dsB3 are of virus origin and related to Q-CMV RNA2

• To determine whether the minor dsRNAs are of CMV origin or not, the isolated minor dsRNAs were hybridised to a 3'-end probe which can detect all genomic and subgenomic RNAs. Hybridisation of the isolated minor dsRNAs with a 3'-common end probe indicated that these dsRNAs are of viral origin (data not shown).

• Furthermore, to determine whether the minor dsRNAs in CMV infected plants are of viral origin or host origin, total dsRNAs were extracted two weeks post inoculation and the dsRNA molecules of interest were isolated from a non-denaturing polyacrylamide gel as described in Section 3.2.4 d. The isolated minor dsRNAs were subjected to northern blot analysis using ^{32}P-labelled RNA probes specific to CMV RNAs 1, 2 or 3. In addition, total dsRNAs extracted from infected and healthy leaves were used as controls.

Northern analysis showed that all three minor dsRNAs hybridised specifically to the RNA 2 specific probe (probe C which is complementary to nucleotides 2410-2712 of Q-CMV RNA 2) (Fig. 3.7). Of the viral RNAs, dsRNAs 2 and 4A also hybridised to probe C. The RNA 1-derived probe, however, which is complementary to nucleotides 1618-2427 of RNA 1, hybridised only to the dsRNA 1 of infected total ds-RNA and no hybridisation signal was detected with the minor dsRNAs (data not shown). Furthermore, the RNA 3 specific probe (complementary to nucleotides 239-535 of RNA 3) that is capable of detecting Q-CMV RNA 3 and 4, only hybridised to the dsRNAs 3 and 4 of infected total ds-RNA and also showed no reaction with the minor dsRNAs (data not shown).

These data demonstrate that the minor dsRNA molecules in the CMV infected plants share sequence homology with Q-CMV RNA 2 but have no homology with genomic RNAs 1 and 3. Lack of cross reaction with other genomic RNA probes also confirmed the purity of the isolated ds-RNA molecules.

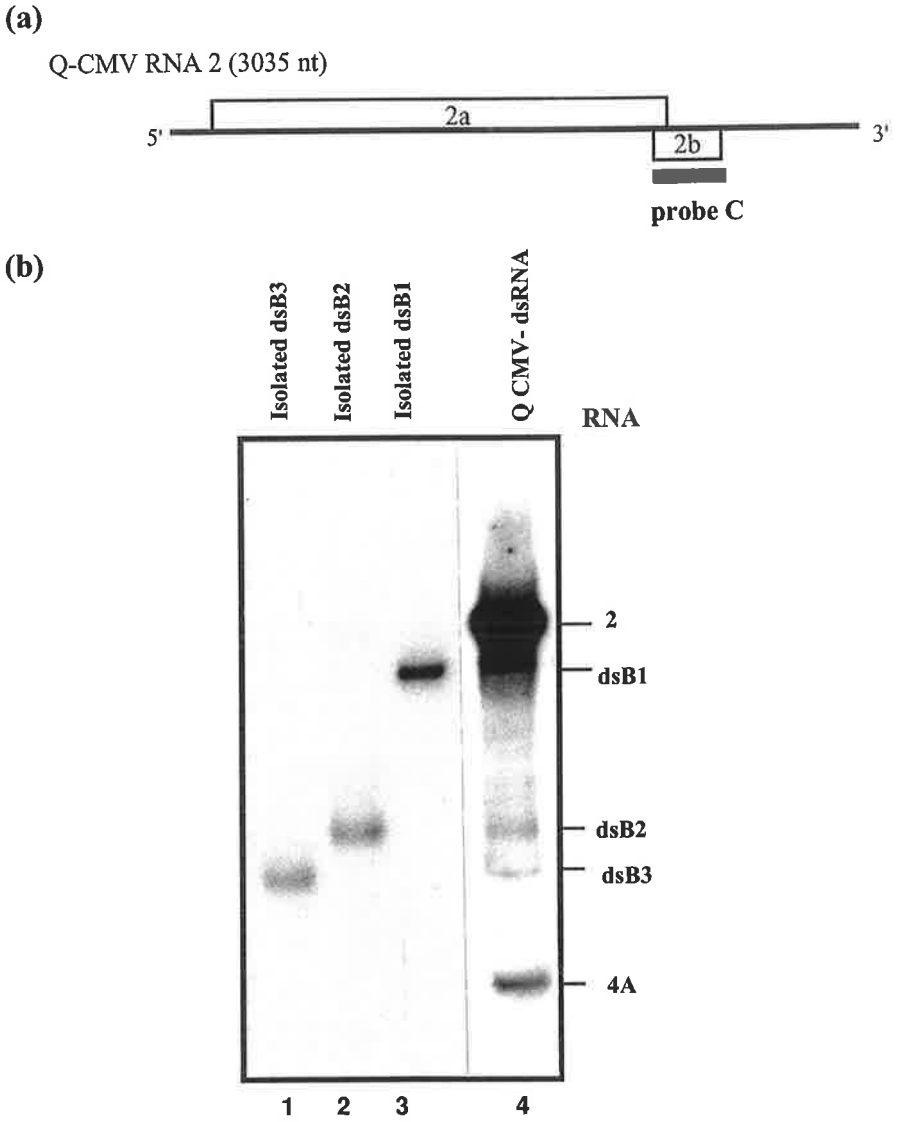

Fig. 3.7 Northern hybridisation analysis of the purified minor ds-RNAs.
(a) Overall structure of Q-CMV RNA 2. The dark box (▬) indicates the position of a strand specific RNA probe (probe C) which is complementary to nucleotides 2410-2712 of Q-CMV RNA 2. **(b)** Northern analysis of the purified minor dsRNAs. The purified dsB1, dsB2 and dsB3 with the BAC method (lanes1-3) and total dsRNAs extracted from QCMV infected cucumber leaves (lane 4) were hybridised with probe C.

3.4 Discussion

3.4.1 Isolation methods of dsRNA fragments and their efficiency

The presence of contaminating nucleic acids in the total ds-RNA samples, extracted from Q-CMV infected plants (Section 3.3.1), implied that the method used for extraction of total ds-RNA (Smith et al., 1991) was not been able to remove contaminant nucleic acids efficiently. In order to remove these contaminants, the total ds-RNA extraction procedure (Section 3.2.1; Fig. 3.1) was supplemented with another cycle of CF-11 cellulose and DNase treatment, since it has been shown that, in the presence of 16% ethanol, dsRNA binds to cellulose powder whereas other nucleic acids pass through (Franklin, 1966). These modifications provided more efficient removal of contaminant nucleic acids from the dsRNA preparations and reduced their concentrations to non detectable levels.

Four methods for the isolation of the minor ds-RNAs from agarose or acrylamide gels were tested in the present work, in order to find the most efficient. These methods were mainly based on the adsorption of dsRNAs to CC-41 cellulose. Specific binding of dsRNAs to CC-41 cellulose provides a useful further step in the purification of dsRNA (Dulieu and Bar-Joseph, 1989).

Isolation of the minor dsRNA fragments from agarose by adsorption to CC-41 cellulose was shown to be efficient and simple; however, the approach has two disadvantages. First, due to lower resolution in agarose than in polyacrylamide gels, the minor segments are not very clear and it is possible that the final eluted dsRNAs contain ss-RNA contaminants. Therefore this method is not suitable for isolation of minor bands, although in the case of genomic ds-RNAs with high concentrations it is an easy and efficient method. Secondly, agarose can non-specifically interact with nanogram amounts of double-stranded RNA molecules during native gel electrophoresis, leading to a change in their electrophoretic mobilities (Sun and Kao, 1995).

In the electroelution method from acrylamide gel slices under the conditions suggested by the manufacturers of the two types of used electroeluters: BIOTRAP (Schleicher & Schuell Inc.) and the Model 422 (Bio-Rad), dsB2 and dsB3 were eluted easily with reasonable recovery, but in the case of dsB1 recovery was very poor with the two types of apparatus tested (Section 3.2.3.3). Changes in electroelution conditions such as the duration of electroelution did not improve the recovery. The reason of the low recovery of dsB1 by electroelution is not clear.

Although the recovery of nucleic acids from agarose was efficient and much easier than from acrylamide, poor resolution was a serious disadvantage, especially for the isolation of minor bands with small amounts of nucleic acid. On the other hand acrylamide gels provided good resolution, allowing visualisation and access to the minor bands of interest in a population of nucleic acids. The beauty of the agarose embedding method (Section 3.2.3.2) is the ease of recovery of nucleic acids in agarose coupled with the high resolution of acrylamide. The isolation of the three minor dsRNAs has shown that this method can be considered as an alternative to the BAC cross linker method (Section 3.2.4 d) for isolation of dsRNAs, especially when they are too close to the other dominant fragments to isolate them from agarose alone.

The work described in this chapter shows that the isolation of minor ds-RNA bands was most efficiently achieved using the BAC method of Dulieu and Bar-Joseph (1989) with some modifications. This approach was therefore used in subsequent experiments described in this thesis. This method utilises BAC, a disulfide-containing analogue of bis-acrylamide which, after polymerisation and electrophoresis, allows the gel to be dissolved by reducing the disulfide bond following the addition of a reducing agent such as 2-mercaptoethanol (Hansen et al.1980). Dissolving the acrylamide and BAC at less than 50°C, keeping the mixture of dsRNA and acrylamide at 50°C during the whole isolation process and increasing the washing times, were found to be efficient modifications which allowed elimination of undissolved acrylamide gel pieces in the eluted dsRNA samples.

3.4.2 The relationship of the new ds-RNAs with genomic RNAs

Analysis of dsRNA from CMV infected plants by PAGE and silver staining enabled the 5 known major CMV RNAs to be visualised, comprising three genomic (RNAs1, 2 and 3) and 3 subgenomic RNAs (RNAs 4, 4A and 5). Three additional dsRNA bands of low staining intensity were consistently observed in the dsRNA profile.

Similar fragments have previously been seen in the dsRNA profiles of some isolates of CMV. In an attempt to use dsRNA analysis for differentiation of CMV isolates, Wang et al. (1988) observed that some isolates (in addition to the replicative form dsRNAs that were twice the size of viral genomic ssRNAs) contained additional minor dsRNAs between dsRNA 2 and 3 as well as RNAs 3 and 4 (Fig. 3 and 4). Pares et al. (1992) also found some isolates of CMV with additional minor dsRNAs between dsRNAs 2 and 4 (Fig. 2 and 3). Similar bands are also visible in the dsRNA profiles of two CMV isolates (as shown in Fig. 1A lanes 4 and 5 of Dodds, 1993). The size of these additional dsRNAs are similar to those of the dsB1 and dsB2.

Therefore, the results of this chapter further confirmed the presence of additional dsRNAs in CMV-infected plants but the structure of these minor dsRNAs and whether they are virus specific or related to the host needs to be determined. The results of northern analysis with genomic specific riboprobes revealed that dsB1 and dsB2 and dsB3 are of viral origin and related to CMV genomic RNA2. The next chapter will describe the results of cloning and sequencing of these minor dsRNAs.

Chapter 4

Characterisation of New ds-RNA Fragments in Cucumber Mosaic Virus Infected Plants

4.1 Introduction

The previous Chapter described that, in addition to the known genomic and subgenomic RNAs of CMV, three new minor dsRNAs were present in the dsRNA profiles of CMV infected plants. Northern analysis showed that these new dsRNA species have sequence homology with RNA 2 of CMV. Moreover, to facilitate the further study of these ds-RNAs and due to their low concentrations, an efficient method for their isolation was developed.

There is a growing interest in using ds-RNAs as useful alternatives to indicate genetic relatedness of viral genomes, for analysing functions, and to obtain sequence information (Dolja and Atabekov, 1987; Dulieu and Bar-Joseph, 1989; Nuss and Dall, 1990).

Methods for synthesising complementary DNA (cDNA) using ds-RNA template were previously reported (Cashdollar et al., 1982; Imai et al., 1983; Asamizu et al., 1985; Le Gall et al., 1988; Jelkman et al., 1989; Nuss and Dall, 1990). However, most procedures depend both on the knowledge of the terminal sequences and on the availability of quantities of dsRNA which are not accessible for most viruses.

The aim of the work presented in this Chapter was to find an efficient method for cDNA synthesis from small amounts of the target ds-RNAs when there is no information about their termini. Amplification of the cDNAs and their subsequent cloning and sequencing would help to elucidate the structure and the origin of the minor ds-RNA fragments associated with CMV infected plants.

4.2 Materials and methods

4.2.1 Comparison of three methods for dsRNA denaturation

In order to determine the best method for denaturation of target dsRNA species prior to cDNA synthesis, CMV dsRNA 2 (corresponds to CMV genomic RNA2) was used since more of that can be obtained. CMV genomic dsRNA 2 was isolated from polyacrylamide gels by the BAC method (Section 3.2.4 d) and then was used as template for the comparison of denaturation methods as follows.

a. Heating at 100°C In this method 1 µl of the isolated CMV genomic dsRNA2 (in water) mixed with 90 ng of reverse primer (SD6) in a total volume of 6 µl was drawn into a plastic capillary tip (Tri-Continent, USA) and sealed in a Bunsen flame. Denaturation was carried out by putting the tube in boiling water for 5 minutes. The tube was then allowed to cool to 42°C over a period of 30 minutes for annealing the primer (SD6) to the template (CMV genomic dsRNA 2). The rest of the methods including first strand complementary synthesis and PCR amplification were carried out as in Section 4.2.1 d.

b. Methylmercuric hydroxide

For denaturation with CH_3HgOH, 1 µl of purified genomic dsRNA2 was added to 90 ng SD6 primer. After addition of 1µl of methylmercuric hydroxide (Johnson Matthey), the tube was incubated at room temperature for 20 minutes. The reaction was stopped by adding 2 µl of 350 mM 2-mercaptoethanol. The reverse transcription and PCR amplification were performed as in Section 4.2.1 d.

c. Heating (100°C) + Methylmercuric hydroxide

In this method about 50 ng of purified dsRNA2 fragment was denatured by heating as described before (Section 4.2.1.a). After rapid cooling on ice, 1 µl of 100 mM CH_3HgOH was added and, following an incubation at room temperature for 10

minutes, the reaction was stopped by adding 2 µl of 350 mM 2-mercaptoethnol. The first strand complementary DNA synthesis and PCR amplification were performed as in Section 4.2.1 d.

d. Amplification of the 3' end of CMV dsRNA2 by specific primers

After extraction of total dsRNA from CMV infected plants, genomic dsRNA 2 was isolated as described in Chapter 3 (Section 3.2.4.d) and denatured by methylmercuric hydroxide, heating or heating and methylmercuric hydroxide as described above (Section 4.2.1 a, b and c). Using 1 µl of the denatured dsRNA 2, and SD6 as primer, reverse transcription was carried out in a 2 µl reaction volume as described before (Section 2.2.9). The amplification reactions were set up in 0.5 ml microfuge tubes as mentioned earlier (Section 2.2.10). Amplification was carried out in an MJ Minicycler (MJ Research Inc., USA, Model PTC 150) using the following program: 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, for 30 cycles followed by a final elongation step of 5 minutes at 72°C. PCR reaction products were separated by electrophoresis on 1.2% agarose gels.

4.2.2 cDNA synthesis of the minor dsRNAs using random primer-PCR

Synthesis of cDNA using purified dsRNAs as templates was carried out with some modifications to the method (Fig 4.1) described by Froussard (1992). First strand cDNA was synthesised using a 20 nucleotide universal primer containing a random hexamer at its 3' end (Universal random primer-dN6; 5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3').

For this purpose, 5 µl of purified dsB1, dsB2 or dsB3 segments were added to 1 µl of the primer and incubated at room temperature for 20 minutes after addition of 1 µl 100 mM MeHgOH. The reaction was deactivated by the addition of 1 µl 700 mM ß-mercaptoethanol (Cf≈30 mM) and incubation at room temperature for 5 minutes. The transcriptase mixture was composed as follows: 0.5 µl (20 units) RNAsin inhibitor

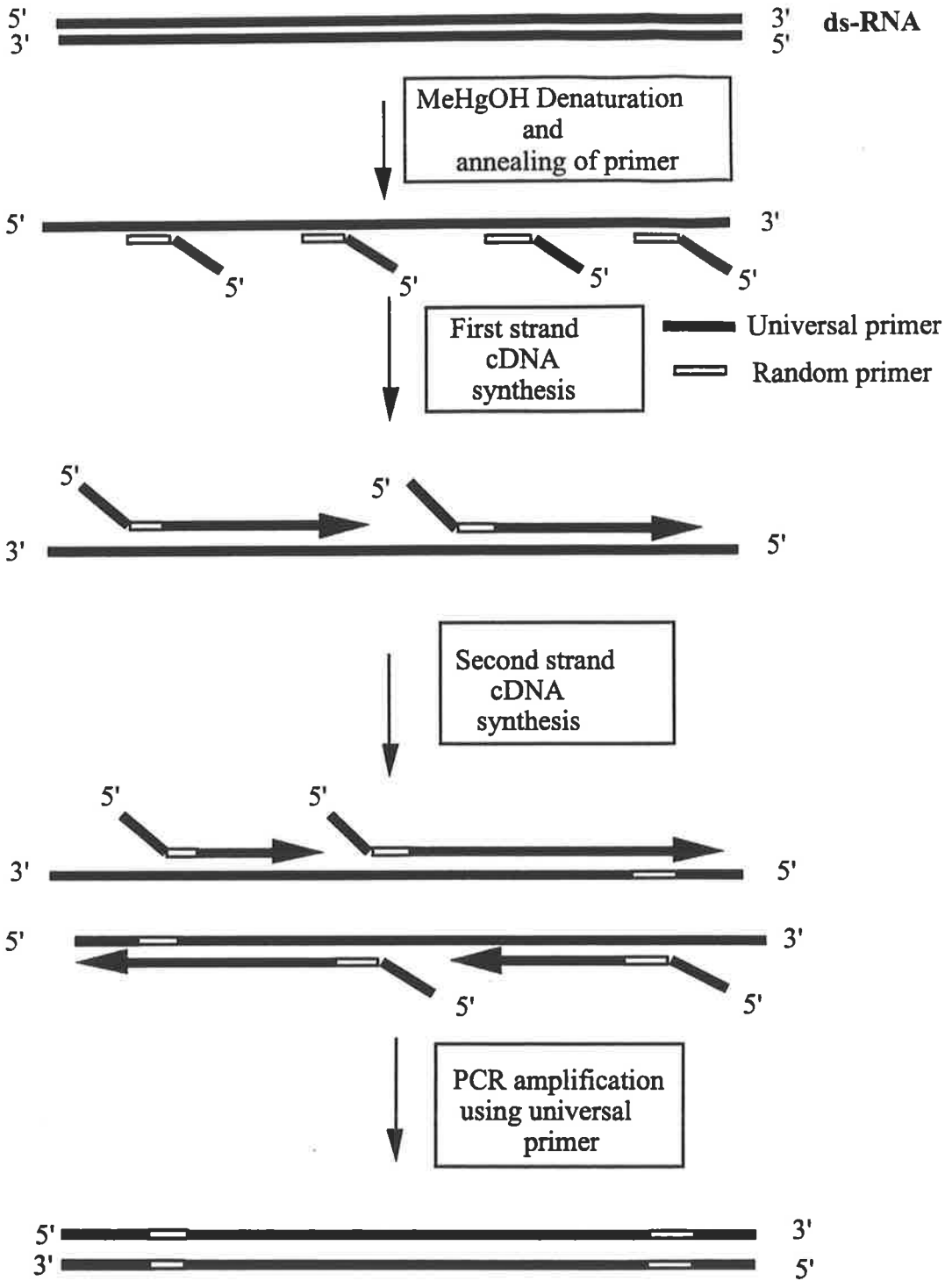

Fig.4.1 Schematic representation of Random-primer PCR method for amplification of dsRNA fragments.

(Promega), 1.25 µl 10x reverse transcription buffer supplied with the enzyme (500mM Tris-HCl pH8.3 at 43°C, 800 mM NaCl, 80 mM $MgCl_2$, 50 mM DTT), 1 mM of each of the dNTPs (Pharmacia) and 16 units AMV reverse transcriptase. After incubation at 43°C for 1 hour, the reaction mixture was heated at 100°C for 2 minutes and cooled on ice immediately. For second strand synthesis, the following reagents were added to the 12.5 µl of the first strand synthesis to reach a final volume of 30 µl : 5 µl of 10x Klenow buffer (500 mM Tris-HCl pH 7.2, 100mM MgS04, 1mM DTT), 2 µl of Klenow fragment enzyme (8 units). After 30 minutes incubation at 37°C, the cDNA was purified from the excess of Universal primer-dN6 by passing through a Sephacryl S-400 column (Promega).

4.2.3 cDNA amplification by PCR

a. Amplification of minor dsRNAs using Random primer-PCR method (rPCR).

Amplification of the double-strand cDNA library synthesised using the Universal-random primer (Section 4.2.2) was performed on a 2 µl aliquot with 0.8 µM of the universal primer. The PCR reactions were set up in 0.5 ml microfuge tubes and 50 µl volumes as described earlier (Section 2.2.10). Amplification was catalysed by 2 units of Taq polymerase and carried out for 40 cycles in an MJ minicycler (MJ Research Inc., USA, Model PTC 150) using the following program: 94°C for 1 minute, 55°C for 1 minutes, 72°C for 3 minutes, for the first five cycles, followed by 94°C for 1 minute, 60°C for 1 minute and 72°C for 3 minutes for the remainder of cycles and a final elongation step of 5 minutes at 72°C. Reaction products were electrophoresed on a 1.2% agarose /1xTAE minigel and visualised by ethidium bromide/UV.

b. Amplification of 5' ends of the minor dsRNAs by RACE-PCR This was performed largely as described by Frohman (1990; see Fig. 4.2 for a description of the RACE procedure). One µl samples of the isolated dsB1and dsB2 (Section 3.2.4.d)

were denatured as previously described (Section 4.2.1.b), and 3 µl of the denatured RNA reverse transcribed (Section 2.2.9) using the SD61 primer, complementary to nucleotides 2692-2709 of the Q-CMV RNA2. The reaction was carried out using AMV reverse transcriptase (Promega) according to the manufacturer's specifications in a volume of 20 µl, and was stopped by incubation of the mixture at 80°C for 3 minutes. Then the reverse transcription mixture was purified from the primer and reaction components using a Sephacryl S-400 column (Promega). The cDNA was tailed with poly (dA) using terminal deoxynucleotidyl transfrase, then the solution was cleaned up by using Bresaclean kit (Bresatec). The tailed cDNAs were diluted to 100 µl with water and stored at -20°C. RACE-PCR was performed on 1 µl of the tailed cDNA using H30 as the sequence-specific primer (see Table 5.1), and RACE 5 (5'-GACTCGAGATCGA[T]17 -3') as the non- specific primer. The PCR reaction was catalysed by Taq polymerase (Promega) according to the manufacturer's direction, with a thermal cycle profile of 94°C for 3 minutes, [55°C/1 minute, 72°C/1 minute 30s, 94°C/40 s] for 30 cycles and a final cycle of 5 minutes at 72°C, 25°C/5 minutes on a DNA thermal sequencer (Corbett). Reaction products were resolved on 1.2 % agarose 1xTAE minigels and visualised with ethidium bromide.

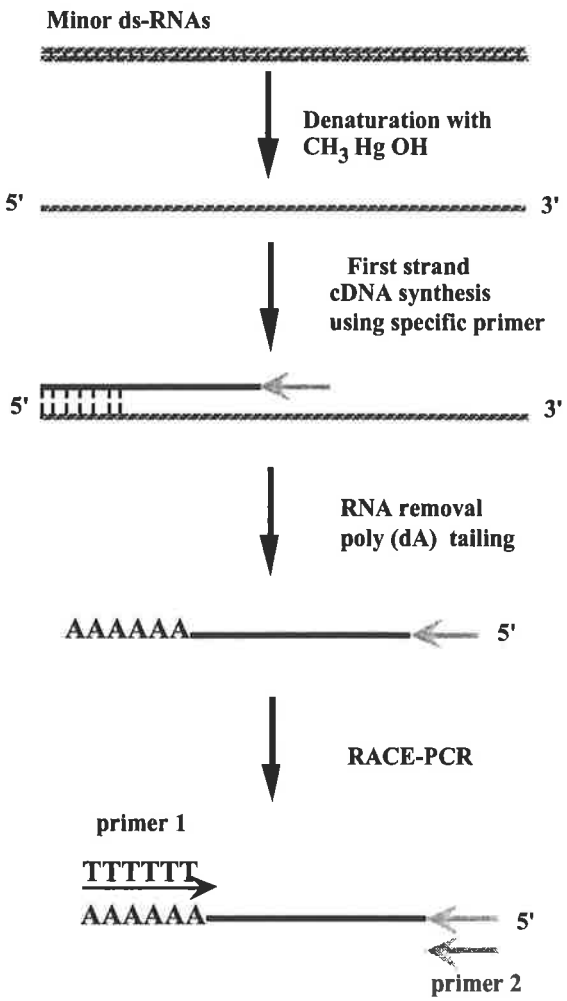

Fig. 4.2 Schematic diagram of RACE-PCR method for the amplification of 5' end of minor ds-RNAs. cDNA synthesis was primed from the desired ds-RNAs (following the denaturation of two strands) with a sequence specific primer (gray arrow). The cDNA was purified from the RNA template and excess first- strand primer, then tailed with poly (dA) using terminal deoxynucleotidyl transferase (TdT). The tailed cDNA was then used as a template for PCR, using a non specific $d(T)_n$ and a specific primer.

c. Amplification of 3'-ends of minor dsRNAs using RT-PCR The PCR method using specific primers was used to determine the 3' terminal nucleotide sequences of dsB1, dsB2 and dsB3. For this purpose, 5 µl of isolated ds-RNA species were denatured using CH_3HgOH as described in Section 4.2.1b and SD6 (Table 5.1) was then employed for first strand cDNA synthesis (Section 2.2.9). PCR amplification was performed using SD6/SD53, specific primer pair as described in Section 2.2.10. The

major PCR products were excised from the gel and purified using a Qiagen II Kit before cloning into the pGEM-T vector (Promega) (Section 4.2.4 c).

Clones were identified by dideoxy sequencing on double-stranded templates as described in Chapter 2 (Section 2.2.18).

4.2.4 Cloning and sequencing

a. Cloning and sequencing of cDNAs obtained from rPCR The obtained PCR products of the minor dsRNAs (Section 4.2.3.a) were separated on a 1.2 % agarose gel and the desired DNA species excised after ethidium bromide staining/UV visualisation. The DNAs were extracted from the gel slices as in Section 2.2.6. The purified DNAs were then digested with *EcoRI* followed by purification on a Sephacryl S-400 column (Promega). After ethanol precipitation, the pelleted DNAs were resuspended in 15 µl water and ligated into the *EcoRI* digested pBSK$^+$ vector. Faithful incorporation of the PCR products was verified by dideoxy sequencing on both strands, using the T3 and T7 primers that flanked the multicloning site (Section 2.2.18).

b. Cloning and sequencing of the 5'-ends of the minor dsRNAs The major reaction products of ≈300 nucleotides obtained by RACE-PCR method (Section 4.2.3.b) were excised from the gel and purified using a QIAEX II Agarose gel extraction kit (QIAGEN) before end-filling for blunt end cloning. End filling of PCR products was performed in a 100 µl reaction mixture containing : 25 µl of purified PCR product, 10 µl of 10x polI buffer (0.5 M Tris pH 7.5, 0.1 M MgCl2, 10 mM DTT, O.5 mg/ml BSA, 200 µM dNTPs), ATP to 1 mM, and about 10 units each of T4 polynucleotide kinase and DNA polymerase I (Promega). After adjusting the final volume to 100 µl, the reaction mixture was incubated at 37°C for 1 hour followed by addition of 1 µl of 0.5 M EDTA pH 8 to stop the reaction. The solution was purified once again using a QIAEX II Agarose gel extraction kit to remove enzymes and salts before blunt end cloning into the *EcoRV* site of pGEM Z 5(-) as in Section 2.2.11 and 2.2.12. Clones

were identified by dideoxy sequencing on double-stranded templates as described in Chapter 2 (Section 2.2.18).

c. Cloning and sequencing of the 3'-ends of the minor dsRNAs

The major PCR products obtained using RT-PCR and specific primer pairs (Section 4.2.3.c) were excised from agarose gels and purified using a Qiagen II kit before cloning into the pGEM-T vector (Promega). Ligation was carried out in a 10 µl volume with 50 ng of vector DNA and the DNA insert fragment in a molar ratio of 1:3 and 1:5, respectively, using conditions suggested by the manufacturer. Transformation of bacteria with plasmids was performed by electroporation, using the *E. coli* strain DH5α and the Gene-Pulser (Bio-Rad, USA) as described before (Section 2.2.13).

Double-stranded DNAs prepared from these clones were sequenced as described in Chapter 2 (Section 2.2.18).

4.3 Results

4.3.1 PCR amplifications and cloning

Further studies of ds-RNAs such as cDNA synthesis and *in vivo* translation, required the melting of ds-RNA to ss-RNA. Due to the poor preliminary results obtained by heat denaturation, three different denaturation methods using isolated CMV RNA 2 dsRNA (can get more of this than desired ds-RNA molecules), were compared to find an efficient method for denaturation of ds-RNA species. The results showed that the CMV RNA 2 dsRNA (dissolved in water) denatured by heat treatment at 100°C (Section 4.2.1a) generated no specific PCR products (Fig. 4.3 lane 1). However, when denaturation was performed by CH_3HgOH or CH_3HgOH+heating (Section 4.2.1 b &c) a single band corresponding to the size of expected of the SD6/SD53 fragment of CMV-RNA2 was obtained after the PCR reaction (Fig. 4.3 lanes 3 and 4). These results indicated that CH_3HgOH was a useful agent for denaturation of the CMV-

RNA2 ds-RNA Therefore, due to the sequence homology of these minor dsRNA species with CMV-RNA 2, CH_3HgOH was selected for denaturation of the minor dsRNAs prior to cDNA synthesis.

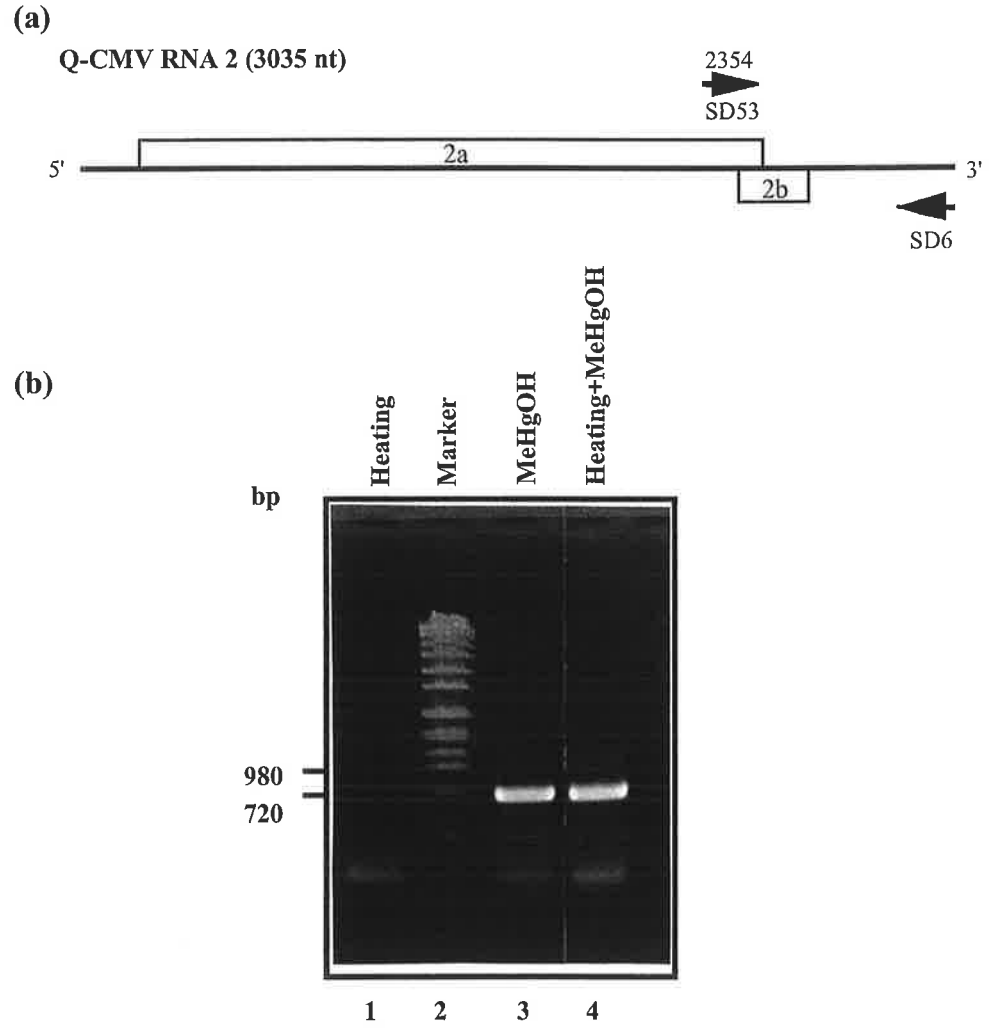

Fig. 4.3 Comparison of three methods for denaturation of dsRNA

(a) The arrowhead lines indicate the positions of the primers which used for amplification of Isolated ds-RNA2 (correspond to the Q-CMV-RNA2). **(b)** PCR amplification of dsRNA2 using SD53/ SD6 primer pair and the parameters given in Section 4.2.1 d . Samples were , in lane 1, ds-RNA2 denatured by heating for 5 min at 100°C; in lane 3, ds-RNA2 denatured by CH_3HgOH and in lane 4, ds-RNA2 denatured by CH_3HgOH+heating at 100°C. The expected size of the PCR product was 680 bp. The DNA size markers were SPP1/ *Eco R1* fragments.

4.3.2 Amplification of dsB1, dsB2 and dsB3 segments

Due to the lack of enough information about terminal sequences of the minor dsRNAs and their low concentrations, the random priming-PCR method which Froussard (1992) used for making a cDNA library from ssRNA was adopted (Fig. 4.1) to determine the nucleotide sequences of isolated dsB1, dsB2 and dsB3 fragments. For determination of the 5' and 3' terminal sequences which were not present in the cDNA library, RACE-PCR or PCR with specific primers were used.

4.3.2.1 rPCR method

Southern analysis showed that the synthesis of cDNAs was successful with a range of 450-900 bp length (Fig. 4.4).

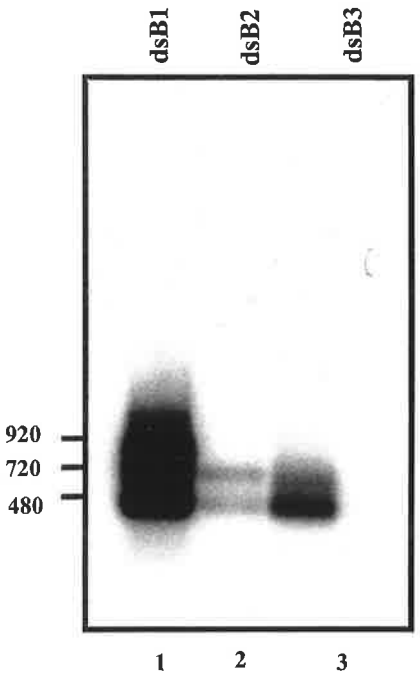

Fig. 4.4 Agarose gel electrophoretic analysis of the rPCR products prepared from minor ds-RNAs. For Southern analysis, 5 µl of the PCR products were electrophoresed in a 1.2% agarose gel, and tranfered to nitrocelloluse and hybridised with a ^{32}P-labeled probe prepared using a random hexamer and a full length Q-CMV RNA2 ds DNA. Lanes 1 to 3 correspond respectively to the rPCR products of dsB1, dsB2 and dsB3. The size markers are shown in bp.

Screening of the cDNA clones by southern analysis (Fig. 4.5), revealed the generation of twelve independent clones covering a large proportion of the dsB1 fragment.

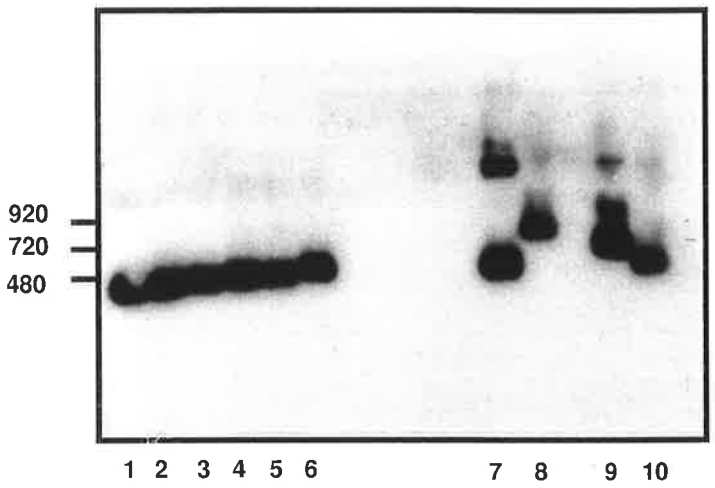

Fig. 4.5 Southern analysis of cDNA clones of the minor ds-RNAs.
Southern hybridisation analysis was carried out on the *Eco R1* digestion products of the minor ds-RNAs' cDNA clones using a ^{32}P-labeled probe prepered using a random hexamer and a full length Q-CMV RNA2 ds DNA . The size markers are shown in bp.

Five of these twelve clones contained an insert of about 900 base pairs, four clones contained an insert of around 850 base pairs, three clones contained an insert of 750 base pairs and four clones contained an insert of about 700 base pairs (Table 4.1). Sequence analysis of obtained clones revealed some overlapping parts which allowed the assembling of the sequences into two large contigs (Fig. 4.9).

For the dsB2 fragment ten independent clones covering most of the fragment were produced (Table 4.1). Four clones with an insert of 480, three clones with an insert of 517 base pairs and three clones with an insert of 470 base pairs were obtained (Table 4.1). On the basis of sequence analysis, dsB2 sequences were assembled into two contigs. However, in the case of dsB3, sequence analysis of six independent clones revealed the presence of just one block (Fig. 4.9).

Table 4.1 Clons and sequences of minor ds-RNA fragments

ds-RNA	Name of clone	Lenght	position [a]
dsB1	p^{DB1}_1	900bp	nt 11-9011
	p^{DB1}_2	900bp	11-9011
	p^{DB1}_3	900bp	11-9011
	p^{DB1}_4	900bp	11-9011
	p^{DB1}_5	900bp	11-9011
	p^{DB1}_6	850bp	717-1567
	p^{DB1}_7	850bp	717-1567
	p^{DB1}_8	850bp	717-1567
	p^{DB1}_9	850bp	717-1567
	p^{DB1}_10	750bp	2048-2798
	p^{DB1}_11	750bp	2048-2798
	p^{DB1}_12	750bp	2048-2798
	p^{DB1}_13	700bp	2227-2927
	p^{DB1}_14	700bp	2227-2927
	p^{DB1}_15	700bp	2227-2927
	p^{DB1}_16	700bp	2227-2927
dsB2	p^{DB2}_1	480bp	30-510
	p^{DB2}_2	480bp	30-510
	p^{DB2}_3	480bp	30-510
	p^{DB2}_4	480bp	30-510
	p^{DB2}_5	517bp	50-567
	p^{DB2}_6	517p	50-567
	p^{DB2}_7	517bp	50-567
	p^{DB2}_8	470bp	2420-2890
	p^{DB2}_9	470bp	2420-2890
	p^{DB2}_10	470bp	2420-2890
dsB3	p^{DB3}_1	637bp	30-667
	p^{DB3}_2	637bp	30-667
	p^{DB3}_3	637bp	30-667
	p^{DB3}_4	567bp	100-667
	p^{DB3}_5	567bp	100-667
	p^{DB3}_6	567bp	100-667

[a] position of each clone are given with compare to RNA2 sequence

4.3.2.2 Determination of the 5'-terminal sequences of the dsB1, dsB2 and dsB3

Using the obtained sequence information (Section 4.3.2.1) one primer was designed for 5'-RACE to get the clones representing the 5' ends of the minor dsRNAs. The RACE method (Forhman, 1990) employs PCR amplification of first-strand cDNA with only one specific primer (Fig. 4.2). Thus, first-strand cDNA synthesised from a sequence-specific primer at the 5' end must first be tailed with poly (dA) using terminal transferase, before synthesis of the second cDNA strand and subsequent PCR amplification using a non-specific $d(T)_n$ oligonucleotide as the second-strand primer. RACE of the 5' ends of dsB1 dsB2 and dsB3 using a non-specific (RACE 5) and specific (H30) primer pairs produced three single bands of 314 nt (Fig. 4.6 a and b).

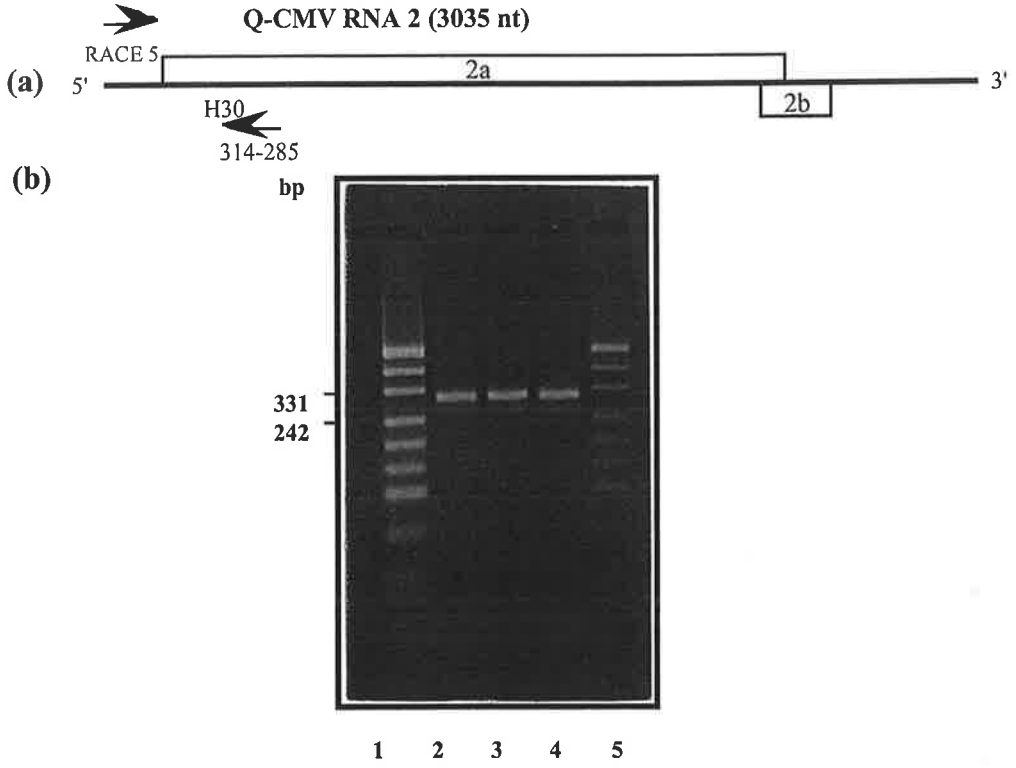

Fig. 4.6 RACE-PCR amplification of the 5'end of the minor ds-RNAs.
(a) The arrowheads indicate the positions of the primers (RACE 5/H30) used in RACE-PCR compared to the Q-CMV RNA2. **(b)** RACE-PCR was carried out on the denatured dsB1 (lane 2), dsB2 (lane 3) and dsB3 (lane 4). Amplification was carried out using the H30 as the specific primer and RACE 5 (5'-GACTCGAGATCGA$[T]_{17}$-3'), as the non-specific primer. The expected size of the PCR product was 314 bp. The DNA size markers (lanes 1& 5) were pUC 19/*HpaII* fragments.

Cloning and sequencing of these three bands revealed that they were identical to the first 314 nt of the CMV RNA2 and that they were overlap the existing sequences (Fig. 4.8).

4.3.2.3 Determination of the 3'-terminal sequences of dsB1 and dsB2

In order to determine the 3' terminal sequences of dsB1 and dsB2, on the basis of sequence information obtained from rPCR method (Section 4.3 2 1) one primer (SD53, Table 5.1) was designed to use in combination with the SD6 primer (complementary to the last 340 nt of all genomic and subgenomic of Q-CMV RNAs). PCR reactions with SD6/SD53 primer pairs and dsB1 and dsB2 as templates, produced a major product of 680 bp (Fig. 4.7) which sequencing revealed that the sequenced clones correspond to

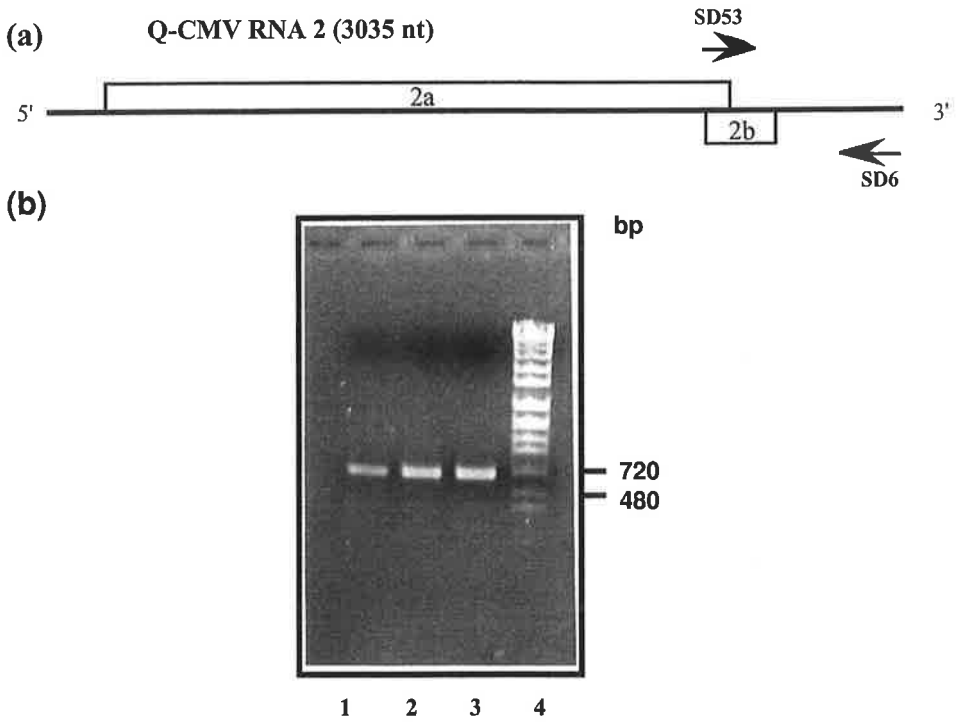

FIG. 4.7 3'-end amplification of the minor ds-RNAs.
(a) The arrowheads indicate the positions of the primers (SD6/SD53) used in RT-PCR. **(b)** RT-PCR assay for 3'-end amplification of the minor ds-RNAs. PCR reaction was performed using purified dsB1 (lane 1), dsB2 (lane 2) and dsB3 (lane 3) as templates and SD53/SD6 primer pair. The expected size of the PCR product was 680 pb. The DNA size markers (lane 4) were SPP-1/*Eco RI* fragments. The relevant size markers are shown in bp.

the last 680 nt of CMV RNA 2 and that they overlap the existing sequences at the 3' ends of dsB1 and dsB2 (Fig. 4.8).

4.3.3. The overall structure of minor ds-RNAs

Sequencing of all clones obtained for dsB1 (produced by all employed methods; rPCR, 5'-RACE and PCR with specific primers) revealed a total nucleotide sequence of 2554 nucleotides (Fig. 4.8) which is close to its estimated size (about 2600bp). Furthermore the results implied that, compared to the size of the Q-CMV RNA 2 (3035 nt), there is a deletion of about 410 nucleotides in the central region of the dsB1.

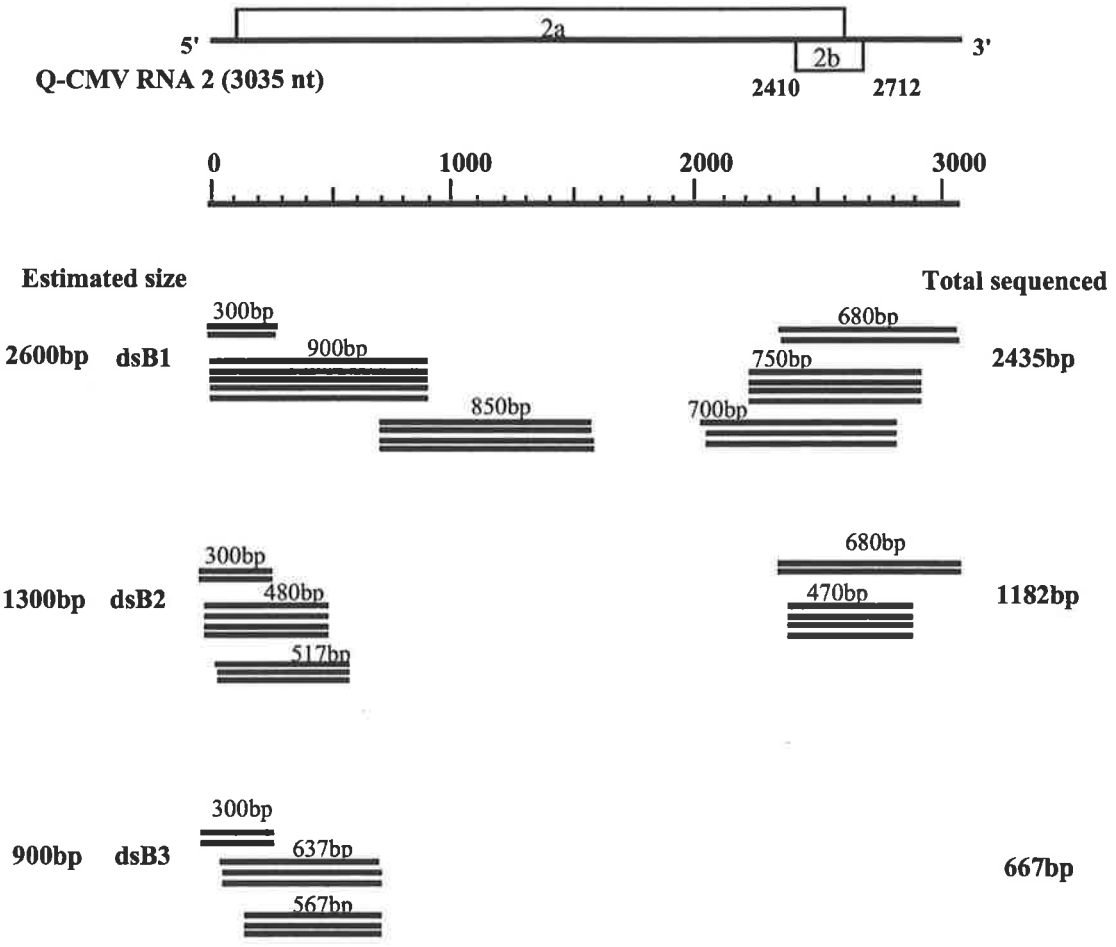

Fig. 4.8 The summary of sequence data obtained for the minor ds-RNAs using rPCR, RACE-PCR and RT-PCR . The minor dsRNAs were sequenced in both directions from several overlapping clones. The lentgh of sequence from each clone is shown in relation to the Q-CMV RNA2.

Sequence determination of the junction sites will provide the exact number of deleted nucleotides. In the case of dsB2, sequence analysis of clones from all three strategies showed a total nucleotide sequence of 1197 nucleotides, compared to the estimated size of the fragment (about 1300bp); hence these sequences represent about 92% of the dsB2 with around 118 nucleotides missing in the central region of the molecule (Fig. 4.8). Sequence analysis of clones of dsB3 generated from both rPCR and 5'-RACE revealed a total nucleotide sequence of 667 nucleotides, compared to the estimated size of the fragment (900bp); these sequences represent 74% of the RNA and also a part of the fragment (around 233 nucleotides) is missing (Fig. 4.8).

4.3.4 Comparison of the minor ds-RNA sequences with CMV RNA2 sequences

Fig 4.9 shows a summary of the regions of the dsB1, dsB2 and dsB3 RNAs which were obtained either by rPCR, RACE or PCR with specific primers. On the basis of these results, dsB1 is made up of two blocks (A and B) entirely derived from the sequence Q-CMV RNA 2 (Rezaian et al., 1984). Block A composed of the first 1567 nucleotides of the RNA2 including 5' leader sequence and the initiation codon of the ORF 2a. Block B composed of 987 nucleotides represents the 3' terminus and corresponds to the nucleotides 2048 to the 3' end of Q-CMV RNA2 and includes the RNA 4A region (Fig. 4.9). dsB2 was also made up of two blocks (C &D) and, like dsB1 , its sequences are identical to those of Q-CMV RNA2. Block C which represent the 5' terminus, is composed of 567 nucleotides and contains the 5' region of ORF 2a. Block D is composed of 680 nucleotides, corresponding to the 3' terminal 680 residues (2354-3035). As can be seen (Fig. 4.9), dsB3 is composed of just one block (E), representing the first 667 nucleotides of Q-CMV RNA2. However with regard to the northern analysis with a 3' common end probe, and strong signal of the dsB3, it is most likely that dsB3 also share some sequences (F) with the 3' end of the Q-CMV RNA2.

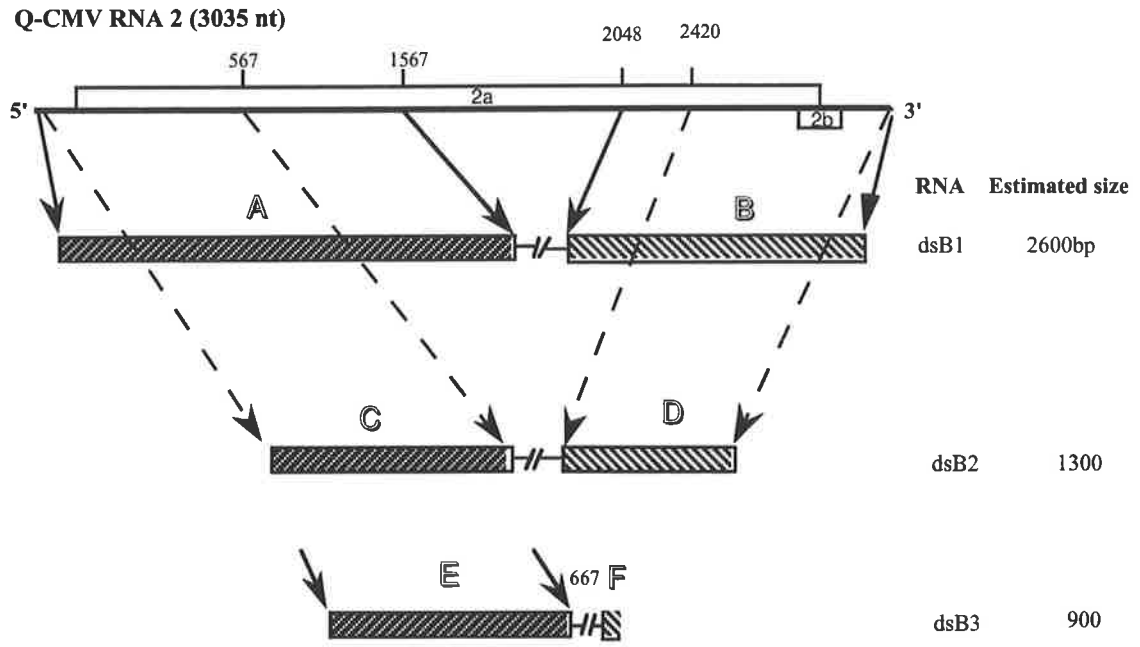

Fig.4.9 Diagram of the overall structure of the minor ds-RNAs in relation to QCMV-RNA2. The arrowheads show the position of different blocks compared to Q-CMV-RNA2. The possible deletion sites are shown in the centre of each dsRNAs. The approximate size of minor dsRNAs are given in bp.

4.4 Discussion

4.4.1 The advantages of rPCR method for cDNA synthesis of dsB1, dsB2 and dsB3

The presence of dsRNA molecules in hosts infected with either ssRNA or dsRNA viruses is now considered as a biologically important feature in terms of diagnosis (Coffin and Coutts 1992; Dale et al., 1986; Aramburu et al., 1991; Rezaian et al., 1991) or molecular analysis of viral diseases (Jelkmann, 1995; Zhang et al., 1991; German et al., 1992; Habili and Rezaian 1995; Smith et al., 1992; Mawassi et al., 1995). However, the difficulty in producing cDNA from dsRNA is an obstacle in molecular analysis of dsRNAs. This problem is mainly related to the physical structure of dsRNA which, unlike the double-helical B form usually adopted by dsDNA, adopts the more

compact and significantly more stable A form of double helix (Lewin, 1990; Coffin and Cutts, 1992).

The random priming-PCR method which Froussard (1992) used for making a cDNA library for ssRNA was adopted with some modifications (Fig. 4.1) to determine the nucleotide sequences of isolated dsB1 and dsB2 fragments. In this method, after denaturation of dsRNA with CH_3HgOH (instead of heating at 65°C which Froussard used) the first strand cDNA was synthesised from a Universal primer containing a random hexamer at its 3' end and the second strand cDNA was made using the Klenow fragment of DNA polymerase I. Then PCR was employed to amplify the double strand cDNA.

The adopted method described here provides a quick, simple and reliable method for constructing cDNA libraries from small amounts of dsRNA molecules extracted from host plants. The combination of reverse transcription using a Universal primer containing a random 3'-hexamer together with PCR, facilitates the amplification of nucleic acids without any specific primers. The lack of laborious steps such as homopolymeric addition or ligation of oligonucleotides, have increased the potential of the method especially when there is no information regarding viral sequences and when a limited amount of dsRNA is available for generating a cDNA library.

4.4.2 The nature of minor dsRNAs

Before drawing any conclusion about the nature of these dsRNAs, the following aspects should be considered:

- These dsRNA fragments have been consistently seen in the Q-CMV dsRNA profile. Moreover a review of the literature revealed that in many cases similar dsRNA bands were present regardless of the CMV strain and method of dsRNA extraction (Wang *et al.*, 1988; Pares *et al.*, 1992; Dodds, 1993).

- These dsRNA components have been isolated from polyacrylamide gels and their purities were checked by northern analysis.

- Northern analysis using different genomic RNA probes proved that the dsRNA segments share sequence homology only with RNA 2 and no cross reaction was observed with other genomic RNA probes.

- Analysis of these new dsRNAs by random priming PCR, cloning and sequencing revealed that, in the case of dsB1 and dsB2, they have both 3' and 5' termini identical to the Q-CMV RNA2. In addition, with regard to their sizes and in comparison with the sequence of RNA 2, it appears that some internal sequences of dsB1 and dsB2 are missing as a consequence of deletion. In the case of dsB3, the sequences obtained correspond to the first 667 nucleotides of Q-CMV RNA2. Moreover, hybridisation of probes (corresponding to the 3' and 5' ends of Q-CMV RNA2) to dsB3 confirmed that the dsB3 fragment also has at least some sequence similarity to the 3' and 5' ends of Q-CMV RNA2 and most likely it is similar to the dsB1 and dsB2, in that the 3' and 5' termini are conserved and possibly a deletion has occurred in the 3'-terminal region.

- It is feasible that these minor dsRNAs with intact 5' and 3' termini are replicable molecules because they maintain the putative promoter elements that are recognised by the viral polymerase.

• Taken together, these results revealed that these molecules are derived from Q-CMV RNA2 retaining the 5' and 3' terminal sequences. Moreover due to the deletion of some sequences they are smaller than full length RNA2. In addition, although drawing the final conclusion requires full length sequences of the dsRNA molecules, on the basis of present data their structures are similar to the "internal deletion" class of DI RNAs (Lazzarini et al., 1981; Perrault, 1981). These molecules have also been termed 5'-3' by Nayak et al. (1985) since both the 5' and 3' terminal sequences are retained.

This type of deletion is found in DI RNAs derived from several plant viruses. Similar to the dsB1, dsB2 and dsB3, in broad bean mottle bromovirus, which like CMV has a tripartite RNA genome (and both belong to the Bromoviridae family), there is a DI RNA derived by a single in-frame central deletion of 448 to 537 nt in the corresponding genomic RNA 2 (Romero et al., 1993). Similarly, tomato spotted wilt tospovirus and cymbidium ringspot virus support the replication of DI RNAs, the sequence of which are derived from genomic RNA, and both 5' and 3' termini are conserved (Burgyan et al., 1991). In conclusion, it is possible that the minor dsRNAs are internally deleted versions of the full length CMV genomic RNA2.

Chapter 5

Characterisation of New ss-RNAs Associated with Cucumoviral Infection

5.1 Introduction

The previous chapters showed that three additional ds-RNA bands were consistently seen in the ds-RNA profile of the CMV infected plants. Sequence analysis showed that the 3' and 5' terminal sequences of these ds-RNA fragments are largely identical to the same region of RNA2. Hence, it was important to see if there are additional RNAs in the ss-RNA profile of CMV infected plants.

Six ss-RNA species which are associated with replication and gene expression of the Q-CMV RNA genome have been fully characterised (Paulukatis et al., 1992; Blanchard et al., 1996). These six RNAs that are encapsidated inside the virions, include three genomic RNAs (RNAs 1, 2 and 3), two subgenomic RNAs (RNAs 4 and 4A) and RNA 5. Recently, two RNAs were added to the previous CMV RNA profile. Graves and Roossink (1995) characterised two defective RNAs in the Fny-CMV strain, 3α and 3ß. Sequence analysis revealed that these two RNAs are derived from RNA3 by a single in-frame deletions (159 nucleotides for 3α and 309 nucleotides for 3ß) within the ORF 3a. These defective RNAs had no effect on their helper virus yield or symptom induction although an in vitro translational product of one of them (3ß) was detected.

Our data indicated that there are several additional ssRNA species associated with Q-CMV infection but which are apparently not encapsidated inside the virions (Ding *et al.*, 1995a). The nature of these minor ss-RNAs (which in this thesis are referred to as TB1, TB2 and TB3 in descending order) is not clear. Similar ssRNA bands are present in two other cucumoviruses, V-TAV and a subgroup I strain of CMV (Bu-Jun Shi, 1997).

The aims of this chapter were to find out whether these ss-RNAs are derived from the CMV genome? Secondly, if they are CMV related molecules, what is their structure?

5.2 Methods and materials

5.2.1 Extraction of total RNA from CMV infected plants

Total RNA was extracted from cotyledons of cucumber and systemic leaves of *Nicotiana glutinosa*, 4 to 5 days post inoculation as described by Verwoerd et al. (1989) (Section 2.2.5). After resuspension of the final pellet in 0.1 mM EDTA (pH 7.0), the integrity of the RNA was checked by agarose gel electrophoresis and ethidium bromide staining (Section 2.2.16).

5.2.2 DNase treatment of total RNA

The total RNA was digested with RNase-free DNase (RQ-1 grade, Promega) before isolation of the target RNAs. Total RNAs (50 μg) were treated with DNase I (1U/3 μg RNA) in a 100 μl reaction volume containing 50 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, 200 U rRNasin (Promega) at 37°C for 30 minutes followed by extraction with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1). RNAs were recovered by precipitation with 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol. The RNA pellet was washed with 70% ethanol and, after vacuum drying for 5 minutes, it was resuspended in distilled water.

5.2.3 Northern blot analysis

Virion and total plant RNAs were electrophoresed in low-formaldehyde agarose gels and transferred to membranes as described in Chapter 2 (Section 2.2.20). The membranes were subjected to northern blot analysis using the following probes. (i) Probe D with sequence complementary to the 3' terminal 340 nucleotides of Q-CMV RNA2. (ii) Probe H corresponds to the 2366-2579 nt of RNA1, (iii) Probe B

corresponds to the 14503-2049 nt of RNA 2, (iv) Probes I and K correspond to the nt 239-535 and 1523-1845 of RNA3 respectively, (v) Probe A hybridises to the first 295 nt of CMV-RNA2. All probes except probe A were riboprobes prepared as described in Section 2.2.19. Probe A was prepared by random priming of a cDNA clone as in Section 2.2.21. After overnight prehybridisation and hybridisation at 42°C, the membranes were washed as follows: twice in 2xSSC, 0.1% SDS at room temperature for 5 minutes; 2xSSC, 0.1% SDS for 20 minutes at 60°C and a final wash in 0.2xSSC, 0.1 SDS at 65°C for 30 minutes. Autoradiography was carried out by overnight exposure to X-ray film at -80°C using an intensifying screen.

5.2.4 Isolation of RNA fragments from formaldehyde-containing agarose gels

Total plant RNAs extracted from CMV-infected plants were separated in a 1.2% formaldehyde agarose gel (Section 2.2.20) while CMV virion RNAs were run in parallel sides as size markers. Isolation of target RNA fragments was carried out using the RNaid Kit (BIO 101) according to the manufacturer's directions as outline below. Briefly, 20 µg of purified virion RNAs were heated at 65°C for 10 minutes in the presence of 50% deionised formamide, 29.4% formaldehyde, 1X gel buffer (200 mM MOPS, pH 7.0; 10 mM EDTA, pH 7.0; 10 mM NaOAc, pH 5.2) and 0.02 µg/µl ethidium bromide and then loaded on a 1.2% agarose gel containing 6.6% formaldehyde and 1X gel buffer. After electrophoresis (5V/cm for three hours) and ethidium bromide staining, by considering the RNAs 3 and 4 of CMV in the virion RNA lane, the similar region in the total RNA lanes, were divided into three parts and the gel slices containing individual desired RNAs were excised and transferred to 1.5 ml Eppendorf tubes. After adjusting the pH of the gel slices to pH 5.0 by adding 0.02% ACETIC ACID, 3 volumes of RNA BINDING SALT were added and the tubes incubated at 37°C for 10 minutes with occasional mixing. When the gel slice was completely liquefied, 1-2 µl of RNA MATRIX™ was added per µg of RNA and incubated at room temperature with periodic mixing for a further 10 minutes. Pellets of isolated RNAs were collected by centrifugation for 1 minute in an Eppendorf

microcentrifuge at 14000rpm and resuspended in 500 µl RNA WASH solution. After centrifugation and the removal of all traces of liquid, the RNA pellets were resuspended in 10-20 µl of autoclaved MQ water per 5 µl RNA MATRIX™. RNAs were eluted by incubation at 80°C for 10 minutes followed by centrifugation for 1 minute at 14000 rpm. The eluted RNAs were heated at 80°C for another 10 minutes to further dissociate residual formaldehyde from RNA.

5.2.5 RT-PCR amplification of the minor ssRNAs

Table 5.1 lists the primers used for RT-PCR. These primers were designed either to amplify the full-length cDNA sequence of CMV-RNA2 (Ding et al., 1995a) or to give partial-length PCR products.

The first strand cDNA synthesis from the isolated minor RNAs (5 µl; Section 5.2.4), was carried out using either primers SD6 or SD61 (Table 5.1) to amplify nt 2692-3035 in a 20 µl reaction volume as described before (Section 2.2.9). PCR was performed using Taq DNA polymerase (Bresatec or Biotec International Ltd.) and the following primer pairs : primers SD6 and SD9 to amplify nt 1-3035; SD6 and SD53 to amplify nt 2354-2376; SD9 and H32 to amplify nt 1- 1923; SD9 and AF2 to amplify nt 1- 1750 and SD9 and AF3 to amplify nt 1-1705 of CMV-RNA2. The amplification reactions were set up in 0.5 ml microfuge tubes as mentioned earlier (Section 2.2.10) and carried out in an MJ minicycler (MJ Research Inc., USA, Model PTC 150) using the following program: 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, for 30 cycles followed by a final elongation step at 72°C for 5 minutes. Reaction products were resolved by electrophoresis on 1.0% agarose 1xTAE gels and visualised with ethidium bromide/UV.

Southern hybridisation analysis of the PCR products using a ^{32}P-dCTP labelled probe, which corresponds to the 2b gene region of Q-CMV RNA 2, was performed as described before (Section 2.2.22). Membranes were washed after overnight hybridisation at 65°C as follows: twice in 2xSSC, 0.1% SDS at room temperature for 5

minutes; twice in 0.2xSSC, 0.1%SDS at 65°C; this wash was continued until the temperature reached room temperature. Autoradiography was carried out by overnight exposure to X-ray film.

Table 5.1 *Primers used for RT-PCR studies and location on Q-CMV RNA 2*

Primer	Primer sequence	Primer position
SD6	5'- ACGGATCCTGGTCTCCTTATGGAGAACCTGTGG -3'	Q-CMV RNA2 nt 3035-3010
SD9	5'- GTTTATTCTCAAGAGCGTATGG -3'	Q-CMV RNA2 nt 1-22
SD53	5'- GTTTTGTATATCTGAGTTCCGGGT -3'	Q-CMV RNA2 nt 2354-2376
SD61	5'- ATCAGCTAGCAAACGACCCTTCGGCCCA -3'	Q-CMV RNA2 nt 2709-2692
H30	5'- TGATGTAACAGGAGCTGCAGTGACGGTTTG -3'	Q-CMV RNA2 nt 314-285
H31	5'- CTTTTCTGGTAGCGATTCTCTGGCC -3'	Q-CMV RNA2 nt 1901-1923
H32	5'- GGCCAGAGAATCGCTACCAGAAAAG -3'	Q-CMV RNA2 nt 1923-1901
AF1	5'- AGAGCACATTCTCAACGATCTTGGT -3'	Q-CMV RNA2 nt 1682-1706
AF2	5'- AACCTATGAAAGTCACACCACCAT -3'	Q-CMV RNA2 nt 1750-1727
AF3	5'- CCAAGATCGTTGAGAATGTGCT -3'	Q-CMV RNA2 nt 1705-1684

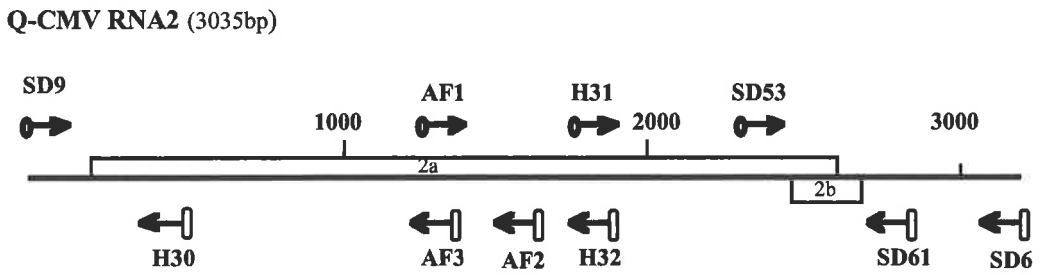

Fig. 5.1 Positions and polarities of the primers used for amplification of the minor RNAs as compared to Q-CMV RNA2.

5.2.6 Cloning and sequencing

The major PCR products obtained in Section 5.2.5, were excised from agarose gels after electrophoresis and purified using a Qiagen II kit before cloning into the pGEM-T vector (Promega). Ligation was carried out in a 10 µl volume with 50 ng of vector DNA

and the DNA insert fragment in a molar ratio of 1:3 and 1:5, respectively, using conditions suggested by the manufacturer (Promega). Transformation of bacteria with plasmids was performed by electroporation, using the *E. coli* strain DH5α and the Gene-Pulser (Biorad, USA) as described before (Section 2.2.13). Clones were identified by dideoxy sequencing on double-stranded templates as described in Chapter 2 (Section 2.2 18).

5.2.7 Amplification of the 5'-ends of the minor ssRNAs using RACE-PCR

For amplification of the 5'-ends of the minor ssRNAs, the RACE-PCR method (Frohman, 1990; see Fig. 4.2 for a schematic diagram of the RACE procedure) was used. 5 µl of isolated minor ssRNAs (TB1, TB2 and TB3; as in Section 5.2.4) were annealed to 6 pmoles of the SD61 (complementary to residues 2692-2709 of the Q-CMV RNA2) by heating at 80°C for 2 minutes and snap-cooling on ice for 1 minute. The rest of the method including reverse transcription, poly (dA) tailing of cDNAs and PCR amplification were carried out as described in Section 4.2.3b. The single major reaction product was resolved by electrophoresis on a 2% agarose gel and blunt-end cloned into the *EcoRV* site of pGEM Z 5(-) as described in Section 4.2.4 b. The viral cDNA inserts of obtained clones were sequenced as described in Section 2.2.18.

5.3 Results

5.3.1 Three novel ss-RNA species found in Q-CMV infected plants

Probe D was used in northern blot hybridisation to determine whether the minor ssRNAs are related to CMV. Probe D, transcribed from pSP72Q-3', is complementary in sequence to the 3' terminal 340 nt of Q-CMV RNA 2 and was expected to hybridise to all Q-CMV RNAs that contain the 3' conserved region. This probe detected six major RNA species (Fig. 5.2; RNAs 1, 2, 3, 4, 4A and 5) both in total RNAs extracted from Q-CMV infected plants (lane 1) and in the encapsidated viral RNAs (lane 3). However in the infected total RNA lane the probe hybridised to three additional minor

RNAs between RNAs 3 and 4 (Fig. 5.2, lane 1). These molecules were not detected in the uninfected control (Fig 5.2, lane 2).

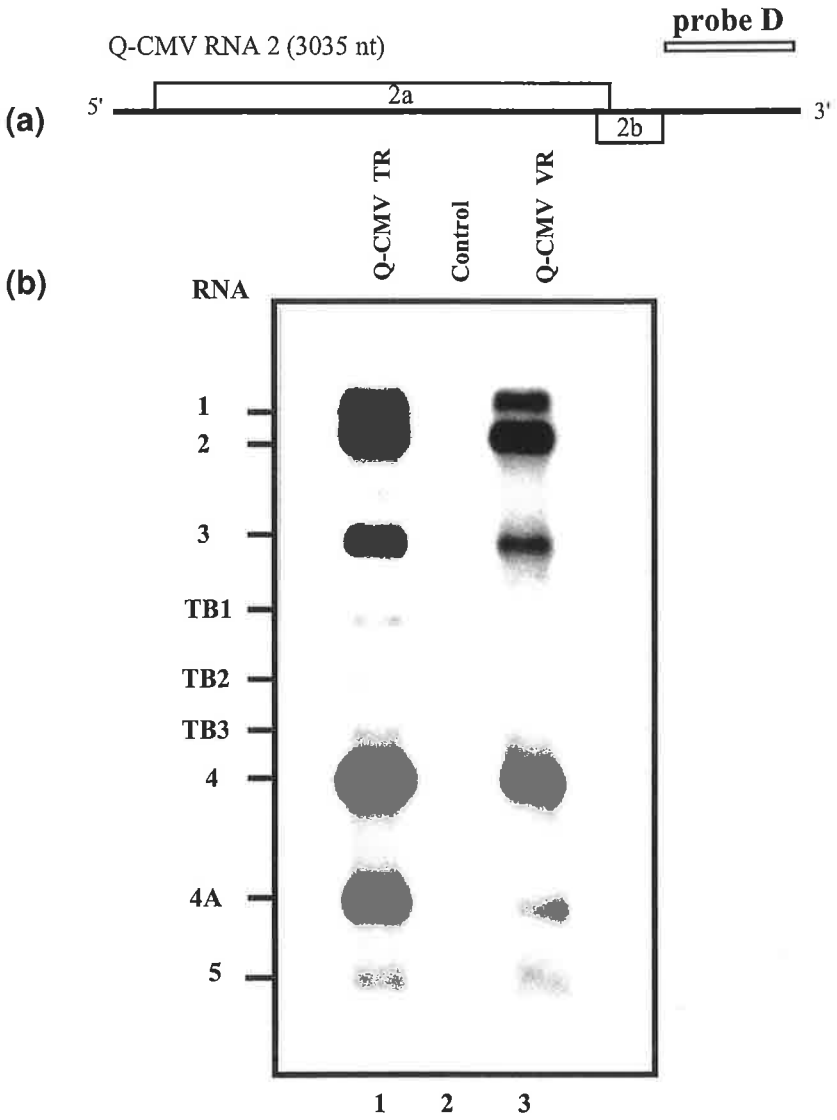

Fig. 5.2 Presence of three additional minor RNAs in total RNAs of Q-CMV infected plants. (a) Position of the positive -strand-specific probe used for northern blot hybridisation . Probe D corresponds to the 3'-terminal 340 nt of Q-CMV RNA2. **(b)** Northern blot analysis of viral RNAs of CMV. Total RNAs extracted from *N. glutinosa* inoculated with Q-CMV (lane 1), sterile water (lane 2) and virion RNAs of Q-CMV (lane 3). The positions of viral RNAs 1, 2, 3, 4, 4A, and three new minor RNAs, TB1, TB2 and TB3 which were detected in total RNAs extracted from Q-CMV infected plants, are indicated.

These results indicated that these three RNA species are derived from Q-CMV and that they contain the 3'-end sequences shared by all known Q-CMV RNAs.

The sizes of the three RNAs was determined by northern analysis under denaturing conditions and were found to be approximately 1800 (TB1), 1400 (TB2) and 1200 nt (TB3) respectively.

Such RNAs were hardly detectable in the virion RNA lane (Fig. 5.2 lane 3) although in longer exposure of the northern membranes they are visible, indicating their inefficient encapsidation.

Furthermore, in order to elucidate the relationship of the new RNAs with Q-CMV genomic RNAs, northern blot analysis was performed using specific probes for RNAs1, 2 and 3. The results indicated that the RNA 2 specific probe, which corresponds to 1403-2094 residues of CMV-RNA2, hybridised to all three target RNAs (Fig. 5.3). However, the RNA1 specific probe only picked up genomic RNA1 and did not hybridise to the new ssRNAs (Fig. 5.4 lanes 1 and 2). Similarly, the RNA 3 specific probe did not hybridise to the minor ssRNAs (Fig. 5.5). In addition, a probe (A; Fig. 5.6 a) which corresponds to the first 219 nt of the Q-CMV RNA2 was used to examine the 5'-terminal sequences of the minor ssRNAs. Northern analysis using total RNAs extracted from healthy and Q-CMV infected plants showed that the three minor ssRNAs, TB1, TB2 and TB3, hybridised strongly with probe A (Fig. 5.6 b), indicating that the minor RNAs share some sequence homology with the 5'-end of Q-CMV RNA2.

In conclusion, the additional RNAs share sequence homology only with RNA 2 and no hybridisation was found with other genomic specific probes. Moreover these RNAs have at least some sequence similarities with both 3' and 5' termini of CMV-RNA 2.

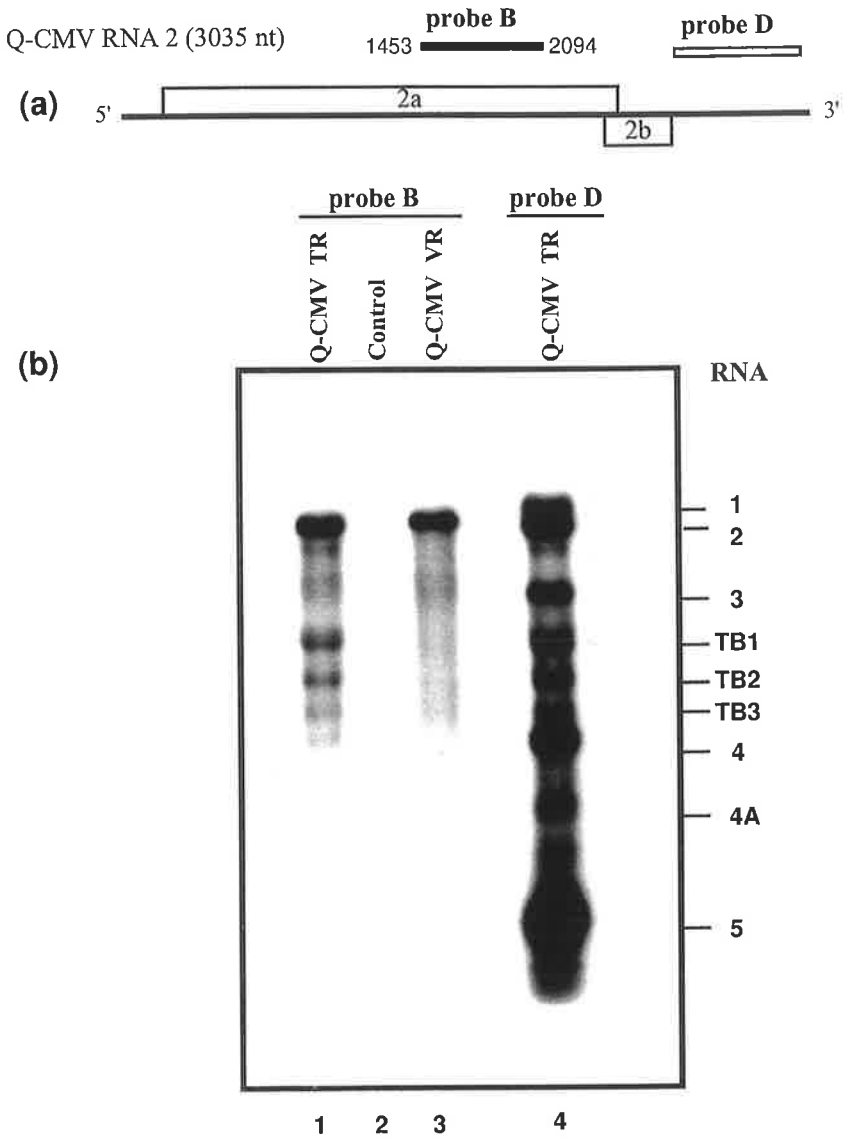

Fig. 5.3 The three minor ssRNAs are related to Q-CMV RNA 2.

(a) Position of the positive-strand-specific probe used for northern blot hybridisation . Probe B is a Q-CMV RNA2 specific probe and probe D corresponds to the 3'-terminal 340 nt of Q-CMV RNA2. **(b)** Northern blot analysis of viral RNAs of CMV. Total RNAs extracted from *N. glutinosa* inoculated with Q-CMV (lane 1), sterile water (lane 2) and virion RNAs of Q-CMV (lane 3). The positions of viral RNAs 1, 2, 3, 4, 4A, and three new minor RNAs, TB1, TB2 and TB3 which were detected in total RNAs extracted from Q-CMV infected plants, are indicated.

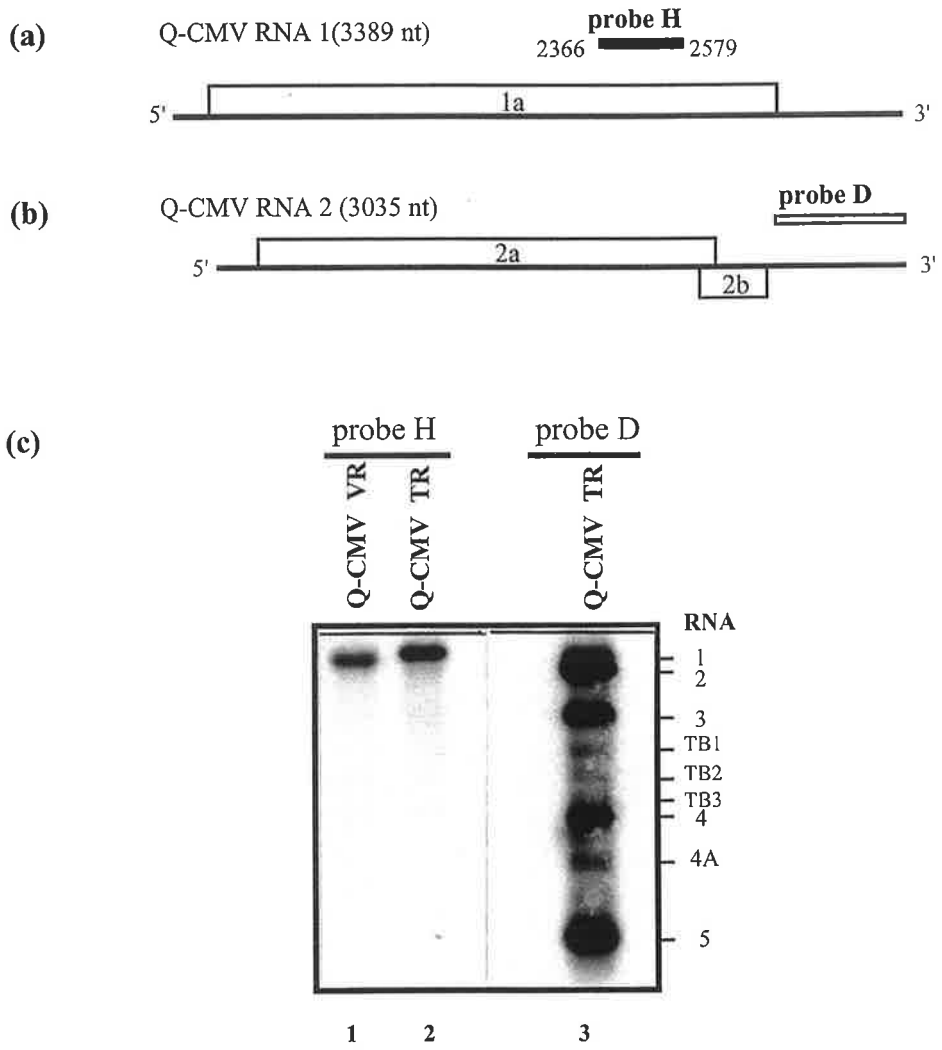

Fig. 5.4 Northern blot analysis of the minor ss-RNAs with QCMV RNA 1 specific probe. **(a)** and **(b)** positions of the specific probes used for northern blot analysis. Probe H is correspond to nt 2366-2579 of RNA 1 of Q-CMV **(a)**, while probe D **(b)** is correspond to the last 340 nt of QCMV-RNA2. **(c)** Northern blot analysis of CMV viral RNAs. Q-CMV virion RNAs (lane 1) and total RNAs extracted from cucumber plants inoculated with Q-CMV (lanes 2-3) were hybridised with probe H (lanes1-2) or probe D (lane 3). The positions of viral RNAs1, 2, 3, 4, 4A, 5 and the three minor RNAs, TB1, TB2 and TB3 are indicated.

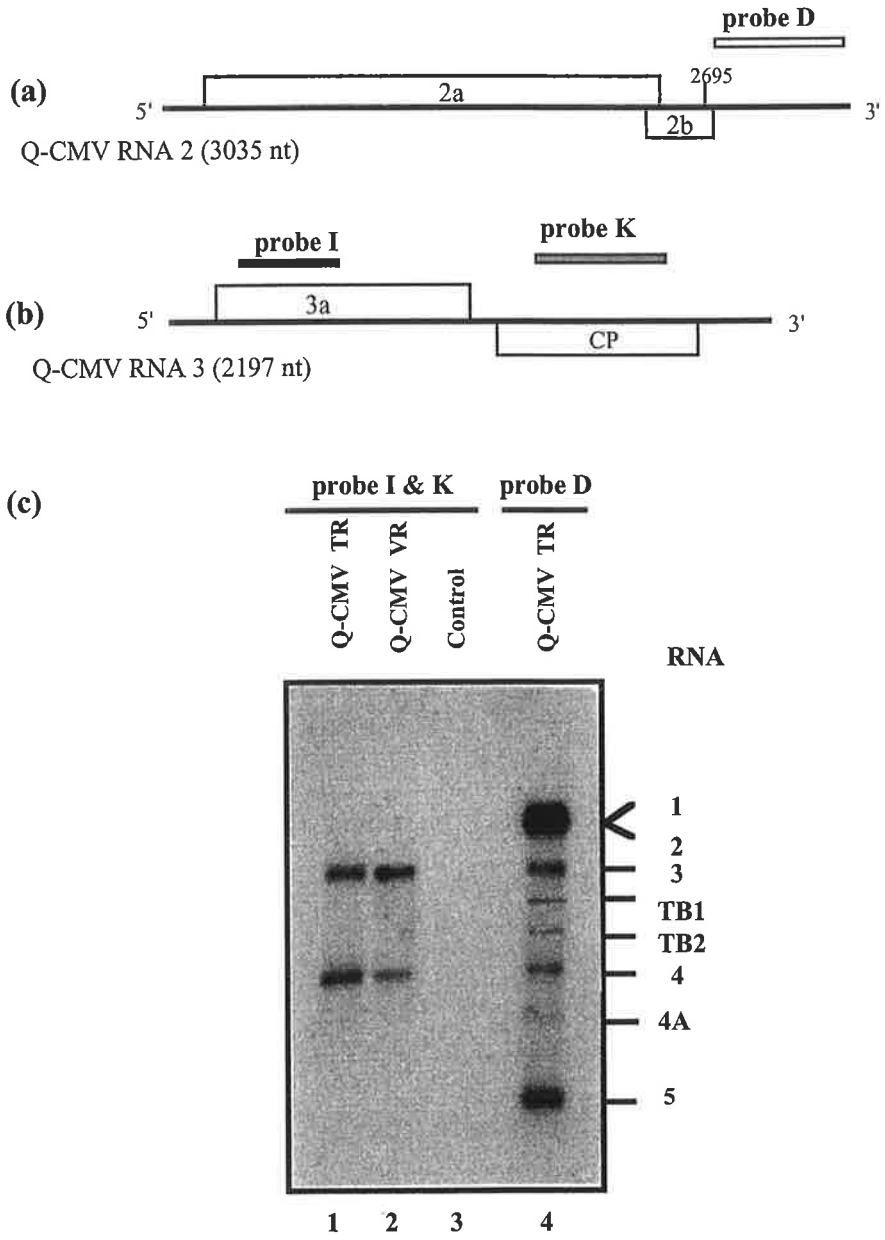

Fig. 5.5 Northern blot analysis of the minor ss-RNAs with QCMV RNA 3 and 4 specific probe. **(a)** and **(b)** positions of the specific probes used for the analysis. **(a)** Probe I and K are correspond to nt 239-535 and 1523-1845 of Q-CMV RNA 3 respectively. **(b)** probe D is correspond to the last 340 nt of QCMV-RNA2. **(c)** Northern blot analysis of CMV viral RNAs. Total RNAs extracted from cucumber plants inoculated with Q-CMV (lanes 1 and 4), sterile water (lane 3) and Q-CMV virion RNAs (lane 2), were hybridised with probe I and K (lanes1 to 3) or probe D (lane 4).

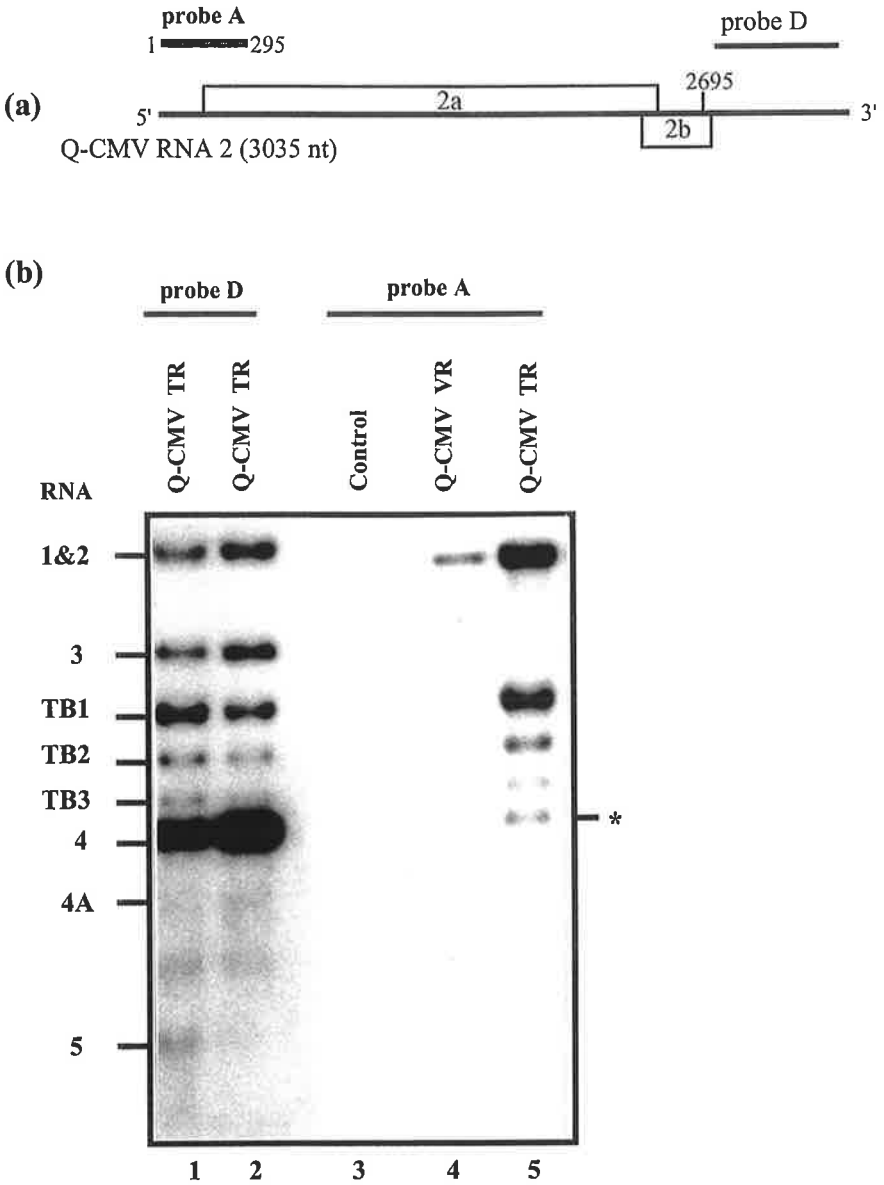

Fig. 5.6 Northern analysis of Q-CMV total RNA with RNA 2 5'-end probe.

(a) Position of the positive-strand-specific probes used for northern blot hybridisation . Probe A corresponds to the first 295 nt of Q-CMV RNA 2 and probe D corresponds to the 3'-terminal 340 nt of Q-CMV RNA2. **(b)** Northern blot analysis of viral RNAs of CMV. Total RNAs extracted from *N. glutinosa* inoculated with Q-CMV (lanes 1, 2 & 5), sterile water (lane 3) and virion RNAs of Q-CMV (lane 4). The samples were hybridised to probe D, (lanes 1 & 2) or probe A, (lanes 3 to 5). The positions of viral RNAs 1, 2, 3, 4, 4A, and three new minor RNAs, TB1, TB2 and TB3 which were detected in total RNAs extracted from Q-CMV infected plants, are indicated. *The identity of the band bellow TB3 in lane 5 are not clear.

5.

5. 3.2 Primary structure of TB1, TB2 and TB3

For further analysis, the minor ssRNAs were isolated from infected total RNAs (as in Section 5.2.4) and their purities were checked subsequently by northern analysis using a Q-CMV RNA 2 specific probe (probe B; Fig. 5.7 a). Probe B, which is complementary to nt 1453-2094 of Q-CMV RNA2, is capable of hybridising to only Q-CMV RNA 2. The presence of three discrete bands (Fig. 5.7 b) implied that the minor ssRNAs were isolated efficiently and that they were not contaminated with other Q-CMV RNA species. At the next step, each of the isolated minor ssRNAs (Section 5.2.4) were used for amplification using the RT-PCR method (Section 5.2.5).

a. TB1. PCR reaction with SD6 /SD53 (Table 5.1) primer pairs produced a major product of 680 bp (Fig. 5.8 lane 2). However, several attempts to amplify TB1 with the SD6/SD9 primer pairs for production of full length DNA or with SD9/H30, SD9/H32, SD9/SD33, SD9/AF2 and SD9/AF3 primer pairs for getting partial length amplifications were unsuccessful (data not shown).

b. TB2. Using first strand cDNAs synthesised with SD6 as primer for subsequent PCR reaction with the SD6/SD53 primer pair, a major product of 680 bp (Fig. 5.8 lane 3) was produced, but attempts at amplification of TB2 with a series of primer pairs including SD9/H30, SD9/SD33, SD9/AF2 and SD9/AF3 were not successful (data not shown)..

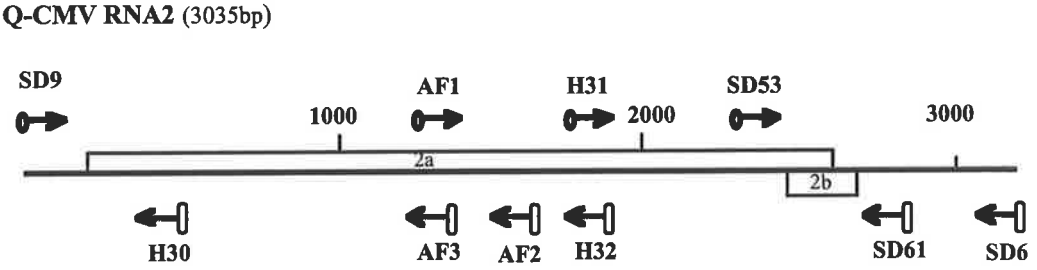

Fig. 5.1 Positions and polarities of the primers used for amplification of the minor RNAs as compared to Q-CMV RNA2.

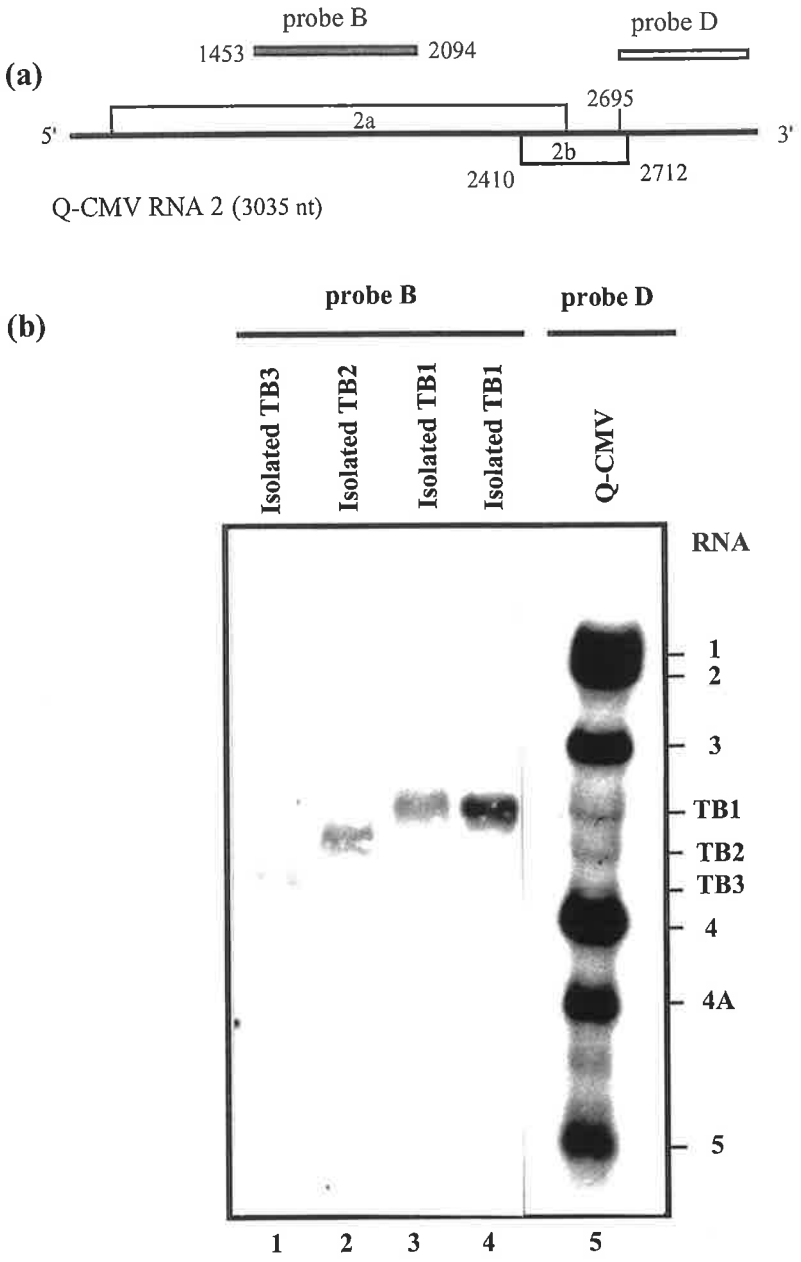

Fig. 5.7 Analysis of purified minor ssRNAs by agarose gel electrophoresis. **(a)** Position of the positive-strands specific probes used in the northern blot hybridisation. probe B and D are correspond to nt 1453-2094 and the last 340 nt of the Q-CMV RNA2. **(b)** Northern blot analysis of isolated minor RNAs. the samples were, isolated TB3 (lane 1), TB2 (lane 2), TB1 (lanes 3 & 4) and Q-CMV total RNAs extracted from infected plant (lane 5), which were hybridised with probe B (lanes 1-4) or probe D (lane 5).

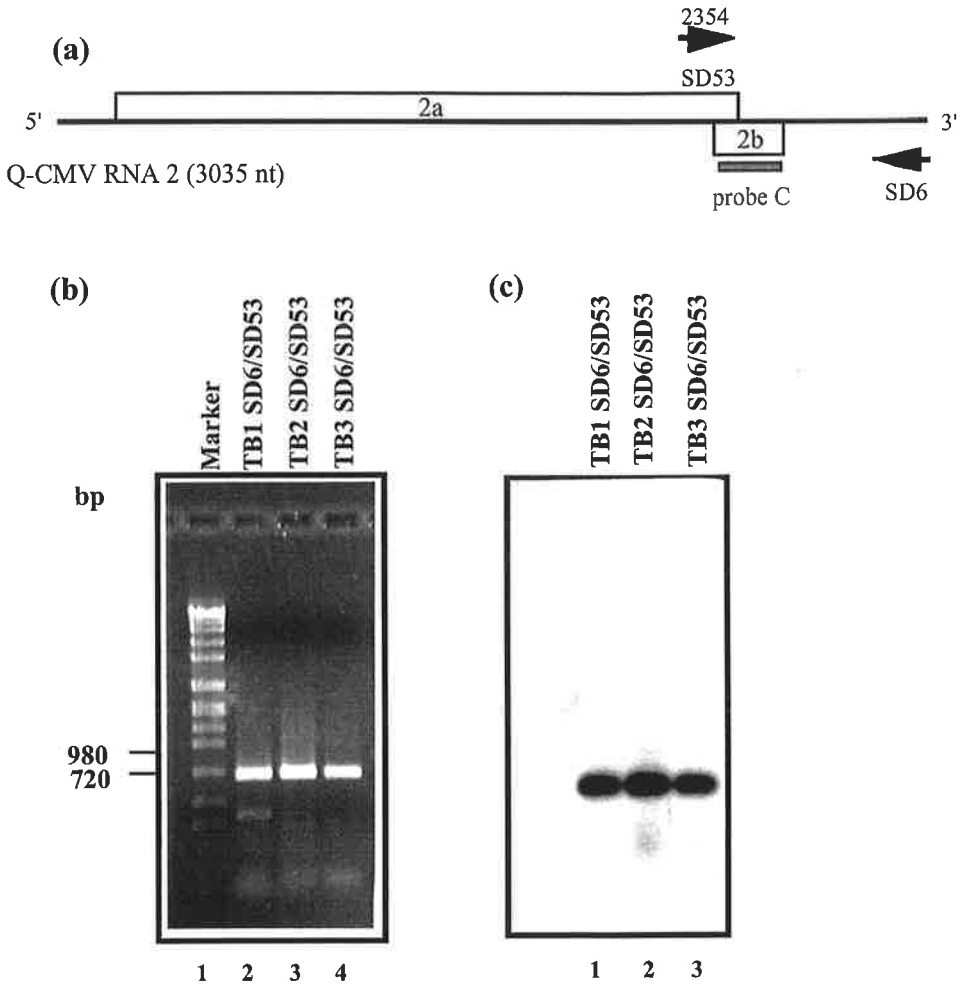

Fig. 5.8 RT-PCR amplification of the 3' region of the minor ssRNAs.
(a) Primers binding sites in relation to the Q-CMV RNA 2. The arrowheads indicate the positions and polarities of the primers. **(b)** PCR amplification of 3'-ends of the minor RNAs. The isolated minor ss-RNAs were amplified using SD53/SD6 primer pair and the parameters given in Section 5.2.5. Samples were, purified TB1 (lane 2), TB2 (lane 3) and TB3 (lane 4). The expected size of the PCR product was 680 bp and the DNA size markers were SPP-1/ *Eco R1* fragments. **(c)** Southern hybridisation analysis was carried out on the PCR products using probe C (specific to the Q-CMV 2b gene region) as indicated in **(a)**.

c. TB3. PCR reaction using TB3 first strand cDNA and the SD6/SD53 primer pair generated a major product of 680 bp (Fig. 5.8 lane 4) while further attempts for amplification of TB3 with other primer pairs (SD9/H30, SD9/SD33, SD9/AF2 and

SD9/AF3) did not produce any products (The binding site of all primers is given in Table 5.1).

With the same PCR conditions and primer pairs (Section 5.2.5), isolated Q-CMV genomic RNA2 (as in Section 5.2.4), was used as a positive control and, produced the expected fragment sizes, indicating that the PCR conditions were optimised (data not shown).

The identity of the above PCR products were confirmed by Southern hybridisation analysis (Fig. 5.8 c) using ^{32}P-labelled Q-CMV 2b specific probe C which corresponds to the Q-CMV 2b gene region (Fig. 5.8 a). Sequence analysis of the cloned PCR products of TB1, TB2 and TB3, (Fig. 5.11) revealed that the 3'-terminal sequences of the minor ssRNAs are identical to the same region of RNA 2.

5'-End amplification of TB1 TB2 and TB3 using RACE-PCR and RACE5/H30 primer pair (Section 5.27) produced a major band of equal size (300 nt) to that produced with Q-CMV RNA2 (Fig. 5.9). The PCR product of TB1 was cloned (Section 4.2.2 b) and sequenced by the dideoxy sequencing method as described in Chapter 2 (2.2.18). The results of sequencing revealed that the 5' terminal sequence of TB1 is identical to that of RNA 2.

The reason for the failure to amplify full length TB1, TB2 and TB3 is unknown. The nucleotide sequence in the 5' region may be sufficiently different from the published sequence to prevent primer hybridisation during RT-PCR. A stable secondary structure in the RNA template which lead to premature chain termination can be another cause of problems in reverse transcription and subsequent PCR.

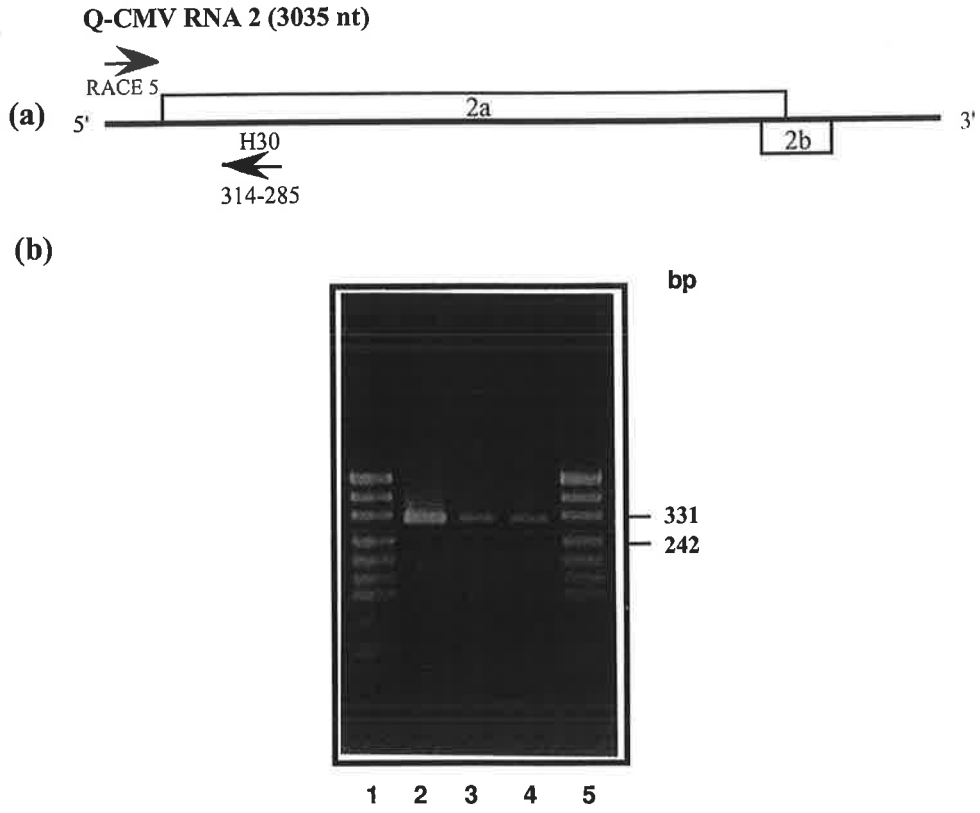

Fig. 5.9 RACE-PCR amplification of the 5'end of the minor ds-RNAs.
(a) The arrwheads indicate the positions of the primers (RACE 5/H30) used in RACE-PCR compared to the Q-CMV RNA2. **(b)** RACE-PCR was carried out on the isolated TB1 (lane 2), TB2 (lane 3) and TB3 (lane 4). Amplification was carried out using the H30 as the specific primer and RACE 5 (5'-GACTCGAGATCGA$[T]_{17}$-3'), as the non-specific primer. The expected size of the PCR product was 314 bp. The DNA size markers (lanes 1& 5) were pUC 19/*HpaII* fragments.

5.4 Discussion

5.4.1 Possible structure and method of production

5.4.1.1 Defective RNAs

(1) The results of sequencing and northern hybridisation analysis obtained in this chapter (Sections 5.3.1; 5.3.2; Fig. 5.10) revealed that the first 300 nucleotides (5'-end) and the last 680 nucleotides (3'-end) of TB1 are identical to the same regions of RNA 2. Similarly the 3' terminal sequences of TB2 and TB3 are identical to the 680 nucleotides of the CMV RNA 2 and although due to the time limitation, 5'-RACE products of the

TB2 and TB3 were not sequenced but with regard to the strong hybridisation signals in northern analysis (Fig.5.6 b) with probe A (Fig. 5.6 a; complementary to the 5' terminus of CMV-RNA2), it can be concluded that the 5' termini of TB2 and TB3 most likly contain the first 295 nucleotides of CMV RNA 2.

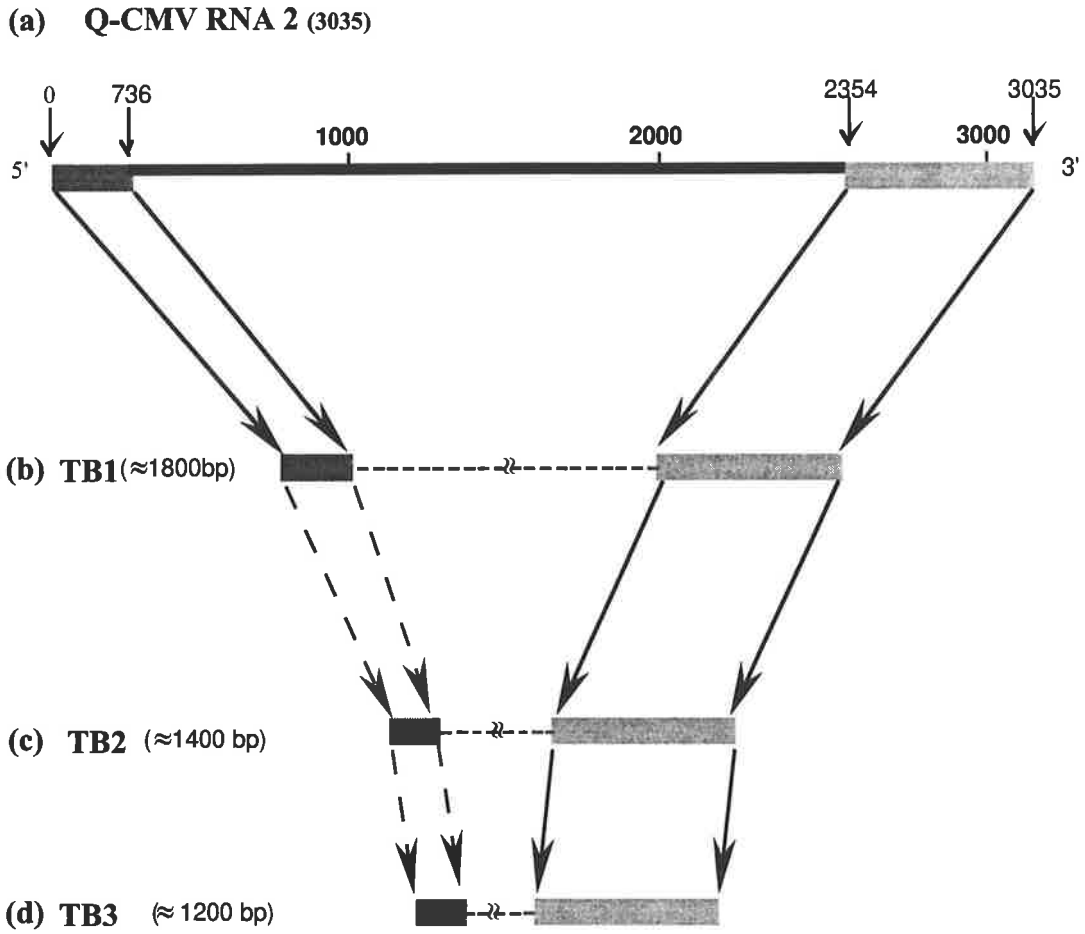

Fig. 5.10 Shematic representation of Q-CMV genomic RNA2 and the minor ss-RNAs associated with Q-CMV infected plants. Q-CMV RNA 2 is shown in (a) with gray boxes which indicate the positions of the amplified regions of the minor RNAs. Amplified regions of the TB1 (b), TB2 (c), and TB3 (d), are represented below as shaded blocks , non-amplified regions depicted as dashed lines and (≈) indicate the possible deletion sites.

In addition, the results showed that the partial amplification of new RNA species using AF3/SD9, AF2/SD9, H32/SD9 for the 5' half and AF1/SD6, H31/SD9 primer pairs for the 3' half (data not shown) were also not successful. However, with regard to the amplification of the positive control (isolated RNA2) with all mentioned primer pairs, it is feasible that there were no hybridisation sites for the mentioned primers. In other words with regards to the PCR products and hybridisation studies, it is also possible that TB1,TB2 and TB3 represent partially deleted molecules, lacking a different number of nucleotides in the middle of RNA 2 (Fig. 5.10).

In conclusion, the results showed that the minor ss-RNAs have identical 3' and 5' termini to the same regions of Q-CMV RNA 2 (indicating that the 3' and 5' terminal sequences of Q-CMV RNA 2 are conserved in the minor ssRNAs) and that their sizes are shorter than RNA2. Taken together these results implied that some deletions occurred in the central region of the RNAs. These features are similar to those of the "internal deletion " class of defective RNAs (Lazzarini et al., 1981). In this type of DI RNAs which have been identified in many animal and plant RNA viruses, both 3' and 5' termini are retained while a portion of the central region was deleted. Moreover, the presence of replicable ds-RNAs in the CMV infected plants, which were identified in chapter 4, support the above conclusions.

(2) How are the additional RNAs produced? The polymerase "copy choice" originally proposed by Huang (1977) is the generally accepted mechanism for defective RNA formation (Holland, 1990; Graves and Roossinck, 1995). It is suggested that DI RNA produced as a result of viral replicase "leaping" or skipping from one viral RNA template to another or from one segment of a template to another. During this leaping the RNA replicase carries the incomplete nascent strand to a new template or template segment, then uses this strand as a primer to start chain elongation at the new template site (Roux et al., 1991). Although present data favour replicase leaps as the major mechanism for the generation of DI particles, other mechanisms such as aberrant splicing events might be involved (Roux et al., 1991).

In the copy choice model the sequences of junction sites are of vital importance since local complementarities and the secondary structure of the replicating RNA, can induce the template switching (Bujarski and Dzianott, 1991; Resenada et al., 1992). In a DI RNA from BBMV (which was derived from genomic RNA 2 by a central deletion) it was found that stable stem-and-loop elements could be folded at both deletion borders while the border sequences could form intramolecular double-stranded regions. This could bring the RNA 2 sequences together and facilitate internal RNA polymerase switches (Romero et al., 1993). So, the copy choice mechanism also explains why only DI RNAs of the 5'-3' type seem to be generated (Resenda et al., 1992).

Further studies toward the understanding of the mechanisms involved in the generation and evolution of DI RNAs suggested that the formation of these molecules in some viruses do not occur as a single event but instead arise as the consequence of stepwise deletions of genomic RNAs. In other words, in these cases the smaller DIs may be derived from the larger ones and the larger DIs act as an intermediate for the formation of smaller ones by internal deletion (White and Morris, 1994; Burgyan et al., 1991).

The resolution of this puzzle clearly requires complete sequences of the minor ssRNAs. Since the minor ss-RNAs show different sizes, while retaining similar 3' and 5' termini (300 and 680 nt respectively), it is possible that the smaller RNAs are generated from CMV RNA 2 by a series of progressive deletions.

5.4.1.2 Degradation products

Harsh treatment of nucleic acids and releasing a large amount of host-ribonucleases during the isolation of nucleic acids from the cells, could cause breaks in the nucleic acids and especially in the RNA molecules. This reaction may lead to the production of less-than full length RNAs or dead end products (Dougherty, 1983; Palukaitis et al., 1983). Tenllado et al. (1995) solved the problem by using a lysis buffer with less cell

disruption and denaturation properties (guanidinium hydrochloride instead of guanidinium isothiocyanate). We also tried guanidinium hydrochloride for extraction of total RNA from CMV infected plants. However, northern hybridisation analysis revealed that the minor ss-RNAs were still present (data not shown).

In conclusion, it is possible that the minor RNAs could be a population of degraded fragments with a mixture of 5'-terminal and 3'-terminal pieces but of similar sizes. However, since these RNAs (minor ds-RNAs and ss-RNAs) were consistently present with a constant and reproducible pattern (in terms of size), specific deletion reactions of some kind should be involved. Specific 3'-terminal ribonuclease degradation products have been observed in other viruses (Dasgupta and Kaesberg, 1977; Florentz et al., 1982). Specific degradation of RNAs 2 and 3 was also suggested as a possible mechanism for the production of RNA 5 of CMV (Blanchard et al., 1996).

5.4.2 Possible function

(1) DI RNAs are replicative entities and usually compete with the helper virus; however, their effect on the accumulation of the helper virus and induction of symptoms are different. Many defective RNAs such as DI RNAs of cymbodium ring spot virus (Burgyan et al., 1989), soil-born wheat mosaic virus (Chen et al., 1994) and turnip crinckle virus (Li et al., 1989) can affect helper virus replication and symptom production. However, in some cases like defective RNAs of Fny-CMV, although they are packaged in the virion at the same level, apparently they do not affect the replication of the helper virus and symptom expression of the virus (Graves and Roossink , 1995).

Defective RNAs exert various effects on plant disease symptoms, causing reduction of symptoms (e.g., cymbidium ring spot virus on *Nicotiana clevelandii)* (Burgyan et al., 1991), no effect on symptoms (e.g., clover yellow mosaic virus on broad bean) (White et al., 1991) and fny-CMV in tobacco, or mild to profound intensification of disease

severity, e.g. turnip crinkle virus on turnip plants (Li et al., 1989) and soil-born wheat mosaic virus on wheat plants (Chen et al., 1994).

(2) It is also possible that these ss-RNAs can be used as mRNAs to translate defective 2a protein in order to down-regulate the replication rate at the later stage of viral infection or to regulate the (+)/(-) strand ratio by switching off synthesis of the minus-strand template RNA. In this line, it was shown that all DI-like RNAs of BBMV which were derived from RNA2 code for truncated 2a proteins (Pogany et al., 1995). Since these shortened 2a proteins retained the N-terminal portion analogous to the one involved in the interaction between 1a and 2a of BMV, they could compete with wild type 2a for binding with BBMV 1a protein, so decreasing the concentration of active replication complex.

5.4.3 Conclusion

The final conclusion about the nature of the minor ss-RNAs in CMV infected plants can only be drawn after completion of their sequences. The observations that these ss-RNAs can be consistently found and that the deletions occurred specifically indicate that it is possible that these minor RNAs may have an important role in the CMV biology

Chapter 6

Further Studies of an Interspecies Hybrid Virus and its Interaction with CMV in Plants

6.1 Introduction

There is no information about the biological effects of the minor RNAs, considered in the previous chapters, on the replication and symptom expression of the CMV genomic RNAs. For such investigations full characterisation of these ss- and ds-RNAs at the sequence level is required. However, as a prelude to such work, it was decided to investigate the further biological characterisation of the 2b gene, another RNA 2-derived subgenomic RNA.

The 2b gene is a newly identified cucumovirus-specific gene which is encoded by RNA2 (Ding et al., 1994). The coding capacity of open reading frame 2b (ORF 2b; Fig. 6.3 a) overlaps the C-terminal 69 codons of ORF 2a encoding the RNA polymerase protein. The *in vivo* expression of the 2b gene, which is most likely from the subgenomic RNA 4A, has been shown for three cucumoviruses (Ding et al., 1994; Shi et al., 1996). A mutational analysis revealed that the 2b gene of CMV is an important virulence determinant in cucumber and *Nicotiana glutinosa* and that it facilitates long-distance movement of the virus in infected plants (Ding et al., 1995b).

By replacing the 2b gene of CMV with its homologue from V-TAV, a hybrid virus called CMV-qt, was described (Ding et al., 1996). The hybrid virus was found to be significantly more virulent, induces earlier onset of systemic symptoms and accumulates to a higher level than the both parents (Ding et al., 1996). It was the first example of an interspecies hybrid which was more efficient in systemic infection of a number of hosts than the parental viruses (Ding et al., 1996). Further analysis showed that the increased virulence resulted from a synergistic interaction between the V-TAV 2b protein and CMV. This type of virus synergy is a new one, since CMV and V-TAV have many homologies and no synergy occurs in double infections with CMV and V-TAV.

Although the hybrid virus is considerably more virulent and induced earlier systemic symptoms in single infection than either parental viruses, its interaction with the parental viruses in mixed infection is not clear.

The objective of the work described in this chapter was to investigate its relative fitness with the parental viruses and to study the genetic stability of the hybrid virus.

6.2 Methods and materials

6.2.1 Mixed inoculation using viral particles

Methods for plant inoculation were as described in Chapter 2 (Section 2.2.3). The inocula were Q-CMV, CMV-qt and V-TAV viral particles at 0.2 mg/ml which were purified from infected *Nicotiana glutinosa* plants as described before (Section 2.2.4). From each plant two leaves were inoculated with Q-CMV/CMV-qt or V-TAV/CMV-qt mixed at 1:1 ratio. Q-CMV, CMV-qt and V-TAV were also inoculated individually as controls. The infection was monitored in three different ways:

a. Symptoms. The symptoms of inoculated plants were checked during a two weeks period.

b. Northern-blot hybridisation. Twelve days post-inoculation, systemic leaves were harvested from 10 plants and total RNAs were isolated as described in Chapter 2 (Section 2.2.5). The extracted total RNAs were then analysed by northern hybridisation using specific riboprobes specific for 2b gene regions of Q-CMV or V-TAV as described in Chapter 2 (Section 2.2.20).

c. Enzymatic digestion of 2b gene region. Isolated total RNAs were used as template for amplification of the 2b gene region using RT-PCR as described before (Section 5.2.5). First-strand cDNA synthesis (Section 2.2.9) was carried out using 1μg total RNA and SD6 as a primer. Two μl of the cDNA was amplified in a PCR reaction employing SD6 and SD53 as PCR primers. The reaction was performed in the MJ minicycler and catalysed by Vent DNA polymerase (Biolab), according to the

manufacturer's directions, with a termal cycle profile of 92°C/1min, 50°C/1min, 72°C/2 min for 30 cycles, followed by a final elongation of 5 min at 74°C. Reaction products were resolved on 1% agarose 1xTAE and the major reaction product of 680 nt was excised from the gel and purified using QIAEX II Agarose gel extraction kit (QIAGEN). The purified DNA was then digested with *Asp718* (Bresatec) using conditions suggested by the manufacturer and the pattern of produced fragments was determined after gel electrophoresis and ethidium bromide staining.

d. Cloning and sequencing

The 2b gene regions were amplified using isolated total RNAs (of mixed inoculated plants as described in the introduction of this Section), SD6/SD53 primers, vent DNA polymerase and the conditions described in Section 6.2.1c. The amplified 2b gene regions were then blunt-end cloned into the *EcoRV* site of pBSK$^+$ and sequenced as described in chapter 2 (Sections 2.2.12 and 2.2.18 respectively).

6.2.2 Mixed inoculation using infectious cDNAs

Plasmids pQCD1 pQCD2 pQCD3 and pQCD2qt (Ding et al., 1995a; 1996) were purified by Superose 6 gel filtration chromatography as described by Skingle et al. (1990). Then the purified plasmid DNAs were digested to release the viral cDNA expression cassette consisting of the full-length CMV cDNA flanked by the 35S promoter and terminator. For this purpose digestion was performed with the following endonucleases; *NdeI* and *SalI* for pQCD1; *PvuII* and *BglII* for pQCD2, pQCD2qt and pQCD3. The reaction mixtures were extracted with phenol:chloroform (1:1 v:v) and DNAs precipitated with 2.5 volumes ethanol in the presence of 0.3 M sodium acetae (pH 5.2).

Nicotiana glutinosa plants were grown to the six-leaf stage under natural lighting conditions, and were placed in the dark for 24 hours prior to inoculation. For each plant, 10 µg each of the pQCD1 and pQCD3 were mixed with 5 µg of pQCD2 and 5 µg of pQCD2qt in a final volume of 10 µl sterile water and mechanically inoculated onto the fourth leaf dusted previously with carborandum powder. Two control inoculations were

performed in the same manner as follows: pQCD1, pQCD2 and pQCD3 and the second one pQCD1, pQCD2qt and pQCD3.

6.2.3 Leaf detachment assay

A leaf detachment assay (Gal-On et al., 1994) was used to compare the relative rate of virus movement between the hybrid and its parental viruses. In this method the half of a leaf distal to the stem was inoculated on individual plants with purified Q-CMV, TAV and CMV-qt virions at 0.2 mg/ml. The inoculated leaves (one leaf inoculated per plant) were detached at the times given in Table 6.1 (five plants were inoculated for each time treatment). For each virus 5 plants were also inoculated as positive controls in which the inoculated leaves were not detached. Symptom expression as well as a tissue print technique (McClure and Guilfoyle, 1989; Lin et al., 1990) were used to determine if inoculated plants were infected. Briefly, two weeks after inoculation, systemic leaves were removed separately and subjected to tissue printing. *Nicotiana glutinosa* leaves were first rolled into a tight cylinder, with the midrib parallel to the long axis of the cylinder, and then cross-sections were cut perpendicular to the midrib axis. Using a new razor blade for each plant, cross-sections were manually cut three times for each rolled leaf and each cut surface was pressed onto a Hybond-N membrane (Amersham) side by side to obtain one print for each leaf sample. Then the membrane was allowed to air dry and RNA was fixed to the membrane by cross linking in the Gene Linker UV chamber (BIO-RAD), using the CL program. The membrane was subjected to prehybridisation and hybridisation as described before (section 2.2.20).

6.2.4 Protoplast infection experiment

Protoplasts were prepared using a method similar to that described by Aryan *et al.* (1991). Three days after inoculation of CSV medium (See appendix I) with tobacco (*N. tabacum*) NT-1 suspension cells and incubation at 26°C with shaking, the suspension culture was precipitated by centrifugation at 100g for 2 minutes. After removal of the supernatant, the pellets were resuspended in a protoplast enzyme solution [0.1% Pectolyase Y-23

(Kikoman), and 1% Cellulase (Onozuka RS), in 0.4 M mannitol and 20 mM MES,pH 5.8], and incubated in dark for 30 minutes at 37°C without shaking following by 60 minutes incubation at 26°C with shaking at 50 rpm. After incubation, the protoplast mixture was pelleted at 100g for 5 min. The protoplasts were then washed three times by resuspending in 20 mM MES and 400 mM manitol, pH 5.5, followed by centrifugation. The washed pellets were resuspended in 0.4 M manitol and the density was adjusted to 10^6/ml by counting aliquots using a haemocytometer and divided into 1 ml aliquots and centrifuged (100g, 5 minutes) again.

The protoplasts in each pellet were inoculated with 4 μg viral RNAs isolated from purified virions (4 μg in single infection and 2 μg of each virus, 2 μg+2 μg, in double infection) and transfected using 100 ml PEG solution (40% PEG and 3 mM $CaCl_2$) as described by van der Vossen *et al.* (1993). After 30 seconds swirling, the mixture was diluted with three volumes (4.5 ml) of 0.4 M mannitol and then the transfected protoplasts were incubated on ice for 20 min. The protoplasts were pelleted and resuspended in 1 ml CSV medium and 0.3 M manitol and incubated for 24 hours at 26°C.

The RNAs were extracted from the protoplasts using TRIzol Reagent (GibcoBRL). The transfected protoplasts were centrifuged (100g/5 min) and the pellet were resuspended in 0.25 ml TRIzol Reagent and lysed by repeated pipetting. Following an incubation at room temperature for 5 minutes, 0.05 ml chloroform was added and the tube shaken vigorously for 15 sec followed by incubation at room temperature for 3 minutes. After a centrifugation (100g/10 min at 4°C), the upper aqueous colourless phase was recovered and mixed with 0.125 ml iso-propanol and incubated for 10 minutes at room temperature followed by a 10 min spin at 100g/ 4°C. After 70% ethanol washing, the RNA pellet was dissolved in 10 μl of 0.1 mM EDTA and analysed by northern blot hybridisation (Section 2.2.20).

6.2.5 Serial passages of the hybrid virus (CMV-qt) in plants

Several *Nicotiana glutinosa* plants were inoculated with CMV-qt and a plant shown tocontain CMV-qt by dot blot was selected as a source of virus for further inoculations.

Tissue from systemically infected leaves was ground with water and the extract inoculated to young test plants. At each passage, in addition to checking the symptoms for any variation, total RNAs were extracted from systemic tissues of 10 infected plants and after RT-PCR amplification of the 2b gene region as described in Section 5.2.5, any possible change in this region was checked by three different ways :

a. Symptomatology : After each passage the symptoms were checked during a two weeks period.

b. Enzymatic digestion of 2b gene region. This method was performed as described in Section 6.2.1 c.

c. Cloning and sequencing

The samples which showed different digestion patterns than CMV-qt (see Fig. 6.8 c2) were selected to clone their 2b gene regions by blunt-end cloning into the *EcoRV* site of $pBSK^+$ and sequenced as described in chapter 2 (Section 2.2.18).

6.3. Results

6.3.1 Competitiveness of the hybrid virus (CMV-qt) versus its parental viruses in whole plants

In single infections, Q-CMV, CMV-qt and V-TAV produced different symptoms. Systemic infection of tobacco plants (*Nicotiana glutinosa*) with Q-CMV produced only light mottling that was hardly visible (Fig. 6.1; top-right). However tobacco plants infected with CMV-qt showed severe symptoms (Fig. 6.1; top-middle); leaves were distorted and plants dwarfed. CMV-qt was also more virulent than V-TAV, although TAV induced clearly visible symptoms (Fig. 6.1; top-left) (Ding et al., 1996). Importantly, Q-CMV and V-TAV produced no obvious symptoms on inoculated leaves while some ringspots were visible on the leaves which were inoculated with CMV-qt.

Fig. 6.1 Symptoms of plants mixed-infected with QCMV+CMV-qt and TAV+CMV-qt. Bottom: *Nicotiana glutinosa* inoculated with Q-CMV+CMV-qt and TAV+CMV-qt compared to healthy tobacco or inoculated with Q-CMV, CMV-qt and V-TAV viruses (top row). The infected plants were photographed two weeks post-inoculation.

In mixed inoculation with viral particles, a few days after inoculation some ring spots appeared on the inoculated leaves of plants co-infected with Q-CMV and CMV-qt.. However two weeks after inoculation the symptoms in the systemic leaves were mild mosaic which is very similar to the symptoms of Q-CMV (Fig. 6.1; top-right). In the plants co-inoculated with V-TAV and CMV-qt , while some ring spot patterns were visible on the inoculated leaves, two weeks post inoculation, the symptoms in the systemic leaves were severe mosaic, different from V-TAV but similar to those developed by the hybrid virus (Fig. 6.1; bottom-left). It seems that the hybrid virus was less

competitive than Q-CMV but more competitive than V-TAV in establishing systemic infection in mixed inoculation.

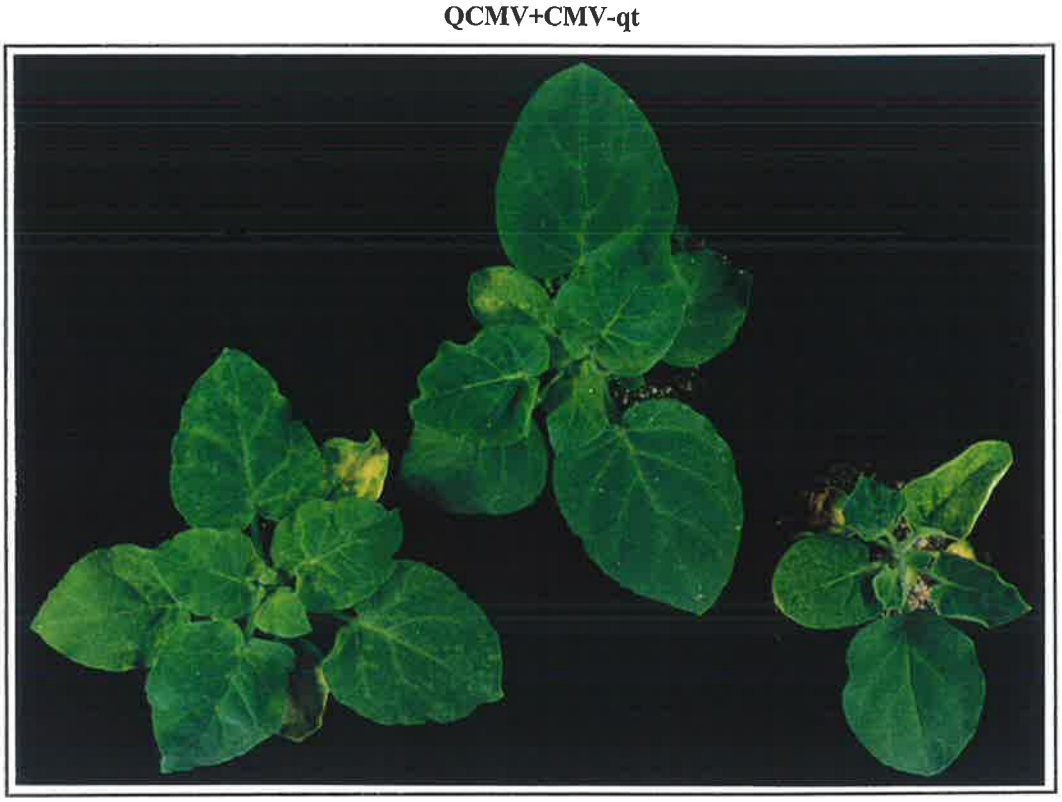

Fig. 6.2 Symptoms of plants mixed-infected with the infectious cDNAs of Q-CMV+CMV-qt. *Nicotiana glutinosa* plants inoculated with infectious cDNAs of QCMV+CMV-qt (top), Q-CMV. (bottom left) and CMV-qt (bottom right). The infected plants were photographed three weeks post-inoculation.

In the mixed infection experiment using infectious clones of Q-CMV and CMV-qt (Section 6.2.2), while ringspots were clear on inoculated leaves of *N. glutinosa* (Fig. 6.2, top) three weeks post-inoculation, the symptoms of systemic leaves were mild mosaic similar to those of Q-CMV (Fig. 6.2, left) and different from the hybrid virus symptoms (Fig. 6.2, right). Thus, the results of mixed inoculation using plasmids as inocula confirmed the above results where viral particles were used as inocula for mixed infection

Northern analysis (Section 2.2.20) was used to determine which virus was present in inoculated and systemic leaves. Total RNAs were extracted from inoculated and systemic

leaves (Section 2.2.5), one and three weeks post inoculation. Northern blots were hybridised with different probes as follows: in the mixed inoculation of Q-CMV and Q-CMV-qt, probe B specific for Q-CMV RNA2 and probe F specific for the 2b gene of TAV (Fig. 6.3 a & b respectively), were used. Probe B is capable of detecting RNAs 2 of Q-CMV and CMV-qt while probe F is capable of detecting RNAs 2 and 4A of V-TAV.

With probe B, RNAs 2 was detected in plants mixed inoculated with Q-CMV+CMV-qt (Fig. 6.3, lanes 2 and 3) as well as in controls which plants inoculated with Q-CMV (lane 1) or CMV-qt (lane 4). However, with probe F no RNAs were detected in the plants mixed infected with Q-CMV+CMV-qt (Fig. 6.3, lanes 7 & 8) and just RNAs 2 and 4A of the control plants (inoculated with CMV-qt; lane 9) were detected. The results indicated the presence of just Q-CMV RNAs in the systemic leaves of plants which were mixed-inoculated with Q-CMV+CMV-qt, while both viruses were detected in the inoculated leaves (data not shown). The presence of just Q-CMV in the systemic leaves of plants coinoculated with the infectious cDNAs of Q-CMV and CMV-qt (Fig. 6.4, lane 2), was further confirmed by the above results. Thus, although both viruses can coinfect plants, Q-CMV is more invasive than CMV-qt.

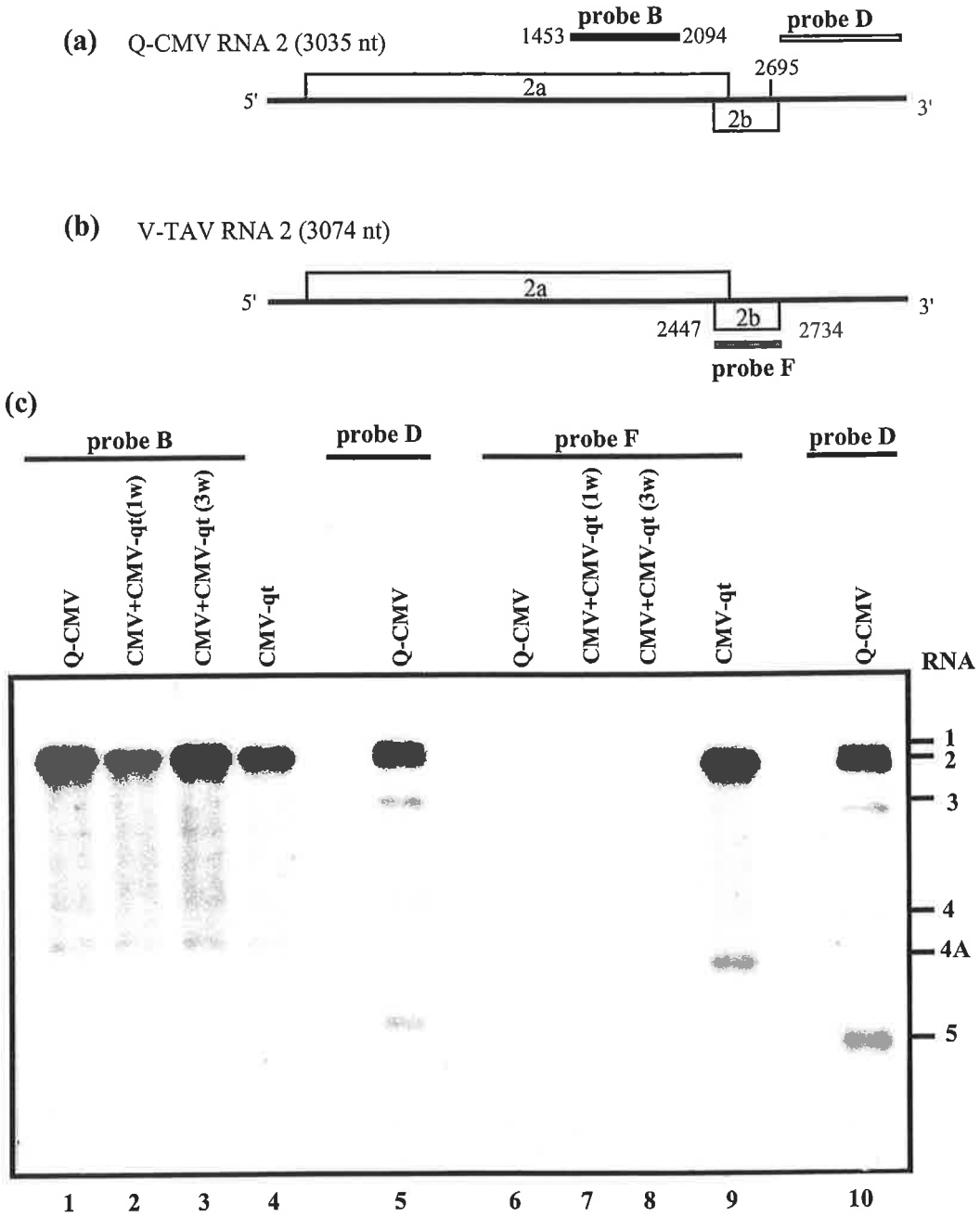

Fig. 6.3 Northern blot analysis of progeny RNAs in mixed-infection of Q-CMV and the hybrid virus (CMV-qt **) in *N. glutinosa*.** **(a)** and **(b)** Positions of the specific RNA probes used for northern blot hybridisation. **(a)** ProbeB is a Q-CMV RNA2 specific probe while, probe F is a V-TAV 2b-specific probe **(b)** and probe D is a 3'-common end Q-CMV probe as used in Chapter 5 and shown in **(a)**. For northern analysis **(c)** the samples were total RNAs isolated from tobacco leaves inoculated with Q-CMV (lanes 1, 5, 6 and 10), Q-CMV+CMV-qt (one week (1w) post inoculation; lanes 2 & 7), (three weeks (3w) post inoculation; lanes 3 & 8) and CMV-qt hybrid virus (lanes 4 & 9). These samples were hybridised with probe B (lanes 1to 4), probe F (lanes 6 to 9) or probe D (lanes 5 & 10). The positions of viral RNAs 1, 2, 3, 4, 4A and 5 are indicated.

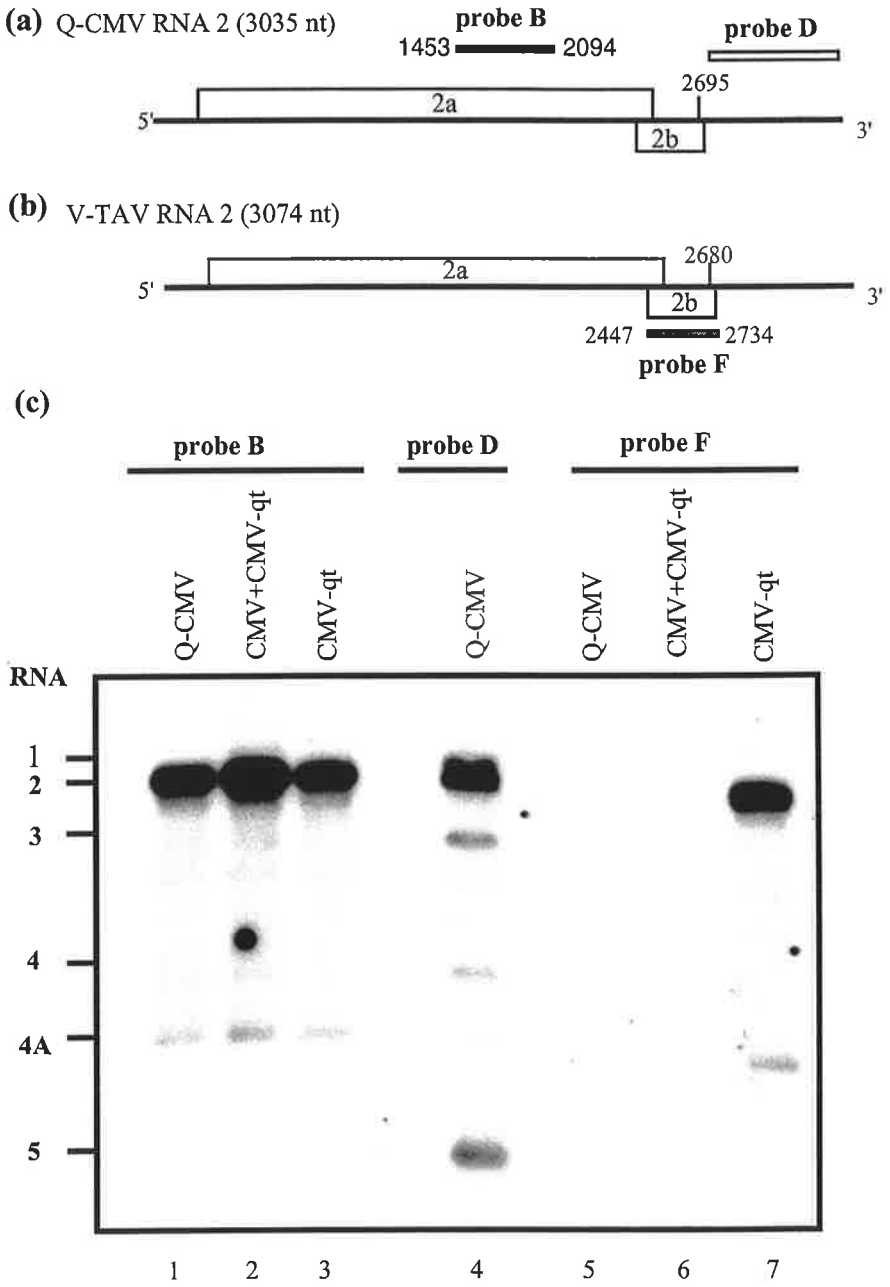

Fig. 6.4 Northern blot analysis of progeny RNAs in mixed-inoculation of plants with infectious cDNAs of Q-CMV and CMV-qt hybrid virus. Positions of the specific RNA probes used for northern blot hybridisation are indicated in **(a)** and **(b)**. Probe C is a Q-CMV 2b specific probe **(a)**, while probe F is a V-TAV 2b specific probe and probe D is correspond to the last 340 nt of all Q-CMV genomic RNAs. For northern analysis **(c)** the samples were total RNAs isolated from tobacco leaves inoculated with V-TAV (lanes 4, 5, 6 and 10), V-TAV+CMV-qt (one week post inoculation; lanes 2 & 7), (three weeks post inoculation; lanes 3 & 8) and CMV-qt hybrid virus (lanes 1 & 9). These samples were hybridised with probe B (lanes 1to 4) or probe G (lanes 5 to 10) . The positions of viral RNAs 1, 2, 3, 4, 4A and 5 are indicated.

In addition, to determine which virus was present in the plants mixed inoculated with V-TAV and Q-CMV-qt, probes B (Fig. 6.5 a), and G (Fig. 6.5 b; complementary to the 3'-terminal 128 nt of V-TAV) were used. Probe B can detect RNAs 2 of Q-CMV and CMV-qt while probe G can detect all V-TAV RNAs. The results (Fig. 6.5 c) showed that in the mixed inoculated plants, RNA 2 were only hybridised to the probe B and no signal was obtained with probe G (except in the controls; lanes 5, 6 & 10), indicating that, from mixed inoculation of TAV and CMV-qt, only hybrid virus was present in the systemic leaves of infected plants while both viruses were detected in inoculated leaves. These results implied the failure of V-TAV to invade the systemic leaves of *N.glutinosa* in the presence of CMV-qt.

Furthermore, the *Asp*718 digestion pattern of the amplified 2b gene region was used to determine which virus was present in plants mixed inoculated with Q-CMV+CMV-qt or V-TAV+CMV-qt as described in Section 6.2.1 c. In the plants which were coinoculated with Q-CMV+CMVqt the pattern of digestion was similar to that of Q-CMV while in the plants inoculated with V-TAV+CMV-qt, the pattern of digestion was similar to that of CMV-qt (data not shown; an example of these patterns were shown in Fig. 6.9. In addition, the amplified 2b gene region of the viruses which were present in the mixed inoculated plants was cloned and sequenced as described in section 6.2.1 d. The results showed that in the systemic leaves of plants mixed inoculated with Q-CMV+CMV-qt no clones were obtained from CMV-qt while in the mixed inoculation of V-TAV+CMV-qt no clones were obtained from V-TAV.

The results of all mixed infection experiments of the hybrid virus with its parental viruses clearly showed that, although coinfection of Q-CMV and CMV-qt as well as of CMV-qt and V-TAV are possible, Q-CMV is more invasive than CMV-qt while CMV-qt is more competitive than V-TAV. A question raised here was whether the different invasiveness of these viruses was a reflection of their abilities to establish themselves in the systemic leaves faster than other viruses or a reflection of their replication levels which enabled them to outcompete other viruses?.

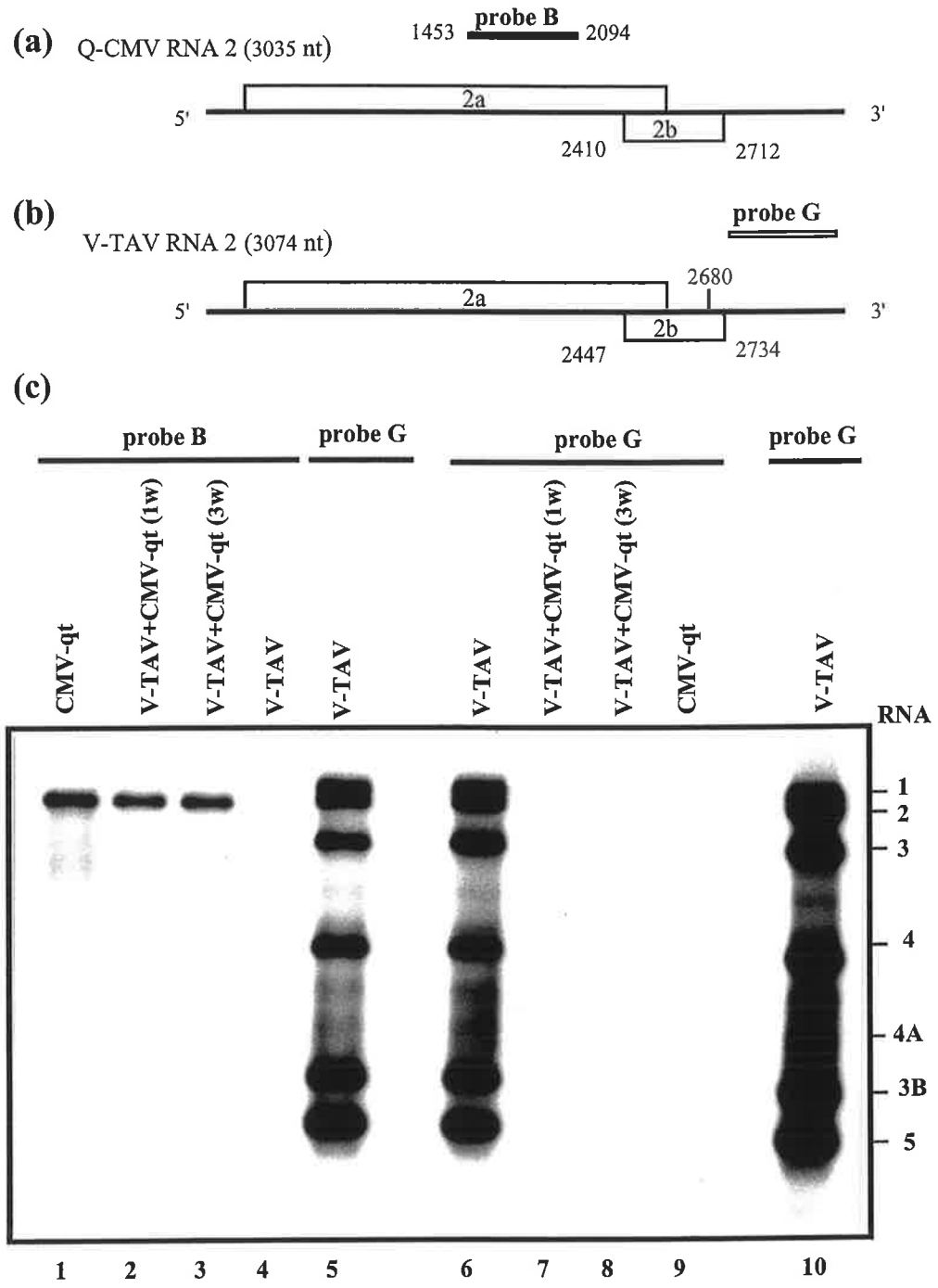

Fig. 6.5 Northern blot analysis of progeny RNAs in mixed-infection of V-TAV and hybrid virus (CMV-qt**). (a) &(b)** Positions of the specific RNA probes used for northern blot hybridisation. Probe B is a Q-CMV RNA 2 specific probe **(a)**, probe G is a strand-specific RNA probe complementary to the 3' terminal 128 nt of all three V-TAV genomic RNAs **(b)**. **(c)** For northern analysis the samples were total RNAs isolated from tobacco leaves inoculated with V-TAV (lanes 4, 5, 6 and 10), V-TAV+CMV-qt (one week (1w) post inoculation; lanes 2 & 7), (three weeks (3w) post inoculation; lanes 3 & 8) and CMV-qt hybrid virus (lanes 1 & 9). Thses samples were hybridised with probe B (lanes 1to 4) or probe G (lanes 5 to 10) . The positions of viral RNAs 1, 2, 3, 4, 4A and 5 are indicated.

6.3.2 Systemic movement of Q-CMV, CMV-qt and V-TAV in tobacco plants

To compare the movement rate of Q-CMV, CMV-qt and V-TAV in plants, a leaf detachment assay (Gal-On et al., 1994) was used since this method can determine how long a particular virus will take to move out of the inoculated leaf.

The systemic movement rates of Q-CMV, CMV-qt and V-TAV were examined under the same inoculation conditions (Section 6.2.2). In this method *N. glutinosa* leaves were inoculated with purified virus particles, however, the inoculated leaves were detached at 12-hours intervals from 24 to 84 hours and two weeks after inoculation, the inoculated and systemic leaves were used for tissue printing hybridisation assay (an example is shown in Fig. 6.6). Table 6.1 shows the total number of plants infected at 14 days post inoculation. As can be seen, the plants inoculated with all three viruses were systemically infected by 48 hours post inoculation. In other words, it took 48 hours for Q-CMV, V-TAV and the hybrid viruses to move out of the inoculated leaves and there were no significant differences in the movement rates of the compared viruses at 12-hours intervals.

Table 6.1 Time course of systemic movement of Q-CMV, CMV-qt and V-TAV

Virus	24 hr[a]	36 hr	48 hr	60 hr	72 hr	84 hr	C[c]
QCMV	0/5[b]	0/5	4/5	3/5	5/5	5/5	5/5
QCMV-qt	0/5	0/5	2/5	4/5	5/5	5/5	5/5
TAV	0/5	0/5	3/5	2/5	5/5	5/5	5/5

[a] Hours (hr) interval between inoculation and removal of the inoculated leaves
[b] Number of infected plants over number inoculated at 14 days post inoculation
[c] Control plants in which the inoculated leaves were not detached

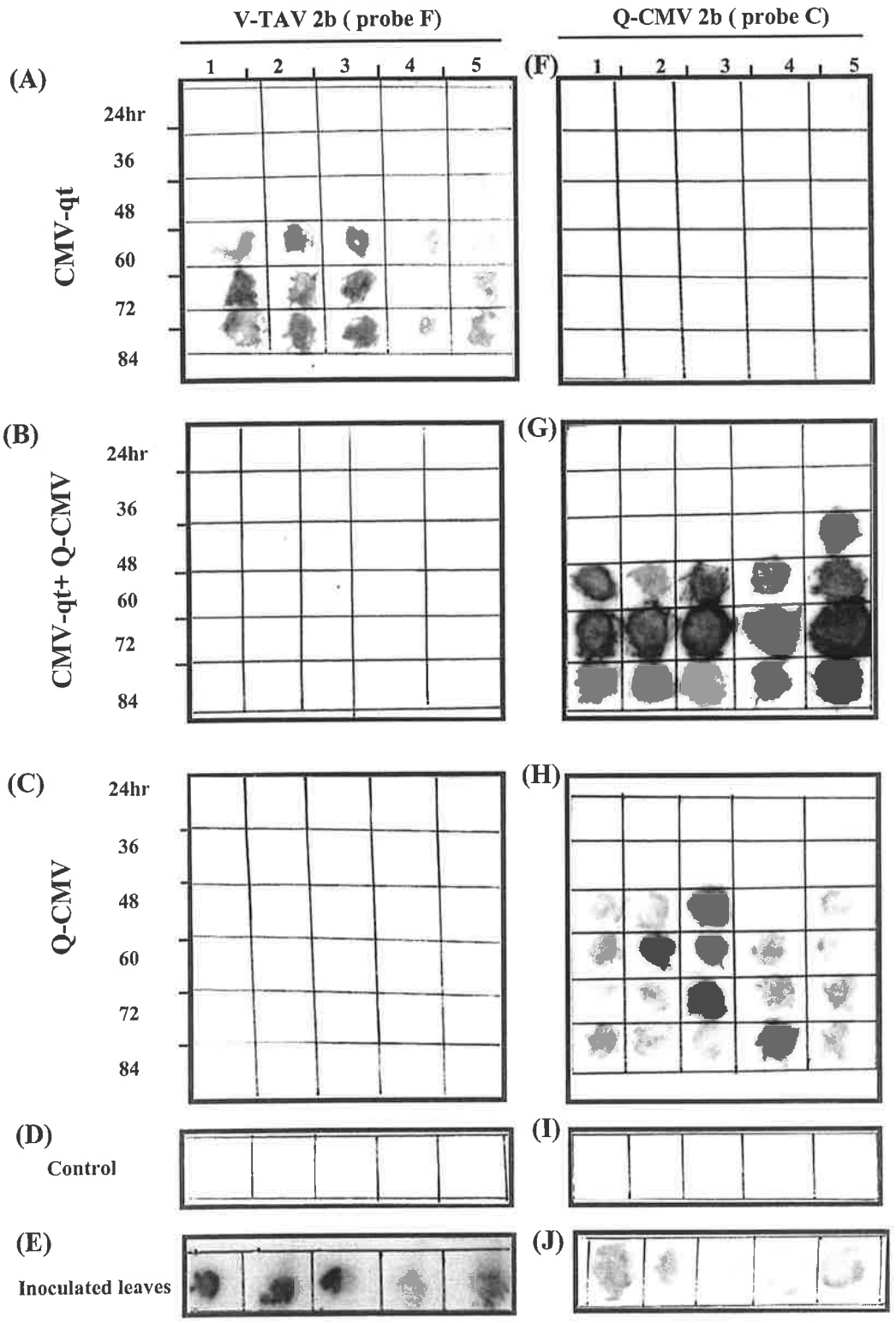

Fig. 6.6 Leaf-detachment assays for movement rate analysis of Q-CMV and the hybrid virus (CMV-qt) . Inoculated leaves from *N.glutinosa* plants were detached 24, 36, 48, 60 and 72 hours after inoculation with Q-CMV **(C)** & **(H)**, CMV-qt **(A)** & **(F)**, Q-CMV+CMV-qt **(B)** & **(G)** with 5 replicates as described in Section 6.2.3. Fourteen days post inoculation the systemic leaves were removed and subjected to tissue printing. Moreover for each virus 5 plants were inoculated and their inoculated leaves were removed after two weeks and used for tissue printing **(E)** & **(J)**. 5 healthy *N. glutinosa* plants were also used as negative controls **(D)** & **(I)**. The blotted samples were hybridised to the probe **(F)**, specific probe for V-TAV 2b region, (**A**, **B**, **C**, **D** and **E**) or probe (**C**), specific for Q-CMV 2b region, (**F**, **G**, **H**, **I** and **J**).

In conclusion, these results implied that there is a decrease in the relative fitness associated with CMV-qt as compared to Q-CMV. However, in comparison with V-TAV, there is an increase in the relative fitness associated with CMV-qt.

6.3.3 The accumulation levels of Q-CMV+CMV-qt and V-TAV+CMV-qt in protoplasts

The accumulation levels of Q-CMV, CMV-qt and V-TAV were compared in tobacco protoplasts inoculated with equal amounts of RNAs (4 μg in single and 2 μg of each virus (2 μg + 2 μg) in double infection) prepared from purified viral particles (Section 6.2.3). Northern blot analysis (Fig. 6.7) was performed using total RNAs extracted from the protoplasts after 24 hours incubation at 26°C and specific probes as follow: in group 1, where protoplasts were mixed inoculated with RNAs of Q-CMV and CMV-qt, probe C (Q-CMV 2b gene region) was used for estimation of Q-CMV RNAs and probe F (V-TAV 2b gene region) for quantification of the CMV-qt RNAs in the mixed infection as compared to the control (single inoculation of Q-CMV and CMV-qt respectively) (Fig. 6.7). In group 2 where RNAs of TAV and CMV-qt were used for mixed inoculation of protoplasts, probes B and J (complementary to the 3a and coat protein region of V-TAV) (Fig. 6.7) were employed to study the RNA accumulation levels of CMV-qt and V-TAV in mixed inoculation respectively (for comparison to the single infection of the viruses). To determine the accumulation level of progeny RNAs, probed membranes were exposed to a storage phosphor screen (Molecular Dynamics) for 1 hour at room temperature and then the intensity of hybridised radiolabelled RNAs (RNA 2 of Q-CMV or CMV-qt and RNA 3 of V-TAV; see Fig. 6.7) was quantified on a phosphorimager (Storm 860, Molecular Dynamics).

The results of northern analysis showed no hybridisation signal in control lanes (Fig. 6.7 lanes 3, 6, 7 and 12) indicating the specificity of the probes C, F, B and J. For quantification of the hybridised RNAs, the hybridised filter which was used for northern analysis in Fig. 6.7, was exposed to a phosphor screen and the intensity of the first top bands of each column counted using a phosphorimager.

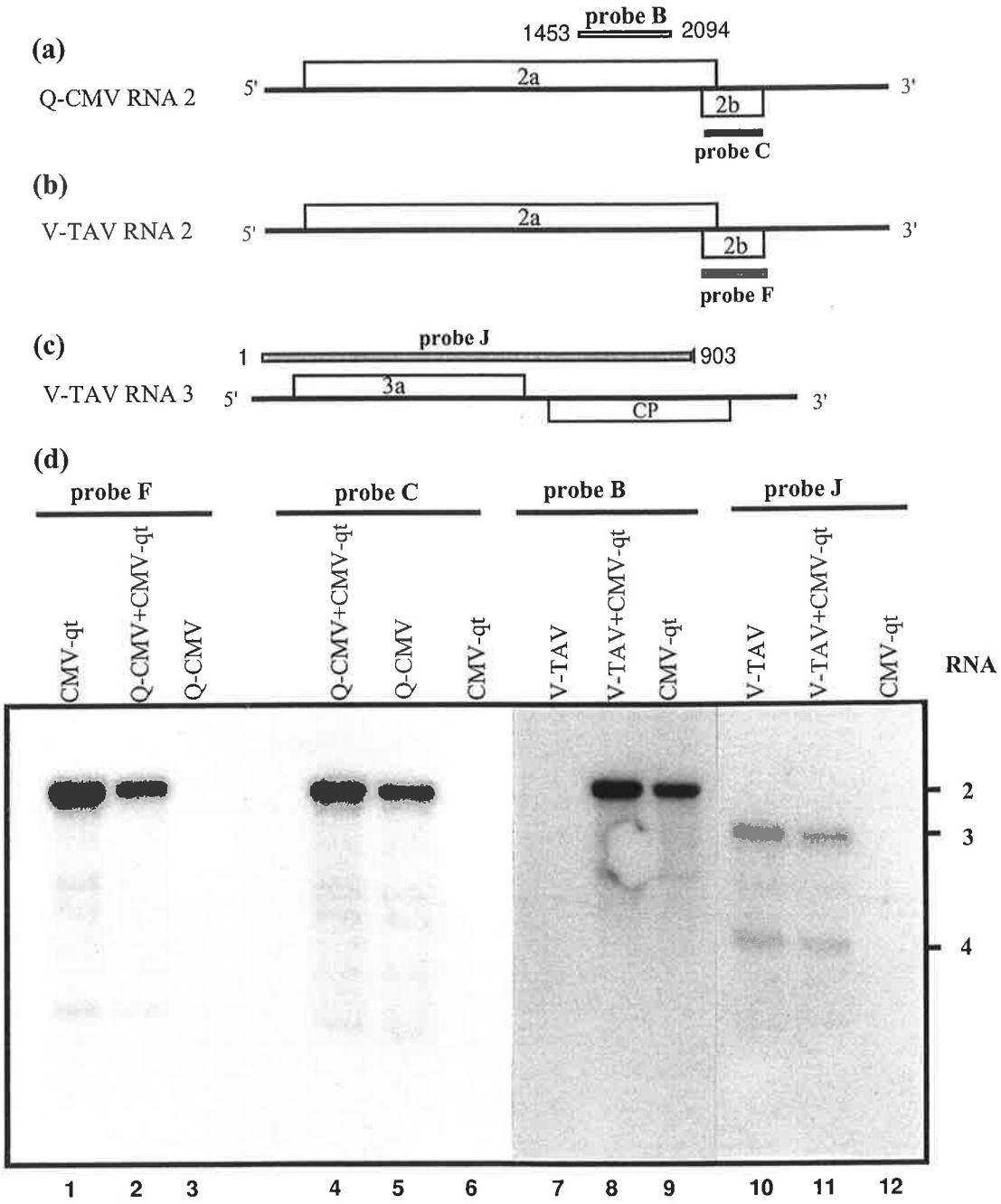

Fig. 6.7 Accumulation of the viral RNAs of CMV-qt and its parental viruses in tobacco protoplasts. (a), (b) & **(c)** positions of the probes used for northern blot analysis. Probes C and F are 2b gene regions specific for Q-CMV and V-TAV, respectively. Probe B is a specific probe of Q-CMV RNA2 as indicated in **(a)**. Probe J corresponds to the first 1903 nt of V-TAV RNA3 and is capable of detecting RNAs 3 and 4 **(c)**. **(d)** Northern analysis of accumulated RNAs in tobacco protoplasts. Each lane contains RNA extracted from about 200,000 protoplasts after inoculation and 24 hours of incubation. Total RNAs extracted from tobacco protoplasts inoculated with the hybrid virus (lanes 1, 4, 9 &12), Q-CMV (lanes 3 & 6), V-TAV (lanes 7 & 10), CMV+CMV-qt (lanes 2 & 5) and TAV+CMV-qt (lanes 8 & 11) were hybridised with probe C (lanes 1 to 3) or B (lanes 7 to 9) as shown in (a), or probe F as shown in (b) (lanes 4 to 6), or probe J as shown in (c) (lanes 10-12). The positions of viral RNAs 2, 3 and 4 are indicated.

Since the specific activities of the different probes were not determined, we were not able to do direct comparison between the intensities of the RNAs hybridised to the different probes (C, F, B and J). Hence a comparisons could only be made between the intensities of the RNAs (in the mixed inoculation and in single infection) which hybridised to each probe within each of the four experiments in Fig. 6.7 and Fig. 6.8.

The results of this quantification showed (Fig. 6.8) that CMV-qt replication was reduced in the presence of Q-CMV because the amount of viral RNA (CMV-qt) signal in column 2 was less than 1/2 of that in column 1; in contrast, the replication of Q-CMV was actually increased in the presence of CMV-qt because the amount of Q-CMV-signal in column 4 was significantly greater than for Q-CMV alone (column 5). Thus Q-CMV is a better template as compared to the CMV-qt. These results also revealed that the replication of V-TAV was reduced in the presence of the hybrid virus (CMV-qt) since the intensity of the hybridised V-TAV (column 11) was only 57% of that in column 10 while the replication of CMV-qt was increased in the presence of V-TAV(column 8) since its RNA showed an increase of 30% compared to that of CMV-qt in single inoculation (column 9). Therefore CMV-qt is a better template than V-TAV.

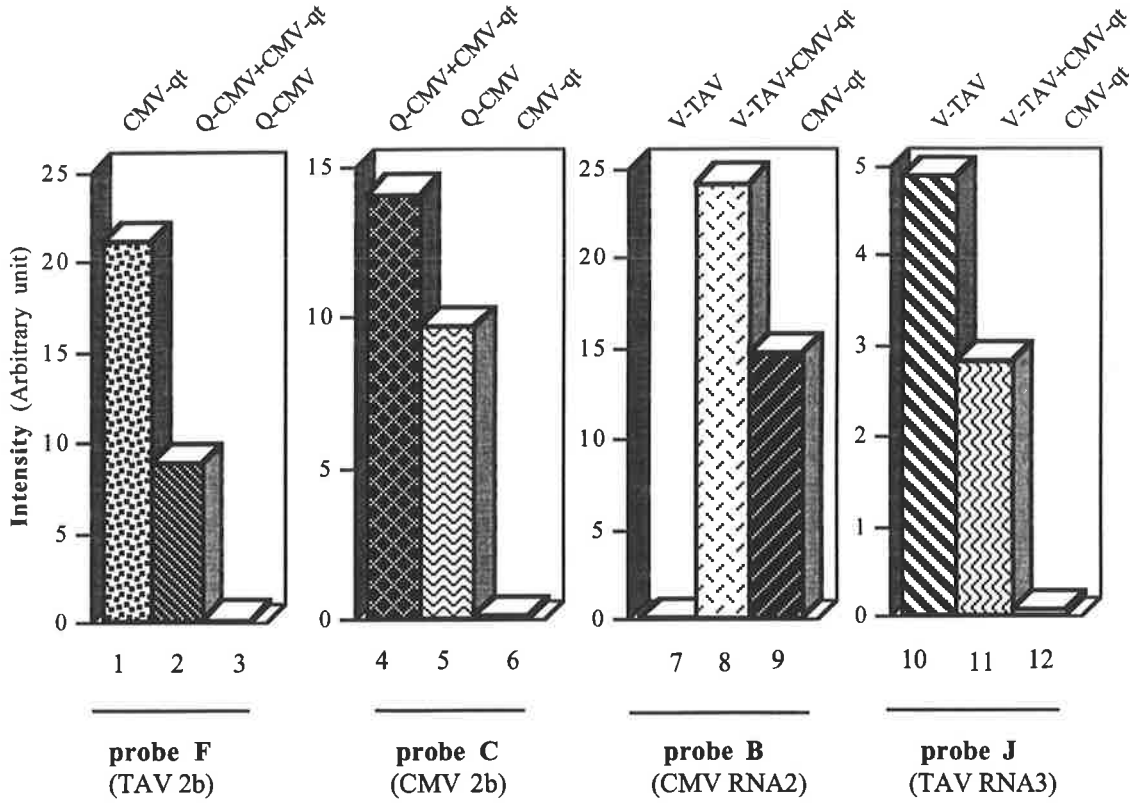

Fig.6.8 Quantification of the accumulated RNAs of the hybrid virus and its parental viruses in the mixed infected protoplasts. The hybridised filter which was used for northern analysis in Fig. 6.7 was exposed to a phosphor screen and the first top bands of each lane counted using a phosphorimager and the values used for comparison of the RNA accumulation levels. Each column is equal to the total RNA from 200,000 protoplasts. Positions of the probes are shown in Fig. 6.7 (a), (b) and (c). Probe F was used to detect CMV-qt and probe C was used to detect Q-CMV in the mixed infection of Q-CMV+CMV-qt. While probe B was used to detect CMV-qt and probe J was used to detect V-TAV in the mixed infection of V-TAV+CMV-qt.

6.3.4 Genetic stability of the hybrid virus in plant

The genetic stability of the CMV-qt hybrid virus was studied by serial passages, investigation of the symptoms and possible sequence variation of the ORF 2b. Total RNAs were extracted (Section 2.2.5) and the 2b gene region was amplified using RT-PCR (Section 5.2.5) and the product was cloned and sequenced as in Section 6.2.5 c. The results showed that after ten passages no changes were found in symptom expression

and sequence of the 2b gene region of the progeny virus (Fig. 6.9) as compared with CMV-qt.. These results indicated that the hybrid virus is genetically stable at least under greenhouse conditions (Section 2.2.3).

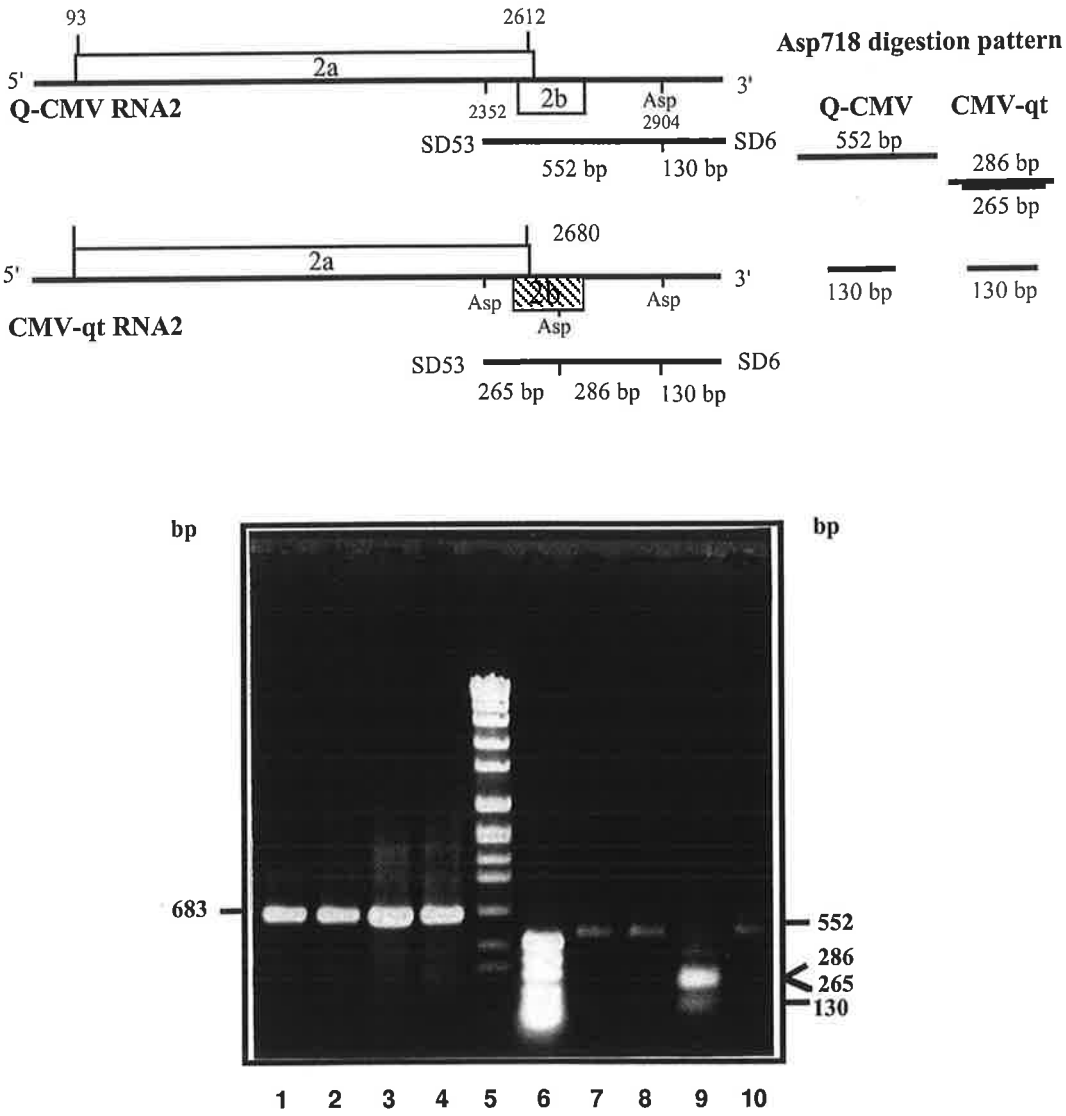

Fig 6.9 *Asp*718 digestion pattern of the 2b gene regions of Q-CMV and CMV-qt. (a) and **(b)** shows the *Asp*718 sites in RNA2 of Q-CMV and CMV-qt respectively. **(c)** Represents the schematic Asp718 digestion products and their sizes in Q-CMV and CMV-qt 2b gene regions. **(d)** Agarose gel electrophoresis of amplified and digested 2b gene regions of Q-CMV and CMV-qt. The samples were PCR amplified 2b gene regions of Q-CMV (lanes 1, 2 and 4) and CMV-qt (lane 3) and their relative *Asp*718 digestion products (2b of Q-CMV lanes 7, 8 and 10; 2b of CMV-qt lane 9). The expected size of the PCR products was 683 bp and the DNA size markers were SPP-1/*Eco RI* (lane 5) and pUC19/*HpaII* (lane 6) fragments.

6.4 Discussion

The CMV-qt virus studied in this chapter is a hybrid virus made by exchange of the 2b coding sequence between Q-CMV and V-TAV (Ding et al., 1996). It has been shown that CMV-qt was more virulent than either of the parental viruses. This is the first example of an interspecies hybrid made from plant or animal RNA viruses (De Jong and Ahlquist, 1992; Hilf and Dawson, 1993; Lopes et al., 1994; Peng et al., 1995; Kuhn et al., 1996) which is more efficient than the naturally selected parents. Due to the epidemiological importance of simultaneous infection of plants with two or more viruses, it was of interest to study the interaction of hybrid virus (CMV-qt) with its parental viruses (Q-CMV and TAV) in mixed infection.

The results of mixed infection experiments (Section 6.3.1) showed that when a mixture of Q-CMV and CMV-qt purified virions were used as inoculum (Table 6.2), although both viruses accumulated and produced characteristic symptoms in inoculated leaves for CMV-qt, CMV-qt was not able to infect *Nicotiana glutinosa* plants systemically. In the systemic leaves just Q-CMV was detectable even when the concentration of the Q-CMV virions was 64 times less than that of CMV-qt virions in the original inoculum (data not shown). These results were further confirmed by another mixed inoculation experiment (Section 6.3.1) in which infectious cDNAs were used as inocula for both CMV-qt and Q-CMV.

Table 6.2 ***A summary of the mixed infection experiment***

Inoculum	Inoculated leaves	Systemic leaves
Q-CMV+CMV-qt	Q-CMV and CMV-qt	Q-CMV
V-TAV+CMV-qt	V-TAV and CMV-qt	CMV-qt

When *Nicotiana glutinosa* plants were mixed inoculated with CMV-qt and V-TAV purified virions (Table 6.2), while both viruses were detected in the inoculated leaves,

only CMV-qt hybrid virus could invade systemic leaves, since only CMV-qt was found in the systemic leaves.

Mixed infection of viral isolates may have profound effects on their epidemiology and evolution through structural interaction between them (Stackey and Francki 1990; Fernandez-Cuartero et al. 1994)). Indeed mixed infections are a condition for, and may lead to, genetic exchange between different genetic types, and genetic exchange both by reassortment and by RNA recombination was seen to occur (Fraile et al., 1997). The results described in this chapter revealed that the interspecies hybrid virus reacted differently, in terms of relative fitness, as compared to its parental viruses (Q-CMV and V-TAV) in mixed infection . Fernandez-Cuartero et al. (1994) showed an increase in the relative fitness associated with a naturally occurring recombinant virus (having RNAs1 and 2 from TrK7-CMV and RNA3 from V-TAV) over its parental viruses, CMV and V-TAV. This recombinant virus, which was obtained after serial passages of a pseudorecombinant virus consisting of RNAs 1 and 2 from CMV and RNA 3 from V-TAV, contained a hybrid RNA 3 in which most of the 3' nontranslated region was derived from RNA 2 by recombination.

Following viral RNA entrance into host cells, the infection cycle will be continued through two main stages, replication of the RNA in the infected cells and spread of the amplified virus through the infected plants. Therefore in competition between two viruses in mixed infections, replication and movement rates are determinant factors. The results here showed that there was no detectable differences between the movement rate of Q-CMV, V-TAV and the hybrid virus (CMV-qt) at 12 hour intervals (Section 6.3.2), although the possibility of different movement rates at lower time intervals cannot be ruled out. The protoplast system was used to compare the replication levels of the RNAs of Q-CMV, V-TAV and the hybrid virus. There were clear differences between the

replication levels of the hybrid virus with its parental viruses in the mixed infection; in the coinoculation of the hybrid with Q-CMV, Q-CMV replication and in the mixed inoculation of the hybrid with V-TAV, the hybrid virus replication were stimulated indicating that Q-CMV is a better template compared to the CMV-qt and CMV-qt is a better template than V-TAV. These results were consistent with the mixed inoculation experiments which were performed with viral particles (Section 6.3.1) and infectious cDNA clones (Section 6.3.1) of the viruses. Therefore it seems that the replication level is the key factor in the competition of the hybrid virus with its parental viruses and it can be concluded that the hybrid virus is a better template for RNA replication as compared to the V-TAV RNAs while in mixed infection with the second parental virus (Q-CMV), Q-CMV utilises this advantage.

Chapter 7

General Discussion

7.1 Subviral RNAs should not be ignored

Recent developments in molecular virology have provided us with insight into the structural organisation of many plant virus RNA genomes as well as with information on the variability in these genomes at the nucleotide level. These studies revealed that diversity, widespread occurrence and host range of RNA viruses reflect their evolutionary success. In reality this success is the consequences of employment of a wide variety of strategies and complex mechanisms in their replication and disease induction process.

In addition to the genomic RNAs, non-genomic or less than full-length fragments are present in the RNA profile of some RNA viruses. Each of these molecules may play a crucial role(s) in the life cycle of viruses, i.e. as a template for replication or as mRNAs which may act in protein synthesis or as a secondary RNAs (i.e. satellite or DI RNAs) that can modulate viral diseases (Kaper and Collmer, 1988; Huang, 1988; Ding et al., 1994). RNA 4A is a good example of such RNAs which, 21 years after the first report of its presence in Q-CMV RNA profile (Peden and Symons, 1973), it was found that as a subgenomic RNAs it plays crucial roles in movement, virulence determination and host specificity of the virus (Ding et al., 1994; 1995b; 1996). Therefore, characterisation of these minor RNAs provides valuable information regarding the life cycle of viruses and their subsequent controls and they should not be ignored or their investigations blocked by simply regarding all of them as the consequences of viral RNA degradation.

7.2 Relation of the minor ds-RNAs and ss-RNAs

On the basis of the results obtained from chapter 4, three minor ds-RNAs (dsB1, dsB2 & dsB3) are consistently present in the CMV-RNA profile. The important features of these RNAs are their intact 5' and 3' termini (which are identical to RNA2) and their

estimated sizes are 2600, 1300 and 900 base pairs. Furthermore, the results of chapter 5 indicated the presence of three single strand minor RNAs in the CMV RNA profile (in addition to the known RNAs) with estimated sizes of 1800, 1400, and 1200 nt.

The three minor ss-RNAs are similar to the three minor ds-RNAs in the following features: firstly, all of these minor RNAs are derived from RNA2, secondly, all of them have intact 3' and 5' termini (identical to the RNA 2) and thirdly, the central regions of these RNAs did appear to be different length deletions of RNA2.

The structure of these minor RNAs is very similar to the structure of DI RNAs characterised in broad bean mottle virus (BBMV) (Pogany et al., 1995), which due to the similar genome organisation, their replication cycle have many common features. Similar to the minor RNAs, the DI RNAs of BBMV are derived from RNA2 with an internal deletion which consequently their 5' and 3' termini are retained. Moreover, in the DI RNAs of BBMV, deletion junction sites are between nucleotides 1152 and 2366 which are very close to the suspected deletion regions of the minor RNAs (Fig. 4.8)

On the other hand, although all of the minor ds- and ss-RNAs are shorter than Q-CMV RNA2, there is not a close correlation in terms of size, between minor ds- and ss-RNAs. Of these RNAs just dsB2 and TB2 have almost similar size (1300-1400bp) while there is no such similarity between the sizes of TB1 and TB3 with dsB1 and dsB3. Since size estimation of minor dsRNAs was carried out in non-denaturing conditions and ss-RNAs in denaturing condition, size comparison is difficult and possibly the difference in electrophoresis conditions may be a good explanation for the discrepancy. In fact, since replicating viral RNA is only partly double stranded and contains single strand tails (nascent product strands) (Matthews , 1991), it is possible that the secondary structures of such single strand tails could affect the electrophoretic mobilities of the minor ds-RNAs. For example, in a study of TMV ds-RNA 3 in non-denaturing conditions, the molecular weight was reported as 2.3×10^5 and this was considered to be too low for a dsRNA form of the LMC (the coat protein mRNA of TMV; Zelcer et al., 1981).

Further study under denaturing conditions showed that ds-RNA-3 is a dsRNA counterpart of the LMC and obviously the previous molecular weight reported for dsRNA-3 had been in error (Palukaitis et al., 1983).

In conclusion, there are some structural similarities between minor ds-RNAs and ss-RNAs and it is possible that the all minor ds-RNAs or just one of them may be the replicative form of their corresponding ss-RNAs. However, sequence completion of the minor RNAs is necessary to resolve these uncertainties.

7.3 Why are the minor RNAs exclusively derived from the RNA2?

By whatever mechanism these minor RNAs (ss- and ds-RNAs) are produced, an important question is why are these RNAs exclusively derived from the CMV RNA2? One possibility is the cis-correlation between replication (of minor RNAs) and translation (2a protein as a crucial part of CMV polymerase) which is called cis-preferential replication (Weiland and Dreher, 1993). In other words, 2a protein is one of the essential replication proteins and is made from RNA2 and is could assemble most efficiently into a replication complex on that same RNA molecule (or related molecules), facilitating the replication process. Cis-preferential replication has been observed for poliovirus (Johnson and Sarnow, 1991), turnip yellow mosaic virus (Weiland and Dreher, 1993) and also suggested for the formation of RNA2-derived DI like RNAs of BBMV (Pogany et al., 1995).

7.4 Increased fitness of an interspecies hybrid

The findings presented in chapter 6 are relevant to two aspects of virology.

(1) RNA-RNA recombination Genetic variation, an intrinsic property of replicating entities, has been widely reported for RNA viruses. This property has been associated primarily with high error rates of RNA-dependent RNA polymerases (Domingo and Holland, 1988). One important way of genetic material exchanging is RNA-RNA recombination, a process that has been reported to occur in different groups of bacterial,

animal and plant RNA viruses (see review by Lai, 1992). RNA-RNA recombination can have an important role in determining the genetic structure of RNA virus populations by the generation of new genetic variants (Fernandes-Cuartero et al., 1994). Our results which showed that the hybrid virus was more invasive than TAV (one of the parental viruses), further supported the concept that RNA-RNA recombination may lead to the increased fitness of the recombinant RNA. The recombinant RNA will then become fixed in the viral population. Therefore, even if infrequent, RNA-RNA recombination will have important consequences for the genetic structure and evolution of viral populations.

(2) Transgenic plants Production of transgenic plants expressing virus-derived sequences is fast becoming routine for a number of crop species. It has been shown that recombination can occur between a challenge virus and a transgene (Greene and Allison, 1994) The results described in this chapter raise an important point which should be considered in virus-resistant transgenic plants assessments. Recombinant viruses generated in transgenic plants may be very stable and fitter than the parental viruses so it will be important to determine whether a virus derived transgene interacts with a second virus occurring in the field.

7.5 Conclusion

The results presented in this thesis indicated that Q-CMV produces three RNA2-derived RNA species during infection in addition to the known RNAs. A question which rises is why defective RNAs are only produced from RNA2?. In BBMV all of the characterised DI RNAs code for internally deleted 2a proteins. Furthermore since these shortened proteins retained the N-terminal portion analogous to the one involved in the interaction between 1a and 2a proteins, so truncated 2a proteins could compete with wild type 2a for binding with 1a and 2a protein and subsequently decrease the concentration of active replication complexes (Pogany et al., 1995).

Along these lines, Gordon and Symons (1985) showed a few RNA2-related translational products, which may correspond to the minor ss-RNAs. Therefore in the CMV system which is very similar to the BBMV system, it is possible that these defective RNAs or their defected proteins have some regulatory roles such as down-regulation of replication rate at the later stages of viral infection or regulation of the plus and minus strand RNA ratio.

7.6 Future work

1. The first priority should be to determine the sequences at the deletion junction sites in both the minor ss- and ds-RNAs. In other words, in order to define the nature of the minor RNAs, completion of their sequences is crucial.

2. To study the molecular basis of interference, determination of the exact nucleic acid sequence and proteins affecting these minor RNAs will be necessary. These studies will reveal a great deal about replication strategies of different viral nucleic acids and their specific regulatory mechanisms.

3. A third direction will be experiments designed to understand the possible role (s) of these RNAs in viral pathogenesis. It can be performed by construction of infectious cDNA clones of the minor RNA species which, together with the infectious cDNA clones of CMV, will allow the investigation of any functional roles of the minor RNAs in the CMV infection cycle.

4. The fourth direction of research will be the manipulation of these RNAs so that they may be used to inhibit ongoing viral infection.

Literature Cited

Aramburu, J., Navas-Castillo, J., Moreno, P., and Cambra, M. (1991). Detection of double-stranded RNA by ELISA and dot immunobinding assay using an antiserum to synthetic polynucleotides. *J. Virol. Methods.* **33,** 1-11.

Aryan, A. P., An, G. and Okita, T.W. (1991). Structural and functional analysis of promoter from gliadin, an endosperm-specific storage protein gene of *Triticum aestivum* L. *Mol. Gen. Genet.* **225,** 65-71.

Asamizu, T., Summers, D., Motica, M. B., Anzola, J. V., and Nuss, D. L. (1985). Molecular cloning and characterisation of the genome of wound tumor virus: A tumor inducing plant reovirus. *Virology* **144,** 398-409.

Bar Joseph, M., Rosner, A., Moscovitz, M., and Hull, R. (1983). A simple procedure for the extraction of double-stranded RNA from virus-infected plants. *J. Virol. Methods* **6,** 1-8.

Blanchard, C., Boyce, P. M., and Anderson, B. J. (1996). Cucumber mosaic virus RNA 5 is a mixed population derived from the conserved 3'-terminal regions of genomic RNAs 2 and 3. *Virology* **217**(2), 598-601.

Blencowe, J. W., and Caldwell, J. (1949). Aspermy-a new virus disease of the tomato. *Ann. Appl. Biol.* **36,** 320-326.

Boccard, F., and Baulcombe, D. (1993). Mutational analysis of cis-acting sequences and gene function in RNA 3 of cucumber mosaic virus. *Virology* **193,** 563-578.

Bouzoubaa, S., Niesbach-Klosgen, U., Jupin, I., Guilley, H., RichardS, K. and Jonard, G. (1991). Shortened forms of beet necrotic yellow vein virus RNA-3 and RNA-4: Internal deletions and a subgenomic RNA. *J. Gen. Virol.* **72,** 259-266.

Bujarski, J. J., and Dzianott, A. M. (1991). Generation and analysis of nonhomologous RNA-RNA recombinant in brome mosaic virus: sequence complementarities at crossover sites. *J. Virol.* **65,** 4153-4159.

Burgyan, J., F. Gerieco, and M. Russo. (1989). A defective interfering RNA molecule in cymbidium ringspot virus infections. *J. Gen. Virol.* **70,** 235-239.

Burgyan, J., Rubino, L. and Russo, M. (1991). *De novo* generation of cymbidium ring spot virus defective interfering RNA. *J. Gen. Virol.* **72,** 505-509.

Burman, A. J., Osman, T. A. M., and Buck, K. W. (1994). Detection of the 3a Protein of Cucumber Mosaic Virus in a Cell Wall Fraction from Infected Nicotiana clevelandii Plants. *Journal of Phytopathology* **142**(3-4), 317-323.

Candelier Harvey, P. R. Y. (1993). Cucumber mosaic virus genome is encapsidated in alfalfa mosaic virus coat protein expressed in transgenic tobacco plants. *Transgenic Research* **2**(5), 277-285.

Carr, J. P., Gal On, A., Palukaitis, P., and Zaitlin, M. (1994). Replicase-mediated resistance to cucumber mosaic virus in transgenic plants involves suppression of both virus replication in the inoculated leaves and long-distance movement. *Virology* **199**(2), 439-447.

Cashdollar, L. M., Esparza, J., Hudson, G. R., Chmelo, R., Lee, P. W. K., Joklik, W. K. (1982). Cloning the double-stranded genes of reoviru: Sequence of the cloned S2 gene. *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7644-7648.

Chen, B., and Francki, R. I. B. (1990). Cucumovirus transmission by the aphid *Myzus persicae* is determined solely by the viral coat protein. *J. Gen.Virol.* **71,** 939-944.

Chen, J., S. A. MacFarlane, and T. M. A. Wilson. (1994). Detection and sequence analysis of a spontaneous deletion mutant of soil-borne wheat mosaic virus RNA2 associated with increased symptom severity. *Virology* **202,** 921-929.

Coffin, R. S., and Coutts, R. H. A. (1992). DsRNA cloning and diagnosis of beet pseudo-yellows virus by PCR and nucleic acid hybridisation. *Intervirology* **33,** 197-203.

Cooper, B., Schmitz, I., Rao, A.L.N., Beachy, R.N. and Dodds, J.A. (1996). Cell-to-cell transport of movement-defective cucumber mosaic and tobacco mosaic viruses in transgenic plants expressing heterologous movement protein genes . *Virology* **216,** 208-213.

Dale, J. L., Philips, D. A., and Parry, J. N. (1986). Double-stranded RNA in banana plants with bunchy top disease. *J. Gen. Virol.* **67,** 371-375.

Dasgupta., R., and Kaesberg, P. (1977). Sequence of an oligonucleotide derived from the 3' end of each of the four brome mosaic viral RNAs. *Proc. Natl. Acad. Sci. U. S. A.* **74,** 4900-4904.

Davies, C., and Symons, R. H. (1988). Further implications for the evolutionary relationships between tripartite plant viruses based on cucumber mosaic virus RNA 3. *Virology* **165,** 216-224.

De Jong, W., and Ahlquist, P. (1992). A hybrid plant RNA virus made by transferring the noncapsid movement protein from a rod-shaped to an icosahedral virus is competent for systemic infection. *Proc. Natl. Acad. Sci. USA* **89,** 6808-6812.

Ding, B. A., Li, Q.B., Nguyen, L., Palukaitis, P. and Lucas, W.J. (1995). Cucumber mosaic virus 3a protein potentiate cell-to-cell trafficking of CMV RNA in tobacco plants . *Virology* **207,** 345-353.

Ding, S. W., Rathjen, J.P., Li, W.X., Swanson, R., Healy, H. and Symons, R.H. (1995a). Efficient infection from cDNA clones of cucumber mosaic cucumovirus RNAs in a new plasmid vector. *J. Gen.Virol.* **76,** 459-464.

Ding, S. W., Li, W.X. and Symons, R.H.. (1995b). A novel naturally occuring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J.* **14,** 5762-5772.

Ding, S. W., Anderson, B. J., Haase, H. R., and Symons, R. H. (1994). New overlapping gene encoded by the cucumber mosaic virus genome. *Virology* **198**(2), 593-601.

Ding, S. W., Shi, B. J., Li, W. X., and Symons, R. H. (1996). An interspecies hybrid RNA virus is significantly more virulent than either parental virus. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7470-7474.

Dodds, J. A., Morris, T. J., and Jordan, R. L. (1984). Plant viral double-stranded RNA. *Ann. Rev. Phytopathol.* **22,** 151-168.

Dodds, J. A., Lee, S. Q., and Tiffany, M. (1985). Cross protection between strains of cucumber mosaic virus: effects of host and type of inoculum on accumulation of virions and double-stranded RNA of the challenge strain. *Virology* **144,** 301-309.

Dodds, J. A. (1993). dsRNA in diagnosis. In "Diagnosis of plant vrus diseases" (R. E. F., Matthews, Eds.), pp. 273-294. CRC Press, Inc.

Dolja, V. V., and Atabekov, J. G. (1987). The structure of barley stripe mosaic virus double-stranded RNAs. *FEBS Lett.* **2,** 313-316.

Dougherty, W. G. (1983). Analysis of viral RNA isolated from tobacco leaf tissue infected with tobacco etch virus. *Virology* **131,** 473-481.

Duggal, R., Lahser, F.C. and Hall, T.C. (1994). Cis-acting sequences in the replication of plant viruses with plus-sense RNA genomes. *Annu. Rev. Phytopathol.* **32,** 287-309.

Dulieu, P., and Bar-Joseph, M. (1989). Rapid isolation of double stranded RNA segments from disulfide crosslinked polyacrylamide geles. *J. Virol Methods* **24,** 77-84.

Edwards, M. C., Gonsalves, D. and Provvidenti, R. (1983). Genetic analysis of cucumber mosaic virus in relation to host resistance: Location of determinants for pathogenicity to certain legumes and *Lactuca saligna*. *Phytopathology* **73,** 269-273.

Edwardson, J. R. and Christie, R.G. (1991). CRC Handbook of Viruses Infecting Legumes. *pp. 293-319. CRC Press, Boca Raton.*

Fernandez Cuartero, B., Burgyan, J., Aranda, M. A., Salanki, K., Moriones, E., and Garcia Arenal, F. (1994). Increase in the relative fitness of a plant virus RNA associated with its recombinant nature. *Virology* **203**(2), 373-377.

Fernandez-Cuartero, B., Burgyan, J., Aranda, M. A., Salanki, K., Moriones, E., and Garcia-Arenal, F. (1994). Increase in the relative fitness of a plant virus RNA associated with its recombinant nature. *Virology* **203,** 373-377.

Finch, J. T., Klug, A. and Van Regenmortel, M.H.V. (1967). The structure of cucumber mosaic virus. *J. Mol. Biol.* **24,** 303-305.

Finnen, R. L. a. R., D. M. (1993). Sequence and structure of defective interfering RNAs associated with cucumber necrosis virus infections. *J. Gen. Virol.* **74,** 1715-1720.

Florentz, C., Briand, J. P., Romby, P., Hirth, L., Eble, J. P., and Giege, R. (1982). . *EMBO J.* **1,** 269-276.

Fraile, A., Alonso-Prados, J. L., A., A. M., Bernal, J. J., Malpica, J. M., and Garcia-Arenal, F. (1997). Genetic exchange by recombination or reassortment is infrequent in natural populations of a tripartite RNA plant virus. *J. Virol.* **71,** 934-940.

Fraile, A., Garcia Arenal, F., and Palukaitis, P. (1993). In vitro interactions between cucumoviral satellite RNAs and RNAs of helper and non-helper viruses. *Phys. Mol. Plant Pathol.* **42**(3), 195-204.

Francki, R. I. B. (1964). Inhibition of cucumber mosaic virus infectivity by leaf extracts. *Virology* **21,** 193-199.

Francki, R. I. B., Randles, J.W., Chambers, T.C. and Wilson, S.B. (1966). Some properties of purified cucumber mosaic virus (Q strain). *Virology* . **28,** 729-741.

Francki, R. I. B., Mossop, D.W. and Hatta, T. (1979). CMI/AAB Description of Plant Viruses No 213. Commonwealth Agricultural Bureau, Kew, Surrey, England.

Francki, R. I. B., Milne, R.G. and Hatta, T. (1985). An Atlas of Plant Viruses. *pp. 53-68. CRC Press, Florida.*

Francki, R. I. B., Fauquet, C. M., Knudson, D. L., and Brown, F. (1991). Classification and Nomenculture of Viruses. Fifth report of the International Committee on Taxanomy of Viruses, *Arch. Virol. Suppl.* 2., Springer Verlag, New York, 1991, 450.

Franklin, R. M. (1996). Purification and properties of replicative intermediates of the RNA bacteriophage R17. *Proc. Natl. Acad. Sci. U. S. A.* **55,** 1504-1511.

French, R., Janda, M. and Ahlquist, P. (1986). Bacteria gene inserted in an engineered RNA virus: efficient expression in monocotyledonous plant cells . *Science* **231,** 1294-1297.

Frohman, M. A. (1990). RACE: Rapid amplification of cDNA ends. *In* "PCR Protocols" (M. A. Innes, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 28-38. Academic Press, San Diego.

Froussard, P. (1992). A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. *Nucleic Acids Res.* **20,** 2900.

Gal On, A., Kaplan, I., and Palukaitis, P. (1995). Differential Effects of Satellite RNA on the Accumulation of Cucumber Mosaic Virus RNAs and Their Encoded Proteins in Tobacco vs Zucchini Squash with Two Strains of CMV helper virus. *Virology* **208**(1), 58-66.

Gal-On, A., Kaplan, I., Roossinck, M.J. and Palukaitis, P. (1994). The kinetics of infection of zucchini squash by cucumber mosaic virus indicate a function for RNA 1 in virus movement. *Virology* **205,** 280-289.

German, S., Candresse, T., Le Gall, O., Lanneau, M., and Dunez, J. (1992). Analysis of the dsRNAs of apple chlorotic leaf spot virus. *J. Gen. Virol.* **73,** 767-773.

Ghaffari, S. H., Dumbar, T. S., Wallace, M. O., and Olson, M.O.J. (1988). Isolation of proteins for peptide mapping, amino acid analyses, and sequencing using disulfide crosslinked polyacrylamide gels. *Anal. Biochem.* **171,** 352-359.

Gordon, K. H. J., and Symons, R. H. (1985). Subgenomic RNA with nucleotide sequences derived from RNA 1 and RNA 2 of cucumber mosaic virus can act as messenger RNA in vitro. *Virology* **142**(1), 144-157.

Graves, M. V., and Roossinck, M. J. (1995). Characterization of defective RNAs derived from RNA 3 of the Fny strain of cucumber mosaic cucumovirus. *J. Virol.* **69**(8), 4746-4751.

Green, A. E., and Alison, R. F. (1994). Recombination between viral RNA and transgenic plant transcripts. *Science* **263,** 1423-1425.

Gubler, U., and Hoffman, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25,** 263-269.

Habili, N., and Francki, R. I. B. (1974a). Comparative studies on tomato aspermy and cucumber mosaic viruses. I. Physical and chemical properties. *Virology* **. 57,** 392-401.

Habili, N., and Francki, R. I. B. (1974b). Comparative studies on tomato aspermy and cucumber mosaic viruses. III. Further studies on relationship and construction of a virus from parts of the two viral genomes. *Virology* **61,** 443-449.

Habili, N., and Rezaian, M. A. (1995). Cloning and Molecular Analysis Of Double-Stranded Rna Associated With Grapevine Leafroll Disease. *Annals of Applied Biology* **127**(1), 95-103.

Hampton, R. O., and Francki, R. I. B. (1992). RNA-1 dependent seed transmissibility of cucumber mosaic virus in *Phaseolus vulgaris*. *Phytopathology* **82,** 127-130.

Hanada, K., and Tochihara, H. (1980). Genetic analysis of cucumber mosaic, peanut stunt and chrysanthemum mild mottle viruses . *Ann. Phytopath. Soc. Japan* **46,** 159-168.

Harrison, B. D., Finch, J.T., Gibbs, A.J., Hollings, M., Shephered, R.J., Valenta, V. and Wetter, C. (1971). Sixteen groups of plant viruses. *Virology* **45,** 356-363.

Harrison, S. C. (1984). Multiple modes of subunit association in the structures of simple spherical viruses. *Trends Biochem. Sci.* **9,** 345-351.

Hayes, R. J., Pereira, V.C.A., Mcquillin, A. and Buck, K.W. (1994). Localisation of functional regions of the cucumber mosaic virus RNA replicase using monoclonal and polyclonal antibodies. *J. Gen. Virol.* **75,** 3177-3184.

Hayes, R. J., and Buck, K. W. (1990). Infectious cucumber mosaic virus RNA transcribed in vitro from clones obtained from complementary DNA amplified using the polymerase chain reaction. *J. Gen. Virol.* **71**(11), 2503-2508.

Hilf, M. E., and Dawson, W. O. (1993). The tobamovirus capsid protein functions as a host-specific determinant of long-distance movement. *Virology* **193,** 106-114.

Hillman, B. I., Carrington, J.C. and Morris, T.J. (1987). A defective interfering RNA that contains a mosaic of a plant virus genome. *Cell* **51,** 427-433.

Holland, J. J. (1990). Defective viral genomes. *In* "Virology" (B. N. F. a. D. M. Knipe, Ed.), pp. 151-165. Raven Press, New York.

Hollings, M., and Stone, O. M. (1971). C.M.I./A.A.B. Descriptions of Plant Viruses, No. 79. 4pp. Commonwealth Agricultural Bureau, Kew, Surrey, England.

Huang, A. S. (1988). Modulation of viral disease processes by defective interfering particles. *In* "RNA Genetics" (E. Domingo, J. J. Holland, and P. Ahlquist, Eds.), Vol. 3, pp. 195-208. CRC press, Boca Raton, FL.

Huang, A. S. (1997). Viral pathogenesis and molecular biology. *Bacteriol. Rev.* **41,** 811-821.

Imai, M., Richardson, M. A., Ikegami, N., Shatkin, A. J., and Furuichi, Y. (1983). Molecular cloning of double-stranded RNA virus genomes. *Proc. Natl. Acad. Sci. U. S. A.* **80,** 373-.

Ishikawa, M., Meshi, T., Motoyoshi, F., Takamatsu, N. and Okada, Y. (1986). *In vitro* mutagenesis of the putative replicase genes of tobacco mosaic virus . *Nucleic Acids Res.* **21,** 8291-8305.

Jelkmann, W. (1995). Cherry virus A: cDNA cloning of dsRNA, nucleotide sequence analysis and serology reveal a new plant capillovirus in sweet cherry. *J. Gen. Virol.* **76,** 2015-2024.

Jelkmann, W., Martin, R. R., and Maiss, E. (1989). Cloning of four plant viruses from small quantities of double-stranded RNA. *Phytopathology* **79**(11), 1250-1253.

Johnson, K. L., and Sarnow, P. (1991). Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent domonance over wild-type poliovirus. *J. Virol.* **65,** 4341-4349.

Jorda, C., Alfaro, A., Aranda, M. A., Moriones, E., and Garcia Arenal, F. (1992). Epidemic of cucumber mosaic virus plus satellite RNA in tomatoes in eastern Spain. *Plant Disease* **76**(4), 363-366.

Joshi, R. L., and Haenni, A. L. (1986). Search for tRNA-like properties in tomato aspermy virus RNA. *FEBS Lett.* **194,** 157-160.

Kaper, J. M., Dienner, T.O. and Scott, H.A. (1965). Some physical and chemical properties of cucumber mosaic virus (strain Y) and its isolated ribonucleic acid. *Virology* **27,** 54-72.

Kaper, J. M. (1973). Arrangement and identification of simple isometric viruses according to their dominating stabilising interactions. *Virology* . **55,** 299-304.

Kaper, J. M. (1975). The Chemical Basis of Virus Structure, Dissociation and Reassembly. pp. 485. Elsevier/North-Holland Press, New York.

Kaper, J. M., Gallitelli, D. and Tousignant, M. E. (1990). Identification of a 334-ribonucleotide viral sattelite as principal aetiological agent in a tomato necrosis epidemic. *Research in Virology* **141,** 81-95.

Kaper, J. M., Geletka, L.M., Wu, G.S. and Tousignant, M.E. (1995). Effect of temperature on cucumber mosaic virus satellite-induced lethal tomato necrosis is helper virus dependent. *Arch. Virol.* **140,** 65-74.

Kaper, J. M., and Collmer, C. W. (1988). Modulation of viral plant diseases by secondary RNA agents. In RNA genetics, Vol. III: Variability of RNA genomes, eds. Domingo, E., Holland, J. & Ahlquist, P. pp. 171-194. Boca Raton, FL. CRC Press. Inc.

Kaper, J. M., and Geelen, J. L. M. C. (1971). Studies on the stabilising forces of simple RNA viruses. II Stability, dissociation and reassembly of cucumber mosaic virus. *J. Mol. Biol.* **56,** 277-294.

Kaper, J. M., and Waterworth, H. E. (1977). Cucumber mosaic virus associated RNA 5: causal agent for tomato necrosis. *Science* **196,** 429-431.

Kaper, J. M., and Waterworth, H. E. (1981). Cucumoviruses. Handbook of Plant Virus Infections and Comparative Diagnosis (E. Kurstak, Ed.), Elsevier/North-Holland Press, New York.

Kaplan, I. B., Shintaku, M. H., Li, Q., Zhang, L., Marsh, L. E., and Palukaitis, P. (1995). Complementation of virus movement in transgenic tobacco expressing the cucumber mosaic virus 3a gene. *Virology* **209**(1), 188-199.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* **12,** 857-872.

Lai, M. M. C. (1992). RNA recombination in animal and plant viruses. *Microbiol. Rev.* **56,** 61-79.

Lakshman, D. K., and Gonsalves, D. (1985). Genetic analyses of two large-lesion isolates of cucumber mosaic virus. *Phytopathology* **75,** 758-762.

Lazzarini, R. A., Keene, J. D., and Schubert, M. (1981). The origins of defective interfering particles of the negative-strand RNA viruses. *Cell* **26,** 145-154.

Le Gall, O., Candresse, T., and Dunez, J. (1988). Nucleotide sequence of the double-stranded RNAs of grapevine chrome mosaic nepovius. *J. Gen. Virol.* **69,** 423-428.

Levis, R., Weiss, B. G., Tsiang, M., Huang, H., and Schlesinger, S. (1986). Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. *Cell* **44,** 137-145.

Lewin, B. (1990). "Genes." (4, Ed.) Oxford University Press, Oxford.

Li, Q., and Palukaitis, P. (1996). Comparison of the nucleic acid- and NTP-binding properties of the movement protein of cucumber mosaic cucumovirus and tobacco mosaic tobamovirus . *Virology* **216,** 71-79.

Li, X. H., Heaton, L.A., Morris, T.J. and Simon, A.E. (1989). Turnip crinkle virus defective interfering RNAs intensify viral symptoms and are generated *de novo*. *Proc. Natl. Acad. Sci. USA* **86,** 9173-9177.

Lin, N. S., Hsu, Y. H., and Hsu, H. T. (1990). Immunological detection of plant viruses and a mycoplasmalike organism by direct tissue blotting on nitocellulose membranes. *Phtopathology* **80,** 824-828.

Lopez, S., Yao, J-S., Kuhn, R.J., Strauss, E.G. and Strauss, J.H. (1994). Nucleocapsid-glycoprotein interactions required for assembly of alphaviruses. *J. Virol.* **68,** 1316-1323.

Lot, H., Marrou, J., Quiot, J.B. and Esvan, C. (1972). Contribution a l'étude du virus de la mosaique du concombre (CMV). II. Méthode de purification rapide du virus. *Ann. Phytopathol.* **4,** 25-38.

Lot, H., Marchoux, G., Marrou, J., Kaper, J.M., West, C.K., van Vloten-Doting, L. and Hull, R. (1974). Evidence for three functional RNA species in several strains of cucumber mosaic virus. *J. Gen.Virol.* **22,** 81-93.

Manohar, S. K., Guilley, H., Dollet, M., Richards, K., and Jonard, G. (1993). Nucleotide sequence and genetic organisation of peanut clump virus RNA2 and partial characterisation of deleted forms. *Virology* **195,** 33-41.

Marchoux, G., Marrou, J. and Quiot, J. B. (1974). Complementation entre ARN de differentes souches du virus de la mosaique du concomber. Mise en evidence d'une interaction entre deux ARN pour determiner un type de symptom. *Compt rendus hebdomadaires des seances de l'Academie des scinces, Serie D* **278,** 1943-1946.

Marsh, L. E., Pogue, G. P., Connell, J. P., and Hall, T.C. (1991). Artificial defective interfering RNAs derived from brom mosaic virus. *J. Gen. Virol.* **72,** 1787-1792.

Matthews, R. E. F. (1991). "Plant Virology.", 3rd ed., Academic Press, San Diego, California.

Mawassi, M., Gafny, R., Gagliardi, D., and Barjoseph, M. (1995). Populations Of citrus tristeza virus contain smaller-than-full-length particles which encapsidate sub-genomic RNA molecules. *J. Gen. Virol.* **76,** 651-659.

McClure, B. A., and Guilfoyle, T. J. (1989). Tissue print hybridisation. A simple technique for detecting organ- and tissue-specific gene expression. *Plant Mol. Biol.* **12,** 517-524.

McGarvey, P. B., Montasser, M. S., and Kaper, J. M. (1994). Transgenic tomato plants expressing satellite RNA are tolerant to some strains of cucumber mosaic virus. *Journal of the American Society for Horticultural Science* **119,** 642-647.

Moriones, E., Roossinck, M.J. and Garcia-Arenal, F. (1991). Nucleotide sequence of tomato aspermy virus RNA 2. *J.Gen.Virol.* **72,** 779-783.

Morris, T. J., Dodds, J. A. (1983). Double-stranded RNA: Fnctional role in RNA virus infection. In Plant Infectious Agents, ed. H. E. Robertson et al, pp. 80-83. Cold Springer Harbor;Cold Spring Harbor Labs.

Mossop, D. W., and Francki, R. I. B. (1977). Association of RNA 3 with aphid transmission of cucumber mosaic virus. *Virology* **81,** 177-181.

Nassuth, A. a. B., J.F. (1983). Altered balance of the synthesis of plus- and minus-strand RNAs induced by RNAs 1 and 2 of alfalfa mosaic virus in the absence of RNA 3. *Virology* **124,** 75-85.

Nayak, D. P., Chambers, T. M., and Akkina, R. K. (1985). Defective-interfering (DI) RNAs of influenza viruses: origin, structure, expression, and interference. *Curr. Top. Microbiol. Immunol.* **114,** 103.

Nitta, N., Takanami, Y., Kuwata, S. and Kubo, S. (1988). Comparative studies on the nucleotide sequence of cucumber mosaic virus RNA 3 between Y strain and Q strain. *Ann. Phytopathol. Soc. Japan* **54,** 516-522.

Nuss, D. L., and Dall, D. J. (1990). Structural and functional properties of plant reovirus genomes. *Adv. Virus Res.* **38,** 249-306.

Owen, J., and Palukaitis, P. (1988). Characterisation of cucumber mosaic virus: I. molecular heterogeneity mapping of RNA 3 in eight CMV strains. *Virology* **166,** 495-502.

Palukaitis, P., Roossinck, M.J., Dietzgen, R.G. and Francki, R.I.B. (1992). Cucumber mosaic virus. *Adv.Virus Res.* **53,** 487-492.

Palukatis, P., Garcia-Arenal, F., Sulzinski, M. A., and Zaitlin, M. (1983). Replication of tobacco mosaic virus. VII. Further characterisation of single- and double-stranded virus related RNAs from TMV-infected plants. *Virology* **131,** 533-545.

Pares, R. D., Gillings, M. R., and Gunn, L. V. (1992). Differentiation of biologically distinct cucumber mosaic virus isolates by PAGE of double-stranded RNA. *Intervirology* **34**(1), 23-29.

Pattnaik, A. K., LeGrone, A. W., and Wertz, G. W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69,** 1011-1020.

Peden, K. W. C., and Symons, R. H. (1973). Cucumber mosaic virus contains a functionally divided genome. *Virology* **53,** 487-492.

Peng, D., Koetzner, C.A., McMahon, T., Zhu, Y. and Masters, P.S. (1995). Construction of murine coronavirus mutants containing interspecies chimeric nucleocapsid proteins. *J. Virol.* **69,** 5475-5484.

Perrault, J. (1981). Origin and replication of defective interfering particles. *Curr. Top. Microbiol. Immunol.* **93,** 151.

Perry, K. L., Zhang, L., Shintaku, M.H. and Palukaitis, P. (1994). Mapping determinants in cucumber mosaic virus for transmission by *Aphis gossypii*. *Virology* **205,** 591-595.

Pleij, C. W. A., Abrahams, J.P., Van Belkum, A., Rietveld, K. and Bosch, L. (1987). The spatial folding of the 3' noncoding region of aminoacylatable plant viral RNAs. *In* "Positive Strand RNA Viruses." (M. Brinton and R. Rueckert, Eds),, pp. 299-316. Liss Press, New York.

Pleij, C. W. A. (1995). Structure and function of RNA pseudoknots. *In* "Genetic Engineering." (J.K. Setlow, Ed.), pp. 67-80. Plenum Press, New York.

Pogany, J., Romero, J., Huang, Q., Sgro, J., Shang, H., and Bujarski, J. (1995). De novo generation of defective interfering-like RNAs in broad bean mottle Bromovirus. *Virology* **212,** 574-586.

Rao, A. L. N., and Francki, R. I. B. (1981). Comparative studies on tomato aspermy and cucumber mosaic viruses. VI. partial compatibility of genomes from the two viruses. *Virology* **114,** 573-575.

Rao, A. L. N., Hatta, T., and Francki, R. I. B. (1982). Comparative studies on tomato aspermy virus and cucumber mosaic virus: 7. Serological relationships reinvestigated. *Virology* **116**(1), 318-326.

Resende, R. D. O., De Haan, P., Van De Vossen, E., Avila, A. C. D., Goldbach, R., and Peters, D. (1992). Defective interfering L RNA segments of tomato spotted wilt virus retain both virus genome termini and have extensive internal deletions. *J. Gen. Virol.* **73,** 2509-2516.

Resnede, R. O., De Avila, A. C., Kitajima, E. W., Kormelink, R., Goldbach, R., and Peters, D. (1991). Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. *J. Gen. Virol.* **72,** 2375-2383.

Rezaian, M. A., Williams, R.H.V. and Symons, R.H. 1985. (1985). Nucleotide sequence of cucumber mosaic virus RNA 1: Presence of a sequence complimentary to part of the viral satellite RNA and homologies with other viral RNAs. *Eur. J. Biochem.* **150,** 331-339.

Rezaian, M. A., Krake, L. R., Cunying, Q., and Hazzalin, C. A. (1991). Detection of virus-associated dsRNA from leafroll infected grapevines. *J. Virol. Methods.* **31,** 325-334.

Rezaian, M. A., and Symons, R. H. (1986). Anti-sense regions in satellite RNA of cucumber mosaic virus from stable complex with the viral coat protein gene. *Nucleic Acids Res.* **14,** 3229-3239.

Rezaian, M. A., Williams, R. H. V., Gordon, K. H. J., Gould, A. R., and Symons, R. H. (1984). Nucleotide sequence of cucumber mosaic virus RNA 2 reveals a translation product significantly homologous to corresponding proteins of other viruses. *Eur. J. Biochem.* **143,** 277-284.

Rizos, H., Gunn, L. V., Pares, R. D., and Gillings, M. R. (1992). Differentiation of cucumber mosaic virus isolates using the polymerase chain reaction. *J. Gen. Virol.* **73,** 2099-2103.

Rizzo, T. M., and Palukaitis, P. (1988). Nucleotide sequence and evolutionary relationships of cucumber mosaic virus (CMV) strains: CMV RNA 2. *J. Gen. Virol.* **69**(8), 1777-1788.

Rizzo, T. M., and Palukaitis, P. (1989). Nucleotide sequence and evolutionary relationships of cucumber mosaic virus (CMV) strains: CMV RNA 1. *J. Gen. Virol.* **70,** 1-11.

Romero, J., Huang, Q., Pogany, J., and Bujarski, J. J. (1993). Characterization of defective interfering RNA components that increase symptom severity of broad bean mottle virus infections. *Virology* **194,** 576-583.

Roossinck, M. J. (1991). Temperature-sensitive replication of cucumber mosaic virus in muskmelon (*Cucumis melo*, cultivar Iroquois), maps to RNA 1 of a slow strain . *J. Gen. Virol.* **72,** 1747-1750.

Roossinck, M. J., Sleat, D. and Palukaitis, P. (1992). Satellite RNAs of plant viruses: Structures and biological effects . *Microbiol. Rev.* **56,** 265-279.

Roossinck, M. J., and Palukaitis, P. (1990). Rapid induction and severity of symptoms in zucchini squash (*Cucurbita pepo*) map to RNA 1 to cucumber mosaic virus. *Mol. Plant-Microbe Interact.* **3,** 188-192.

Roux, L., Simon, A. E., and Holland, J. J. (1991). Effects of defective interfering viruses on virus replication and pathogenesis *in vitro* and *in vivo*. *Adv. Virus Res.* **40,** 181-211.

Ryu, K. H., and Park, W. M.,. (1995). Complementary DNA hybridisation analysis of double-stranded RNAs associated with cucumber mosaic virus-As strain. *J. Phytopathology* **143,** 683-687.

Sackey, S. T., and Francki, R. I. B. (1990). Interaction of cucumoviruses in plants: Persistence of mixed infections of cucumber mosaic and tomato aspermy viruses. *Physio. Mol. Plant Pathol.* **36**(5), 409-420.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbour Laboratory,, Cold Spring Harbour, New York.

Schwinghamer, M. W. a. S., R.H. (1975). Fractionation of cucumber mosaic virus RNA and its translation in a wheat embryo cell-free system. *Virology* **63,** 252-262.

Scott, H. (1963). Purification of cucumber mosaic virus. *Virology* **20,** 103-106.

Shi, B. J., DING, S.W., and Symons, R. H. (1997). *In vivo* expression of an overlapping gene encoded by the cucumoviruses. *J.Gen.Virol.* **78,** 237-241.

Shintaku, M. H., Zhang, L., and Palukaitis, P. (1992). A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. *Plant Cell* **4**(7), 751-757.

Skingle, D. C., McInnes, J. L., and and Symons, R. H. (1990). An improved method for eliminating RNA contaminating of plasmid DNA preparations. *BioTechniques* **9,** 314-317.

Sleat, D. E., Zhang, Li and Palukaitis, P. (1994). Mapping determinants within cucumber mosaic virus and its satellite RNA for the induction of necrosis in tomato plants. *Mol. Plant-Microbe Interact.* **7,** 189-195.

Smith, G. R., van de Velde, R., and Dale, J. L. (1992). PCR amplification of a specific double-stranded RNA region of Fiji disease virus from diseased sugarcane. *J. virol. Methods* **39,** 237-246.

Smith, O. P., Hunst, P. L., Hewings, A. D., Stone, A. L., Tolin, S. A., and Damsteegt, V. D. (1991). Identification of dsRNAs associated with soybean dwarf virus-infected soybean. *Phytopathology* **81,** 131-134.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503-517.

Sun, J., and Kao, C. C. (1995). Double-stranded RNA is nonspecifically bound by agarose. *Anal. Biochem.* **230,** 185-187.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N. and Takanami, T. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA 3 using an *in vitro* transcription system . *Virology* **183,** 106-113.

Suzuki, M., Kuwata, S., Masuta, C., and Takanami, Y. (1995). Point mutations in the coat protein of cucumber mosaic virus affect symptom expression and virion accumulation in tobacco. *J. Gen. Virol.* **76,** 1791-1799.

Symons, R. H. (1975). Cucumber mosaic virus RNA contains 7-methylguanosine at the 5'-terminus of all four RNA species. *Mol. Biol. Rep.* **2,** 277-285.

Symons, R. H. (1978). The two-step purification of ribosomal RNA and plant viral RNA by polyacrylamide slab gel electrophoresis. *Aust. J. Biol. Sci.* **31,** 25-37.

Takahashi, H., Goto, N and Ehara, Y. (1994). Hypersensitive response in cucumber mosaic virus-inoculated *Arabidopsis thaliana. Plant J.* **6,** 369-377.

Takahashi, H., and Ehara, Y. (1993). Severe chlorotic spot symptoms in cucumber mosaic virus strain Y-infected tobaccos are induced by a combination of the virus coat protein gene and two host recessive genes. *Mol. Plant-Microbe Interact.* **6,** 182-189.

Taliansky, M. E., and Garcia Arenal, F. (1995). Role of cucumovirus capsid protein in long-distance movement within the infected plant. *J. Virol.* **69**(2), 916-922.

Tenllado, F., Garcia-Luque, I., Serra, M. T., and Diaz-Ruiz. (1995). Nicotiana benthamiana transformed with the 54-KDa region of the pepper mild mottle tobamovirus replicase gene exhibit two types of resistance responses against viral infection. *Virology* **211,** 170-183.

Tolin, S. A. (1977). Cucumovirus (cucumber mosaic virus) group. In "The Atlas of Insect and Plant Viruses: Including Mycoplasma Viruses and Viroids." (K. Maramorosch, Ed.), pp. 303-307. Academic Press, New York.

Turner, D. R., Lorie, E.J. and Butler, P.J.G. (1988). The tobacco mosaic virus assembly origin RNA: Function characteristics defined by directed mutagenesis. *J. Mol. Biol.* **203,** 531-547.

Van der Vossen, E. A. G., Neeleman, L. and Bol, J.F. (1993). Role of the 5' leader sequence of alfalfa mosaic virus RNA 3 in replication and translation of the viral RNA. *Nucleic Acids Res.* **21,** 1361-1367.

Vaquero, C., Turner, A. P., Demangeat, C., Sanz, A., Serra, M. T., Roberts, K., and Garcia Luque, I. (1994). The 3a protein from cucumber mosaic virus increases the gating capacity of plasmodesmata in transgenic tobacco plants. *J. Gen. Virol.* **75,** 3193-3197.

Verwoerd, T. C., Dekker, B.M.M. and Hoekema, A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* **17,** 2362.

Wang, W. Q., Natsuaki, T., and Okuda, S. (1988). Comparison of cucumber mosaic virus isolates by double-stranded RNA analysis. *Ann. Phytopath. Soc. Japan* **54,** 536-539.

Weiland, J., and Dreher, T. W. (1993). Cis-preferential replication of the turnip yellow mosaic virus RNA genome. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6095-6099.

Weiss, B., H. Nitschko, I Ghattas, R. Wright, and S. Schlesinger. (1989). Evidence for specifity in the encapsidation of Sindbis virus RNAs. *J. Virol.* **63,** 5310-5318.

White, K. A., Bancroft, J.B. and Mackie, G.A. (1991). Defective RNAs of clover yellow mosaic virus encode nonstructural/coat protein fusion products. *Virology* **183,** 479-486.

White, K. A., and Morris, T. J. (1994). Nonhomologous RNA recombination in Tombusviruses: generation and evolution of defective interfering RNAs by stepwise deletions. *J. Virol.* **68,** 14-24.

Wilson, P. A., and Symons, R. H. (1981). The RNAs of Cucumoviruses: 3'-terminal sequence analysis of two strains of tomato aspermy virus. *Virology* **112,** 342-345.

Wood, K. R. (1991). Organisation and expression of the genome of cucumber mosaic virus. *In* "Biochemistry and Molecular Biology of Plant-Pathogen Interactions." (C.J. Smith, Ed.).pp. 26-49. Clarendon Press, Oxford.

Wu, G., and Kaper, J. M. (1995). Competition of viral and satellite RNAs of cucumber mosaic virus for replication in vitro by viral RNA-dependent RNA polymerase. *Research in Virology* **146,** 61-67.

Zelcer, A., Weaber, K. F., Balazs, E., and Zaitlin, M. (1981). The detection and characterization of viral-related double-stranded RNAs tobacco mosaic virus-infected plants. *Virology* **113,** 417-427.

Zhang, L., Zitter, T.A., and Palukaitis, P. (1991). Maize white line mosaic virus double-stranded RNA, replication structure, and *in vitro* translation product analysis. *Phytopathplogy* **81,** 1875-.

Zitter, T. A., and Gonsalves, D. (1991). Differentiation of pseudorecombinants of two cucumber mosaic virus strains by biological properties and aphid transmission . *Phytopathology* **81,** 139-143.

Appendix

Media recipies for protoplasts experiment (Section 6.2.4)

CS V Medium components

	Per litre stock
CS Macro (20x)	50 ml
CS Micro (100x)	1 ml
CS Iron (1000x)	1 ml
CS Organic (1000x)	1 ml
Sucrose	30 g
myoinositol	1 g
2,4-D (0.1mg/ml)	20 ml
Kinetin (0.1mg/ml)	500 µl

Adjust pH to 5.8 with KOH and autoclave

CS Stocks

CS Macro (20x)

	Per litre stock
NH_4NO_3	24.8 g
KNO_3	50.1 g
$(NH_4)\ H_2PO_4$	9.2 g
$CaCl_2.H_2O$	4.0 g
$MgSO_4.7H_2O$	8.0 g

CS Oraganics (1000x)

	Per 500 ml stock
Thiamine HCl	1000 mg
Nicotianic acid	1000 mg
Pyridoxinic acid	1000 mg

CS Micro (1000x)

	Per 500 ml stock
$MnSO_4$	6.5 g
H_3BO_3	2.5 g
$ZnSO_4.7H_2O$	0.5 g
KI	0.5 g
$CuSO_4.5H_2O$	100 mg
$Na_2MoO_4.2H_2O$	50 mg
$CoCl_2.6H_2O$	50mg

CS Iron (1000x)

	Per 100 ml stock
Na_2EDTA	2.0 g
$FeSO_4.7H_2O$	1.5 g