ENCAPSIDATION OF NUCLEIC ACIDS BY CUCUMOVIRUS COAT PROTEINS

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Summary

An improved method with high efficiency for reassembly of both CMV and TAV was developed and physico-chemical, serological, and biological analyses showed that the reassembled particles were indistinguishable from the native viruses.

In vitro reassembly of CMV (T strain, TCMV) particles with separated CMV RNA species demonstrated that four types of particles encapsidating different RNA content were indistinguishable both in morphology and sedimentation rate in sucrose density gradients from native TCMV. These particles encapsidated: 1) one molecule of RNA 1; 2) one molecule of RNA 2; 3) one molecule each of RNAs 3 and 4; and 4) 3-4 molecules of RNA 4, respectively.

When heterologous virus particles were assembled with coat protein from the V strain of TAV (VTAV) or M strain of CMV (MCMV), all particles were cucumovirus-like, irrespective of RNAs from tobacco mosaic virus (TMV), velvet tobacco mottle virus (VTMoV), galinsoga mosaic virus (GMV), MCMV and VTAV. They were all infectious and able to induce symptoms characteristic of the encapsidated viral RNAs. The assembled particles with VTAV coat and TMV RNA sedimented faster than VTAV in sucrose density gradient.

Coat proteins of VTAV and TCMV encapsidated *in vitro* a range of single-stranded RNAs or DNAs, which were either linear or circular. The minimum length required for encapsidation was about 35 but not 20 nucleotides and the maximum length was possibly less than 7200 nucleotides. Double-stranded RNA and DNA were not encapsidated. The heterologously assembled particles retained the size of 28-30 nm in diameter. VTAV coat protein preferred homologous VTAV RNA to heterologous TMV RNA in competitive assembly.

Particles with mixed capsid were assembled when coat proteins of VTAV and TCMV were mixed and assembled with VTAV RNA. These particles reacted with antisera to VTAV and TCMV.

In experiments to investigate the encapsidation of satellite RNA in cucumovirus particles, native B1 satellite RNA (B1 satRNA) of CMV was encapsidated by either VTAV or TCMV coat proteins *in vitro* . The assembled particles were cucumovirus-like and had the same sedimentation rate as native TCMV. Assembly of ^{32}P-labelled satellite transcripts in the presence of viral genomic RNAs of VTAV with VTAV protein showed that satRNA was co-

encapsidated with the viral genomic RNA. The coat protein of TCMV was better than that of VTAV in encapsidating satRNA into cucumovirus-like particles.

The aphid transmissibility of VTAV and MCMV was shown to be determined solely by the properties of their coat proteins. The non aphid-transmissible viruses, TMV and MCMV were readily transmitted by the aphid *Myzus persicae* when their RNAs were encapsidated by coat protein from the aphid-transmissible VTAV. Conversely, aphids could not transmit infection with particles reassembled from coat protein of the non aphid-transmissible MCMV, and RNAs from VTAV and TMV.

In an experiment done to determine whether the non aphid-transmissible VTMoV could be transmitted by aphids from plants co-infected with VTMoV and VTAV, a low rate of transmission was observed. However, no transmission was found in three other subsequent experiments conducted. On the other hand, satellite RNA of VTMoV was transmitted by aphids in a relatively higher rate when the plants co-infected with VTAV and VTMoV plus its satellite RNA were used as virus sources.

STATEMENT

This thesis contains no material which has been previously presented for any other degree or diploma in any university and to the best of my knowledge and belief, does not contain material previously published or written by another person, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

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Chapter 1 General Introduction

The following review covers literature up to 1989 when the research project described in this thesis commenced.

1. Cucumovirus group

Cucumoviruses are icosahedral particles, 28-30 nm in diameter, with a tripartite genome (Francki, 1985b). Members of this group include cucumber mosaic virus (CMV), the type member, with cryptogram R/1:1.3/18+1.1/18+(0.8+0.3)/18: S/S: S/C,Ve/Ap (Francki *et al.*, 1979), tomato aspermy virus (TAV) (Hollings and Stone, 1971) and peanut stunt virus (PSV) (Mink, 1972). In addition, cowpea ringspot virus (CPRSV) has been included as a possible member (Matthews, 1982). Two other viruses, black locust true mosaic and soybean stunt viruses are also listed as distinct cucumoviruses (Boswell and Gibbs, 1983). However, the status of these two viruses has been questioned since it seems that some previously published data do no favour it (Francki, 1985b; Richter *et al.*, 1979; Hanada and Tochihara, 1982).

The three formal members are all distantly related serologically (Devergne and Cardin, 1975; Devergne *et al.*, 1981) but do not appear to have any significant base sequence homologies between their respective RNAs (Gonda and Symons, 1978; Piazzolla *et al.*, 1979). The affinities of CPRSV with the approved members are not yet clear (Phatak *et al.*, 1976).

2. Genome structure of CMV

Of the cucumovirus group, CMV (Q strain, Q-CMV) is the only virus whose genome sequence has been completely determined (Davies and Symons, 1988; Rezaian *et al.*, 1984, 1985). In Q-CMV, RNA 1 and 2 consist of 3389 and 3035 nucleotides (nt), which have open reading frames (ORF) of 2973 and 2517 nt, repectively (Rezaian *et al.*, 1985, 1984); RNA 3 consists of 2197 nt with two ORFs, the 3a (837 nt) and coat protein gene (654 nt) (Davies and Symons, 1988). RNA 4 (1030 nt) (Davies and Symons, 1988) is transcribed from RNA 3 and identical to the 3' half of the RNA 3. This subgenomic RNA serves as mRNA for coat protein translation (Gould and Symons, 1982). For infection, only the three largest genomic RNAs are required (Peden and Symons, 1973; Lot *et al.*, 1974). Genetic mapping studies with

several strains of CMV have indicated that RNA 1 may be involved mainly in the regulation of viral RNA synthesis while RNA 2 is involved in replication as well as in determining pathogenicity and host range (Marchoux *et al.*, 1974; Hanada and Tochihara, 1980; Rao and Francki, 1982; Edwards *et al.*, 1983; Lakshman and Gonsalves, 1985). Similarities in the nucleotide sequence of RNA 1 and 2, respectively, between CMV, brome mosaic virus and alfalfa mosaic virus have led to suggestions that RNA 2 encodes the polymerase subunits of the replicase, while RNA 1 encodes a nucleotide-binding protein that may also be a helicase (Kamer and Aogos, 1984; Hodgman, 1988). The 3a gene in RNA 3 is thought to be involved in virus cell to cell movement (Davies and Symons, 1988).

3. Physico-chemical properties of cucumovirus virions

3.1 Virion structure

Cucumoviruses use 180 identical protein subunits to build a virus particle which has the triangulation number T = 3 (Finch *et al.*, 1967; Hollings and Stone, 1971; Mink, 1972). The molecular weights of coat protein subunits of CMV, TAV and PSV are all about 26,000 daltons (Habili and Francki, 1974a; Peden and Symons, 1973; Mink, 1972). Virus particles sediment as a homogeneous component in analytical equibilium centrifugation with a sedimentation coefficient of 98-99 S and RNA contents in particles account for 16% of PSV (Mink, 1972) and 18-19% for CMV and TAV (Kaper *et al.*, 1965; Francki *et al.*, 1966; van Regenmortel, 1967; Hollings and Stone, 1971; reviewed by Francki, 1985b).

Neutron small-angle scattering studies of CMV particles have revealed that the RNA is deeply embedded into protein subunits (15-20 Å) leaving an empty hole in the centre. The particle surface is not completely covered by the subunits, leaving room for holes representing about 15% of the surface (Jacrot *et al.*, 1977). TAV or PSV has not been studied by this technique.

3.2 Stabilizing force

Encapsidated RNAs of both CMV and TAV are sensitive to pancreatic RNase, although TAV is more resistant than CMV to enzymatic degradation (Francki, 1968; Habili and Francki, 1974b). Particles of these viruses are readily disrupted in the presence of low concentrations of sodium dodecyl sulphate (SDS), an anionic detergent (Kaper, 1973; Habili and Francki, 1974b) and in high concentrations of neutral chloride salts (Kaper *et al.*, 1965; Francki *et al.*,

1966) indicating that their capsid structure depends on RNA-protein interactions. However, there are differeces between CMV and TAV. Whereas TAV is stabilized by Mg^{2+}, CMV precipitates in the presence of the cation; CMV is stabilized, but TAV is degraded by EDTA (Habili and Francki, 1974b). PSV is less stable than CMV when treated with trinitrobenzenesulfonic acid (TNBS), a reagent reacting specifictly with lysine (Lot and Kaper, 1976a). There are differences among CMV strains in their reactions with TNBS, in that the D strain is relatively more stable (Lot and Kaper, 1976a)

3.3 Distribution of the viral genome in the virus particle population

Molecular weights of CMV and TAV particles are about 5.3-5.8 x 10^6 daltons (Kaper and Re, 1974; Francki *et al.*, 1966; van Regenmortel *et al.*, 1972; Stace-Smith and Tremaine, 1973) and the virus particles sediment as homologous components in analytical (Kaper *et al.*, 1965; Francki *et al.*, 1966) and in sucrose density gradient centrifugation (Habili and Francki, 1974a). 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 not encapsidated in a single but rather in three different particles. That is, the first particle contains a molecule of RNA 1, second particle contains a molecule of RNA 2, and the third particle contains one molecule each of RNA 3 and 4 (Peden and Symons, 1973). A comparative study of CMV and TAV also led Habili and Francki (1974a) to the conclusion that TAV RNA is also encapsidated in the same way as CMV.

According to the above encapsidation model, there should be some differences in the molecular weight of different types of particles. However, differentiation of these particles has not been achieved due to the instability of cucumoviruses in high salt solution such as CsCl used in isopycnic gradient centrifugation (Lot and Kaper, 1973; Habili and Francki, 1974a; Lot and Kaper, 1976b). Particles of CMV and TAV fixed with formaldehyde can resist CsCl degradation, but the particles show only slight heterogeneity in isopycnic density gradients (Lot and Kaper, 1973, Habili and Francki, 1974a) and RNA has not been recovered from the formaldehyde-treated TAV and CMV preparations after centrifugation (Habili and Francki, 1974a; Lot and Kaper, 1976a). Using CMV-D, a strain which is more resistant to disruption in RbCl, Lot and Kaper (1976b) demonstrated that particles containing RNA 1 were richer in the heavier gradient fractions, whereas those containing RNA 2 were predominantly in the lighter fractions. RNA 3 and 4 were slightly more abundant in the middle of the gradient peak area.

Such a distribution pattern may resemble that of the bromoviruses, in which it is proposed that three types of particles are used to house the four RNA pieces, that is, RNA 1 in one particle, RNA 2 in a second particle and one each of RNA 3 and 4 in the third particle (Lane and Kaesberg, 1971; Hull, 1972; Bancroft and Flack, 1972). However, the occurence of RNA 3 and 4 as well as 5 (possibly a satellite RNA) throughout the peak area suggested that in CMV the encapsidation of genomic RNA is much more complicated (Lot and Kaper, 1976b). Since RNAs 3 and 4 are often not present in equimolar ratios in virus preparations, it has been suggested that other particle types containing various combinations of RNA 2, 3, 4 or 5, may exist (Lot and Kaper, 1976a,b; Hull, 1976; Kaper and waterworth, 1981).

4. Satellite RNA of cucumoviruses

In some strains of CMV, in addition to the genomic and subgenomic RNAs, a fifth small RNA (about 0.1 x 10^6 daltons) has been found to be encapsidated in virus particles (Kaper *et al.*, 1976). This small RNA is not found in healthy plants and it does not share significant sequence homology with CMV (Kaper and Waterworth, 1977; Gould *et al.*, 1978). It was subsequently found that this RNA could replicate only in the presence of CMV (Kaper *et al.*, 1976; Mossop and Francki, 1978). Hence, the concept of a satellite RNA (satRNA) was established (Murant and Mayo, 1982; Francki, 1985a).

In cucumoviruses, satRNA has been found in CMV and PSV (Kaper *et al.*, 1978). The satRNA of PSV (393 nucleotides, Collmer *et al.*, 1985) is larger than any satRNA of CMV which range from 335 to 386 nucleotides (Francki, 1985a; Hidaka *et al.*, 1988).

4.1. Structure of satRNA of CMV

All satRNA variants sequenced have a cap structure (m^7Gppp) at their 5'- and a hydroxyl group at their 3'-terminus, the same as CMV RNA (e.g., Richards *et al.*, 1978, Hidaka *et al.*, 1988). One to three open reading frames (ORFs) with potential encoding capability of about 27-38 amino acids are found in the molecules (Kaper *et al.*, 1988) and some of these peptides have been translated *in vitro* (Avila-Rincon *et al.*, 1986). The *in vivo* expression of satRNA-coded proteins has not been reported and the role of these potential proteins in pathogenesis seems to be not important based on experiments with the site-directed mutation of certain satRNA variants (Collmer and Kaper, 1988).

satRNA of CMV has a high degree of secondary structure (52% residues involved) and

could possibly form a tRNA-like secondary structure at its 3'-end, showing considerable sequence and structural homology with parts of the structure formed by CMV genomic RNA (Gordon and Symons, 1983). This feature may account for its stability both *in vitro* and *in vivo* and for its relatively high infectivity (Mossop and Francki, 1978).

4.2 Replication and encapsidation

It is believed that the satRNA replicates in the same way as its helper CMV, using the same replicase but on its own template (Mossop and Francki, 1978; Kaper, 1982). The satRNA can not infect whole plants (Kaper *at al.*, 1976; Mossop and Francki, 1978) or protoplasts (Linthorst and Kaper, 1985) by itself. A satRNA isolated from one strain of CMV could be supported by some other strains (Kaper and Tousignant, 1977) or even by another related virus (TAV) (Mossop and Francki, 1979). The amount of satRNA produced is highly variable depending on both the host species and the strain of helper virus (Kaper and Tousignant, 1977). On a given host plant, the efficiency of satRNA replication and encapsidation is governed by its helper virus (Mossop and Francki, 1979). By using pseudorecombinants as a helper virus, Mossop and Francki (1979) showed that the rate of satRNA replication was controlled by helper-virus RNA 1 and/or 2.

Since the first trace of the existence of the satRNA was from the RNA preparation extracted from the purified virus preparations, there is no doubt that the satRNA is encapsidated by the viral coat protein. The satRNA was found throughout the virus peak fractions in density gradient following equilibrium density gradient centrifugation (Lot and Kaper, 1976b). It is probably packaged in different types of particles together with viral RNA. It is also possible that it is encapsidated alone. The broad distribution of satRNA in gradients could be the result of the particles encapsidating different copy numbers of satRNA.

4.3 Interaction with helper virus and symptom regulation

With a few exceptions, satRNA reduced the yield of its helper virus (Kaper and Tousignant, 1977; Mossop and Francki, 1979; Gonsalves, *et al.*, 1982; Jacquemond and Leroux, 1982). With some strains CMV satRNA can account for 25-50% of the total RNA encapsidated in the virus particles (Mossop and Francki, 1979). Accumulation of large amounts of double-stranded (ds) satRNA in infected plants is a striking phenomenon (Habili and Kaper, 1981). These data suggest that the satRNA competes with CMV genomic RNA for replication and depresses the replication of the latter. But how satRNA competes with CMV is

not clear.

It appears that the effect of satRNA on the disease induced by CMV is the result of its interaction with the host plant and helper virus genome. The infection of tomato with CMV normally produces mosaic symptoms, however, the addition of D-satRNA (D-CARNA 5) will result in the necrosis of the plants. But in all other plants tested, symptoms remained unchanged or were attenuated (Kaper and Waterworth, 1977; Waterworth *et al.*, 1979). On the other hand, most satRNA variants tend to attenuate the symptoms caused by their helper virus (Garcia-Arenal *et al.*, 1987; Mossop and Francki, 1979; Jacquemond and Leroux, 1982; Simon, 1988).

The mechanism of satRNA in attenuating CMV pathogenicity is not yet fully understood. Sequence analysis reveals that CMV RNA 1 and 2 have a highly conserved 5' leader sequence which forms part of a hairpin structure and this structure appears in the complementary sequence of CMV satRNA. Base-paring of the complementary satRNA to CMV RNA 1 and 2 may be one mechanism by which satRNA regulates both viral and satRNA replication (Rezaian *et al.*, 1985). The anti-sense regions in CMV satRNA bind specifically to the viral coat protein gene through an unconventional knot-like structure and this may be another mechanism by which satRNA regulates viral coat protein translation (Rezaian and Symons, 1986). Other mechanisms such as the competive regulation based on the assumption that satRNA out-competes the viral genomic RNAs for replicase has also been proposed (Kaper, 1982).

5. Aphid transmission of plant viruses

According to Harris (1981), there are approximately 383 known species of animal vectors of plant viruses. About 94% of these vectors are arthropods, and the remainder are nematodes. Of the 358 known arthropod vectors, 356 are insects and 2 are mites. About 273 of the insect vectors belong to the order Homoptera: 214 species in the suborder Sternorrhyncha and 59 in the suborder Auchenorrhyncha (Harris, 1983). There are approximately 321 known, animal-borne plant viruses, of which, 298 are vectored by arthropods and 23 by nematodes. About 55% of the arthropod-borne viruses are transmitted by aphids. Therefore, aphids are the most important vectors of plant viruses (Harris, 1983).

5.1 Relationships between aphids and viruses

Different terms have been used to describe the ways in which aphids transmit viruses, and

the viruses that are transmitted by aphids. For example, based on the transmission behaviour, virus-vector associations can be classified as nonpersistent, in which virus can only be retainted in the aphid for a very short time for inoculativity, and persistent, in which virus can be retainted in the aphid for a long period but a latent period is required before the aphid can transmit the virus (Watson and Roberts, 1939). Semipersistent is used to describe the transmission mode that is between nonpersistent and persistent, in which virus infectivity can be retained in the aphid for several hours and without a latent period before transmission (Sylvester, 1958). Based on whether a virus has to circulate within the organs of its vector before it can be transmitted by the vector, Harris (1977) introduced the terms "circulative" and "noncirculative". In this category, the noncirculative (synonymous with nonpersistent) mode of transmission is characterized by the absence of a detectable latent period, loss of vector inoculativity through moulting, no virus entering the hemocoel and exiting via the vector's salivary system and a very short acquisition time; the circulative mode (synonymous with persistent), in contrast, requires a latent period following acquision feeding, translocation of the virus, and retention of inoculativity for a much longer time. The circulative mode may be further subcategorized as propagative (virus propagates in the vector) or nonpropagative (virus does not propagate in the vector) (Harris, 1983).

Among 29 groups of plant viruses (Francki, personal communication), 9 groups contain viruses which are transmitted by aphids (Shepherd, 1977). These groups are caulimovirus, luteovirus, pea enation mosaic virus, cucumovirus, alfalfa mosaic virus, carlavirus, potyvirus, closterovirus, and the family Rhabdoviridae (plant rhabdovirus).

5.2 Viruses transmitted in a nonpersistent manner

These viruses are divided into two types according to the ways they interact with aphid vectors. With the first type of virus, aphid transmission can be achieved by using highly purified virus. Therefore, only aphid and virus are invloved in the association. Viruses of this type include alfala mosaic virus, carlavirus, cucumovirus and closterovirus (Pirone and Harris, 1977; Harris, 1983). Another type of virus includes the potyviruses and caulimoviruses, in which a virus-aphid association requires the presence of a third substance -- helper component (HC) to achieve transmission (Pirone, 1977; Pirone and Harris, 1977). The HC has been purified from plants infected with tobacco vein mottling mosaic virus (TVMV) or potato virus Y (PVY) and identified as a virus-encoded peptide with M*r* of 53 K or 58 K (Thornbury *et al.*,

1985). The genes for the HC as well as the whole genome of PVY (Robaglia *et al.*, 1989), TVMV (Domier *et al.*, 1986) and tobacco etch virus (Allison *et al.*, 1985, 1986) have been determined. In cauliflower mosaic virus (CaMV), the HC is the product of ORF II in the viral genome (Gordon *et al.*, 1988). It has been shown that the HC activity of CaMV is associated with the viroplasms (Rodriguez *et al.*, 1987).

5.3 Aphid transmission of cucumoviruses

It has been reported that CMV is transmitted by over 60, TAV by 10 (Kennedy *et al.*, 1962) and PSV by 3 species of aphid (Hebert, 1967). Transmission efficiency varies with the aphid species (Simons, 1959) and host plant species (Simons, 1955; 1957). Some strains of CMV are more readily transmitted than others(Simons, 1957, Gera *et al.*, 1979). It has been reported that spinach and M strains of CMV lose their transmissibility by *Myzus persicae* (Badami, 1958; Mossop and Francki, 1977). Pirone and Megahed (1966) demonstrated that *M. persicae* could acquire purified CMV through Parafilm membrane and transmit it to a plant. The transmission by aphids of purified TAV and PSV through membrane feeding has not been reported. By the construction of psudorecombinants using aphid-transmissible and non aphid-transmissible strains, Mossop and Francki (1977) demonstrated that CMV aphid transmissibility was determined by RNA 3 of the virus. *In vitro* transcapsidation of the RNA and coat protein from two strains of CMV, one with high (CMV-T) and the other with low (CMV-6) transmission rates by *Aphis gossypii*, showed that the transmission efficiency was determined by the viral coat proteins (Gera *et al.*, 1979).

6. *In vitro* reassembly of plant viruses

6.1 A brief outline

Some of our knowledge of virus structure, particularly the protein-protein and RNA-protein interactions in virus particles, derives from studies of *in vitro* reassembly of the viruses. Tobacco mosaic virus (TMV) is the first plant virus employed in reassembly studies (Fraenkel-Conrat and Williams, 1955) and is also the most extensively studied. So far, about 15 viruses covering rod-shaped, filamentous and spherical viruses have been successfully reassembled *in vitro* (Table 1-1). The process of reassembly consists of dissociation of virus particles into their nucleic acid and coat protein and reconstitution of the separated components into virus particles. Different viruses may vary in their requirements for reassembly. Viruses which have

Table 1-1 Plant viruses which have been reassembled *in vitro*

Virus[1]	Morphology	Dissociation[2]	Reassembly[3]	Reference
TMV	Rod-shaped	67% acetic acid	0.1 M pyrophosphate pH 7.3, 25 ^{0}C using disks of protein	Butler & Klug (1971)
TRV	Rod-shaped	50-67% formic	0.25 M glycine, pH 8.0	Semancik & Reynolds (1969)
BSMV	Rod-shaped	$CaCl_2$	0.01 M Tris-HCl, pH 7.2	Atabekov *et al.* (1970)
PMV	Filamenous	67% acetic acid	0.01 M Tris, pH 8.0 25 ^{0}C	Erickson & Bancroft (1978)
PVX	Filamentous	2 M LiCl	0.01 M Bicine, pH 6-6.2 20 ^{0}C	Goodman *et al.* (1975)
PVY	Filamentous	67% acetic acid or 2 M LiCl	0.01 M MES, pH. 7	McDonal & Bancroft (1977)
BMV, BBMV, CCMV	Spherical	1 M NaCl	0.01 M Tris, 0.01M KCl, 1 mM DTT, pH 7.4 5 mM Mg Cl_2	Hiebert *et al.* (1968)
AMV	Spherical	1.5 NaCl, 1.5 M LiCl, 0.5 M $MgCl_2$	0.02 M Tris, 0.08 M KCl 1 mM glutathione, pH 7.0	Hull (1970)
CMV	Spherical	2 M LiCl	20 mM Tris, 80 mM KCl, 1 mM DTT, pH 7.2 (TKC)	Kaper & Geelen (1971)
TAV	Spherical	2 M LiCl	TKC plus 1 mM $MgCl_2$	Habili (1974)
TCV	Spherical	0.5 M NaCl, EDTA, pH 8.5	0.05 M NaCl, 0.1 M PIPES, 1 mM MgCl2, pH 6, 2 mM mecaptoethanol	Sorger *et al.* (1986)
SBMV	Spherical	2 M LiCl, EDTA, pH 8.0	15 mM $MgCl_2$, 5 mM CaCl, 10 mM Tris-HCl, pH 9	Savithri & Erickson (1983)
SoMV	Spherical	1 M NaCl, EDTA, pH 7.5	Two step dialysis, first in 0.1 M Tris, 20 mMg^{2+}, pH 7, then in the same buffer added with 5 mM Ca^{2+}, 0.5 mM Ni^{2+}	Tremaine & Ronald (1977)

1. TMV = tobacco mosaic virus; TRV = tobacco rattle virus; BSMV = barley strip mosaic virus; PMV = papaya mosaic virus; PVX = potato virus X; BMV = brome mosaic virus;

BBMV = broad bean mosaic virus; CCMV = cowpea chlorotic mosaic virus; AMV = alfalfa mosaic virus; CMV = cucumber mosaic virus; TAV = tomato aspermy virus; TCV = turnip crinkle virus; SBMV = southern bean mosaic virus; SoMV = sowbane mosaic virus

2. Optimum conditions for dissociation of virus particles into their RNA and protein components.
3. Optimum conditions for reassembly of RNA and protein components into virus-like particles.

been reassembled or have been studied on reassembly are listed in Table 1-1. Among these viruses, reassembled TMV and all the spherical viruses have fulfilled the requirement of infectivity, but the reassembled filamentous viruses have either low infectivity (PVX) or are not infectious (PVY). Criteria used to assay the reassembled virus normally include the following parameters: morphology, sedimentation rate, RNA and protein content, serological properties, sensitivity to RNase and infectivity.

6.2 The reassembly of spherical viruses

The reassembly of all spherical plant viruses known so far follows the same principle: dissociate in high salt and assemble in low salt (Table 1-1). Many of the concepts and techniques of *in vitro* reassembly of the spherical viruses came from the early work on CCMV (Bancroft and Hiebert, 1967; Hiebert *et al.*, 1968). A fresh CCMV preparation was dissociated by dialysis in 1 M NaCl buffered in 0.02 M Tris, pH 7.4, in the presence of 1 mM Cleland's reagent (DTT), and nucleic acid was sedimented away from protein by ultracentrifugation (Bancroft *et al.*, 1967). When the protein preparation was mixed with CCMV RNA prepared by phenol extraction in a ratio of 3 : 1 (by weight) and dialyzed against TKMC buffer (0.01 M Tris, pH 7, 0.01 M KCl, 0.005 M MgCl2, and 0.001 M DTT) for an hour, CCMV-like particles were observed from the reaction mixture and these particles were infectious when inoculated to the host plants (Bancroft and Hiebert, 1967). A further study showed that the assembled particles had the same properties, such as morphology, sedimentation rate, net charge of particles as well as resistance to digestion by snake venom phosphodiesterase, as the native CCMV (Hiebert *et al.*, 1968). Adolph and Butler (1977) reported the assembly of CCMV under mild conditions (0.1-0.2 M phosphate buffer, pH 6.0, 25 ^{0}C) and that nearly 70% of the RNA was encapsidated as infectious CCMV. BMV and BBMV were also reassembled efficiently from their separated RNA and protein components under the same

conditions (Hiebert *et al.*, 1968).

Heterologous assembly with coat protein from CCMV and RNAs from BMV and BBMV or *vice versa*, were also sucessful (Hiebert *et al.*, 1968). Reassembly of CCMV protein with yeast sRNA resulted in the formation of a heterologous group of nucleoproteins with an average sedimentation coefficient of about 75S; with TMV RNA particles were 110 S, and were CCMV-like, and had twice the infectivity as free TMV RNA on an equimolar basis (Hiebert *et al.*, 1968). In a study of AMV reassembly, it was found that irrespective of the type of nucleic acids, the AMV coat protein could polymerize with all the nucleic acids, either natural or synthetic, to form different types of components under the optimal condition of pH 6 and low ionic strenght (I = 0.1 M). For example, with poly (A), the assembled products were cylindral or approximately spherical, and with calf thymus DNA (double-stranded DNA) they were long tubules (Driedonks *et al.*, 1978).

In assembly using single viral RNA and mixed viral coat proteins from two or three bromoviruses (BMV, CCMV and BBMV), Wagner and Bancroft (1968) obtained reassembled virus particles with mixed capsids. These particles differed from their parental viruses in their electrophoretic mobility in electrophoresis.

The S strain of CMV was dissociated in 2 M LiCl containing 2.5 mM DTT and could be reassembled by dialysing against 0.02 M Tris-HCl, pH 7.2, 0.08 M KCl amd 0.001M DTT (TKC) (Kaper and Geleen, 1971). V strain of TAV also dissociated and reassembled under the same conditions (Habili, 1974). The difference between CMV and TAV in the requirement for reassembly is that Mg^{2+} enhances the assembly of TAV but inhibits the assembly of CMV (Habili, 1974). The reassembled CMV could react with antiserum to CMV in immunogel diffussion tests (Kaper and Geelen, 1971). Heterologous assembly of CMV protein with turnip yellow mosaic virus (TYMV) RNA (Kaper and Geelen, 1971), and peanut stunt virus RNA (Lot and kaper, 1976a) or with TAV protein and CMV RNA (Habili, 1974) has been successful.

6.3 Application of *in vitro* assembly

a. Elucidation of assembly mechanism

Although heterologous RNA can assemble with TMV coat protein *in vitro* (Fraenkel-Conrat and Singer, 1959; Matthews, 1966), the homologous TMV RNA nucleates assembly more than

an order of magnitude faster than either poly (A) or poly (I) (Butler and Klug, 1971). It has been identified that a sequence (AGAAGAAGUUGUUGAUGA) in TMV RNA is responsible to this rapid nucleation of assembly (Zimmern, 1977; Jonard *et al.*, 1977). This sequence is designated as the origin of assembly (OAS) (Zimmern, 1977) and has been found to be just over 900 nucleotides from the 3' end of TMV RNA (Goelet *et al.*, 1982). The assembly efficiency was incresed greatly when the OAS was inserted into the heterologous RNA molecule (Sleat *et al.*, 1986). Deletion or alteration of the unpaired sequence in the loop of the OAS abolishes rapid packaging (Turner and Butler, 1986; Turner *et al.*, 1988). The *in vivo* function of the OAS has also been demonstrated by the encapsidation of BMV RNAs which have been inserted with the OAS sequence near the 3' ends by the expressed TMV protein (Sacher *et al.*, 1988). After the initial nucleation, the particle elongates in both the 5'- and 3'- directions. It has been demonstrated that the elongation to the 5'- end is much faster than to the 3'- end (Zimmern, 1977; Fukuda and Okada, 1985). However, the elongation to the 3'- end is independent of that to the 5'- end, since it has been shown that when the 5'- elongation could not be completed due to the blockage of the viral RNA by a cDNA fragment, the elongation to the 3'- end could still be completed (Fairall *et al.*, 1986).

It has been found that cleavage of the first 25 amino acids from the N- terminal region of the AMV coat protein peptide will result in the conversion of the bacilliform particles to spherical structures (Bol *et al.*, 1974). In TCV, the assembly of T=1 particles is the result of cleavage of the arms of the coat protein subunit (Sorger *et al.*, 1986). The assembly study also suggested that in TCV, the rp-complex, in which three dimers of coat protein subunit bind stably to the viral RNA, is the initiation complex form of assembly (Sorger *et al.*, 1986).

Studies with PMV suggest that the assembly process involves two stages: nucleation and elongation, and the assembly only starts from the 5'- end at pH 8.0 in low ionic strength buffer (AbouHaidar and Erickson, 1985).

b. Investigation of viral coat protein-aphid transmission relationship

There are some viruses, including TMV, which can not be transmitted by aphid vectors. The mechanism for this is not known. By using the *in vitro* transcapsidation (heterologous assembly) and membrane feeding techniques, Gera *et al.* (1979) demonstrated that the difference in the aphid transmission rate of two strains of CMV, one with a low aphid transmission rate (CMV-6) and another with a high aphid transmission rate (CMV-T) were due

to the properties of their viral coat proteins .

c. Delivery vehicle for foreign genes

The possibility of using plant viruses as vectors to deliver foreign genes into plants was an attractive concept a decade ago (Howell, 1982). Although it seems that there has not been any successful example of using a plant virus to mediate gene transfer, the successful delivery of mRNA of chloramphenicol acetyltransferase (CAT) into plant protoplasts using the *in vitro* assembly technique to encapsidate the mRNA into TMV-like particles before inoculation has been reported (Gallie *et al.*, 1987).

7. *In vivo* interactions between RNA viruses

7.1 Multi-infection events

The co-infection of plants with more than one virus or strain of virus under natural conditions has been known for a long time. For example, two strains of TMV coinfected the same tobacco plant (McKinney, 1929), and two viruses (carrot mottle dwarf virus and parsnip mottle virus) infected the same carrot plants (Watson *et al.*, 1964). Multi-infection of plants with two or more viruses sometimes changes the symptom expression, e.g. tomato plants showed mottling and slight dwarfing when they were infected with TMV, or showed only mild mottling when infected with PVX, however, the plants showed extensive necrosis when infected with both viruses (Bennet, 1953).

7.2 Interaction of viruses in infected plants

a. The relative content of viruses

When plants are infected with two unrelated viruses, the viruses can normaly co-exist together. The most usual situation is that the presence of one virus decreases the concentration reached by another or that they have little effect on one another (Gibbs and Harrison, 1976). For example, the presence of PVY or CMV reduced the infectivity of Nicotiana virus 6 from their host plant *Nicotiana sylvestris* (McKinney, 1941). Two distantly related viruses, TAV and CMV, could co-exist independently without intefering with each other's concentration in *Nicotiana clevelandii* (Sackey, 1989). In a few instances, the presence of one virus may considerably increase the concentration attained by the other, e.g., PVX reached a greater concentration in tobacco when the plant was co-infected with PVY than in those infected by

PVX alone (Rochow and Ross, 1955).

When plants are infected with two related viruses, interference usually occurs. The number of necrotic lesions produced in tobacco and *Nicotiana sylvestris* by the tomato aucuba mosaic strain of TMV was decreased by adding to the inoculum the type strain of TMV, which does not itself produce necrotic lesions in these plants (Sadasivan, 1940). In particular, if the plants are infected previously with a virus, the second related virus or strain would sometimes not be able to infect the plants. Such a phenomenon (termed "cross protection") was first observed on tobacco with strains of TMV (Mckinney, 1929) and then with strains of PVX (Salaman, 1933). Indeed, cross protection has been found with many plant viruses and it has been and is being used for controlling virus diseases (Sequeira, 1984; Beachy, 1988).

b. Recombination and pseudorecombination of genomic viral RNAs

There has not been any direct evidence that two unrelated viruses exchange their genetic information through RNA recombination *in vivo*. However, recombination between the 3' end-mutated RNA 3 and RNA 1 or 2 of BMV to resume a normal RNA 3 has been demonstrated (Bujarski and Kaesberg, 1986). Angenent *at al.* (1989) found that RNA 2 of tobacco rattle virus (TRV) strain PLB consisted of a 5'-terminal sequence of 1376 nucleotides identical to the 5'-sequence of RNA 2 of TRV strain PSG and a 3'-terminal sequence of 820 nucleotides that is identical to the 3'-sequence of RNA-1 of strain PLB. These suggest that in strains PLB and PSG the same coat protein gene is fused to different RNA 1 derived 3'-termini (Angenent *et al.*, 1989).

Pseudorecombinants of tripartite viruses involving an exchange of RNAs 3 between BMV and CCMV (Bancroft, 1972) or between CMV and TAV (Rao and Francki, 1981) have been constructed *in vitro* and they were shown to be stable *in vivo*. However, no such or similar pseudorecombinats have been reported to occur in plants co-infected with two tripartite viruses.

c. Transcapsidation

The phenomenon that aphid transmission of one virus is dependent on another virus was first observed by Smith (1945). He found that the "mottle" virus could not be transmitted by *Myzus persicae* from tobacco plants, unless it was accompanied in the plant by the aphid-transmissible "vein-distorting" virus (Smith, 1945). The first clear demonstration that the genomic RNA of a virus (or strain) could be encapsidated in the capsid of another virus during their co-infection of the same plants (termed transcapsidation or phenotypic mixing, Kassanis,

1964), came from the work of Rochow (1970). In his experiment, two serologically unrelated isolates of barley yellow dwarf virus (BYDV), RPV and MAV, each having a different aphid vector (*Rhopalosiphum padi* L.for RPV and *Macrosiphum avenae* Fabricius for MAV) and transmissible in the persistent manner, were co-inoculated to oats. It was found that MAV was transmitted by *R. padi* together with RPV from the co-infected plants to healthy plants. Purified MAV, RPV and a mixture of both could not be transmitted by their aphid vectors if the preparations were treated with their homologous antisera prior to acquisition by aphids through membrane feeding. Treatment with MAV antiserum of virus extracted from doubly infected plants only prevented the transmission of MAV by *M. avenae* but not by *R. padi*. However, neither RPV nor MAV was transmitted by *R. padi* after the virus preparations were treated with antiserum to RPV. Thus, the transmission of MAV by *R. padi* must be due to some MAV RNA being coated with the RPV capsid protein during simultaneous synthesis of the two strains in the doubly infected plants (Rochow, 1970; Rochow, 1977). Transcapsidation of RNA of carrot mottle virus (CMotV) into the capsids of carrot red leaf virus (CRLV) and the aphid transmission of CMoV with the CRLV specific aphid vector from doubly infected plants has also been demonstrated (Waterhouse and Murant, 1983).

d. Mixed capsids

Another type of virus-virus interaction *in vivo* is the viral coat protein interaction that would result in the formation of mixed capsids. When protoplasts were inoculated with TMV together with tomato mosaic Tobamovirus, some of the individual progeny particles were found to be coated with a mixture of the two coat proteins (Otsuki and Takebe, 1978). The biological significance of such mixed capsids is not known.

Scope of this thesis

Encapsidation of nucleic acids with the coat protein of two cucumoviruses, TAV and CMV, is the theme of this thesis. An efficient *in vitro* reassembly system suitable for both TAV and CMV was first developed from previously reported methods. Upon the establishment of the reassembly and its analysis system, this technique was applied to the investigation of a number of diverse objectives: *in vitro* encapsidation capacity of CMV and TAV, CMV genome distribution in virus particle population, relationship between satellite and genomic RNAs of CMV and TAV with regard to encapsidation, as well as the interaction between coat proteins of

TAV and CMV in assembly. The viral coat protein-aphid transmissibilty relationship was investigated using this defined system. Finally, the concepts developed during *in vitro* encapsidation studies were extended to an *in vivo* system, in which the interaction of an aphid-transmissible TAV or CMV and a non aphid-transmissible virus, velvet tobacco mottle virus with its satellite RNA, was used to focus on the events of transcapsidation and aphid transmission.

Part of the work reported in this thesis has been published (see Appendix II).

Chapter 2 General Materials and Methods

1. Materials

1.1 Viruses

The viruses used in this study were cucumber mosaic cucumovirus (CMV), strains M (MCMV) and T (TCMV); tomato aspermy cucumovirus (TAV), V strain (VTAV); tobacco mosaic tobamovirus (TMV), U1 strain; velvet tobacco mottle virus (VTMoV); and galinsoga mosaic virus (GMV). The sources of virus isolates, the propagation hosts and the relevant references are listed in Appendix I-1.

1.2 Clone of CMV satellite RNA

pBsat 5, which was constructed from the transcribable PBS plasmid vector (3204 bp) and the full length cDNA of B1 satellite RNA (337 nt) of CMV (Garcia-Arenal *et al.*, 1987) was a gift from Dr. G. Kurath of Cornell University, U.S.A.. B1 satellite cDNA was inserted at the plasmid nucleotide position 897 between EcoR I and Sma I restriction sites. The transcription of plus sense satellite RNA was controlled by a T7 promotor (downstream) and that of minus sense RNA by a T3 promotor (upstream). The plus sense satellite RNA transcript is infectious (Kurath, personal communication).

1.3 Plants

All plants unless otherwise specified were grown in a glasshouse at 20-25 ^{0}C.

1.4 Bio-chemicals

The main bio-chemicals used in this study are listed in Appendix I-2.

1.5 Miscellaneous chemicals

The main chemicals used in this study are listed in Appendix I-3. Other chemicals not listed were all analytical grade.

1.6 Water

Autoclaved double distilled water (DDW) was used in all experiments except that autoclaved NANO-purified water (NPW) was used in RNA transcription experiments.

2 Methods

2.1 Precautions against RNase activity

Whenever possible all equipment, glassware, buffers and chemicals were autoclaved to inactivate RNase. Where autoclaving was not applicable, items were washed with alcoholic KOH (100 g KOH, 100 ml DDW and 900 ml ethanol) and rinsed thoroughly with DDW.

2.2 Virus propagation and purification

All viruses were inoculated mechanically to their propagation host plants and harvested 10-14 days post inoculation for purification.

MCMV was purified as described by Mossop *et al.* (1976). Leaf tissue was homogenized in a Waring blender in 3 volumes (w/v) of pre-cooled 0.1 M Na_2HPO_4, pH 9.0, containing 0.1% each of thioglycollic acid and Na-diethyldithiocarbamate. Triton X-100 was added to the homogenate to 2% and stirred for 20 min at 4 ^{0}C. After clarification by centrifugation at 10,000 g for 10 min, the virus was pelleted from the supernatant by centrifugation at 223,000 g for 50 min. The pellet was resuspended in 1/10 of the original volume of extraction buffer. Particulate material was removed by centrifugation at 10,000 g for 10 min and the supernatant was then layered over 8 ml of a 15% (w/v) sucrose cushion prepared in 0.1 M Na_2HPO_4 and centrifuged in a Beckman 65 rotor at 223,000 g for 50 min. The virus pellet was resuspended in 0.1 M Na_2HPO_4. The virus was futher purified by rate zonal centrifugation in a Beckman SW28 rotor at 25,000 rpm for 150 min at 4 ^{0}C through a 5-25% (w/v) sucrose density gradient prepared in 0.1 M Na_2HPO_4. The gradient was fractionated with the aid of an ISCO Model 640 coupled with a UA-5 recorder and the virus peak was collected and diluted appropiately with 0.1 M Na_2HPO_4. The virus was sedimented by ultracentrifugation and resuspended in 10 mM borate buffer, pH 7.6, and stored at 0 ^{0}C.

TCMV was purified as described by Francki *et al.* (1979). Leaf tissue was homogenised in a Waring blender in 2 volumes (w/v) of 0.5 M sodium citrate buffer, pH 6.5, with 0.5% thioglycollic acid and 5 mM Na_2-EDTA and 1 volume of chloroform and centrifuged at 10,000 g for 10 min. PEG_{6000} was added to the supernatant to 10% (w/v) and the mixture was stirred for 45 min at 4 ^{0}C. The precipitated virus was sedimented by centrifugation at 10,000 g for 10 min and suspended in extraction buffer containing 1% Triton X-100 by stirring for 45 min. The suspension was clarified by centrifugation at 15,000 g for 20 min and subjected to 2 cycles of high- and low speed centrifugation at 223,000 g for 50

min, and 10,000 g for 5 min respectively. The virus pelleted by ultracentrifugation was resuspended in 5 mM sodium borate containing 5 mM Na_2-EDTA. The virus was further purified by rate zonal centrifugation as above in 5-25% sucrose density gradient prepared in 20 mM phosphate buffer, pH 7.5. The virus was resuspended in 20 mM phosphate buffer, pH 7.6 and stored at 0 ^{0}C.

VTAV was purified by the above procedure except that no Na_2-EDTA was included in the buffers at any stage of purification.

Concentrations of MCMV, TCMV, and VTAV were determined spectrophotometrically assuming $E_{260}^{0.1\%}$=5 (Francki *et al.*, 1966; Stace-Smith and Tremaine, 1973).

TMV was purified as described by Boedtker & Simmons (1958). Leaf tissue was ground to powder in liquid nitrogen before adding Na_2HPO_4 (dried salt) to 4% (w/w). Two volumes of 0.1 M phosphate buffer, pH 7.0, containing 0.2% TGA was added to the powder. After thawing and centrifugation at 10,000 g for 10 min, the supernatant was filtered through two layers of cheesecloth and PEG_{6000} was added to 10% (w/v) and Triton X-100 to 2% (v/v). The mixture was stirred at 4 ^{0}C for 30 min and then subjected to centrifugation at 10,000 g for 10 min. The pelleted virus was resuspended in extraction buffer (1/5 of original volume). The supernatant was clarified by centrifugation at 10,000 g for 10 min and then layered over 8 ml of a 20% sucrose cushion prepared in DDW, pH 7.6 (adjusted with NaOH-HCl) and centrifuged at 223,000 g for 40 min. The pelleted virus was resuspended in DDW and subjected to another two cycles of high- and low speed centrifugation through a sucrose cushion. The final virus pellet was resuspended in double distilled water and stored at 4 ^{0}C. The concentration of virus was determined spectrophotometrically assuming $E_{260}^{0.1\%}$=2.7 (Knight, 1962).

VTMoV was purified as described by Randles *et al.* (1981). Leaf tissue was extracted with two volumes (w/v) of 0.1 M phosphate buffer, pH 7.4, containing 0.1% thioglycollic acid and an equal volume of a mixture of chloroform and butanol (v/v). The slurry was stirred at 4 ^{0}C for 20 min and clarified by centrifugation at 10,000 g for 10 min. The supernatant fluid was then centrifuged at 78,000 g for 90 min and the pellet was resuspended in 20 mM phosphate buffer, pH 7.4. After clarification by centrifugation at 10,000 g for 10 min, the supernatant fluid was centrifuged at 270,000 g for 30 min, and the pellet was resuspended in 20 mM phosphate buffer. The preparation was again clarified at 10,000 g for 10 min and then centrifuged at 270,000 g as described above. The final virus pellet was resuspended in 20 mM

phosphate buffer, pH 7.4. VTMoV concentration was examined spectrophotometrically assuming $E_{260}^{0.1\%}$=5 (Randles *et al.*, 1981).

GMV was purified as described by Behncken *et al.* (1982). French bean leaves with local lesions were homogenized with two volumes (w/v) each of 0.1 M phosphate buffer, pH 7.4, containing 0.1% thioglycollic acid and a mixture of chloroform and n-butanol (1:1, v/v). After clarification at 10,000 g for 10 min, the supernatant was ultracentrifuged at 200,000 g for 50 min. The pellet was resuspended in 20 mM phosphate, pH 7.4 and then centrifuged at 10000 g for 10 min. The virus was then subjected to two cycles of ultracentrifugation through a 15% (w/v) sucrose cushion alternating with low speed centrifugation for clarification. The virus was finally resuspended in 20 mM phosphate buffer, pH 7.4. The concentration of virus was determined spectrophotometrically assuming $E_{260}^{0.1\%}$=5.

2.3 Infectivity assay

For local lesion infectivity assay, *Chenopodium amaranticolor* plants at the 5-6 leaf stage were used for MCMV and VTAV, *N. glutinosa* plants at the 5-7 leaf stage were used for TMV, and *Phaseolus vulgaris* at the primary leaf stage was used for GMV. Lesions were counted 2-8 days after inoculation.

2.4. Isolation of viral RNA from purified virus preparation

Viral RNAs were isolated by phenol-SDS extraction (Peden & Symons, 1973). Equal volumes of virus preparation, extraction buffer (0.6 M sodium acetate, 0.6% SDS, 20 mM EDTA, in 20 mM tris-HCl buffer, pH 7.2) and extraction buffer-saturated phenol containing 0.1% 8-hydroxyquinoline were mixed and shaken for 20 min at room temperature. The aqueous phase was recovered after centrifugation at 10,000 g for 10 min and re-extracted with one half volume of phenol. RNA was precipitated with 2.5 volumes of cold redistilled ethanol and left at -20 ^{0}C overnight, recovered by centrifugation and washed twice with 80% ethanol. The RNA pellets were vacuum-dried and resuspended in DDW and kept at -20 ^{0}C. The RNA concentration was determined spectrophotometrically assuming $E_{260}^{0.1\%}$=25.

2.5. Isolation of total RNAs from plant leaf tissues

One gram of young leaf tissue of healthy or virus-infected *N.clevelandii* was ground to powder in liquid nitrogen. Four volumes (w/v) of extraction buffer (50 mM Tris, pH 7.6, containing 0.1 M NaCl, 1 mM Na_2-EDTA and 0.5% SDS) and two volumes of phenol-chloroform (1:1, v/v) were added to the powder. The mixture was shaken for 20 min at room

temperature then centrifuged at 10,000 g for 10 min. The aqueous phase was re-extracted with one half volume of phenol-chloroform. The nucleic acids were precipitated with 2.5 volume of cold ethanol and washed twice with 80% ethanol. The RNA pellets were vacuum-dried and resuspended in 1 ml TE (10 mM Tris-HCl, 1 mM Na_2-EDTA, pH 8.0) (Carrington & Morris, 1984).

2.6. Separation and purification of satellite and viral RNAs

RNAs extracted from purified CMV preparations with or without satellite RNA were subjected to electrophoresis in 1.5% horizontal agarose gels in TAE buffer (0.4 M Tris-HCl, 0.2 M sodium acetate, 20 mM Na_2-EDTA, adjusted to pH 8.0 with glacial acetic acid). The gels were stained with aqueous ethidium bromide (100 ug/l) and viewed in a UV transilluminator to localize the RNA band positions. Gel slices containing individual RNA species was transferred into a punctured sterile Eppendorf centrifuge tube that had been plugged with about 2 mm of siliconized sterile glass wool. The tube was put on the top of another tube in a two-tiered system and centrifuged at 10,000 g for 10 min. The eluate containing the RNA was collected in the bottom tube. After precipitatation in 2.5 volumes of cold ethanol, the RNA was resuspended in DDW (Heery *et al.*, 1990).

2.7. *In vitro* transcription of CMV satellite RNA

a. Propagation and isolation of satellite clone pBsat 5

A fresh single colony of *E. coli* (strain HB 101) hosting a plasmid with the CMV satellite clone, pBsat 5, was first cultured in 3 ml LB medium (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, per litre, pH 6.5) with Ampicillin (50 g/l) at 37 ^{0}C overnight with shaking. One ml of this overnight culture was then used to inoculate 500 ml of LB medium with Ampicillin in a 1-litre loosely capped bottle. Chloramphenicol was added to the culture (150 ug/l) 8 h later and it was incubated for another 16 h to amplify the plasmid. The bacteria were pelleted by centrifugation at 13,200 g for 10 min at 4 ^{0}C and resuspended in TNE (10 mM Tris-HCl, 100 mM NaCl, 1mM Na_2-EDTA, pH 7.5) in 1/5 the original volume. The bacteria were sedimented as above. For every gram of bacteria, 10 ml of solution I (50 mM glucose, 10 mM Na_2-EDTA, 25 mM Tris-HCl, pH 8.0) and 20 mg lysozyme were added. The bacteria were resuspended and kept on ice for at least 5 min. For every 10 ml batch of bacteria in lysozyme, 20 ml of solution II (0.2 M NaOH, 1% SDS) was added and the solution mixed by inverting gently until all brown bacterial cells had been lysed and a buoyant precipitate became

visible. Fifteen ml of 5 M acidic potassium acetate (with 11.5% of acetic acid, v/v) was added to the solution and inverted gently until viscosity dropped and a flocculent precipitate had formed. The precipitate was removed by centrifugation at 13,200 g for 10 min at 4 ^{0}C and discarded. The supernatant containing the plasmid DNA was added to 2.5 volumes of cold ethanol and 1/10 volume of 5 M NaCl and left at 20 ^{0}C for 15 min. The precipitated plasmid was pelleted by centrifugation at 13,200 g for 20 min at 4 ^{0}C and dissolved in TE (1-2 ml for 500 ml of original bacterial culture) (Sambrook *et al.*, 1989).

b. Purification of pBsat 5 by caesium chloride density gradient centrifugation

One to two ml of plasmid DNA preparation were transferred into a 10 ml tube, made up to 10 ml with 9.5 g caesium chloride and TE and then transferred into a 16x76 mm Polyallomer Quick-Seal centrifuge tube together with 0.4 ml of ethidium bromide (10 mg /ml). After filling and balancing, the tubes were sealed and centrifuged at 230,000 g for 16 h at 20 ^{0}C. The plasmid band was recovered with an 18 gauge needle and syringe and transferred to a 10 ml tube. The ethidium bromide was removed by 6 extractions with equal volumes of water-saturated n-butanol. The aqueous phase was then transferred to a fresh tube and 3 volumes of TE was added to dilute the CsCl. The plasmid DNA was precipitated by adding 2 volumes of ethanol, 1/10 volume of 3 M sodium acetate, left at -20 ^{0}C for 10 min and centrifuged at 15,000 g for 20 min at 4 ^{0}C. The pellet was vacuum-dried and resuspended in 1-2 ml TE buffer. The concentration of DNA was determined spectrophotometrically assuming $E_{260}^{0.1\%}$=20 (Sambrook *et al.*, 1989).

c. Linearization of pBsat 5

To prepare templates for transcription of plus-strand (+) satRNA, 40 units of restriction endonuclease Sma I were added to 20 ug pBsat5 DNA and incubated in 50 µl of medium salt incubation buffer (Boehringer) at 25 ^{0}C for 24 h. The reaction was stopped by heating at 65 ^{0}C for 5 min then chilling on ice. Linearized DNA was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v) and precipitated with 2.5 volumes of cold ethanol after adding 1/10 volume of 3 M sodium acetate and left at -20 ^{0}C overnight. The DNA was pelleted by centrifugation at 10,000 g for 10 min. After a brief cycle of vacuum-drying, the DNA was resuspended in 20 µl TE.

To prepare templates for transcription of minus strand (-) satRNA, 50 units of restriction endonuclease EcoR I was added to 25 µg of plasmid DNA and incubated in 50 µl of

high salt incubation buffer (Boehringer) for 4 h. The DNA was extracted with phenol/chloroform as described above.

d. Transcription

A typical reaction was done in a volume of 50 µl in a 1.5 ml centrifuge tube. The reaction mixture contained 1.2 µg Sma I linearised template pBsat 5 DNA, 10 µl of 5x- reaction buffer, 6 µl of a mixture containing 3.3 mM each of ATP, GTP, and CTP, 3 µl of 2 mM UTP, 1 µl of ^{32}P-labelled UTP (3000 Ci/mmol; 10 µCi/µl), 5 µl of 0.1 M DTT, and 40 units of T7 RNA polymerase and 20 µl of NPW. The mixture was incubated at 37 ^{0}C for 60 min and then 22 units of RNase-free DNase I (RQ1 DNase, Promega) was added and incubated at the same temperature for 10 min to digest the DNA template. Five µl of 0.1 M EDTA was added to terminate the reaction. After adding 50 µl of NPW the mixture was extracted with 100 µl of phenol: chloroform: isoamyl alcohol. The aqueous phase containing transcript RNA was separated by centrifugation in a microfuge for 5 min and transferred to a fresh sterile tube. One hundred µl of NPW was added to the remaining phenol phase, mixed well and centrifuged for 5 min. The aqueous phase was removed and mixed with the previous one and 22 µl of 3 M sodium acetate was added. The RNA was precipitated with 3 volumes of cold ethanol at -20 ^{0}C overnight, briefly vacuum-dried, resuspended in 50 µl of NPW, then stored at -20 ^{0}C (Kurath and Palukaitis, 1987).

For transcription of minus-strand satRNA, the pBsat 5 template linearised with EcoR I, and T3 RNA polymerase were used.

2.8 Isolation of viral coat proteins

CMV and TAV coat proteins were isolated from highly purified virus preparations by LiCl dissociation (Kaper, 1969) with the following modifications. Virus at a concentration of 2-3 mg/ml was mixed with an equal volume of 6 M LiCl and placed at -70 ^{0}C for 20 min. After thawing at 4 ^{0}C, the precipitated RNA was removed by centrifugation at 10,000 g for 10 min and discarded. The supernatant containing viral coat protein was dialysed against 2 M LiCl, then against 1 M LiCl, both in 0.5 M Tris-HCl, pH 8.4, and finally, against 0.5 M LiCl in 0.2 M Tris-HCl, pH 8.4, with 2.5 mM DTT, each for 1 h respectively. The protein preparation was clarified by centrifugation at 370,000 g for 25 min. Concentration of protein was determined spectrophotometrically assuming $E_{280}^{0.1\%}$=1.25 (Chen & Francki, 1990).

2.9 *In vitro* reassembly

Protein and RNA were mixed in the required ratios in autoclaved dialysis tubes either 15 or 10 mm in diameter and dialysed overnight against reassembly buffer TCKMg (20 mM Tris-HCl, pH 7.2, containing 80 mM KCl, 1mM DTT and 1 mM $MgCl_2$) with gentle stirring at 4 ^{0}C. Insoluble materials were removed by centrifugation at 10,000 g for 10 min leaving the reassembled virus-like particles in the supernatant (Kaper and Geleen, 1971; Habili, 1974).

The nomenclature used to describe the assembled particles was as follows: protein was identified as P with a prefix to indicate the kind of protein and RNA was identified as R with a prefix to indicate the source of RNA. For example, MP-VR stands for MCMV protein-VTAV RNA and VP-TR for VTAV protein-TMV RNA combinations.

An assembly containing a stoichiometric 1 part of RNA and 4 parts of viral coat protein (RNA:protein=1:4, by weight) was designated as standard RNA ratio assembly. Thus a non standard RNA ratio assembly ment a variation of RNA-protein ratio. For example, a double RNA ratio assembly meant 2 parts of RNA to 4 parts of protein, and triple RNA ratio assembly meant 3 parts of RNA to 4 parts of protein.

2.10. Electrophoresis

a. Agarose gel

TAE-buffered agarose gels of 1.5% (w/v) for RNA and 0.75% (w/v) for plasmid DNA were used for electrophoresis at a constant 100 volts. Samples were mixed with 2% (w/v) bromophenol blue prepared in 50% (v/v) glycerol in TAE immediately before electrophoresis. The gel was stained with ethidium bromide (500 µg/l).

b. Urea-PAGE

Denaturing 4% (w/v) acrylamide gels containing 7 M urea and TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.5) (Peacock and Dingman, 1968) were used to identify the quality and quantity of satRNA transcript or native satRNA preparations. The gel was prepared from 14.7 g of urea, 2.8 ml of 50% (w/v) of acrylamide-bisacrylamide (30:1, w/w) in DDW, 3.5 ml of 10x- TBE buffer, 0.35 ml of 10% (w/v) ammonium persulphate, 0.035 ml of TEMED and 15.8 ml of DDW. The gel was allowed to polymerize for 3 hour then pre-run at 35 mA for 20-30 min. Lanes were washed with TBE before loading the samples. The running buffer was TBE and the current 35 mA. The gel was stained with ethidium bromide and autoradiographed if required.

c. PAGE for protein

Viral protein samples were mixed with Laemmli sample buffer (3.1% SDS (w/v), 10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), in 63 mM Tris-HCl, pH 6.8) and analysed in a 12% discontinuous acrylamide-bisacrylamide gel (Laemmli, 1970) in a vertical gel apparatus (Bio-Rad). The upper running buffer contained 125 mM Tris-HCl and 0.1% (w/v) SDS, pH 6.8. The lower running buffer contained 375 mM Tris-HCl and 0.1% SDS, pH 8.8. After loading the samples, it was run at 100 volts. When samples had entered the separation gel, 180 volts was applied. The gel was stained with silver (Wray *et al.*, 1981) as follows: the gel was removed from the apparatus and soaked in 50% (v/v) methanol overnight with three changes on a rocking platform. Silver nitrate of 0.8 g was dissolved in 4 ml water and added dropwise to a 21 ml solution of 0.36% NaOH containing 1.4 ml of fresh 14.8 M ammonium hydroxide with vigorous stirring and water added to the final 100 ml. The gel was quickly rinsed twice with water and soaked in this solution for 15 min with constant agitation and then washed twice with water each for 5 min, and then developed in a solution containing 2.5 ml of 1% (w/v) citric acid and 0.25 ml of 38% (w/v) formaldehyde in 500 ml water with gentle agitation till the bands appeared. The gel was washed 3 times in water and incubated in Ilford rapid fix (1:5 dilution with water) for 5-10 min to remove background. After rinsing 3 times in water the gel was soaked in Kodak hypoclearing agent for 30 min with agitation. The gel was rinsed in water and placed in a solution of 10% (v/v) methanol, 5% (v/v) glycerol in water and photographed.

2.11 cDNA probe preparation

The cDNA to B1 satellite of CMV was prepared with a random primer and M-MLV RNA reverse transcriptase (Taylor *et al*, 1976; Palukaitis & Symons,1980). Three µl of dNTP (a mixture of 8.3 mM of d GTP, dATP, and dTTP at equal volumes), 5 µl of buffer (200 mM Tris-HCl, pH8.3, 700 mM KCl, 100 mM $MgCl_2$), 5 µl of 200 mM DTT, 5 µl (about 1-2 µg) of random primer, 10 µl of ^{32}P-dCTP (3,000 Ci/mmol; 10 µCi/µl), 5 µl of 40 mM sodium pyrophosphate, and 1 µl (200 units) of AMV reverse transcriptase were mixed in an Eppendorf tube and incubated at 42 ^{0}C for 60 min. The reaction was stopped by adding 5 µl of 0.4 M Na_2-EDTA, 5 µl of 5% SDS, 125 µl of DDW, and 15 µl of 4 M NaOH. The mixture was then left at 37 ^{0}C to digest the RNA template. cDNA fragments were separated from unincorporated free ^{32}P-dCTP by column chromotography (1 ml of fine G50 sephadex with TE as washing buffer) in a Pasteur pipette. Fractions were assayed by Cerenkov counting and the cDNA

peaks pooled. The cDNA was used directly or stored at -20 ^{0}C for a short period.

2.12. Dot bot, Northern blot, and RNA-cDNA hybridization

a. Dot bot

Nitrocellulose membrane was soaked in 20x- SSC (175.3 g of NaCl, 93.3 g of Na_3-citrate, and 0.2 ml of 0.2 N HCl/litre in DDW) and dried in air. Purified RNA or RNA extracted from plants with one cycle of phenol-chloroform treatment and concentrated by ethanol precipitation were dotted on 20-40 cm^2 pieces of nitrocellulose membrane (1 µl of sample was dotted at a space of 0.5 x 0.5 cm). The membrane was then baked in a vacuum oven at 85 ^{0}C for two hour (Palukaitis *et al*, 1985).

b. Northern blot

RNAs were electrophoresed in 1.5% agarose gels which were then trimmed after visualising the ethidium bromide stained bands in UV light. The gel was placed on top of two sheets of Whatman 3MM chromotography paper (pre-soaked in 20 x SSC and connected with a 20x SSC reservoir of 150 ml below the gel supporter) and the edges of the gel were surrounded with plastic membrane. The gel was then covered by a piece of nitrocellulose of the same size (pre-soaked in 20 x SSC) without leaving any air bubble in between. Three sheets of the dry Whatman paper were placed above the nitrocellulose membrane. A stack of paper towels with a glass sheet and a weight of about 500 g was placed on the Whatman papers. The transfer was allowed to proceed overnight. The membranes were baked as above (Sambrook *et al.*, 1989).

c. Hybridization

For pre-hybridization, the blotted membrane was transferred into a plastic bag and 3 ml/50 cm^2 of Maule's buffer (3x- SSC, containing 0.08% (w/v) each of bovine serum albumin, Ficoll, and polyvinyl-pyrrolidone, 1 mM Na_2-EDTA and 250 ug/ml yeast RNA) (Maule *et al.*, 1983) was added. The bag was heat-sealed after air bubbles had been removed and immersed in a water bath with constant shaking for 24 h at 42 ^{0}C.

For hybridization, the labelled cDNA probe was boiled for 5 min, chilled, and added to the plastic bag containing the membrane and 3 ml of hybridization buffer (cDNA was added at the ratio of 1 million cpm/50 cm^2 of membrane). The hybridization was done at 65 ^{0}C in a water bath for 24 h. The membrane was released from the bag and washed twice at 65 ^{0}C, each for 30 min and twice at 25 ^{0}C in 2x- SSC containing 0.5% SDS, each for 5 min, and then

twice in 0.1x- SSC containing 0.5% SDS at 65 ^{0}C, each for 15 min (Palukaitis *et al.*, 1983). The membrane was then drained and wrapped in two sheets of Gladwrap, and exposed to X-ray film at -70 ^{0}C. The signal was enhanced by using a cassette with an intensifying screen.

2.13 ^{35}S labelling of TCMV coat protein

N. clevelandii plants infected with TCMV showing the first sign of symptoms (5-7 days post inoculation) were removed from the pots and roots were washed free of soil. The roots of 6 plants were put together and placed in a 4 cm beaker. ^{35}S-sulphate in water solution (2.5 mCi, 1ml) was applied to the roots when the plants were about to dehydrate. The plants were kept in light with a small amount of water at the bottom of the beaker for 6-8 days in a screened area. The virus was purified as described above (Hajimorad, 1989).

2.14 Measurement of radioactivity

^{32}P was measured directly and ^{35}S was measured in the presence of toluene-based scintillation fluid (3.5 g of 2, 5- diphenyloxazole (PPO) and 0.35 g of 1, 4-bis 2, 5-diphenyloxazolyl (POPOP), per litre) (Mossop, 1978) in a Beckman LS 5000 TD Liquid Scitillation System. The relative radioactivity was expressed as count per minute (CPM).

To locate the radioactivity in an agarose gel after immunodiffusion test, the gel was soaked and washed in 0.85% NaCl for 2 days, and then dried at 37 ^{0}C in a dry incubator. The gel was exposed to a Fuji X-ray film (RX) at -70 ^{0}C.

2.15 Electron microscopy

Copper grids of 400 mesh coated with formvar and a carbon film were briefly discharged with a ST 4 M Spark tester before use. Cucumoviruses, VTMoV, and GMV were stained with 1% uranyl acetate, pH 6.5. TMV was stained with 2% phosphotungstic acid (PTA), pH 4.5 (Hatta and Francki, 1984). A drop of about 20 µl of appropriately diluted virus preparation was pipetted on to a grid for 1 min and extra liquid removed with a piece of filter paper. The grid was then stained with uranyl acetate or PTA for 1 min and extra liquid removed as above, then placed on a Whatman filter paper to dry. The leaf dip method (Noordam, 1973) was used to examined TMV particles in an infected plant. This was done by cutting the leaf and touching the cut edge to a grid with a drop of stain on it for about 30 seconds. The viruses were viewed in a JEM 100 CX electron microscope.

2.16 Sedimentation analysis

Native or reassembled particles were analysed for their sedimentation properties by 5-

25% sucrose density gradient centrifugation in a Beckman SW41 rotor. Virus samples were loaded on to the tops of gradients. The gradients were fractionated with the aid of an ISCO fractionator Model 640 coupled with a UA-5 recorder. The sedimentation rates were expressed as a migration rate, relative to that of virus peaks of known sedimentation rate in the gradients. For comparative studies, a more precise result was obtained by mixing two viruses of interest and co-centrifuging them in the same gradient.

2.17 Spectrophotometry

Ultraviolet absorption spectra were determined using a Beckman DU-8B spectrophotometer with a 1 cm-path quartz cuvette.

2.18 Densitometry

For the determination of relative ratios of RNA species, samples were electrophoresed in agarose gels. The gels were stained with ethidium bromide and photographed. The strips of prints of the photographs containing RNA bands were then scanned in a JOYCE LOEBL CHROMOSSAN densitometer. The relative ratio of each RNA was calculated by measuring the area of corresponding peak recorded.

2.19 Aphid transmission

A single aphid of *Myzus persicae* which had been raised on Chinese cabbage (*Brassica pekinensis* Rupr.) which is not a host of CMV and TAV, so as to eliminate any possible virus contamination, was transferred to *N. tabacum* cv. Xanthi and maintained in a growth chamber with 12 h nights at 20 ^{0}C and 12 h days at 25 ^{0}C and 15,000 lx.

In experiments on transmission of virus by aphids from diseased plants to healthy plants, apterous aphids were removed with a fine brush to a 6 cm beaker lined with damp paper towel, fasted for 3- 4 h, then given access to virus source plants. Aphids were placed on the detached leaves of virus source plants and individual aphids were monitored and allowed to probe or feed for about 1 min and then transferred to healthy small seedlings of *N. clevelandii* or *N. tabaccum* cv. White Burley covered with cages. Aphids were killed with Pyrethrum insecticide 24 h after transmission.

In experiments using membrane feeding system (Rochow, 1960; Pirone, 1964), sucrose was added to virus preparations to a final 5% concentration. The virus preparations (about 250 to 300 µl) in phosphate buffer were sandwiched between two layers of stretched Parafilm membrane (Parafilm M, American Can Company) secured at the top of a feeding

chamber consisting of a plastic tube 15 mm in diameter. Fasted aphids were placed on the membrane and each aphid was observed and removed after probing for 1-2 min. The aphids were then caged on small seedlings of *N. clevelandii* or *N. tabacum* for 24 h and then killed with insecticide. The plants were maintained in the glasshouse and examined for symptom expressions over 3 weeks.

2.20 Cross absorption of antiserum to VTAV with TCMV

The antiserum to VTAV cross-reacted with TCMV in gel immunodiffution tests. Such a cross reaction was eliminated by two cycles of incubation of the antiserum with TCMV (1 mg of TCMV was added to 200 µl of the antiserum) at 25 ^{0}C for 3 h. Excess virus and virus-antibody aggregates were removed by ultracentrifugation at 370,000 g for 25 min.

Chapter 3 *In Vitro* Reassembly of Viral Particles

Introduction

Cucumovirus particles are stabilized by RNA-protein interactions (Kaper and Geleen, 1971; Kaper and Waterworth, 1981). It has been shown that at high concentrations of neutral chloride salts CMV and TAV can be readily dissociated into their RNA and protein components (Francki *et al.*, 1966; Kaper, 1969; Habili, 1974). The reassociation of the RNA and protein into particles can be achieved by diluting or dialyzing the mixture against low salt concentration buffers (Kaper and Gellen,1971; Habili, 1974).

Because this technique of *in vitro* reassembly was to be used to study the characteristics of *in vitro* encapsidation of nucleic acids into cucumoviruses, and the dependence of aphid transmissibility on virus coat protein, a study was done to develop an efficient reassembly system suitable for both VTAV and MCMV.

Results and discussion

1. Preparation of viral coat proteins

1.1 LiCl-mediated dissociation of virus particles

Dissociation of virus particles with 2 M LiCl (Francki *et al.*, 1966) gave good protein quality and a high recovery for VTAV, but MCMV protein was not completely dissociated from its viral RNA. Both viruses disassociated completely in 3 M LiCl with $O.D_{280/260}$ 1.39 for VTAV and 1.48 for MCMV, respectively (Fig. 3-1).

1.2 LiCl concentrations essential for reassembly

Proteins in 3 M LiCl could not be used directly in reassembly because high molecular weight single stranded RNAs (ss RNAs) were precipitated when added to the protein preparations and therefore resulted in very poor assembly. A range of LiCl concentrations were tested to determine their effect on protein stability. The criteria examined were the amount of soluble protein remaining and the degree of RNA precipitation when it was added to the protein preparations. With a direct dilution method (Kaper and Geleen, 1971), it was found that VTAV RNA precipitated visually at 1.0 M but not 0.5 M LiCl and the solubility of protein at 0.5 M

LiCl was about 50% of that at 3 M LiCl. Therefore, 0.5 M LiCl was chosen as the standard salt concentration of coat protein preparation for reassembly. The loss of soluble protein due to the direct dilution of LiCl was minimized by a dialysis procedure which included sequential dialysis at 2 M, 1 M, and then 0.5 M LiCl, at pH 8.4 and with DTT added at the 0.5 M LiCl dialysis stage. Thus, with this procedure about 80% of VTAV coat protein and about 60% of MCMV coat protein were recovered in 0.5 M LiCl solution buffered in 0.1 M Tris-HCl, pH 8.4, containing 2.5 mM DDT.

1.3 Characteristics of viral coat protein preparations

a. UV absorption profile. The UV absorption profile of either VTAV or MCMV coat protein preparations showed a typical protein spectrum with an absorbance maximum at 278 nm and with an $O.D_{280/260}$ =1.64-1.66 (Fig. 3-2).

b. Infectivity. No infection was caused on indicator plants (systemic host *N. clevelandii* and local lesion host *C. amaranticolor*) when the protein preparations of VTAV and MCMV were inoculated mechanically to the plants.

2. Preparation of viral RNAs

Viral RNAs were extracted from purified virus preparations using the phenol-SDS procedure (2.2.4). RNAs of TMV, VTMoV, GMV, VTAV and MCMV were all intact as shown by agarose gel electrophoresis (Fig. 3-3).

3. Viral particle reassembly

3.1. Homologous reassembly

The coat protein and RNA from the same virus [VTAV coat protein (VP) and VTAV RNA (VR)] were mixed at ratios of 4 : 1, 4 : 2 and 8 : 1 by weight to determine the optimum ratio for assembly. Analysis by sucrose density gradient centrifugation showed that the stoichiometric mixture of 4 parts of protein and 1 part of RNA produced the highest yield of reassembled particles (Fig. 3-4). Thus the stoichiometric ratio was chosen as the standard ratio of assembly for either homologous or heterologous assembly. Elcctron microscopy showed that the homologously assembled VTAV (VP-VR) and MCMV (MP-MR) were cucumovirus-like particles of 28-30 nm in diameter , indistinguishable from native VTAV and MCMV (Fig.

Fig. 3-1. Spectra of coat proteins of VTAV (VP) and MCMV (MP) in 3 M LiCl. Virus preparations (3 mg/ml) were dissociated with an equal volume of 6 M LiCl (prepared in water). The supernatants containing viral coat protein were diluted 1 : 5 with 3 M LiCl.

Fig. 3-2. Spectra of coat proteins of VTAV (VP) and MCMV (MP) in 0.5 M LiCl buffered in 0.1 M Tris-HCl, pH 8.4, containing 2.5 mM DTT.

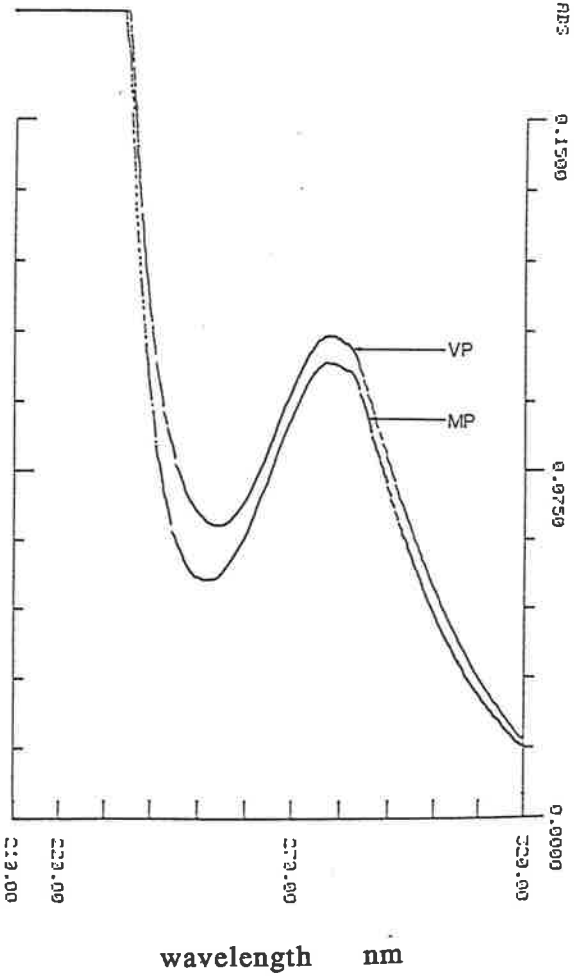

ABS
0.1500
0.0750
0.0000
VP
MP
219.00
223.00
279.00
323.00
wavelength nm

Fig. 3-2

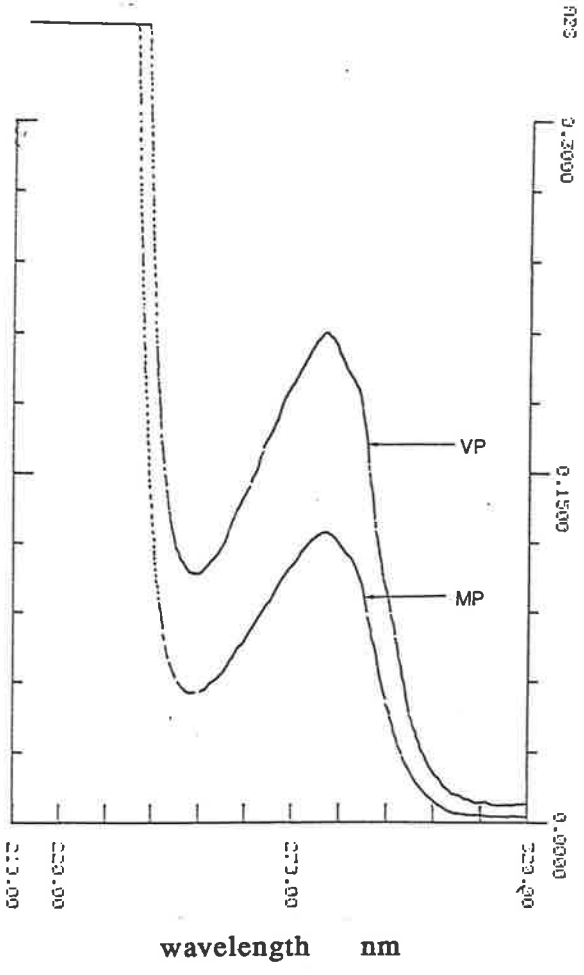

ABS
0.2000
0.1500
0.0000
VP
MP
219.00
223.00
279.00
323.00
wavelength nm

Fig. 3-1

Fig. 3-3 RNAs used in assembly. The RNAs (1 = TMV RNA, 2 = GMV RNA, 3 = VTMoV RNA, 4 = VTAV RNA, and 5 = MCMV RNA) were analyzed by electrophoresis in a 1.5% agarose gel in TAE buffer.

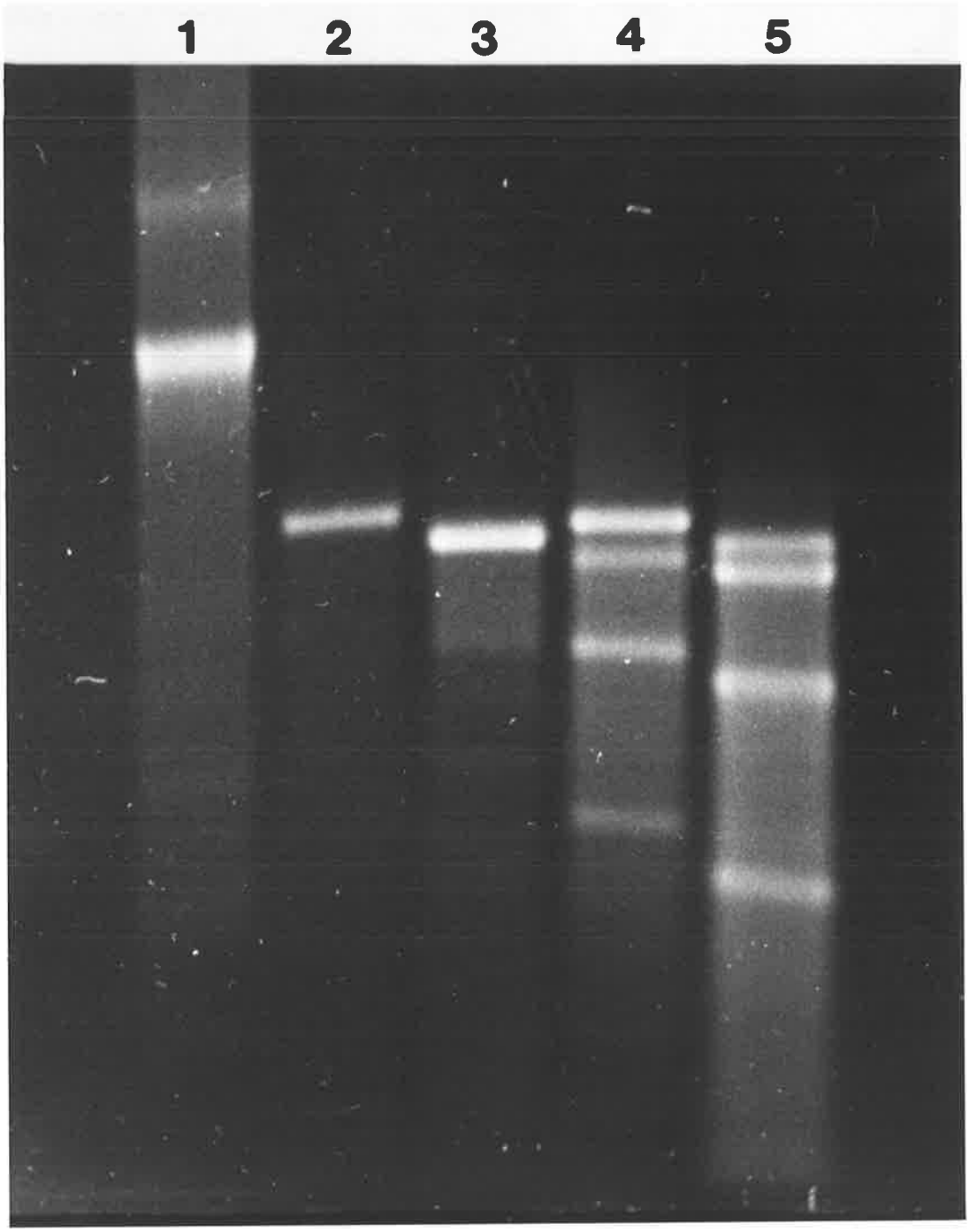
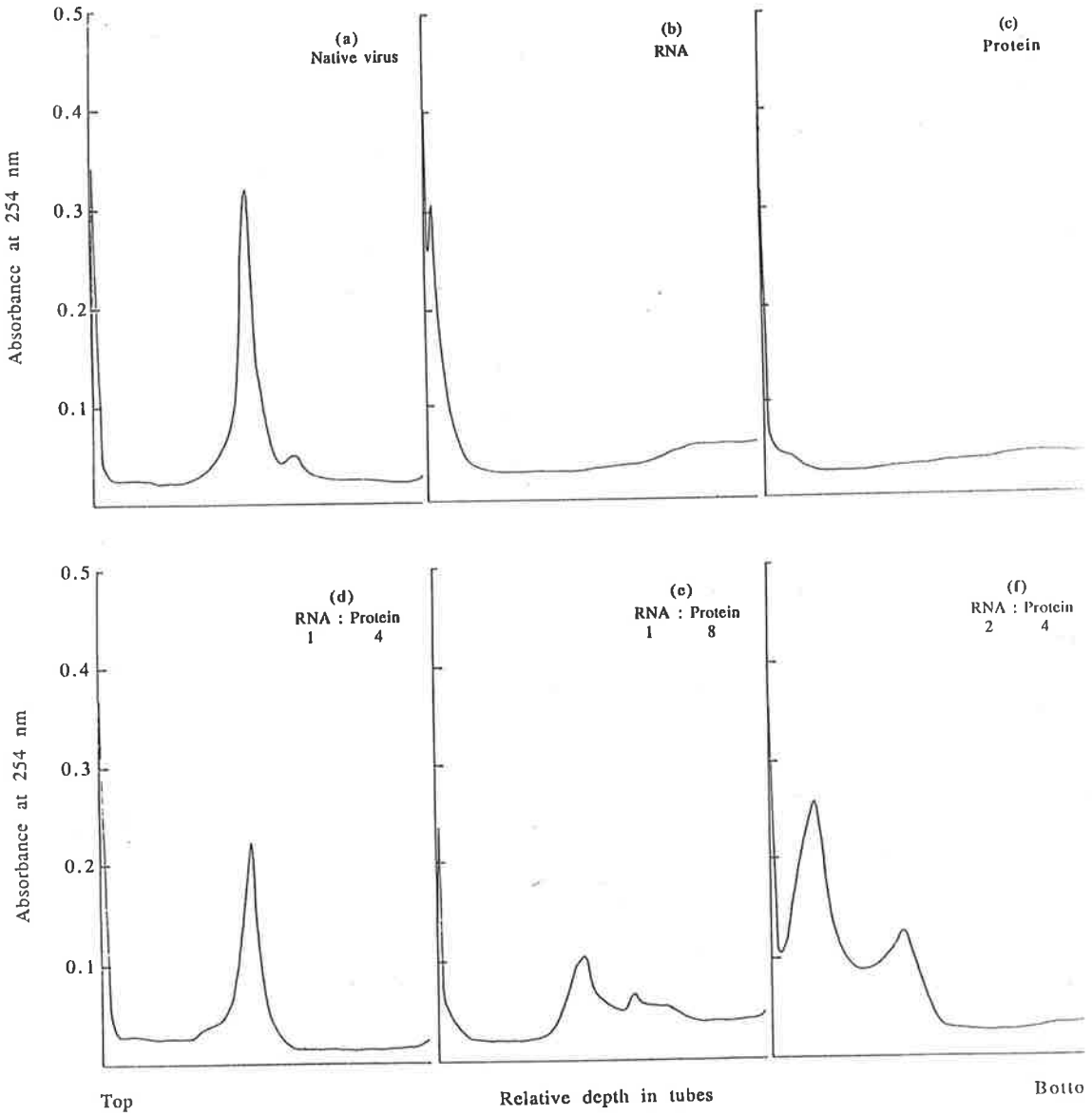

1
2
3
4
5

Fig. 3-4 Effects of RNA/protein ratio on virus assembly efficiency. The assembly efficiency was evaluated by centrifuging each RNA-protein assembly mixture through a linear 5-25% (w/v) sucrose density gradient and analysing the particle content by comparing it with a known amount of virus. Homologous VR and VP were mixed at three different ratios as follows: 200 μg of VR plus 800 μg of VP (RNA:protein=1:4), 200 μg of VR plus 1,600 μg of VP (RNA:protein=1:8) and 400 μg of VR plus 800 μg of VP (RNA:protein=2:4). A volume of assembly mixture containing RNA-protein equivalent to 150 μg VTAV (assuming 100% input RNA and protein were assembled into particles) was loaded on a sucrose gradient. The amount loaded were: in mixtures of RNA:protein=1:4, a volume containing 150 μg RNA-protein (d); of RNA:protein=1:8, a volume containing 30 μg of RNA (e); and of RNA:protein=2:4, a volume containing 120 μg of protein (f). The three controls were native VTAV (a, 150 μg), VR (b, 30 μg) and VP (c, 120 μg). Centrifugation was done in a Beckman SW 41 rotor at 35,000 rpm for 2 h. The gradients were fractionated as described in 2.2.16. Sedimentation was from left to right.


(a)
Native virus
(b)
RNA
(c)
Protein
(d)
RNA : Protein
1 4
(e)
RNA : Protein
1 8
(f)
RNA : Protein
2 4
Absorbance at 254 nm
0.5
0.4
0.3
0.2
0.1
Top
Relative depth in tubes
Botto

3-5). The reassembly efficiency for VTAV was about 75% and for MCMV 65% (efficiency was calculated from the ratio of reassembled particles recovered to the protein-RNA input, e.g., 0.7 mg of assembled particles recovered from an assembly mixture containing 0.8 mg of protein and 0.2 mg of RNA, the efficiency is 70%). No effect of protein-RNA concentration on assembly efficiency was observed in experiments with protein-RNA concentrations varied from 0.5 to 1.5 mg/ml.

3.2 Heterologous reassembly

The coat protein from one virus [VP or MCMV protein (MP)] and RNA from another virus [VR, MCMV RNA (MR), GMV RNA (GR), and TMV RNA (TR)] was mixed stoichiometrically and dialysed against reassembly buffer. Regardless of the various origins of the viral RNAs, all particles reassembled were cucumovirus-like with a diameter of 28-30 nm (Fig. 3-6). The heterologous reassembly efficiency relative to VP was from 65% for VP-MR and 70% for VP-TR and those relative to MP was 70% for MP-VR and MP-TR.

4. Physico-chemical properties of the reassembled viral particles

4.1 Sedimentation properties in sucrose density gradient centrifugation

Homologously reassembled VTAV (VP-VR) and MCMV(MP-MR) had exactly the same sedimentation rates as the native VTAV and MCMV when analysed by sucrose density gradient centrifugation. The peak of VP-VR co-sedimented with that of VTAV (Fig. 3-7 i) as did that of MP-MR with MCMV (Fig. 3-7 ii).

Heterologous reassembly within the cucumoviruses (e.g., VP-MR) produced particles which sedimented at the same rate as native VTAV (Fig. 3-8 i). In contrast, heterologous reassembly with VP and the relatively high molecular weight TMV RNA (TR) produced particles which sedimented faster than VTAV when compared in the same gradient (Fig. 3-8 ii).

4.2 Infectivity and identities of protein and RNA of reassembled viral particles

All reassembled viral particles were infectious when inoculated mechanically to their local or systemic host plants. VP-VR, VP-MR or MP-MR and MP-VR caused local lesions on leaves of *C. amaranticolor* and systemic infection on *N. clevelandii*. VP-GR induced local

Fig. 3-5 Electron micrographs of homologously reassembled virus particles of VP-VR (a), MP-MR (b) and native VTAV (c) and MCMV. Bar represents 100 nm.

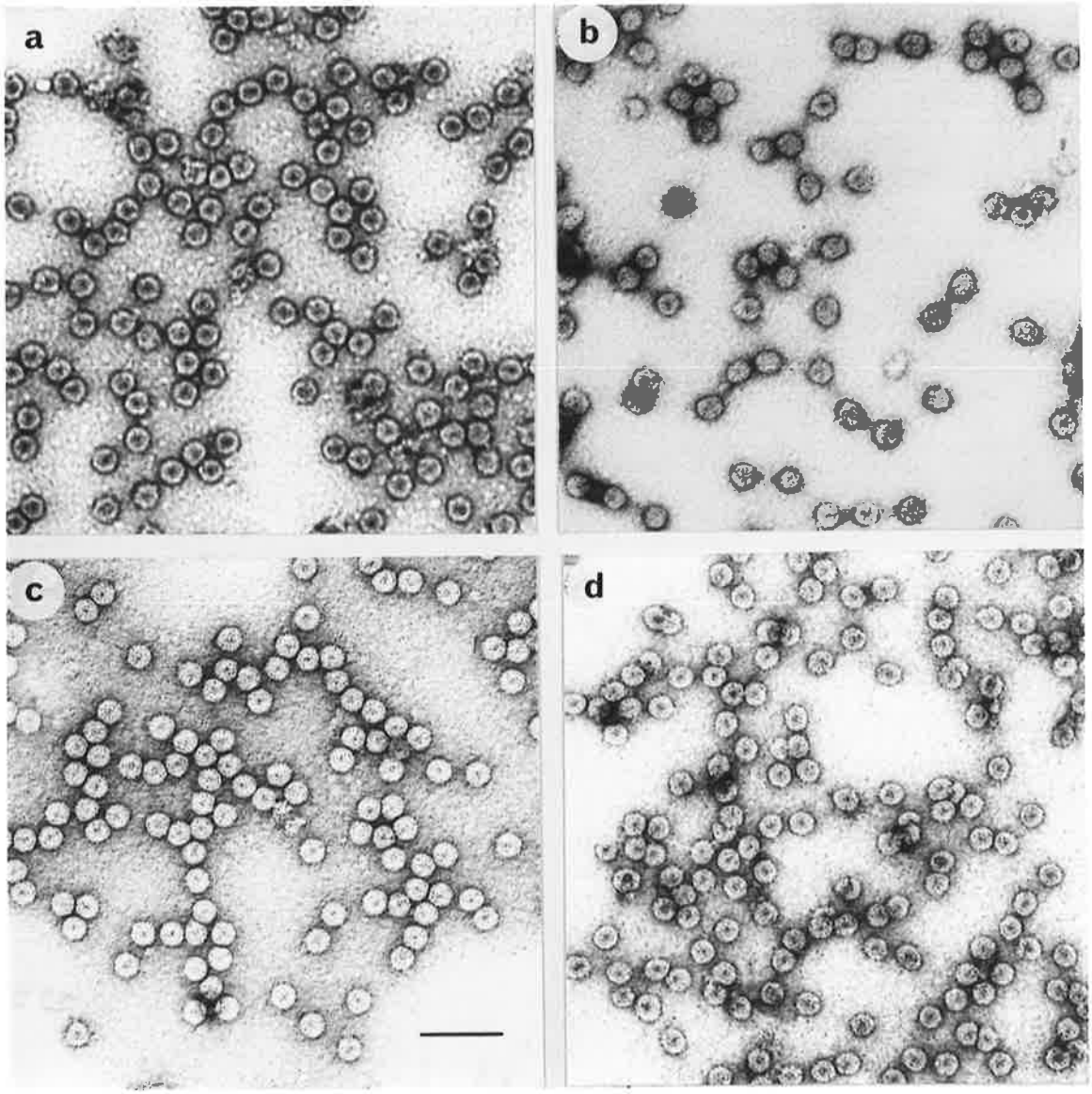

a
b
c
d

Fig. 3-6 Electron micrographs of heterologously reassembled viral particles of VP-MR (a), VP-VTR (b), VP-TR (c), VP-GR (d), MP-VR (e) and MP-TR (f). The individual reassembled particles were pelleted through a 15% sucrose cushion at 370,000 g for 20 min from their assembly mixtures after clarification at 10,000 g for 10 min. The particles were resuspended in 20 mM phosphate buffer, pH 7.5. Bar represents 100 nm.

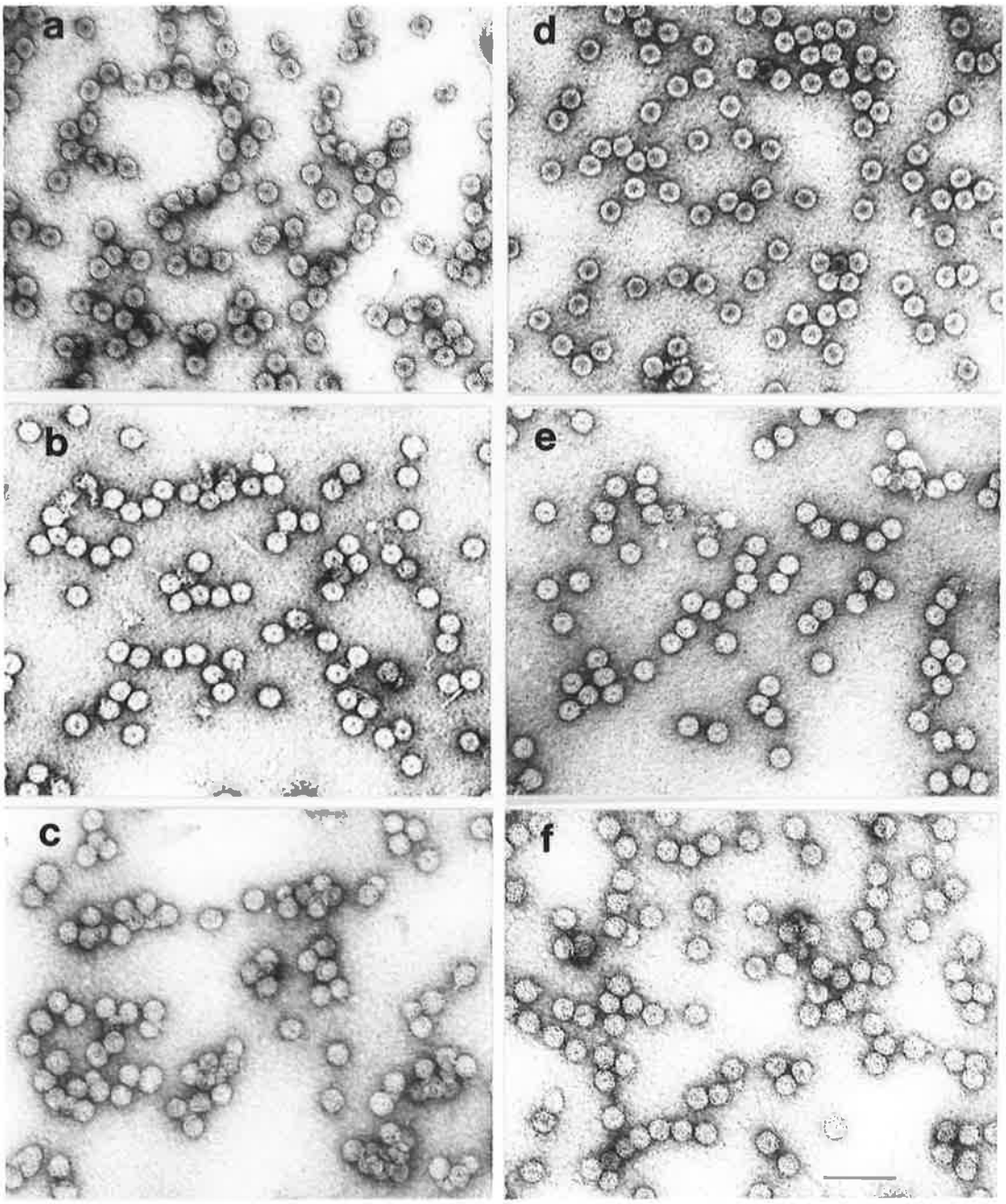
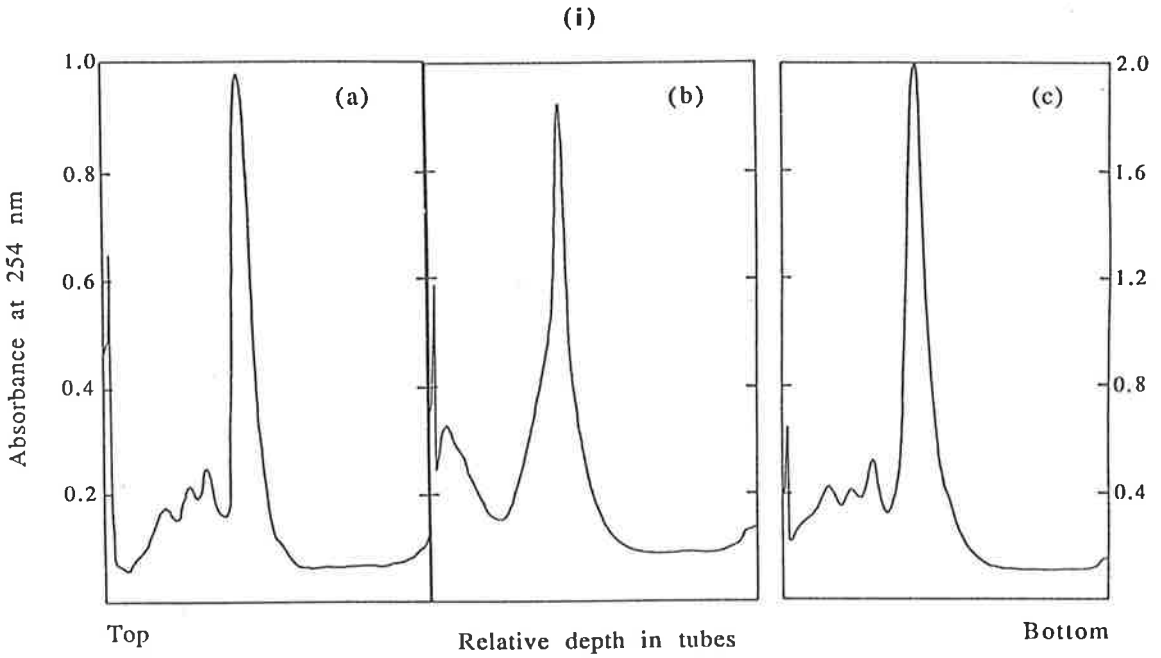

a
d
b
e
c
f

Fig. 3-7 Comparison of sedimentation properties of homologously reassembled virus particles and native viruses in sucrose density gradient centrifugation. After centrifugation, the gradients were fractionated as described in 2.2.16.

(i). Comparison of VP-VR and native VTAV. Native VTAV (a) and VP-VR (b), 300 μg each, and a mixture of 150 μg each of native VTAV and VP-VR (c) were layered on 5-25% sucrose density gradients and centrifuged at 35,000 rpm for 80 min.

(ii). Comparison of MP-MR and native MCMV. Native MCMV (a) and MP-MR (b), 300 μg each, and a mixture of 150 μg each of native MCMV and MP-MR were analysed as above (i).


(i)
(a)
(b)
(c)
1.0
0.8
0.6
0.4
0.2
2.0
1.6
1.2
0.8
0.4
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom

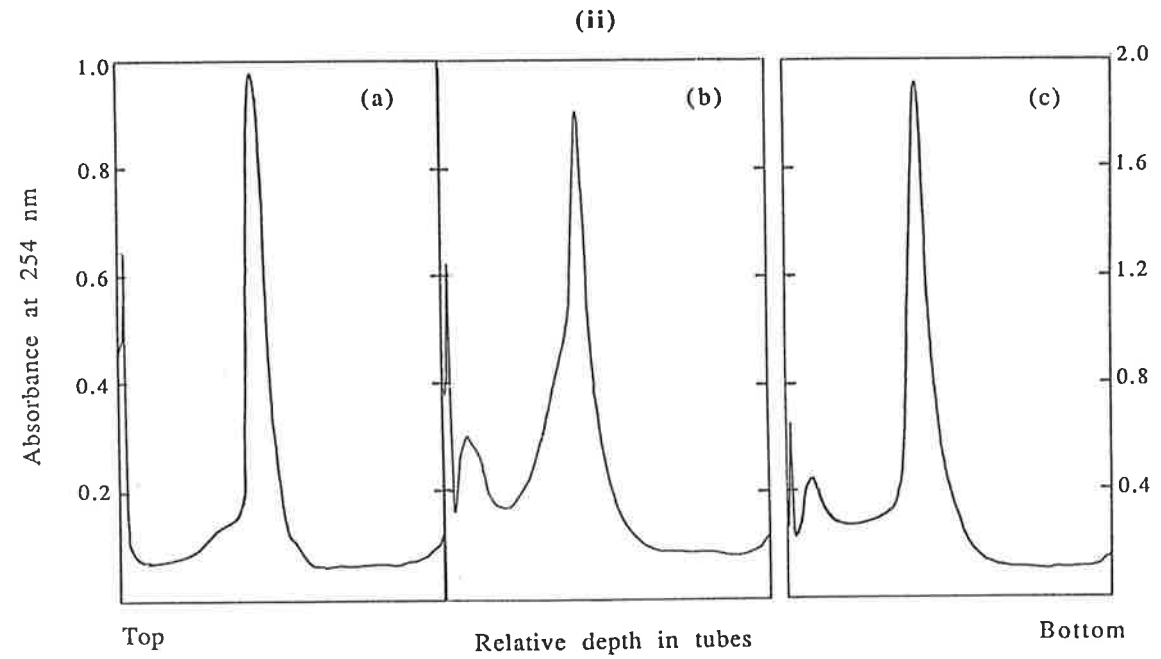
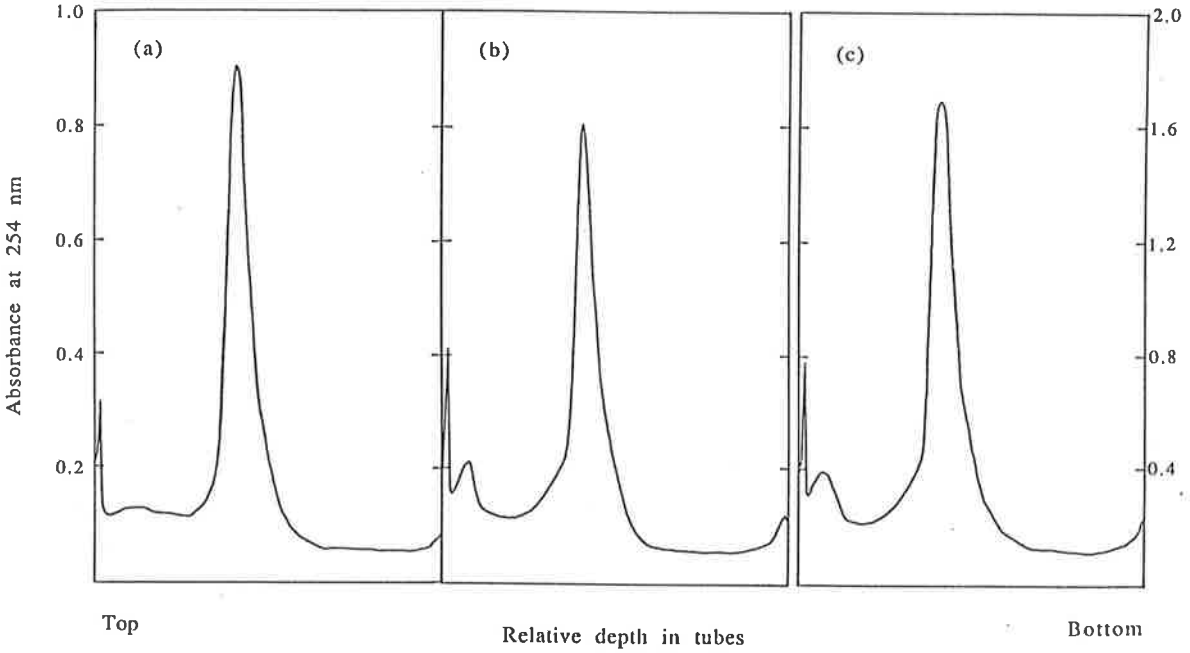

(ii)
(a)
(b)
(c)
1.0
0.8
0.6
0.4
0.2
2.0
1.6
1.2
0.8
0.4
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom

Fig. 3-8 comparison of sedimentation properties of heterologously reassembled virus particles and native VTAV in sucrose density gradients.

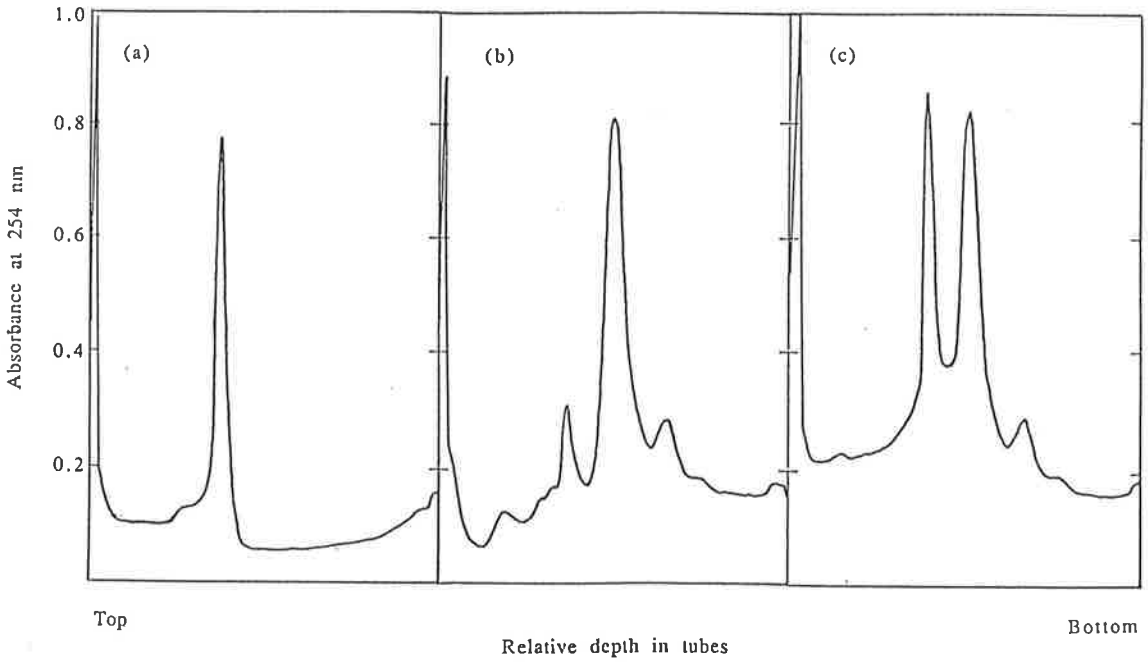
(i). Comparison of native VTAV and VP-MR. Native VTAV (a) and VP-MR (b), 300 μg each, and a mixture of 150 μg each of native VTAV and VP-MR (c) were analysed as in Fig. 3-7

(ii). Comparison of native VTAV and VP-TR. Native VTAV (a) and VP-TR (b), 300 μg each, and a mixture of each of 300 μg and VP-TR (c) were analysed as in Fig. 3-7.

(i)


1.0
0.8
0.6
0.4
0.2
(a)
(b)
(c)
2.0
1.6
1.2
0.8
0.4
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom

(ii)


1.0
0.8
0.6
0.4
0.2
(a)
(b)
(c)
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom

lesions on *Phaseolus vulgaris*. VP-TR or MP-TR produced local lesions on leaves of *N. glutinosa* and systemic infection on *N. clevelandii* and *N. tabacum*. VP-VTR (VP and VTMoV RNA, satellite-free) caused systemic infection on *N. clevelandii*. The infections were confirmed by characteristic symptom expression, serological tests or electron microscopy showing that the infections were determined by the viral RNAs encapsidated within the particles.

The infectivity of heterologously reassembled particle VP-TR was compared with that of TMV RNA and native TMV particles. The concentration of each inoculum was adjusted to 0.005 mg RNA/ml. By using half-leaf local lesion tests on *N. glutinosa*, the number of local lesion caused by VP-TR was about twice that by TMV RNA but about one third of that by TMV particles. The number of local lesion caused by of TMV particles was about seven times that by TMV RNA (Table 3-1). The assembled particles and naked TMV RNA were significantly different in causing the local lesion on the plants as determined by t test ($t = 4.9$, $p = 0.0078$). Thus encapsidation by VP increased the infectivity of TMV RNA.

To evaluate the quality of assembled particles, the particles were centrifuged through a 5-25% sucrose density gradient. Fractions from the gradient were inoculated to local lesion host plants. Assembled VP-VR and VP-MR produced local lesions on cowpea in the fractions corresponding to the virus peaks in the gradients (Fig. 3-9).

Infectivity was found to be associated only with the fractions from the UV-absorption peaks of the gradients loaded with the heterologously reassembled particles VP-TR (Fig 3-10 a) and VP-GR (Fig. 3-11 a).

The viral coat protein and RNA content of fractions from the sucrose density gradients was analysed. With VP-TR, TMV RNA was found in fractions corresponding to the UV-absorption peak and infectivity (Fig. 3-10 b). With VP-GR, VTAV protein and GMV RNA were found distributed from the middle to bottom position of the gradients (Fig. 3-11 b). This was probably due to the aggregation of particles since the UV absorption profile and infectivity (Fig. 3-11 a) also indicated the broad distribution of the RNA-protein. The protein and RNA of VP-MR were mainly in the positions where the UV-absorption peak was located (Fig 3-12).

Proteins from several reassembled particles were compared by PAGE. As shown in Fig. 3-13, protein from VTAV, VP preparation, VP-VR, VP-MR, VP-GR and VP-TR had the same mobility in the gel indicating that there was no degradation of the coat protein.

RNAs recovered from reassembled particles were also compared with the RNAs used for

Fig. 3-9 Analyses of infectivity of homologously reassembled viruses VP-VR and MP-MR from sucrose density gradient fractions. Reassembled virusVP-VR (a) or MP-MR (b), 300 μg each, was centrifuged as in Fig. 3-7. The gradients were fractionated into 12 x 1 ml fractions. Fractions were diluted four times with 20 mM phosphate buffer, pH 7.5 and inoculated to *Chenopodium amaranticolor*. For each fraction, three leaves were inoculated. The local lesions were counted 3-4 days post inoculation and expressed as means for the three leavies inoculated.

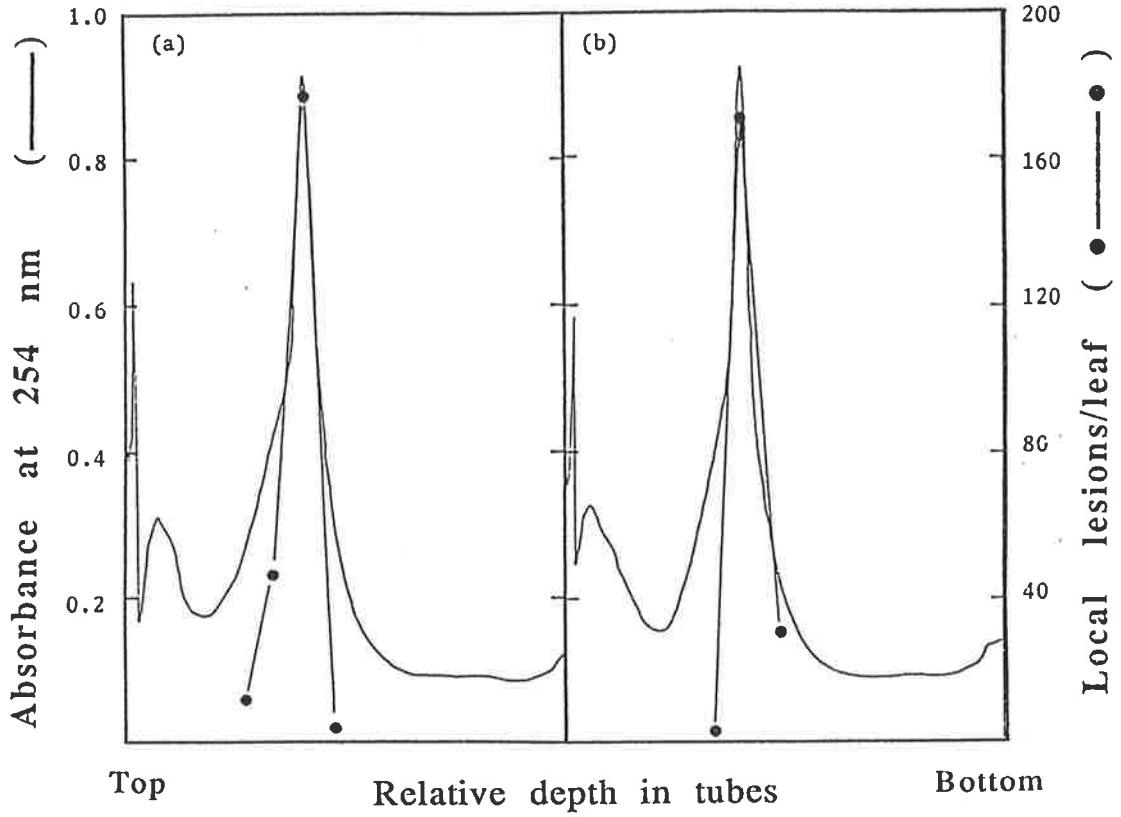

(a)
(b)
1.0
0.8
0.6
0.4
0.2
200
160
120
80
40
Absorbance at 254 nm (——)
Local lesions/leaf (●——●)
Top
Relative depth in tubes
Bottom

Fig. 3-10 Infectivity and RNA identity of heterologously assembled VP-TR.

(a). UV-absorption profile of the VP-TR in gradient (—) and infectivities of fractions (•—•). VP-TR particles (150 µg) were centrifuged and fractionated as in Fig. 3-9. Fractions were diluted three times with 20 mM phosphate buffer and for each fraction three leaves of *N. glutinosa* were inoculated. Local lesions were counted 3 days post inoculation and expressed as the mean for three leaves.

(b). RNA distribution in the gradient fractions. From each fraction, 47.5 µl was transferred to an Eppendorf tube and incubated at 25 ^{0}C for 1.5 h in 0.5% SDS (Lot and Kaper, 1976a). The sample was mixed with 5 µl of 2% bromophenol blue in 50% glycerol and electrophoresed in a 1.5% agarose gel as described in 2.2.10.

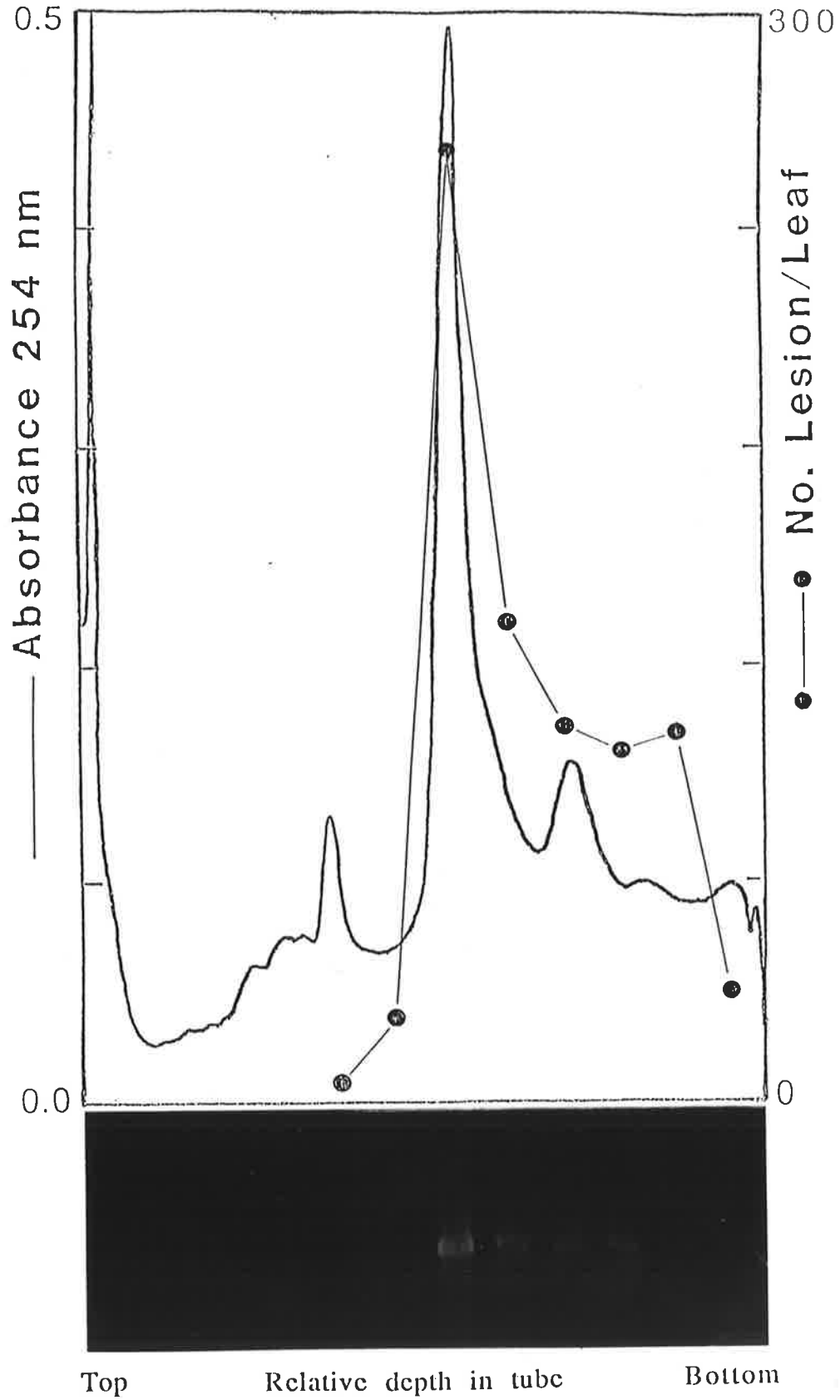

0.5
0.0
Absorbance 254 nm
300
0
No. Lesion/Leaf
Top
Relative depth in tube
Bottom

Fig. 3-11 Infectivity, protein and RNA identities of heterologously assembled VP-GR.

(a). UV-absorption profile (——) and infectivity (•—•) of VP-GR in the gradient. After clarification at 10,000 g for 10 min, 300 μg of protein-RNA from the assembly mixture was centrifuged and fractionated as in Fig. 3-7. Fractions were diluted three times with 20 mM phosphate buffer and for each fraction, three leaves of *Phaseolus vulgaris* were inoculated. Local lesions were counted and expressed as mean for three leaves.

(b). Protein distribution in the gradient fractions. From each fraction, 20 μl was transferred to an Eppendorf tube and incubated with 20 μl of water, 40 μl of sample buffer and 20 μl of dye solution at 90 ^{0}C for 5 min. Five μl of this mixture was loaded on a 12% polyacrylamide gel and electrophoresed. The gel was stained with silver as described in 2.2.10.

(c). RNA distribution in the gradient fractions. The sample preparation and electrophoresis were the same as described in the caption for Fig. 3-10 b.

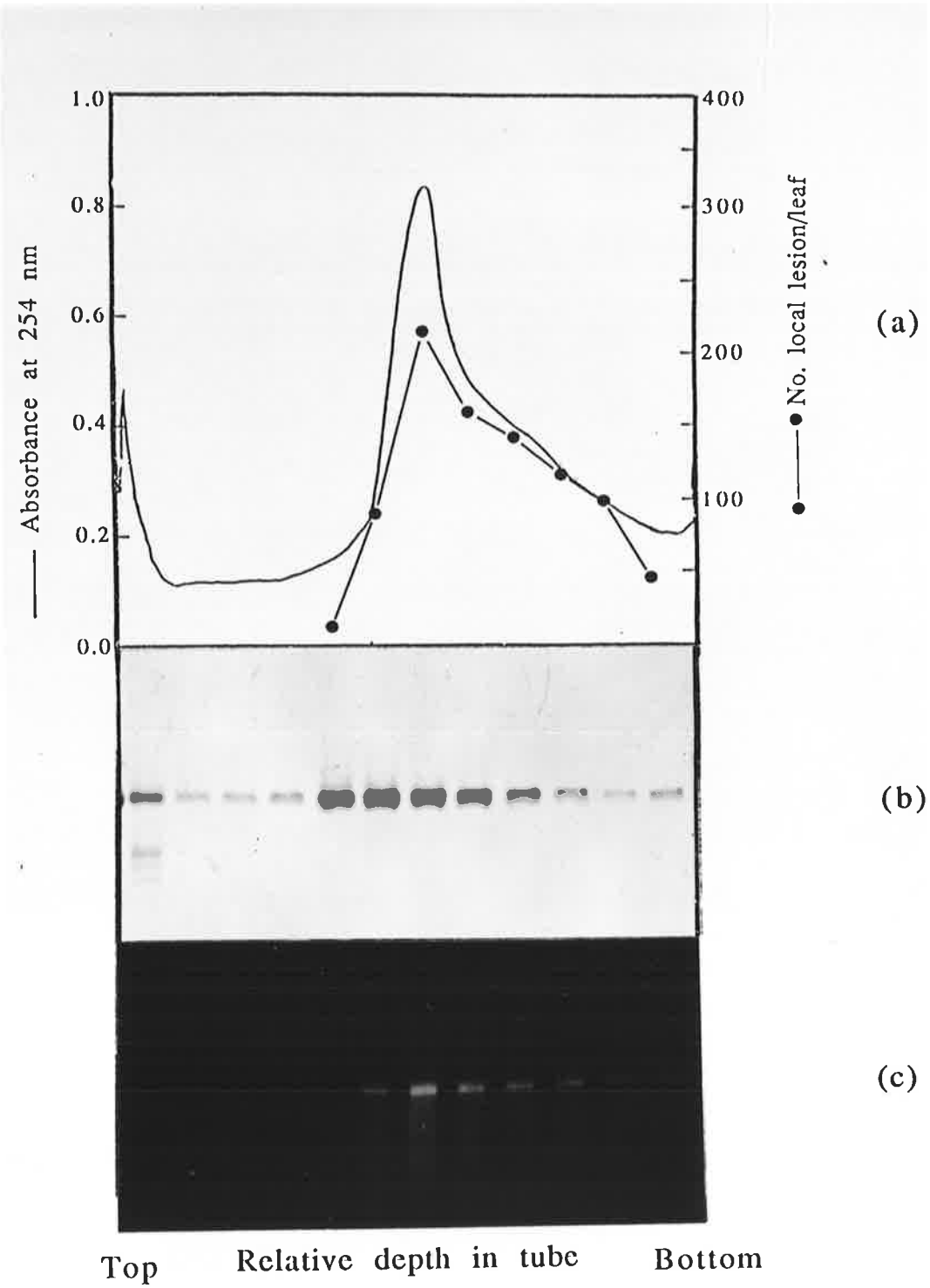

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 254 nm
400
300
200
100
No. local lesion/leaf
(a)
(b)
(c)
Top
Relative depth in tube
Bottom

Fig. 3-12. UV-absorption profile, protein and RNA identities of heterologously assembled VP-MR in 5-25% sucrose density gradient.

(a). UV-absorption profile. A sample from the assembled particle preparation containing 300 μg protein-RNA was centrifuged and fractionated as Fig. 3-9.

(b). Protein distribution in the gradient fractions. The sample preparation and electrophoresis were the same as described in the caption for Fig. 3-11 b.

(c). RNA distribution in the gradient fractions. The sample preparation and electropheresis were done the same as described in the caption for Fig. 3-10 b.

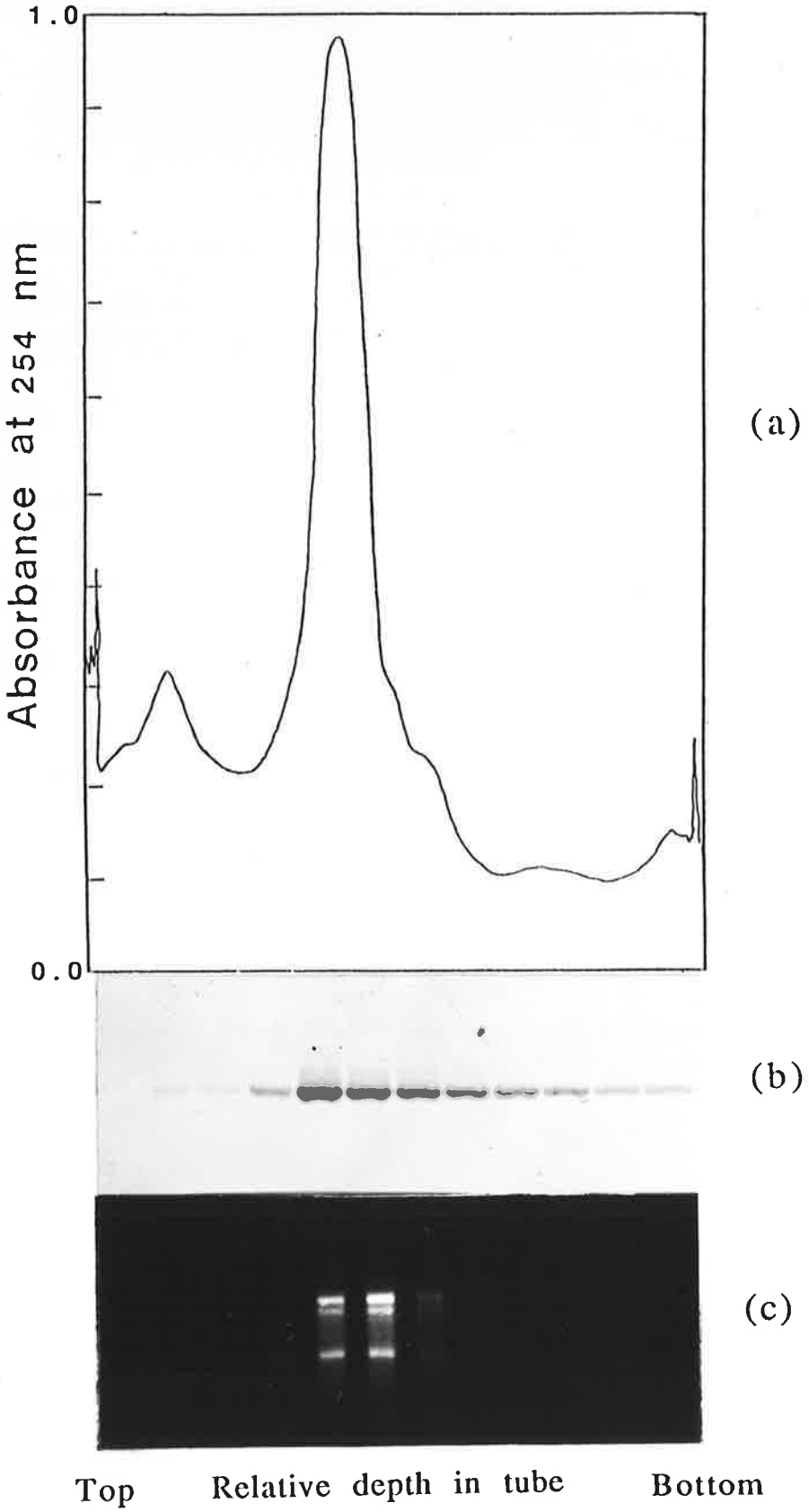

1.0
Absorbance at 254 nm
0.0
(a)
(b)
(c)
Top
Relative depth in tube
Bottom

Table 3-1. Comparison of infectivity of VP-TR , TMV RNA and TMV by local lesion assay on opposite half leaves of *N. glutinosa*

Leaf No.	Inoculum					
	VP-TR	TMV RNA	VP-TR	TMV	TMV RNA	TMV[a]
1	97[b]	38	41	175	30	171
2	63	35	75	289	23	267
3	77	21	78	208	42	303
4	59	39	98	295	45	372
5	69	38	101	381	44	197
Mean	73 ± 15[c]	34.2 ± 7.5	78.6 ± 24	269.6 ± 80.9	36.8 ± 9.8	262 ± 81.1
t	t=4.938		t=-6.871		t=-6.461	
P	p=0.0078		p=0.0024		p=0.003	

a. Underline indicates pairs compared on each leaf

b. Number of local lesions counted 2 days post inoculation

c. Standard error of mean

reassembly. As shown in Fig. 3-14, there were no differences in mobility between the input RNAs (VR, MR and TR) and the RNAs recovered from the assembled particles (VP-TR, VP-MR and VP-VR). There were no significant differences between the relative amount of individual RNA species of VATV or MCMV in the RNA extracted from the reassembled particles as compared with the RNA used (Fig. 3-14) indicating that there was no significant selection of RNA species during the assembly.

4.3 UV absorption spectrum

There were no differences in UV absorption spectra (210-320 nm) between native VTAV and homologously reassembled VP-VR (Fig. 3-15) indicating that the reassembled VP-VR contained the same amount of RNA as native VTAV. However, heterologously reassembled particles VP-TR showed a spectrum different from that of TMV but similar to that of VTAV (Fig. 3-16) with $O.D_{260/280}$=1.70 indicating that the percentage of RNA in VP-TR was greater than that of TMV.

Fig. 3-13. Comparison of protein from assembled particles with VP and native viruses whose RNAs were used for reassembly by PAGE. Native VTAV (lane V), GMV (lane G), MCMV (lane M) and TMV (lane T) were compared with assembled VP-GR (lane a), VP-VR (lane b), VP-MR (lane c) and VP-TR (lane d). VP preparation in 0.5 M LiCl (lane vp) was used as a control. The loadings for lanes V, G, M and T were 50 ng virus, for lane vp 40 ng VP and for lanes a, b, c and d, 20 μl of peak fraction of assembled viruses in sucrose gradients (Figs. 3-9 a, 3-10 a, 3-11 a and 3-12 a) each. Arrows indicate the positions of coat proteins from the native viruses in the gel.

Fig. 3-14. Comparison of RNAs used for assembly (TMV, lane 1; MCMV, lane 2; and VTAV, lane 3) and RNAs isolated from the assembled virus particles (VP-TR, lane 4; VP-MR, lane 5; and VP-VR, lane 6). Each lane was loaded with 2 μg of TMV RNA or 3 μg of VTAV or MCMV RNA.

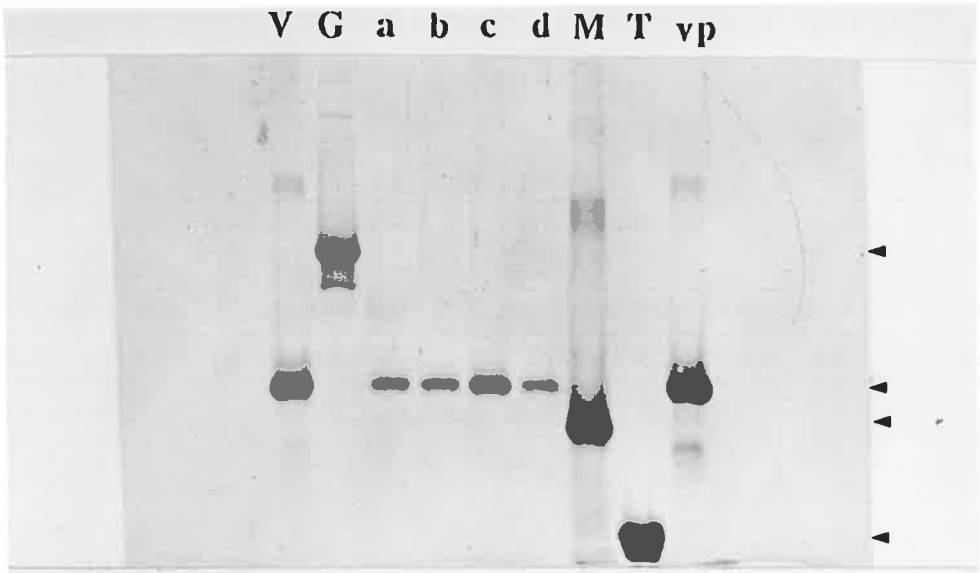


Fig. 3-13

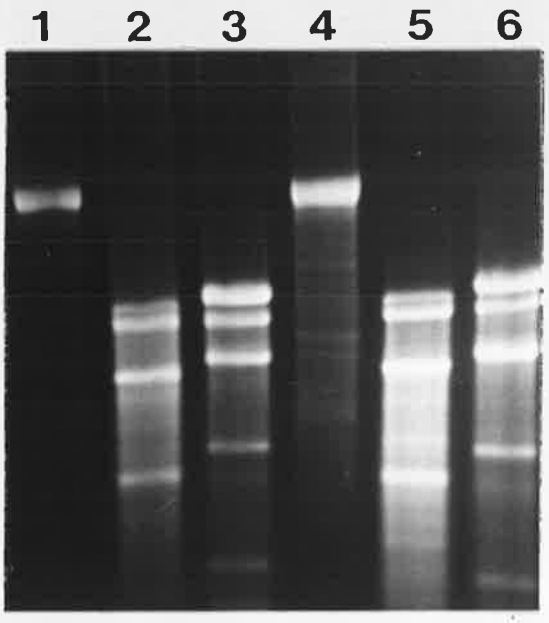
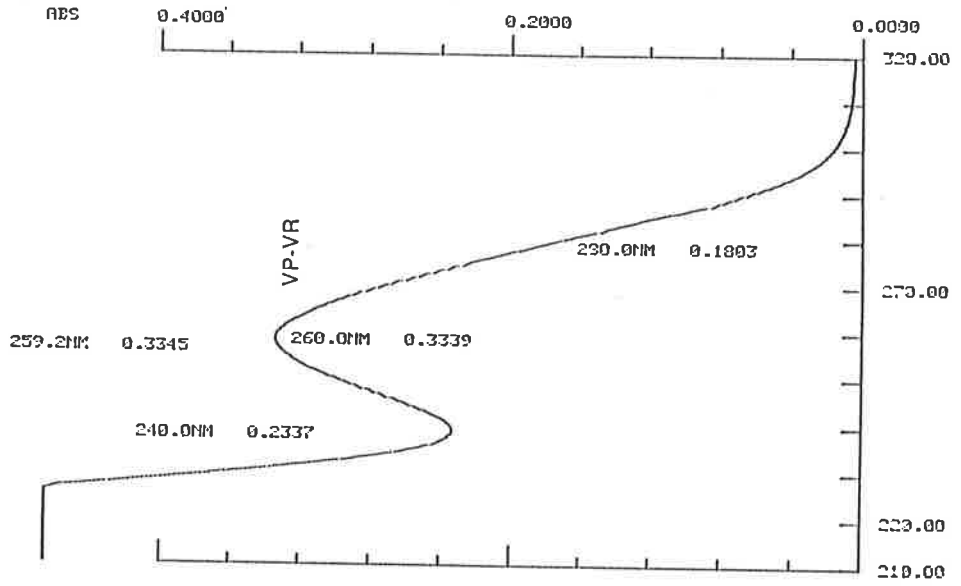


Fig. 3-14

Fig. 3-15. UV absorption spectra of native VTAV (i) and assembled VP-VR (ii).

(ii)


ABS
0.4000
0.2000
0.0000
320.00
VP-VR
230.0NM 0.1803
270.00
259.2NM 0.3345
260.0NM 0.3339
240.0NM 0.2337
220.00
210.00
wavelength nm

(i)

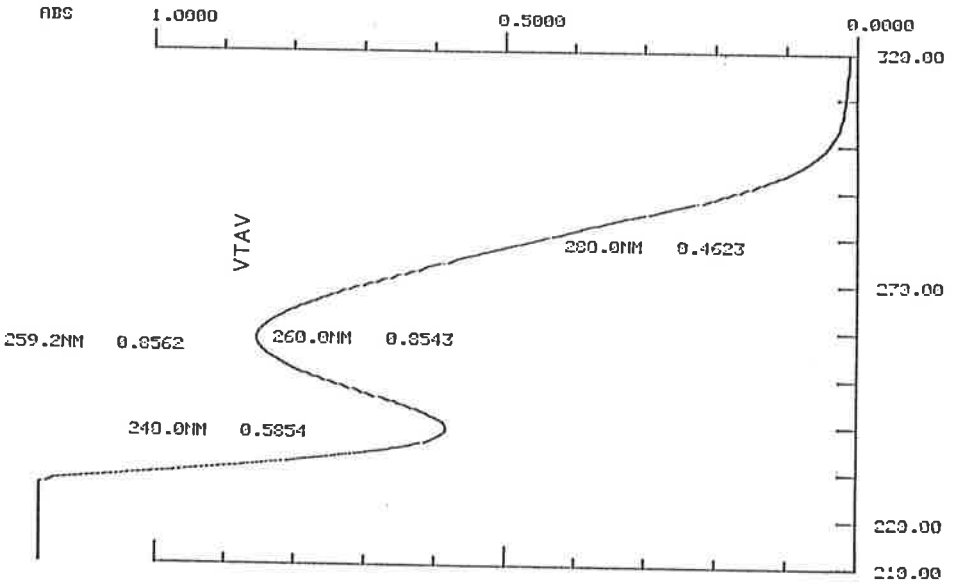
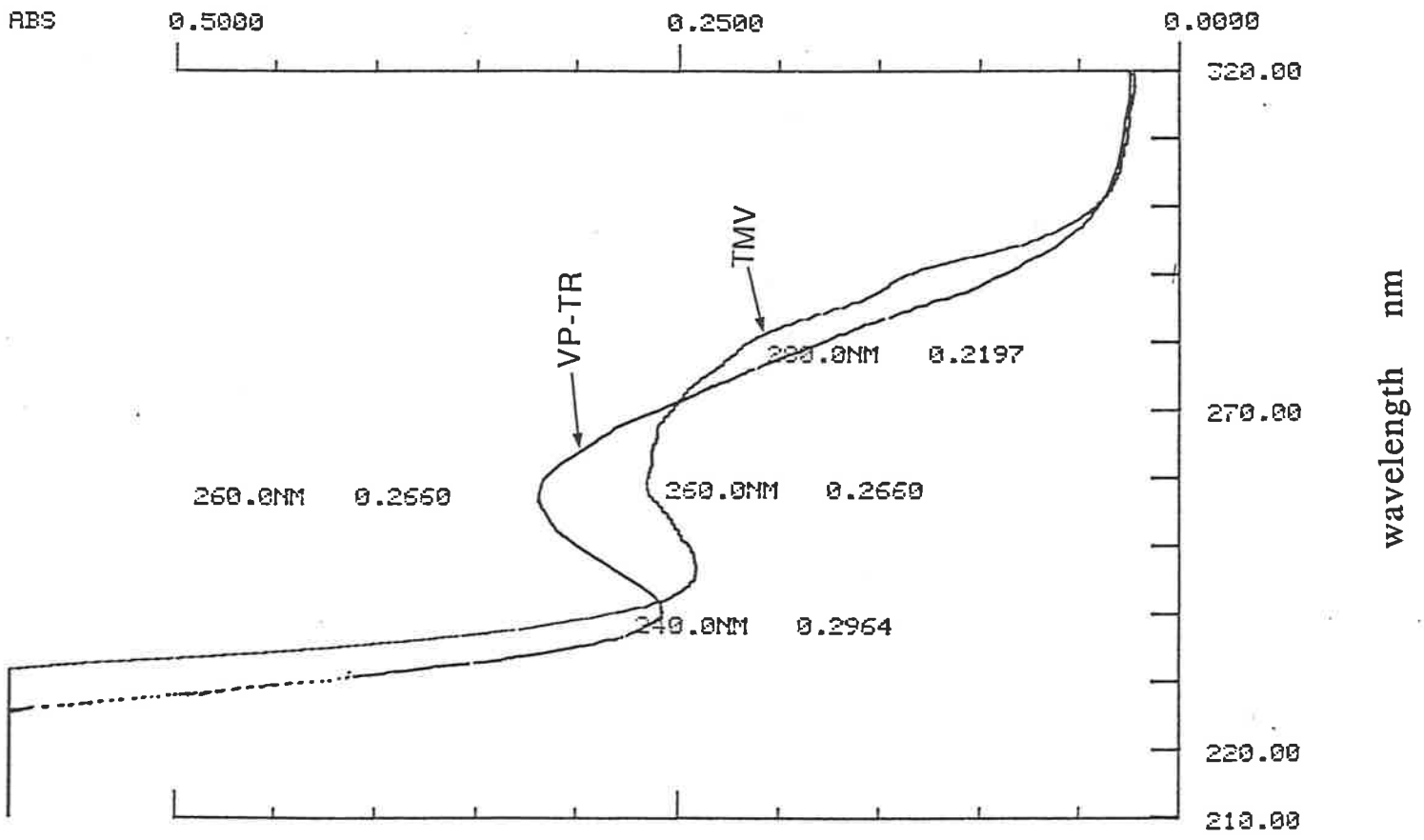

ABS
1.0000
0.5000
0.0000
320.00
VTAV
280.0NM 0.4623
270.00
259.2NM 0.8562
260.0NM 0.8543
240.0NM 0.5854
220.00
210.00
wavelength nm

Fig. 3-16. UV absorption of spectra of native TMV and assembled VP-TR.


ABS
0.5000
0.2500
0.0000
320.00
270.00
220.00
210.00
wavelength nm
VP-TR
TMV
280.0NM 0.2197
260.0NM 0.2660
260.0NM 0.2660
240.0NM 0.2964

5. Serological identity

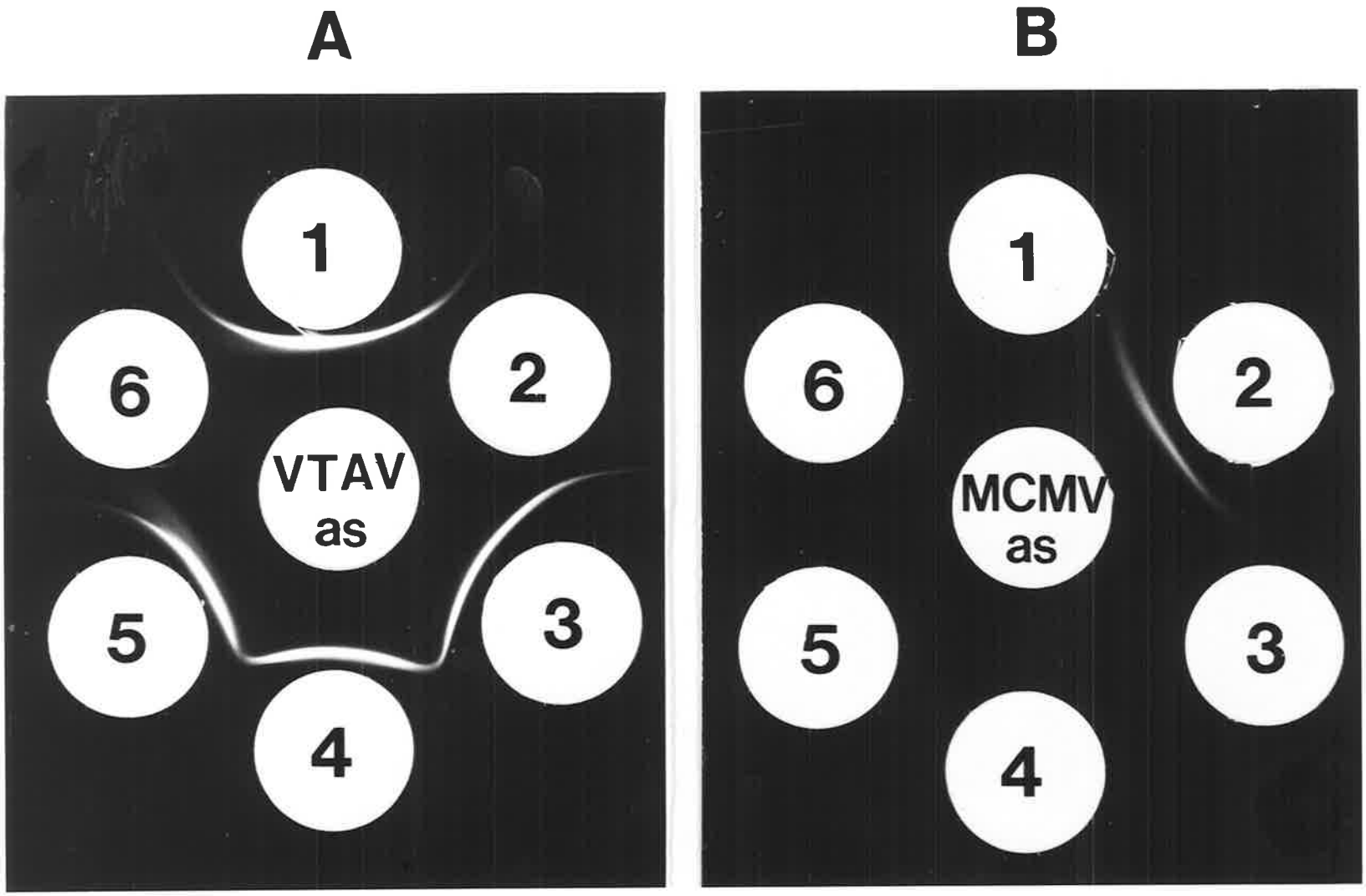
The serological properties of the reassembled viral particles were analyzed. In agar gel immunodiffusion tests, all reassembled viral particles containing VTAV coat protein reacted with antiserum against VTAV but not with antiserum against MCMV, and the precipitates merged with that of native VTAV without a spur (Fig. 3-17).

Conclusion

The results demonstrated that the *in vitro* reassembled homologous cucumovirus particles were indistinguishable from those of the native virus in morphology, sedimentation rate , RNA and coat protein compositions and serological identity. The heterologously reassembled particles with VTAV or MCMV coat protein retain cucumovirus-like morphology and serological properties. In particular, the biological identity of the heterologous viral RNAs in the reassembled particles remains unchanged.

The high efficiency of viral coat protein preparation and viral protein-RNA reassembly establishes the possibility of using this *in vitro* reassembly system to study some other phenomena relevant to cucumovirus particle structure and biological aspects, which are covered in the following chapters.

Fig. 3-17. Immunogel double diffusion tests of assembled virus particles. The peripheral wells contained 20 μg each of VP-MR (1), native MCMV (2), VP-VR (3), native VTAV (4), VP-TR (5) and native TMV (6). The central wells were loaded with antisera (As) to either VTAV (plate A) or MCMV (plate B).


A
1
2
3
4
5
6
VTAV
as
B
1
2
3
4
5
6
MCMV
as

Chapter 4 *In vitro* encapsidation of CMV RNA species

Introduction

Cucumoviruses sediment as one homologous component with a $S_{20,w}$ of 99 (Habili and Francki, 1974a; Francki *et al.*, 1979). Based on the molecular weights of virus particle, viral RNA and the coat protein subunit, it has been calculated that the genome of cucumovirus must be encapsidated in more than one type, possibly in three types of particles (Habili and Francki, 1974a). Attempts have been made previously by some workers to differentiate these expected particles, but no conclusive separation has been achieved due to the instability of cucumoviruses in high salt solutions (Habili and Francki, 1974a; Lot and Kaper, 1976b). Therefore the overall picture of encapsidation of viral RNAs in cucumovirus, particularly the encapsidation of RNAs 3 and 4, remains unclear.

Construction of particles with known RNA content by *in vitro* reassembly may help to develop an understanding of *in vivo* encapsidation of cucumovirus. This chapter presents the results of experiments on the *in vitro* encapsidation of the TCMV RNAs.

Results and discussion

1. Preparation of TCMV RNA species and TCMV coat protein

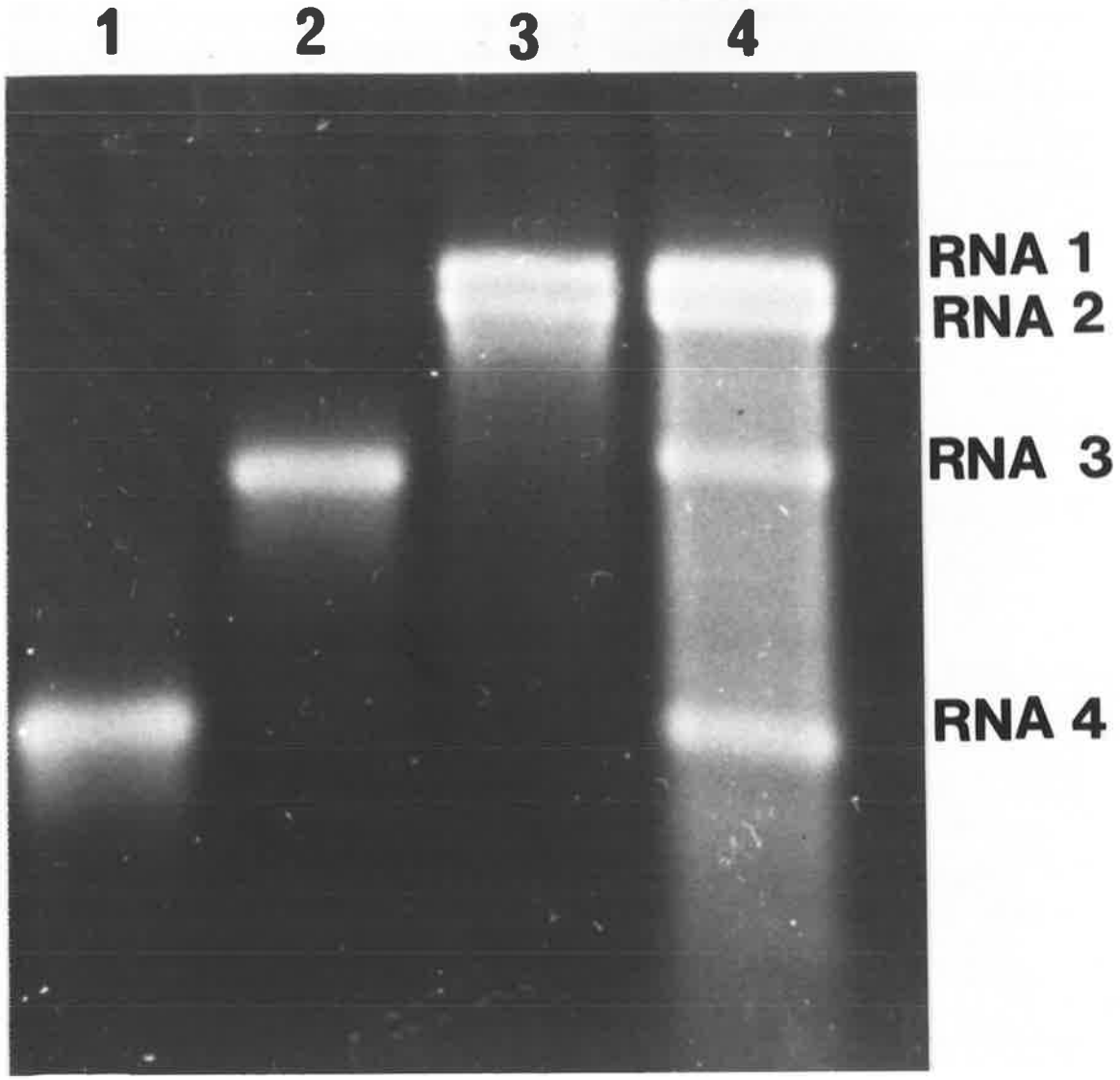
With the method described by Heery *et al.* (1990), TCMV RNAs 1+2, 3 and 4 are well separated from each other by one cycle of gel electrophoresis (Fig. 4-1). RNA was recovered from the gel at rates of 80-90%. RNAs 1 and 2 were used as a mixture throughout the experiments because of the difficulty of separating adequate amounts of sufficient purity. The TCMV coat protein was prepared as described in 2.2.8.

2. Properties of assembled particles with different RNA contents

2.1 Properties of particles encapsidating RNA 1 or 2

Particles assembled with TCMV coat protein (TcP) and a mixture of RNAs 1 and 2 sedimented as a major and two minor peaks in sucrose density gradients (Fig. 4-2 a ①). The major peak had the same sedimentation rate as native TCMV (Fig. 4-2 a ②). When the assembled particles were mixed with native TCMV and centrifuged in the same tube, the

Fig 4-1. The RNA species of TCMV used for *in vitro* assembly. Purified RNA species (lanes 1-3) were compared with TCMV genomic RNAs (lane 4) from which single species was separated and purified. The loadings were 0.6 μg RNA in lanes 1 and 2 , 1 μg in lane 3 and 2.5 μg in lane 4. Electrophoresis was in a 1.5% agarose gel in TAE buffer and the gel was stained with ethidium bromide and photographed under UV illumination.


1
2
3
4
RNA 1
RNA 2
RNA 3
RNA 4

mixture produced a single, enhanced peak (Fig. 4-2 a ③) indicating that the components had the same sedimentation rates. Assembled particles recovered from the gradient peak were typically TCMV-like with the size of 28-30 nm in diameter when viewed in the electron microscope (Fig. 4-2 b). RNAs 1 and 2 were recovered from the assembled particles (Fig. 4-2 c). One of the two fast migrating minor peaks of the assembled particles co-sedimented with the fast migrating peak of native TCMV indicating that it was probably due to the formation of particle dimers. The composition of these minor peaks was not further analysed. Since the assembled particles have the same sedimentation rate as native TCMV, RNAs 1 and 2 must be encapsidated separately otherwise the particles would be heavier and thus sediment faster as demonstrated by the assembled particles with TMV RNA and VTAV coat protein (3.4.1).

2.2 Properties of particles encapsidating RNA 3

Particles from the assembly mixture of TcP and RNA 3 sedimented as four peaks in sucrose density gradients (Fig. 4-3 a ①). As shown in Fig. 4-3 a ①, the minor peaks (1 and 2) sedimented slower but the major peak (3) slightly faster than native TCMV (Fig. 4-3 a ②). The major peak in ① became a shoulder to the peak of native TCMV while the fourth peak in ① merged with the fast-migrating component of native TCMV when assembled particles (①) were mixed with native TCMV (②) (Fig. 4-3 a ③). Samples prepared from the assembly mixture revealed TCMV-like particles predominantly of 28-30 nm in diameter, but some larger particles (33-35 nm) were also observed (Fig. 4-3 b). RNA 3 was recovered from all four peaks of the sucrose gradients shown in Fig. 4-3 a ① (Fig. 4-3 c).

2.3 Properties of particles encapsidating RNA 4

The particles assembled with RNA 4 sedimented as four peaks in sucrose density gradients (Fig. 4-4 a ①). The first and second peaks migrated significantly slower than the native TCMV (Fig. 4-3 a ②). The fourth peak was a "shoulder" of the third (Fig. 4-4 a ①), which sedimented at the same position as native TCMV. However, the fourth peak disappeared when the assembled particles were mixed with native TCMV at an equal ratio and centrifuged in the same tubes (Fig. 4-4 a ③). Possibly it was within the range of sedimentation of native TCMV. Electron microscopy showed that the assembly mixture contained particles of mostly 28-30 nm in diameter (Fig. 4-4 b). RNA 4 was recovered from all peaks except the first one detected

Fig. 4-2

a. UV absorption profiles of assembled particles encapsidating RNAs 1 and 2 (①), native TCMV (②), and a mixture of assembled particles and native TCMV (③). The amounts layered on the tops of 5-25% sucrose gradients were 200 μg of RNA-protein from assembly mixture (calculated from the input, assuming 100% assembly efficiency) (①), 100 μg of native TCMV(②), and 100 μg of RNA-protein from the assembly mixture plus 50 μg native TCMV(③). Centrifugation was in a Beckman SW41 rotor at 35,000 rpm for 120 min. Sedimentation was from left to right.

b. Electron micrograph of assembled particles recovered from the main UV-absorbing peak in the gradient (Fig. 4-2 a ①). Particles were pelleted from the gradient fractions after dilution of the sucrose with 20 mM phosphate buffer, pH 7.5 and resuspended in 10 mM borate buffer, pH 7.6. Bar represents 100 nm.

c. Gel electrophoresis of RNA extracted from the assembled particles recovered from the UV-absorbing peak in the gradients (Fig. 4-2 a ①) (lane 1, approximately 1 μg) and TCMV genomic RNAs (lane 2, 2.5 μg). The conditions for electrophoresis were the same as described in the caption of Fig. 4-1.

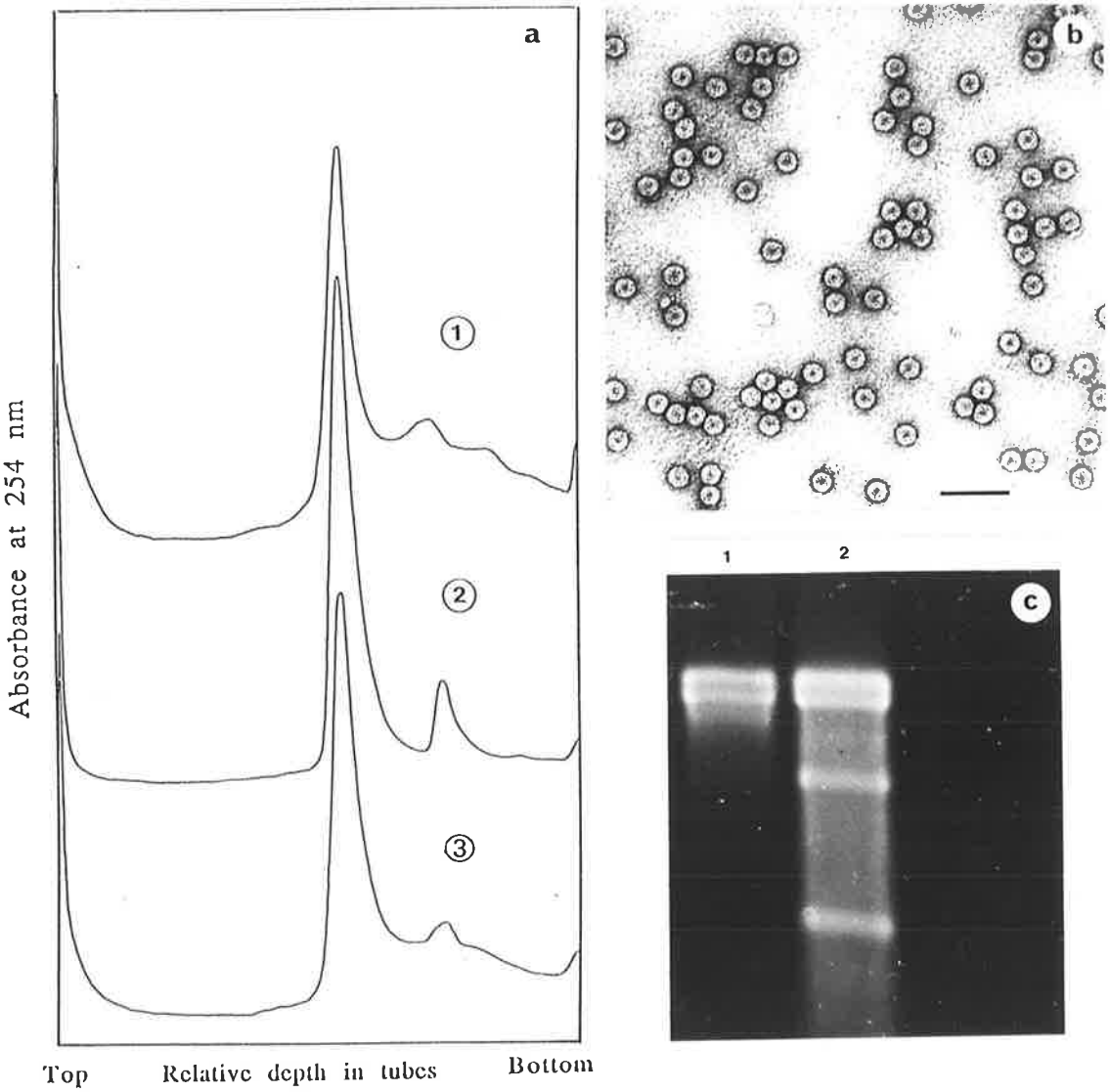
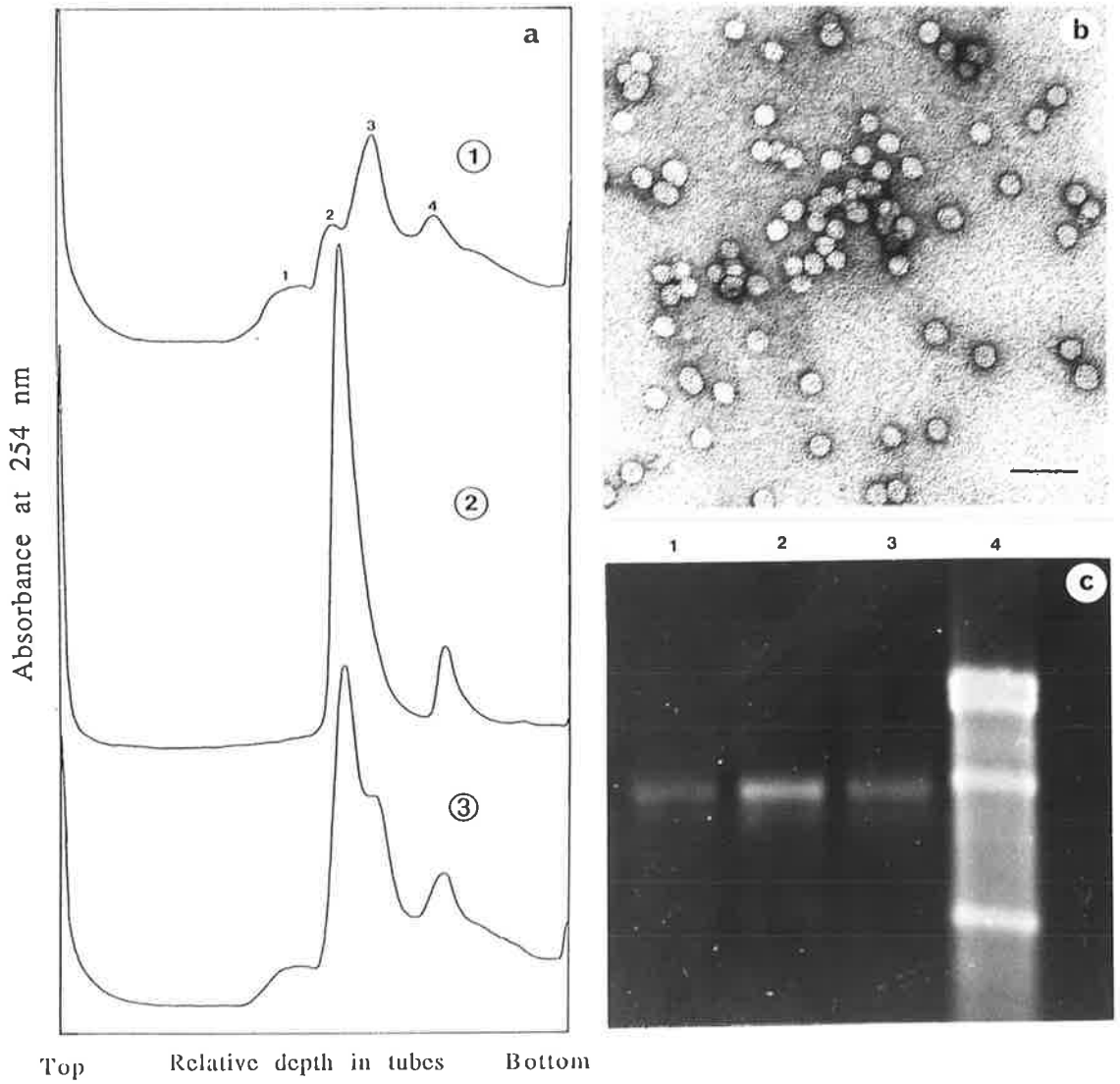

a
b
c
1
2
3
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom
1
2

Fig. 4-3

a. UV absorption profiles of assembled particles encapsidating RNA 3 (①), native TCMV (②), and a mixture of the assembled particles and native TCMV (③). The amount of virus layered on the 5-25% sucrose gradients was 200 μg of RNA-protein from assembly mixture (calculated from the input, assuming 100% assembly efficiency) (①), 100 μg of native TCMV(②), and 100 μg of RNA-protein from the assembly mixture plus 50 μg native TCMV(③). The centrifugation was conducted as stated in the caption of Fig. 4-2 a.

b. Electron micrograph of assembled particles recovered from second and third UV-absorbing peaks in the gradients (Fig. 4-3 a ①). Samples were prepared as described in the caption of Fig. 4-2 b. Bar represents 100 nm.

c. Gel electrophoresis of RNA extracted from the assembled particles recovered from the first (lane 1), second and third (lane 2) and fourth (lane 3) UV-absorbing peaks in the gradients (Fig. 4-3 a ①) and TCMV genomic RNAs (lane 4). RNA extracted from each peak (s) was dissolved in 20 μl DDW. A volume of 6 μl from the first and fourth and 3 μl from the second plus third peaks were loaded on the gel. The control TCMV RNA was 1.5 μg in 2 μl DDW. The conditions for electrophoresis were the same as those in Fig. 4-1.


a
b
c
1
2
3
4
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom

(i.e. the lightest) in the sucrose density gradients (Fig. 4-4 c) showing that the components contained intact RNA molecules. These results may indicate that particles sedimenting in the second peak contained either 1 or 2 molecules of RNA 4, the third peak 3 molecules, and the fourth peak 4 molecules of RNA 4. It is not known whether any RNA was encapsidated in the first peak.

2.4. Properties of particles encapsidating RNAs 3 and 4

Particles were assembled with TcP and a mixture of RNAs 3 and 4 in equal weight ratio or 1 : 2 molar ratio. Analyses by centrifugation through sucrose density gradients showed that there were four components in the assembly mixture (Fig. 4-5 a ①). The major and the fastest-migrating peaks matched those of native TCMV (Fig. 4-5 a ②). Two small peaks were also found at the upper position of the gradient (Fig. 4-5a ①). When compared with native TCMV in the same gradients, the major and fastest migrating peaks merged with those of native TCMV producing stronger peaks (Fig. 4-5 a ③). Such a sedimentation pattern differed from that of assembled particles containing only RNA 3 (Fig. 4-3 a ①). It also differed from the sedimentation pattern of assembled particles containing only RNA 4 (Fig. 4-4 a ①) in that the "shoulder" was not found. Nevertheless similarities were observed between the two patterns because both contained two small peaks at the upper position in the sucrose gradients (Fig. 4-4 a ① and Fig 4-5 a ①). This similarity may be due to the excess RNA 4 assembled independently with TcP. Particles recovered from the major peak fractions in the gradients contained TCMV-like particles, 28-30 nm in diameter (Fig. 4-5 b). RNAs 3 and 4 were recovered from these particles in about equal amount (Fig. 4-5 c). These results suggest that the assembled particles contain both RNAs 3 and 4, one molecule of each, in a particle, or some particles contained 3-4 molecules of RNA 4 as demonstrated in 4.2.3.

Conclusion

Thee results presented above allow the following conclusions to be drawn:

1. Particles assembled with RNAs 1 and 2, or with a mixture of RNAs 3 and 4, confirm the encapsidation models of cucumoviruses proposed by Peden and Symons (1973) and Habili and Francki (1974a) for cucumoviruses, i.e., three types of particle in a cucumovirus preparation, the first with a molecule of RNA 1, the second with a molecule of RNA 2, and the

Fig. 4-4

a. UV absorption profiles of assembled particles encapsidating RNA 4 (①), native TCMV (②), and a mixture of the assembled particles and native TCMV (③). The amounts of virus layered onto the tops of 5-25% sucrose gradients were 200 μg of RNA-protein from assembly mixture (calculated from the input, assuming 100% assembly efficiency) (①), 100 μg of native TCMV(②), and 100 μg RNA-protein from the assembly mixture plus 50 μg native TCMV(③). The centrifugation was conducted in the same condictions as stated in the caption of Fig. 4-2 a.

b. Electron micrograph of assembled particles recovered from the third and fourth UV-absorbing peaks in the gradients (Fig. 4-4 a ①). Samples were prepared as described in the caption of Fig. 4-2 b. Bar represents 100 nm.

c. Gel electrophoresis of RNA extracted from the assembled particles recovered from the first (lane 1), second (lane 2) and third plus fourth (lane 3) UV-absorbing peaks in the gradients (Fig. 4-4 a ①) and TCMV genomic RNAs (lane 4). RNA extracted from each peak (s) was dissolved in 20 μl DDW. A volume of 6 μl from the first and second and 1 μl from the third plus fourth peaks were loaded into the gel. The control TCMV RNA was 1.5 μg in 2 μl DDW. The conditions for electrophoresis were the same as those in Fig. 4-1.

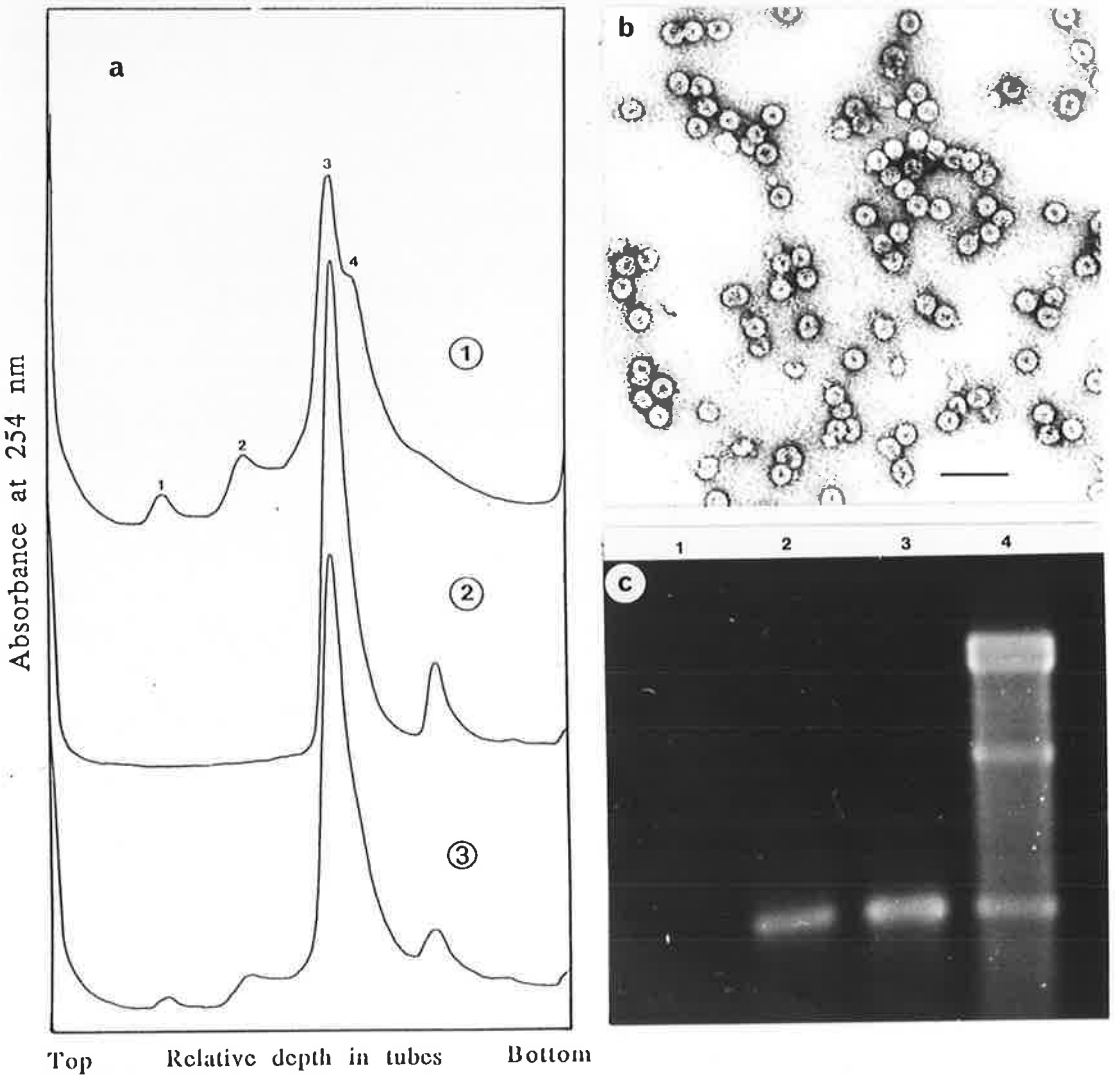
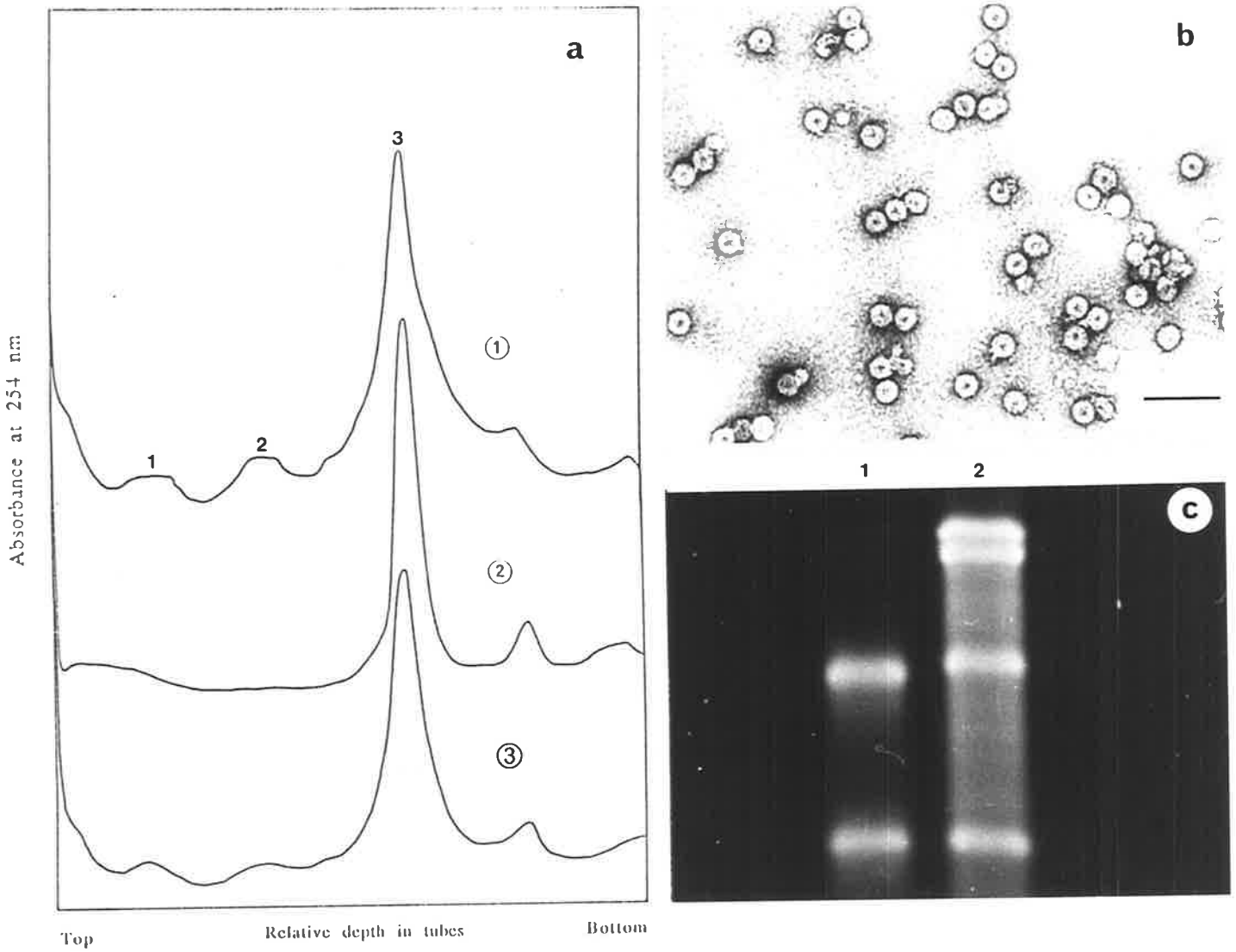

a
b
c
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom
1
2
3
4
1
2
3
4

Fig. 4-5

a. UV absorption profiles of assembled particles encapsidating RNAs 3 and 4 (①), native TCMV (②), and a mixture of the assembled particles and native TCMV (③). The amounts of virus layered on 5-25% linear sucrose density gradients were 200 μg of RNA-protein from assembly mixture (calculated from the input, assuming 100% assembly efficiency) (①), 100 μg of native TCMV(②), and 100 μg RNA-protein from the assembly mixture plus 50 μg native TCMV(③). The centrifugation was conducted as stated in the caption of Fig. 4-2 a.

b. Electron micrograph of assembled particles recovered from the major UV-absorbing peak in the gradients (Fig. 4-5 a ①). Samples were prepared as described in the caption of Fig. 4-2 b. Bar represents 100 nm.

c. Gel electrophoresis of RNA extracted from the assembled particles recovered from the major UV absorbing peak in the gradient (Fig. 4-5 a ①) (lane 1, approximately 1 μg) and TCMV genomic RNAs (lane 2, 2.5 μg). The conditions for electrophoresis were the same as those in Fig. 4-1.


a
b
c
Absorbance at 254 nm
1
2
3
①
②
③
Top
Relative depth in tubes
Bottom
1
2

third with a molecule each of RNA 3 and 4.

2. The observation that particles encapsidating RNA 4 alone have the same sedimentation rate in sucrose density gradient as native TCMV presumably indicates that, each particle contains 3 molecules of RNA 4. This suggests that encapsidation of pure RNA 4 could also be expected *in vivo*.

3. Particles encapsidating RNA 3 alone do not sediment at the same rate as native TCMV. This suggests that it is unlikely that such encapsidation would occur *in vivo*.

Chapter 5 *In vitro* non-specific encapsidation of nucleic acids by cucumovirus coat proteins

Introduction

In Chapter 3 it was skown that both VTAV and MCMV coat proteins can encapsidate a variety of plant viral RNAs. While the reassembled particles have cucumovirus-like morphology, they maintain the biological properties of the encapsidated viral RNAs.

To further characterize this *in vitro* encapsidation, the ability of cucumovirus coat protein to encapsidate a wider range of nucleic acid molecules was studied.

Results and discussion

1. Encapsidation of host plant total RNAs

The assembly of a standard stoichiometric mixture (see 2.2.9) of total RNAs isolated from healthy plant *N. clevelandii* and VTAV coat protein (20% of RNA to 80% of protein, by weight) produced VTAV-like particles (Fig. 5-1 b).

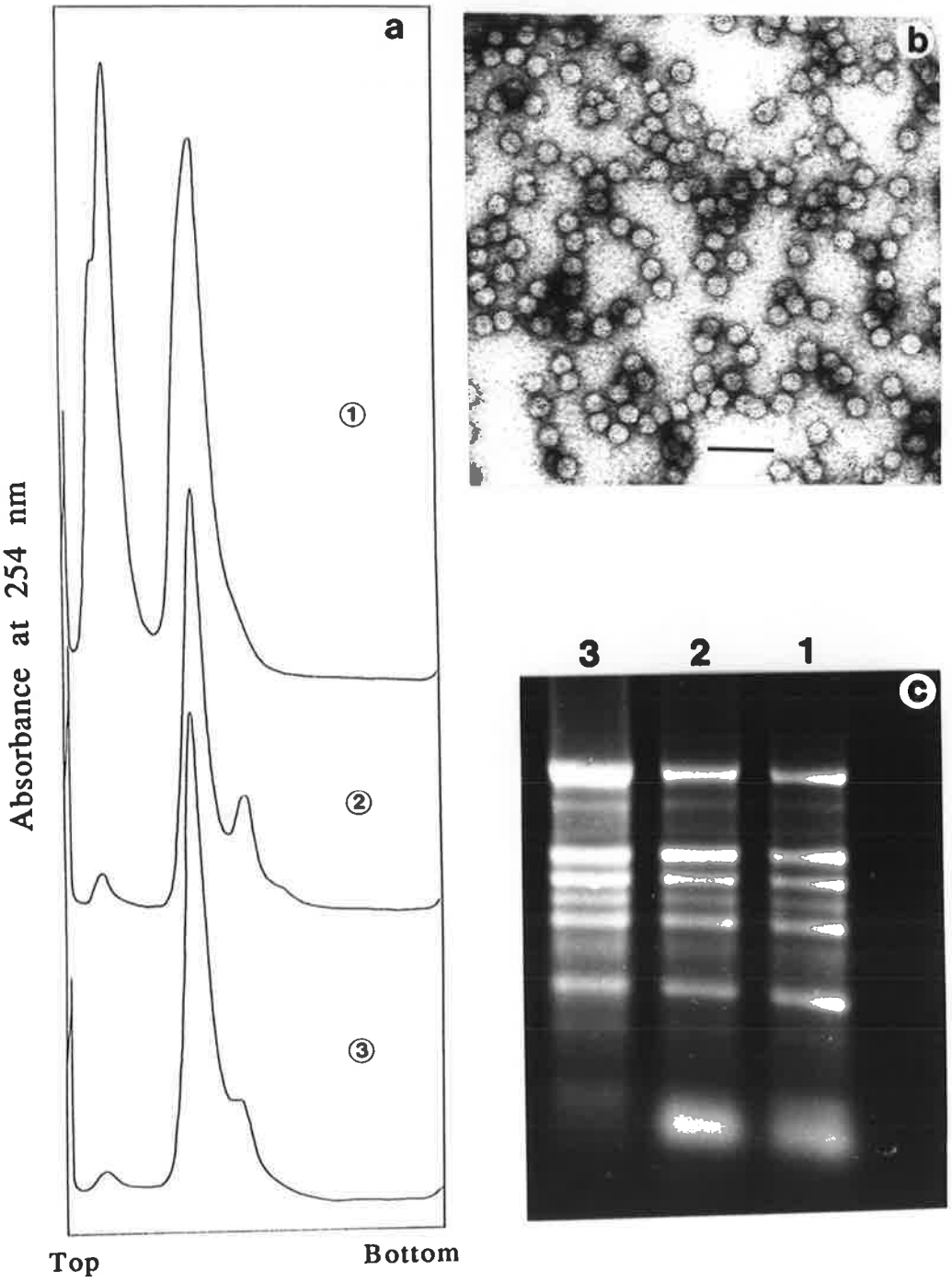
To investigate whether there was a size preference for the RNA molecules encapsidated, a competition pressure was created by adding extra RNA to the assembly mixture (see 2.2.10 for definition). Standard stoichiometric assembly was used as a control. After assembling overnight, samples of the assembly mixture were analysed by sucrose density gradient centrifugation. Following assembly with the double RNA ratio, compared with that of the stoichiometric assembly, two major components were found: one near the top and another close to the middle of the gradient (Fig. 5-1 a ①). The peak near the top of the gradient contained RNA but not particles. Cucumovirus-like particles were recovered from the faster-moving peak. In contrast, stoichiometric assembly yielded a single major component (Fig. 5-1 a ②) which sedimented at a rate similar to the assembled VTAV (Fig. 5-1 a ③). Particles were collected from the gradient peaks and RNA was extracted from the particles. Analysis of the encapsidated RNAs revealed that the RNA species contained in particles from the stoichiometric assembly (Fig. 5-1 c, lane 2) were the same as the RNA used for assembly (Fig. 5-1 c, lane 1) but the relative amount of the smallest RNA molecules was significantly reduced in particles from the double RNA ratio assembly (Fig. 5-1 c, lane 3). These results suggest that there is no

Fig. 5-1 Characterisation of particles assembled from plant total RNA and VP

a. UV absorption profiles of assembly mixtures containing VP and double (①) and stoichiometric (②) ratios of total plant RNAs. Native VTAV (③) was used as control. A content of 160 μg viral protein (equivalent to 200 μg of virus) from the assembly mixtures was loaded on a 5-25% sucrose density gradient and then centrifuged in a Beckman SW41 rotor at 35,000 rpm for 100 min. The gradients were fractionated with the aid of an ISCO fractionator. Sedimentation was from left to right.

b. Electron micrograph of assembled particles recovered from the peak areas of stoichiometric assembly mixture (a, ②). Bar represents 100 nm.

c. Agarose gel electrophoresis of RNAs extracted from assembled particles recovered from the stoichiometric (lane 2) and double RNA ratio (lane 3) assembly. Total plant RNA (lane 1) was used as control. Loadings were 3 μg of RNA per lane. The gels were stained with ethidium bromide.


a
b
c
Absorbance at 254 nm
①
②
③
Top
Bottom
3
2
1

significant difference among RNA species in the encapsidation by VTAV coat protein with the exception of the smaller molecule which is disadvantaged when extra RNA is present.

2. Encapsidation of yeast transfer RNA

The observation that total plant RNA was encapsidated by VTAV protein raised another question: what was the nature of the particles with only the small RNA molecule? As the smallest RNA from plant total RNAs is transfer RNA (tRNA, approximately 80 nucleotides), yeast tRNA was chosen for the experiment.

When tRNA was assembled stoichiometrically with VTAV protein, typical cucumovirus-like particles were formed (Fig. 5-2 a). These particles sedimented somewhat slower than native VTAV in sucrose density gradients (Fig. 5-2 b). When the mixture of the assembled particles and native VTAV was centrifuged in a gradient, the UV-absorption profile showed that there was a small peak which sedimented close to but slower than the major peak (Fig. 5-2 c). The RNA patterns recovered from the gradient fractions indicated that tRNA was present in a relatively higher concentration in the upper fractions (6-8, from top) and VTAV RNA was at a relatively higher concentration in the lower fractions of the gradient (7-9, from top) (Fig. 5-2 d). These data suggest that the assembled particles containing tRNA have the same dimensions as cucumovirus but that they are slightly lighter than native VTAV in weight.

3. Encapsidation of linear and circular RNA molecules

Circular and linear single stranded satellite RNA of VTMoV was assembled with VTAV coat protein. VTAV-like particles were formed (Fig. 5-3 b). To investigate whether there was any preference for linear or circular molecules for encapsidation, a competitive encapsidation with the double RNA ratio (twice the amount for the stoichiometric assembly, as defined above) was attempted. Particles of the same dimension were formed in both stoichiometric and double RNA assembly as VTAV. The assembled particles sedimented at a rate similar to that of native VTAV (Fig. 5-3 a). RNA was recovered from the assembled particles (Fig. 5-3 c). As shown in Fig. 5-3 c, there was no significant difference in the relative amount of circular and linear molecules in the double RNA ratio assembly (lane 3) as compared with stoichiometric assembly (lane 2) and the RNA used for assembly (lane 1). These suggest that single stranded circular and linear molecules are encapsidated by VTAV coat protein with the same efficiency.

Fig. 5-2 Characterisation of particles assembled from yeast tRNA and VP

a. Electron micrograph of particles assembled with yeast tRNA and VP. Bar represents 100 nm.

b. UV absorption profiles of assembled particles containing tRNA (①) and VR (②). Loadings were 200 μg of RNA-protein. Sedimentation was from left to right. Conditions of centrifugation and fractionation of the gradients were the same as described in the caption of Fig. 5-1 a.

c. UV absorption profile of a mixture of native VTAV and assembled particles containing tRNA. An amount of 100 μg of RNA-protein each of VTAV and the assembled particles was loaded on a sucrose gradient. Sedimentation was from left to right. Conditions for centrifugation were the same as those described in the caption of Fig. 5-1 a. The gradient was fractionated into 12 x 1 ml fractions.

d. Agarose gel electrophoresis of RNAs recovered from the fractions of the gradient in (c). SDS was added to each fraction (50 μl) to a final concentration of 2% (w/v) and the mixture was incubated at 25 ^{0}C for 2 h before loading on a 1.5% agarose gel for electrophoresis. The arrow indicates the position of tRNA bands.

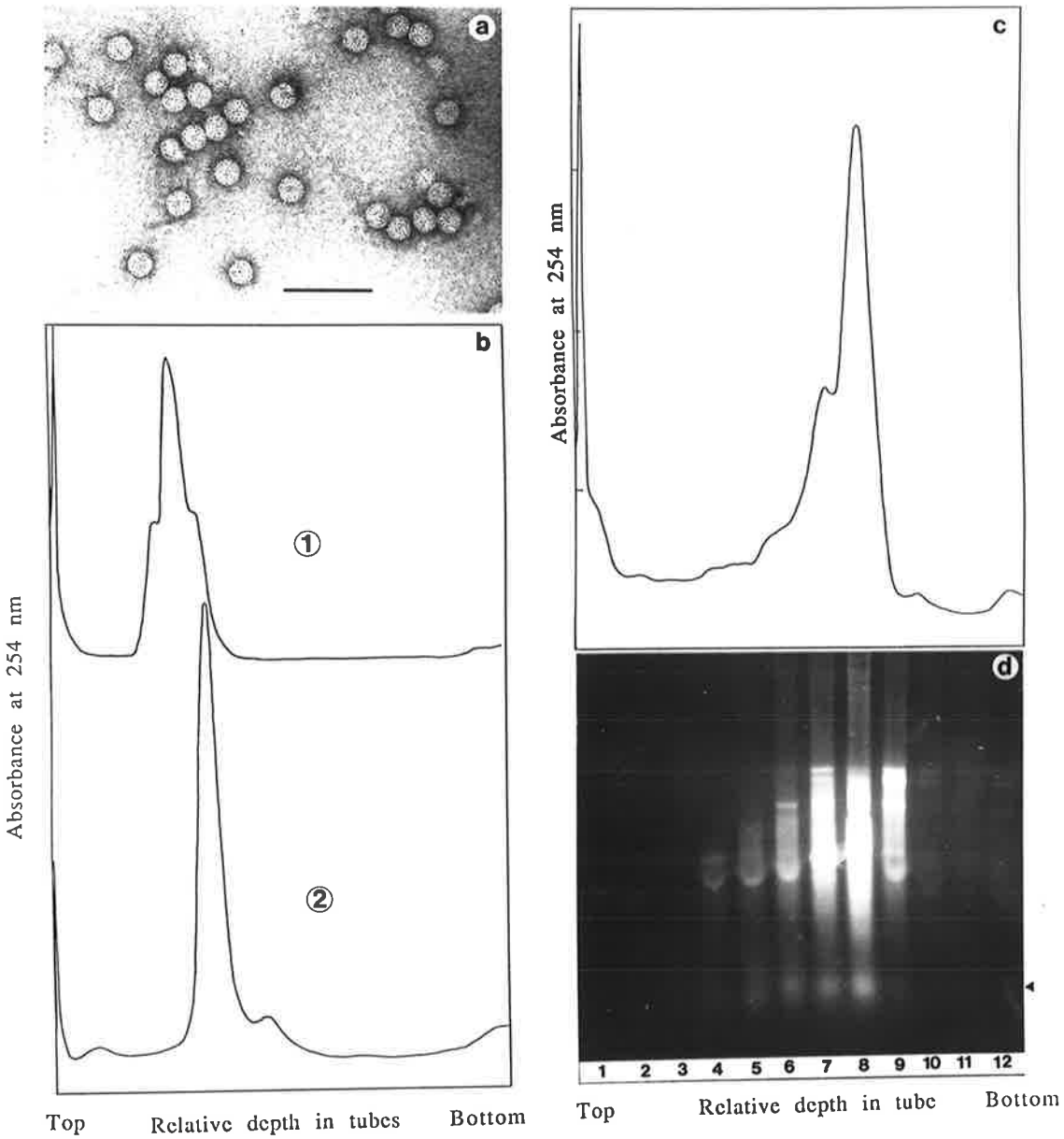
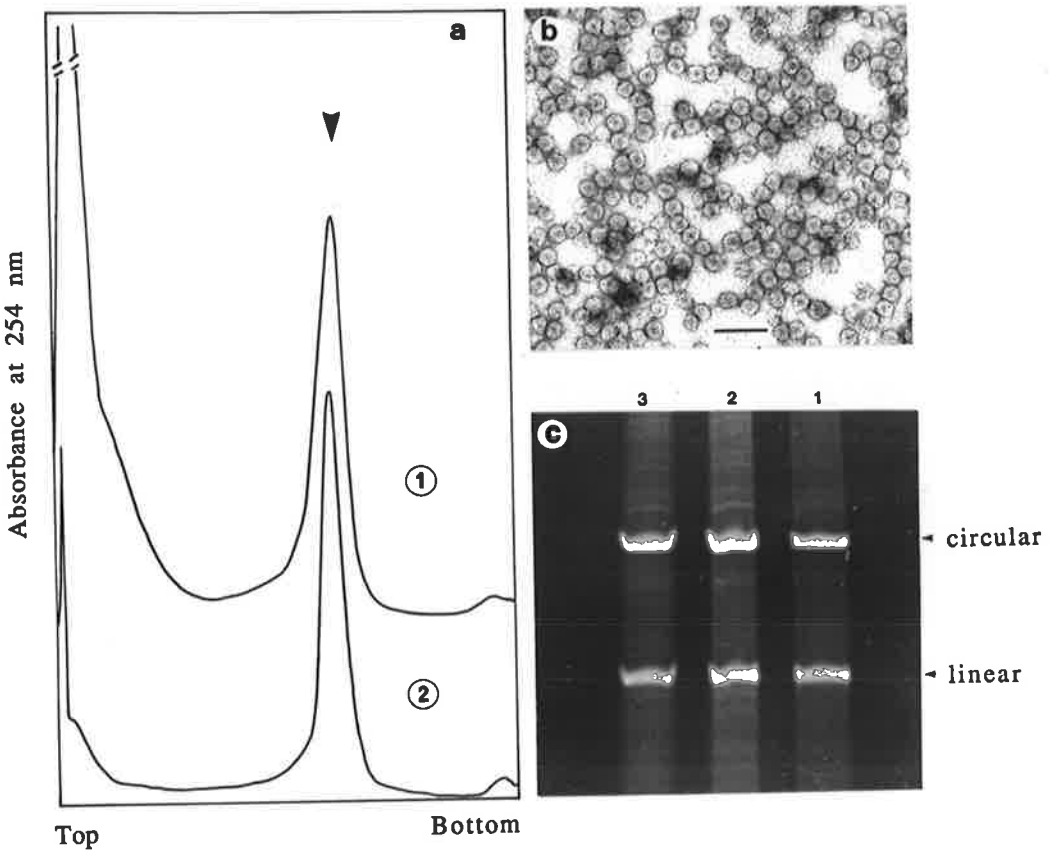

a
b
①
②
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom
c
Absorbance at 254 nm
d
1 2 3 4 5 6 7 8 9 10 11 12
Top
Relative depth in tube
Bottom

Fig. 5-3 Characterisation of particles assembled from satellite RNA of VTMoV and VP

a. UV absorption profiles of assembly mixtures with VP and satRNA of VTMoV at double (①) or standard stoichiometric (②) RNA ratio. The assembly mixture loaded on the gradient contained 160 μg of protein (equivalent to 200 μg of VTAV). Sedimentation was from left to right. Conditions of centrifugation and fractionation of gradients were the same as described in the caption of Fig. 5-1 a.

b. Electron micrograph of assembled particles from the stoichiometric assembly mixture containing VP and satRNA of VTMoV. Bar represents 100 nm.

c. RNA content of assembled particles recovered from the stoichiometric (lane 2) and double RNA ratio (lane 3) assembly.


a
b
c
Absorbance at 254 nm
Top
Bottom
①
②
3
2
1
circular
linear

4. Encapsidation of polynucleotides

Particles were assembled when poly (C) (size not determined) was mixed with VTAV coat protein (Fig. 5-4). However, attempts to assemble particles using poly (I) (size not determined) and VTAV coat protein were unsuccessful. Poly (I) precipitated immediately when it was added to VTAV coat protein preparation. The reason for this was not investigated.

5. Encapsidation of maize rough dwarf virus RNA

Maize rough dwarf virus (MRDV) (Boccardo and Milne, 1984) contains 10 segments of double stranded genomic RNA (ds RNAs). None of these segments could form particles with VTAV protein. However, when the RNA was boiled for 20 min and chilled promptly in iced water to separate the double stranded into single stranded forms and then mixed with VTAV protein, VTAV-like particles were assembled (Fig. 5-5). This suggests that only single stranded RNA can be encapsidated by VTAV coat protein.

6. Encapsidation of DNA

Chimeric plasmid pBsat 5 (3400 bp) is similar in length to that of CMV RNA 1. When mixed with TCMV coat protein (TcP) the double stranded molecules were not encapsidated, as determined by electron micrography and by sucrose density gradient analysis (Fig. 5-6 a). However, after thermal denaturation into single stranded molecules and then mixing with TcP, particles were formed as analysed by gradient centrifugation (Fig 5-6 b). Denatured linearised plasmids (Fig. 5-6 b ①) were more efficient in assembly than denatured unlinearised ones (Fig. 5-6 b ②). Cucumovirus-like particles were found from the assembled mixture of denatured linearised plasmid and TcP (Fig. 5-7 a). The assembly mixture of denatured unlinearised plasmid and TcP contained cucumovirus-like particles as well as some larger particles of about 40-45 nm (Fig. 5-7 b). The assembled particles containing denatured linearised plasmid (Fig. 5-7 a) co-migrated with native TCMV in sucrose density gradients (Fig. 5-8).

7. Minimum and maximum length of nucleotide for encapsidation

Oligonucleotides were used to determine the minimum length of nucleotides that TcP

Fig. 5-4 Electron micrograph of particles assembled with VP and poly (C). Bar represents 100 nm.

Fig. 5-5 Electron micrograph of particles assembled with VP and single stranded RNAs of maize rough dwarf virus. Bar represents 100 nm.

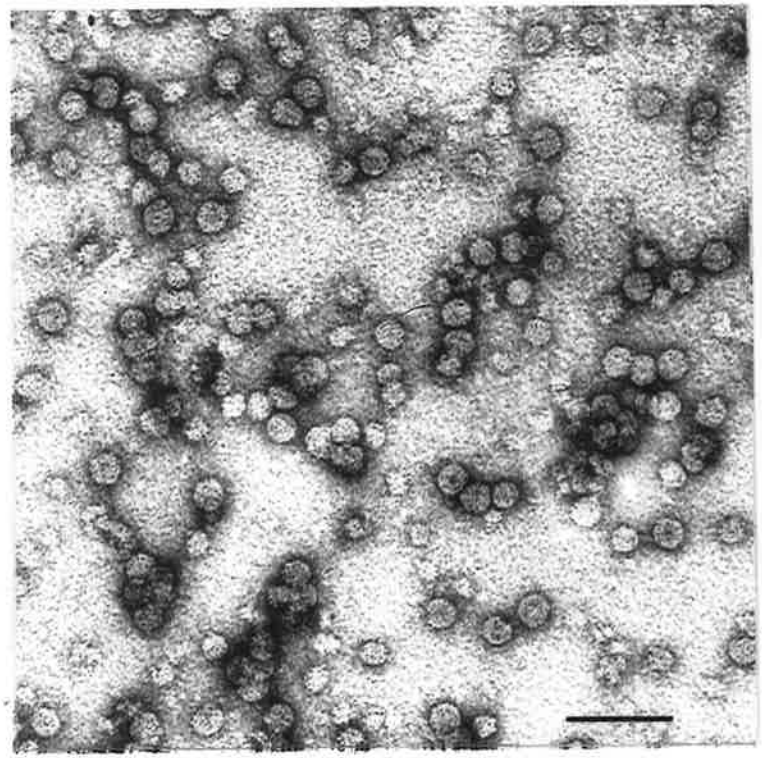


Fig. 5–4

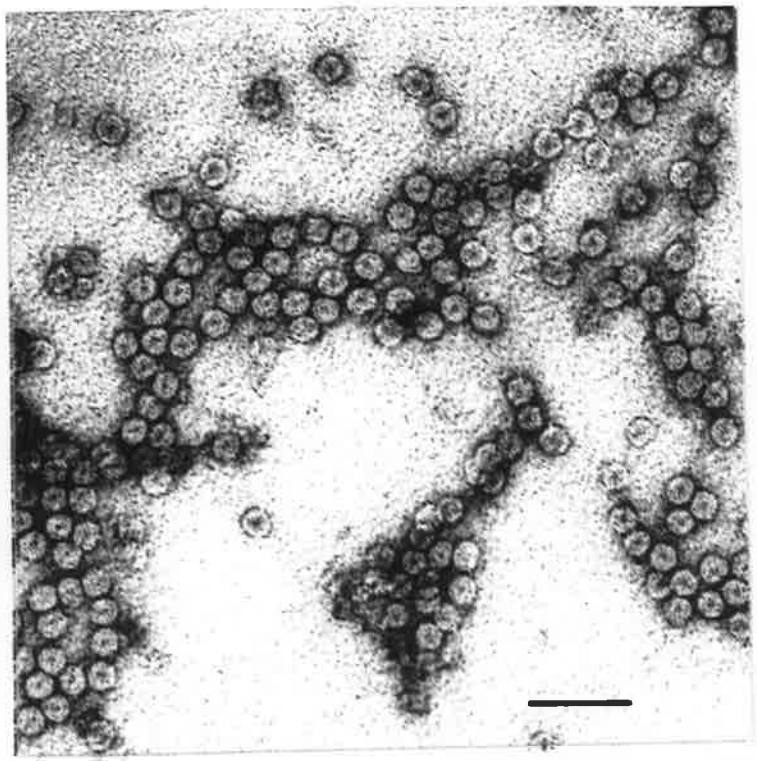


Fig. 5–5

Fig. 5-6 UV absorption profiles of assembly mixtures containing TCMV protein and double stranded DNA (a) and single stranded DNA (b) of pBsat 5. Assembly with linear single stranded DNA (b, ①) was compared with circular single stranded DNA (b, ②) of pBsat 5. After clarification at 10,000 rpm for 10 min, an amount of assembly mixture containing 150 μg of DNA-protein (assuming 100% of the input DNA and protein remained in the supernatant) was loaded on sucrose gradients. Sedimentation was from left to right. The conditions of centrifugation and fractionation of the gradient were the same as described in the caption of Fig. 5-1 a.

Fig. 5-7 Electron micrographs of assembled particles containing linearized ss DNA (a) and circular ss DNA (b) of pBsat 5 recovered from the gradient peak areas of Fig. 5-6 b, ① and ②, respectively. Arrows indicate the larger particles (b). Bar represents 100 nm.

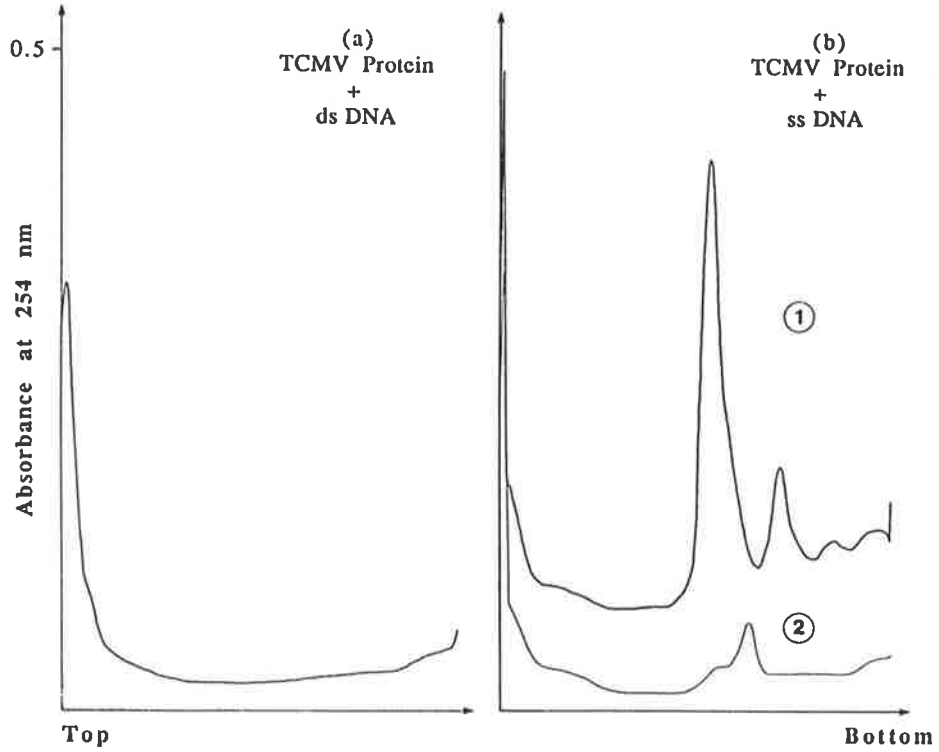

0.5
Absorbance at 254 nm
(a)
TCMV Protein
+
ds DNA
(b)
TCMV Protein
+
ss DNA
1
2
Top
Bottom

Fig. 5-6

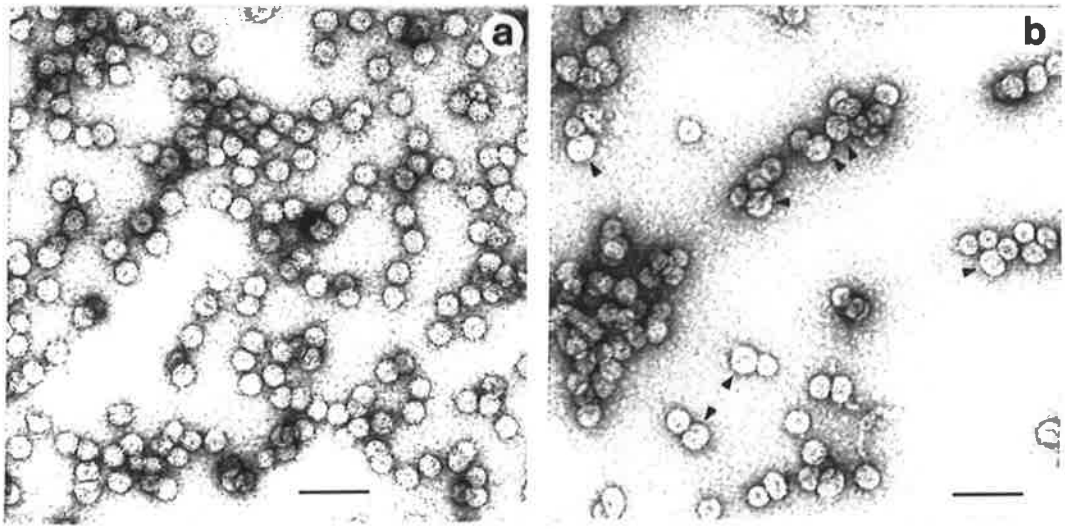
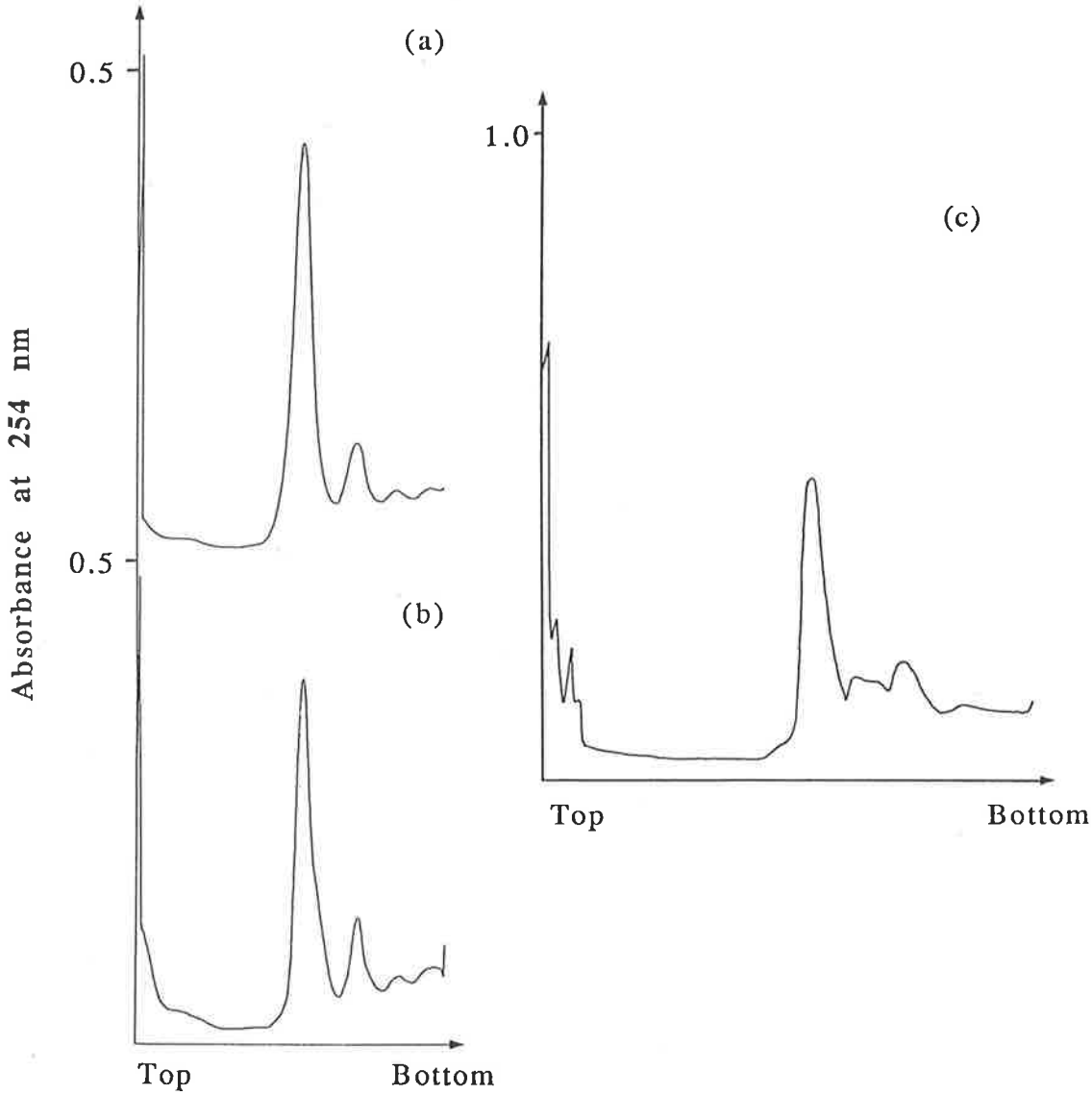

a
b

Fig. 5-7

Fig. 5-8 Comparison of sedimentation rates of assembled particles containing linear ss DNA of pBsat 5 (a) and assembled homologous VTAV virus (b). An amount of 100 µg of DNA-protein or RNA-protein was loaded on the gradient. A mixture containing 50 µg each of the assembled DNA-containing particles and VTAV was analysed in the same gradient (c). The sedimentation was from left to right. Conditions of centrifugation and fractionation of gradients were the same as described in the caption of Fig. 5-1 a.


(a)
0.5
0.5
(b)
Absorbance at 254 nm
Top
Bottom
1.0
(c)
Top
Bottom

could encapsidate. Particles were assembled using TcP and a 35 nucleotide ss DNA sequence (5' GGATCCGTCGACGCATGCGTTTATT$^{T}_{C}$ACAAGAGCG 3') (Fig. 5-9 c) but not a 20-mer (5' ACATCCTCGGGAGTGTCGAC 3'). When viewed under the electron microscope, the shapes of the assembled particles were less regular than TCMV, ranging from 22 to 30 nm. Sucrose density gradient centrifugation analysis showed that the reassembled particles sedimented significantly slower than native TCMV (Fig. 5-9 a). The assembly mixture of TcP and the 20-mer did not form RNA-protein components, and all the DNA remained at the top of the gradient (Fig. 5-9 b).

Larger molecules derived from DNA plasmids were used to determine the upper limit for encapsidation by VTAV. At the first attempt, a denatured linearised chimeric plasmid of 11.7 kb (pKU 83) was mixed with VP. No particle was found. Cucumovirus-like particles were formed using a denatured, linearised plasmid of 7020 nucleotides, pUK88 (Fig. 5-10 c, i) and VP (Fig. 5-10 b). The assembled mixture, however, showed two components with the faster-moving one sedimenting at a rate similar to native VTAV while the slower one was distributed as a broad shoulder on the main peak in the gradient (Fig. 5-10 a). DNA recovered from the assembled particles was immobile when electrophoresed in a 0.75% agarose gel (Fig. 5-10 c, ii). No further effort was made to solve this problem and the identity of the encapsidated DNA remains unknown. Since the assembled particles did not sediment faster than native VTAV, it seems that these particles do not contain full length plasmid DNA.

8. Competitive encapsidation between VTAV RNA and TMV RNA

The TMV RNA (TR) and VTAV RNA (VR) were mixed with a limited amount of VP in a series of competitive encapsidation experiments. Assembled particles were then recovered by ultracentrifugation through a 20% sucrose cushion. RNA was extracted from the particles and analysed by electrophoresis in agarose gels. As shown in Fig. 5-11, in assembly with a double RNA ratio (one part each of VR and TR), VR was more abundant than TR (about 1.7 : 1, as determined by densitometry of photographs of gels) (a, lane 3). In assembly with a triple RNA ratio (two parts of TR and one part of VR, by weight), the relative amount of TR to that of VR was increased (about 1 :1) (a, lane 4). In assembly with four times the amount of RNA (two parts each of of VR and TR), VR were significantly more abundant than TR (about 4 :1) (b, lane 2). In assembly with six times the amount of RNA (three parts each of VR and TR), about

90% of encapsidated RNA was VR (b, lane 3). These results indicate that there is preference for homologous VR to heterologous TR in encapsidation by VP.

9. Assembly in presence of heparin

Since an RNA-protein interaction through salt bonds is the prevailing stabilizing force in cucumoviruses, blockage of charged groups in the protein would possibly affect the assembly of the virus. As heparin can bind to cationic amino acid residues of virus coat protein (Pesce *et al.*, 1986), it was used to test whether it could affect the assembly of VTAV. Homologous VTAV RNA and protein were assembled in the presence of heparin at various concentrations and the assembly efficiency was evaluated by sucrose density gradient centrifugation. Data of two experiments were summarised in Table 5-1. As shown in Table 5-1, the assembly of virus was not affected by heparin at concentrations of 0, 10 and 100 μg/ml (the average efficiencies were all around 65%). However, the yield of assembled particles was significantly reduced by heparin at concentration of 1000 μg/ml (with efficiency of 12-14%).

Table 5-1 Effect of heparin on assembly of homologous VTAV†

No. expt	Heparin (μg/ml)	Assembly efficiency (%)*
	0	62
1	10	65
	100	72
	1000	14
	0	67
2	10	63
	100	62
	1000	12

†. The concentration of VR-VP in the stoichiometric assembly mixture was 1 mg/ml. Heparin was mixed with VP and incubated at 4 ^{0}C for 16 h before VR was added. Assembly conditions were described in 2.2.9.

*. Assembly efficiency was calculated by comparing the virus-containing peak areas of assembled particles in sucrose density gradients with that of known quantity of native VTAV.

In all treatments, assembled particles were all cucumovirus-like (Fig. 5-12). These results suggest that heparin inhibits virus assembly only when it is at about the same concentration as the VP.

Fig. 5-9 Determination of minimum length of nucleotide for encapsidation by TcP. Oligonucleotides comprising either a DNA 35-mer or a 20-mer were mixed with TcP stoichiometrically. After clarification an amount of 150 μg of DNA-protein from the assembly mixtures (assuming 100% of them remained in the solution) was loaded on a sucrose density gradient. Centrifugation and fractionation were done as described in the caption of Fig. 5-1 a. The arrow indicates the position of native TCMV in the gradient.

a. UV absorption profile of assembly mixture containing the 35-mer.

b. UV absorption profile of assembly mixture containing the 20-mer.

c. Particles observed from the assembly mixture containing the 35-mer. Bar represents 100 nm.

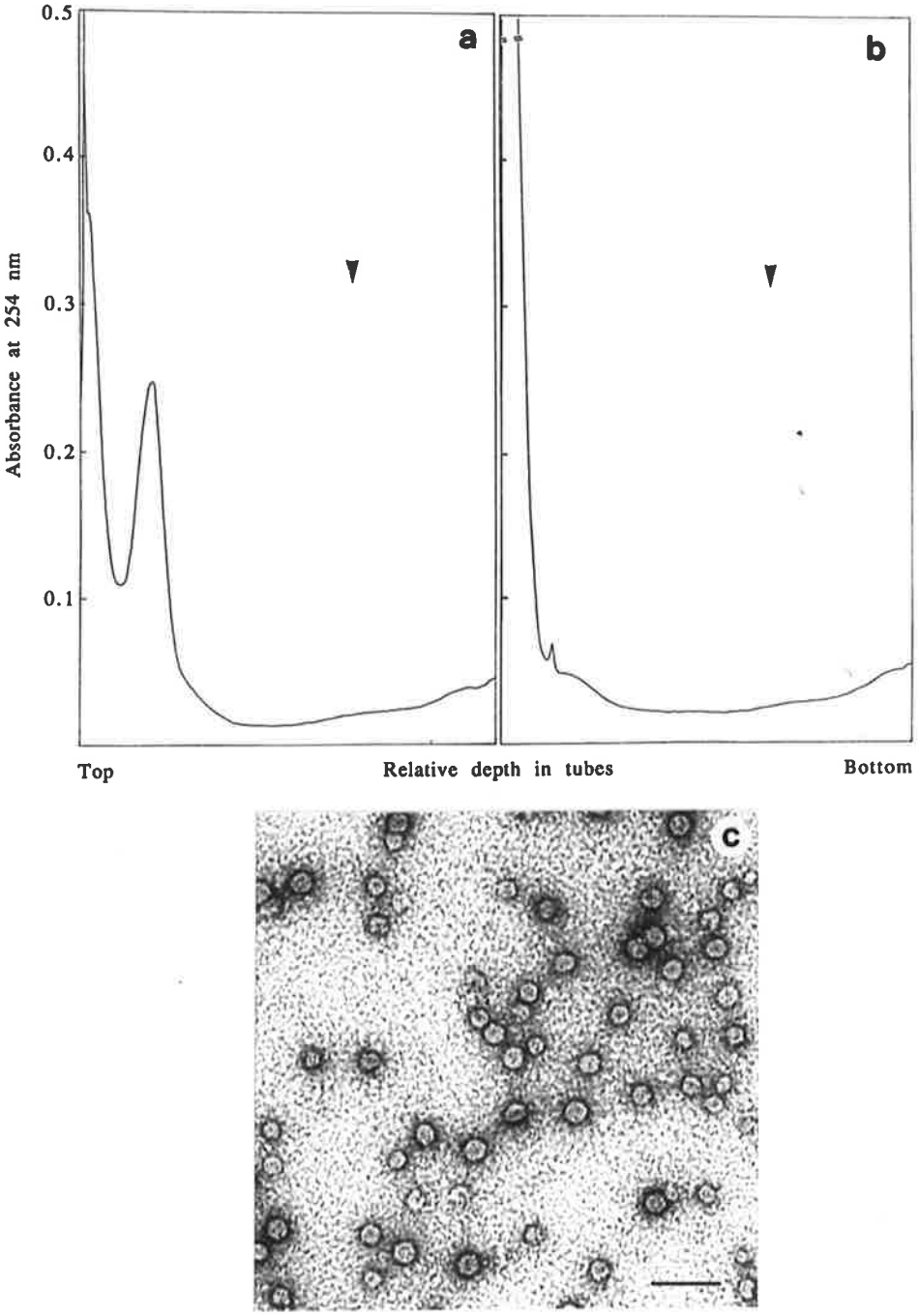

0.5
0.4
0.3
0.2
0.1
Absorbance at 254 nm
a
b
Top
Relative depth in tubes
Bottom
c

Fig. 5-9

Fig. 5-10 Analysis of the particles assembled from VP and boiled pKU 88.

a. UV absorption profiles of the assembly mixture containing VP and boiled pKU 88 (①) and assembled homologous VTAV (②). An amount of 200 μg of DNA-protein or RNA-protein was loaded on the sucrose density gradient. Conditions of centrifugation and gradient fractionation were the same as described in the caption of Fig. 5-1 a.

b. Electron micrograph of the assembled particles recovered from the peak area in the gradient as shown in Fig. 5-10 a ①. Bar represents 100 nm.

c. Agarose gel electrophoresis of pKU 88 DNA.

(i) Linearized molecules (lane 2, 0.5 μg) migrated more slowly than circular ones (lane 3, 0.5 μg) in the 0.75% gel. The size marker was λ DNA (lane 1, 1 μg) restricted with Hind III endonuclease to give 23, 9.4, 6.6, 4.4, 2.3, and 1.9 kb fragments.

(ii) DNA recovered from the top area (lane 2) and peak area (lane 3) of the gradient (Fig. 5-10 a ①) was compared with the boiled linearized pKU 88 (lane 1) which was used in assembly. The arrow indicates the position where the recovered DNA located in the gel.

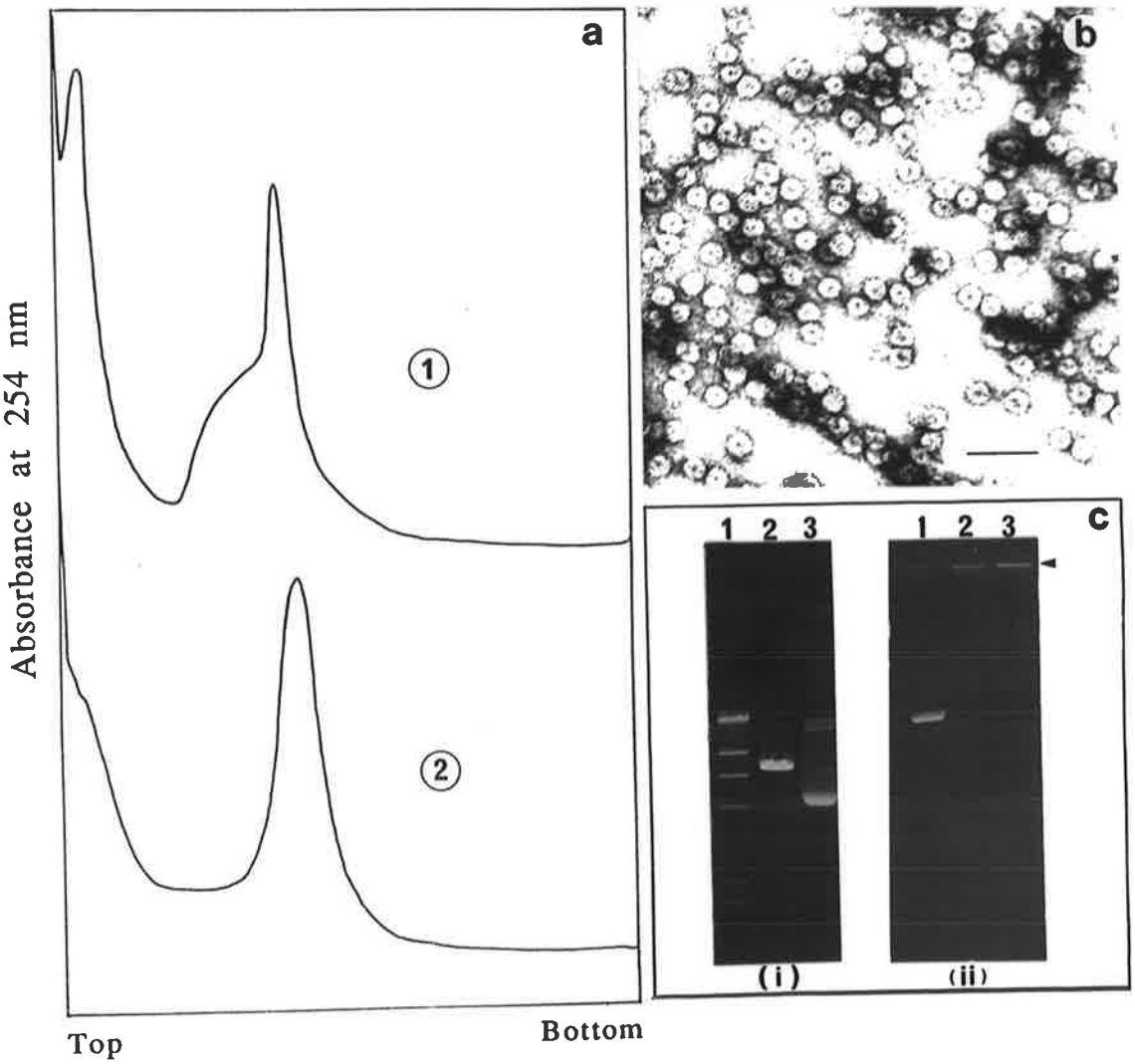
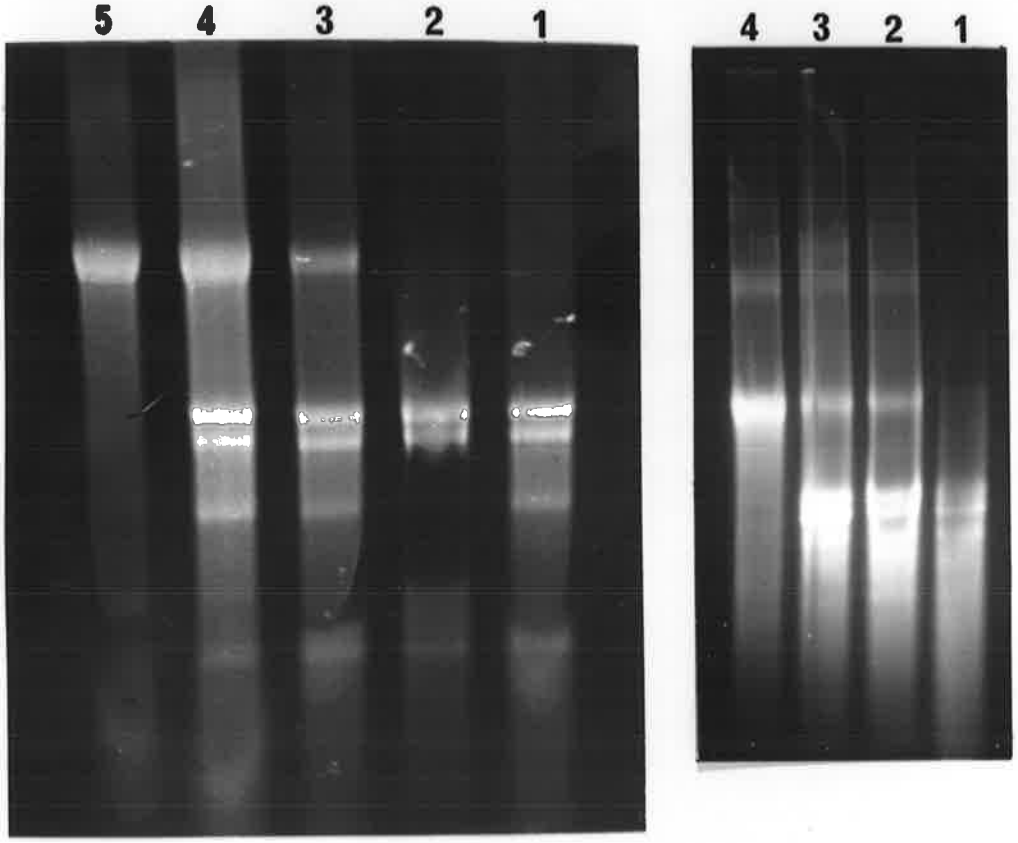

a
b
c
Absorbance at 254 nm
1
2
Top
Bottom
1 2 3
1 2 3
(i)
(ii)

Fig. 5-11 Analysis of encapsidated VTAV and TMV RNAs from competitive encapsidation assemblies. Assembled particles were pelleted by ultracentrifugation through 20% sucrose cushions. RNAs were extracted from the assembled particles. After electrophoresis, gels were stained with ethidium bromide and photographed. Relative amounts of the viral RNAs were calculated by scanning the RNA bands in photographs.

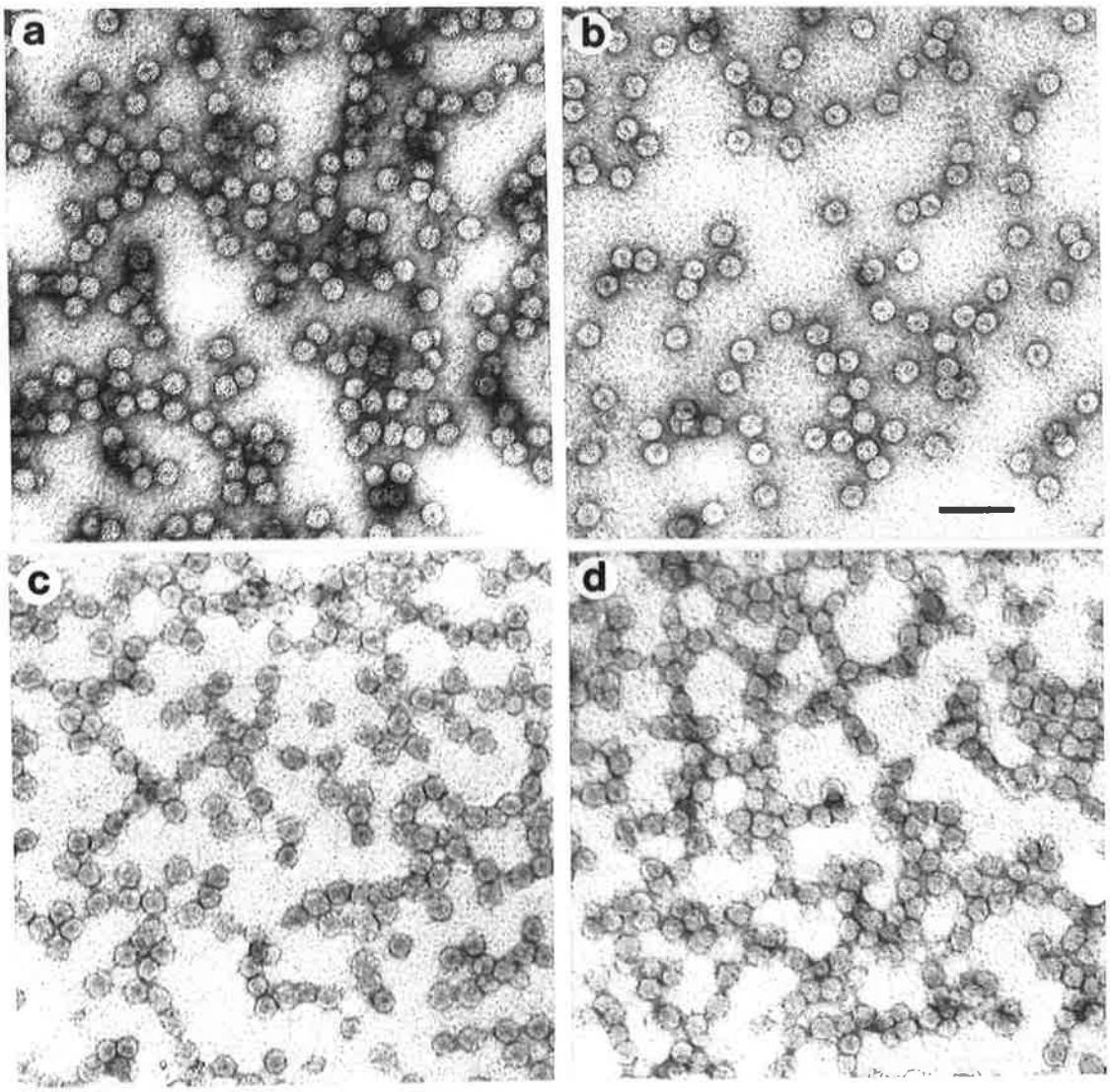
a. Gel electrophoresis of RNAs recovered from double RNA ratio (0.2 mg each of VTAV RNA and TMV RNA was mixed with 0.8 mg of VP) (lane 3) and from triple RNA ratio (0.2 mg of VTAV RNA and 0.4 mg of TMV RNA with 0.8 mg of VP) (lane 4), stoichiometric assemblies with TMV RNA (0.2 mg of TMV RNA with 0.8 mg of VP) (lane 5) and with VTAV RNA (0.2 mg of VTAV RNA with 0.8 mg VP) (lane 2) and VTAV RNA which was used in assembly (lane 1). The loading of RNAs from assembled particles was not quantified. VTAV RNA of 2 μg was loaded (lane 1).

b. Gel electrophoresis of RNAs recovered from VTAV RNA stoichiometric assembly (lane 1), four-time RNA ratio (0.4 mg of VTAV RNA and 0.4 mg of TMV RNA with 0.8 mg of VP) (lane 2), six-time RNA ratio (0.6 mg of VTAV RNA and 0.6 mg of TMV RNA with 0.8 mg of VP) (lane 3) and TMV RNA stoichiometric (lane 4) assemblies. Loadings of RNA were not quantified.



(a) (b)

Fig. 5-12 Electron micrographs of homologous VTAV particles assembled in the presence of heparin at different concentrations: 0 μg/ml (a), 10 μg/ml (b), 100 μg/ml (c) and 1000 μg/ml (d). The particles were collected from the peak areas in sucrose density gradients. Bar represents 100 nm.


a
b
c
d

Conclusion

1. *In vitro*, cucumovirus coat proteins can encapsidate a wide range of linear and circular RNA and DNA from 35 to about 7000 nucleotides long.

2. Cucumovirus coat protein did not encapsidate double stranded RNA and DNA.

3. Cucumoviruses or virus-like particles assembled *in vitro* tended to conserve particle dimension and molecular weight, i.e, 28-30 nm in diameter and 5.5 x 10^6 daltons (or 99S). In assembly with small nucleic acid species such as VTMoV satellite and tRNA, about 15 and 50 molecules respectively could have been encapsidated in a single particle.

4. The observation that VTAV *in vitro* assembly was interfered with by heparin only at the higher concentration suggest that heparin may be a weak inhibitor of cucumovirus assembly. The interaction between viral RNA and viral coat protein is far more stronger than that between heparin and viral coat protein.

5. VTAV RNA has a stronger affinity with VTAV coat protein than does TMV RNA when *in vitro* assembly is done in the presence of both.

Chapter 6 Construction of hybrid capsids containing coat protein subunits of both VTAV and TCMV *in vitro*

Introduction

It was shown in chapter 5 that the encapsidation of nucleic acids by cucumovirus coat proteins is non specific. One question which arises is whether coat protein subunits of two different cucumoviruses can combine in the same capsid to produce a hybrid particle. Since the particle structures and molecular weights of coat protein subunits of cucumoviruses are similar (Francki, 1985), VTAV and TCMV were chosen as model viruses to address the above question. The *in vitro* assembly system was taken as an approach.

Results and discussion

1. Strategy

In an *in vitro* assembly with isotope-labelled TCMV protein (TcP) and unlabelled VTAV protein (VP) and RNA from VTAV (VR), three types of particle could possibly be formed: VP-VR, TcP-VR and a hybrid (VP +TcP)-VR. In a gel immunodiffusion test, VP-VR will specifically react with antiserum to VTAV and TP-VR with antiserum to TCMV. Hence, if the hybrid (VP+TcP)-VR is formed, it should react with antisera to both VTAV and TCMV because it contains subunits of both viruses. If the gel is carefully washed to remove radioactive background and exposed to X-ray film, positive signals of the isotope should only be located in the antibody-antigen precipitin line (s). Positive radioactive signals in the precipitate formed between antiserum to VTAV and the mixture of assembled particles would indicate the existence of such hybrid particles.

2. *In vitro* reassembly

The TCMV used in the assembly was a mixture of ^{35}S-labelled (see 2.2.14) and unlabelled virus preparations in the ratio of 1:4 (by weight). VTAV was unlabelled. Proteins of VTAV and TCMV were mixed in equal amount and assembled with VR stoichiometrically (see 2.2.10 and the caption of Fig. 6-1). After assembly overnight, the mixture was analysed by sucrose density gradient centrifugation. As shown in Fig. 6-1 a, the mixture contained mainly a major

component. Cucumovirus-like particles were recovered from the peak fractions of the gradients (Fig. 6-1 b).

3. Gel immunodiffusion tests and autoradiography

The gradient-purified reassembled virus particles were allowed to react with antisera to VTAV and TCMV in a 0.75% agarose gel prepared in 20 mM phosphate buffer, pH 7.5. The reassembled particles reacted with antisera to both VTAV and TCMV indicating that proteins of both VTAV and TCMV were assembled into particles (Fig. 6-1 c). Autoradiography derived from this gel revealed the positive radioactive signal at the precipitin line between VTAV antiserum and assembled particles (Fig. 6-1 d) indicating that ^{35}S-labelled TCMV coat protein had incorporated into particles with VTAV protein. Thus, the hybrid capsids with proteins of both VTAV and TCMV had been assembled with VR.

Conclusion

The demonstration that particles with a hybrid capsid containing coat protein subunits of both VTAV and TCMV can be assembled *in vitro* suggests that there is no specificity in assembly between coat protein subunits of these two viruses. This observation also suggests that the spatial structure of coat protein subunits of VTAV and TCMV may be similar.

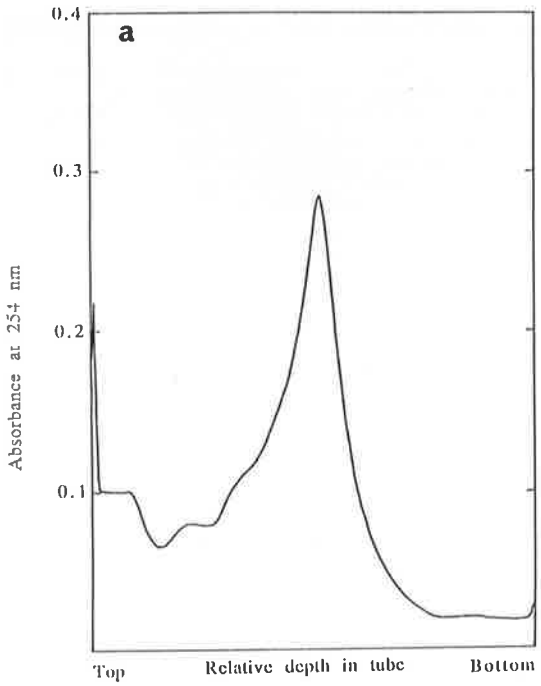
Fig. 6-1. Analysis of particles with a hybrid capsid containing coat protein subunits of both VTAV and TCMV. TcP was prepared from 1.5 mg TCMV (0.3 mg of ^{35}S-labelled and 1.2 mg of unlabelled virus) and VP from 1.5 mg of VTAV. An amount of 0.7 mg each of TcP and VP was mixed with 0.35 mg of VR in a total volume of 3.32 ml. The mixture was dialysed against TKCMg (see 2.2.9) overnight.

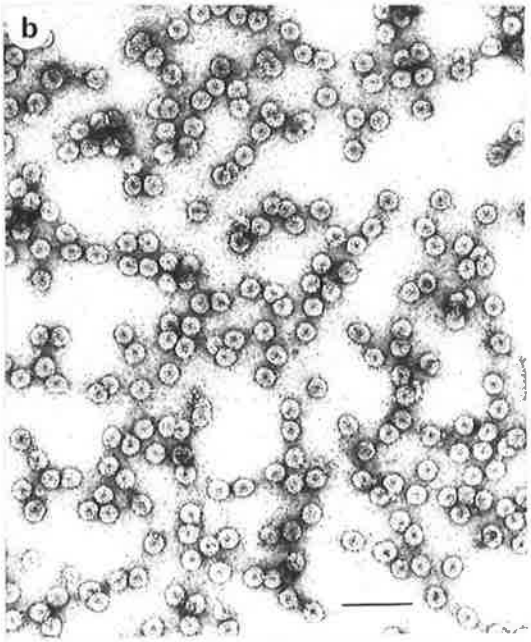
(a). UV absorption profile of assembly mixture containing VR and a mixture of VP and TcP. An amount of 200 µg of RNA-protein from assembly mixture was loaded on a 5-25% sucrose density gradient and centrifuged in a Beckman SW 41 rotor at 35,000 rpm for 2 h. The gradient was fractionated with an ISCO fractionator. The sedimentation was from left to right.

(b). Electron micrograph of assembled particles recovered from the UV absorption peak in the sucrose gradient of (a).

(c). Results of gel immunodiffusion test of the assembled particles with antisera to VTAV (V As, titer 1/128) and TCMV (T As, titer 1/100). An amount of 5 µg of VTAV (V) or TCMV (T) or 12 µg of assembled particles (R) was loaded in a well. The radioactivity of ^{35}S-labelled native TCMV was 10820 CPM/µg and that of assembled particles 457 CPM/µg of virus. TCMV used here was a mixture of ^{35}S-labelled and unlabelled preparation at a rate of 1 : 9 by weight. The reaction was conducted at 25 ^{0}C for 24 h.

(d). The autoradiograph of (c). The gel was washed in 0.8% NaCl for two days with 6 changes to remove unreacted viral antigens and then dried at 37 ^{0}C in an incubator overnight. The dried gel was exposed to a Fuji X-ray film (RX) at -70 ^{0}C for 4 days.


a
0.4
0.3
0.2
0.1
Absorbance at 254 nm
Top
Relative depth in tube
Bottom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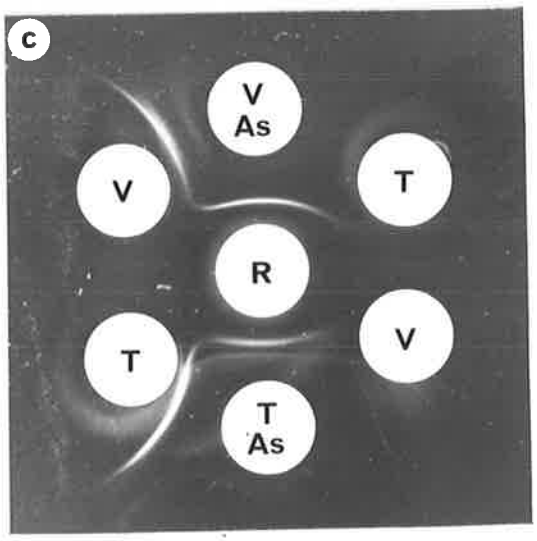
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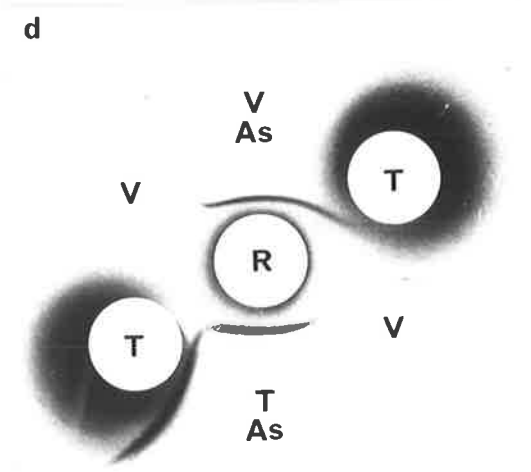
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Chapter 7 Transmission of Reassembled Viral Particles by *Myzus persicae*

Introduction

CMV can be transmitted by the aphid *Myzus persicae* following acquisition through a membrane of highly purified virus preparations (Pirone and Megahed, 1966). Therefore, transmissibility of CMV seems to be the property of the virus itself without the involvement of helper components (HC) such as those of the potyviruses or cauliflower mosaic virus (Harrison and Murant, 1984).

The cucumoviruses VTAV (aphid-transmissible) and MCMV (non aphid-transmissible) (Mossop and Francki, 1977b) would make an ideal system for testing the effect of providing a virus with a specific coat protein on its transmissibility by aphids.

In chapter 3, it was shown that both VTAV and MCMV coat proteins could encapsidate homologous as well as heterologous viral RNAs and that the encapsidated viral RNA retained its biological identity. If the conformation of the viral capsid required for aphid acquisition did not change, any viral RNA encapsidated in such a particle should be transmissible by a given vector. This hypothesis was tested by constructing assembled particles *in vitro* and using the membrane feeding technique for acquisition.

Results and discussion

1. Some characteristics of the viruses used

Some charateristics of the viruses used in this study are compared in Table 4-1. Of the four viruses VTAV is the only one reported to be transmitted by aphids. These characteristics of aphid transmissibility were confirmed by using *M. persicae* as a vector (Table 4-2).

2. Establishment of a colony of *M.persicae*

It was desirable to establish a colony of *M. persicae* with the ability to transmit VTAV at a high rate. A test of infectivity was done by placing single aphids on test plants after a brief acquisition period on a VTAV-infected plant. Of 10 aphids, two inoculated plants with VTAV. An aphid from the colony derived from one of these two aphids was transferred to the VTAV-immune host Chinese cabbage (*Brassica pekinensis* Rupr.) and allowed to colonize. After

Table 7-1 The viruses used for reassembly experiments

Virus	Aphid Transmissibility	Genome ($Mr \times 10^6$)	Coat protein ($Mr \times 10^3$)	Particle (nm)	Reference
VTAV	+	RNA 1 (1.26) RNA 2 (1.10) RNA 3 (0.90) RNA 4 (0.34)	26.1	28	Habili & Francki (1974a)
MCMV	-	RNA 1 (1.27) RNA 2 (1.18) RNA 3 (0.82) RNA 4 (0.35)	25.8	28	Mossop (1978)
VTMoV	-	(1.5)	37	30	Randles & Francki (1986)
TMV	-	(2.1)	18	Rod shape 18 x 300	Gibbs (1977)

proving the host plants to be VTAV-free by immunodiffusion tests with antiserum to VTAV, individuals of the colony were transferred to *N. tabacum* var. Xanthi to establish a colony.

3. Enhancement of the rate of aphid transmission of VTAV

For tests of membrane feeding for the transmission of purified virus preparation by aphids, a highly aphid transmissible VTAV would be desirable. It was speculated that there might be some individual virus particles in plants which could be preferentiably transmitted by aphid vector. Continuous selection by aphid transmission might then result in a relative increase in the proportion of such particles in the virus population and thus the aphid transmission rate should be boosted. To test this hypothesis, an experiment using continuous transmission of VTAV with *M. persicae* was conducted. A plant infected with virus by aphid transmission (designated by the number of the passage) was used as the virus source for the next aphid transmission.

Table 7-2 Transmissibility of VTAV, MCMV, VTMoV and TMV by *M. persicae*†

Experiment[a]	Virus	Transmission[b]
1	VTAV	17/36
	MCMV	0/36
	VTMoV	0/34
	TMV	0/36
2	VTAV	14/34
	MCMV	0/35
	VTMoV	0/32
	TMV	0/36

†. Plants of *N. clevelandii* infected with these viruses were used as virus sources. Fasted aphids were given an acquisition feed of about 1 min on the symptomatic leaves and an inoculation feed of 24 h on *N. clevelandii* plants at the 3-4 leaf age. Results were scored after 3 weeks both by symptoms and serological tests. As shown in Table 4-2, VTAV was the only virus to be transmitted by the aphid.

a. Aphids given an acquisition feed on a source plant leaf were placed on *N. clevelandii* seedlings at the rate of 10 per test plant.

b. The numerator indicates the number of plants infected and the denominator the number of plants used in the trial.

Results shown in Table 4-3 did not support the hypothesis. The transmission rates were low for the first few passages. It increased from the 5th passage (4/12) and reached the highest at 6th (11/15), then stabilized at around 50% (5-6/10). However, the rates dropped after the 10th passage. Since the experiments were done under glasshouse conditions over a long period of time, perhaps some other environmental factors, such as. temperature which affected the aphid probing behaviour, or light which might affect the virus concentration in the plant tissue could have contributed to this fluctuation. Since the virus passaged by aphid transmission was used as the inoculum for the large scale virus propagation, it was assumed that the highest possible aphid transmission probability was obtained.

4. Aphid transmission of reassembled virus particles following acquisition through membranes

Native or assembled viral particles in required concentrations were sandwiched between two layers of Parafilm membrane after adding sucrose to a final concentration of 5% (w/v), and this was secured on the top of a 10 mm plastic tube. Fasted aphids were allowed acquisition feeds for 1-2 min and then caged on young *N.clevelandii* test plants for 24 h. The plants were scored for infection 2-3 weeks later.

Data presented in Table 4-4 showed that native, as well as homologously assembled VTAV particles, were readily transmitted by *M. persicae* acquired through membranes. Moreover, heterologously assembled particles from VTAV protein and either MCMV or TMV

Table 7-3 Effect of aphid passage on VTAV transmission rates

Passage	Aphid/plant	Transmission rate*
1	10	1/6
2	10	2/6
3	10	2/22
4	10	2/22
5	10	4/12
6	5	11/15
7	5	6/10
8	5	5/10
9	5	6/9
10	5	3/12
11	5	2/24
12	5	2/16

* The numerator indicates the number of infected plants and the denominator the number of plants used in the trial.

Table 7-4. Transmission of virus particles reassembled from VTAV protein by *Myzus persicae* probing through membranes

Trial	Virus particle composition		Virus concentration	Transmission*
Number	Protein	RNA	(mg/ml)	
1	VTAV	VTAV(native)	2.0	6/15†
2			2.0	4/10
3	VTAV	VTAV(reassembled)	2.0	5/10
4			2.0	1/24
5			4.0	20/40
6	VTAV	MCMV(reassembled)	2.0	1/24
7			4.0	10/32
8			4.0	10/22
9	VTAV	TMV(reassembled)	2.0	5/10
10			2.0	7/20
11			4.0	9/40
12			4.0	18/24
13	VTAV	VTMoV(reassembled)	4.0	3/23
14	MCMV	MCMV(native)	4.0	0/20
15	MCMV	VTAV(reassembled)	2.0	0/10
16	MCMV	TMV(reassembled)	2.0	0/10
17			4.0	0/40
18	TMV	TMV(native)	2.0	0/10
19			4.0	0/20

*. Transmission by the aphids was tested on young *N. clevelandii* seedlings except in trials 9, 10, 16 and 18 in which N. tabacum cv. White Burley was used. Ten aphids were fed on each plant except in trials 1 and 18 where 5 and 20 aphids per plant were used, respectivily.

†. The numerator indicates the number of plants infected and the denominator the number of plants in the trial.

as well as VTMoV RNAs were also transmitted. On the other hand, the aphids failed to transmit either native MCMV, TMV or particles with RNAs of VTAV, TMV and VTMoV encapsidated in protein from MCMV (Table 4-4). The infections were confirmed by symptom expression, serological tests or electron microscopy:

On *N. clevelandii* plants infected with heterologously reassembled particles with MCMV RNA and VTAV protein, yellowing mosaic was first seen about 8 days after aphid inoculation. Some plants showed symptoms a few days later than others. After 18 days the MCMV specific symptom of brilliant yellow mosaic was fully developed (Fig. 7-1 a) and MCMV antigen was detected by agar gel diffusion tests.

A mild mosaic appeared at 8 to 10 days after aphid inoculation of *N. clevelandii* with heterologously reassembled particles containing VTMoV RNA and VTAV protein. The symptom remained mild resembling to that described for satellite-free K1-VTMoV (Francki *et al.*, 1985). Samples taken from these symptom-showing plants reacted with VTMoV antiserum in gel diffusion tests but those from symptomless plants did not (Fig. 7-1 b).

N. clevelandii plants infected with heterologously reassembled particles with TMV RNA and VTAV protein showed yellowing mosaic on young leaves in about one week. Symptom severity increased, growth ceased and plants normally died within three weeks post infection. Infected *N. tabacum* cv. White Burley plants showed symptoms in 7 to 10 days, then the symptoms developed into typical mosaic with distortions on leaves. Leaf-dip samples prepared from these plants for electron microscopy revealed abundant TMV particles (Fig. 7-1 c). Inoculum from these plants produced local lesions on *N. glutinosa* confirming the presence of TMV.

Conclusion

Results presented in this chapter demonstrate that only the coat protein determines aphid transmissibility of VTAV and MCMV, two member viruses of cucumoviruses.

The lack of specificity with which cucumovirus proteins encapsidate RNAs *in vitro* raises some interesting questions regarding cucumovirus particle assembly *in vivo*. For example, could transcapsidation and aphid transmission occur *in vivo*? This topic is addressed in Chapter 9.

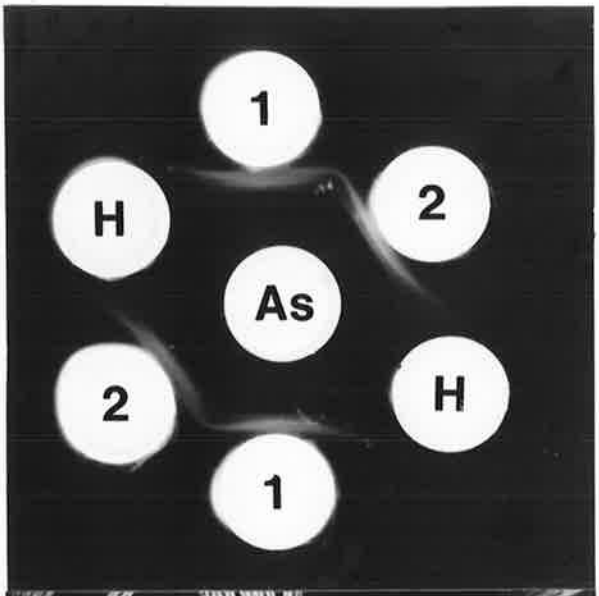
Fig. 7-1. Identification of viruses from plants infected with assembled viral particles which were transmitted by aphids through membrane feeding.

a. A *N. clevelandii* plant showing bright yellow mosaic symptom characteristic of MCMV, induced by assembled particle VP-MR.

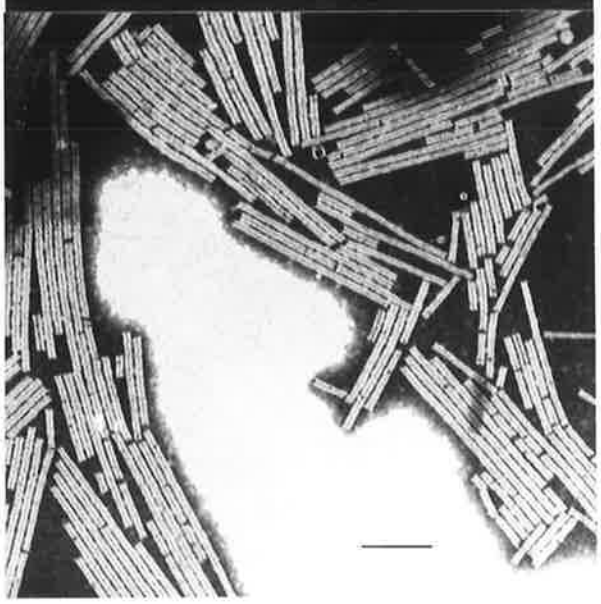
b. Result of a gel immunodiffusion test of the infected plant with assembled viral particle VP-VTR transmitted by aphids (1), with VTMoV transmitted mechanically (2) and the healthy plant (H). VTMoV antiserum (As) reacted uniformly and only with the sap extracted from the VP-VTR and VTMoV infected plants.

c. TMV particles observed in an extract of a plant infected with assembled particle VP-TR. Bar represents 200 nm.

a



b



c

Chapter 8 Comparative Studies on the Encapsidation of CMV Satellite RNA with VTAV and TCMV Coat Proteins

Introduction

The satellite RNA (satRNA) of cucumber mosaic virus (CMV) was first recognized as a low molecular weight RNA encapsidated with its helper's genomic RNAs, and which had the ability to modulate disease symptom expression on certain host plants (Kaper and Waterworth, 1977). It has been proposed that the replication of satRNA is controlled by the helper-virus RNA 1 and/or 2 (Mossop and Francki, 1979; Francki, 1985). As the proportion of satRNA encapsidated compared with the helper-virus genomic RNA varies with the strain of helper-virus (Mossop and Francki, 1979), it is also possible that one helper-virus might be more efficient than another in encapsidating the satRNA. To examine the encapsidation hypothesis, the encapsidation behaviour of a specific satellite RNA by two different cucumoviruses, VTAV and TCMV, was compared.

Results and discussion

1. *In vivo* replication and encapsidation of satellite RNA

A satellite RNA (B1-satRNA) was transcribed from the satellite cDNA clone, pBsat 5, using T 7 RNA polymerase (see 2.7). The satRNA transcript was mixed with TCMV RNA and VTAV RNA separately to a final concentration of 20 µg satRNA + 200 µg viral RNA/ml and then inoculated to young *N. clevelandii*. Viruses were purified from the infected plants 14 days after inoculation and RNA was extracted from the purified virus preparations. Agarose gel electrophoresis and northern blot hybridizations with cDNA probe specific to satRNA showed that the satRNA was encapsidated in both virions of TCMV and VTAV and that the encapsidated satRNA/genomic RNA ratio in TCMV (23%) was about 6 times that of VTAV (4%) as determined by densitometry of photographic prints of gels stained with ethidium bromide (Fig. 8-1 a). These results established that both TCMV and VTAV could support the replication but differed in the level of encapsidation of the satRNA. A further experiment was done to analyse the relationship between the satRNA pool in plants and the amount of satRNA encapsidated in the virions. The satRNA was mixed with TCMV RNA and VTAV RNA,

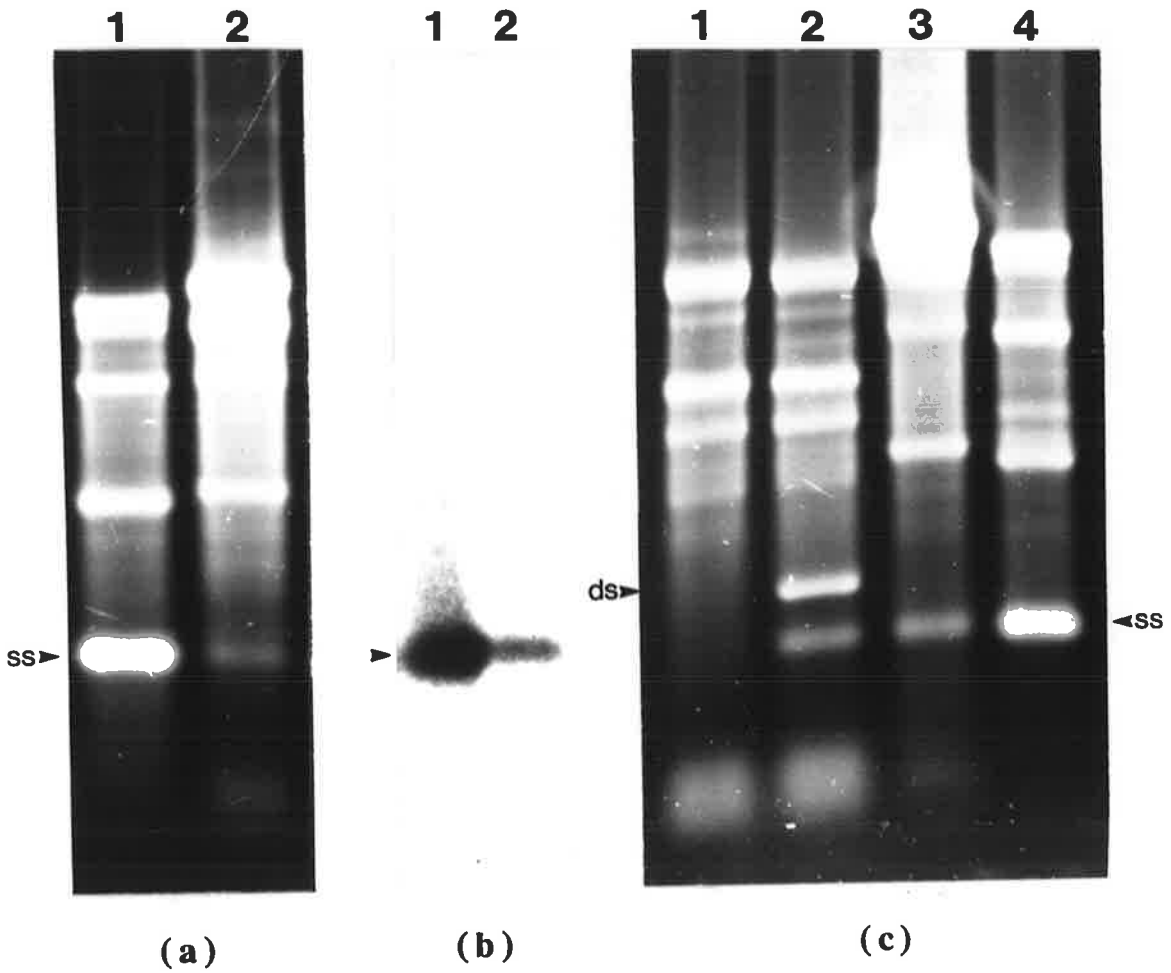
Fig. 8-1 Analysis of satRNA replication and encapsidation *in vivo*. *N. clevelandii* was co-infected with satRNA and TCMV or satRNA and VTAV. RNA was either isolated from plants (total RNA) or from purified virus preparations (encapsidated RNA). ss stands for single stranded and ds for double stranded RNA.

a. Agarose gel electrophoresis of encapsidated RNA (lane 1, from TCMV, 3 μg and lane 2, from VTAV, 5 μg). Beside the four viral RNA species, a fifth fastest-migrating species (indicated by the arrow) appeared in RNA preparations extracted from both TCMV (lane 1) and VTAV (lane 2). The amount of this small molecule in TCMV (lane 1) was much more than that in VTAV (lane 2).

b. Northern blot hybidization of (a). After electrophoresis the RNA was transferred to nitrocellulose membrane and hybridized with a cDNA probe reverse transcribed from the B1 satRNA transcript (2.11). The cDNA specifically bound to the fastest-migrating spcies in the gel (indicated by the arrow) suggesting that the small molecule is B1 satRNA. The signal from TCMV (lane 1) was much stronger than that from VTAV (lane 2) confirming the results from (a).

c. Comparison of the satRNA in total and in encapsidated RNAs. Under the experimental conditions, there was not detectable satRNA found in total RNA from plants co-infected with the satRNA and VTAV (lane 1), but the satRNA was found in encapsidated RNA, although its ratio to the VTAV genomic RNA was very low (lane 3). The satRNA was detected in both total RNA from plants co-infected with the satRNA and TCMV (lane 2) and in encapsidated RNA (lane 4). A strong band associated with the satRNA was found in the total RNA (lane 2, indicated by "ds" plus an arrow). This RNA band disappered in the gel after denaturation by glyoxal-DMSO and this resulted in an enhanced satRNA band. A similar RNA species was previously found in total RNA from plant co-infected with CARNA 5 and CMV-D, where it was identified as double stranded (ds) satRNA (Diaz-Ruiz and Kaper, 1977; Habili and Kaper,1981). Thus, the satRNA-associated RNA found here (lane 2) was identified as ds satRNA.

Loadings were 1 μl (lane 1 and 2, equivalent to 1 μg of plant tissue, see 2.2.5), 5 μg (lane 3) and 3 μg (lane 4).


1
2
ss
1
2
1
2
3
4
ds
ss
(a)
(b)
(c)

separatively, and inoculated to *N. clevelandii*. Analysis of the total RNA of the infected plants showed that the satRNA concentration in plants infected with TCMV was higher, with the accumulation of a large amount of double stranded satRNA (Fig. 8-1 c, lane 2), than in plants infected with VTAV, in which the satRNA was under the detectable level (Fig. 8-1 c, lane 1). The RNA patterns of the viruses purified from the infected plants showed that there was much more satRNA encapsidated in TCMV capsids than in VTAV capsids (Fig. 8-1 c, lane 3 and 4). Therefore, the abundance of satRNA in TCMV preparations may be due to the larger pool of satRNA in plants but it is also possible that TCMV has a higher efficiency of encapsidation of the satRNA.

2. Encapsidation of ^{32}P-labelled satRNA transcripts *in vitro*

To improve the sensitivity of detection and to elucidate the relationship between satRNA and viral genomic RNA in encapsidation, ^{32}P-labelled satRNA transcripts (^{32}P-satRNA) were used in this experiment.

2.1 The transcripts

The ^{32}P- labelled plus (+) and minus (-) stranded transcripts were 358 (lane 2) and 396 (lane 3) nucleotides (nt) respectively (Fig. 8-2 a). Autoradiography showed that the (+) transcript (lane 2) was in the position corresponding to that in the polyacrylamide gel indicating that the molecules were intact, but the (-) transcript (lane 3) was a mixture of intact and specifically degraded molecules (Fig. 8-2 b).

2.2 Encapsidation of satRNA transcripts with VTAV and TCMV coat proteins

a. Encapsidation with VTAV coat protein

(+) or (-) ^{32}P-satRNAs were mixed stoichiometrically with VTAV coat protein (Vp) and dialysed against reconstitution buffer (see 2.10). Spherical particles with a diameter of 22-32 nm were assembled with (+) satRNA transcript (Fig. 8-3 a). These particles fell into mainly two groups: 25 and 28-30 nm in diameter (Fig. 8-4 a). The particles sedimented slower than VTAV but as a single peak in a sucrose density gradient (Fig. 8-4 b).

Since the formation of non cucumovirus-like particles did not comply with the results obtained from the previous non specific encapsidation (Chapter 5), experiments were done to

Fig. 8-2 satRNA transcripts used in assembly studies. The transcription was described in 2.7.

a. Polyacrylamide gel electrophoresis of (+) (lane 2) and (-) (lane 3) sense ss satRNA transcripts. Satellite RNA of velvet tobacco mottle virus (lane 1) was used as a size marker. The arrow indicates the position of the linear molecule of VTMoV satellite RNA (366 nt). The gel composition and conditions of electrophoresis were described in 2.10.

b. Autoradiograph of the gel (a) showing that both (+) and (-) satRNA (lane 2 and 3, respectively) were labelled with ^{32}P. The (+) satRNA was intact but specific degradation of the (-) satRNA was observed.

Fig. 8-3 Electron micrographs of particles assembled with VP and (+) satRNA transcript (a), VP and (-) satRNA transcript (b) and with TcP and (+) satRNA transcript (c). Bar represents 100 nm.

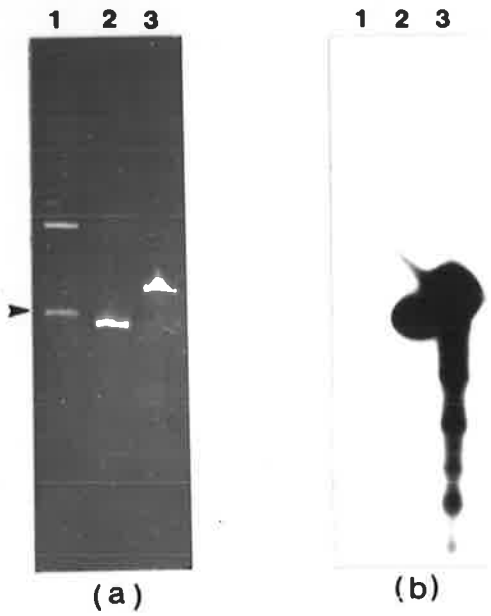


Fig. 8-2

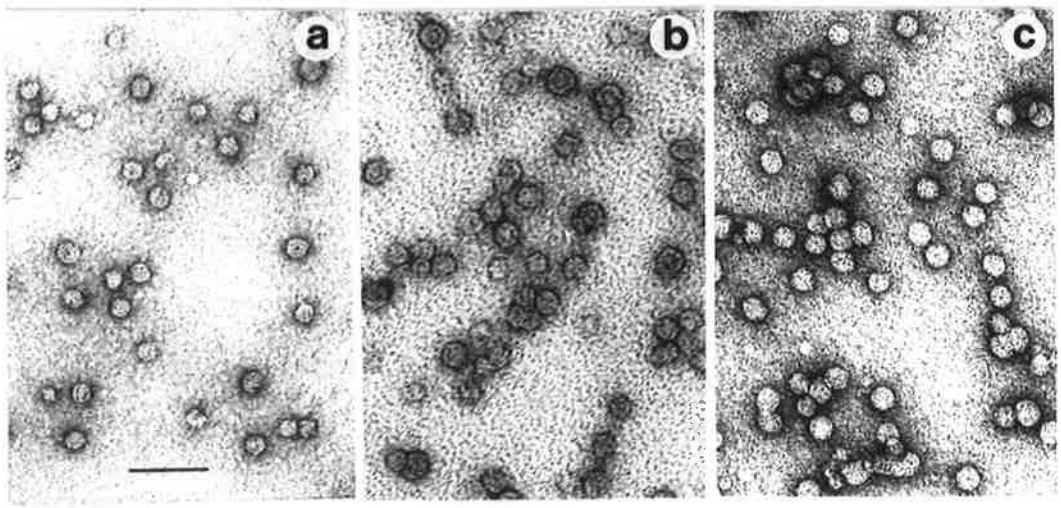


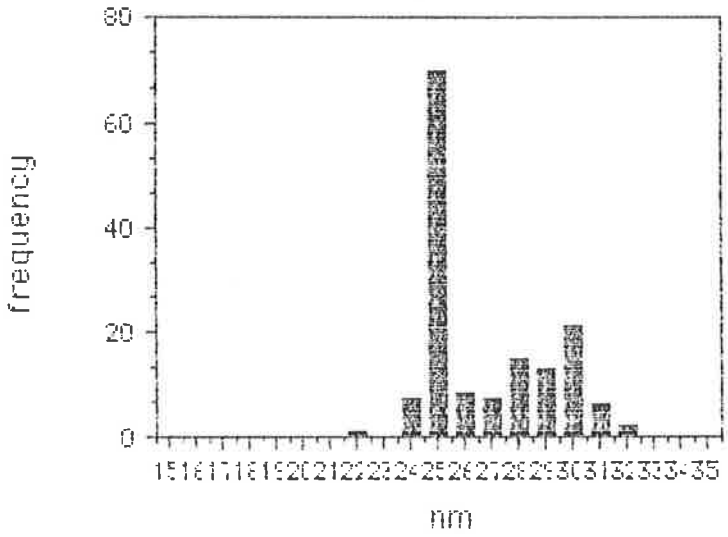
Fig. 8-3

Fig. 8-4. Some properties of particles assembled with (+) ^{32}P-satRNA and VP.

a. Frequency distribution of the size of particles assembled with the ^{32}P-satRNA and VP (see Fig. 8-3 a).

b. Sedimentation profile of particles assembled with the (+) ^{32}P-satRNA (see Fig. 8-3 a). A volume of 100 µl of the assembly mixture containing 31 µg of ^{32}P-satRNA-protein and 75 µg native VTAV were loaded on top of a 5-25% (w/v) sucrose density gradient and centrifuged in a Beckman SW41 rotor at 35,000 rpm for 150 min. Sedimentation was from left to right. The gradient was auto-fractionated to 30 x 0.4 ml fractions. Radioactivity was determined by Cerenkov counting. The arrow indicates the position of the peak of native VTAV in the gradient.

(a)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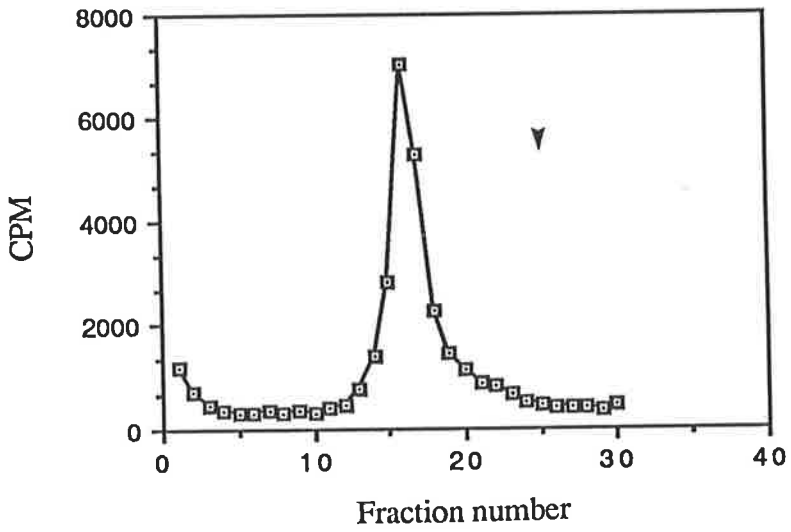
40
20
0
frequency
nm

(b)


8000
6000
4000
2000
0
CPM
0
10
20
30
40
Fraction number

investigate conditions favouring the assembly of the different particles. In the first trial, ^{32}P-satRNA was mixed with different amounts of protein to yield a series of RNA/ protein ratios. Two types of particles (small particle, 27 nm and smaller; large particle, 28 nm and above) were always found under all satRNA/protein ratios (Table 8-1). As shown in Table 8-1, in three independent assemblies with a standard stoichiometric mixture (satRNA : protein = 1 : 4), the number of small and large particles was about the same. With a decrease in the satRNA/protein ratio (expt. No. 4 and 5, satRNA/protein = 1 : 6 and 1 : 8, respectively), the ratio of small particles to large particles increased. However, when the satRNA/protein ratio reached 1 to 40, the large particles were dominant. It was noticed that at this ratio, the assembly efficiency was very low (<3%).

To investigate whether the concentration of the satRNA and protein in assembly reaction mixtures had any effect on the types of particles assembled, a series of concentrations varying from 0.05 to 1.5 mg satRNA-protein/ml, all in standard stoichiometric assembly conditions, was tested. Large particles were dominant at higher concentrations (1.5 and 0.8 mg/ml) while small particles were dominant at lower concentrations (0.4 mg/ml or less) (Table 8-2).

Particles assembled with the (-) satRNA transcripts were mainly 28-30 nm in diameter (Fig. 8-2 b). No further investigations were done to characterise them.

b. Encapsidation with TCMV coat protein

Particles of 28-30 nm in diameter were assembled using (+) ^{32}P-satRNA and TCMV coat protein (TcP) (Fig. 8-3 c). The particles sedimented slightly more slowly than TCMV in sucrose density gradients (Fig. 8-5 b).

2.3 Encapsidation of the satRNA transcript in the presence of VTAV or TCMV genomic RNAs

To study whether the satRNA could be co-encapsidated into particles with viral genomic RNA, ^{32}P-satRNA was first mixed with VTAV or TCMV RNA (VR or TR) and then added to protein preparations for assembly. The assembled particles were then analysed for their sedimentation properties.

In stoichiometric assembly, a mixture of (+) ^{32}P-satRNA and VR was assembled with VP. Analysis of the sedimentation profiles and their radioactivity distribution showed that about 40% of the satRNA was encapsidated in particles which sedimented at the same rate as

Table 8-1. Effects of SatRNA/protein Ratio on the Particle Structure

No. Expt	SatRNA : Protein		Assembly[a] efficiency (%)	No. Particles[b] 27 nm ≤	≥ 28 nm
1	1	4	20	85	57
2	1	4	15	49	107
3	1	4	20	66	12
4	1	6	28	80	50
5	1	8	9	100	34
6	1	40	2	5	155

a. Assembly efficiency was calculated by comparing the radioactivity from peak fractions of the sucrose density gradient with the radioactivity of the amount loaded (assuming no radioactive material was lost during the assembly and clarification procedures).

b. The particle structure was examined by electron microscopy. Particles were grouped according to their diameter.

those of assembled VTAV while about 60% was encapsidated in particles which sedimented slightly faster than particles containing pure ^{32}P-satRNA (Fig. 8-5 a).

Similar experiments were done by mixing the (+) ^{32}P-satRNA and genomic TCMV RNA (TcR) in assembly reactions with TCMV protein (TcP). In the presence of TcR, all encapsidated satRNA was found in a position on the sucrose density gradient where assembled TCMV was recovered (Fig. 8-5 b, Sat+TcR-TcP). In a parallel gradient, assembled particles containing pure satRNA sedimented slightly more slowly than assembled TCMV (Fig. 8-5 b, Sat-TcP).

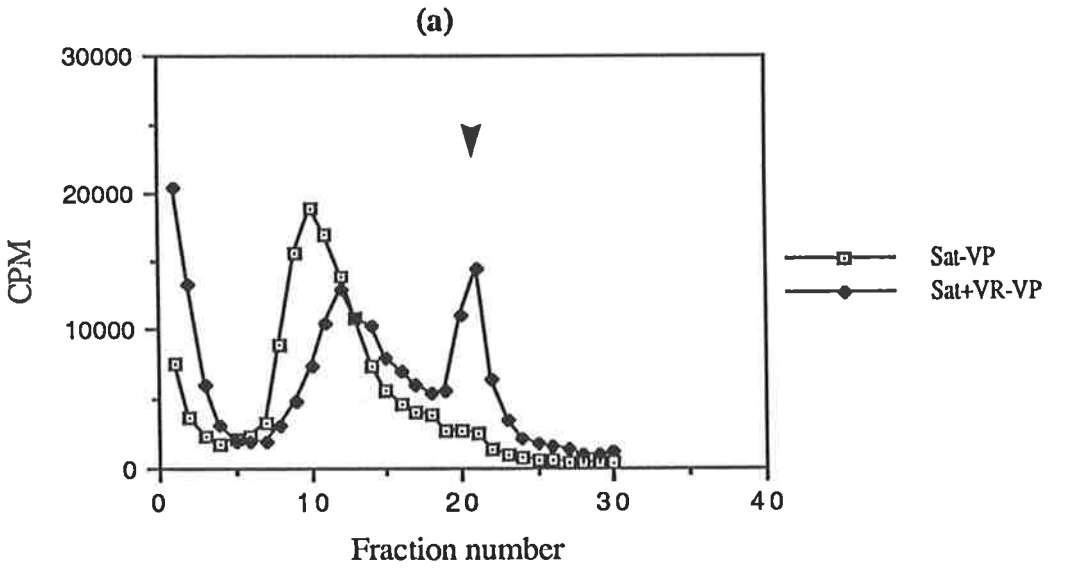
3. Encapsidation of native satellite RNA

Native B1 satRNA isolated from satRNA-containing TCMV preparations by a gel separation method (2.6) was used to investigate its encapsidation behaviour. The relatively larger amounts of native satRNA available made it possible to perform the assembly on a larger scale and so minmise the errors between experiments.

Fig. 8-5. Effects of viral genomic RNA on encapsidation of the (+) ^{32}P-satRNA.

(a). Assembly of the ^{32}P-satRNA and VP in the presence of VR. The ^{32}P-satRNA (30 μg) was mixed with VR (60 μg) and then assembled with VP (360 μg) (Sat+VR-VP) in a total volume of 900 μl . The control assembly reaction (Sat-VP) with ^{32}P-satRNA (30 μg) and VP (120 μg) was in a volume of 300 μl. One-third of the assembly mixture either Sat+VR-VP or Sat-VP was loaded on a 5-25% sucrose density gradient. The conditions of centrifugation and fractionation of gradients was the same as described in the legend of Fig. 8-4 b. The arrow indicates the position of the peak of native VTAV in the gradient.

(b). Assembly of the ^{32}P-satRNA and TcP in the presence of TcR. The treatments were as in (a) except that VR was replaced by TcR and VP by TcP to give the treatments Sat+TcR-TcP and Sat-TcP. The arrow indicates the position of the peak of native TCMV in the gradient.


(a)
30000
20000
10000
0
CPM
0
10
20
30
40
Fraction number
Sat-VP
Sat+VR-VP

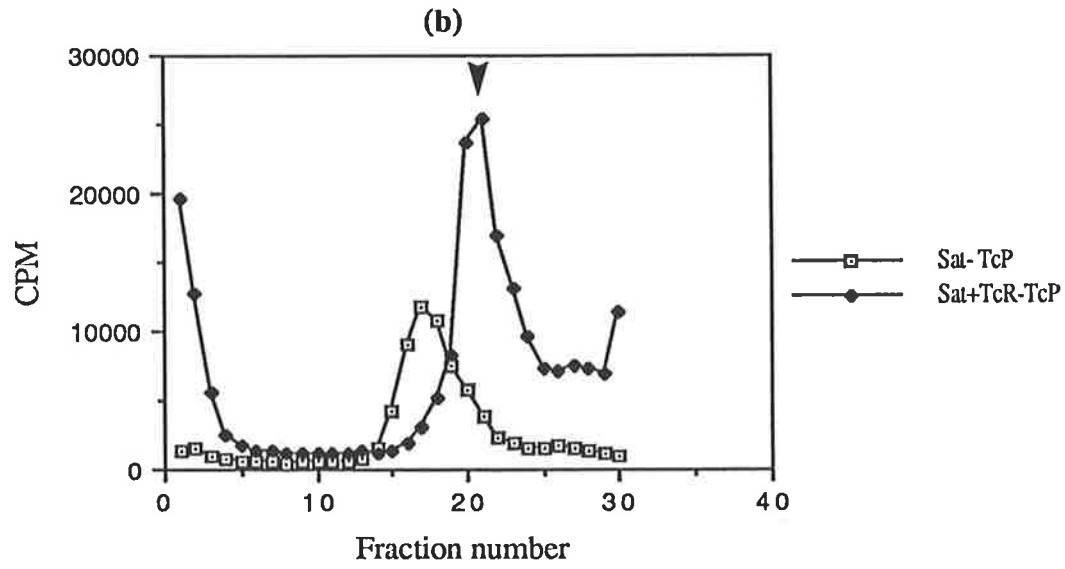

(b)
30000
20000
10000
0
CPM
0
10
20
30
40
Fraction number
Sat-TcP
Sat+TcR-TcP

Table 8-2. Effects of satRNA-protein concentration on the particle structure†

No. Expt	SatRNA-protein mg/ml	Assembly efficiency (%)	No. particle 27 nm ≤	≥28 nm
1	1.5	16	32	103
2	0.8	15	46	104
3	0.4	20	115	67
4	0.17	24	39	22
5	0.07	12	111	71
6	0.05	2.5	34	5

†. Assembly efficiency and particle groupings were examined as described in Table 8-1.

3.1 Encapsidation by VTAV coat protein

Typical cucumovirus-like particles, 28-30 nm in diameter, were assembled when the native satRNA was mixed stoichoimetrically with VP (Fig. 8-6 b). Sucrose density gradient centrifugation analyses showed that the assembly mixture contained three components (Fig. 8-6 a, ①) with the major one (peak 3) co-sedimenting with native VTAV (Fig. 8-6 a, ②). The lightest component was near the top (Fig. 8-6 a, ①, peak 1) and the third component sedimented slightly more slowly than the major one and showing as a broad "tail" (Fig. 8-6 a, ①, peak 2). However, when mixed with native VTAV, peak 2 disappeared while peak 1 remained near the top and peak 3 merged with the peak of VTAV (Fig. 8-6 a, ①+②) indicating that it may be within the peak range of VTAV. SatRNA was recovered from the fractions of peak 3 (Fig. 8-6 a, ①) in the gradient (Fig. 8-6 c, lane 1). No attempt was made to identify the RNA in peaks 1 and 2 in the gradient (Fig. 8-6 a). The assembly efficiency was about 30%.

3.2. Encapsidation by TCMV coat protein

Typical cucumovirus-like particles were formed when native satRNA was assembled with TcP (Fig. 8-7 b). There were two peaks in the gradient on which the assembly mixture was loaded: a small peak at the top and a major one midway (Fig. 8-7 a, ①). The major peak sedimented at a position corresponding to that of the control native TCMV (Fig. 8-7 a, ②). A precise comparison by centrifuging the mixture of the assembled particles and native TCMV in the same gradient showed that the two types of particles sedimented at the same rate (Fig. 8-7

a, ③). The assembly efficiency was about 60%.

Conclusion

1. Both TCMV and VTAV can support the replication of B1 satellite RNA of CMV and encapsidate it into their capsids *in vivo*.

2. TCMV supports the replication of B1 satellite RNA *in vivo*.better than VTAV.

3. Both coat proteins of TCMV and VTAV can encapsidate satRNA transcript *in vitro*. The assembled particles with TcP are cucumovirus-like but particles with VP are a mixture of cucumovirus-like and small ones (about 25 nm). The assembled particles with VP are significantly lighter than VTAV but particles with TcP are only slightly lighter than TCMV.

4. The change in sedimentation profile of particles containing satRNA transcripts assembled in the presence of viral genomic RNAs indicates that molecules of the transcript may have been co-encapsidated with viral genomic RNAs, possibly with RNAs 3 or 4. Coat protein of TCMV is more efficient than that of VTAV in encapsidating the satRNA transcript into cucumovirus-like particles.

5. In assembly with native satRNA, coat proteins of both TCMV and VTAV can encapsidate satRNA into cucumovirus-like particles with the same sedimentation rate in sucrose density gradients as that of native TCMV or VTAV.

6. TCMV protein is more efficient than VTAV coat protein in encapsidating native satRNA *in vitro*.

Fig. 8-6 Analysis of particles assembled with native satRNA and VP.

a. UV-absorption profiles on sucrose density gradient of assembled particles encapsidating native satRNA (①), native VTAV (②), and a mixture of assembled particles and native VTAV (③). The amounts of particles layered on 5-25% sucrose gradients were 140 μg of RNA-protein from the assembly mixture (calculated from the input, assuming 100% assembly efficiency) (①), 70 μg of native VTAV(②), and 70 μg of RNA-protein from the assembly mixture plus 35 μg native VTAV(③). Centrifugation was in a Beckman SW41 rotor at 35,000 rpm for 120 min. Sedimentation was from left to right.

b. Electron micrograph of assembled particles recovered from the main UV-absorption peak in the gradients (Fig. 8-6 a ①). Particles were sedimented from the gradient fractions after dilution of the sucrose with 20 mM phosphate buffer, pH 7.5 and resuspended in 10 mM borate buffer, pH 7.6. Bar represents 100 nm.

c. Agarose gel electrophoresis of RNA extracted from the assembled particles recovered from the UV-absorption peak in the gradients (Fig. 8-6 a ①) (lane 1, approximately 0.6 μg) and TCMV genomic RNA plus satRNA (lane 2, 2.5 μg). The electrophoresis was done in a 1.5% agarose gel and stained with ethidium bromide.

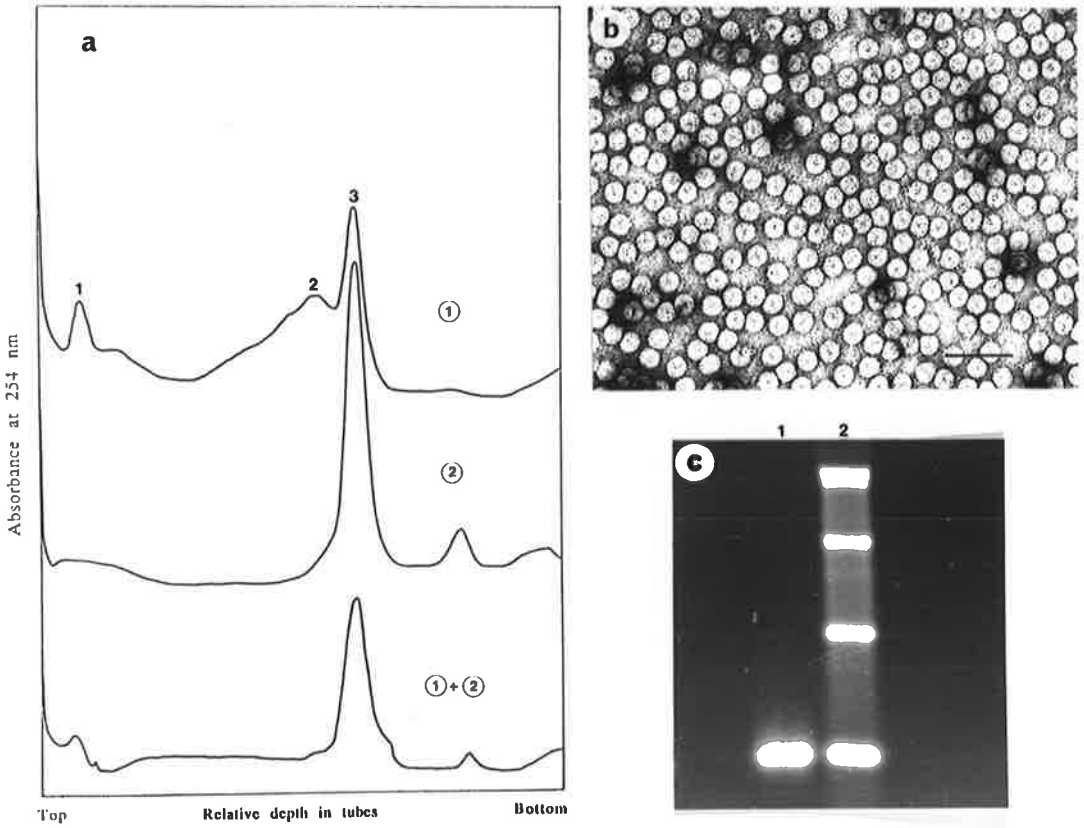
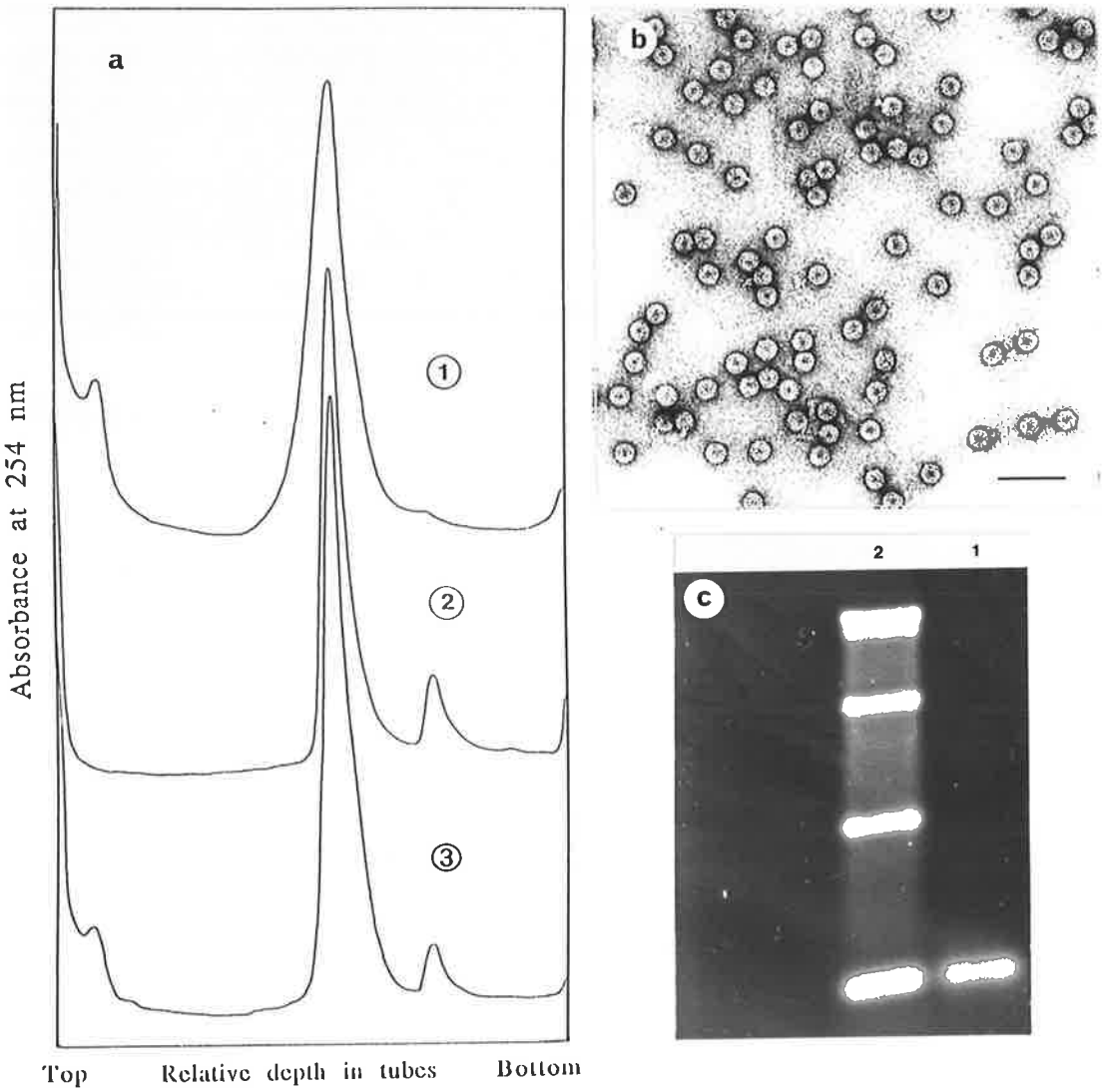

a
b
c
1
2
3
①
②
①+②
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom
1
2

Fig. 8-7 Analysis of particles assembled with native satRNA and TcP

a. UV absorption profiles of assembled particles encapsidating native satRNA (①), native TCMV (②), and a mixture of the assembled particles and native TCMV (③). An amount of 200 μg of RNA-protein from the assembly mixture (calculated from the input, assuming 100% assembly efficiency) (①), 100 μg of native TCMV(②), and 100 μg of RNA-protein from the assembly mixture plus 50 μg native TCMV(③) was layered on 5-25% sucrose gradients. The centrifugation was as described in the caption of Fig. 8-6 a.

b. Electron micrograph of assembled particles recovered from the main UV absorption peak in the gradients (a, ①). Samples were prepared as described in the caption of Fig. 8-6 b. Bar represents 100 nm.

c. Agarose gel electrophoresis of RNA extracted from the assembled particles recovered from the main UV absorption peak (a, ①) (lane 1) and TCMV genomic RNA plus satRNA (lane 2). About 0.5 μg (lane 1) and 2.5 μg (lane 2) of RNA were loaded. Conditions for electrophoresis were the same as described in the caption of Fig. 8-6 c.


a
b
c
Absorbance at 254 nm
Top
Relative depth in tubes
Bottom
1
2
3
2
1

Chapter 9 Transcapsidation and Aphid Transmission of VTMoV and its Satellite RNA from Source Plants Co-infected with VTAV or TCMV

Introduction

In Chapter 3 it was shown that the coat protein of tomato aspermy virus (V strain, VTAV) was able to encapsidate a number of plant viral RNAs into cucumovirus-like particles. This coat protein also encapsidated satellite RNA (satRNA) of VTMoV into particles which were indistinguishable from VTAV both in morphology and sedimentation rate in sucrose gradient (Chapter 5). It was further demonstrated in Chapter 7 that *in vitro* heterologously assembled viral particles were transmitted by *M. persicae* after acquisition of the particles through membrane feeding. The question asked here is whether such events of transcapsidation and aphid transmission occur *in vivo*?

Velvet tobacco mottle virus (VTMoV) and its satRNA seem to be suitable for the investigation of *in vivo* transcapsidation with VTAV. VTMoV cannot be transmitted by aphid vectors (Table 7-2; Randles, personal communication). Its monopartite genome is about the size of VTAV RNA 1. The satRNA of VTMoV, a very infectious and highly structured molecule 358 nucleotides long, can account for about 50% of the RNA encapsidated in VTMoV capsids (Randles *et al.*, 1981). The features of VTMoV and its satRNA would appear to be fovarable for encapsidation by VTAV capsids when they co-infect the same plants and therefore, aphid transmission of VTMoV or its satRNA could be expected if transcapsidation does occur *in vivo*.

Results and discussion

1. Co-infection of *N. clevelandii* with VTMoV and VTAV or TCMV

Inoculation of satellite-free VTMoV ($VTMoV^{-S}$) (Francki *et al.*, 1986) with either VTAV or cucumber mosaic virus (T strain, TCMV) to young *N. clevelandii* plants resulted in a severe mosaic differing from that induced by $VTMoV^{-S}$, VTAV or TCMV separately. Tests with antisera showed that both VTMoV and either VTAV or TCMV had co-infected the diseased plants (Fig. 9-1).

2. Transmission of VTMoV by *M. persicae*

Plants co-infected with VTMoV and either VTAV or TCMV were used as a virus-source for aphid transmission. Apterous aphids were allowed to probe on detached leaves of diseased plants and then placed and caged on young *N. clevelandii* plants. After three weeks individual plants were examined for the presence of antigen (s) of both viruses. The experiments were done four times and only in one experiment was VTMoV transmitted by the aphids at a rate of 2 out of 15; in that particular experiment, the transmission rate of VTAV was much higher (12/15) than in the other experiments (Table 9-1). Since transmission of VTMoV was only observed in one experiment, insufficient evidence was available to conclude that transcapsidation of VTMoV by VTAV coat protein had occurred.

3. Transmission of satellite RNA of VTMoV by *M. persicae*

In experiments similar to those described above, plants of *N. clevelandii* were co-infected with satRNA-containing VTMoV ($VTMoV_{+S}$) and VTAV. The diseased plants containing both viruses were used as a source of inoculum for aphids transmission experiments. Recipient plants were *N. clevelandii* pre-inoculated with $VTMoV^{-S}$ so that they could express satRNA if it was transmitted in association with VTAV.

Three weeks after aphid transmission, the recipients were analysed serologically for the presence of VTAV antigen and by dot-blot hybridization with a cDNA probe specific to satRNA of VTMoV. As shown in Table 9-2, transmission of satRNA was detected in all three experiments from the source plants co-infected with VTAV and $VTMoV_{+S}$ but not from the source plants infected only with $VTMoV_{+S}$. In experiment 1, the transmission rate of VTAV was 12 out of 28 and that of satellite 4 out of 28 (Fig. 9-2). Interestingly, one satRNA transmission (Fig. 9-2 b, No. 8) occurred without concurrent transmission of VTAV (Fig. 9-2 a, No. 8) while the other three transmissions were associated with that of VTAV. A similar phenomenon was also observed in experiment 3 (Table 9-2) where two satRNA transmissions were associated with infection by VTAV and two were independent of infection with VTAV.

Conclusion

In experiments where plants were co-infected with VTMoV and either VTAV or TCMV, aphid transmisssion of VTMoV was rare. This result indicates that the transcapsidation of

Table 9-1 Transmission of viruses from source plants co-infected with VTMoV^{-S} and either VTAV or TCMV by *M. persicae*†

Experiment	Source	Virus recovered		
		VTAV	TCMV	VTMoV
1	VTMoV^{-S} + VTAV	10/15		2/15*
	VTMoV^{-S}			0/15
2	VTMoV^{-S} + VTAV	14/56		0/56
	VTMoV^{-S}			0/28
3	VTMoV^{-S} + VTAV	6/34		0/34
	VTMoV^{-S} + TCMV		8/34	0/34
	VTMoV^{-S}			0/34
4	VTMoV^{-S} + TCMV		12/36	0/36
	VTMoV^{-S}			0/36

† 10 aphids per plant except in experiment 3 where 5 aphids per plant were used. The conditions of aphid transmission were described in 2.19.

* The numerator indicates the number of infected plants and the denominator the number of plants used in the trial.

VTMoV RNA into either VTAV or TCMV capsids occurs at a very low frequency. However, in experiments devised to see whether the satellite RNA of VTMoV could be transcapsidated by VTAV capsid, the satellite RNA was transmitted by *M. persicae* at a reasonably high frequency (an average of about 25% of the transmission of VTAV in the three experiments done). Satellite RNA occur at relatively high concentration in infected plants (Randles *et al.*, 1981) and may therefore occur at assembly site (s) of VTAV and thus become encapsidated with VTAV capsid.

Table 9-2 Transmission of satellite RNA of VTMoV by *M. persicae* through plants co-infected with VTAV†

Experiment	Source	Recovered virus and satellite	
		VTAV	Sateliite
1	$VTMoV_{+S}$ + VTAV	12/28	4/28*
	$VTMoV_{+S}$		0/28
2	$VTMoV_{+S}$ + VTAV	17/28	1/28
	$VTMoV_{+S}$		0/28
3	$VTMoV_{+S}$ + VTAV	6/28	4/28
	$VTMoV_{+S}$		0/28

† 10 aphids per plant except in experiment 3 where 5 aphids per plant were used. The conditions of aphid transmission were described in 2.19.

* The numerator indicates the number of infected plants and the denominator the number of plants used in the trial.

Fig. 9-1 Immunodiffusion tests of plants co-infected with VTMoV and either VTAV or TCMV. Plants were tested three weeks after mechanical inoculation. Leaf was extracted by crushing in plastic bags with one volume (w/v) of 20 mM phosphate buffer, pH 7.5. The loading was 12 μl per well.

a. Sap from plants infected with VTMoV^{-S} and VTAV (Cv), VTMoV^{-S} alone (1) and VTAV alone (2) were allowed to react with antisera to VTAV (A) and VTMoV (B).

b. Sap from plants infected with VTMoV and TCMV (Ct), VTMoV^{-S} alone (1) and TCMV alone (3) were allowed to react with antisera to VTMoV (B) and TCMV (C).

Fig. 9-2 Identification of VTAV and satellite RNA of VTMoV from recipient plants after aphid transmission.

a. Results of immunodiffusion tests for 28 plants inoculated by aphids as in Table 9-2, experiment 1. Sample preparation and experimental conditions were the same as those described in the caption of Fig. 9-1. Numbers represent individual recipient plants. ⊕ represents positive for VTAV and ⊖ negative, respectively.

b. Dot blot hybridization. Preparation of sample and cDNA, and the conditions for hybridization are described in 2.2.11-12. The template for cDNA synthesis was linear satellite RNA which was separated by one cycle of electrophoresis in a 4% polyacrylamide gel containing 7 M urea. The band containing the linear molecules of the satellite RNA was excised and then embedded in a 1.5% agarose gel. The RNA was subjected to electrophoresis and recovered by the centrifugation method described in 2.2.6. Numbers represent individual recipient plants the same as in (a). Purified satellite RNA of VTMoV (S), total RNAs of plants infected with VTAV (V), VTMoV^{-S} (K1), and VTMoV$_{+S}$ (SV) were used as controls.

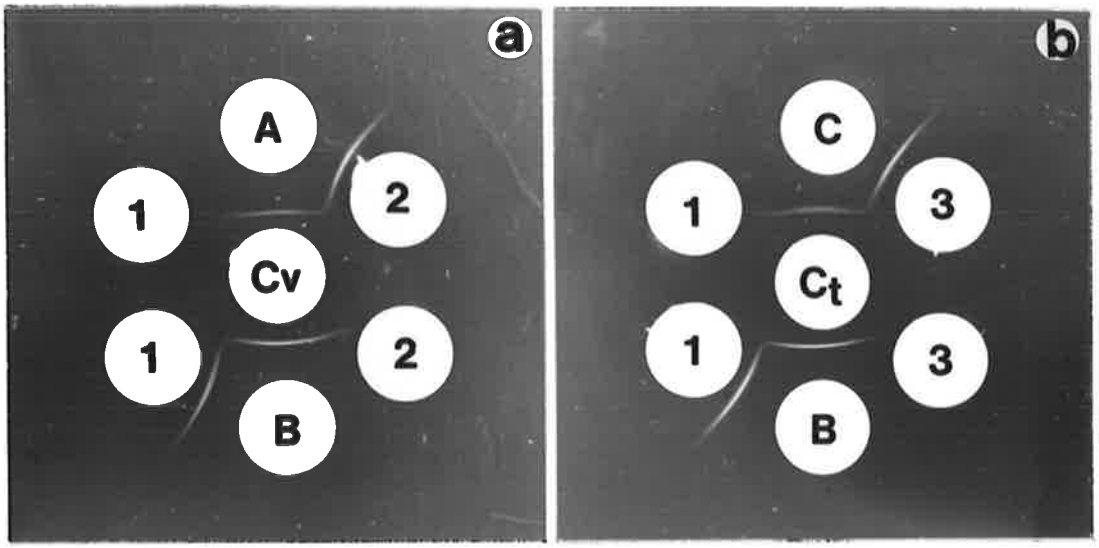


Fig. 9-1

Fig. 9-2

Chapter 10 General Discussion

1. The reassembly method

In Chapter 2 and 3, the method developed for the reassembly of VTAV and MCMV partices was described in detail.

a. A comparison of the existing methods with the method used in this study

All reassembly methods for spherical plant viruses follow the same principle established through the reassembly study of cowpea chlorotic mosaic virus (CCMV) (Bancroft *et al.*, 1967; Bancroft and Hiebert, 1967; Hiebert *et al.*, 1968), that is, dissociation in high salt and reassembly in low salt solutions. Modifications of reassembly conditions are necessary for some other viruses, e.g. cucumber mosaic virus (CMV) (Kaper and Geelen, 1971), turnip crinkle virus (TCV) (Sorger *et al.*, 1986) and sowbane mosaic virus (SoMV) (Tremaine and Ronald, 1977). Within CMV, different strains also vary in their response to dissociation (Lot and Kaper, 1976a) and reassembly (Kaper, 1969; Kaper and Geelen, 1971). In all these reports, coat protein preparations in high salt solution were mixed directly with RNA and successful assembly was obtained (Kaper and Geelen, 1971; Habili, 1974; Gera *et al.*, 1979; Hiebert *et al.*, 1968).

MCMV differs from other CMV strains in its requirement for a unique purification procedure (Mossop *et al.*, 1976). It did not dissociate completely in 2 M LiCl, but it did in 3 M LiCl. Compared with MCMV, VTAV was easily dissociated in either 2 or 3 M LiCl. For comparative purposes in this study, both VTAV and CMV (MCMV and TCMV) were dissociated in 3 M LiCl (Fig. 3-1).

MCMV and VTAV could not be efficiently reassembled by mixing viral coat protein in 3 M LiCl and viral RNA either by direct dilution or by dialysis against a buffer with low ionic strength. Gradual lowering of salt concentration in protein solutions by sequential dialysis against buffers with lower salt concentration could maintain about 85% of VTAV and 70% of MCMV protein in solutions in the 0.5 M LiCl. High pH in low salt is important to keep coat protein soluble (Sorger *at al.*, 1986). When these protein preparations were assembled with homologous or heterologous viral RNA, a reassembly efficiency of 60-75% was achieved. PAGE analysis of VTAV coat protein preparation showed that the protein peptide was intact without degradation (Fig. 3-13).

b. Assay criteria used for examining assembled particles

A set of comprehensive criteria were used to assay the reassembled products under the reassembly conditions described. Morphology and sedimentation properties were usually taken as the primary approaches. As shown in Figs. 3-5 and 3-6, homologous and heterologous assembled virus particles were all cucumovirus-like, 28-30 nm in diameter, regardless of the viral RNA used. The homologously assembled VTAV and MCMV had the same sedimentation rates in sucrose density gradients as their native viruses (Fig. 3-7). Heterologously assembled particles with VTAV protein and MCMV RNA co-sedimented with native VTAV (Fig. 3-8 i) but particles with VTAV protein and TMV RNA sedimented faster than native VTAV (Fig. 3-8 ii) in sucrose density gradients. The distribution of infectivity, RNA, protein and UV absorption patterns in the sucrose gradients faithfully corresponded with each other (Fig.3-9, 3-10, 3-11 and 3-12). Analyses of RNA and protein recovered from homologously and heterologously assembled virus particles showed that they were both intact (Fig. 3-13, 3-14). Spectral analysis indicated that the homologously assembled VTAV was indistinguishable from native VTAV (Fig. 3-15). The reassembled particles retained the immunological properties of the coat protein capsids (Fig. 3-17). All these data suggest that the reassembled particles behave like normal spherical viruses, biologically, physico-chemically and immunologically. Thus, it is concluded that the reassembly system is functional.

The introduction of RNA and protein identification as assay criteria seems to be important. A number of early reports on virus reassembly claiming high reassembly efficiency but with low infectivity (Semancik and Reynold, 1969; Goodman *et al.*, 1975; McDonal and Bancroft, 1977; Kaper, 1969) may well be the result of degradation of viral RNA.

RNase resistance was not taken as a critical criterion in assaying the reassembled particles in this study, because native CMV particles themselves are sensitive to the enzyme (Francki, 1968; Habili and Francki, 1974b).

The $O.D._{260/280}$ of heterologously assembled particles with VTAV protein and TMV RNA was 1.70 (Fig. 3-16), similar to but not greater than that of VTAV. Since TMV RNA is about 90% larger than CMV RNA 1, particles containing full length TMV RNA should give a higher UV absorption at 260 nm. A possible explanation for this is that the tightly packed TMV RNA in the limited space of a particle may behave like double stranded RNA and therefore may have a lower extinction coefficient, as all other data suggest that the reassembled

particles must contain full length TMV RNA.

2. Possible types of particle in cucumovirus particle populations

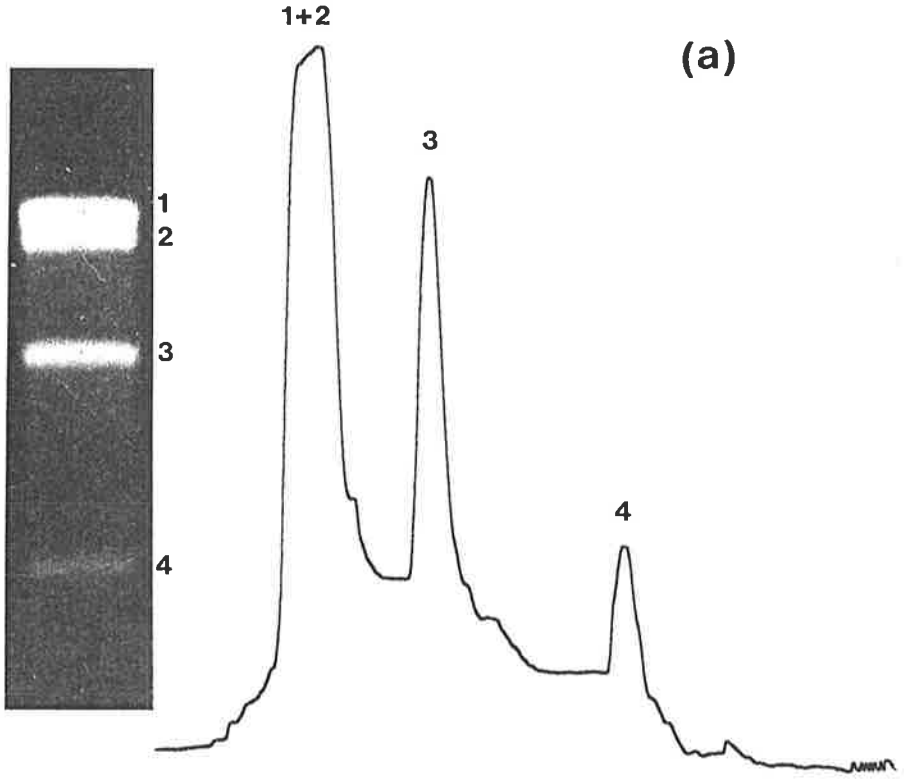
Because of its instability in high salt solution, the particle pattern of CMV has not been well differentiated. Evidence obtained from the novel experiment presented in Chapter 4 suggested that four types of virus particles rather than the three proposed previously (Peden and Symon, 1973) may exist in CMV populations .

The criteria used to evaluate the particle pattern were the morphology, relative sedimentation rate in sucrose density gradient and RNA integrity. Homologously assembled particles containing RNA 1 or 2 were indistinguishable from native CMV in both morphology and sedimentation rate in sucrose gradients; RNA 1 and 2 were recovered from the assembled particles without showing any degradation (Fig. 4-2). This suggests that RNA 1 and 2 must have been encapsidated separately otherwise heavier particles would have been detected if the two RNAs were encapsidated in the same particles. Assembled particles encapsidating pure RNA 3 did not share sedimentation properties with native TCMV (Fig. 4-3) and therefore the possibility that many such particles occured in preparation of native CMV was ruled out. Particles encapsidating either pure RNA 4 (Fig. 4-4) or a mixture of RNAs 3 and 4 (Fig. 4-5) were indistinguishable from native TCMV both in size and sedimentation rate, and RNA recovered from these two types of particles was intact . Therefore, they are considered as likely to be part of a native TCMV particle population. Thus, this study shows that there are four possible types of particles in which cucumovirus genomic RNAs can be encapsidated: the first particle encapsidates one molecule of RNA 1; the second particle encapsidates one molecule of RNA 2; the third particle encapsidates one molecule each of RNAs 3 and 4 and the fourth particle encapsidates 3 to 4 molecules of RNA 4. This four particle pattern may or may not apply to all strains of cucumovirus. The concept of the existence of the fourth type of particle can also be supported by the observation that RNA 4 is much more abundant than RNA 3 in some CMV preparations (Fig. 10-1). As shown in Fig. 10-1, in one preparation (a, VTAV), the molar ratio of RNA 3 to 4 is 1 to 0.68 indicating that the two molecules may have been encapsidated together although some RNA 3 seems to be in excess; in another preparation (b, TCMV), the molar ratio of RNA3 to 4 is about 1 to 2.3 indicating a large excess of RNA 4. Thus, the extra RNA 4 must have been encapsidated alone in the virus particles. Indeed, when

Fig. 10-1. Comparison of encapsidated RNA patterns of VTAV and TCMV. The RNA was extracted from purified virus preparations and individual species was separated by electrophoresis in 1.5% agarose gels . The gels were stained with ethidium bromide and photographed under UV light. The photographic prints were scanned with a densitometer. The relative amount (weight) of each RNA species in the gels was expressed as the area under its peak as determined by densitometry. To compare the molar ratio of the two RNA species, the peak area of each species was divided by its molecular weight before comparison. The molecular weights of VTAV RNAs 3 and 4 were taken as 0.9 and 0.4 x 10^6, and those of TCMV RNAs 3 and 4 were 0.82 and 0.35 x 10^6 daltons (Habili and Francki, 1974a).

a. VTAV RNA. The peak area ratio of RNA 3 : 4 = 1 : 0.3. The molar ratio of RNA 3 : RNA 4 = 1 : 0.68.

b. TCMV RNA. The peak area ratio of RNA 3 : 4 = 1 : 1. The molar ratio of RNA 3 : RNA 4 = 1 : 2.3.


1+2
(a)
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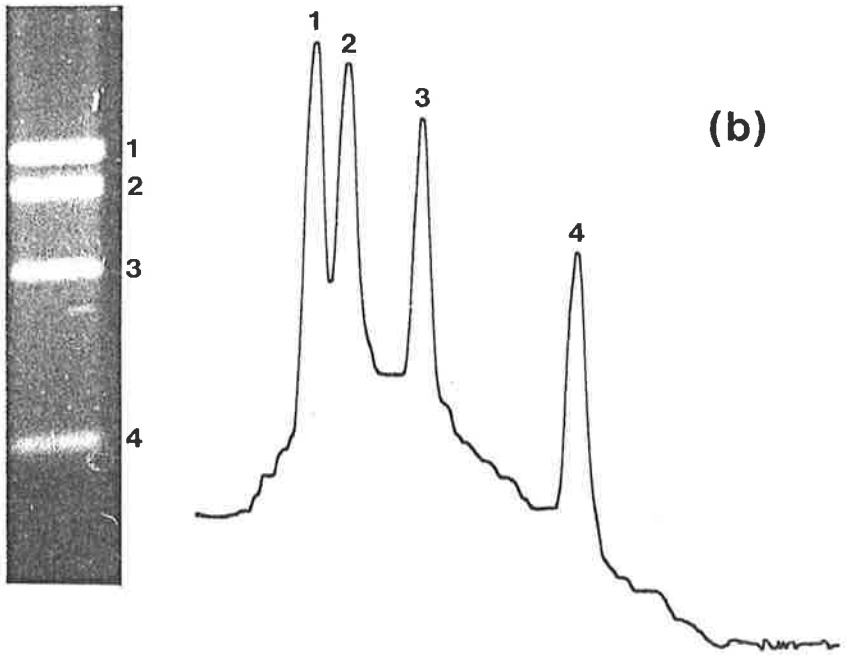
1
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(b)
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4

reexamining CMV RNA patterns in some published work, dominance of RNA 4 over RNA 3 is also found (e.g., Garcia-Luque *et al.*, 1983; Piazzolla *et al.*, 1979). It is not known why some strains of CMV overproduce RNA 4 *in vivo*. Ecologically, the production of the fourth type of virus particle would not seem to confer any advantage on the virus itself, because the mutiplication of the virus only need RNAs 1-3.

3. Characterisation of cucumovirus encapsidation *in vitro*

a. Morphology determination and conservation of total length of nucleotide

It was reported previously that encapsidation of fragmented TYMV RNA (M_r 60,000, about 200 nt) and full length TYMV RNA (M_r 1.9 x 106, about 5400 nt) with CMV protein resulted in the formation of a 93 S and a 130 S components, respectively, (Kaper and Geelen, 1971). But the dimentions of these components were not given. The observation was made in this study that regardless of the different nucleic acids used, e.g. long (TMV RNA, 6395 nt) or short (tRNA, 80 nt), or with mixtures of RNA with different lengths (plant total RNAs), particles assembled with either VTAV, TCMV or MCMV coat protein were always cucumovirus-like in morphology (Figs. 3-5, 5-1, 5-2). These common features suggest that the particle shape under these defined conditions is determined solely by the coat protein subunits. Another common feature observed was that the particles assembled with smaller RNAs (e.g. tRNA and satellite RNA of VTMoV) or mixtures of RNAs (plant total RNA, about 80-3000 nt) tended to retain both cucumovirus-like morphology and molecular weight (Figs. 5-2, 5-3, 5-1). This charateristic led me to the conclusion that cucumovirus preferentially encapsidate nucleic acid of a total length of about 3000 to 3400 nucleotides, in a capsid of 180 protein subunits. Therefore, this length of nucleotide can be considered as the structurally required optimum length.

b. RNA-protein, RNA-RNA and protein-protein interactions and cooperative encapsidation

In cucumovirus, an average of 3200 nt of viral RNA was encapsidated by 180 protein subunits giving a ratio of 17-18 nt per subunit (Kaper *et al*, 1965; Francki *et al*, 1966; Habili and Francki, 1974a; Francki *et al.*, 1979). It is believed that some of this RNA is not used to build the particle (Tikchonenko, 1975). In this study it was found that ss DNA of 20 nt could not form particles with VTAV protein but ss DNA of 35 nt could although the particles were

less uniform and much lighter than normal VTAV (Fig. 5-9). However, tRNA of about 80 nt could form cucumovirus-like particles and were only slightly lighter than VTAV (Fig. 5-2) and satellite RNAs of VTMoV (366 nt, Fig. 5-3) or CMV (337 nt, Figs. 8-6, 8-7) could form particles indistinguishable from VTAV or TCMV both in size and weight. The correlation between the length of nucleic acid and ability for particle to assemble suggests that there is a minimum requirement for the length of a nucleic acid molecule to build a cucumovirus-like particle and it is concluded that this length is between 35 and 80 nt.

In an experiment using BMV coat protein, it was found that particles which were superficially similar to T = 1 particles and sedimented at 32 S were assembled with oligouridylic acid with average size of 23 residues (Bancroft *et al.*, 1969). Obviously, VTAV coat protein differed from BMV coat protein in that it did not form T =1 particles with nucleic acids of the minimum length.

Since a minimum length of each RNA molecule is required for successful assembly, interactions between RNA molecules may be important, or alternatively a basic RNA-protein complex could have to form before a full-scale particle can be assembled. The assembled products of 35 nt DNA oligomer and VTAV protein sedimented much more slowly than native VTAV in sucrose gradients and they were irregular and with many small spherical structures as viewed under the electron microscope (Fig. 5-9 a and c). This might suggest that 35 nt DNA could only form an incomplete complex with VTAV protein which could not proceed further to form a complete particle. On the other hand, a 20 nt DNA oligomer could not form any kind of structure with VTAV protein (Fig. 5-9 b). This might be due to the nucleotide being too short to bind protein subunits.

A particle which encapsidates tRNA but with cucumovirus-like dimensions and a slightly lower sedimentation rate compared with native cucumovirus will contain about 40 molecules of the RNA. Therefore an average of 4-5 subunits would be assigned to a tRNA molecule. The bringing together of so many RNA molecules and assembling them into a defined particle with 180 subunits must occur in an ordered manner. How this is achieved is not known. Referring to the binding model proposed for AMV assembly (Houwing and Jaspars, 1982), it may be postulated that encapsidation of tRNA by cucumovirus includes two stages. In the first stage a tRNA molecule is bound by 4-5 protein subunits to form a structural unit and then 40 such units aggregate, driven by both protein-protein (hydrophobic and hydrophilic) and RNA-RNA

(hydrogen bonding) interactions. Of course, further experiments must to be done before a final conclusion can be drawn.

The cooperative binding of coat protein to unsaturated protein-nucleic acid molecules rather than to a free nucleic acid molecule was previously observed for AMV coat protein, and it was concluded that the polymerization of the protein resulting in a nucleoprotein particle was a cooperative process (Driedonks *et al.*, 1978). The observation that smaller RNA species (e.g. tRNA, satRNA) could assemble with cucumovirus protein to form typical cucumovirus-like particles (Figs. 5-2, 5-3) could create another concept -- cooperative encapsidation, in which both protein and RNA would tend to bind to the unsaturated RNA-protein complex, then form a complete particle.

c. Maximum encapsidation capacity

The maximum encapsidation capacity of cucumoviruses has not been precisely determined in this study. However, since full length TMV RNA (6395 nt) was encapsidated but not a ss DNA molecule of 11700 nt, it is concluded that the maximum length of nucleotide a cucumovirus can encapsidate is less than 11700 nt. The particles assembled with VTAV coat protein and ss DNA (7020 nt) did not sediment faster than VTAV indicating that possibly the particles did not encapsidate full length DNA. Therefore, it is possible that the maximum length of nucleic acid may be between 6395 and 7020 nt. Further experiments to characterize the encapsidated DNA are needed to address this.

d. Why are ds nucleic acids not encapsidated?

AMV coat protein can form tubular complexes with a ds DNA (calf thymus DNA) (Driedonks *et al.*, 1978). Tubular, bacilliform, and spherical structures were formed when coat proteins of CCMV, BMV and BBMV were mixed with calf thymus DNA, respectively (Bancroft *et al.*, 1969). However, cucumovirus coat protein cannot assemble with ds DNA or RNA (see 5.5 and Fig. 5-6). The reason why ds RNA or DNA could not be encapsidated is not known. Kaper (1976) has suggested that lysine is the amino acid likely to interact with the RNA, probably through uridine and guanosine phosphate residues. It is possible that the double helix of nucleic acid results in a shortage of "free" phosphates by altering the spatial conformation of the molecule so that they can no longer interact with the viral protein.

The inability of cucumovirus coat protein to encapsidate ds RNA or DNA may be a possible strategy employed by viruses to encapsidate selectively the (+) but not (-) sense viral

RNA *in vivo*. Since most if not all (-) viral RNA is in ds or RI (replicative intermediate) form, the double-strandedness would effectively prevent the (-) sense viral RNA from being encapsidated. In brome mosaic virus, the free (-) sense viral RNA in infected plants might be very rare due to the asymmetric replication of (+) and (-) sense viral RNA, by which the ratio of (+) over (-) sense RNA could be as high as 100 to 1000 times (Nassuth and Bol, 1983; Marsh *et al.*, 1991). Thus, the limited amount of (-) sense viral RNA would possibly be neutralized by forming ds molecules with (+) sense viral RNA.

e. Why specific encapsidation *in vivo*?

In contrast with non specific encapsidation *in vitro*, *in vivo* cucumoviruses encapsidate their own viral RNA rather than that of any other host RNA (Hull and Maule, 1985; Kaper and Waterworth, 1981). Site specificity for replication and assembly is thought to be one of the reasons (Hull & Maule, 1985). Virus-specific tonoplast-associated vesicles containing ds RNAs have been observed in CMV and TAV-infected leaf cells and these structures are suggested as possible sites of virus replication and assembly (Hatta and Francki, 1981). As noted by Francki (1987) virus-induced vesicles have been found to be associated with membranes of the endoplasmic reticulum (e.g. tobacco ringspot virus), with nuclei (e.g. pea enation mosaic virus), with chloroplast (e.g. turnip yellow mosaic virus) and with mitochondria (e.g. cucumber green mottle mosaic virus). Virus-specific dsRNA and replicase activity have been detected in organelle fractions containing some of these structures (Assink *et al.*, 1973; De Zoeten *et al.*, 1974; Zabel *et al.*, 1974; De Zoeten *et al.*, 1976; Powell and De Zoeten, 1977). The lack of a link between ultrastructure and biochemical activity makes it difficult to identify the precise site(s) of virus replication and encapsidation. However, indirect evidence from mixed infections of tobacco protoplasts suggests that two related viruses (CCMV and BMV) replicate at different intracellular sites (Sakai *et al.*, 1983). The observation that BMV RNAs were encapsidated in TMV coat in barley protoplasts infected with BMV RNA 1, 2 and the hybrid RNA 3 (BMV RNA 3 with its coat gene replaced by TMV coat gene) suggests that the TMV coat is expressed in the BMV assembly site (Sacher *et al.*, 1988).

4. Hybrid capsids: are they biologically significant?

The demonstration of the formation of hybrid capsids comprising coat protein subunits both of VTAV and TCMV (Fig. 6-1) indicates that the spatial structure of these two subunits

may be compatible. Hybrid capsids have previously been constructed between bromoviruses (CCMV, BBMV, and BMV) (Wagner and Bancroft, 1968). Since bromovirus coat protein can form empty particles or some other structures in the absence of RNA (Adolph and Butler, 1974; Pfeiffer, P., and Hirth, L., 1974), it seems that the protein-protein interactions in these viruses is stronger than that of the cucumoviruses in which no empty particles or similar aggregated structure have ever been found when assembly is done in the absence of RNA (Kaper and Geelen, 1971; Habili, 1974; and chapter 3 of this work). Thus, given the weak protein-protein interaction in cucumovirus particles, a hybrid capsid would require some spatial similarities in the two different subunits in order to form a stable particle. The evidence for a distant serological relationship between TAV and CMV may be evidence in support of such a proposal. This will need to be confirmed by tertiary structural analysis of the subunits. Overall, the characteristics of hydrid capsid formation in cucumoviruses and bromoviruses is different from that of TMV, a rod shaped virus. Subunits of different strains of TMV, or from closely related viruses normally do not co-assemble (Sarkar, 1960) or they give aggregates with anomalous properties (Novikov *et al.*, 1974). The reason proposed is that the altered lateral interaction between subunits causes a spatial problem (Bloomer *et al.*, 1981; Bloomer & Bulter, 1986). However, *in vivo* mixed capsids which comprise subunits of both TMV and tomato mosaic tobamovirus have been reported (Otsuki and Takebe, 1978).

It is not clear whether the aphid transmissibility of a virus is determined by the whole capsid or by only part of it. Sequence analysis of an aphid-transmissible mutant of MCMV reveals that only a few nucleotides have changed as compared with that of the non aphid-transmissible MCMV (S.F. Zhu, 1990, personal communication). In tobacco vein mottling potyvirus, it has been shown that a point mutation in viral coat protein gene abolished aphid transmissibility of the virus (Atreya *et al.*, 1990). This evidence seems to favour the notion that perhaps only part of the whole virus particle determines the virus-aphid relationship. If this hypothesis is correct, then in the event of co-infection with an aphid-transmissible and a non aphid-transmissible cucumovirus, a hybrid capsid may possibly contribute to the transcapsidation and aphid transmission of the non aphid transmissible virus. Thus, the concept of transcapsidation could include two situations: RNA of one virus encapsidated in the capsid of another virus or in a hybrid capsid with proteins from two viruses. A non aphid transmissible virus would, therefore benefit from the formation of a hybrid capsid in that it

could become capable of spread by aphids. Transcapsidated particles between two strains (MAV and RPV) of barley yellow dwarf virus have been identified in doubly infected plants by immunohybridization, but hybrid capsids have not been detected (Creamer and Falk, 1990).

5. The relationship between cucumovirus coat protein and aphid transmissibility

a. Coat protein determines aphid transmissibility in cucumovirus

More than two decades ago, Hiebert *et al.* (1968) suggested that *in vitro* assembly techniques would be useful for investigating the mechanism of aphid transmission of plant viruses.

Results presented in Chapter 7 demonstrated that coat protein alone determines the aphid transmissibility of cucumovirus. This was previously suspected from results of pseudorecombination experiments showing that aphid transmissibility was determined by RNA 3 (Mossop & Francki, 1977). However, the data did not preclude the possibility that the other protein with M*r* of approximately 30,000 (3a protein) specified by RNA 3 was responsible for the aphid transmissibility. This protein is now thought to be involved in cell-to cell movement (Davies & Symons, 1988). In experiments similar to the one described in chapter 7, Gera *et al.* (1979) showed that the transmission efficiency of two CMV strains (CMV-T & CMV-6) by *Aphis gossypii* was determined by their coat protein.

For successful transmission by an aphid vector, virus particles must be taken up and released by aphids without inactivating their infectivities. It is not known at which stage VTAV and MCMV differ in the process of aphid transmission. The elucidation as to whether MCMV can be taken up and released by *M. persicae* would be important to further understand the mechanism of cucumovirus-aphid recognition.

b. Why can TMV not be transmitted by aphids?-- an old and new explanation

The transmission of tobacco mosaic virus (TMV) by *M. persicae* after TMV RNA had been encapsidated with VTAV coat protein raises the question as to why native TMV is not transmitted by aphids (Harris, 1977; Gooding, Jr., 1986). It is known that TMV can be acquired from plants or through membranes (Ossiannilsson, 1958; Kikumoto and Matsui, 1962; Pirone 1967; Takahashi and Orlob, 1972) and transmitted it to water or buffer droplets by aphids (Matsui *et al.*, 1963; Pirone, 1967). The particles released through membranes have

been shown to be infectious when inoculated mechanically to tobacco plants (Pirone, 1967). It appears that the non aphid-transmisibility of TMV lies in the inoculation phase (Pirone, 1967). Data presented in Chapter 7 (Table 7-4) suggests that aphids can pick up the assembled particles and release them into plant cells; the released particles can uncoat and cause infections. An explanation of this may be that aphid-delivered native TMV cannot uncoat in plant cells and therefore not cause infection. Such an explanation may not conflict with the observation that TMV particles released into buffer from aphid mouthparts were still infectious (Pirone, 1967) because in that experiment the released virus was inoculated mechanically to plants. The violent rubbing may help break the particles and release the encapsidated RNA which then could replicate. If the uncoating blockage hypothesis is correct, what substance (s) could inhibits the uncoating ? Since it has been shown that TMV is transmitted by aphids after treating with poly-L-ornithine (PLO) (Pirone and Shaw, 1973; Pirone, 1977), perhaps PLO overcomes an inhibility of TMV to dissociate *in vivo* or it may neutralize the inhibitor (s) in the aphid. Further experiments should be done on this aspect to help elucidate the TMV puzzle.

6. Encapsidation of CMV satellite RNA

satRNA alone can form cucumovirus-like particles with TCMV or VTAV coat protein *in vitro* (Figs. 8-6, 8-7). Mixed encapsidation of satRNA and the viral genomic RNA in the same particles was also demonstrated *in vitro* in this study (Fig. 8-5). Therefore it is possible that these two types of encapsidation may occur *in vivo*. With some strains of CMV, satRNA can account for up to about 50% of encapsidated RNAs (Mossop and Francki, 1979). In such a situation, satRNA may be encapsidated both alone and with genomic viral RNA because of the abundance of satRNA. With VTAV (see 8.1), satRNA only accounted for about 4% of the encapsidated RNA in VTAV preparations. In this particular case, it is probable that the satRNA was encapsidated together with its helper viral RNA, because the satRNA showed the tendency to be jointly co-encapsidated with VTAV RNA *in vitro* (Fig. 8-5 a).

It is not known why some lighter and smaller particles were assembled using VTAV coat protein and satRNA transcript (Fig. 8-3 a). Particles of about 25 nm were larger than T=1 particles. The difference between the (+) satRNA transcript and the native B1 satRNA was that the former had an extra 22 nt polylinker sequence, comprising 16 nt at the 5'- and 6 nt at the 3'-termini.

There was a difference between TCMV and VTAV coat protein in the encapsidation of satRNA. TCMV protein was better than VTAV protein in encapsidating satRNA into cucumovirus-like particles (Fig. 8-5). Analysis of the satRNA pool *in vivo* suggested that TCMV was a better helper for satRNA replication than VTAV. TCMV particles also encapsidated much more satRNA than VTAV (Fig. 8-1). However, these data are insufficient to conclude that TCMV is better than VTAV in encapsidating satRNA *in vivo* because the total TCMV RNA/satRNA and VTAV RNA/satRNA ratios have not been determined. The small amount of satRNA encapsidated in VTAV could also possibly be due to the small satRNA pool *in vivo* (Fig 8-1).

There are two criteria which can be used to describe the helping efficiency of a virus for a satRNA: the proportion of satRNA in the RNA encapsidated in the purified virus preparation plus virus yield, or the absolute amount of satRNA produced in the host plants. Previous publications adopted the first criterion (Kaper and Tousignant, 1977; Mossop and Francki, 1979). Perhaps there could be such a situation that a virus is efficient in helping the replication but inefficient in helping the encapsidation of its satRNA.

7. *In vivo* transcapsidation: differences between genomic and satellite RNAs

When plants co-infected with the aphid-transmissible VTAV or TCMV and the non aphid transmissible, satRNA-free VTMoV ($VTMoV^{-s}$) were used as virus sources, $VTMoV^{-s}$ was transmitted by aphids at a very low frequency (Table 9-1). In contrast, the satRNA of VTMoV was transmitted by aphids at a much higher frequency than $VTMoV^{-s}$ from the plants co-infected with VTAV and satRNA-containing VTMoV (VTMoV+s) to the recipient plants which were pre-infected with $VTMoV^{-s}$ (Fig. 9-2). Since there was no transmission of the satRNA from the control plants (infected with VTMoV+s only), it is concluded that the transmission was due to the transcapsidation of the satRNA into the VTAV capsids which then became aphid transmissible.

The low frequency of aphid transmission of $VTMoV^{-s}$ possibly reflects the low level of transcapsidation between this virus and VTAV. This may be due to the difference in cellular sites of replication and encapsidation between the two viruses rather than the imbalance of the virus concentrations, since both viruses were easily detected *in vivo* by a relatively insensitive immunodiffusion serological test (Fig. 9-1).

The relatively high transmission rate of the satRNA by aphids suggests that the transcapsidation frequency of satRNA into VTAV capsids may also be at a high level. The possible reasons for the high transcapsidation of the satRNA could be its very high concentration in plants (Randles *et al.*, 1981) and it is also likely, that the highly base paired structure of the satRNA (Haseloff and Symons, 1982) may confer on it some mobility in the cells and increase its chance of encapsidation by VTAV. The latter point of view could be supported by the fact that potato spindle tuber viroid was encapsidated by VTMoV particles when the two pathogens co-infected the same plants (Francki *et al.*, 1986b).

8. Some general considerations in using viral coat protein genes in transgenic plants

The demonstration of *in vitro* transcapsidation and aphid transmission of virus particles with cucumovirus coat proteins illuminates the risk involved in using coat protein genes in transgenic plants to gain protection against viral infection (Cuozzo *et al.*, 1988). A study of heterologous coat protein gene expression and *in vivo* encapsidation showed that TMV coat protein was expressed from a hybrid RNA 3 of brome mosaic virus in which the coat protein gene was replaced by that of cowpea strain of TMV plus its assembly origin. The hybrid as well as RNA 1 or 2 in which the TMV assembly origin sequence had been inserted were shown to be encapsidated in rod-shaped particles when barley protoplasts were infected (Sacher *et al.*, 1988). The transmission of VTMoV satRNA by aphids from source plants co-infected with VTMoV+s and VTAV further prove the reality of such a risk. If a viral coat protein gene is integrated randomly into a plant genome, it might by chance express at a site where encapsidation of a non aphid-transmisible virus can take place. Therefore, transcapsidation by the expressed protein and aphid transmission of the virus could possibly occur. Although such a possibility has not been demonstrated in transgenic plants, due caution by selecting coat protein genes from non aphid-transmissible strains or mutants of viruses can certainly minimise the risk.

9. Potential of *in vitro* transcapsidation

a. A vehicle to deliver ss RNA or DNA.

Successful delivery of TMV RNA, MCMV RNA and VTMoV RNA into plants by *M.*

persicae after *in vitro* transcapsidation of the viral RNAs into VTAV capsids was shown in Chapter 7. It is likely that other non aphid transmissible viruses may also be transmitted by *M. persicae* if their RNA are transcapsidated with coat protein from an aphid transmissible cucumovirus. Therefore this *in vitro* transcapsidation system may be useful in identifying suspected viral agents for which transmission has not been successful. For example, a small virus-like particle with ss DNA has been found associated with banana bunchy top disease (Thomas and Dietzgen, 1991; Harding *et al.*, 1991). However, mechanical transmission of the purified virus to banana plants has not been achieved. Since the yield of the virus is very low, the use of the banana aphid vector (*Pentalonia nigronervosa* Coq.) to transmit the virus through membrane feeding could be unsuccessful. Alternatively, with the polymerase chain reaction technique, a large amount of full length viral DNA could be easily obtained. Using the *in vitro* transcapsidation system the viral DNA could be easily encapsidated into a cucumovirus capsid (such as that of banana strain of CMV). Then *M. persicae* or perhaps even banana aphids could be used to transmit the heterologously assembled virus particles to banana plants through a membrane feeding system.

A cereal non mechanically transmissible gemini virus DNA (ss DNA), e.g. maize streak virus (MSV) (Bock, 1974) would be ideal in testing the suitability of cucumovirus as a delivery vehicle for ssDNA through *in vitro* transcapsidation. The delivery of the DNA could easily be determined by the infectivity of the virus.

In another encapsidation system, using TMV assembly *in vitro* , it has been shown that pseudovirus particles of mainly 60 nm long can be assembled using TMV coat protein and chimeric single-stranded RNA transcripts encoding an assayable enzyme, chloramphenicol acetyltransferase (CAT). Significantly higher levels of CAT activity were detected with packaged than with naked CAT messenger RNA after inoculation of plant protoplasts in the presence of polyethylene glycol or abrasive inoculation of intact leaf surfaces (Gallie *et al.*, 1987). The particles therefore appeared to favour delivery of the nucleic acid. Three features of the cucumovirus *in vitro* encapsidation system would make it superior to TMV system: its greater encapsidation capacity, its non specific encapsidation and its dual aphid and mechanical transmissibility. Therefore, the potential of cucumovirus *in vitro* transcapsidation as a transient gene delivery system is worth testing.

b. The possibility of delivering ds DNA. The inability of cucumovirus to encapsidate ds

DNA or RNA makes it less promising as a gene delivery vector, because most of the foreign genes to be introduced into plants are ds DNA. However, if the separated ds DNA could reanneal *in vivo*, cucumovirus encapsidation might be useful as a vector system, particularly in monocotyledous, in which a highly efficient vector system is still waiting to be developed. As shown in Fig. 5-8, a ds DNA was easily separated into ss form by thermal denaturation and then encapsidated by TCMV coat protein. Thus a ds DNA fragment of interest could be first separated into ss DNA and encapsidated with cucumovirus coat protein, then the assembled particles used to inoculate a plant. Such a proposed system would be simple to be tested.

10. Conclusion

This thesis makes the following original contributions to knowledge of cucumoviruses:

1). The development of an efficient reassembly and its analysis methods suitable for both CMV and TAV;

2). The minimum length of nucleotide a cucumovirus coat protein could encapsidate was between 35 to 80 nt; the maximum encapsidation capacity of a cucumovirus was possibly between 6395-7020 nt; the total length of about 3200 nt was preferred by cucumovirus coat protein in forming a cucumovirus-like particle *in vitro*; encapsidation of small nucleic acid molecules could be a cooperative process involving protein-protein, RNA-RNA, and protein-RNA interactions; the inability to encapsidate ds RNA/DNA could be one of the strategies cucumoviruses use *in vivo* to encapsidate selectively (+)-sense viral RNA;

3). Coat proteins of two cucumoviruses, TAV and CMV, could form hybrid capsids in *in vitro* assembly;

4). CMV may contain four types rather than three types of particles proposed previously;

5). Cucumovirus transmission by *Myzus persicae* is determined solely by the property of its viral coat protein. The non aphid transmissibility of TMV may be due to uncoating blockage of the aphid-delivered particles;

6). Satellite RNA of CMV could be encapsidated by CMV or TAV coat protein alone or co-encapsidated with TAV genomic RNA. There is difference in encapsidation efficiency of satellite RNA between TCMV and VTAV coat proteins *in vitro*;

7). The establishment of a system to detect the *in vivo* transcapsidation and aphid transmission of satellite RNA of VTMoV into VTAV capsids revealed that in co-infection with

VTAV, the satellite RNA of VTMoV was transmitted by aphids in a much higher frequency than its helper VTMoV.

Appendix I-1: Viruses used in this study

Virus	Source of isolate	Propagation host	Reference
Cucumber mosaic virus M strain (MCMV)	A mutant of strain CMV-Price No. 6	*Nicotiana clevelandii* A. Gray	Mossop *et al.* (1976)
T strain (TCMV)	Capsicum	*N. clevelandii*	Mossop and Francki (1979)
Tomato aspermy virus V strain (VTAV)	Waite Institute collection	*N. clevelandii*	Hollings and Stone (1971)
Tobacco mosaic virus (TMV) U1 strain	Waite Institute collection	*N. tabaccum* L. cv. White burley	
Velvet tobacco mottle virus (VTMoV)	*N. velutina* L.	*N. clevelandii*	Randles and Francki (1986)
Galinsoga mosaic virus (GMV)	*Galinsoga parviflora* Cav.	*Phaseolus vulgaris* cv. Hawkesbury Wonder	Behncken *et al.* (1982)

Appendix I-2: Main biochemicals used in this study

Biochemical	Source
ATP	Boehringer-Mannhaim, FGR
CTP	" "
GTP	" "
TTP	" "
dATP	" "
dCTP	" "
dGTP	" "
dTTP	" "
^{32}P-CTP (3000 Ci/mmol)	Bresatec, Australia
^{32}P-UTP (3000 Ci/mmol)	" "
^{35}Sulphur(as sulphate in aqueous solution, 10 MCi/mmol)	Anersham Internatioal plc., England
EcoR I restriction endonuclease	Boehringer-Mannheim
Sma I restriction endonuclease	" "
T3 RNA polymerase	" "
T7 RNA polymerase	" "
DNase I (RQ1 DNase)	Promega, U.S.A.
M-MLV RNA reverse transcriptase	Bethesda Research Laboratory, U.S.A.
Lysozyme	Sigma, U.S.A.
Synthesized random DNA primer	" "
Transfer RNA (Torula yeast)	" "
Poly (C)	" "
Poly (I)	" "

Appendix I-3: Some chemicals used in this study

Chemicals	Source
2-Mercaptoethanol	BDH Chemicals Ltd, Australia
Acrylamide (99.9%)	Bio-Rad Laboratories, U.S.A.
Agar (Bacto-agar)	Difco Laboratories, U.S.A.
Agarose (Type II)	Sigma
Bromophenol blue	BDH
DL-Dithiothreitol (DTT)	Sigma, U.S.A.
DMSO	" "
Ethidium bromide	" "
Formaldehyde solution (formalin)	Ajax Chemical Ltd. Australia
Fuji X-ray film (RX)	Fuji Photo Film Co. Ltd, Japan
G50 Sephadex (fine)	Pharmacia, Sweden
Glyoxal	Sigma
Heparin (sodium salt, grade I)	" "
Hypoclearing agent	Kodak Pty Ltd., Australia
Ilford rapid fixer	Ilford Pty Ltd., Australia
Kodak developer	Kodak Pty Ltd.
N,N'-Methylene-bis-acrylamide(Bis)	Bio-Rad laboratories, U.S.A.
N,N,N'-N'-tetramethyl ethylenediamine (TEMED)	Sigma
N-ethyl-morpholine	Sigma
Polyethylene glycol (PEG_{6000})	ACE Chemical Co., Australia
Silver nitrate	Ajax
Sodium diethyldithiocarbamate	Sigma
Sodium dodecyl sulphate (SDS)	BDH
Thioglycollic acid (TGA)	Ajax Chemical Ltd
Triton X-100	BDH Chemicals, England

Appendix II. Published work

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, B. & Francki, R. I. B. (1990). Cucumovirus transmission by the aphid Myzus persicae is determined solely by the viral coat protein. *Journal of General Virology, 71*(4), 939-944.

References

AbouHaidar, M.G., and Erickson, J.W. (1985). Structure and *in vitro* assembly of papaya mosaic virus, In: *Molecular plant virology* (J.W. Davies, ed.), Vol. **1**, pp. 85-121, CRC Press, Boca Raton

Adolph, K.W., and Butler, P.J.G. (1974). Studies on the assembly of a spherical plant virus, I. Stages of aggregation of the isolated protein, *J. Mol. Biol.* **88**, 327-341

Adolph, K.W., and Butler, P.J.G. (1977). Studies on the assembly of a spherical plant virus, *J. Mol. Biol.* **109**, 345-357

Allison, R.F., Johnston, R.E., and Dougherty, W.G. (1986). The nucleotide sequence of the coding regiongsof tobacco etch virus genomic RNA: Evidence for synthesis of a single polyprotein, *Virology* **154**, 9-20

Allison, R.F., Sorenson, J.C., Kelly, M.E., Armstrong, F.B., and Dougherty, W.G. (1985). Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: Evidence for synthesis and processing of a polyprotein in potyvirus genome expression, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3969-3972

Angenent, G.C., Posthumus, E., Brederode, F.T., and Bol, J. (1989). Genome structure of tobacco rattle virus strain PLB: further evidence on the occurence of RNA recombination among tobraviruses, *Virology* **171**, 271-274

Assink, A.M., Swaans, H., and Van Kammen, A. (1973). The localization of virus-specific double-stranded RNA of cowpea mosaic virus in subcellular fractions of infected *Vigna* leaves, *Virology* **53**, 384-391

Atabekov, J.G., Novikov, V.K., Vishnichenko, V.K., and Kaftanova, A.S. (1970). Some properties of hybrid viruses reassembled *in vitro*. *Virology* **41**, 519-532

Atreya, C.D., Raccah, B., and Pirone, T.P. (1990). A point mutation in the coat protein abolishes aphid transmissibilty of a potyvirus, *Virology* **178**, 161-165

Avila-Rincon, M.J., Collmer, C.W., and Kaper, J.M. (1986). *In vitro* translation of cucumoviral satellites. II. CARNA5 cDNA from cucumber mosaic virus strain S and SP6 transcripts of cloned (S) CARNA5 cDNA produce electrophoretically comigrating protein products, *Virology* **152**, 455-458

Badami, R.S. (1958). Changes in the transmissibility by aphids of a strain of cucumber

mosauc virus. *Ann. appl. Biol.* **46**, 554-562

Bancroft, J.B. (1972). A virus made from parts of the gemone of brome mosaic and cowpea chlorotic mottle viruses, *J.gen. Viol.* **14**, 223-228

Bancroft, J.B., and Flack, I.H. (1972). The behaviour of cowpea chlorotic mottle virus in CsCl, *J. gen. Virol.* **15**, 247-251

Bancroft, J.B., and Hiebert, E. (1967). Formation of an infetious nucleoprotein from protein and nucleic acid isolated from a small spherical virus, *Virology* **32**, 354-356

Bancroft, J.B., Hiebert, E., and Bracker, C.E. (1969). The effects of various polyanions on shell formation of some spherical viruses, *Virology* **39**, 924-930

Bancroft, J.B., Hills, G.J., and Markham, R. (1967). A study of the self-assembly process in a small spherical virus: Formation of organized structures from protein subunits *in vitro*, *Virology* **31**, 354-379

Beachy, R.N. (1988). Virus cross-protection in transgenic plants, In: *Temporal and Spatial Regulation of Plant Genes* (D.P.S. Verma and R.B. Goldberg, eds.), pp. 313-331

Behncken, G.M., Francki, R.I.B., and Gibbs, A.J. (1982). Galinsoga mosaic virus, *CAB/AAB Descriptions of Plant Virusrs,* No 252

Bennet, C.W. (1953). Interactions between viruses and virus strains, *Adv. Virus. Res.* **1**, 39-67

Bloomer, A.C. and Butler, P.J.G. (1986). Tobacco mosaic virus, structure and self-assembly, In: *The Plant Viruses, vol. 2: The Rod-shape plant Viruses* (M.H.V. van Regenmortel and H. Fraenkel-Conrat, eds.), pp.19-57, Plenum Press, New york.

Bloomer, A.C., Graham, J., Hovmoller, S., Butler, P.J.G., and Klug, A. (1981). Tobacco mosaic virus: interaction of the protein disk with nucleotides and its implications for virus structure and assembly, In: *Structural Aspects of Recognition in Biological Macromolecules* (M. Balaban, J. Sussman, W. Traub, and A.A. Yonath, eds.), pp. 851-864, Balaban ISS, Rehovot.

Boccardo, G., and Milne, R.G. (1984). Plant reovirus group, *CMI/AAB Descriptions of Plant Viruss*, No. 294

Bock, K.R. (1974). Maize streak virus, *CMI/AAB Descriptions of Plant Viruses No. 133*

Boedtker, H., and Simmons, N.S. (1958). The preparation and characterization of essentially uniform tobacco mosaic virus particles, *J. Am. Chem. Soci.* **80**, 2550

Bol, J.F., Kraal, B., and Brederode, F.J. (1974). Limited proteolysis of alfalfa mosaic virus: Influence on the structural and biological fuction of the protein, *Virology* **58**, 101-110

Boswell, K.F., and Gibbs, A.J. (1983). Viruses of legume 1983, *Descriptions and keys from VIDE.* Canberra Publishing and Printing Co., Canberra.

Bujarski, J.J., and Kaesberg, P. (1986). Genetic recombination between RNA components of a multipartite plant virus, *Nature* **321**, 528-531

Butler, P.J.G., and Klug, A. (1971). Assembly of the particle of tobacco mosaic virus from RNA and disks of protein, *Nature* (London) New Biol. **229**, 47-50

Carrington, J.C., and Morris, T.J. (1984). Complementary DNA cloning and analysis of carnation mottle virus RNA, *Virology* **139**, 22-31

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

Collmer, C.W., and Kaper, J.M. (1988). Site-directed mutagenesis of potential protein-coding regions in expressible cloned cDNAs of cucumber mosaic virus satellites, *Virology* **163**, 293-298

Collmer, C.W., Hadidi, A., and Kaper, J.M. (1985). Nucleotide sequence of the satellite of peanut stunt virus reveals structural homologies with viroid and certain nuclear and mitochondrial introns, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3110-3114

Creamer, R. and Falk, B.W. (1990). Direct detection of transcapsidated barley yellow dwarf luteoviruses in double infected plants, *J. gen. Virol.* **71**, 211-217

Cuozzo, M., O'Connel, K.M., Kaniewski, W., Fang, R.-X, Chua, N.-H., and Tumer, N.E. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein and its antisense RNA, *Bio/Technology* **6**, 549-557

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 Zoeten, G.A., Assink, A.M., and Van Kammen, A. (1974). Association of cowpea mosaic virus induced double-stranded RNA with a cytopathic structure in infected cells, *Virology* **59**, 341-355

De Zoeten, G.A., Powell, C.A., Gaard, G., and German, T.L. (1976). *In situ* localisation of pea enation mosaic virus double-stranded ribonucleic acid, *Virology* **70**, 459-469

Devergne, J.C., and Cardin, L. (1975). Relations serologiques entre cucumoviruses (CMV, TAV, PSV), *Ann. Phytopathol.* **7**, 255-276

Devergne, J.C., Cardin, L., Burckard, J., and van Regenmortel, M.H.V. (1981). Comparison of direct and indirect ELISA for dectecting antigenically related cucumoviruses, *J. Virol. Method* **3**, 193-200

Diaz-Ruiz, J.R., and Kaper, J.M. (1977). Cucumber mosaic virus-associated RNA 5. III. Little nucleotide sequence homology between CARNA 5 and helper RNA, *Virology* **80**, 204-213

Domier, L.L., Franklin, K.M., Shahabuddin, M., Hellmann, G.M., Overmeyer, J.H., Hiremath, S.T., Siaw, M.F.E., Lomonossoff, G.P., Shaw, J.G., and Rhoads, R.E. (1986). The nucleotide sequence of tobacco vein mottle mosaic virus RNA, *Nucleic Acid Res.* **14**, 5417-5430

Driedonks, R.A., Krijigsman, P.C.J., and Mellema, J.E. (1978). A characterization of alfalfa mosaic virus protein polymerization in the presence of nucleic acids, *Eur. J. Biochem.* **82**, 405-417

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

Erickson J.W., and Bancroft, J.B. (1978). The self-assembly of papaya mosaic virus, *Virology* **90**, 36-46

Fairall, L., Finch, J.T., Hui, C.-F., Cantor, C.R., and Butler, P.J.G. (1986). Studies of tobacco mosaic virus reassembly with an RNA tail blocked by a hybridised and cross-linked probe, *Eur. J. Biochem.* **156**, 459-465

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

Fraenkel-Conrat, H., and Singer, B. (1959). Reconstitution of tobacco mosaic virus. III. Improved methods and use of mixed nucleic acids. *Biochim. Biophys. Acta* **33**, 359-370

Francki, R.I.B. (1968), Inactivation of cucumber mosaic virus (Q strain) nucleoprotein by pancreatic ribonuclease, *Virology* **34**, 694-700

Francki, R.I.B. (1985a). Plant virus satellites, *Ann. Rev. Microbiol.* 39, 151-174

Francki, R.I.B. (1985b). The viruses and their toxonomy, In: *The Plant Viruses, vol. 1., Polyhedral Virions with Tripartite Genomes* (R.I.B. Francki, ed.), pp.1-18, Plenum Press, New York.

Francki, R.I.B. (1987). Response of plant cells to virus infection with special reference to the sites of RNA replication, In: *Positive Strand RNA Viruses* (M.A. Brinton and R.R. Rueckert, eds.), pp. 423-436, Alan R. Liss, New York.

Francki, R.I.B., Grivell, C.J., and Gobbs, K.S. (1986a). Isolation of velvet tobacco mottle virus capable with and without a viroid-like RNA, *Virology* **148**, 381-384

Francki, R.I.B., Mossop, D.W., and Hatta, T. (1979). Cucumber mosaic virus, *CMI/AAB Descriptions of Plant Viruses,* No. 213.

Francki, R.I.B., Randkes, 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., Zaitlin, M., and Palukaitis, P. (1986b). *In vivo* encapsidation of potato spindle tuber viroid by velvet tobacco mottle virus particles, *Virology* **155**, 469-473.

Fukuda, M., and Okada, Y. (1985). Elongation in the major direction of tobacco mosaic virus assembly. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3631-3634

Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C., and Wilson, T.M.A. (1987). *In vivo* Uncoating and efficient expression of foreign mRNAs packeged in TMV-like particles, *Science* **236,** 1122-1124

Garcia-Arenal, F., Zaitlin, M., and Palukaitis, P. (1987). Nucleotide sequence analysis of six satellite RNAs of cucumber mosaic virus: Primary sequence and secondary structure alternation do not correlate with difference in pathogenicity, Virology 158, 339-347

Garcia-Luque, I, Diaz-Ruiz, J.R., Rubio-Huertos, M., and Kaper, J.M. (1983). Cucumovirus survey in Spanish economically important crops, *Phytopath. medit.* **22**, 127-132

Gera, A., Loebenstein, G., and Raccah, B. (1979). Protein coats of two strains of cucumber mosaic virus affect transmission of *Aphis gossypii, Phytopathology* **69**, 396-399.

Gibbs, A.J. (1977). Tobamovirus group, *CMI/AAB Descriptions of Plant Viruses No. 184*

Gibbs,A., and Harrison, B. (1976). *Plant virology: The principles*. pp. 292. Edward Arnold, London

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J., and Karn, J.

(1982). Nucletide sequence of tobacco mosaic virus RNA, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5818-5822

Gonda, T.J., and Symons, R.H. (1978). The use of hybridization analysis with complememtary DNA to determine the RNA sequence homology between strains of plant viruses: Its applications to several strains of Cucumoviruses, *Virology* **88**, 361-370

Gonsalves, D., Provvidenti, R., and Edwards, M.C. (1982). Tomato white leaf: the relation of an apparant satellite RNA and cucumober mosaic virus, *Phytopathology* **72**, 1533-1538

Goodings, G.V., Jr. (1986). Tobacco mosaic virus: epidemiology and control, In: *The Plant Viruses, vol. 2, The Rod-Shaped Plant Viruses* (M.H.V. van Regenmortel and H. Fraenkel-Conrat, eds.), pp. 133-152., Plenum Press, New York.

Goodman, R.M., Horne, R.W., and Hobart, Y.M. (1975). Reconstitution of potato virus X *in vitro*. II. Charaterization of the reconstituted product, *Virology* **68**, 299-308

Gordon, K.H.J., and Symons, R.H. (1983). Satellite RNA of cucumber mosaic virus forms a secondary structure with partial 3'-terminal homology to genomic RNAs, *Nucleic Acid Res.* **11**, 947-960

Gordon, K.H.J., Pfeiffer, P., Fuetterer, J., and Hohn, T. (1988). *In vitro* expression of cauliflower mosaic virus gene. *EMBO J.* **7**, 309-317

Gould, A.R., and Symons, R.H. (1982). Cucumber mosaic virus RNA 3: Deternination of the nucleotide sequence provides the amono acid sequences of protein 3A and viral coat protein. *Eur. J. Biochem.* **126**, 217-226

Gould, A.R., Palukaitis, P., Symons, R.H., and Mossop, D.W. (1978). Characterization of a satellite RNA associated with cucumber mosaic virus, *Virology* **84**, 443-455

Habili, N. (1974). *Comparative studies on tomato aspermy and cucumber mosaic viruses*, Ph.D. thesis, The University of Adelaide, South Australia

Habili, N. and Francki, R.I.B. (1974a). Comparative studies on tomato aspermy and cucumber mosaic virus. 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 vieuses. II. Virus stability, *Virology* **60**, 29-36

Habili, N., and Francki, R.I.B. (1974c). 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 Kaper, J.M. (1981). Cucumber mosaic virus-associated RNA5. VII. Double-stranded form accumulation and disease attenuation in tobaccco, *Virology* **112**, 250-261

Hajimorad, M.R. (1989). *Variation in alfalfa mosaic virus*, PhD Thesis, The University of Adelaide, South Australia

Hanada, K. and Tochihara, H. (1980). Genetic analysis of cucumber mosaic, peanut stunt and chrysanthemum mild mottle viruses, *Annals of the Phytopathological Society of Japan* **46**, 159-168.

Hanada, K., and Tochihara, H. (1982). Some properties of an isolate of the soybean stunt strain of cucumber mosaic virus, *Phytopathology* **72**, 761-764

Harding, R.M., Burns, T.M., and Dale, J.L. (1991). Virus-like particles associated with babana bunchy top disease contain small single-stranded DNA, *J. gen. Virol.* **72**, 225-230

Harris, K.F. (1977). An ingestion-egestion hypothesis of noncirculative virus transmission, In: *Aphids as Virus Vectors* (K.F. Harris and K. Maramorosch, ed.), pp. 166-220. Academic Press, New York.

Harris, K.F. (1981). Arthropod and nematode vectors of plant viruses, *Ann. Rev. Phytopathol.* **19**, 391-426

Harris, K.F. (1983). Sternorhynchous vectors of plant viruses: virus-vector interactions and transmission mechanisms, *Adv. Virus Res.* **28**, 113-140

Harrison, B.D., and Murant, A.F. (1984). Involvement of virus-coded proteins in transmission of plant viruses by vectors, In: *Vectors in virus biology* (M.A. Mayo and K.A. Harrop, eds.), pp. 1-36, Academic Press, London

Haseloff, J. & Symons, H.R. (1982). Comparative sequence and structure of viriod-like RNAs of two plant viruses, *Nucleic acids Res.* **10**, 3681-3691

Hatta, T., and Francki, R.I.B. (1981). Cytopathic structures associated with tonoplasts of plant cells infected with cucumber mosaic and tomato aspermy viruses, *J. gen. Virol.*, **53**, 343-346

Hatta, T., and Francki, R.I.B. (1984). Differences in the morphology of isometric particles of some plant viruses stained with uranyl acetate as an aid to their identification, *J. Virol.*

Methods **9**, 237-247

Hebert, T.T. (1967). Epidemiology of the peanut stunt virus in North Carolina, *Phytopathology* **57**, 461

Heery, D.M., Gannon, F., and Powell, R. (1990). A simple method for subcloning DNA fragments from gel slices, *Trends in Genetics* **6**, 173

Hidaka, S. Hanada, K., Ishikawa, K., and Miura, K. (1988). Complete nucleotide sequence of two new satellite RNAs associated with cucumber mosaic virus, *Virology* **164**, 326-333

Hiebert, E., Bancroft, J.B., and Bracker, C.E. (1968). The assembly *in vitro* of some small spherical viruses, hibrid viruses, and other nucleoproteins, *Virology* **34**, 492-508

Hodgman, T.C. (1988). A new superfamily of replicative proteins, *Nature* (London) **333**, 22-23

Hollings, M., and Stone, O.M. (1971). Tomato aspermy virus, *CMI/AAB Descriptions of Plant Viruses* No. 79.

Houwing, C.J. and Jaspar, E.M.J. (1982). Protein binding sites in nucleation complexes of alfalfa mosaic virus RNA 4, *Biochemistry* **21**, 3408-3414

Howell, S.H. (1982). Plant molecular vehicles: potential vectors for introducing foreign DNA into plant. *Ann. Rew. Plant Physiol.* **33**, 609-650

Hull, R. (1970). Studies on alfalfa mosaic virus. III. Reversible dissociation and reconstitution studies, *Virology* **40**, 34-47

Hull, R. (1972). The multicomponent nature of broad bean mottle virus and its nucleic acid, *J. gen. Virol.* **17**, 111-117

Hull, R. (1976). The behaviour of salt-labil plant viruses in gradients of cesium sulphate, *Virology* **75**, 18-25

Hull, R. and Maule, A.J. (1985). Virus multiplication, In: *The Plant Viruses,* vol. **1.**, *Polyhedral Virions with Tripartite Genomes*, (R.I.B. Francki, ed.), pp.83-116, Plenum Press, New York.

Jacquemond, M., and Leroux, J.P. (1982). L'ARN satellite du virus de la mosaique du concombre. II. Etude de la relation virus-ARN satellite chez diers hôtes, *Agronomie* **2**, 55-62

Jacrot, B., Chauvin, C., and Witz, J. (1977). Comparative neutron small-angle scattering

study of small spherical RNA viruses, *Nature* (London) **266**, 417-421

Jonard, G., Richards, K.E., Guilley, H., and Hirth, L. (1977). Sequence from the assembly region of TMV RNA, *Cell* (Cambridge, Mass.) **11**, 483-493

Kamer, G., and Argos, P. (1984). Primary structural comparison of RNA-dependent RNA polymerases from plant, animal and bacterial viruses, *Nucleic Acid Res.* **12**, 7269-7282

Kaper, J.M. (1969). Reversible dissociation of cucumber mosaic virus (strain S), *Virology* **37**, 134-139

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

Kaper, J.M. (1976). Molecular organization and stabilizing forces of simple RNA viruses. V. The role of lysyl residues in the stabilization of cucumbe mosaic virus strain S, *Virology* **71**, 185-198

Kaper, J.M. (1982). Rapid synthesis of double-stranded cucumber mosaic virus-associated RNA 5: mechanism controlling viral pathogenesis? *Biochem. Biophys. Res. Commun.* **105**, 1014-22

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

Kaper, J.M., and Re, G.G. (1974). Redetermination of the RNA content and the limiting RNA size of the three strains of cucumber mosaic virus, *Virology* **60**, 308-311

Kaper, J.M., and Tousignant, M.E. (1977). Cucumber mosaic virus-associated RNA5. I. Role of host plant and helper strain in determining amount of associated RNA5 with virions, *Virology* **80**, 186-195

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

Kaper, J.M., and Waterworth, H.E. (1981). Cucumoviruses, In: *Handbook of Plant Virus Infections: Comparative Diagnosis* (E. Kurstak, ed.), pp. 257-332, Elsevier/North-Holland, Amsterdam

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

Kaper, J.M., Tousignant, M.E., and Lot, H. (1976). A low molecular weight replicating RNA associated with a divided genomic plant virus: defective or satellite RNA? *Biochem. Biophys. Res. Commun.* **72**, 1237-1243

Kaper, J.M., Tousignant, M.E., and Steen, M.T. (1988). Cucumber mosaic virus-associated RNA5. XI. Comparison of 14 CARNA5 variants relates ability to induce tomato necrosis to a conserved nucleotide sequence, *Virology* **163**, 284-292

Kaper, J.M., Tousignant, M.E., Diaz-Ruiz, J.R., Tolin, S.A. (1978). Peanut stunt virus-associated RNA 5: second tripartide genome virus with an associated satellite-like replicating RNA. *Virology* **88**, 166-170

Kennedy, J.S., Day, M.F., and Eastop, V.F. (1962). A conspectus of aphids as vectors of plant viruses. Commonwealth Institute of Entermology, London.

Kikumoto, T., and Matsui, C. (1962). Electron microscopy of plant viruses in aphid midguts. *Virology* **16**, 509-510.

Knight, C.A. (1962). Tobacco mosaic virus, In: *Biochemical Preparations,* (M.J. Coon, ed.), Vol. **9**, pp. 132-136

Kurath, G., and Palukaitis, P. (1987). Biological activity of T7 transcripts of a prototype clone and a sequence variant clone of a satellite RNA of cucumber mosaic virus, *Virology* **159**, 199-208

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680-685

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

Lane, L.C., and Kaesberg, P. (1971). Multiple genetic components in bromograss mosaic virus, *Nature* (London) New Biol. **232**, 40-43

Linthorst, H.J.M., and Kaper, J.M. (1985). Cucumovirus satellite RNAs cannot replicate autonomously in cowpea protoplasts. *J. gen. Virol.* **66**, 1839-1842

Lot, H.H., and Kaper, J.M. (1973). Comparison of the buoyant density heterogeneity of cucumber mosaic virus and brome mosaic virus, *Virology* **54**, 540-543

Lot, H., and Kaper, J.M. (1976a). Physical and chemical differentiation of three strains of cucumber mosaic virus and peanut stunt virus, *Virology* **74**, 209-222

Lot, H., and Kaper, J.M. (1976b). Further studies on the RNA component distribution

among the nucleoproteins of cucumber mosaic virus, *Virology* **74**, 223-226

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

Marchoux, G., Marou, 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, *Compts rendus hebdomadaires des seances de l'Academie des scinces, Serie D* **278**, 1943-1946

Marsh, L., Huntley, C.C., Pogue, G.P., Connell, J.P., and Hall, T.C. (1991). Regulation of (+) : (-)-strand asymmetry in replication of brome mosaic virus RNA, *Virology* **182**, 76-83.

Matsui, C., Sasaki, T., and Kikumoto, T. (1963). Electron microscopy of tobacco mosaic virus particles from aphid stylets, *Virology* **19**, 411-412.

Matthews, R.E.F. (1966). Reconstitution of turnip yellow mosaic virus RNA with TMV protein subunits, *Virology* **30**, 82-96

Matthews, R.E.F. (1982). Classification and nomenclature of viruses: Fourth report of the International Committee on Toxanomy of Viruses, *Intervirology* **17**, 1-199

Maule, A.J., Hull, R., and Donson, J. (1983). The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues, *J. Virol. Methods* **6**, 215-224

McDonal, J.G., and Bancroft, J.B. (1977). Assembly studies on potato virus Y and its coat protein, *J. gen. Virol.* **35**, 251-263

McKinney, H.H. (1929). Mosaic diseases in the Canary Islands, W. Africa and Gibraltar, *J. Agric. Res.* **39**, 557-578

Mink, G.I. (1972). Peanut stunt virus, *CMI/AAB Descriptions of Plant Viruses* No. 92.

Mossop, D.W. (1978). *Studies on cucumoviruses*, PhD Thesis, The University of Adelaide, South Australia

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

Mossop, D.W., and Francki, R.I.B. (1978). Survival of a satellite RNA *in vivo* and its dependence on cucumber mosaic virus for replication, *Virology* **86**, 562-566

Mossop, D.W., and Francki, R.I.B. (1979). Comparative studies on two satellite RNAs of

cucumber mosaic virus. *Virology* **95**, 395-404

Mossop, D.W., Francki, R.I.B., and Grivell, C.J. (1976). Comparative studies on tomato aspermy and cucumber mosaic virus. V. Purification and properties of a cucumber mosaic virus inducing severe chlorosis, *Virology* **74**, 544-546

Murant, A.F., and Mayo, M.A. (1982). Satellites of plant viruses, *Ann. Rev. Phytopathol.* **20**, 49-70

Nassuth, A., and Bol, 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.

Noordam, D. (1973). *Identification of Plant Viruses: Methods and Experiments*. Pudoc, Wageningen

Novikov, V.K., Sarukhan-Bek, K.K., and Atabecov, J.G. (1974). Anomalous stable aggregates in mixture of TMV and cucumber virus 3 proteins, *Virology* **62**, 134-144.

Ossiannilsson, F. (1958). Is tobacco mosaic virus not inbibed by aphids and leafhoppers? *Kungl. Lantbrushogsk. Ann.* **24**, 369-374.

Otsuki, Y., and Takebe, I. (1978). Production of mixedly coated particles in tobacco mesophyll protoplasts doubly infected by strains of tobacco mosaic virus, *Virology* **84**, 162-171

Palukaitis, P., and Symons, R.H. (1980). Nucleotide sequence homology of thirteen tobamovirus RNAs as determined by hybridization analysis with complementary DNA, *Virology* **107**, 354-361

Palukaitis, P., Cotts, S., and Zaitlin, M. (1985). Detection and indentification of viriods and viral nucleic acids by "dot-blot" hybridization, *Acta Hort.* **164**, 109-118

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

Peacock, A.C., and Dingman, C.W. (1968). Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels, *Biochemistry* **7**, 668-674

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

Pesce, A.J., Apple, R., Sawtell, N., and Michael, J.G. (1986). Cationic antigens. Problems associated with measurement by ELISA, *J. Immunol. Methods*, **87**, 21-27

Pfeiffer, P., and Hirth, L. (1974). Aggregation states of brome mosaic virus protein, *Virology* **61**, 160-167

Phatak, H.C., Diaz-Ruiz, J.R., and Hull, R. (1976). Cowpea ringspot virus: A seed transmitted cucumoviruses. *Phytopathol. Z.* **87**, 132-142

Piazzolla, P., Diaz-Ruitz, J.R., and Kaper, J.M. (1979). Nucleic acid homologies of eighteen cucumber mosaic virus isolates determined by competitive hybridization, *J. gen. Virol.* **45**, 361-369

Pirone, T.P. (1964). Aphid transmission of a purified stylet-borne virus acquired through a membrane, *Virology* **23**, 107-108

Pirone, T.P. (1967). Acquisition and release of infectious tobacco mosaic virus by aphids, *Virology* **31**, 569-571.

Pirone, T.P. (1977). Accessory factors in nonpersistent virus transmission, In: *Aphids as Virus Vectors* (K.F. Harris and K. Maramorosch, eds.), pp. 221-235. Academic Press, New York.

Pirone, T.P., and Harris., K.F. (1977). Nonpersistent transmission of plant viruses by aphids. *Ann. Rew. Phytopathol.* **15**, 53-73

Pirone, T.P., and Megahed, E. (1966). Aphid transmissibility of some purified viruses and viral RNAs, *Virology* **30**, 631-637

Pirone, T.P., and Shaw, J.G. (1973). Aphid stylet transmission of poly-L-ornithine treated tobacco mosaic virus, *Virology* **53**, 274-276

Powell, C.A. and De Zoeten, G.A. (1977). Replication of pea enation mosaic virus RNA in isolated pea nuclei, *Proc. Natl. Acad. Sci. USA*, **74**: 2919-2922

Randles, J.W., Davies, C., Hatta, T., Gould, A.R., and Francki, R.I.B. (1981). Studies on encapsidated viroid-like RNA. I. Characterization of velvet tobacco mottle virus, *Virology* **108**, 111-122

Randles, J.W., and Francki, R.I.B. (1986). *Velvet tobacco mottle virus, AAB Descriptions of Plant Viruses* No.317

Rao, A.L.N. (1982). Genetic studies of cucumoviruses, PhD Thesis, Adelaide University

Rao, A.L.N. and Francki, R.I.B. (1981). Comparative studies on tomato aspermy and

cucumber mosaic viruses. VI. Partial compatibility of genome segments from the the two viruses. *Virology* **114,** 573-575.

Rao, A.L.N., and Francki, R.I.B. (1982). Distribution of determinants for the symptom production and host range on the three RNA components of cucumber mosaic virus, *J. gen Virol.* **61,** 197-205

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

Rezaian, M.A., Williams, R.H.V., and Symons, R.H. (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,** 1331-1339

Rezaian, M.A., Williams, R.H.V., Gordon, K.H.J., Gould, A.R., and Symons, R.H. (1984). Necleotide 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

Richards, K.E., Jonard, G., Jacquemond, M, and Lot, H. (1978). Nucleotide sequence of cucumber mosaic virus-associated RNA 5, *Virology* **89,** 395-408

Richter, J., Proll, E., and Musil, M. (1979). Serological relationships between robinia mosaic, clove blotch and peanut stunt viruses, *Acta Virol.* **23,** 489-

Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S., and Casse-Delbart, F. (1989). Nucleotide sequence of potato virus Y (N strain) genomic RNA. *J. gen Virol.* **70,** 935-947

Rochow, W.F. (1960). Transmission of barley yellow dwarf virus acquired from liquid extracts by aphids feeding through membranes, *Virology* **12,** 223-232

Rochow, W.F. (1970). Barley yellow dwarf virus: phenotypic mixing and vector specificity, *Science* **167,** 875-878

Rochow, W.F. (1977). Dependent virus transmision from mixed virus infection, In: *Aphids as Virus Vectors* (K.F. Harris and K. Maramorosch, eds.), pp. 253-273. Academic Press, New York.

Rockow, W.F., and Ross, A.F. (1955). Virus multiplication in plants doubly infected by

potato viruses X and Y. *Virology* **1**, 10-27

Rodriguez, D., Lopez-Abella, D., and Diaz-Ruiz, J.R. (1987). Viroplasms of an aphid-transmissible isolate of cauliflower mosaic virus contain helper component activity, *J. gen Virol.* **68**, 2063-2067

Sacher, R., French, R., and Ahlquist, P. (1988). Hybrid brome mosaic virus RNAs express and are packaged in tobaco mosaic virus coat protein *in vivo*, *Virology* **167**: 15-24.

Sachey, S.T. (1989). *Interactions of two cucumoviruses.* PhD thesis, Adelaide University

Sadasivan, T.S. (1940). A quantative study of the interaction of viruses in plants, *Ann. appl. Biol.* **27**, 359-367

Sakai, F., Dawson, J.R.O., and Watts, J.W. (1983). Interaction in infections of tobacco protoplasts with two bromoviruses, *J. gen Virol.*, **64**, 1347-1354

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: A labotatory manual*, (2nd edition), Cold Spring Harbor Laboratory Press, USA

Sarkar, S. (1960). Interaction and mixed aggregation of proteins from tobacco mosaic virus strains, *Z. Naturforsch*, **156**, 778-786

Savithri, H.S., and Erickson, J.W. (1983). The self-assembly of the cowpea strain of southern bean mosaic virus: Formation of T = 1 and T = 3 nucleoprotein particles. *Virology* **126**, 328-335

Semancik, J.S., and Reynolds, D.A. (1969). Assembly of protein and nucleoprotein particles from extracted tobacco rattle virus protein and RNA. *Science* **164**, 559-560

Sequeira, L. (1984). Cross protection and induced resistance: their potential of plant disease contral, *Trends in Biotechnology* **2**, 24-29

Shepherd, R.J. (1977). Intrinsic properties and taxonomy of aphid-borne viruses, In: *Aphids as Virus Vectors*, (K.F. Harris and K. Maramorosch, eds.), pp. 121-136. Academic Press, New York.

Simon, A.E. (1988). Satellite RNAs of Plant Viruses, *Plant Molecular Biology Reporter* **6**, 240-252

Simons, J.N. (1955). Some plant-vector-virus relationships of southern cucumber mosaic virus. *Phytopathology* **45**, 217-219

Simons, J.N. (1957). Three strains of cucumber mosaic virus affecting bell pepper in the Everglades area of South Florida. *Phytopathology* **47**, 145-150

Simons, J.N. (1959). Variation in efficiency of aphid transmission of southern cucumber mosaic virus and potato virus Y in pepper, *Virology* **9**, 612-623

Sleat, D.E., Turner, P.C., Finch, J.T., Butler, P.J.G., and Wilson, T.M.A. (1986). Packaging of reconbinant RNA molecules into pseudovirus particles directed by the origin-of assembly sequence from tobacco mosaic virus RNA, *Virology* **155**, 299-308

Smith, K.M. (1945). Transmission by insects of plant virus complex, *Nature* **155**, 174

Sorger, P.K., Stockley, P.G., and Harrison, S.C. (1986). Structure and assembly of turnip crinkle virus. II. Mechanism of reassembly *in vitro*, *J. Mol. Biol.* **191**, 639-658

Stace-Smith, R., and Tremaine, J.H. (1973). Biophysical and biochemical poperties of tomato aspermy virus, *Virology* **51**, 401-408

Sylvester, E.S. (1958). Aphid transmission of plant viruses, *Proc. 10th Int. Congr. Entermol.* **3**, 195-200

Takahashi, T., and Orlob, G.B. (1972). Tobacco mosaic and brome mosaic viruses in aphids, *Phytopathology* **62**, 429-434.

Taylor, J.M., Ilimensee, R., and Summers, J. (1976). Efficient transcription of RNA into DNA by avian sarcoma virus polymerase, *Biochem Biophys Acta* **442**, 324-330

Thomas, J.E., and Dietzgen, R.G. (1991). Purification, characterization and serological detection of virus-like particles associated with banana bunchy top disease in Australia, *J. gen. Virol.* **72**, 217-224

Thornbury, D.W., Hellmann, G.M., Rhoads, R.E., and Pirone, T.P. (1985). Purification and charaterisation of potyvirus helper component, *Virology* **144**, 260-267

Tikchonenko, T.I. (1975). Structure of viral nucleic acids *in situ*, In: *Comprehensive Virology* (H. Fraenkel-Conrat and R.R. Wagner, eds), vol. **5**, pp.2-118. Plenum Press, New York.

Tremaine, J.H., and Ronald, W.P., (1977). Assembly of particles from southern bean mosaic virus and sowbane mosaic virus components. *Can. J. Bot.* **55**, 2274-2277

Turner, D.R., and Butler, P.J.G. (1986). Essential features of the assembly origin of tabocco mosaic virus RNA as studied by directed mutagenesis. *Nucleic Acids Res.* **14**, 9229-9242

Turner, D.R., Joyce, L.E., and Butler, P.J.G. (1988). The assembly origin of tobacco mosaic virus RNA: Functional characteristics defined by directed mutagenesis, *J. Mol.*

Biol. **203**, 531-547

van Regenmortel, M.H.V. (1967). Biochemical and biophysical properties of cucumber mosaic virus, *Virology* **31**, 391-396

van Regenmortel, M.H.V., Hendry, D.A., and Baltz, T. (1972). A reexamination of the molecular size of cucumober mosaic virus and its coat protein, *Virology* **49**, 647-653

Wagner, G.W., and Bancroft, J.B. (1968). The self-assembly of spherical viruses with mixed coat protein, *Virology* **34**, 748-756

Waterhouse, P.M. and Murant, A.F. (1983). Further evidence on the nature of the dependence of carrot mottle virus on carrot red leaf virus for transmission by aphids, *Ann. appl. Biol.* **103**, 455-464

Waterworth, H.E., Kaper, J.M, and Tousignant, M.E. (1979). CARNA 5, the small cucumber mosaic virus--dependent replicating RNA, regulates disease expression. *Science* **204**, 845-847

Watson, M., Serjeant, E.P., and Lennon, E.A. (1964). Carrot mottle dwarf and parsnip mottle viruses, *Ann. appl. Biol.* **54**, 153-166

Watson, M.A., and Roberts, F.M. (1939). A comparative study of the transmission of *Hyocyamus* virus 3, potato virus Y, and cucumber virus 1 by the vectors *Myzus persicae* (Sulz.), *M. circumflexus* (Buckton) and *Macrosiphum gei* (Koch). *Proceedings of the Royal Sociaty of London.* Series B **127**, 543-576.

Wray, W.T., Boulikes, T., Wray, V.P., and Hancock, R. (1981). Silver staining of proteins in polyacrylamide gel, *Analytical Biochemistry* **118**, 197-203

Zabel, P., Weenen-Swaans, H., and Van Kammen, A. (1974). *In vitro* replication of cowpea mosaic virus RNA. I. Isolation and properties of the membrane-bound replicase, *J. Viol.* **14**, 1049-1055

Zimmern, D. (1977). The nucleotide sequence at the origin for assembly on tobacco mosaic virus RNA. *Cell* **11**, 463-482