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GENETIC STUDIES OF CUCUMBER MOSAIC AND TOMATO ASPERMY VIRUSES

by

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SUMMARY

I. Eight cucumovirus isolates were examined with respect to their host range, antigenic properties and nucleic acid composition. All the isolates are readily distinguishable by their reactions on selected host plant species and contained four major RNA components, designated RNAs 1-4 in order of decreasing molecular weight.

II. It was demonstrated that TAV and CMV are serologically distantly related. The antigenic relationship between the two viruses was investigated by three serological methods: radial double immunodiffusion in agar, enzyme-linked immunosorbent assays (ELISA) and immune-electron microscopy (IEM) using the antibody decoration technique. The relative merits of various tests used for the detection of minor and major antigenic differences among viruses has been discussed.

III. Three strains of CMV, (UCMV, MCMV and KCMV) and one of TAV (VTAV) were selected as suitable candidates for genetic analysis by _in vitro_ pseudorecombination. The three largest RNA components of the selected cucumoviruses were separated by electrophoresis in agarose gels. All attempts to construct pseudorecombinants involving the exchange of RNA-1 and RNA-2 between CMV and TAV were unsuccessful. It was demonstrated that only RNA-3 is readily interchangeable between the two viruses. However, all three RNA segments were interchangeable between all the CMV strains tested.

IV. Eighteen pseudorecombinants were constructed in all possible combinations between pairs of three CMV strains. The pseudorecombinants were checked for authenticity by regenerating parental viruses by back crossing appropriate pairs of pseudorecombinants. From detailed studies on the pseudorecombinants it was demonstrated that infection and symptom expression of CMV in a plant is a complex interaction of the genetic material of the virus with that of the host genome. Some host reactions were shown to be determined by a single RNA species. For example, systemic necrosis in N. x edwardsonii, ability to infect maize systemically and leaf blistering and distortion in several host species are determined by RNA-2. Similarly, the production of chlorotic local lesions in G. globosa and brown necrotic local lesions in V. faba are determined by RNA-3. Some host reactions were determined by a combination of RNA-2 and RNA-3; for example the induction of yellow mosaic in several host species examined and the production of chlorotic local lesions in D. stramonium and S. melongena. In some instances the symptom expression appears to involve interaction between genetic information on any two or perhaps even all the three RNA segments of the virus.

V. Three pseudorecombinants constructed between CMV and TAV by exchange of RNA-3 confirmed that the symptom expression in Nicotiana spp. examined and in C. sativus, is due to the genetic information located on RNA-1 and/or RNA-2 of TAV.

STATEMENT

This thesis has not previously been submitted for an academic award at this or any other University, and is the original work of the author, except where due reference is made in the text.

A.L.N. Rao

TO MY PARENTS

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ABBREVIATIONS

AMV	alfalfa mosaic virus
BMV	brome mosaic virus
CCMV	cowpea chlorotic mottle virus
CarMV	carnation mottle virus
CMV	cucumber mosaic virus
CMMV	chrysanthemum mild mottle virus
PSV	peanut stunt virus
RRV	raspberry ringspot virus
TAV	tomato aspermy virus
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
TRSV	tobacco ringspot virus
VTMoV	velvet tobacco mottle virus
EDTA	ethylenediaminetetraacetic acid
mA	milliamperes
PBS	Phosphate buffered saline
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
ss	single-stranded
TEMED	N,N,N',N" - tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminoethane
UV	ultraviolet

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CHAPTER 1

GENERAL INTRODUCTION

I THE CUCUMOVIRUS GROUP

The cucumovirus group received its name from cucumber mosaic virus (CMV), the type virus and the best known member of the group. Other members of the group include tomato aspermy virus (TAV; Hollings and Stone, 1971) and peanut stunt virus (PSV; Mink, 1972) while cowpea ringspot virus (CpRSV; Phatak et al, 1976) is considered to be a possible member of the group (Matthews, 1979). Although robinia mosaic virus (Schmelzer, 1971) and clover blotch virus (Musil et al, 1975) were described as distinct viruses also belonging to the group, recent studies indicate that they can be considered as strains of PSV (Richter et al, 1979).

II STRUCTURE AND COMPOSITION OF CUCUMOVIRUSES

1. Particle Structure

Members of the cucumovirus group have particles with icosahedral symmetry of 28-30 nm diameter (Francki et al, 1979) and are not distinguishable from each other in the

electron microscope (Tolin, 1977). Cucumovirus capsids are composed of 180 protein sub-units arranged in pentamer-hexamer clusters with T = 3 surface lattice symmetry (Finch et al, 1967). The protein subunit has a molecular weight of about 24,500. (Habili and Francki, 1974a). Cucumovirus infectivity is sensitive to ribonuclease (RNase; Francki 1968; Habili and Francki, 1974a ; Mink, 1972) and virions are dissociated into protein and RNA by sodium dodecyl sulphate (SDS) and high salt concentrations which led Kaper (1975 a, b) to postulate that the structure of these viruses is mainly stabilized by protein-RNA interactions.

2. Structure of the Cucumovirus Genome

Viruses which have their genetic information distributed over several RNAs and in which these RNAs are encapsidated in separate nucleoprotein particles are collectively known as viruses with divided (multipartite) genomes (reviewed by Jaspars, 1974; Van Vloten-Doting and Jaspars, 1977; Lane 1979). Members of the cucumovirus group contain functionally divided genomes since the largest three of the four major RNA species found in purified preparations are needed for infectivity (Peden and Symons, 1973; Lot et al, 1974). RNA-4 is genetically redundant but is regenerated upon infection (Habili and Francki, 1974b). In addition a number of other sub-genomic RNAs have also been reported in some strains (Kaper and West, 1972; Peden and Symons, 1973;).

The exact molecular weights of the CMV-RNAs is a subject of debate. Values ranging between 1.01-1.35, 0.89-1.21, 0.68-0.93 and 0.33-0.38 X 10^6 daltons for RNAs 1-4 respectively have been reported (Kaper and West, 1972; Peden and Symons, 1973; Marchoux et al, 1973). However, electrophoretic mobilities of RNA species may differ slightly between strains (Mossop, private communication; Hanada and Tochihara, 1980). In TAV, RNA-1 and RNA-2 have molecular weights similar to those of CMV, but RNA-3 and RNA-4 are somewhat larger and have molecular weights of 0.9 and 0.43 X 10^6 respectively (Habili and Francki, 1974a). Minor differences in the molecular weights of RNA-2 of PSV and CMV have also been reported (Lot and Kaper, 1976a).

Several CMV and PSV strains were shown to contain small, encapsidated, single stranded-RNA (ss-RNA) molecules of about 1 X 10^5 daltons (Kaper et al, 1976, Mossop and Francki, 1978, Gould et al 1978, Kaper and Tousignant 1978). Their total dependence on cucumoviruses for replication and their unique base sequences indicated that they are satellites (Lot et al, 1977; Diaz-Ruiz and Kaper, 1977; Mossop and Francki, 1978; Gould et al, 1978). One satellite RNA (sat-RNA) referred to as CARNA 5 (Kaper and Waterworth 1977) has been found to be responsible for a lethal disease of tomato. However, it has been demonstrated that sat-RNAs reduce severity of symptoms on most host plant species (Mossop and Francki, 1979).

3. Encapsidation of the Cucumovirus Genome

The multicomponent nature of cucumovirus particles is masked by their sedimentation homogeneity. Purified preparations of infectious cucumoviruses sediment as a single component (90-100 S, Francki et at, 1966; Hollings and Stone, 1971; Mink, 1972) with a molecular weight of 5.8-6.1 X 10^6 (Francki et al, 1979) and having 18% RNA (Francki et al, 1966; Kaper, 1975b). On the basis of the chemical analysis of the RNA content of CMV, Kaper and Re (1974) suggested that the amount of RNA encapsidated per virus particle cannot exceed 1 X 10^6 daltons. Hence under no circumstances can all the RNA components, usually found in CMV preparations occur together in one virus particle. Attempts to resolve the different particle types expected by isopycnic density gradient centrifugation have been hampered by the extreme structural instability of cucumoviruses in solvents with high salt concentrations normally used in these analyses (Lot and Kaper, 1973; Habili and Francki, 1974a; Lot and Kaper, 1976a). Formaldehyde stabilized cucumovirus preparations showed component heterogeneity in these analyses, but isolated RNA from such components would not be infectious (Habili and Francki, 1974a; Lot and Kaper, 1976a). However, Lot and Kaper (1976b) directly analysed the RNA component distribution among virus particles of the salt stable D-strain of CMV and observed that the encapsidation of the CMV genome was similar to that of bromoviruses

(Lane and Kaesberg, 1971). That is, the viral RNAs are encapsidated in three types of particles; one type containing one molecule of RNA-1, a second type containing one molecule of RNA-2 and a third type containing one molecule each of RNA-3 and RNA-4. However, it has been suggested that the encapsidation of cucumovirus RNAs is more complicated than that of bromoviruses since RNA-3 and RNA-4 are often not present in equimolar amounts (Lot and Kaper, 1976a; Hull, 1976).

III BIOLOGICAL PROPERTIES OF CUCUMOVIRUSES

Cucumoviruses have a number of common biological properties such as :

a. They are amenable to mechanical inoculation and are efficiently transmitted by aphid vectors in a non-persistent manner (Francki *et al*, 1979).

b. Cucumoviruses are economically important and pathogenic to an extremely wide range of herbaceous and woody plant species (Francki *et al*, 1979).

c. The viruses occur in the cytoplasm and vacuoles of infected cells (Hatta and Francki, 1979) and some times the particles form crystalline arrays in the vacuoles (Francki *et al*, 1979). The viruses also produce membrane bound vesicles associated with the tonoplasts (Hatta and Francki, 1981).

The biological properties of cucumoviruses overlap to such an extent that no single property is sufficiently reliable for identification. However it is considered that TAV can be distinguished from CMV by its ability to invade chrysanthemum systemically but not cucumber and it also induces enations on leaves of Nicotiana spp. (Hollings and Stone, 1971). PSV has been considered to be distinguished by its ability to invade cowpea, French bean and peanuts systemically, (Mink, 1972).

IV RELATIONSHIPS AMONG CUCUMOVIRUSES

Relationships among cucumoviruses have been a matter of some controversy (Habili and Francki, 1975). More reliance has been placed on the results of serological tests than any other single criterion to trace the degree of relationships between CMV, TAV and PSV (Grogan et al, 1963; van Regenmortel, 1966; Habili and Francki, 1974 a,b; Devergne and Cardin, 1975). Thorough serological analysis of CMV strains indicated the presence of at least two serogroups both of which are related to some strains of TAV and PSV (Devergne and Cardin, 1975). In contrast, Habili and Francki (1975) failed to detect any serological relationship between the Q strain of CMV (Francki et al, 1966) and V strain of TAV. The lack of agreement as to the serological relationship between TAV and CMV has been probably due to the use of different strains of the viruses which appear to

be very variable, and to the lack of high titred antisera due to the poor immunogenicity of the viruses (Francki and Habili, 1972; Habili and Francki, 1975).

V GENETICS OF CUCUMOVIRUSES

The observation that there is no sequence homology between the three genomic RNAs of CMV (Gould and Symons, 1977) suggest that each RNA carries different genes. In vitro recombination is a well adopted method of locating genetic characters on the genome segments of plant viruses with multi-partite genomes (Jaspars, 1974). For those plant viruses in which the RNA components are encapsidated in particles with different sedimentation properties (eg. alfalfa mosaic virus) the particles which are relatively stable can themselves be separated. However, with viruses whose component nucleoproteins cannot be separated, the isolation of genomic components must be done on the isolated RNA.

Progeny viruses obtained by the reassortment of genome parts from related viruses or strains of the same virus have been referred to as 'hybrids' (Bancroft, 1972; Dingjan-Versteegh et al, 1972; Marchoux et al, 1974a) or pseudorecombinants (Harrison et al, 1974; Habili and Francki, 1974c; Gibbs and Harrison, 1976; Mossop and Francki, 1977; Hanada and Tochihara, 1980). Since the term 'hybrid' has connotations of a diploid state, the term 'pseudorecombinant' has been used throughout this thesis.

A number of workers have constructed pseudorecombinants of cucumoviruses, most of which involved the exchange of RNA-3 between different CMV strains. Like that of other viruses which have tripartite genomes (eg. bromoviruses, Lane and Kaesberg, 1971; alfalfa mosaic virus, Bol and Van Vloten-Doting, 1973), it was demonstrated that the coat protein gene of cucumoviruses is located on RNA-3 (Habili and Francki, 1974c; Marchoux et al, 1974 a&b; Mossop and Francki, 1977; Hanada and Tochihara, 1980). From the properties of pseudorecombinants constructed between CMV strains, Marchoux et al (1974 a&b) concluded that most biological properties of CMV reside on RNA-3, one exception was the primary and systemic symptoms produced in *Vigna sinensis* which are controlled by both RNA-2 and RNA-3 and by RNA-2 alone respectively. On the other hand, Mossop and Francki (1977) observed that the symptoms produced on a range of plant hosts by pseudorecombinants constructed between QCMV and MCMV were due to the genetic information located on RNA-1 and/or RNA-2, as well as RNA-3. On the basis of these observations they suggested that symptom development in response to a cucumovirus is a complex interaction involving genetic information sometimes residing on at least two genomic RNAs. The inheritance of transmissibility of CMV by an aphid vector has also been studied. Mossop and Francki (1977) exchanged RNA-3 between two CMV strains (QCMV and MCMV) which differ antigenically and in aphid transmissibility, and demonstrated that RNA-3

determines both properties. This supports a growing body of evidence that with many plant viruses coat proteins play a critical role in vector specificity (Lane, 1979).

Successful pseudorecombination between two different cucumoviruses was also observed. Habili and Francki (1974c) reported that a pseudorecombinant constructed from RNA-1 and RNA-2 of VTAV and RNA-3 of QCMV was serologically indistinguishable from QCMV and produced symptoms on cucumber and several Nicotiana spp. characteristic of VTAV. Thus it was suggested, that RNA-1, RNA-2 or both carried genes determining host reactions. Recently Hanada and Tochihara (1980) confirmed these determinants on RNAs of cucumoviruses, by exchanging RNA-3 between four CMV strains and chrysanthemum mild mottle virus (CMMV, probably closely related to TAV). In addition these authors exchanged RNA-2 between several CMV strains and reported that the symptoms on cowpea are determined by RNA-2, a similar pattern of inheritance observed by Marchoux et al (1974a).

VI OTHER PLANT VIRUSES WITH TRIPARTITIE GENOMES

Apart from cucumoviruses the best studied plant viruses with tripartite genomes include members of the bromoviruses, ilarviruses and alfalfa mosaic virus (Table 1.1). Van Vloten-Doting and Jaspars (1977) divided the tripartite

genomes into isocapsidic and heterocapsidic viruses. Members of the former group have their genome parts in identical capsids (i.e. cucumo and bromoviruses) and the latter groups have spherical or bacilliform capsids of different sizes and sedimentation properties (i.e. ilarviruses and alfalfa mosaic virus). The division is not only based on structural differences but also on the fact that the isocapsidic viruses do not require coat protein for infection (protein-independent) whereas with hetero-capsidic viruses coat protein or RNA-4 is required to initiate infection (protein-dependent).

Members of the hordeivirus group (Jackson and Lane, 1981) also have a divided genome. Working with barley stripe mosaic virus (BSMV) the type member, Lane (1974) demonstrated that three different RNA species derived from three different sized rod-shaped particles constitute the genome. However, BSMV may contain 2 to 4 different length classes of nucleoprotein particles depending on the strain (Jackson and Lane 1981), but the significance of this is not clear. Other members of the hordeivirus group include poa semilatent virus and lychnis ringspot virus (Lane, 1974) which are not adequately characterized. Hence it is still obscure as to whether they have bipartite or tripartite genomes.

Table 1.1

Properties of Particles and RNAs of Plant Viruses with Tripartite Genomes

Virus Group	Virus	Morphology (Vector)	Dimension (nm)	Nucleoprotein $s_{20,w}$	% RNA	Coat Protein 10^3 Daltons	Molecular Weight Genome Part in Component 10^6 Daltons RNA 1	RNA 2	RNA 3	RNA 4	Reference
Bromoviruses	Brome mosaic virus*	Polyhedral (Beetles)	26	85	20	20	1.09	0.99	0.75	0.28	Lane & Kaesberg 1971; Lane 1981
	Cowpea Chlorotic Mottle Virus	Polyhedral (Beetles)	26	85	20	20	1.15	1.00	0.85	0.32	Bancroft, 1971 Lane, 1981
	Broad Bean Mottle Virus	Polyhedral (Beetles)	26	85	20	20	1.10	1.03	0.90	0.36	Hull, 1972 Lane, 1981
	Melandrium Yellow Fleck Virus	Polyhedral (Beetles)	25	88	20	22	1.20	1.10	1.0	0.3	Hollings & Horvath, 1981
Cucumoviruses	Cucumber Mosaic Virus*	Polyhedral (Aphids)	30	98.6	18	24.5	1.27	1.13	0.82	0.35	Habili & Francki, 1974a,b Francki et al, 1979
	Tomato Aspermy Virus	Polyhedral (Aphids)	30	98.6	18	24.5	1.26	1.10	0.90	0.43	Habili & Francki 1974a,b
	Peanut Stunt Virus	Polyhedral (Aphids)	30	98	18	-	1.19	1.02	0.73	0.35	Mink, 1972; Hanada & Tochihara, 1980
Ilarviruses	Tobacco Streak* Virus	Polyhedral	35-30-27-25	109-93-78	14	24	1.30	1.10	0.80	0.40	Lister & Bancroft, 1970 Ghabrial & Lister, 1974
	Citrus Leaf Rugose Virus	Polyhedral	32-31-26-25	105-98-89-79	14	26	1.10	1.0	0.70	0.30	Garnsey & Gonsalves, 1976
	Citrus Variegation Virus	Polyhedral	33-31-28	110-93-83	14	26	1.10	1.10	0.70	0.30	Fulton, 1981
	Elm Mottle Virus	Polyhedral	30-25-22	101-88-83	20	25	1.30	1.15	0.82	0.39	Jones & Mayo, 1973
	Prunus Necrotic Ringspot Virus	Polyhedral	32-30-26	95-90-72	-	25	-	-	-	-	Fulton, 1975
	Tulare Apple Mosaic Virus	Polyhedral	31-30-28	114-108-93	-	19	1.01	0.92	0.74	-	Lister & Saksena, 1976
AMV group	Alfalfa Mosaic Virus	Bacilliform (Aphids)	58x18, 46x18 35x18, 31x18	65-51-43-29	16-15-15-15	24.3	1.10	0.80	0.70	2x0.30	Hull, 1969; Van Vloten-Doting & Jaspars, 1977
Hordeiviruses	Barley Stripe Mosaic Virus*	Rod-Shaped	143, 128, 112 x x x 25 25 25	201-192-182	4	22	1.43	1.24	1.10	0.98	Brakke & Palomar, 1976 Palomar et al, 1977 Jackson & Lane, 1981

* Type Member of the Group

VII SCOPE OF THIS THESIS

When this study began (March, 1978), our knowledge regarding the genetic functions of cucumovirus RNA-1 and RNA-2 was still in its infancy. Therefore, the main objective of this thesis was to investigate the distribution of genetic determinants for symptom production and host range on the three RNA segments of cucumoviruses. With this goal in mind in the first instance, several cucumovirus isolates from the Waite Agicultural Research Institute collection were screened and their host range, nucleic acid composition and serological properties were compared. RNA preparations of selected strains were separated in to components by electrophoresis in agarose gels. The purified RNA components from three strains of CMV and one of TAV were used to construct pseudorecombinants *in vitro*. Isolated pseudorecombinants were checked for the RNA composition by regenerating parental viruses by back crossing. The host range and symptomatology of the pseudorecombinants were studied with the aim of explaining the previous work done by Marchoux *et al*, (1974a,b), Habili and Francki (1974c), Mossop and Francki (1977) and Hanada and Tochihara (1980) which was superficial and in which the results appear to be conflicting.

CHAPTER 2

GENERAL MATERIALS AND METHODS

I. MATERIALS

1. Virus Isolates

The virus isolates used throughout the course of these investigations and their respective sources are listed in Table 2.1.

2. Materials Used For Handling and Separation of Genomic RNAs of Cucumovirus Isolates

All buffers and solutions used for RNA studies were prepared in double distilled water and were autoclaved (121° for 15 min). Glassware was subjected to heat sterilization at 130° for at least 3 hr. Objects sensitive to heat sterilization were rinsed with 0.2M KOH dissolved in 90% ethanol and then rinsed thoroughly with sterile distilled water.

3. Materials Used for Electrophoresis of Viral RNA

Agarose (Sigma Chemical Co., U.S.A., electrophoretic grade) was used for routine analysis and fractionation of viral RNA. Polyacrylamide and N'N' - methylene bis acrylamide were obtained from BDH Chemicals, England.

Table 2.1

List of Virus Isolates Used in this Thesis

Virus	Source	Reference
A. Cucumovirus Isolates		
QCMV	*Capsicum frutescens* L., Queensland	Francki *et al* (1966)
MCMV	A mutant of Price's No. 6 yellow strain, isolated by Dr. K.M. Smith, England	Mossop *et al* (1976)
TCMV	*Capsicum* spp., Western Australia	Dr. G.D. McLean (Personal Communication)
XCMV	*Lactuca sativa* L., Queensland	Dr. J.L. Dale (Personal Communication)
UCMV	*Musa paradisiaca* L., Queensland	Dr. J.L. Dale (Personal Communication)
KCMV	*Centaurea cyanus* L., China	Dr. Tien-Po (Personal Communication)
VTAV	*Chrysanthemum* spp., Victoria	Habili & Francki (1974a)
NTAV	*Nicotiana glauca* Graham., South Australia	Dr. R.I.B. Francki (Personal Communication)
B. Other Virus Isolates Used		
VTMoV	*Nicotiana velutina* L.	Randles *et al* (1981)
CarMV	*Dianthus* spp	Randles *et al* (1981)

4. Materials Used in Enzyme-linked Immunosorbent Assays (ELISA)

The enzyme, alkaline phosphatase (B-grade) and the substrate p-nitrophenyl phosphate (Mol.wt. 371.2) were obtained from Calbiochem, La Jolla, California, U.S.A. Round bottomed microplates used for ELISA tests were obtained from Disposable Products Private Ltd., Adelaide, South Australia.

5. Instruments and Apparatus

High speed centrifugation was done either in a Beckman L2-65 or a L-50 refrigerated centrifuge. Medium speed centrifugation was done in either Sorvall RC-2B or Sorvall RC-3 centrifuges. Sucrose density gradients were scanned at 254 nm, using an ISCO model 183 density gradient fractionator coupled to an ISCO UA-2 absorbance monitor. Absorption spectra were recorded with a Unicam SP 1800 spectrophotometer. For tube gel electrophoresis, the apparatus was made in our laboratory from plastic containers, inserted with 12 plexiglass tubes (15cm X 0.5cm) or 8 tubes (15cm X 1cm).

Electron micrographs illustrated in this thesis were taken with a JOEL JEM 1000 CX electron microscope.

6. Buffers and Solutions

RNA preparations were suspended in TE buffer (10mM Tris-HCl, lmM EDTA, pH 8.3). Buffer used in analytical and preparative gel electrophoresis was TBE pH 8.3 (Peacock and Dingman, 1968) and contained 10.8g Tris, 5.5g boric acid, 0.93g EDTA, in one litre of distilled water.

All buffers used in ELISA tests contained 0.02% sodium azide. Phosphate buffered saline (PBS, pH 7.4) was prepared by dissolving 8g NaCl, 0.2g KH_2PO_4, 2.9g Na_2HPO_4. 12 H_2O, 0.2g KCl, 0.2g NaN_3 in one litre of distilled water. Microplates were washed with PBS buffer containing 0.5ml of Tween-20 per litre. The γ - globulins were diluted in coating buffer (pH 9.6) containing 1.59g Na_2CO_3; 2.93g $NaHCO_3$; 0.2g NaN_3 per litre of distilled water. Substrate buffer (pH 9.8) was prepared by dissolving 97 ml diethanolamine and 0.2g NaN_3 in 800 ml distilled water and made up to one litre.

7. Miscellaneous Materials

All chemicals not listed in Table 2.2 were of standard analytical reagent grade.

Table 2.2

Chemicals and Biochemicals Used in this Thesis

Chemical	Source
Adjuvant, Freund's complete	Calbiochem. U.S.A.
Bovine serum albumin (BSA)	Sigma Chemical Co. U.S.A.
Cellulose DE 22	Whatman Biochemicals Ltd. England.
Diethanolamine	Ajax Chemicals Ltd. Sydney.
Glutaraldehyde	Taab Laboratories, England.
Ovalbumin	Sigma Chemical Co. U.S.A.
Polyethylene glycol (PEG 6000)	Union Carbide, U.S.A.
Polyvinylpyrollidone (PVP), Mol.wt 40,000	General Biochemicals, U.S.A.
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co. U.S.A.
N,N,N',N" - tetramethyl-ethylenediamine (TEMED)	Sigma Chemical Co. U.S.A.
Thioglycollic acid (TGA)	Ajax Chemicals Ltd. Sydney.
Tris (hydroxymethyl) aminomethane (Tris)	Sigma Chemical Co. U.S.A.
Triton X-100	Ajax Chemicals Ltd. Sydney.
Tween 20	Drug Houses of Australia Ltd. Australia.
Urea	BDH Chemicals, England.

II METHODS

1. Plants, Inoculations and Virus Propagation

All cucumoviruses were routinely maintained in *Nicotiana glutinosa* L and propagated in *N. clevelandii* A. Gray. Plants were lightly dusted with carborandum (500 mesh) and mechanically inoculated by rubbing crude sap from infected leaves or viral RNA suspension on the leaves with a forefinger enclosed in a rubber fingerstall. Excess inoculum was washed off with tap water. Unless otherwise stated, plants were maintained in an insect-proof glasshouse with natural illumination and an average temperature of 25°.

2. Virus Purification

Except for MCMV, all CMV isolates were purified by the method of Peden and Symons (1973). All steps were done at 4°. Infected leaf tissue, 12-14 days after inoculation, was ground in a mortar and pestle with 2 volumes (W/V) of 0.5M sodium citrate buffer, pH 6.5 containing 0.5% thioglycollic acid and 5mM EDTA, in the presence of acid washed sand. The juice was strained through a double layer of cheesecloth and clarified by centrifugation at 10,000g for 15 min. The supernatant was then emulsified with an equal volume of chloroform (v/v) and the emulsion was broken by centri-fugation at 10,000g for 15 min. The virus was precipitated

from the buffer phase with polyethylene glycol, which was added to a final concentration of 10% (w/v) and the mixture was stirred for 45-60 min at 4°. The precipitate was collected by centrifugation at 15,000g for 15 min. The resulting pellets were suspended in 5mM sodium borate buffer containing 0.5mM EDTA (pHc. 9.0) and stirred for 30 min at 4°, after adding Triton X-100, to a final concentration of 2%. The suspension was clarified by centrifugation at 10,000g for 10 min and subjected to two cycles of high and low speed centrifugation at 144,000g for 60 min and 5,000g for 10 min respectively. When required, the virus was further purified by centrifugation in a 5%-25% sucrose density gradient containing 20mM phosphate buffer, pH 7.5 (Beckman SW 27 rotor, 100,000 g for 120 min at 4°). The recovered virus was diluted with sodium borate buffer and sedimented by centrifugation at 78,000g for 150 min. The final pellets were suspended in borate buffer and stored at 4° until required.

VTAV and NTAV were purified by the same procedure as described above, except EDTA was not included in buffers at any stage of purification, since it has been shown to disrupt the virus (Habili and Francki, 1974b).

MCMV was purified from infected N. clevelandii as described by Mossop et al (1976). The purification method involves the extraction of infected tissue in 3 volumes

(w/v) of 0.1M Na_2HPO_4 containing 0.1% each of thioglycollic acid and diethyldithiocarbamate (pHc. 8.0). The extract was strained through a double layer of cheese-cloth and clarified by centrifugation at 15,000g for 15 min. Triton X-100 was added to 1%-2% to the clarified supernatant and stirred for 15-20 min at 4°. The mixture was subjected to high speed centrifugation at 78,000g for 120 min and the pellets were suspended in one-tenth of the original volume of extraction buffer. Particulate material was removed by centrifugation at 5,000g for 10 min and the supernatant was layered over 5ml of 10% sucrose cushions prepared in 0.1M Na_2HPO_4 and subjected to centrifugation at 144,000g for 60 min. The pellets were suspended in 0.1M Na_2HPO_4 and the particulate material was removed by centrifugation at 5,000g for 15 min. The virus was further purified from the supernatant by centrifugation in 5%-25% sucrose density gradients, containing 0.1M Na_2HPO_4 for 150 min at 100,000 g in a Beckman SW 27 rotor. The final pellets were suspended in 10mM sodium borate buffer, pH 7.5

The yield of both TAV and CMV isolates ranged from lg to 700 mg per kg of leaf material. Purified virus preparations of VTMoV and CarMV were kindly provided by Dr. R.I.B. Francki.

3. Serology

a. Preparation of Antigens

Cucumovirus preparations purified by sucrose density gradient centrifugation were used for antiserum production. Prior to immunization virus preparations were fixed with 0.2% glutaraldehyde to enhance immunogenicity (Francki and Hatta, 1980).

b. Immunization of Animals

Antisera to TAV and CMV strains were prepared in rabbits. Each adult rabbit received two subcutaneous injections containing 1 mg of fixed virus, emulsified with an equal volume of Freund's complete adjuvant. Injections were given at weekly intervals. A third injection containing 2 mg of fixed virus was administered intravenously two weeks after the second injection. One week after the final injection blood samples were collected from an ear vein and serum was recovered after clotting and removal of cellular material by centrifugation at 2,000g for 10 min.

c. Storage of Antisera

All antisera were stored at -15° in glass vials. For continuous use, approximately 2-3 ml of antiserum of each cucumovirus was stored at 4° in the presence of 0.02% sodium azide.

Antisera specific to some strains of CMV were prepared by absorption of sera to a heterologous viral antigen. For this purpose, approximately 200-300 μg of heterologous viral antigen was added to 0.5 ml of antiserum and the mixture was incubated at 37° for 30-45 min and then left at 4° overnight. Unreacted protein was recovered in the supernatant after centrifugation at 5,000g for 10 min and stored as described above. After a single absorption step, such antisera failed to react with the heterologous antigen in immunodiffusion tests. Antisera to TMV and CarMV were kindly provided by Dr. R. I. B. Francki.

4. Serological Techniques

a. Agar-gel Immunodiffusion Tests

Immunodiffusion tests were done in glass petri dishes containing 12 ml of 0.75% purified bateriological agar (DIFCO) prepared in 10mM phosphate buffer, pH

7.6, containing 0.02% sodium azide (Francki and Habili, 1972). Wells, approximately 3 mm in diameter and 3.5 mm apart were cut and 10-12 μl of viral antigen or antiserum was dispensed into each well. Plates were then incubated at 25° for 24-48 hr for the development of precipitin lines.

b. Enzyme-linked immunosorbent assays (ELISA)

Procedures employed in this thesis for ELISA were essentially the same as those described by Clark and Adams (1977).

i. Preparation and Purification of γ-globulins

One ml of each cucumovirus antiserum was diluted to 10 ml with distilled water, 10 ml of saturated ammonium sulphate was added and the preparation was left at room temperature for 30 min. The mixture was then subjected to centrifugation (5,000*g* for 10 min) and the precipitate was dissolved in approximately 2 ml of half-strength PBS. The dissolved precipitate was then dialysed against the same buffer (at least 3 changes). The γ-globulins were further purified by DE 22 cellulose column chromatography and the unabsorbed fractions were collected in silicone (Prosil-28,

PCR-Research Chemical Inc., USA) coated tubes. The purified γ-globulin fractions were adjusted to approximately 1 mg/ml (E_{278} = 1.4) and stored in silicone-treated tubes at -15°. These preparations will be referred to as stock solutions of purified γ-globulins.

ii. Conjugation of Enzyme Alkaline Phosphatase with γ -globulins

Approximately 2 mg of alkaline phosphatase was dissolved in 1 ml of stock solution of purified γ -globulins and dialyzed against PBS buffer at 4° (at least 3 changes). Glutaraldehyde was then added to 0.06% final concentration and the mixture was incubated at room temperature for 4 hr. Glutaraldehyde was removed by dialyzing the mixture against several changes of PBS buffer and the conjugate was stored at 4° in the presence of 1% bovine serum albumin.

In most experiments all wells of a plate were used and the plates were incubated in a humid chamber at 37°. After each incubation step, the plates were rinsed three times with PBS buffer containing 0.05% Tween-20, by flooding the wells and allowing the liquid to stay for 3-4 min between each rinse.

iii. Preparation of ELISA Plates

Initially the plates were coated with 200 μl of purified γ-globulins diluted in carbonate buffer, pH 9.0, and incubated for 3 hr. at 37°. Two-fold dilutions of glutaraldehyde fixed antigens were prepared in PBS-Tween containing 2% PVP and incubated (200 μl) in the wells overnight at 4°. Previously enzyme conjugated γ-globulins (200 μl of 1:200 dilution of stock in PBS-Tween containing 2% PVP and 0.02% ovalbumin) were incubated for 5 hr at 37°. Finally, the unreacted conjugate was rinsed away and specific antibody-antigen reactions were detected by adding 250 μl of substrate, di-sodium p-nitrophenylphosphate (1 mg/ml prepared in subtrate buffer, pH, 9.8) and by incubating at room temperature for 1 hr. Reactions were arrested by adding 50 μl of 3M NaOH. The colour reaction in each well was measured by its absorbance at 405 nm.

c. Immune-electron Microscopy (IEM)

Immune-electron microscopy was done by combining the Derrick particle trapping and the antibody decoration procedures (Milne and Luisoni, 1977). In each test a mixture of two viruses was used, usually one whose antigenic properties were being studied and the other as

a control. The method involves application of a drop of a mixture of the two antisera, each diluted 1/1000 in 10mM phosphate buffer, pH 7.0, to a carbon-coated formvar grid which had been ionized. After 5 min incubation in a humid chamber at room temperature, the grid was washed three times by floating for 1 min each time on distilled water. After draining but not drying, the grid was applied to a drop of a mixture of 50 µg/ml of each virus. After incubation at room temperature for 15 min the grid was washed with distilled water and the trapped virus particles were decorated by incubation for 30 min on a drop of test antiserum diluted 1/10 in 10mM phosphate buffer, pH 7.0. The grid was then washed, stained with 2% aqueous uranyl acetate for 30 sec and examined in the electron microscope, at an instrument magnification of 33,000.

5. Isolation of Viral RNA

RNA was isolated from purified preparations of cucumoviruses, using the phenol-SDS procedure described by Peden and Symons (1973). Each virus preparation was emulsified with an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline, and buffer containing 0.6M sodium acetate, 0.6% SDS and 20mM EDTA (1 ml : 1 ml : 1 ml). The mixture was shaken vigorously for 10 min at 25° and the aqueous phase was

separated by centrifugation at 10,000g for 10 min. One-half volume of phenol was added to the aqueous phase and the extraction repeated. Traces of phenol were removed by washing the aqueous phase with 2 volumes of ether and the RNA was precipitated by adding 2.5 volumes of cold, re-distilled ethanol. After leaving the mixture overnight at 15°, the precipitate was sedimented at 5,000g for 10 min and the pellet was washed twice with ethanol. RNA pellets were then dried in a Dynavac freezedrying unit and suspended in TE buffer, pH 8.3 and stored at -15°.

6. Electrophoresis of Viral RNA

a. Analytical Gel Electrophoresis

The cucumovirus RNA preparations were routinely analysed by electrophoresis in 2% agarose gels, prepared in TBE buffer. A piece of dialysis tubing was stretched tightly over the end of each tube and secured with a rubber band. The gel solution was autoclaved for 15 min to ensure inactivation of RNases and dispensed into sterile 15x 0.5 cm plexiglass tubes to a depth of approximately 13 cm. After leaving the gels at room temperature for 1 hr to set, the tops of each gel were trimmed off to produce a flat loading surface

and to maintain a uniform gel length. Gels were prepared immediately prior to use. Before loading, RNA samples (10-15 μg in TE buffer, pH 8.3) were heated at 60° for 10 min or 70° for 5 min and cooled rapidly in an ice-water bath. Sucrose was then added to each sample to a final concentration of 10%. The RNA preparations were allowed to enter the gel by initial electrophoresis at 1 mA/gel for 30 min and then at 2.5 mA/gel (10 volts/cm) for 2½ hr. at 25°. Bromophenol blue was used as a tracking dye. After electrophoresis, the gels were stained for 10-15 min with toluidine blue 0 (0.05% in 50mM sodium acetate, 5mM acetic acid and 0.01M EDTA, pH 8.5; Peden and Symons, 1973) and destained in several changes of 5% acetic acid.

Electrophoresis under denaturing conditions was done in 3% polyacrylamide gels containing 7M urea, as described by Gould (1980). To prepare 50 ml of gel solution, 1.5 g acrylamide monomer, 0.075 g bis-acrylamide, 5 ml 10 x TBE and 20 ml distilled water were added to 21 g urea and the mixture was stirred for 10-15 min. Then, 30 μl TEMED and 0.3 ml freshly prepared 10% ammonium persulphate were added and the volume was adjusted to 50 ml by adding sterile distilled water. The mixture was dispensed in to 15 x 0.5 cm plexiglass

tubes to a depth of 13 cm. A flat loading surface was formed by overlayering the gels with 50% t-butanol. When the gels had polymerised, the t-butanol was removed by gently washing the loading surface with sterile distilled water. Gels were pre-electrophoresed at 2 mA/gel for 1 hr. RNA samples (10-15 μg) were heated in the presence of 7 M urea at 70° for 5 min. and cooled rapidly in an ice-water bath. Electrophoresis was at 25° at 2.5 mA/gel for 5 hrs. Location of RNA bands was the same as that described above.

b. Preparative gel Electrophoresis

Cucumovirus RNA components were fractionated by two cycles of electrophoresis using 2% agarose gels. Conditions of electrophoresis were similar to those described above, except that gels were cast in 15 x 1 cm glass tubes and 100μ g of RNA samples were loaded on each gel. Electrophoresis was done at 4 mA/gel (10 volts/cm) for 3½ to 4 hr at 25°. To minimise changes in pH during electrophoresis the buffer was exchanged between the reservoirs with a peristaltic pump. After electrophoresis, the gels were stained in toluidine blue 0 for 30 sec (0.05% toluidine blue 0 prepared in 0.02M sodium acetate, pH 7.8) and destained by several changes

of the same buffer. RNA fractions were cut out with a sterile scalpel blade and layered in tubes of the same diameter over 2% agarose gel columns (1 gel slice/tube). The fractions were then re-electrophoresed as described above. Gel slices containing RNA after the second electrophoretic step were homogenised in TBE + 0.2M NaCl (0.75 ml/ slice) and the slurry was shaken for 15-20 hrs at 4°. Phenol extraction was repeated twice and the RNA was recovered by ethanol precipitation. The isolated RNA components were freeze-dried, suspended in TE buffer and stored at -15° until required.

The recovery of genomic RNA components by the method described above was found to be approximately 45% by spectrophotometric estimation while that of polyacrylamide gel electrophoresis was approximately 27% (Mossop, private communication).

7. Infectivity of Fractionated RNA Components

To assess the purity of fractionated cucumovirus RNAs 1-3 preparations, each was adjusted to a concentration of about 15 μg/ml in TE buffer, mixed in the required combination and inoculated to half leaves of *Chenopodium amaranticolor* Coste & Reyn, which had

been previously kept in the dark for 48 hr. After inoculation the plants were kept at 25° under continuous light at 12,000 lux. Lesions were counted 4 days later.

8. In Vitro Construction, Isolation and Characterization of Pseudorecombinants

The methods used for *in vitro* construction, isolation and characterization of pseudorecombinants were essentially the same as those used by Habili and Francki (1974 c) and Mossop and Francki (1977). Appropriate dilutions of heterologous genomic RNAs were prepared in TE buffer and inoculated to the expanded leaves of *N. tabacum* cv White Burley and transferred to 25° under continuous illumination of 5,000 lux. In all experiments inocula contained approximately 5 μg/ml each of cucumovirus RNA-1, 2 and 3. Prior to inoculation the plants were kept in the dark for 24 hours to increase their sensitivity and to enhance the development of local lesions. Usually under these conditions faint chlorotic lesions appeared on the inoculated leaves in 3-4 days. Lesions were excised with a sterile cork borer and homogenised in a single drop of sterile 10m*M* phosphate buffer pH 7.0 and inoculated to one tobacco plant. After testing the virus infecting the plant by immunodiffusion, the isolate was further cloned by three more single lesion transfers and was then propagated in *N.*

clevelandii. The pseudorecombinants were purified by the method used for the parental strain providing RNA-3. The RNA composition of each pseudorecombinant was examined by electrophoresis in 2% agarose gels as described above. However, information from this was usually inadequate since some genome segments of the strains used had electrophoretic mobilities which were not distinguishable from each other (see Chapter 3). Thus to confirm that the isolated pseudorecombinants had the expected RNA composition, their RNA components were separated and mixed in vitro so as to regenerate the parental virus strains using the same procedure as that used for isolating pseudorecombinants.

9. Examination of Pseudorecombinants

In all experiments each pseudorecombinant was inoculated to at least six plants of each species. The parental viruses from which the pseudorecombinants originated were always inoculated as controls. Each experiment was repeated at least twice, at different seasons of the year and maintained in an insect proof glass house as mentioned before.

10. Spectrophotometry

The concentrations of purified cucumovirus and RNA preparations were determined from their absorbance at 260 nm using $E_{260}^{0.1}$ = 5.0 and 25.0 respectively (Francki *et al*, 1966; Stace-Smith and Tremaine, 1973).

CHAPTER 3

CHARACTERISTICS OF SEVERAL CUCUMOVIRUSES

I. INTRODUCTION

Attempts to assign genes determining biological properties of cucumoviruses to particular genomic RNA segments necessitates a careful selection of parental strains. In a search for cucumoviruses suitable for in vitro recombination, several isolates of cucumber mosaic virus (CMV) and tomato aspermy virus (TAV) from the Waite Agricultural Research Institute virus collection were screened. In this chapter are described experiments on comparative host range, nucleic acid composition and serological properties of QCMV, TCMV, XCMV, UCMV, MCMV, KCMV, VTAV and NTAV.

II. HOST RANGE AND SYMPTOMATOLOGY

Host ranges of QCMV, TCMV, XCMV, UCMV, MCMV, KCMV, VTAV and NTAV were studied over a protracted period and the results are summarised in Tables 3.1 and 3.2. Of many cucumoviruses examined, MCMV was the most readily distinguishable strain which induced severe chlorosis in all the host plant species tested (Tables 3.1 and 3.2). Moreover, plants infected with MCMV often developed leaf

blistering followed by distortion, 3-4 weeks after inoculation. On the other hand, QCMV, XCMV and UCMV produced mild to moderate mosaic in all the host plants tested (Table 3.1). However, UCMV can be distinguished from all the other CMV strains by its characteristic systemic necrosis in *N*.x *edwardsonii*, 5-7 days after inoculation, while QCMV and XCMV by their reactions on *D*. *stramonium* (Table 3.1). TCMV produced severe symptoms in four *Nicotiana* spp. tested, but unlike the other strains it failed to infect *D*. *stramonium* (Table 3.1). Systemic symptoms produced by KCMV were severe mosaic followed by leaf distortion in four *Nicotiana* spp. tested, *D*. *stramonium* and *G*. *globosa*. KCMV was also distinguishable from the other strains by its ability to infect *Zea mays* L. systemically (Table 3.1). Furthermore, like TCMV and unlike the other strains, KCMV induced reddish brown local lesions in broad beans (*Vicia faba* L.), 5-7 days after inoculation.

VTAV and NTAV produced similar systemic symptoms in the four *Nicotiana* spp. tested, *D*. *stramonium* and *S*. *melongena* (Table 3.1), but are distinguishable by their reactions on *G*. *globosa*. Both the TAV strains, unlike all the CMV strains, failed to infect *C*. *sativus* var Polaris systemically.

Table 3.1

Symptoms Produced in Several Host Species by Seven Parental Cucumoviruses

Host	QCMV	TCMV	XCMV	UCMV	MCMV	KCMV	VTAV	NTAV
Nicotiana glutinosa L.	mild mosaic	severe mosaic	mosaic	mild mosaic	yellow mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion
N. tabacum L.cv White Burley	mild mosaic	severe mosaic	mosaic	mild mosaic	yellow mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion
N.x edwardsonii	mild mosaic	severe mosaic	mosaic	necrosis	yellow mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion
N. clevelandii A.Gray	mild mosaic	severe mosaic	mosaic	mild mosaic	yellow mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion	severe mosaic and leaf distortion
Datura stramonium L.	mild mosaic	not infected[a]	mosaic and green ring spots	mosaic	chlorotic local lesions, yellow mosaic and leaf distortion	chlorotic local lesions, severe mosaic and leaf distortion	necrotic local lesions and severe mosaic distortion	necrotic local lesions and mild mosaic
Gomphrena globosa L.	symptomless infection	symptomless infection	symptomless infection	symptomless infection	chlorotic local lesions, yellow mosaic and leaf distortion	severe mosaic	symptomless infection	mild mosaic
Solanum melongena L.	mild mosaic	mosaic	mild mosaic	mild mosaic	chlorotic local lesions and yellow mosaic	severe mosaic	mild mosaic	mild mosaic
Cucumis sativus L. var. Polaris	chlorotic local lesions and mosaic	chlorotic local lesions and mosaic	chlorotic local lesions and mosaic	chlorotic local lesions and mosaic	chlorotic local lesions and mosaic	chlorotic local lesions and mosaic	not infected[a]	not infected[a]
Zea mays L.	not infected[a]	not infected[a]	not infected[a]	not infected[a]	not infected[a]	mosaic	not infected[a]	not infected[a]
Vicia faba L.	not infected[a]	brown local lesions	not infected[a]	not infected[a]	not infected[a]	brown local lesions	not infected[a]	not infected[a]

[a]Plants produced no symptoms and virus could not be recovered by back-inoculation to susceptible hosts.

Table 3.2

Cucumoviruses Having a Number of Common Hosts[a]

Virus Isolate	QCMV	TCMV	XCMV	UCMV	MCMV	KCMV	VTAV	NTAV
QCMV		7 (E)*	8 (E)	8 (C)	8 (A-H)	8 (I-J)	7 (G)	7 (G)
TCMV			7 (E)	7 (C&E)	7 (A-H)	8 (E&I)	6 (E&G)	6 (E&G)
XCMV				8 (C)	8 (A-H)	8 (I&J)	7 (G)	7 (G)
UCMV					8 (A-H)	8 (C,I&J)	7 (C&G)	7 (C&G)
MCMV						8 (A-H)	7 (A-H)	7 (A-H)
KCMV							7 (G,I&J)	7 (G,I&J)
VTAV								7 (F)
NTAV								-

a The figures in the Table represent the number of host species shared by any pair of cucumoviruses. A total of 10 plant species were inoculated with these viruses.

* The letters in the parentheses correspond to the host(s) listed below on which any pair of viruses can be readily differentiated (A = _N. glutinosa_, B = _N. tabacum_ cv White Burley, C = _N.x edwardsonii_, D = _N. clevelandii_, E = _D. stramonium_, F = _G. globosa_, G = _C. sativus_ var Polaris, H = _S. melongena_, I = _Z. mays_, J = _Vicia faba_)

III. PURIFICATION AND PROPERTIES OF CUCUMOVIRUSES

Previously uncharacterized cucumovirus isolates, XCMV, UCMV and KCMV were readily purified by the procedure employed for QCMV (Chapter 2). The three strains are also serologically closely related to QCMV (Table 3.3).

When RNA preparations of QCMV, TCMV, XCMV, UCMV, MCMV, KCMV, VTAV and NTAV were subjected to electrophoresis in 2% agarose gels, all contained four major RNA components (eg. Fig. 3.1A) referred to as RNAs 1-4 in order of decreasing molecular weight. None of these cucumoviruses were found to contain satellite RNA (sat-RNA, Mossop and Francki, 1978; Gould *et al*, 1978). The presence of sat-RNA might result in misinterpretation of genetic analysis of cucumoviruses, since it has been reported that sat-RNA was capable of modifying the symptoms in several plant species (Kaper and Waterworth, 1977; Mossop and Francki, 1979; Takanami, 1981). Hence, care was taken to maintain all cucumovirus strains used in this study free from sat-RNA, by periodic routine analysis of their RNAs by electrophoresis in 2% agarose gels.

Some of the genomic RNA segments of UCMV, MCMV, KCMV and VTAV could be distinguished by differences in their electrophoretic mobilities in 2% agarose gels. RNA-3 of VTAV can be distinguished from that of UCMV, MCMV and

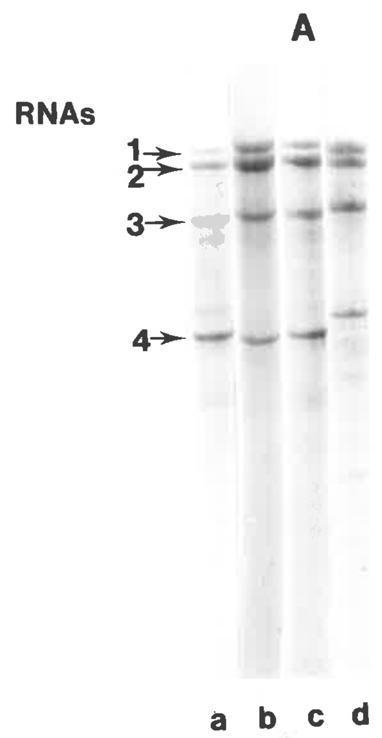
Figure 3.1

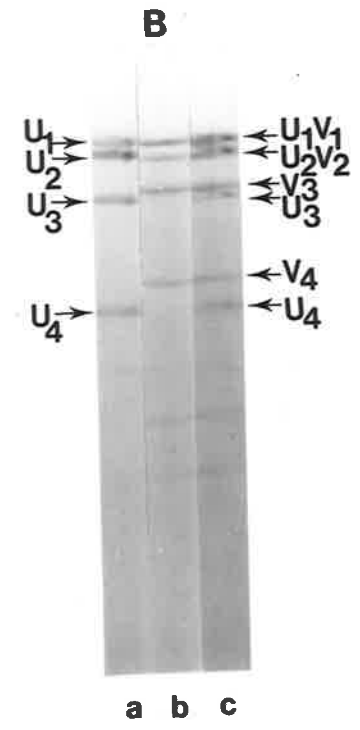
A. Electrophoretic analysis of RNA components of UCMV (a), MCMV (b), KCMV (c) and VTAV (d), in 2% agarose gels under non-denaturing conditions. RNA samples (10-15 μg) were heated at 70° for 5 min. and cooled rapidly at 0° prior to electrophoresis. Electrophoresis was at 25° at 2.5 mA/gel for 2½ hr.

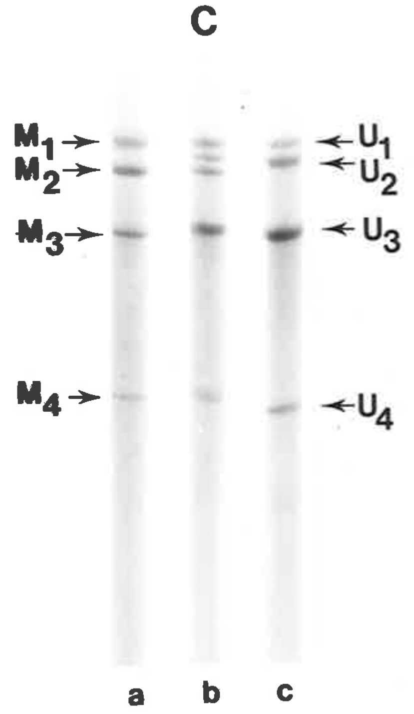
B. Comparison of the electrophoretic mobilities of RNA components from UCMV (a), VTAV (b) and a mixture of UCMV and VTAV (c) in 2% agarose gels. RNA sample preparation and the conditions of electrophoresis were the same as in A. Arrows indicate RNA components e.g. U_1=UCMV RNA-1 and V_1= VTAV RNA-1.

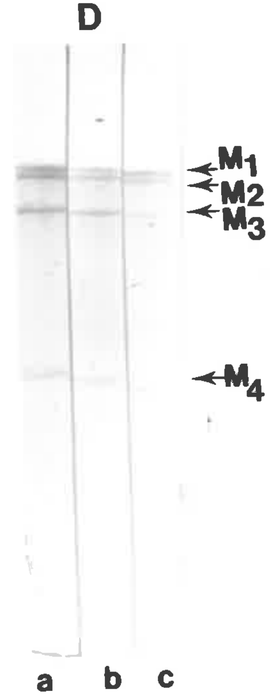
C. Comparison of the electrophoretic mobilities of RNA components of MCMV (a), UCMV (c) and a mixture of MCMV and UCMV (b) in 2% agarose gels as described in A.

D. Comparison of the electrophoretic mobilities of RNA components of UCMV (a), MCMV (c) and a mixture of MCMV and UCMV (b) in 3% polyacrylamide gels containing 7M urea. RNA samples (10-15 μg) were heated in the presence of 7M urea at 70° for 5 min. and cooled rapidly at 0° prior to electrophoresis. Electrophoresis was at 25° at 2.5 mA/gel for 5 hr.


A
RNAs
1
2
3
4
a b c d


B
U1
U2
U3
U4
U1V1
U2V2
V3
U3
V4
U4
a b c


C
M1
M2
M3
M4
U1
U2
U3
U4
a b c


D
M1
M2
M3
M4
a b c

KCMV (e.g. Fig. 3.1B) and RNA-2 of UCMV from that of MCMV and KCMV (e.g. Fig. 3.1C). However, none of the RNAs-1 could be distinguished from each other by this method.

In polyacrylamide gels, under denaturing conditions, the electrophoretic mobilities of RNAs 1, 2, 3 and 4 of XCMV, UCMV and KCMV were indistinguishable from those of MCMV (eg. Fig. 3.1D). Hence, the molecular weights of their RNAs were not determined and they were assumed to be similar to those reported for MCMV (1.27, 1.15, 0.79 and 0.35 x 10^6 for RNAs 1, 2, 3 and 4 respectively, Mossop et al, 1976).

IV. SEROLOGY

Tomato aspermy virus (TAV) and cucumber mosaic virus (CMV) have many properties in common, but opinions about their antigenic relationship to each other are divided (Habili and Francki, 1975). Devergne and Cardin (1975) presented clear evidence that the two viruses are serologically distantly related. Work done previously in our laboratory, using a few strains from Australia and antisera with relatively low titres failed to demonstrate such a relationship (Habili and Francki, 1975; Mossop et al, 1976).

During the course of this study, antisera prepared against several CMV strains showed distant antigenic relationship to TAV in immunodiffusion tests (Table 3.3). In order to confirm these observations, three different serological techniques were used and the results of these experiments are presented below.

1. Properties of Antisera Used

Homologous titres of the TAV and CMV antisera varied from 1/128 to 1/512 in immodiffusion tests (Table 3.3). Additional booster injections of antigens failed to increase the homologous titres significantly. Attempts were made to obtain antisera of QCMV and MCMV with higher titres using two additional rabbits; but neither produced antisera with homologous titres higher than 1/128.

The following serological techniques were used to trace the degree of relationship between TAV and CMV strains.

2. Agar-gel Immunodiffusion Tests

Homologous and heterologous titres of antisera to all the virus strains are presented in Table 3.3 showing that QCMV, TCMV, XCMV, UCMV and KCMV are all closely related since

Table 3.3

Serological Relationships Among TAV and CMV Strains

Test Antigen[a]	Antiserum to:							
	QCMV	TCMV	XCMV	UCMV	MCMV	KCMV	VTAV	NTAV
QCMV	128[b]	512	512	256	64	64	2	4
TCMV	128	512	256	128	128	128	4	4
XCMV	256	256	512	256	128	64	16	16
UCMV	128	256	256	256	128	64	4	4
MCMV	64	128	256	64	128	128	4	2
KCMV	64	128	64	64	64	256	4	4
VTAV	2	4	16	2	4	4	512	256
NTAV	4	4	16	4	4	4	512	256

a All antigen preparations consisting of glutaraldehyde-fixed virus were adjusted to concentrations of 1 mg/ml.

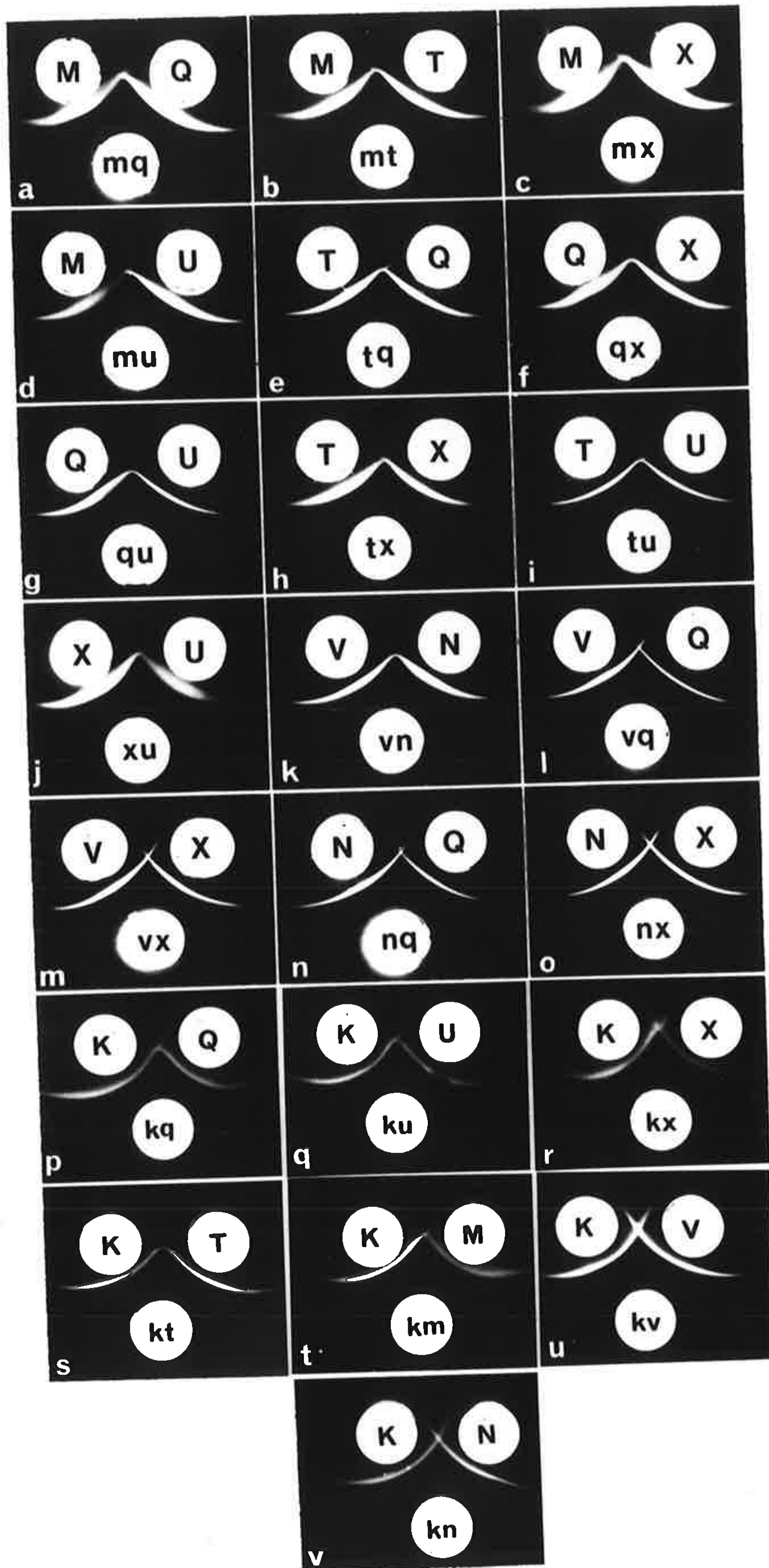
b Reciprocals of maxium antiserum dilution producing visible immuno-precipitin lines. Homologous titres are underlined.

all the heterologous titres are within one two-fold dilution of each other. However, the relationship between MCMV and the other CMV strains appears to be a little more distant (Table 3.3). The relationships between the strains of CMV were also investigated in immunodiffusion tests using mixtures of two antisera in one well reacted against preparations of the homologous viruses loaded in separate wells (Fig. 3.2; Grogan et al, 1964). These tests confirm that MCMV can be distinguished serologically by formation of single or double spurs from all the other CMV strains (Fig. 3.2 a-d). These tests also show that TCMV and KCMV are distinguishable from QCMV, XCMV and UCMV (Fig. 3.2 e,h,-i,p,q,r.) but are not distinquishable from each other (Fig. 3.2 s). Similarly, QCMV, XCMV and UCMV could not be differentiated from each other (Fig. 3.2 f,g,j) in these tests.

Unlike previously reported results with QCMV and VTAV (Habili and Francki, 1975), data presented in Table 3.3 indicated a distant but significant serological relationship of all the strains of CMV with the two strains of TAV. The very close relationship between VTAV and NTAV (Table 3.3) was confirmed by immunodiffusion tests with mixed antisera (Fig. 3.2k). Similar tests shown in Fig. 3.2 l-o demonstrate that the distant relationships between the two TAV strains and QCMV (Table 3.3) could not be distinguished from the somewhat closer relationship between the TAV strains and XCMV (Table 3.3).

Figure 3.2

Immunodiffusion tests between strains of CMV and TAV. Capital letters refer to wells filled with 10 μl of fixed virus preparation adjusted to a concentration of 1 mg/ml. Small letters refer to wells filled with 10 μl of mixture of two antisera, each adjusted to a homologous titre of 1/128. Letter codes of the various virus strains are same as those in Table 3.3



M Q mq a
M T mt b
M X mx c
M U mu d
T Q tq e
Q X qx f
Q U qu g
T X tx h
T U tu i
X U xu j
V N vn k
V Q vq l
V X vx m
N Q nq n
N X nx o
K Q kq p
K U ku q
K X kx r
K T kt s
K M km t
K V kv u
K N kn v

3. Enzyme-linked Immunosorbent Assays (ELISA)

Variations of the double antibody sandwich form of ELISA as used by Clark and Adams (1977) have proved to be sensitive methods of detecting plant viruses as well as differentiating closely related antigens (Koenig, 1978; Bar-Joseph and Salomon, 1980). In this study I have utilized this technique to investigate antigenic variation among strains of CMV which appear to be closely related and CMV and TAV which are apparently only distantly related (Table 3.3, Fig. 3.2).

a. Determination of Optimum Concentrations of Coating and Enzyme Labelled γ-Globulins

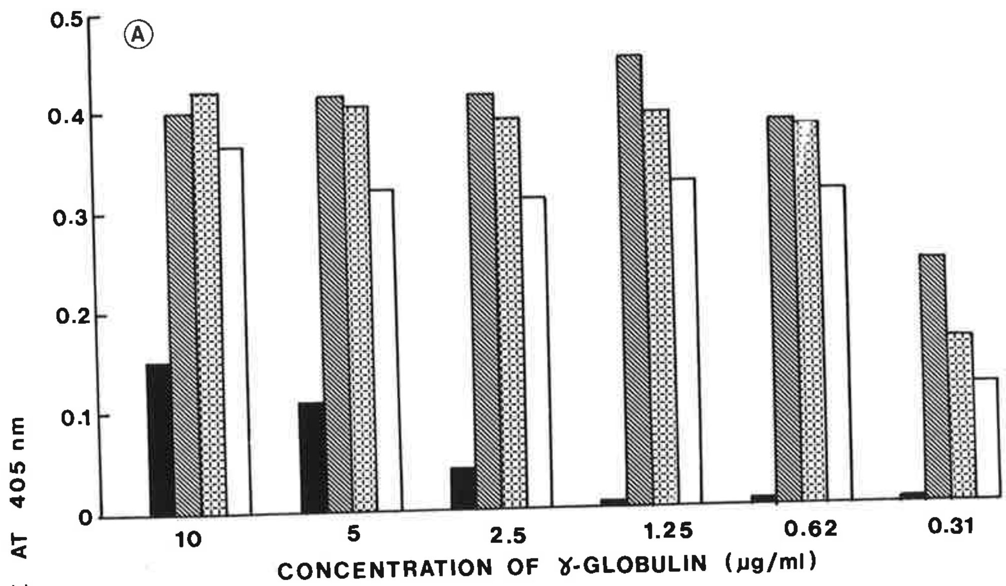
Fig. 3.3A shows the influence of different concentrations of coating γ-globulin of anti-TCMV serum on the amount of colour produced at various dilutions of homologous antigen. It can be observed that the coating γ-globulin concentrations exceeding 1.25 μg/ml reduced the extinction value of virus specific reactions and increased that of non-specific reactions in control wells (PBS).

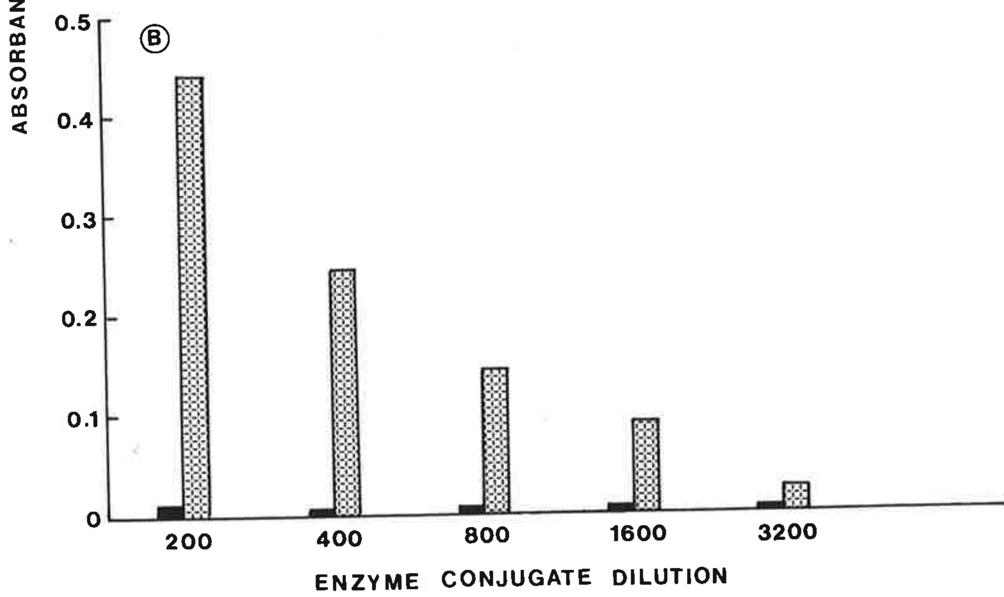
Fig. 3.3B shows the influence of various dilutions of enzyme-antibody conjugate of anti-TCMV serum on the colour production at a constant homologous antigen

Figure 3.3

A. Influence of different concentrations of coating γ-globulins of anti-TCMV serum on the colour production in ELISA. Homologous antigen was diluted beyond its titre (1:4) in immunodiffusion tests to 1:256 (▨), 1:512 (⬚) and 1:1024 (□) in PBS-Tween. Anti-TCMV enzyme conjugate was diluted to 1:200 in PBS-Tween containing 2% PVP and 0.02% ovalbumin. PBS-Tween (■) was used as a control.

B. Influence of various dilutions of anti-TCMV enzyme conjugate on the colour production in ELISA at constant homologous antibody coating (1.25 μg/ml) and antigen dilution (1:512, ⬚). Preparation of reagents was the same as described in A. above. PBS-Tween (■) was used as a control.


A
0.5
0.4
0.3
0.2
0.1
0
10
5
2.5
1.25
0.62
0.31
CONCENTRATION OF γ-GLOBULIN (μg/ml)
ABSORBANCE AT 405 nm


B
0.5
0.4
0.3
0.2
0.1
0
200
400
800
1600
3200
ENZYME CONJUGATE DILUTION

dilution. Two-fold dilutions of enzyme-antibody conjugate in the range of 1:200 to 1:3200 usually halved the extinction values. Hence for coating ELISA plates, 1.25 μg/ml of γ -globulins proved to be the optimum in combination with 1:200 dilution of enzyme-antibody conjugate. Similar optimum parameters were determined for each cucumovirus antiserum and the results are presented in Table 3.4.

b. <u>Serological Relationships between TAV and CMV Strains as determined by ELISA</u>

For distinguishing closely related antigens heterologous antibodies were used for coating the ELISA plate and for conjugating with the enzyme. Results of these experiments are summarised in Fig. 3.4. The tests confirm that all the six strains of CMV are antigenically closely related. (Fig. 3.4 A-F). MCMV which had been distinguished from all other CMV strains by both types of immunodiffusion tests (Table 3.3, Fig. 3.2) was also the most readily distinguishable strain in these tests (Fig. 3.4A). By ELISA, even the CMV strains, QCMV, XCMV, and UCMV and TCMV and KCMV which were not distinguished by immunodiffusion tests (Table 3.3, Fig. 3.2) could be differentiated (Fig. 3.4). On the other hand, the remote relationship between TAV and CMV was not detected. (Fig. 3.4). ELISA tests also indicated that the two strains of TAV are antigenically very similar (Fig. 3.5 A & B).

Table 3.4

Characteristics of Cucumovirus Antisera Used for ELISA Tests

Antiserum	Homologous Titre of Antiserum (Reciprocal)@	Optimum Coating Conc. of γ-globulins (μg/ml)	Optimum Enzyme-antibody Dilution (Reciprocal)
QCMV	128	2.5	200
TCMV	512	1.25	200
XCMV	512	1.25	200
UCMV	256	1.25	200
MCMV	128	2.5	200
KCMV	256	1.25	200
VTAV	512	1.25	200
NTAV	256	1.25	200

@ Determined by immunodiffusion tests in agar.

Figure 3.4

Serological relationship between six strains of CMV and two of TAV as revealed by standard ELISA tests using antisera to TCMV (A), QCMV (B), XCMV (C), UCMV (D), MCMV (E) and KCMV (F). Preparation of reagents was the same as described in Figure 3.3. A preparation of VTMoV was used as the control antigen.

TCMV ○—○

QCMV ●—●

XCMV ✶—✶

UCMV ✫—✫

MCMV ■—■

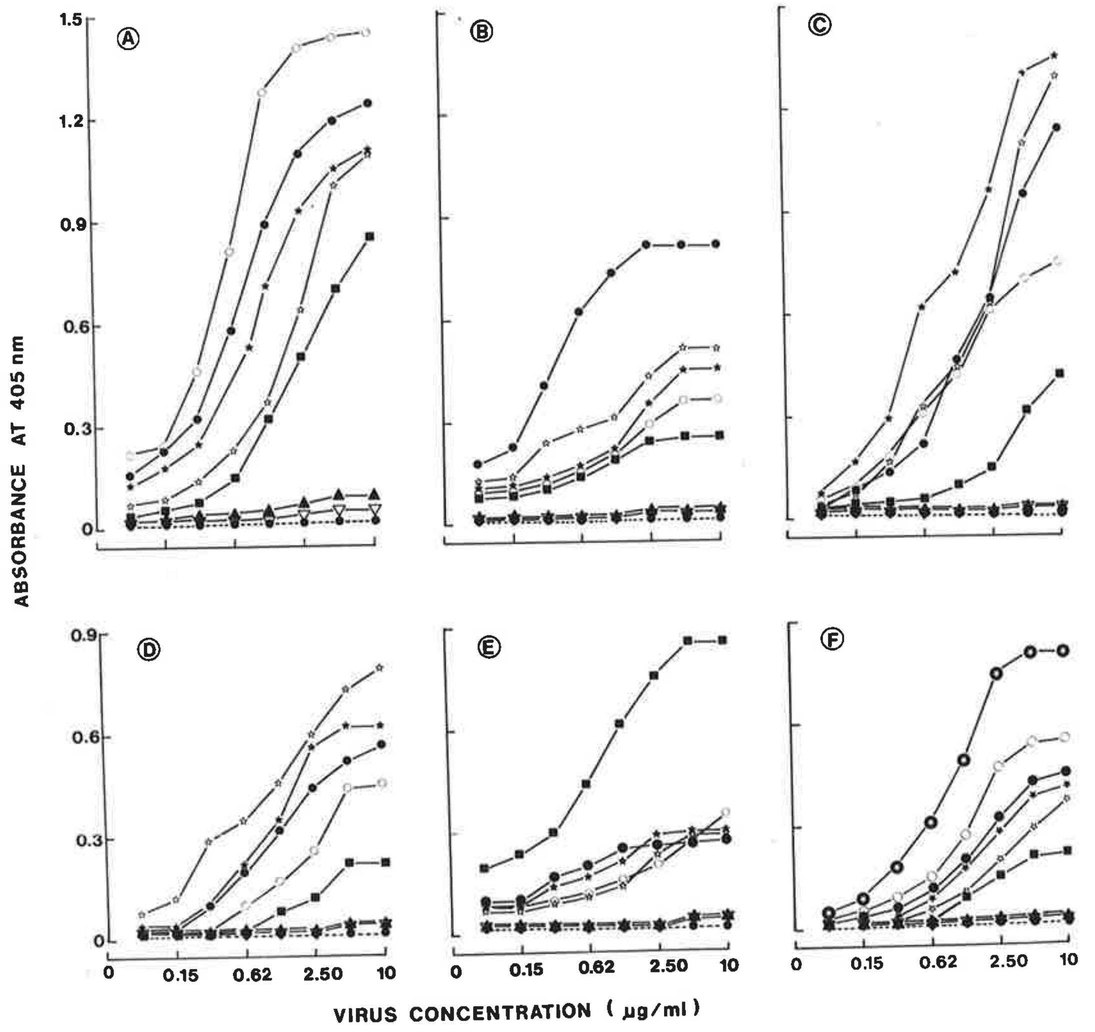
KCMV ✪—✪

VTAV ▲—▲

NTAV ▽—▽

Control ●---●

Note: KCMV (✪—✪) has not been tested against antisera of other CMV isolates (A to E), since it was not available then.



ABSORBANCE AT 405 nm
1.5
1.2
0.9
0.6
0.3
0
A
B
C
D
E
F
0.9
0.6
0.3
0
0
0.15
0.62
2.50
10
VIRUS CONCENTRATION (µg/ml)

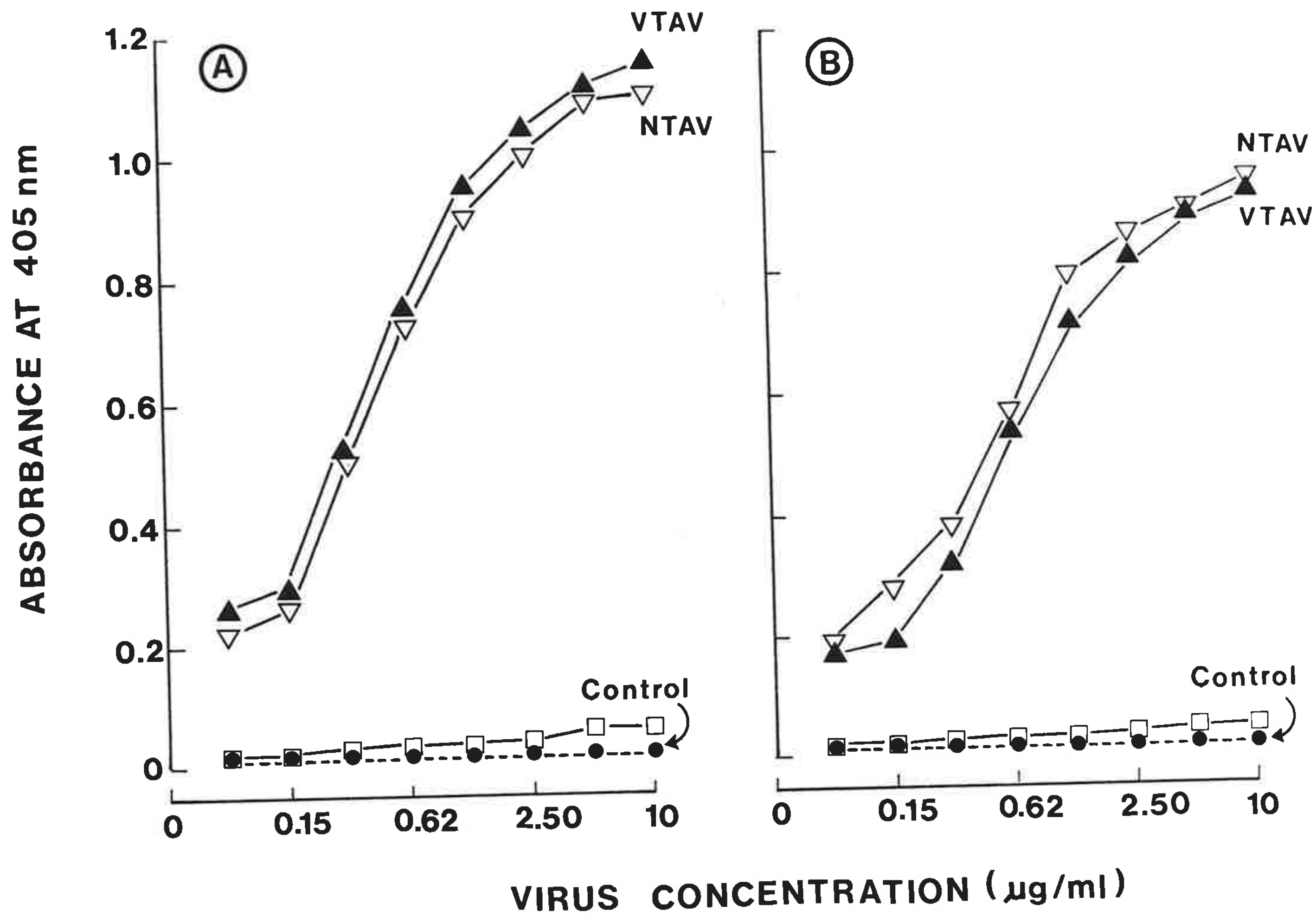
Figure 3.5

Serological relationships between two strains of TAV and six strains of CMV as revealed by standard ELISA tests using antisera to VTAV (A) and NTAV (B). Preparations of six CMV strains (TCMV, QCMV, XCMV, UCMV, MCMV and KCMV) produced similar reactions indicated by (□——□). A preparation of VTMoV was used as the control antigen.

VTAV ▲——▲

NTAV ▽——▽

Control ●---●


A
B
VTAV
NTAV
NTAV
VTAV
Control
Control
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE AT 405 nm
0
0.15
0.62
2.50
10
0
0.15
0.62
2.50
10
VIRUS CONCENTRATION (µg/ml)

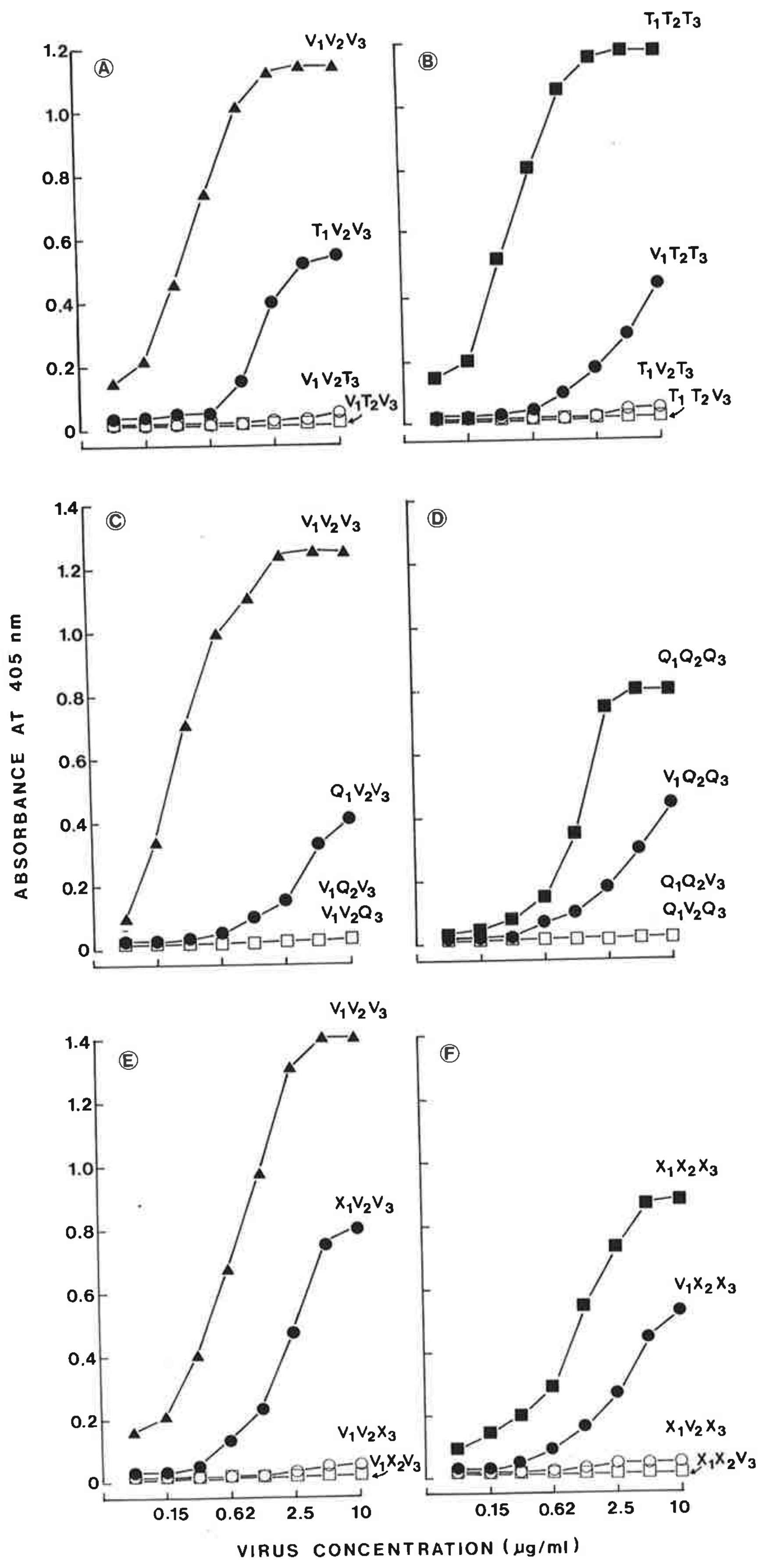
Results presented in Fig. 3.4 and 3.5. indicate that ELISA in its standard form is not suitable for the detection of the distant antigenic relationship between TAV and CMV (Table 3. 3). It has been shown that the high specificity of ELISA depends more on the enzyme-conjugated antibodies than on the coating antibodies used in the tests (Koenig, 1978; Bar-Joseph and Salomon, 1980). I have reached a similar conclusion from experiments with TAV and CMV in which heterologous and homologous antibodies were used for coating and coupling to enzyme respectively. Results of these experiments are summarised in Fig. 3.6 and 3.7. It can be observed from Fig. 3.6 A & B that when VTAV or TCMV preparations were tested with both heterologous coating and enzyme-linked antibodies ($T_1V_2T_3$, $V_1T_2V_3$) or homologous coating and heterologous enzyme-linked antibody ($V_1V_2T_3$, $T_1T_2V_3$) no antigenic relationship was detected between the two viruses. However, with heterologous coating and homologous enzyme-linked antibodies ($T_1V_2V_3$, $V_1T_2T_3$), an antigenic relationship between TAV and CMV became evident. Similar results were obtained in tests with other strains of CMV and TAV (Fig. 3.6 and 3.7).

4. Immune-electronmicroscopy (IEM)

Immune-electron microscopy using the antibody decoration technique (Milne and Luisoni, 1977) has recently been used with apparent success for the

Figure 3.6

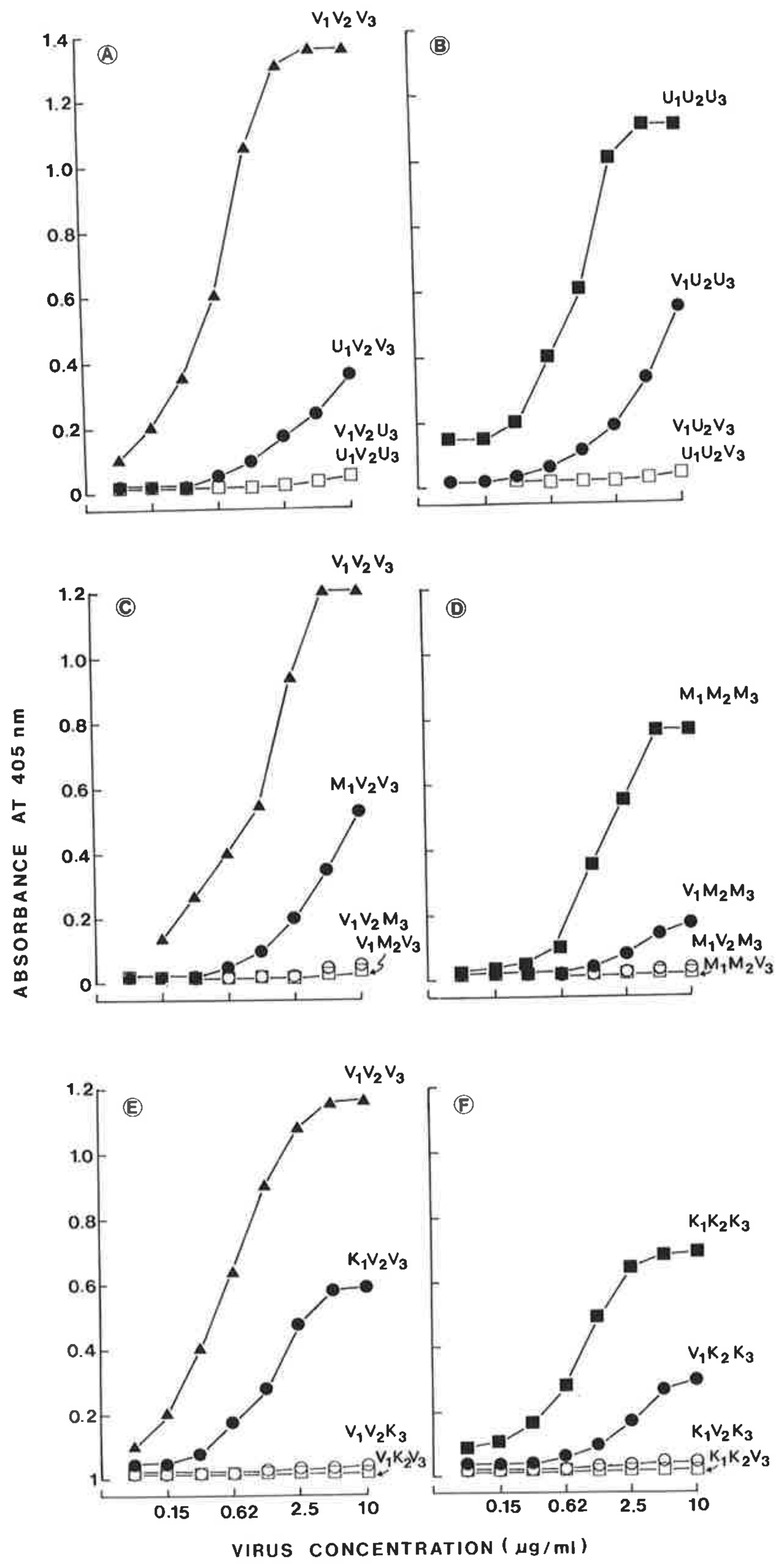
Serological relationships between VTAV and TCMV (A-B), QCMV (C-D) and XCMV (E-F) as revealed by ELISA tests using heterologous and homologous combinations of coating and enzyme-linked antibodies. Letters refer to the virus strains used (e.g. V=VTAV, T=TCMV) and the numbers refer to the nature of reagents (1 = coating antibody, 2 = antigen, 3 = enzyme-linked antibody).



A
B
C
D
E
F
V1V2V3
T1V2V3
V1V2T3
V1T2V3
T1T2T3
V1T2T3
T1V2T3
T1 T2 V3
Q1V2V3
V1Q2V3
V1V2Q3
Q1Q2Q3
V1Q2Q3
Q1Q2V3
Q1V2Q3
X1V2V3
V1V2X3
V1X2V3
X1X2X3
V1X2 X3
X1V2 X3
X1X2V3
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.15
0.62
2.5
10
ABSORBANCE AT 405 nm
VIRUS CONCENTRATION (µg/ml)

Figure 3.7

Serological relationships between VTAV and UCMV (A-B), MCMV (C-D) and KCMV (D-E) as revealed by ELISA tests using heterologous and homologous combinations of coating and enzyme-linked antibodies. Explanations for the letters and the numbers were same as those in Figure 3.6



A
B
C
D
E
F
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE AT 405 nm
VIRUS CONCENTRATION (µg/ml)
0.15
0.62
2.5
10
V1V2V3
U1V2V3
V1V2U3
U1V2U3
U1U2U3
V1U2U3
V1U2V3
U1U2V3
M1V2V3
V1V2M3
V1M2V3
M1M2M3
V1M2M3
M1V2M3
M1M2V3
K1V2V3
V1V2K3
V1K2V3
K1K2K3
V1K2K3
K1V2K3
K1K2V3

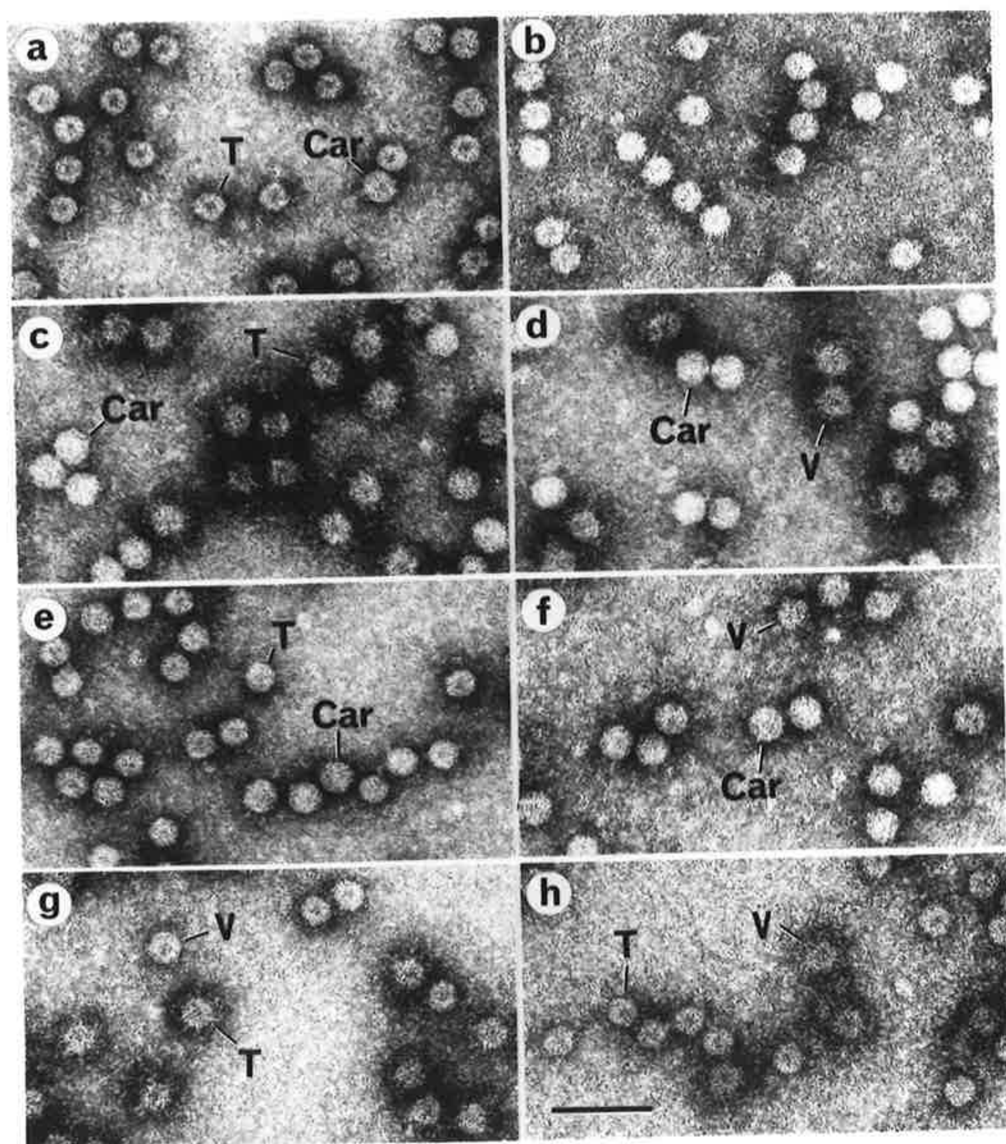
detection of distant antigenic relationships among luteoviruses (Roberts et al, 1980). To confirm the distant antigenic relationship between TAV and CMV (Table 3.3), a similar technique was used in this study. Carnation mottle virus (CarMV), morphologically different (Fig. 3.8 a-b) and serologically unrelated to either TAV or CMV was used as a control in these experiments for which the results are summarised in Fig. 3.8.

It can be observed from Fig. 3.8 c-d, that both TCMV and VTAV particles were heavily decorated by their homologous antibodies where CarMV particles on the same specimen grid failed to bind antibodies to either TCMV or VTAV. In similar tests with heterologous antisera to TCMV and VTAV, the antigen particles were decorated lightly with antibodies (Fig. 3.8 e-f) some of which were not easy to distinguish from particles treated with antisera to completely unrelated viruses such as those to TMV or CarMV (Fig. 3.8 a-b).

The differences in decoration with homologous and heterologous antibodies are illustrated by experiments done with mixtures of TCMV and VTAV preparations mounted on the same specimen grid and treated separately with antisera to each of the two viruses (Fig. 3.8 g-h). It can be seen that the heavy decoration with homologous antibodies were easily observed while

Figure 3.8

Serological relationships between VTAV and TCMV as revealed by IEM using the antibody decoration technique. Particles of VTAV are indicated by V, TCMV by T and CarMV (control antigen) by Car. Antisera used for decoration were as follows:
TMV (a), CarMV (b), TCMV (c, f and g) and VTAV (d, e and h). Bar represents 100 nm.


a
T
Car
b
c
T
Car
d
Car
V
e
T
Car
f
V
Car
g
V
T
h
T
V

decoration with heterologous antibodies was rather difficult to distinguish from the background granularity around particles tested with antisera to completely unrelated antigens, such as those to TMV or CarMV (Fig. 3.8 a-b).

V. CONCLUSIONS

Six isolates of CMV (Q,T,X,U,M,K) and two of TAV (V and N) are readily distinguishable by their reactions on several host species (Table 3.1) and also by the differences in electrophoretic mobilities of their RNA components (Fig. 3.1) in agarose gels. Serologically all CMV isolates are closely related to each other and distantly to TAV (Table 3.3). Immunodiffusion tests were less efficient than ELISA in distinguishing minor antigenic differences between strains of CMV and also distant relationships between TAV and CMV. Decoration of virus particles with antibodies in IEM was the least satisfactory method for investigating antigenic relationships among the viruses.

In view of their distinguishable RNA components under non-denaturing conditions of electrophoresis and the distinctive symptoms they induce in a range of host species, four cucumovirus isolates, UCMV, MCMV, KCMV and VTAV were selected as suitable candidates for the *in vitro* construction of pseudorecombinants.

CHAPTER 4

PURIFICATION AND COMPATIBILITY OF GENOME PARTS FROM TOMATO ASPERMY AND CUCUMBER MOSAIC VIRUSES

I. INTRODUCTION

In vitro construction of pseudorecombinants necessitates the purification of RNA components to the point where they are non-infectious, but become highly infectious when mixed. In this chapter a simple method for the rapid preparative separation of cucumovirus RNA components by electrophoresis in 2% agarose gels is described. Initial attempts to construct pseudorecombinants by exchanging RNA-1 and RNA-2 between CMV and TAV were unsuccessful. Hence work described in the later part of this chapter was aimed at studying the compatibility of genome segments from four strains of CMV and one of TAV.

II. PREPARATIVE FRACTIONATION OF CUCUMOVIRUS GENOMIC RNAs

1. Reasons for using Agarose as a Supportive Medium in Analytical and Preparative Gel Electrophoresis of Cucumovirus RNA Preparations

Unlike polyacrylamide gel (2.4%), agarose forms a highly porous, yet rigid gel at a concentration of 2% and

this facilitates easy handling in both analytical and preparative gel electrophoresis. In preliminary experiments, both polyacrylamide and agarose gels were used to separate and to determine the relative electrophoretic mobilities of RNA preparations from several cucumoviruses. MCMV, a suitable candidate for genetic analysis was readily resolved into four major RNA components under non-denaturing conditions in 2% agarose gels but not in polyacrylamide gels (Mossop et al, 1976). Furthermore, when UCMV and MCMV were co-electrophoresed, the difference in the mobility of RNA-2 of the two virus strains was readily demonstrated in 2% agarose gels (refer Chapter 3 Fig. 3.1 C) but not in polyacrylamide gels (chapter 3., Fig. 3.1 D). Hence, agarose gels were used for routine preparative and analytical gel electrophoresis of cucumovirus RNA preparations.

2. Purification of Cucumovirus Genomic RNAs

Initial attempts to fractionate and purify cucumovirus RNA components by the method of Mossop and Francki (1977) resulted in considerable loss of RNA. They used 2.4% polyacrylamide gels for the first cycle of electrophoresis and localised the RNA bands by staining the gels in 0.1% ethidium bromide. The low recovery of RNA by this method was attributed to the undesirable

migration of RNA between the gel column and plexiglass tubes and the inefficient elution of RNA from the acrylamide gel (Mossop, personal communication). Furthermore, RNA bands stained in 0.01% ethidium bromide were insufficiently clear to allow accurate excision of the CMV RNA-1 and RNA-2 bands from the gels, under normal light conditions. To overcome these problems, 2% agarose gels and toluidine blue 0 stain were used (see Chapter 2). The RNA bands stained clearly in toluidine blue 0 and were easy to detect.

3. Assessment of Infectivity and Purity of Isolated RNA Components

To determine the percentage of infectivity retained by the fractionated RNA components and the acceptable degree of purity of each RNA component suitable for the construction of pseudorecombinants *in vitro*, the following experiements were done.

The infectivities of unfractionated and fractionated RNA components of UCMV were compared on opposite half leaves of *C. amaranticolor*. The concentration of unfractionated UCMV-RNA preparation was adjusted to 15 μg/ml in TE buffer. Fractionated preparations of UCMV-RNAs-1, 2 and 3 were each adjusted to a concentration of 15 μ g/ml and were used for making up all the

inocula by mixing appropriate volumes of each solution. Inocula containing mixtures of RNAs-1, 2 and 3 were prepared so as to contain approximately the same concentration of each component as that present in the unfractionated RNA. The mean number of local lesions on 8 half-leaves produced by unfractionated UCMV-RNA was 102 and by the mixture 74. This indicates that after two cycles of electrophoresis, the fractionated RNA components were approximately 70% as infectious as unfractionated RNA. The slight reduction in infectivity could possibly be attributed to the reaction between RNA and the photoreactive dye toluidine blue 0 (Schuerch et al, 1975), which was used to locate RNA bands in the present study and also due to the different proportions of RNA-1, 2 and 3 in the mixed inocula.

From the data summarised in Table 4.1 it can be observed that the number of local lesions produced by inocula containing single genome segments or a combination of two segments of UCMV, XCMV, MCMV, KCMV and VTAV were always less than 10% and usually much lower than that relative to a combination of all three segments. These results demonstrate that the RNA components of each cucumovirus separated by two step agarose gel electrophoresis were of high purity.

Table 4.1

Infectivity of Fractionated RNA Segments and Mixtures from Four Strains of CMV and One of TAV

Experiment[a]	Combination of RNA Segments in Inoculum[b]	Lesions per half-leaf[c]				
		XCMV	UCMV	MCMV	KCMV	VTAV
1	RNA-1 RNA-1+2+3	0 36	0 59	0 126	0 46	0 18
2	RNA-2 RNA-1+2+3	0 33	0 43	2 97	0 53	1 26
3	RNA-3 RNA-1+2+3	0 34	0 56	0 106	0 37	0 22
4	RNA-1+2 RNA-1+2+3	2 42	2 49	0 82	1 61	3 34
5	RNA-1+3 RNA-1+2+3	1 41	4 114	2 93	4 57	1 83
6	RNA-2+3 RNA-1+2+3	0 38	9 122	0 76	0 73	4 53

a In each experiment the infectivity of the two inocula was compared on 8 opposite half-leaves of _C. amaranticolor_.

b The concentration of each RNA segment was adjusted to approximately 15 µg/ml in TE buffer and inoculated to _C. amaranticolor_ as described by Habili and Francki (1974c).

c Mean number of local lesions produced by each inoculum on 8 half-leaves.

III. COMPATIBILITY OF RNA GENOME SEGMENTS FROM TAV AND CMV

Infectivity of heterologous mixtures of cucumovirus RNAs provides a basis for testing whether parental strains yield viable pseudorecombinants or not. During the course of this study, several attempts to interchange RNA-1 and RNA-2 between strains of CMV and TAV were unsuccessful. Hence it was decided to investigate the compatibility of genome segments from pairs of viruses by local lesion assay. Heterologous mixtures of RNA segments of the 4 strains of CMV and one of TAV were prepared in the required combinations and inoculated to *C. amaranticolor*.

Results summarised in Table 4.2, demonstrate that the infectivity of all inocula containing mixtures of three genomic segments from the four strains of CMV in all combinations were highly infectious and produced similar numbers of lesions. Similarly, inocula containing mixtures of RNAs-1 and 2 of any of the CMV strains and RNA-3 of TAV were also highly infectious. However, the infectivities of mixtures of RNA-1 of TAV with RNAs-2 and 3 of any of the CMV strains or RNA-1 of any of the CMV strains with RNA-2 and 3 of TAV were almost as low as those of the individual or pairs of RNA segments. Similarly, RNA-2 of TAV mixed with RNA-1 and 3 of any CMV strain or RNA-2 of any CMV strain with RNAs-1 and 3 of TAV were poorly or not infectious.

Table 4.2

Compatibility of RNA Genome Segments of TAV and CMV

Combinations of RNA segments in inoculum[a]	Main lesions per half-leaf[b]									
	Expt.1. A=XCMV B=VTAV	Expt.2. A=UCMV B=VTAV	Expt.3. A=MCMV B=VTAV	Expt.4. A=XCMV B=UCMV	Expt.5. A=XCMV B=MCMV	Expt.6. A=UCMV B=MCMV	Expt.7. A=XCMV B=KCMV	Expt.8. A=UCMV B=KCMV	Expt.9. A=MCMV B=KCMV	Expt.10. A=KCMV B=VTAV
(1) $A_1A_2A_3$	87	84	74	69	85	84	76	85	63	75
(2) $B_1B_2B_3$	75	75	86	73	83	78	83	93	71	59
(3) $A_1B_2B_3$	3.2	0.7	0.7	30	82	79	61	86	89	3.5
(4) $B_1A_2A_3$	5.4	9.5	0.7	29	79	79	66	74	93	2.6
(5) $A_1B_2A_3$	4.3	0.4	3.5	85	78	83	57	83	85	1.5
(6) $B_1A_2B_3$	3.6	0.5	1	82	76	81	53	61	86	0.5
(7) $A_1A_2B_3$	50	31.5	27	82	77	58	81	69	73	49
(8) $B_1B_2A_3$	78	56	72	90	88	57	83	66	79	71
(9) A_1- -	0.0	0	0	1	1	0.0	0	0	0	0
(10) B_1- -	0.5	0	0	0.5	0	0.0	0	0	0	0
(11) - A_2-	0.0	0.5	1	1	1	0.5	0.5	0	0	1
(12) - B_2-	0.3	0.3	1	0	1	0.0	0.5	0	0	1
(13) - - A_3	0.0	1	1	0	0	0.5	0.3	0.5	2.5	0
(14) - - B_3	0.5	2	1	0.5	0	0.3	0	1	3.5	2
(15) - A_2A_3	2.3	13	0	2	1	0.3	3.5	2.5	0	1
(16) - B_2B_3	1.0	0.3	0.3	3.5	2	0.8	1.0	0	1	1
(17) A_1- A_3	1	0	6	2	2	1	2	0	1	0
(18) B_1- B_3	1	0.5	0	2	2	1	2	0	0	1
(19) A_1A_2-	1.5	0.5	7	2	1	5	1	1	1	0
(20) B_1B_2-	0.8	1	1	1	2	3	1	1	0	1

a All preparations containing only one segment of virus genomic RNA were adjusted to approximately 15 μg/ml so that each inoculum contained a total RNA concentration of 15 μg/ml. The preparations were inoculated to half-leaves of C. amaranticolour as described by Habili and Francki (1974c).

b Each experiment was done as three separate trials. In each trial 32 leaves were used and only one of the three RNA segments was exchanged. One half of each of 16 leaves was inoculated with preparations containing three segments, one of which was from virus A and two from virus B. Similarly, one half of each of another 16 leaves was inoculated with preparations containing one segment from virus B and two from virus A. The opposite half-leaves were inoculated with the remaining inocula listed. Thus the mean lesion numbers tabulated are from counts on 24 (inocula 1-2), 16 (inocula 3-8) and 4 (inocula 9-20) half-leaves.

These observations demonstrate that whereas RNA-3 of TAV and CMV are readily interchangeable to produce infections resulting in development of lesions, RNA-1 and 2 are not. This would explain my inability to construct pseudorecombinants from TAV and CMV involving the exchange of RNAs-1 and 2.

VI. CONCLUSIONS

The results presented here allow the following conclusions to be drawn:

1. The three genomic RNA segments of cucumoviruses fractionated and purified by two-cycles of electrophoresis in 2% agarose gels were highly infectious and of high purity (Table 4.1)

2. Infectivity assays of heterologous RNA mixtures of tomato aspermy and 4 strains of cucumber mosaic virus demonstrated that whereas RNA-3 is readily interchangeable between the two viruses, RNA-1 and RNA-2 are not, although they are interchangeable between the 4 strains of CMV (Table 4.2).

CHAPTER 5

GENETIC ANALYSIS OF CUCUMOVIRUS GENOME BY IN VITRO PSEUDORECOMBINATION

I. INTRODUCTION

Efforts to locate genes on cucumovirus RNAs-1, 2 and 3 involved in host symptom induction by the construction of pseudorecombinants have led to some contradictory conclusions (see Chapter 1). Results presented in the previous chapter clearly demonstrated that RNA-3 is readily interchangeable between CMV and TAV but not RNA-1 and RNA-2, although they are interchangeable between CMV strains (Chapter 4; Table 4.2). In this chapter are described the *in vitro* construction and characterization of 18 pseudorecombinants by exchanging RNAs in all combinations between pairs of the three strains of CMV and 3 pseudorecombinants by exchanging RNA-1+2 of TAV and RNA-3 of three CMV strains. The biological properties of these pseudorecombinants were compared with that of the parent viruses, to investigate the distribution of determinants for symptom production and host range on the three RNA segments.

II. IN VITRO CONSTRUCTION AND CHARACTERIZATION OF PSEUDORECOMBINANTS FROM THREE CMV STRAINS

The nomenclature used throughout this thesis to identify pseudorecombinants is derived from the origin of their genomic RNAs. For example, the pseudorecombinant constructed from MCMV-RNAs-1+2 and UCMV-RNA-3 is referred to as $M_1M_2U_3$.

Pseudorecombinants in all combinations of RNA-1, 2 and 3 were constructed from pairs of the three strains of CMV and cloned by successive local lesion transfers on *N. tabacum* cv White Burley and propagated in *N. clevelandii*. Using mono-specific antisera produced by cross absorption, all pseudorecombinants were checked and found to react with antisera to the parental CMV strains providing RNA-3 (e.g. Fig 5.1a). This was as expected, because it has been demonstrated that RNA-3 carries the coat protein gene (Marchoux *et al*, 1974a,b; Habili and Francki, 1974c; Mossop and Francki, 1977; Hanada and Tochihara, 1980). The RNA segments of all the pseudorecombinants were analysed by agarose gel electrophoresis to identify their RNA-2 (e.g. Fig. 5.1 b). To further check the authenticity of the pseudorecombinants back crosses were constructed between the appropriate pairs of pseudorecombinants to regenerate the parental virus strains which were compared to the original parental viruses on suitable differential hosts. The

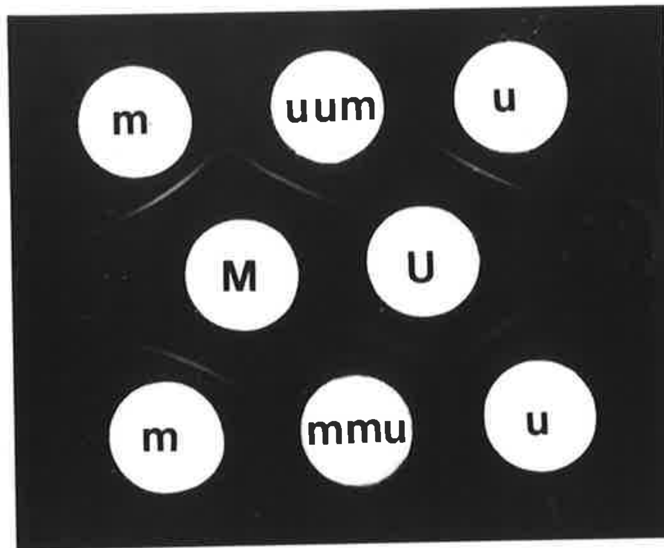
Figure 5.1

Analysis of RNA components and serological relationships between UCMV and MCMV and their pseudorecombinants.

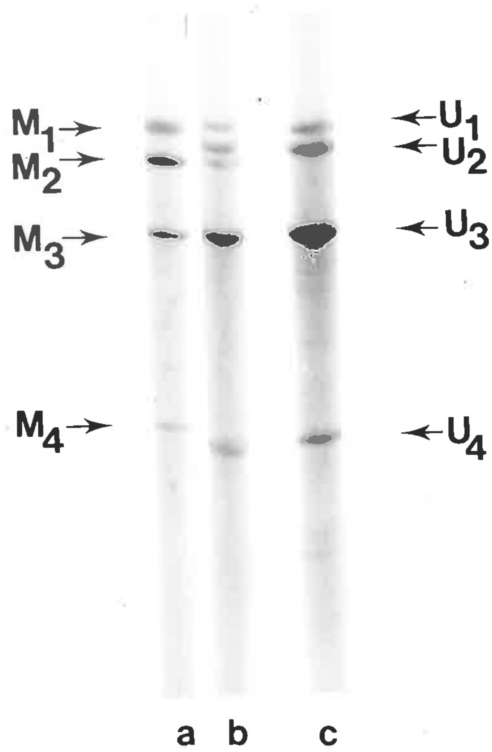
a. Immunodiffusion test demonstrating antigenic identity of $U_1U_2M_3$ (uum) with MCMV (m) and $M_1M_2U_3$ (mmu) with UCMV (u). Antisera specific to UCMV (U) and MCMV (M) with homologous titres 1/32 and 1/8 respectively were prepared by absorption with heterologous antigens.

b. Electrophoretic analysis of the RNA components of $U_1U_2M_3$ in 2% agarose gels. MCMV (a), $U_1U_2M_3$ + MCMV (b) and UCMV (c).

(a)



(b)



four Nicotiana spp., Datura stramonium L., Gomphrena globosa L. and Solanum melongena L. were used to check the back-crossed pseudorecombinants corresponding to UCMV and MCMV. These and two additional hosts, Zea mays L., and Vicia faba L. were used to check the back-crossed pseudorecombinants corresponding to KCMV. In all cases the symptoms induced by the back-crossed pseudorecombinants were indistinguishable from the original parental virus strains, demonstrating that all the pseudorecombinants had the expected RNA compositions and no mutants had been selected inadvertently.

1. Symptomatology and Host Range of Pseudorecombinants

Examination of all the pseudorecombinants constructed by exchanges involving RNA-1 indicate that this segment had little effect on symptom induction on most of the host species examined (Tables 5.1 - 5.3). However, $U_1M_2M_3$ produced symptoms milder than those of MCMV on three of the four Nicotiana spp. tested (Table 5.1, Fig. 5.3). Similarly symptoms induced by $M_1K_2K_3$ on N. glutinosa (Fig. 5.17 b) and S. melongena were milder than those induced by KCMV (Table 5.3) as was $U_1K_2K_3$ on S. melongena (Table 5.2). On the other hand $U_1K_2K_3$ and $M_1K_2K_3$ produced more severe symptoms than those by either of the parental strains on N. tabacum (Fig. 5.10a) and G.

globosa respectively (Tables 5.2 and 5.3). Furthermore, $K_1M_2M_3$ failed to infect D. stramonium systemically (Table 5.3; Fig. 5.22).

From the data summarised in Tables 5.1-5.3 it appears that exchanges of RNA-2 between the strains of CMV have very significant effects on symptom induction. The most severe symptom produced by UCMV was necrosis and subsequent death of N. x edwardsonii (chapter 3, Table 3.1; Fig. 5.2 c). The determinant for this property must reside on RNA-2 since $M_1U_2M_3$ (Fig. 5.4 c) and $K_1U_2K_3$ (Fig. 5.12 c) both induced this symptom. Moreover, all other pseudorecombinants containing U_2 killed N. ex edwardsonii (Fig. 5.2 c; 5.7 c; 5.9 c; 5.13 c).

The determinant for the induction of leaf distortion by CMV appears also to reside on RNA-2. Only UCMV and the pseudorecombinants containing RNA-2 from UCMV, $M_1U_2M_3$ and $K_1U_2K_3$ (Tables 5.1 and 5.2) failed to induce leaf distortion. It is also evident that symptoms produced by some pseudorecombinants involving RNA-2 exchange can be quite different from those induced by either of the parental strains. For example, $M_1U_2M_3$, $K_1U_2K_3$ and $M_1K_2M_3$ failed to infect D. stramonium (Fig. 5.8; 5.15; 5.22) and S. melongena system-ically, although all three parental strains were

capable of systemic movement in this host. This indicates that the ability of CMV to infect *D. stramonium* and *S. melongena* systemically is governed by an interaction of determinants on RNA-2 and on RNA-1 or RNA-3 or both. Although $K_1M_2M_3$ also failed to infect *D. stramonium* systemically, it behaved like MCMV on *S. melongena*. This indicates that the mechanism determining systemic movement of virus in the two hosts is different.

Data presented in Tables 5.1-5.3 show that the ability of CMV to infect maize resides on RNA-2 because pseudorecombinants $U_1K_2U_3$ and $M_1K_2M_3$ were both able to infect maize as were all other pseudorecombinants containing K_2.

From the data summarised in Tables 5.1-5.3 it appears that exchanges of RNA-3 between strains of CMV have significant effects on symptom development. The most characteristic symptom induced by MCMV was the yellow mosaic of all the susceptible host plants examined (Chapter 3, Table 3.1 Fig. 5.2; 5.8 and 5.23 c,d). It appears that the genetic determinant for this property must reside at least in part on RNA-3 because pseudorecombinants $U_1U_2M_3$ (Fig. 5.7 and 5.8) and $K_1K_2M_3$ (Fig. 5.20 and 5.22) induced yellow mosaic of all susceptible host plants examined (Tables 5.1 and 5.3). However, the yellow mosaic induced by the pseudorecombinant $U_1U_2M_3$ in three of the *Nicotiana* spp.

Table 5.1

Characteristics of Pseudorecombinants between UMCV ($U_1U_2U_3$) and MCMV($M_1M_2M_3$)*

Host Plant	Pseudorecombinant					
	Exchange of RNA-1		Exchange of RNA-2		Exchange of RNA-3	
	$M_1U_2U_3$	$U_1M_2M_3$	$M_1U_2M_3$	$U_1M_2U_3$	$M_1M_2U_3$	$U_1U_2M_3$
Nicotiana tabacum cv. White Burley	= U	= M	§M(milder)[a]	§M(milder)	§M(milder)	§M(irregular chlorosis)[a]
N. glutinosa	= U	§M(milder)	§M(milder)[a]	§M(milder)	§M(milder)	§M(irregular chlorosis)[a]
N. x edwardsonii	= U	§M(milder)	= U	§M(milder)	§M(milder)	= U
N. clevelandii	= U	§M(milder)	§M(milder)[a]	§M(milder)	§M(milder)	§M(irregular chlorosis)[a]
Datura stramonium	= U	= M	≠M, ≠U[b]	= M	§M(severe)	§M(irregular chlorosis)[a]
Solanum melongena	= U	= M	≠M, ≠U[b]	§M(milder)	§M(milder)	§M(irregular chlorosis)[a]
Gomphrena globosa	= U	= M	§M(milder)[a]	§M(milder)	§M(milder)	§M(irregular chlorosis)[a]
Cucumis sativus var. Polaris	= U, = M	= U, = M	= U, = M	= U, = M	= U, = M	= U, = M
Zea mays	= U, = M	= U, = M	= U, = M	= U, = M	= U, = M	= U, = M
Vicia faba	= U, = M	= U, = M	= U, = M	= U, = M	= U, = M	= U, = M

* The sign = indicates that symptoms were indistinguishable from the designated parental strain; § indicates that the symptoms were similar but distinguishable from the designated parental strain; ≠ indicates that the symptoms were unlike those of either designated parental strains. Descriptions of symptoms produced by parental strains are presented in Chapter 3, Table 3.1.

a Indicates instances where the pseudorecombinants failed to induce leaf distortion characteristic of MCMV.

b Indicates that the pseudorecombinants produced chlorotic lesions on the inoculated leaves similar to those produced by MCMV but failed to infect the plant systemically.

Figure 5.2

Symptoms produced in four *Nicotiana* species infected with UCMV (left), $M_1U_2U_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

UCMV | $M_1U_2U_3$ | MCMV

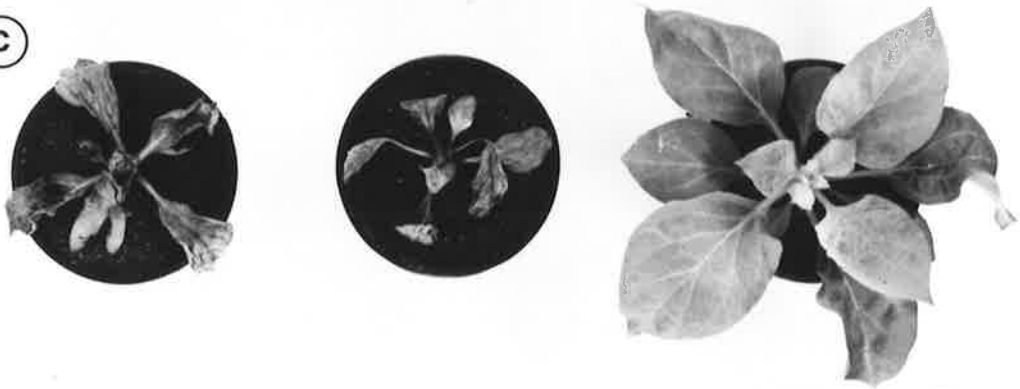
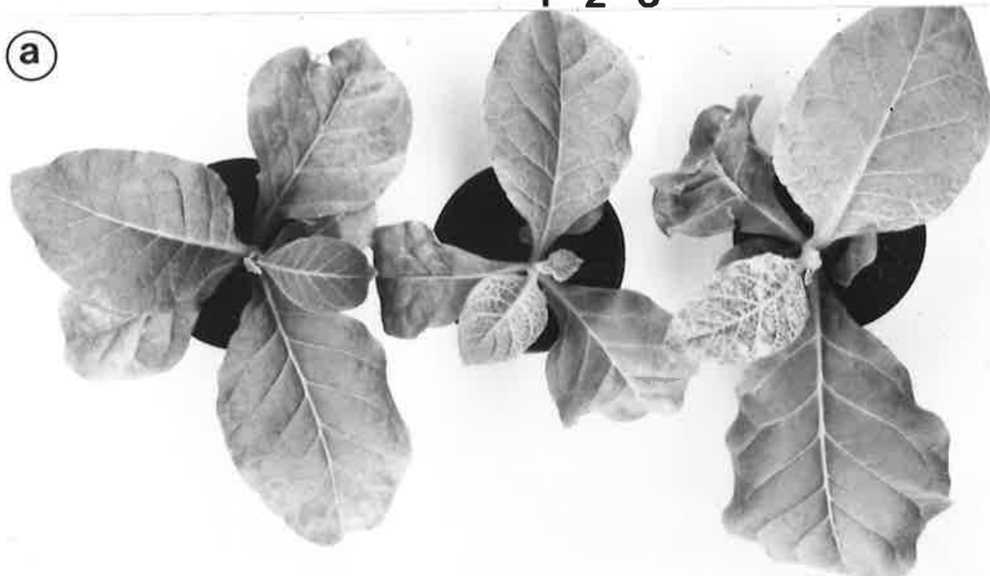
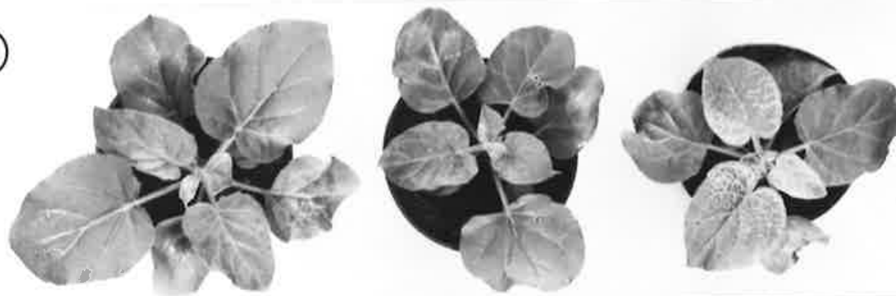


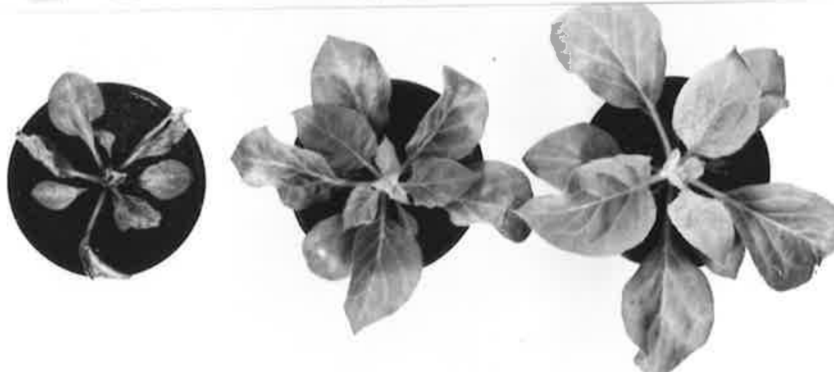
Figure 5.3

Symptoms produced in four *Nicotiana* species infected with UCMV (left), $U_1M_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.


UCMV
$U_1M_2M_3$
MCMV
a


b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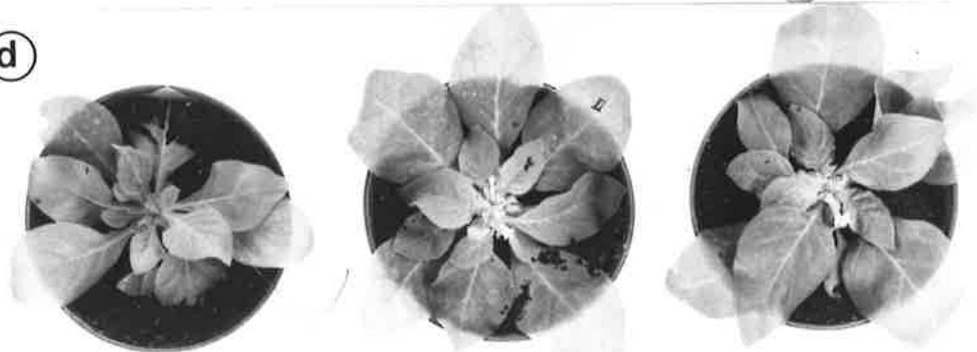
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Figure 5.4

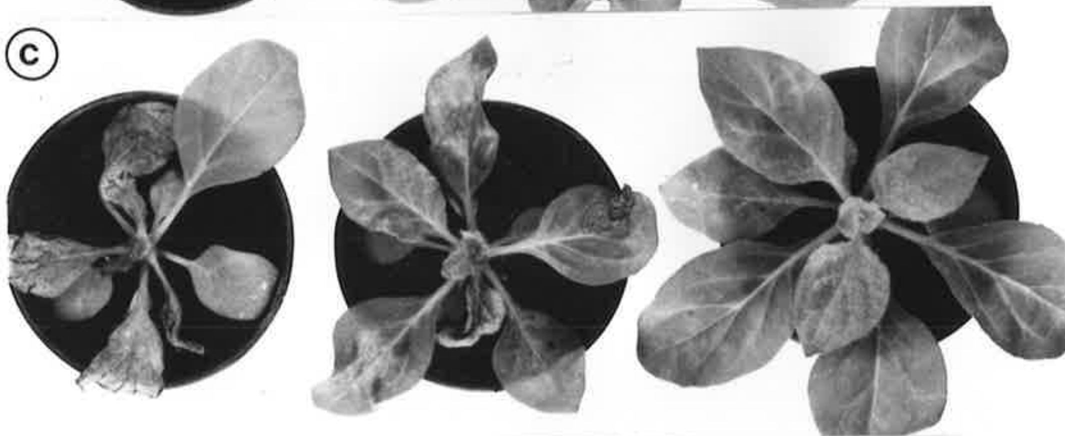
Symptoms produced in four *Nicotiana* species infected with UCMV (left), $M_1U_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

UCMV
$M_1U_2M_3$
MCMV

a

b


c

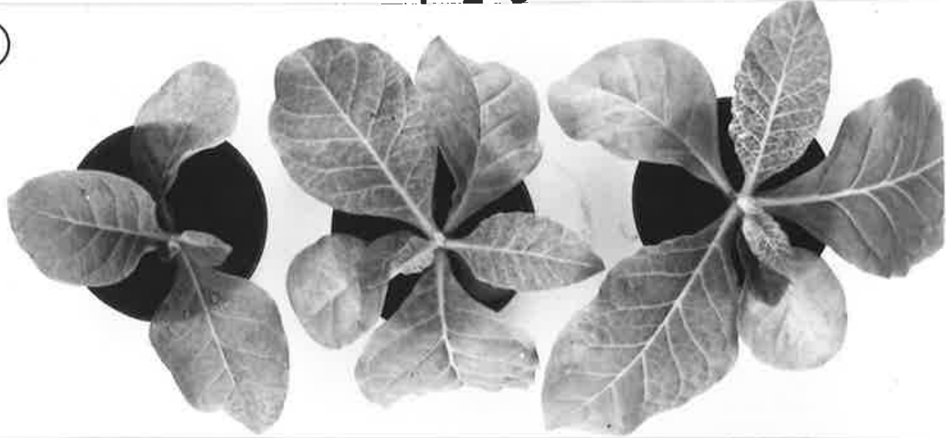
d

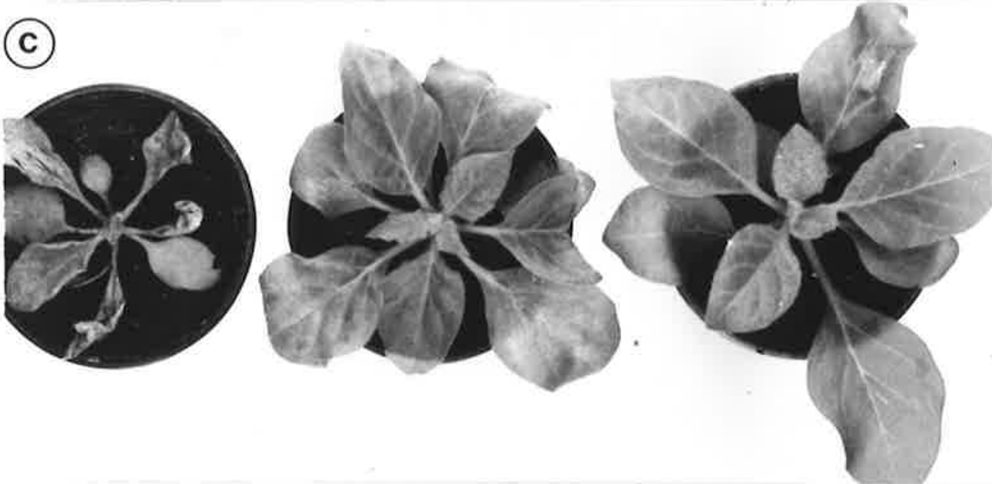
Figure 5.5

Symptoms produced in four *Nicotiana* species infected with UCMV (left), $U_1M_2U_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

UCMV $U_1M_2U_3$ MCMV





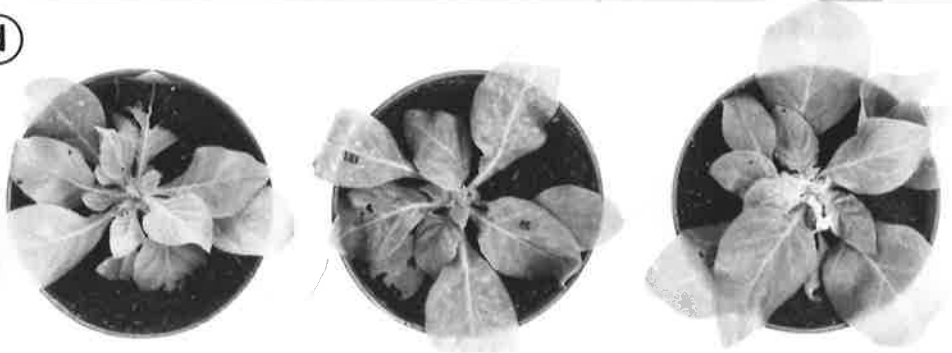


Figure 5.6

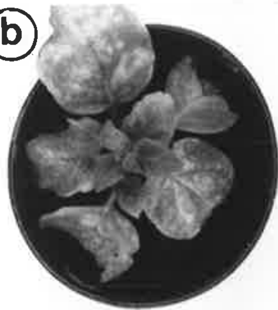
Symptoms produced in four *Nicotiana* species infected with UCMV (left), $M_1M_2U_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

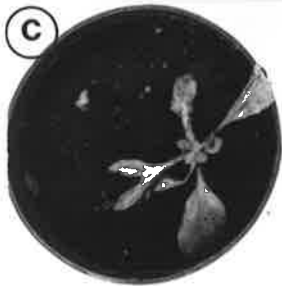
UCMV $M_1M_2U_3$ MCMV

(a)

(b)



(c)



(d)

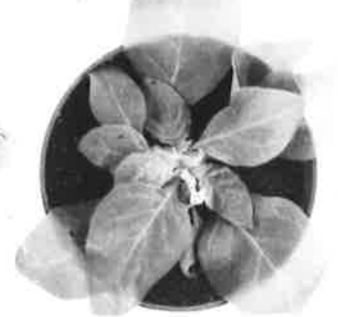


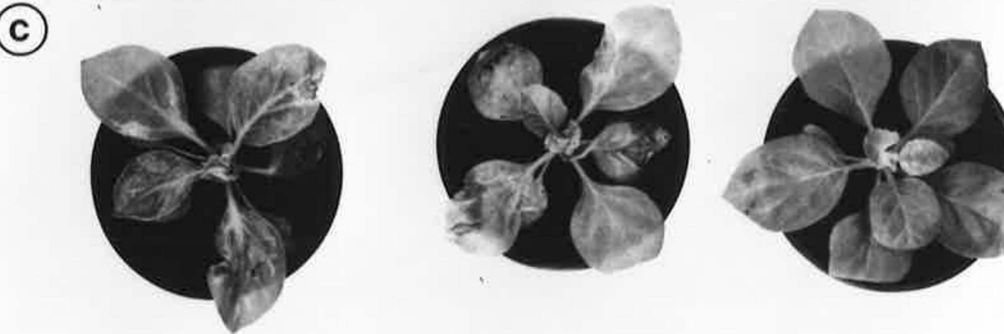
Figure 5.7

Symptoms produced in four *Nicotiana* species infected with UCMV (left), $U_1U_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

UCMV
$U_1U_2M_3$
MCMV
a

b


c

d

Figure 5.8

Symptoms induced on Datura stramonium by UCMV, MCMV and pseudorecombinants constructed in vitro from the three genomic segments of the two virus strains.

UCMV

MCMV

$M_1U_2U_3$

$U_1M_2M_3$

$M_1U_2M_3$

$U_1M_2U_3$

$M_1M_2U_3$

$U_1U_2M_3$

Table 5.2

Characteristics of Pseudorecombinants between UCMV ($U_1U_2U_3$) and KCMV ($K_1K_2K_3$)*

Host Plant	Pseudorecombinant					
	Exchange of RNA-1		Exchange of RNA-2		Exchange of RNA-3	
	$K_1U_2U_3$	$U_1K_2K_3$	$U_1K_2U_3$	$K_1U_2K_3$	$U_1U_2K_3$	$K_1K_2U_3$
Nicotiana tabacum cv White Burley	= U	§K(more severe)	§K(more severe)	= U	= U	= K
N. glutinosa	= U	= K	§K(more severe)	= U	= U	=K
N. x edwardsonii	= U	= K	= K	= U	= U	= K
N. clevelandii	= U	= K	= K	= U	= U	= K
Datura stramonium	= U	= K	= K	≠ U, ≠ K[a]	= U[c]	= K
Solanum melongena	= U	§K(milder)	§K(milder)	≠ U, ≠ K[b]	= U	§K(milder)
Gomphrena globosa	= U	= K	= K	= U	= U	= K
Cucumis sativus var. Polaris	= U, = K	= U, = K	= U, = K	= U, = K	= U, = K	= U, = K
Zea mays	= U	= K	= K	= U	= U	= K
Vica faba	= U	= K	= U	= K	= K	= U

* Signs describing symptoms are the same as in Table 5.1.

a Indicates that the pseudorecombinant produced chlorotic local lesions on inoculated leaves similar to KCMV but failed to infect the plant systemically.

b Indicates that the pseudorecombinant failed to infect the plant systemically.

c Indicates that systemic symptoms were similar to those of UCMV but chlorotic local lesions were induced on the inoculated leaves similar to those induced by KCMV.

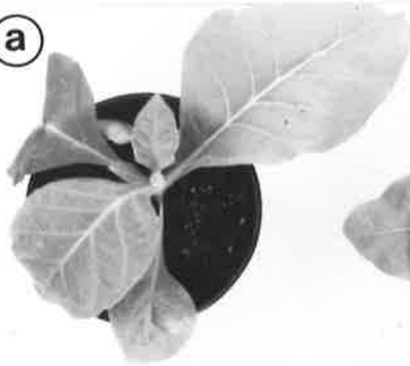
Figure 5.9

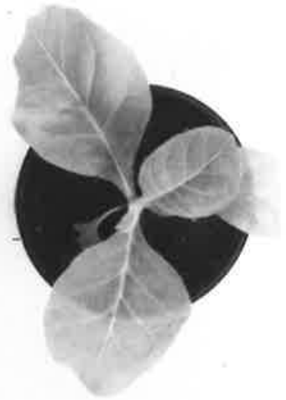
Symptoms produced in four *Nicotiana* species infected with KCMV (left), $K_1U_2U_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

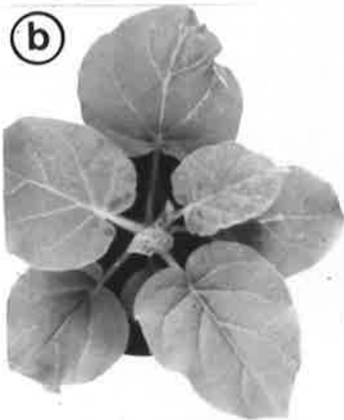
KCMV $K_1U_2U_3$ UCMV

(a)





(b)



(c)

(d)

Figure 5.10

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $U_1K_2K_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

KCMV $U_1K_2K_3$ UCMV

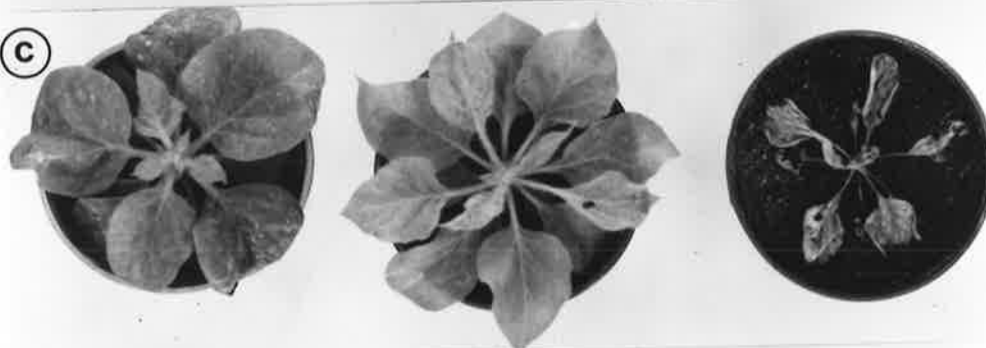


Figure 5.11

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $U_1K_2U_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

KCMV $U_1K_2U_3$ UCMV

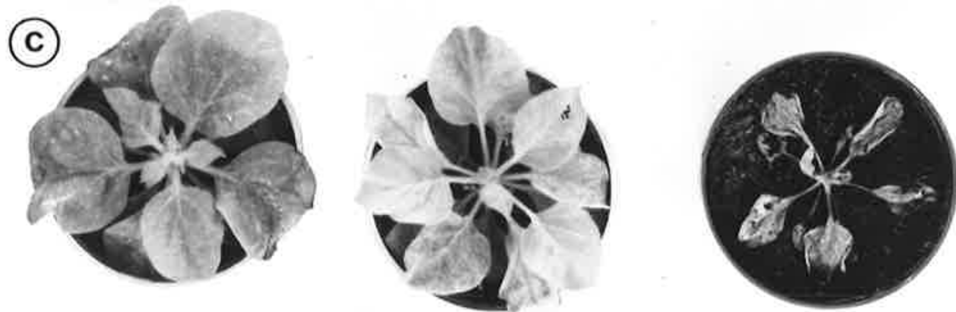
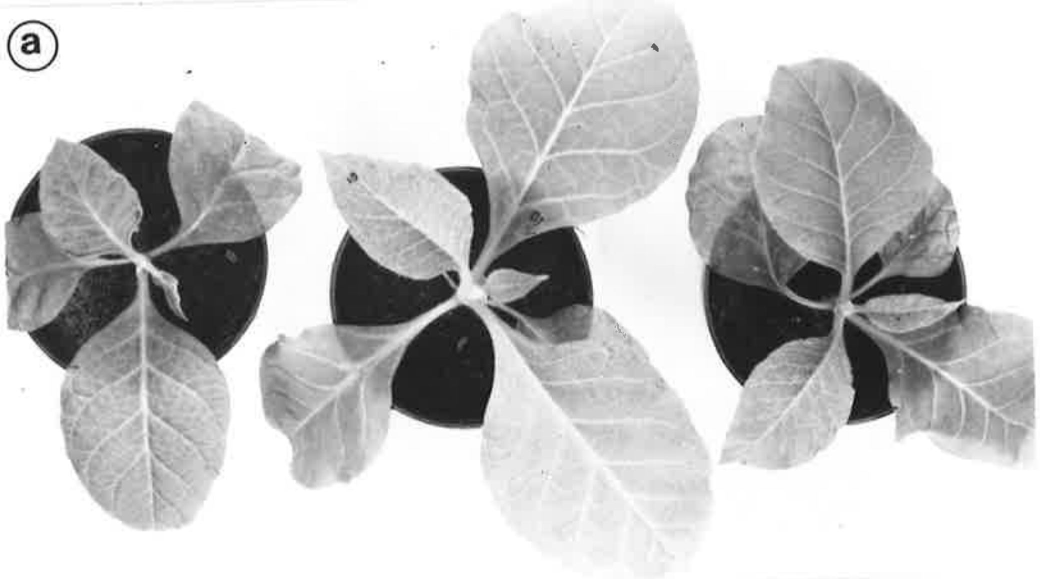
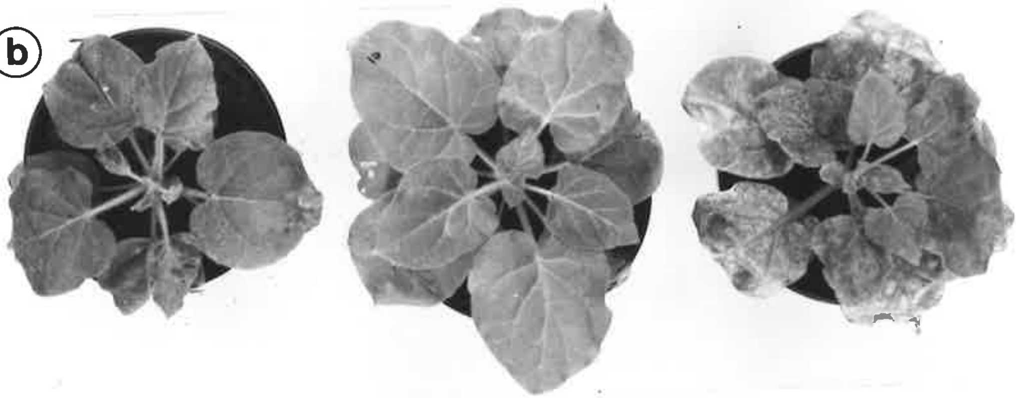


Figure 5.12

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $K_1U_2K_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.


KCMV
$K_1U_2K_3$
UCMV
a


b

c

d

Figure 5.13

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $U_1U_2K_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

KCMV $U_1U_2K_3$ UCMV

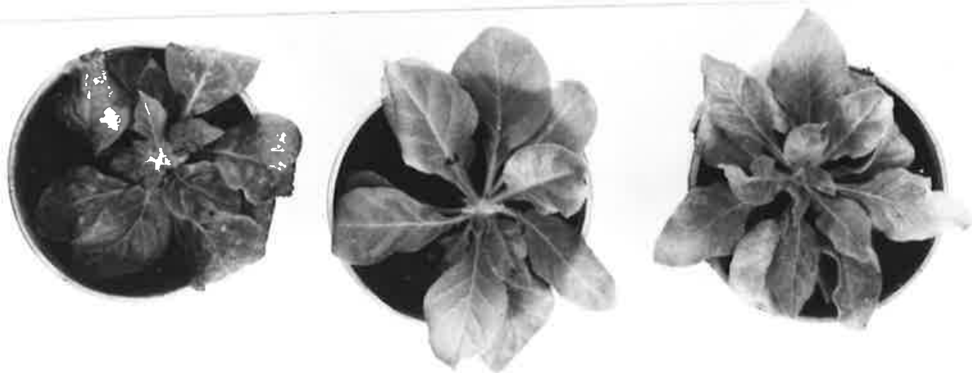
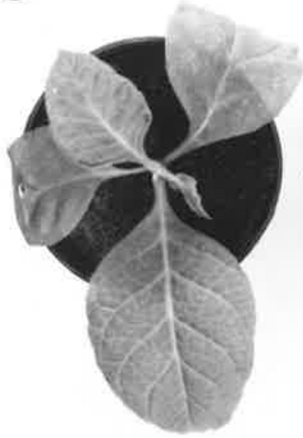
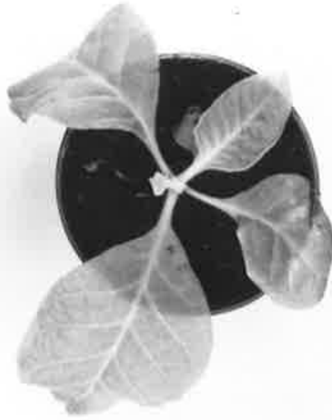


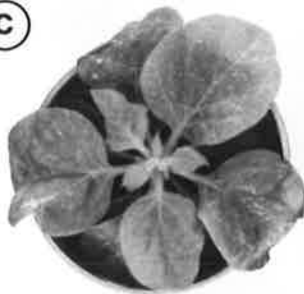
Figure 5.14

Symptoms produced in four Nicotiana species infected with KCMV (left), $K_1K_2U_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, N. glutinosa, N. x edwardsonii and N. clevelandii respectively.


KCMV
$K_1K_2U_3$
UCMV
a


b


c

d

Figure 5.15

Symptoms induced on *Datura stramonium* by UCMV, KCMV and pseudorecombinants constructed *in vitro* from the three genomic segments of the two virus strains.

UCMV

KCMV

$K_1U_2U_3$

$U_1K_2K_3$

$U_1K_2U_3$

$K_1U_2K_3$

$U_1U_2K_3$

$K_1K_2U_3$

Table 5.3

Characteristics of Pseudorecombinants between MCMV ($M_1M_2M_3$) and KCMV ($K_1K_2K_3$)*

Host Plant	Pseudorecombinant					
	Exchange of RNA-1		Exchange of RNA-2		Exchange of RNA-3	
	$K_1M_2M_3$	$M_1K_2K_3$	$K_1M_2K_3$	$M_1K_2M_3$	$K_1K_2M_3$	$M_1M_2K_3$
Nicotiana tabacum cv White Burley	= M	= K	§M(milder)	= M	= M	§M(milder)
N. glutinosa	= M	§K(milder)	§M(milder)	= M	= M	§M(milder)
N. x edwardsonii	= M	= K	§M(milder)	= M	= M	§M(milder)
N. clevelandii	= M	= K	§M(milder)	= M	= M	§M(milder)
Datura stramonium	≠ M, ≠ K[a]	= K	§M(milder)	≠ M, ≠ K[a]	= M	§M(milder)
Solanum melongena	= M	§K(milder)	§K(milder)	≠ M, ≠ K[b]	= M	§M(milder)
Gomphrena globosa	= M	§K(severe)	§M(milder)	= M	= M	= M
Cucumis sativus var. Polaris	= M, = K	= M, = K	= M, = K	= M, = K	= M, = K	= M, = K
Zea mays	= M	= K	= M	= K	= K	= M
Vica faba	= M	= K	= K	= M	= M	= K

* Signs describing symptoms are the same as in Table 5.1.

a Indicates that the pseudorecombinant $K_1M_2M_3$ and $M_1K_2M_3$ were indistinguishable from both the parental strains in producing chlorotic lesions on inoculated leaves, but failed to infect the plants systemically.

b Indicates that the pseudorecombinant produced chlorotic local lesions similar to MCMV but failed to infect the plants systemically.

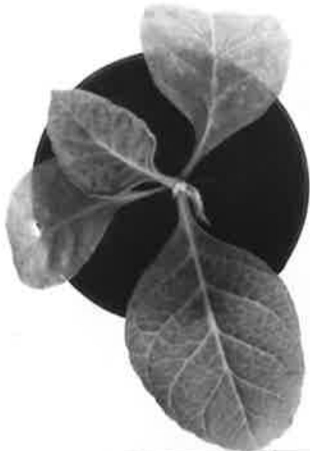
Figure 5.16

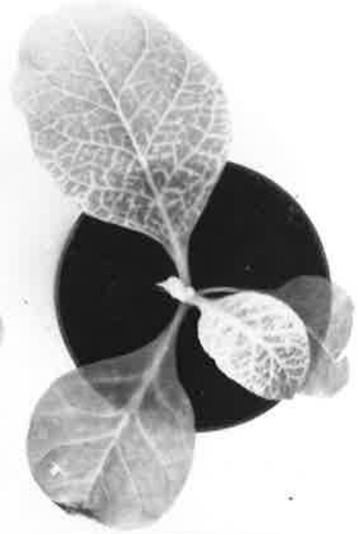
Symptoms produced in four *Nicotiana* species infected with KCMV (left), $K_1M_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

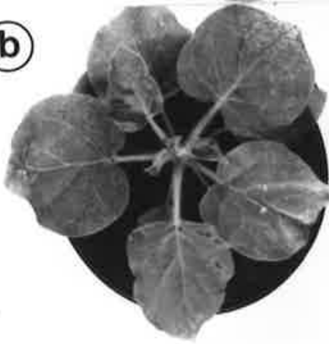
KCMV $K_1M_2M_3$ MCMV

(a)

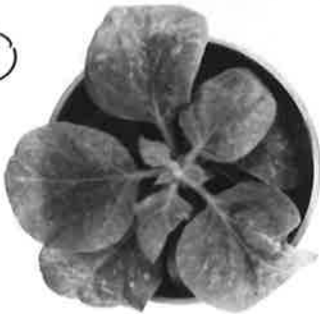




(b)



(c)



(d)

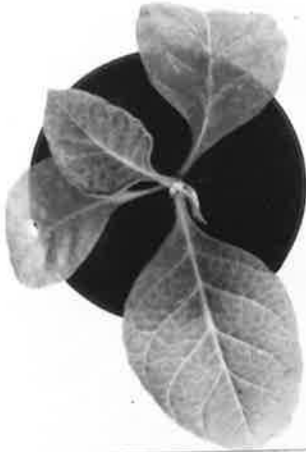
Figure 5.17

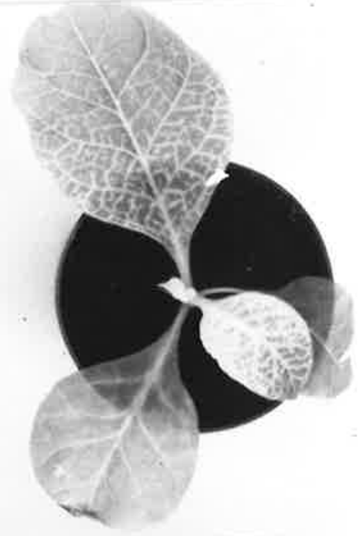
Symptoms produced in four *Nicotiana* species infected with KCMV (left), $M_1K_2K_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

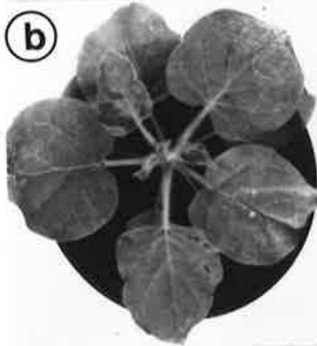
KCMV $M_1K_2K_3$ MCMV

(a)

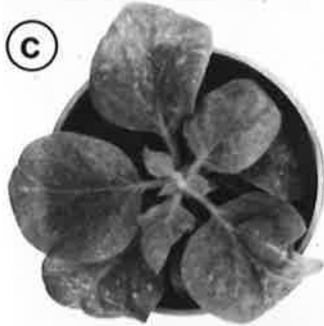




(b)



(c)



(d)

Figure 5.18

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $K_1M_2K_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

KCMV $K_1M_2K_3$ MCMV

Figure 5.19

Symptoms produced in four Nicotiana species infected with KCMV (left), $M_1K_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, N. glutinosa, N. x edwardsonii and N. clevelandii respectively.

KCMV | $M_1K_2M_3$ | MCMV

(a)

(b)

(c)

(d)

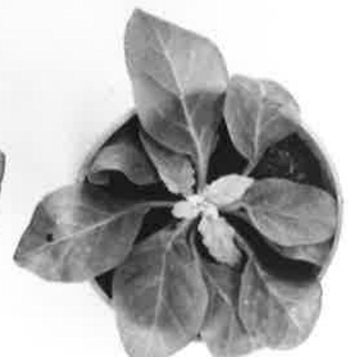


Figure 5.20

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $K_1K_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

KCMV $K_1K_2M_3$ MCMV

(a)

(b)

(c)

(d)

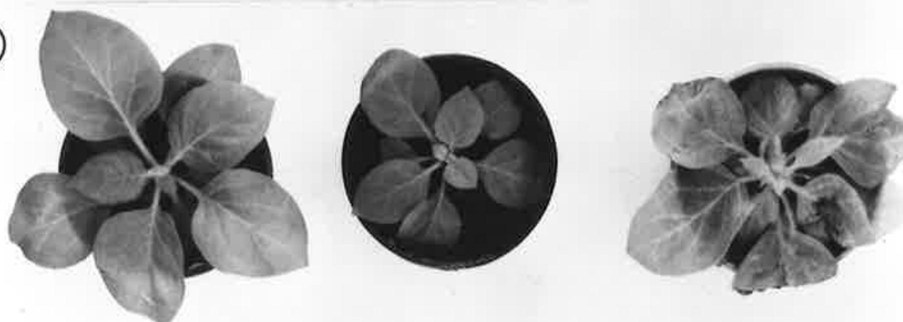
Figure 5.21

Symptoms produced in four *Nicotiana* species infected with KCMV (left), $M_1M_2K_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

KCMV
$M_1M_2K_3$
MCMV
a

b


c

d

Figure 5.22

Symptoms induced on Datura stramonium by KCMV, MCMV and pseudorecombinants constructed in vitro from the three genomic segments of the two virus strains.

KCMV

MCMV

$K_1M_2M_3$

$M_1K_2K_3$

$K_1M_2K_3$

$M_1K_2M_3$

$K_1K_2M_3$

$M_1M_2K_3$

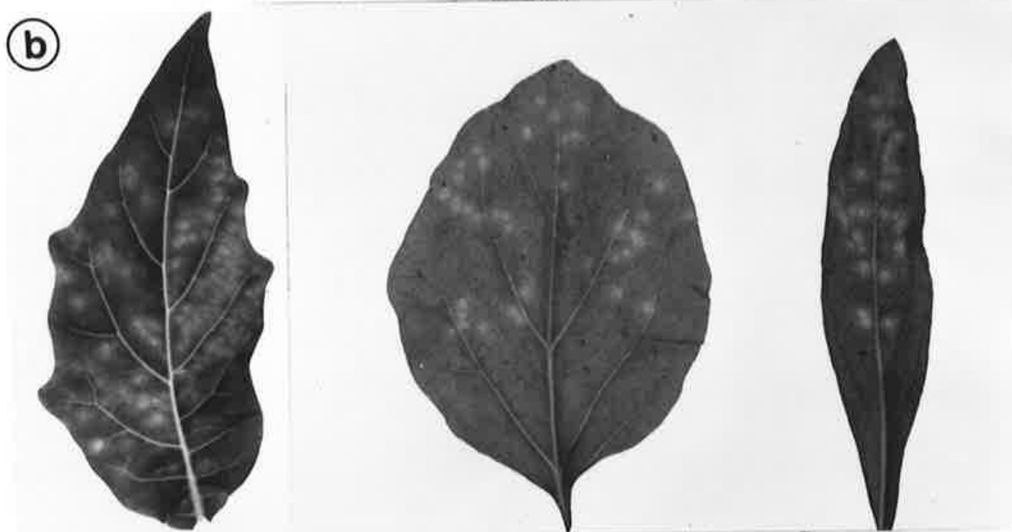
(Fig. 5.7 a, b, d) and in D. stramonium (Fig. 5.8), G. globosa and S. melongena (Table 5.1) was irregularly scattered along interveinal areas, indicating that this symptom is not determined by RNA-3 alone and that yellow mosaic is the result of interactions between genetic information on RNAs-2 and 3.

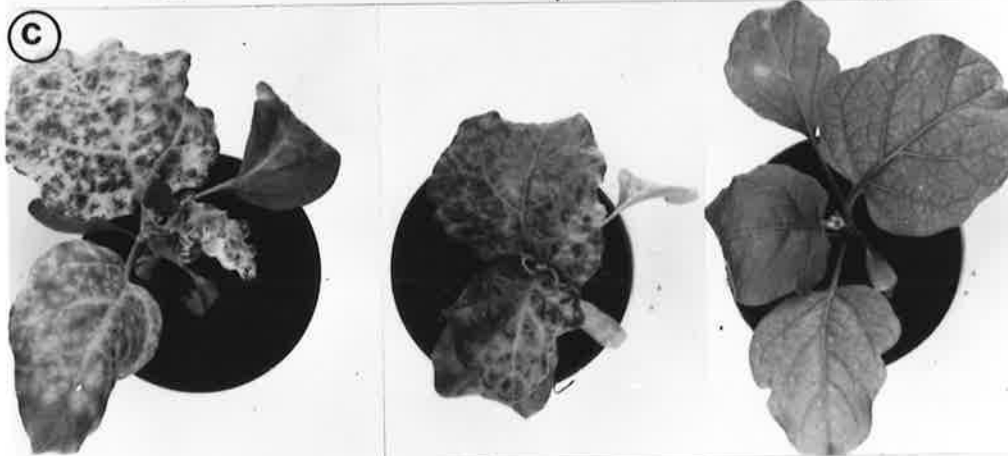
UCMV was the only strain of CMV used which did not induce lesions in any of the hosts other than C. sativus (Chapter 3, Table 3.1). MCMV induced chlorotic local lesions in D. stramonium, G. globosa and S. melongena (Fig. 5.23 b) while KCMV induced chlorotic local lesions in D. stramonium and brown local lesions in Vicia faba (Fig. 5.23 a). Data summarised in Tables 5.1-5.3 indicate that chlorotic lesions in D. stramonium and S. melongena are determined by both RNA-2 and RNA-3 because all pseudorecombinants containing either RNA-2 or RNA-3 or both RNA-2 and RNA-3 of MCMV induced chlorotic lesions in D. stramonium and S. melongena. Pseudorecombinants constructed from KCMV and UCMV confirmed this conclusion by their reactions on D. stramonium (Table 5.2). On the other hand, the ability to induce chlorotic lesions in G. globosa and brown lesions in Vicia faba is determined by RNA-3 alone because pseudorecombinants $U_1U_2M_3$ and $K_1K_2M_3$ induced chlorotic local lesions in G. globosa (Tables 5.1 and 5.3) while pseudorecombinants $U_1U_2K_3$ and $M_1M_2K_3$ induced brown lesions in Vicia faba (Tables 5.2 and 5.3).

Figure 5.23

Local lesions produced by KCMV on *Vicia faba* (a), and by MCMV (left to right) on *D. stramonium*, *S. melongena* and *G. globosa* (b) respectively. Systemic symptoms induced by MCMV, KCMV and UCMV respectively on *S. melongena* (c) and *G. globosa* (d).

a


b


c

d

III. IN VITRO CONSTRUCTION AND CHARACTERIZATION OF PSEUDORECOMBINANTS FROM RNA-1+2 OF VTAV AND RNA-3 OF THREE CMV STRAINS

Following the results obtained from the compatibility of genome segments from CMV and TAV (Chapter 4; Table 4.2) three pseudorecombinants were constructed from RNA-1+2 of VTAV and RNA-3 of UCMV, MCMV and KCMV. Although the infectivity data (Chapter 4; Table 4.2) indicate that VTAV RNA-3 stimulates the infectivity of RNA-1+2 from the three strains of CMV,attempts to induce local lesions in tobacco with mixtures of RNA-1+2 from UCMV, MCMV and KCMV and VTAV RNA-3 were unsuccessful. Moreover, attempts to recover these pseudorecombinants from lesions induced by genomic RNA mixtures on *C. amaranticolor* and *V. signensis* were also unsuccessful. Similar difficulties were also experienced by previous workers in our laboratory (Habili and Francki, 1974c; Mossop, private communication). The three pseudorecombinants, $V_1V_2U_3$, $V_1V_2M_3$ and $V_1V_2K_3$ were found to react with antisera to the parental CMV strains providing RNA-3 (e.g. Fig. 5.24 a). The electrophoretic mobility of RNA 3 of each pseudorecombinant was also identical to the parental strain providing RNA-3 (e.g. Fig. 5.24 b).

1. Symptomatology and Host Range of Pseudorecombinants

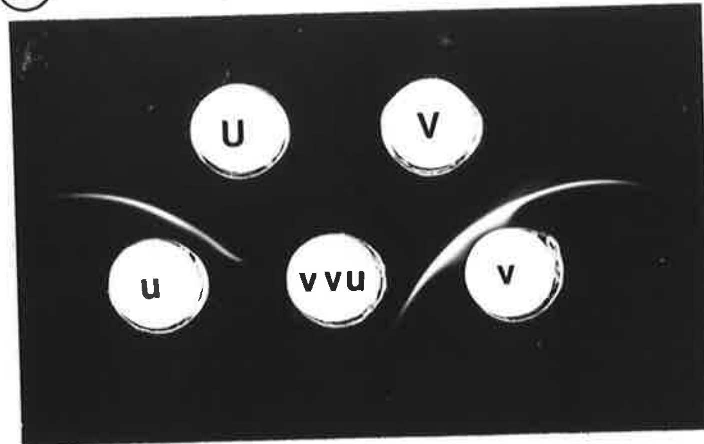
The data summarised in Table 5.4 indicate that the symptoms produced by $V_1V_2U_3$ and $V_1V_2K_3$ in four

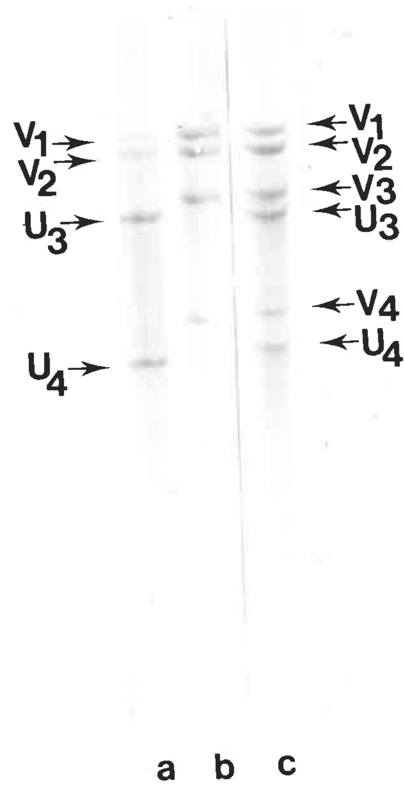
Figure 5.24

Analysis of RNA components and serological relationships between UCMV and VTAV and their pseudorecombinant.

a. Immunodiffusion test demonstrating antigenic identity of $V_1V_2U_3$ (vvu) with UCMV (u). Antisera of UCMV (U) and VTAV (V) were diluted to 1:64.

b. Electrophoretic analysis of the RNA components of $V_1V_2U_3$ in 2% agarose gels, $V_1V_2U_3$ (a), VTAV (b) and $V_1V_2U_3$ + VTAV (c).


a
U
V
u
vvu
v


b
V_1
V_2
U_3
U_4
V_1
V_2
V_3
U_3
V_4
U_4
a b c

Nicotiana spp. tested (Fig. 5.25, 5.27), G. globosa and C. sativus var Polaris were indistinguishable from those of VTAV, suggesting that RNA-1 and/or RNA-2 of VTAV determines the symptom expressions in these host species. On the other hand, the symptoms produced by $V_1V_2M_3$ in N. glutinosa, N. x edwardsonii and N. clevelandii (Fig. 5.26 b, c and d respectively) were more severe but similar to MCMV while those produced in N. tabacum (Fig. 5.26 a) were milder than MCMV and were not detectable until 4-5 weeks after inoculation. These observations confirm the earlier conclusions that determinants for systemic chlorosis also reside on RNA-3 (Table 5.1 and 5.3). Like VTAV, $V_1V_2M_3$ failed to infect C. sativus var Polaris systemically (Table 5.4).

Results presented in Table 5.4 also confirm that RNA 3 determines the lesion production in D. stramonium, S. melongena and G. globosa since $V_1V_2M_3$ induced lesions in these three host species, as did $V_1V_2K_3$ in D. stramonium and V. faba. It is also evident from Table 5.4 that pseudorecombinants between CMV and TAV strains could be quite different from the parent viruses, since $V_1V_2M_3$ and $V_1V_2K_3$ failed to infect D. stramonium and S. melongena systemically as did $V_1V_2U_3$ in S. melongena. However, $V_1V_2U_3$ produced more severe symptoms in D. stramonium than either of the parent viruses (Table 5.4). Like VTAV, $V_1V_2K_3$ failed to infect Z. mays systemically (Table 5.4).

Table 5.4

Characteristics of Pseudorecombinants Involving Exchange of RNA-1+2 of VTAV and RNA-3 of Three Strains of CMV*

	Pseudorecombinant		
Host Plant	$V_1V_2U_3$	$V_1V_2M_3$	$V_1V_2K_3$
Nicotiana tabacum cv White Burley	= V	§M(milder)	= V
N. glutinosa	= V	§M(severe)	= V
N.x edwardsonii	= V	§M(severe)	= V
N. clevelandii	= V	§M(severe)	= V
Datura stramonium	≠ V, ≠ U[b]	≠ M, ≠ V[a]	≠ V, ≠ K[a]
Solanum melongena	≠ V, ≠ U[c]	≠ M, ≠ V[a]	≠ V, ≠ K[c]
Gomphrena globosa	= V, = U	≠ M, ≠ V[a]	= V
Cucumis sativus var. Polaris	= V	= V	= V
Zea mays	= V, = U	= V, = M	= V
Vicia faba	= V, = U	= V, = M	= K

* Signs describing the symptoms are the same as in Table 5.1.

a These pseudorecombinants produced chlorotic local lesions on the inoculated leaves but failed to infect the plants systemically.

b This pseudorecombinant produced more severe mosaic and leaf distortion symptoms than either of the parental viruses.

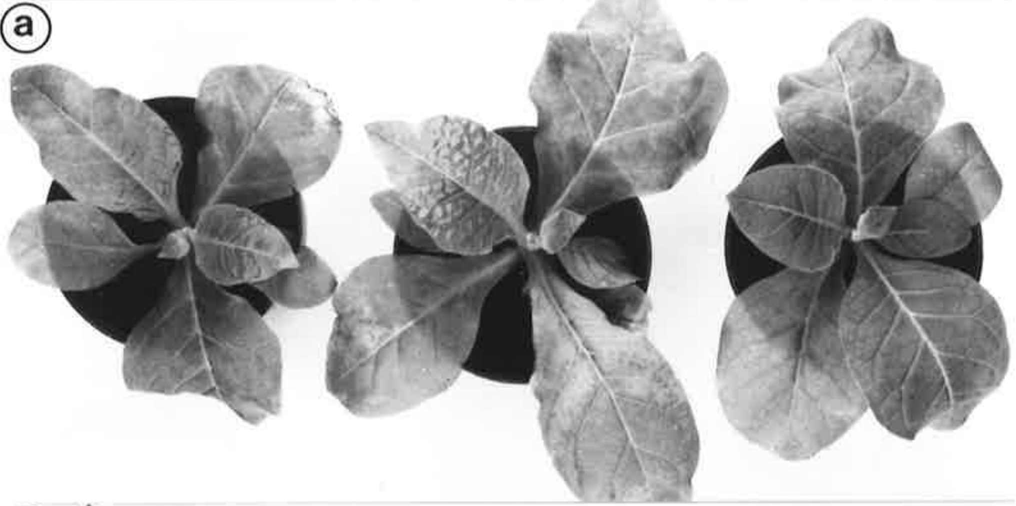
c These pseudorecombinants failed to infect this host systemically.

Figure 5.25

Symptoms produced in four *Nicotiana* species infected with VTAV (left), $V_1V_2U_3$ (middle) and UCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

VTAV $V_1V_2U_3$ UCMV



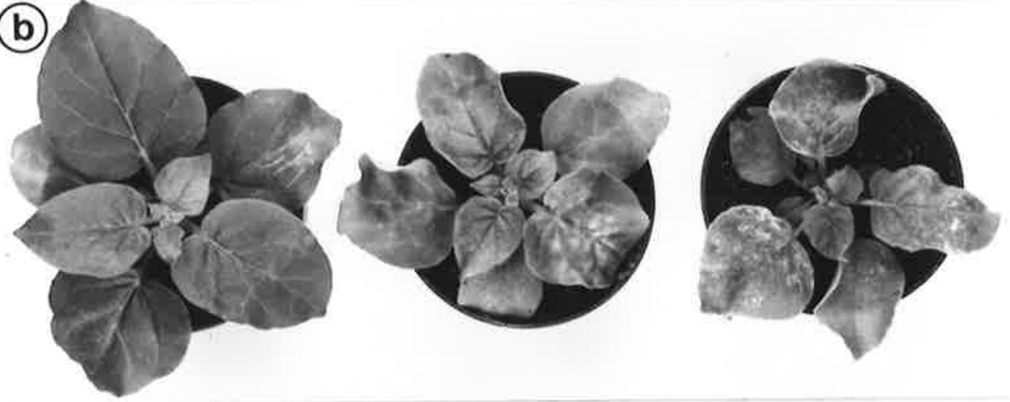


Figure 5.26

Symptoms produced in four *Nicotiana* species infected with VTAV (left), $V_1V_2M_3$ (middle) and MCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N.* x *edwardsonii* and *N. clevelandii* respectively.

VTAV $V_1V_2M_3$ MCMV

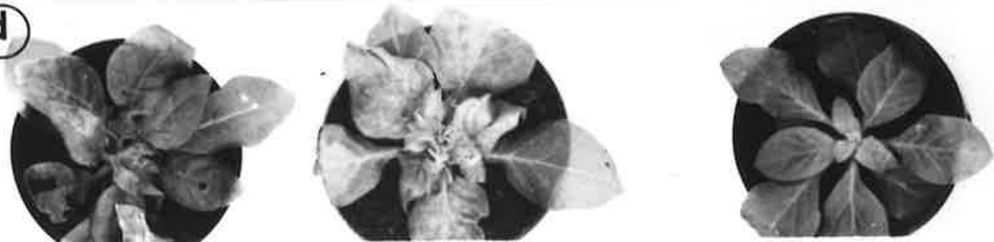


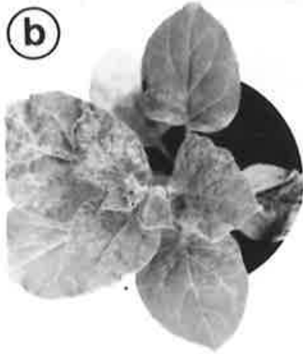
Figure 5.27

Symptoms produced in four *Nicotiana* species infected with VTAV (left), $V_1V_2K_3$ (middle) and KCMV (right), 12 days after inoculation. Host species in rows from top to bottom are:

N. tabacum cv White Burley, *N. glutinosa*, *N. x edwardsonii* and *N. clevelandii* respectively.

VTAV
a

$V_1V_2K_3$

KCMV

b

c

d

IV. CONCLUSIONS

The results presented here enable the following conclusions to be drawn:

1. Table 5.5.

Distribution of Genetic Determinants for Biological Characters of CMV

Character	Location of Determinant(s)
Systemic necrosis in *N. x edwardsonii*	RNA-2
Leaf blistering and distortion	RNA-2
Ability to infect maize systemically	RNA-2
Lesion type in *G. globosa*	RNA-3
Lesion type in *V. faba*	RNA-3
Yellow mosaic in several host species	RNA-2 and RNA-3
Lesion type in *D. stramonium*	RNA-2 and RNA-3
Lesion type in *S. melongena*	RNA-2 and RNA-3

2. In addition to the above, some systemic symptom expression appears to involve interaction of any two or perhaps even all three RNA segments of the virus.

3. Some symptom expression in plants appear to be a complex interaction of the genetic material of the virus with that of the host genome.

4. Symptom expression in Nicotiana spp. by VTAV and pseudorecombinants containing VTAV-RNA 1 + 2 is apparently determined by gene(s) located on RNA 1 and/or RNA 2.

5. The ability of cucumoviruses to infect C. sativus is controlled by RNA 1 and/or RNA 2.

6. Some symptom expressions appear to involve interactions between genomic RNA 1 and/or RNA 2 of TAV and RNA 3 of CMV.

CHAPTER 6

GENERAL DISCUSSION

I. SEROLOGICAL RELATIONSHIPS BETWEEN CMV AND TAV

CMV and TAV have many properties in common but their relationship to each other has been of some controversy (see Chapter 1). The varied conclusions reached by different authors concerning the relationship between the two viruses are largely based on serological and cross protection studies. It is well documented that cross-protection tests are generally less reliable for studying virus relationships (Kassanis, 1963). Hence it has been suggested that serological data would provide the best evidence of virus or strain relationships (Knight, 1959; Cadman and Lister, 1962). However, it must be pointed out that the results of serological tests largely depend on the method of assay and the titre of the antisera used, especially if the two viruses are distantly related.

It has been demonstrated that CMV is a poor immunogen (Francki and Habili, 1972). However it is now possible to produce high titred antisera to cucumoviruses using highly purified virus preparations stabilised with aldehydes. During the course of this study, antisera were produced against several strains of CMV and TAV with which I was

able to demonstrate that the two viruses are serologically distantly related. This conclusion is contrary to that of Habili and Francki (1974a; 1975) who had used one strain each of CMV and TAV. The distant antigenic relationship between CMV and TAV has been established by immunodiffusion tests in agar (Table 3.3) and ELISA (Fig. 3.4 -3.7) while IEM particle decoration method appeared to be the least convincing (Fig. 3.8). Although heavy decoration with homologous antibodies was easily observed, decoration with heterologous antibodies was rather difficult to distinguish from the background granularity around particles reacted with antisera to completely unrelated antigens. On the basis of my experience, I find this technique difficult to recommend as a reliable method for the detection of distant antigenic relationships between CMV and TAV.

The reason why the distant antigenic relationship between CMV and TAV was not detected by Habili and Francki (1974a; 1975) in their immunodiffusion tests is not clear. Although most antisera used by them had relatively low titres, some anti-TAV and anti-CMV sera had titres as high as 1/128 and 1/256 respectively (Habili and Francki, 1975). Judging from my results presented in Table 3.3, they should have produced cross reactions. A possible explanation for the lack of cross reactions in their tests may be that the antisera were produced in mice which could have been more discriminatory than those from rabbits used in

this study. Moreover, unfixed virus preparations were used as test antigens in their immunodiffusion tests which usually produce more diffuse precipitin lines than those found with fixed virus and hence are more difficult to detect (Francki and Habili, 1972).

My work has compared the relative virtues of immunodiffusion tests, ELISA and IEM. Of these, ELISA appears to be a versatile method. In its standard form (direct method) where heterologous antibodies are used for both plate coating and coupling to enzyme, it is very specific as already reported for other viruses (Koenig, 1978; Lister and Rochow, 1979; Bar-Joseph and Salomon, 1980). The superiority of this test in its standard form is illustrated by the detection of antigenic differences between closely related strains of CMV (Fig. 3.4 and 3.5) some of which could not be detected in immunodiffusion tests using mixtures of antisera (Fig. 3.2). Similar results have been reported in ELISA tests for citrus tristeza virus (Bar-Joseph et al, 1979), apple chlorotic leaf spot virus (Detenne et al, 1979), bean yellow mosaic virus (Stein et al, 1979) and potato virus Y (Weidermann and Koenig, 1979). On the other hand, using heterologous antibodies for coating and homologous antibodies for coupling with enzyme, ELISA detected a distant antigenic relationship between CMV and TAV (Fig. 3.6 and 3.7) which was less readily demonstrated in immunodiffusion tests.

Recently Devergne et al. (1981) have compared the relative values of direct and indirect ELISA methods for detecting the antigenic relationships between several cucumovirus strains. As in my studies, they observed that direct ELISA was capable of detecting closely related strains of CMV but not the distant relationships between CMV, TAV and PSV. However, they detected the distant relationships between these viruses by the indirect ELISA method. These observations are similar to those reported for tobamoviruses (Van Regenmortel and Buckard, 1980), bromoviruses (Rybick and Von Wechman, 1981) and baculoviruses (Crook and Payne, 1980).

My studies with CMV and TAV have shown that the results of direct ELISA tests agree with those of Devergene et al (1981) while heterologous and homologous direct ELISA gives similar results to those obtained in indirect ELISA by Devergene et al (1981). However, the implications of these methods are felt to be important in a number of ways. Heterologous and homologous direct ELISA has the advantage that the tests can be done with only one antiserum for each virus to be tested, whereas the indirect form requires two antisera against each virus raised in two different animal species. The disadvantage of direct ELISA is that it necessitates the preparation of different antibody conjugates for each virus to be tested. On the other hand, indirect ELISA requires only one antiglobulin enzyme conjugate for all

tests. However the high specificity of direct ELISA raises problems in detecting closely related viruses. For example, Rochow and Carmichel (1979) suggested that conjugated antibodies in at least five different antisera would be needed to detect the presence of the five major strains of barley yellow dwarf virus. Hence the application of direct ELISA may be of limited use for diagnostic work, although it is very useful for the identification of closely related virus strains (Van Regenmortel and Burckard, 1980).

II. COMPATIBILITY OF GENOME SEGMENTS FROM TAV AND CMV

Work done previously demonstrated the *in vitro* construction of a pseudorecombinant from RNAs-1 + 2 of TAV and RNA-3 of CMV (Habili and Francki, 1974c). During endeavours to construct further pseudorecombinants from the genome segments of the two viruses, I observed that whereas RNA-3 is readily interchangeable between the two viruses, RNA-1 and RNA-2 are not. At present there is no single criterion to predict whether complimentary genome segments of different viruses will form infectious combinations or not. Nevertheless, Lane (1979) suggested that within a taxonomic group, the genome segments of close serological relatives are compatible while those of distant relatives are not. For example, the genome components are freely exchangeable between closely related strains of AMV

(Van Vloten-Doting et al. 1970), CMV (Marchoux et al. 1974a, b; Hanada and Tochihara, 1980, Table 4.2), cherry leaf roll virus (Jones and Duncan, 1980; Haber and Hamilton, 1980) and cowpea mosaic virus (Thongmeearkom and Goodman, 1978). On the other hand, distantly related strains of tobacco rattle virus (TRV; Sanger, 1969) and different viruses in the comovirus group (Van Kammen, 1968; Govier, 1975) are incompatible. However there are indications that among distantly related viruses certain combinations of genome segments are viable and often the interchange is unilateral. For instance, in the case of the Y and Z strains of tobacco rattle virus, a heterologous mixture containing short particles of TRV-Y and long particles of TRV-Z was shown to be infectious while the reciprocal combination was not (Ghabrial and Lister, 1973). Similar observations were recorded between two distantly related isolates of tomato black ring virus (Randles et al, 1977). In the case of viruses which have tripartite genomes, the distant serological relatives are unilaterally compatible and the only segments which interchange are apparently those which carry the coat protein gene (Lane, 1979). Bancroft (1972) reported the construction of a pseudorecombinant from RNAs-1+2 of BMV and RNA-3 of CCMV. But in this case the reciprocal combination is not viable. Similar difficulties were also reported for CMV and TAV (Habili and Francki, 1974c). However, results presented in Table 4.2 clearly demonstrated that all combinations of genomic RNAs-1+2 and RNA-3 from

four strains of CMV or TAV were infectious on C. amaranticolor. It seems likely that the apparent incompatibility of CMV RNA-1+2 and TAV RNA-3 on C. amaranticolor reported by Habili and Francki (1974c) was due to the host plant insensitivity because the lesions produced by this particular combination are extremely small and develop only on the most sensitive leaves (Mossop, 1978; Table 4.2). It is possible that the apparent incompatibility of heterologous genomic RNA mixtures of BMV and CCMV (Bancroft, 1972), RRV and TRSV (Harrison et al, 1972) and strains of TRV (Ghabrial and Lister, 1973) may be explained on the basis that these pseudorecombinants produce extremely small lesions which escaped detection on the indicator plants chosen. Alternatively, Van Vloten-Doting et al. (1977) suggested that the incompatibility results from the fact that a 'replicase subunit' specified by the genomic RNA of one strain does not recognize the complementary genomic RNA of another. However, it is not clear how this suggestion explains why some heterologous mixtures are compatible while the reciprocal combinations are not. Perhaps investigations on synthesis of specific RNAs and proteins in protoplast systems might provide some answers to this question.

In contrast to the partial compatibility observed between CMV and TAV in this study, Marchoux et al. (1974) reported that all combinations of their strains of CMV, TAV and PSV were viable. Since there are no indications regarding

the authenticity of their pseudorecombinants, it must be pointed out that it is necessary to check the pseudorecombinants by back crossing or other suitable techniques to exclude the possibility of isolating the wrong pseudorecombinants or mutants. Hence it would be interesting to extend the work reported here on the compatibility of genome segments to a wider range of cucumoviruses. Most cucumoviruses are serologically related (Devergene et al, 1981) and hence decisions regarding whether two cucumoviruses are distinct viruses or strains of the same virus are rather arbitary. Genome segment compatibility may be a better criterion for making such decisions.

III. GENETIC ANALYSIS OF CUCUMOVIRUSES

It had previously been demonstrated that RNA-3 determines the antigenic properties of CMV and TAV (Habili and Francki, 1974c; Marchoux et al, 1974a,b; Mossop and Francki, 1978; Hanada and Tochihara, 1980) but the genetic functions of RNA-1 and RNA-2 are unknown. The work presented in chapter 5 describes the biological properties of pseudorecombinants constructed in vitro by exchanging all the RNAs between pairs of CMV strains. Although mixtures of RNAs-1+2 from four strains of CMV and RNA-3 from VTAV were infectious on C. amaranticolor (Table 4.2), attempts to recover these pseudorecombinants by local lesion transfer were unsuccessful. Similar difficulties were experi-

enced by Habili and Francki (1974c) and Mossop (1978). The explanation for this problem remains obscure in spite of the fact that CMV-RNA is efficiently encapsidated in vitro with VTAV coat protein and vice versa (Habili, 1974). Hence the conclusions presented in this thesis concerning the distribution of genetic determinants for biological properties on RNAs 1, 2 and 3 were mainly based on the properties of pseudorecombinants constructed between the RNA genome segments of CMV strains.

My observations on the ten host plant species infected with three strains of CMV and the 18 pseudorecombinants constructed from them, indicate that all the three genome segments can be involved in symptom production (Tables 5.1 - 5.3). It was demonstrated that some host reactions are controlled by only one RNA segment. Some reactions were also identified as being controlled by the interaction of two or perhaps even three RNA segments.

The most compelling evidence that a single RNA segment can control a specific symptom comes from pseudorecombinants which carry genome segments such as RNA-2 from UCMV and which invariably produced severe necrosis on N. x edwardsonii (Tables 5.1 and 5.2). Similarly, pseudorecombinants with RNA-2 from KCMV were all able to infect Z. mays (Tables 5.2 and 5.3). Evidence that RNA-3 carries a specific determinant for symptom production comes from

pseudorecombinants carrying RNA-3 from KCMV which were all able to produce brown local lesions in V. faba (Tables 5.2 and 5.3).

Evidence that the same symptom reactions can be controlled by either RNA-2 or RNA-3 is provided by pseudorecombinants containing either of these segments from MCMV and inoculated to D. stramonium and S. melongena; they all produced local chlorotic lesions (Tables 5.1 and 5.3). However, a similar symptom on G. globosa can be produced only by a determinant on RNA-3 since pseudorecombinants carrying RNA-2 from MCMV do not induce local chlorotic lesions on this host (Tables 5.1 and 5.3). This illustrates the importance of the interaction of the host genome with that of the virus in symptom production. In the case of D. stramonium and S. melongena the host genome can interact with either RNA-2 or RNA-3 of MCMV to produce the lesions whereas in G. globosa only RNA-3 is capable of this interaction.

Evidence that some symptoms produced by CMV are the results of the interaction of two or more viral genome segments is demonstrated by the reactions of some of the pseudorecombinants which produce symptoms unlike those of either parental virus strains (Tables 5.1 - 5.3). Several pseudorecombinants behaved in this way on D. stramonium. In three instances pseudorecombinants failed to infect hosts which were susceptible to both parental virus strains (Tables 5.1 - 5.3).

It seems clear from my work that symptom expression in a plant can be a complex interaction of the genetic material of the plant host with that of the virus. It would appear that previous apparent inconsistencies regarding the parts played by the three cucumovirus RNA segments in host symptom expression (Marchoux et al, 1974; Habili and Francki, 1974c; Mossop and Francki, 1977; Hanada and Tochihara, 1980) were due to the relatively few pseudorecombinants tested on too few host plants.

Reactions of ten host plant species infected with three pseudorecombinants constructed by exchanges of RNA-3 between CMV and TAV confirmed that most host reactions are controlled by more than one RNA segment (Table 5.4). These results are similar to those of Habili and Francki (1974c) and Hanada and Tochihara (1980). It was observed that some host reactions are apparently due to the interaction of genomic RNAs of CMV and TAV. Evidence for this is provided by the reactions of some pseudorecombinants on D. stramonium (Table 5.4). It was confirmed that brown local lesions on Vicia faba and chlorotic local lesions on G. globosa are determined by RNA-3 of KCMV and MCMV respectively (Table 5.4). Reactions of $V_1V_2M_3$ on Nicotiana spp. and D. stramonium confirmed that RNA-3 in part carries the genes for systemic chlorosis and chlorotic local lesion production respectively (Table 5.4).

The pseudorecombinant studies described in this thesis are similar to those of other viruses with tripartite genomes (Table 6.1). It appears from Table 6.1 that all these viruses are genetically very similar. For example, in all the cases the gene specifying viral coat protein is located on the smallest genomic RNA segment. However, it has been demonstrated that in BMV, AMV and CMV, RNA-3 does not represent the *in vivo* mRNA for coat protein synthesis while the subgenomic RNA-4, which is derived *in vivo* from RNA-3, is the coat protein mRNA (Shih and Kaesberg, 1973; Mohier, 1975; Schwinghamer and Symons, 1977). The other similarity between these viruses is the location of the genetic determinants for local lesion production. In CMV and AMV, both RNA-2 and RNA-3 determine the lesion development on some of the host plants examined (Table 6.1). Furthermore, my studies indicate that RNA-1 does not carry any demonstratable genetic determinants (Table 6.1). These observations are similar to those reported for bromo and alfalfa mosaic viruses (Table 6.1), except for artifically produced mutants.

TABLE 6.1

Distribution of Genetic Determinants in Plant Viruses with Tripartite Genomes

Virus	RNA-1	RNA-2	RNA-3	Reference
Brome mosaic virus			Lesion type in *C. hybridum* Alterations in electrophoretic mobility of RNA components Coat protein changes	Bancroft and Lane, 1973
Cowpea chlorotic mottle virus	Temperature sensitivity	Lesion type in *C. hybridum*	Coat protein changes Serological specificity Systemic symptom changes	Bancroft and Lane, 1973
			Assembly mechanism	Bancroft *et al*, 1976
	Replicates in resistant cowpeas			Kuhn and Wyatt, 1979
Cucumber mosiac virus		Lesion type in cowpea ←→ Ability to infect cowpea systemically	Lesion type in cowpea Systemic symptoms in tobacco Serological specificity Electrophoretic mobility	Marchoux *et al*, 1974a Marchoux *et al*, 1974b
			Serological specificity Aphid transmissibility	Mossop and Francki, 1977
		Ability to infect cowpea	Serological specificity	Hanada and Tochihara, 1980
		Systemic necrosis in *N. x edwardsonii* Leaf blistering and distortion in several host plants Ability to infect maize systemically Yellow mosaic in several host plants ←→ Lesion type on *D. stramonium* ←→ Lesion type on *S. melongena* ←→	Lesion type on *G. globosa* Lesion type on *V. faba* Serological specificity Yellow mosaic in several host plants Lesion type on *D. stramonium* Lesion type on *S. melongena*	This study
Tomato aspermy virus			Serological specificity	Habili and Francki, 1974c
			Serological specificity	This study
Alfalfa mosaic virus		Lesion type on *P. vulgaris* ←→ Symptoms on *P. vulgaris*	Lesion type on *P. vulgaris* Serological specificity	Majorana and Paul, 1969 Dingjan-Versteegh *et al*, 1972
	Temperature resistance		Symptoms in tobacco Lesion type in tobacco	Frank and Hirth, 1976 Hartmann *et al*, 1976

←→ shows both RNAs have influence on the property

APPENDIX

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