INTERACTIONS OF TWO CUCUMOVIRUSES

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SUMMARY

1. Two cucumoviruses, the M (white mutant) strain of cucumber mosaic virus (MCMV) and the V strain of tomato aspermy virus were selected for studies on mixed infections of RNA plant viruses. MCMV differs from the other cucumoviruses by its characteristic systemic yellow mosaic symptoms and aphid non-transmissibility. The choice of CMV and TAV was based on the need to use two viruses close enough to enable molecular interactions, but sufficiently distant not to cross-protect against each other.

2. Antisera made against MCMV and VTAV fixed virus preparations showed no cross reaction in either gel immunodiffusion or ELISA tests. Molecular hybridization analysis using cDNA probes to total viral RNA showed there was less than 1% sequence homology between the two viruses. It was thus possible to independently detect the presence of each virus in coinfections by ELISA, while hybridization analysis provided a means of determining the genomic composition of mixed infections and variant isolates.

3. MCMV and VTAV were shown to readily coinfect a wide range of plant species systemically. Both viruses were also detected in the same local lesions on hypersensitive hosts. The symptoms induced in systemically infected plants were characteristic of, and dependent on the dominant component of mixed inocula, or which virus was inoculated first. There was no cross-protection between the two viruses, and the coinfections were found to persist to varying extents during passaging in a range of plant species tested.

4. The standard method used for cucumovirus purification was satisfactory for VTAV but not for MCMV. However, MCMV could be purified by an alternative method which was unsuitable for VTAV. When virus was purified from mixedly infected leaves by the VTAV method, no virus encapsidated in MCMV coat protein was recovered. However, when similar material was purified by the MCMV method, small amounts of VTAV were recovered. Virus preparations from mixedly infected leaves purified by the VTAV method were inoculated to *Beta vulgaris*, a local lesion host of both MCMV and VTAV, in an

endeavour to isolate virus variants. Four of the variants isolated were shown to be MCMV-like indicating that they were transcapsidants of MCMV RNAs in VTAV coat protein. A fifth variant, however was shown to consist of VTAV RNAs 1 and 2 and MCMV RNA 3. This indicates that it arose from MCMV RNA 3 encapsidated in VTAV coat protein which associated subsequently with VTAV RNAs 1 and 2.

Most isolates from *Beta vulgaris* inoculated with virus recovered by the MCMV purification method were MCMV-like. However, a few were obtained which induced VTAV-like symptoms but contained mostly MCMV RNA sequences. After passaging through *Cucumis sativus* which is immune to VTAV, these isolates lost their VTAV-like character. This indicates that they contained the complete genome of MCMV and at least some genome segments containig VTAV RNA sequences.

5. Attempts were also made to isolate variants by inoculating hypersensitive hosts with inocula from leaf tissues infected by both MCMV and VTAV. From these experiments, a variant isolate was obtained which consisted of VTAV RNAs 1 and 2 and an RNA 3 encoding both the MCMV coat and 3a proteins but which migrated slower during agarose gel electrophoresis than did the MCMV RNA 3. The mechanism by which this variant arose remains obscure.

6. The third method devised utilised the transmissibility by aphids of VTAV but not MCMV in attempts to isolate variants from coinfected plants. Most of the isolates were indistinguishable from VTAV. A few contained both MCMV and VTAV RNA sequences but the symptoms induced, and antigens detected were VTAV-like. However, the MCMV RNA sequences were lost during passaging in *Nicotiana clevelandii*. Nevertheless, two isolates were obtained which were MCMV-like and could not be transmitted further by aphids, indicating that they were transmitted in VTAV coat protein. Two other MCMV-like isolates in which VTAV RNA sequences were detected by dot-blot hybridization analysis were, however, found to be aphid transmissible. It was unclear whether this property was due to mutation, or had been conferred on them by the VTAV RNA sequences detected.

STATEMENT

I declare that the material presented in this Thesis has not been previously presented for an academic award in this or any other University. Published reports of other researchers cited are gratefully acknowledged.

SAMMY TAWIAH SACKEY

TO THE MEMORY OF MY LATE FATHER, EMMANUEL,

to my mother, Martha Korkor Siau, and my brothers and sisters in Ghana,

and to Susan and Matthew in Adelaide.

CHAPTER ONE

INTRODUCTION

"I suspect that many plant diseases are influenced by associated organisms to a much more profound degree than we have yet realized, not only as to inhibition, but as to acceleration of the processes. It may be that a number of diseases may require an association of organisms for their occurrence and cannot be produced by infection of one organism alone. These considerations appear to indicate an inviting field for much more extended research."

H.S. FAWCETT (1930)

1.0. Mixed infections of plant viruses.

Since Fawcett (1930) made these comments, studies of mixed virus infections have played an important role in the development of plant virology. Identification of diseases caused by more than one virus, interactions among related viruses resulting in cross-protection, and studies of interactions among unrelated viruses have all contributed to current concepts in plant virology. Rochow (1972) gave several reasons for the importance of mixed infections in virus epidemiology. First, research on the role of mixed infections is a useful approach to understanding some basic relationships between viruses and their aphid vectors. Secondly, certain mixed infections represent a place where current interest in heterogeneity in the assembly of viral protein and nucleic acids can focus on a practical or functional role in the plant virus evolution and transmission processes. Best (1961), Thompson (1961), and Watson (1960) were among the earliest exponents of the possibility of recombination arising from coinfections. The discovery of multiple genetic components in virus populations (Bancroft, 1971; Lane and Kaesberg, 1971; Lot et al., 1974; Peden and Symons, 1973; van Kammen, 1967 and 1968) underscores the importance of heterogeneity of plant virus populations even in singly infected plants (Domingo et al., 1985; Domingo and Holland, 1988) while offering potential sources of variability through pseudorecombination. A third reason arises from relationships such as those in which one component of a mixed infection requires the presence of another for its transmission.

1.1. Types of Mixed Infections

It has been known for a long time that plants infected with one virus often retain susceptibility to infection by a second, related or unrelated virus. McKinney (1929) first described mixed infections of two strains of tobacco mosaic virus (TMV) in tobacco plants. Thung (1931) and Salaman (1933) subsequently confirmed these observations in other plant-virus systems. Thus, under field conditions, a plant may, and often does, carry more than one virus. Each virus may multiply and invade the plant according to its own peculiar pattern and produce its own characteristic effects. The multiple infections may result in the formation of "dependent" relationships in which one virus depends on another for its multiplication (Kassanis and Nixon, 1960) or transmission (Kassanis, 1962; Kassanis and Govier, 1971). Alternatively "independent" virus complexes representing various equilibrium concentrations of the components modulated by environmental conditions may be formed. With successive passaging through the same or different host plants, the complex may assume a distinct and unique set of biological and physical properties. Thus, the disease known as carrot motley dwarf and originally attributed to a single virus by Stubbs (1948) was shown by Watson et al., (1964) to be caused by a complex of two viruses. Upon separation, the components of the complex may exhibit symptomatology, host range, mode of transmission and other properties quite different from those associated with the complex.

Bennett(1953) pointed out that under field conditions, beets are often simultaneously infected with beet curly top, beet mosaic, beet yellow net and dodder latent mosaic viruses. Multiple virus infections have also been found in barley (Rochow and Jedlinski, 1970), parsnip (Murant and Goold,1968), potatoes (Badami and Kassanis, 1959; Bagnall, 1956) and many other crops.

The common occurrence of mixed infections in the field provides both the source of most doubly infected systems studied, as well as a focus for experimental work. Bennet (1953) described two types of interactions arising from multiple infections of plant viruses; those between related viruses, and those between unrelated viruses. Interactions between

unrelated viruses may be either antagonistic or synergistic. Interactions between related viruses, however, were almost invariably antagonistic.

1.1.1. *Antagonistic Interactions Between Unrelated Viruses.*

Antagonistic interactions between apparently unrelated viruses vary in the degree of interference, and possibly also in the type of interference involved. McKinney (1941) attempted superinfection of *Nicotiana sylvestris* plants previously inoculated with cucumber mosaic virus, celery mosaic virus, potato vein banding virus or potato virus Y with *Nicotiana* virus 6. He showed that the numbers of lesions produced were reduced, and their appearance delayed in the test plants compared to controls inoculated with *Nicotiana* virus 6 alone. He also showed that previous infections with tobacco ringspot virus resulted in a reduction in the numbers of lesions produced by a yellow mutant of tobacco mosaic virus but did not delay their appearance.

McWhorter (1938) segregated two viruses from tulips showing flower "breaking". One of these, tulip virus I was identified as a "colour removing virus", and the other, tulip virus II as a "colour adding virus". When present together they caused breaks in tulip blossoms. Virus I when present in the higher concentration caused severe injury, but as the concentrations shifted in favour of virus II, the plants become more vigorous and showed various colour patterns determined largely by the proportions of the two viruses in the plant.

Bawden and Kassanis (1945) showed that previous inoculation of tobacco plants with severe potato etch virus (PEV) prevented the multiplication of potato virus Y (PVY) and *Hyoscyanus* virus 3. In some plants PEV was so dominant the other viruses were no longer detectable. They considered this interaction to be different from the antagonistic interactions between related viruses and concluded that PEV was having an effect on cell metabolism resulting in the suppression of some material or enzyme system necessary for the multiplication of the challenge viruses.

1.1.2. *Synergistic interactions between unrelated viruses.*

The first reported case of this type of interaction was found in tomato plants infected by double streak disease, caused by a dual infection of TMV and potato virus X (PVX)

(Bennet, 1953). TMV alone may cause only mottling and a certain amount of dwarfing of tomato plants, while PVX alone induces only mild mottling. Double infection, however, results in increased injury characterized by the production of extensive necrosis of the leaves and stems. Further, the severity of streak disease symptoms is influenced by the virulence of the strain of PVX that is present in the mixture.

Bennett (1949) described the production of necrotic and deformed leaves in tomato plants following inoculation with dodder latent mosaic virus. The necrotic phase was followed by recovery and increase in the virus concentration of recovered tissues. Superinfection by tobacco etch virus (TEV) or TMV, however, resulted in the reappearance of symptoms of dodder latent virus in addition to those of TEV or TMV. Ross (1950) and Rochow and Ross (1954) described a similar relationship between potato viruses X and Y, where leaf extracts of mixedly infected plants contained much greater amounts of PVX than do extracts from plants infected with PVX alone.

Yarwood (1951) reported that dual infection of bean leaves by bean rust and TMV may result in virus concentrations as much as 50 times that present in rust-free plants. Pound and Walker (1945) showed that cabbage virus A and blackring virus occur in higher concentrations in cabbage plants at 28°C than at 16°C. No entirely satisfactory explanation was provided for these observations, and thus the findings that certain environmental influences and even parasitic organisms affect virus concentrations in a significant manner cannot be overlooked. These observations, however, suggest that virus concentrations may be influenced by any one of a number of factors including factors of the environment, and perhaps that any factor that alters the normal physiological processes of the plant may be capable of influencing virus concentrations (Bennet, 1953).

1.1.3. *Interactions between Related viruses.*

McKinney (1929) was the first to report the ability of one virus strain to protect against infection and invasion by a second, related strain. In experiments with TMV, he showed that plants infected with a strain which induced light "green mosaic" showed no change in symptoms after inoculations with a strain causing "yellow mosaic". Thung (1931) subsequently demonstrated that tobacco plants infected with TMV showed no additional

symptoms following inoculation with a variant that induced "white mosaic" symptoms when present alone. Salaman (1933) extended these studies to strains of PVX and showed that tobacco plants previously infected with a mild strain were protected from infection by challenge inoculations of other, more virulent strains. These observations led to the suggestion of the possible economic significance of this type of antagonistic interaction in the protection of field crops with mild virus strains against infection by virulent strains (Salaman, 1937).

This antagonistic interaction between related viruses has been variously described as cross-protection, acquired immunity, antagonism, cross-immunization, prophylactic inoculation, dominance, interference, premunity, and protective inoculation. Fulton (1986) considered the term cross-protection as the most appropriate because of its common usage, as well as being descriptive of the phenomenon of most concern in disease control: prevention of the deleterious effects of other, more severe strains. This and other definitions do not address the question of whether the challenge virus accumulates in the protected plant without being able to express its symptoms (Dodds et al., 1985).

Since these early observations, the phenomenon has been developed into a strategy which has been used in the protection of field crops. Protection of tomato plants from infection by tomato mosaic virus (ToMV) was conferred by prior inoculation with a mild mutant of ToMV produced by nitrous acid (Rast, 1972). The protection was however incomplete, with the appearance of mild to more conspicuous symptoms as the infection progressed (Fletcher and Rowe, 1975). Accumulation of challenge ToMV in tomato plants showing no symptoms or delayed symptoms of the challenge virus has also been described (Cassells and Herrick, 1977), providing a hidden reservoir of the virus strain targeted for control (Broadbent, 1976)

Mild strains of citrus tristeza virus found in Brazil (Grant and Costa, 1951) were used in large scale cross-protection programmes of citrus crops. However, Costa and Muller (1980) and Bar-Joseph (1978) showed that the protection was incomplete, with significant numbers of trees showing mild to fairly severe symptoms.

In Ghana, mild "strains" of cacao swollen shoot virus were used in attempts to cross protect against the virulent strain 1A (Crowdy and Posnette, 1947; Posnette and Todd,

1951). However, no significant cross-protection was found (Anonymous, 1951). Nevertheless, since the virus isolates used had not been characterised and their selection had been based on host range and symptomatology, this was not surprising (Bennet, 1953).

Using mild strains of passionfruit woodiness virus, passion fruit trees have been protected from "woodiness disease" (Simmons, 1959). Also, primary inoculations of mild nitrous acid mutants have been used in cross-protecting papaya plants from papaya ringspot virus (Yeh and Gonsalves, 1984).

Using two strains of cucumber mosaic virus (CMV), Dodds et al., (1985) demonstrated cross-protection in tomato (*Lycopersicon esculentum* cv Rutgers) and *Nicotiana tabacum* cvs Xanthium nc. and Turkish Samsun. The only sign of breakdown was the production of pathogenicity related ds-RNA and, to a lesser extent, virions of the challenge strain when infectious viral RNA was used as the inoculum.

As a tool in plant virus control, cross-protection has not achieved the eminence that immunization has achieved in the control of human and animal diseases (De Zoeten and Fulton, 1975). The reason for this may be due to the absence of humoral and cellular immune responses similar to those in vertebrates and to a lesser extent, a lack of understanding of the mechanism of cross-protection . Indeed, several different theories have been advanced to explain the phenomenon.

Köhler and Hauschild (1947) suggested that the protecting virus monopolised a metabolite essential for the challenge virus. This presumes a different metabolite for each virus. Gibbs (1969) and Ross (1974) suggested that replicase recognition might be the basis of specificity, and that the host component of the replicase complex might be the limiting metabolite. Kavanau (1949) suggested that particles of the challenge virus were adsorbed by aggregates of the protecting virions, thus immobilizing them. De Zoeten and Fulton (1975) hypothesized that free coat protein of the protecting virus might coat the challenge virus and prevent its uncoating. Subsequently, the specific involvement of coat protein in TMV cross-protection was demonstrated by Sherwood and Fulton (1982). Alternatively, Palukaitis and Zaitlin, (1984) proposed that plus sense transcripts of the primary viral RNA may bind to the newly made minus sense transcripts of the challenge strain RNA before it can replicate.

Most of the theories advanced involve assumptions unsupported by experimental evidence (Nelson et al., 1987; Sherwood and Fulton, 1982). Over 35 years ago, Bennet (1953) concluded that "the diversity of the various theories advanced to account for cross-protection between virus strains reflects the uncertain state of knowledge regarding the basic nature of the factors underlying this phenomenon. Each theory appears logical when applied to selected instances of protection. It does not seem possible, however, to correlate all of the observed facts with the requirements of any one of the theories on the basis of the available information. There is no compelling reason, moreover, for assuming that all cross-protection must result from the operation of the same factors; it is possible that more than one kind of defense mechanism is involved". Nelson et al., (1987) came to the same conclusion, that cross-protection could be the result of several mechanisms working sequentially or simultaneously.

1.1.4. *Resistance Genes and Transformed Plants.*.

Although the mechanism of cross-protection remains elusive, several workers (Beachy et al., 1985; Hamilton, 1980; Palukaitis and Zaitlin, 1984; Sanford and Johnston, 1985; Sequira, 1984) have suggested that introducing a part or all of a viral genome into plants may result in resistant plants. The ability to supplement plant genomes with alien genetic material by the *Agrobacterium* Ti plasmid system developed by Bevan and Chilton (1982) and Hoekema et al (1983) presented a suitable system for such developments in plant disease control efforts.

Three different types of genes have since been used to confer cross-protection in plants:

(1) Genes coding for the viral coat protein (Bevan et al., 1985; Hemenway et al., 1988; Loesch-Fries et al., 1987; Powell-Abel et al., 1986; Tumer et al., 1987).

(2) Genes coding for antisense RNA (Cuozzo et al., 1988; Hemenway et al., 1988, Gees et al, 1988).

(3) Genes whose transcripts correspond to a precursor of viral satellite RNA (Bauldcombe et al., 1986; Gerlach et al., 1987; Harrison et al., 1987; Jacquemond et al., 1988).

These approaches differ both in terms of the mechanisms of resistance or tolerance involved, as well as in terms of how resistance is expressed.

Introduction of the viral coat protein gene of TMV (Powell-Abel et al., 1986; and others cited) and alfalfa mosaic virus (Tumer et al., 1987; and others cited) into plants and its subsequent expression prevented or delayed the development of the disease induced by the challenge virus. Although the mechanism(s) by which coat protein induces this protection remains elusive, *in vitro* experiments suggest that the coat protein might inhibit either viral RNA synthesis (Houwing and Jaspars, 1986; Horikoshi et al., 1987) or cotranslational disassembly (Wilson and Watkins, 1986) during the early stages of infection.

Genes encoding coat protein or antisense RNA confer resistance whose effectiveness depends on the level of expression of the genes introduced and the concentration of the virus used as challenge. In many cases these types of resistance are overcome by high levels of inoculum. Except for the protection confered by the capsid protein of PVX (Hemenway et al., 1988), coat protein induced resistance can also be overcome by an inoculum of purified viral RNA (Cuozzo et al., 1988).

When genes based on satellite RNA are used, tolerance is observed regardless of the strain of virus, the form (virus particles or RNA), the concentration or the level of satellite RNA gene expression (Harrison et al., 1987; Gerlach et al., 1987). This resistance persists regardless of the method of transmission of challenge inoculum; i.e., mechanical inoculation or vector (aphid) transmission (Jacquemond et al., 1988). However, the method has the disadvantage that the satellite may be transmitted along with any compatible virus that infects the plant (Cuozzo et al., 1988) in which new host-pathogen interaction the effect is not necessarily innocuous (Kurath, G. personal communication and in paper presented to 1988 Robertson Symposium, Australian National University, Canberra, ACT, December 1988).

1.2. Sites of virus replication.

The occurrence in nature and in experimental systems of multiple infections of plant viruses resulting in a variety of virus-virus interactions suggest that the interacting viruses use common sites for their replication.

At the cellular level, infection by many plant viruses results in the appearance of numerous small vesicles in the infected cells (reviewed by Francki et al., 1985; Martelli and Russo, 1984). In comovirus and nepovirus infected cells, which show similar cytopathic

effects, the vesicles usually aggregate together in the cytoplasm with ribosomes and the endoplasmic reticulum into large vesicular inclusions. These are usually located between, but separate from the nucleus, chloroplasts and mitochondria, and are not membrane-bound. Many of the vesicles contain fibrils which in the case of broad bean true mosaic virus have been shown, by enzyme cytopathological studies, to consist of ds-RNA (Hatta and Francki, 1978). The most convincing evidence that the vesicular inclusions are involved in virus synthesis comes from the work with cowpea mosaic virus. Results of organelle fractionation and autoradiographic experiments showed that the inclusions contained virus-specific ds-RNA and replicase bound to its endogenous template (Assink et al., 1973; De Zoeten et al., 1974; van Kammen, 1984; Zabel et al., 1974). Similar cytoplasmic vesicles have also been observed in cells infected by some closteroviruses, potyviruses and dianthoviruses (reviewed by Francki et al., 1985).

Vesicles with fine fibrils have been seen both in the perinuclear spaces and cytoplasm of cells infected by some luteoviruses, bromoviruses and pea enation mosaic virus (PEMV) (Francki et al., 1985; Martelli and Russo, 1984; Martelli and Russo, 1985). In PEMV-infected cells, the vesicles are located predominantly in the perinuclear spaces, having apparently developed from the inner nuclear membrane (De Zoeten et al., 1972). Autoradiographic and biochemical analysis support the conclusion that the specific ds-RNA and RNA dependent RNA polymerase are associated with the nuclei of the infected cells (De Zoeten et al., 1976; Powell and De Zoeten, 1977).

The development of vesicles in chloroplasts has been observed in cells of plants infected with turnip yellow mosaic virus (TYMV) (reviewed by Francki et al., 1985). Similar structural changes have also been observed in cells infected with all other tymoviruses which have been examined (Hatta and Matthews, 1974; Lesemann, 1977; Ushiyama and Matthews, 1970). Detailed studies have established that the TYMV-induced chloroplast vesicles appear at an early stage of infection (Hatta and Matthews, 1974; Ushiyama and Matthews, 1970), and are formed as small invaginations of both chloroplast membranes with their necks remaining open to the cytoplasm (Hatta et al., 1973). Within the vesicles, fibrillar material with the expected appearance of nucleic acid is often observed (Ushiyama and Matthews 1970). The vesicles are scattered singly or in groups over the

surfaces of the chloroplasts. At the early stages of infection, the endoplasmic reticulum can usually be seen near the vesicles but disappears to be replaced with what appear to be coat protein subunits. Later still, numerous virus particles can be observed between the chloroplasts which are by now swollen and clustered together (Hatta and Matthews, 1974; Hatta and Matthews, 1976). This sequence of events suggests that the chloroplast vesicles are the sites of viral RNA replication and that the RNA is released into the cytoplasm to be encapsidated by viral coat protein synthesized in the cytoplasm.

Large multivesicular bodies appear to be characteristic of infections by most tombusviruses (Francki et al., 1985; Martelli and Russo, 1984). It was at first thought that these multivesicular bodies are derived from membranes of the endoplasmic reticulum and dictyosomes (Russo and Martelli, 1972), and later that they originated from, or were associated with the chloroplasts (Appiano et al., 1978). However, there is reliable evidence that multivesicular bodies in tombusvirus-infected cells develop from peroxisomes (Francki et al., 1985; Martelli and Russo, 1984). It has been demonstrated by cytochemical studies, that in cells infected by several tombusviruses, the multivesicular bodies contain catalase and glycolate oxidase, two enzymes usually associated with peroxisomes (Russo et al., 1983; Martelli et al., 1984).

Plants infected by several viruses belonging to a number of different taxonomic groups develop multivesicular bodies resembling those induced by tombusviruses, which, however, develop in the mitochondria (Francki et al., 1985; Martelli and Russo, 1984). The mitochondria are usually enlarged and misshapen, containing numerous vesicles about 50 to 70nm in diameter which are located in spaces between the outer and inner membranes of the mitochondria, including the intercristal spaces. Many vesicles contain fibrils with the appearance of nucleic acid (Hatta et al., 1971; Hatta and Ushiyama, 1973; Sugimura and Ushiyama, 1975).

Small tonoplast-associated vesicles have been observed in cells infected by all three cucumoviruses (Francki et al., 1985; Hatta and Francki, 1981; Martelli and Russo, 1984). About 50 to 90nm in diameter, the vesicles protrude into the vacuole. Each vesicle is bound by a membrane which in some electron micrographs, can be seen to be continous with the tonoplast membrane, and with its contents in contact with the cytoplasm through a narrow

neck. Some of the vesicles contain electron-dense fibrils which were digested with ribonuclease in low but not high salt buffers, indicating they were ds-RNA (Hatta and Francki, 1981). Similar tonoplast vesiculation has also been seen in cells infected with tobacco necrosis virus, some potexviruses, and a number of tobamoviruses (Francki et al., 1985), as well as in cells infected with carrot mottle, lettuce speckle and bean yellow vein viruses (Cockbain and Jones, 1981; Falk et al., 1979b; Murant et al., 1973).

Fujisawa et al., (1967) investigated interactions between two serologically unrelated viruses, TMV and TEV, in leaves of *Nicotiana tabacum* . Analysis of thin sections of mixedly inoculated leaves by electron microscopy revealed two types of interaction depending on the time between, and sequence of both inoculations. In the first, the challenge virus failed to develop in cells when the primary virus was already fully established. On the other hand, the challenge virus occurred within the same cells when the first virus infection had not been fully established. Both viruses, however, occurred in the same cells when the plants were inoculated simultaneously with them. Further, when TEV and TMV occurred together in the same cells, most masses of the two viruses were entangled with each other within the cytoplasm.

It would seem that not only do viruses from the same group form similar vesicles located in similar regions of plant cells, but there is evidence of viruses from different taxonomic groups occurring in similar vesicles. Together with the evidence of different viruses occurring not only in the same cells but also in physical contact, mixed infections would seem to represent a "gene soup" from which variability in genome composition and gene function could possibly be derived.

1.3. Consequences of Mixed Infections.

Given the widespread occurrence of multiple infections, and the common sites and structures associated with virus replication, a wide range of interactions beyond the antagonistic and synergistic types previously described are possible. These interactions include recombination, pseudorecombination (or reassortment), and transcapsidation (or phenotypic mixing). These "macromolecular" interactions represent possible sources of variation in RNA viruses and supplement mutation as mechanisms of RNA virus evolution.

King (1988) defined genetic recombination in RNA viruses as any process involving an exchange of information between genomic RNA molecules. In so doing, he distinguished between recombination and the analogous process of reassortment (pseudorecombination) which occurs between segmented RNA viruses, as well as the internal rearrangements seen in the genomes of defective interfering particles. King (1988) further defined two types of recombinational processes: homologous and non-homologous. In the former, the parental RNAs are related to each other and the location of the genetic cross-over point is the same in both sequences, thus preserving any open reading frames and producing potentially functional recombinant RNA molecules. In non-homologous recombination, neither of these restrictions apply. Of these two processes, homologous recombination has been the most studied (King, 1988).

1.3.1. *Recombination in RNA viruses.*

The possibility of genetic recombination in RNA viruses was first suggested by Hirst (1962) and Ledinko (1963). Independently, they showed that infection of cells with a mixture of inhibitor-sensitive variants of poliovirus resulted in production of genetically stable resistant progeny. Similar observations were made with foot and mouth disease virus (Pringle, 1965).

Genetic recombination of DNA is one of the fundamental mechanisms underlying the evolution of DNA viruses and higher organisms, and results in their diversity and adaptability. The importance of recombination is far less evident with RNA viruses (Bujarski and Kaesberg, 1986). Recombination has been detected in several groups of animal RNA viruses: picornaviruses, coronaviruses, and retroviruses (reviewed by King, 1988) and has been shown to promote the evolutionary variation of picornaviruses (Cooper, 1968; Emini et al., 1983 King et al., 1982; Tolskaya et al., 1983). It is involved in the creation of defective interfering (DI) RNA of positive and negative strand viruses (Lai et al, 1985; Lazzarini et al., 1981; Jennings et al., 1983; Monroe and Schlesinger, 1984; Stark and Kennedy, 1978). Until recently, the lack of DI RNAs and the inability to demonstrate recombination in mixedly infected plants was taken as evidence that plants do not support recombination of viral RNAs (Bujarski and Kaesberg, 1986).

The characteristics of RNA picornaviruses recombination, according to King (1988), are that it is

(1) homologous: there are never any insertions or deletions.

(2) efficient: a large proportion of genomes undergo recombination during each growth cycle.

(3) general : it occurs anywhere in the genome.

To achieve recombination some mechanism for aligning the parental RNA sequences is required. However, no cellular process resembling this description has been described. Since DI particles have been isolated from nearly every animal RNA virus in which it has been sought, it seems reasonable to expect that most RNA viruses, if not all, should occasionally undergo intermolecular rearrangements of a non homologous nature. Goldbach (1986) has suggested that the absence of reports of such events may simply reflect the difficulties in the design of suitable experiments under which the products of such an event will have a selective advantage and thus be detected.

Notwithstanding the wealth of evidence supporting recombination of animal RNA viruses, there is only one reported case of recombination in a plant RNA virus. Using brome mosaic virus (BMV), a plus stranded, tripartite RNA virus, Bujarski and Kaesberg (1986) showed that a genetically engineered deletion in the 3' terminal region of a single BMV RNA genomic component can be repaired during infection by recombination with the homologous region of either of the remaining wild type RNA components. Indirect evidence supporting possible recombination has also come from the studies of Robinson et al (1987), and Hillman et al (1987). Robinson et al., (1987) showed that the RNA 2 species of two tobravirus isolates, I6 and N5 contained sequences typical of both tobacco rattle virus (TRV) and pea early browning virus (PEBV). As a result, the two viruses had the pathogenicity of TRV while possessing the serological properties of PEBV. They concluded that I6 and N5 were recombinant viruses. Nucleotide sequencing of a symptom modulating RNA associated with tomato bushy stunt virus (TBSV) showed that it was derived from 5', 3' and internal segments of the TBSV genome (Hillman et al., 1987). The identification of this symptom modulating RNA as a deletion mutant of the helper virus established it as the first

definitive defective interfering particle (DI) RNA to be identified in association with a plant virus. These results clearly showed that plant RNA viruses can recombine in plants.

1.3.2. *Pseudorecombination (reassortment).*

Even though pseudorecombination has often been invoked as a probable mechanism of plant virus diversification and adaptability, only indirect evidence, from *in vitro* constructed pseudorecombinants, has been provided to support this assertion (van Vloten-Doting, 1983). Pseudorecombination is confined to multipartite genomic RNA viruses, and appears to be confined to the exchange of genomic RNA segments between related viruses. Pseudo-recombinants have been constructed from strains of cowpea mosaic virus (De Jaeger and van Kammen, 1970); alfafa mosaic virus (Dingjan-Verstegh et al., 1972); tobacco rattle virus (Sanger, 1968; Lister, 1968); and cucumber mosaic virus (Habili and Francki, 1976; Mossop and Francki, 1977; Rao and Francki, 1981). Pseudorecombinants between most of the tripatite viruses studied involved exchanges limited to the RNA 3, but some involved all three genomic RNA segments.

Bancroft (1972) constructed pseudorecombinants involving an exchange of RNAs 3 between brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV). These two viruses are members of the bromovirus group and share many physical properties (Harrison et al., 1971), but are considered serologically distinct (Bancroft et al., 1968) and have few host plants in common. *In vitro* pseudorecombination has demonstrated that only the RNAs 3 of CMV and TAV, two cucumoviruses, can be exchanged to produce viable, infectious virus (Rao and Francki, 1981).

Allison et al., (1988) using infectious *in vitro* transcripts from CCMV and BMV cDNA clones, showed that exchange of RNAs 3 between the two viruses leads to pseudo-recombinants. These replicated and spread sufficiently from cell-to-cell to form macroscopic lesions in *C. hybridium*, but were unable to systemically infect either cowpea or barley, both natural hosts of the parental viruses. They concluded that appropriate adaptation of some factor or factors encoded by RNA 3 must be required for successful systemic infection. However, since RNA 3 substitution is not sufficient to produce changes in the host ranges of the two viruses, systemic infection must also require proper adaptation of factors encoded by

RNA 1 and/or RNA 2, either for direct compatibility with the host or for functional compatibility with the RNA 3 genes.

Preliminary attempts by Hanada and Tochihara, (1975) to construct pseudo-recombinants between peanut stunt virus (PSV) and two other cucumoviruses, CMV and chrysanthemum mild mottle virus (probably closely related to TAV) were unsuccessful.

It has been shown that monocistronic RNAs 1 and 2 of BMV encode genes which are both required and, together, sufficient to induce viral RNA synthesis (French et al., 1986; Kiberstis et al., 1981). This functional compatibility requirement may explain the non-viability of some pseudorecombinants involving exchanges of RNAs 1 or 2. It would seem therefore that in addition to similarity and proximity of replicating sites, *in vivo* pseudo-recombination would also require compatibility of the genomic RNAs.

1.3.3. *Transcapsidation.*

Smith (1945) reported a type of interaction between two unrelated viruses which induced a rosette disease of tobacco. The virus complex consisted of two viruses designated respectively as "vein distorting" virus and "mottle" virus. The vein distorting virus is dependent on an insect vector for its transmission, but the mottle virus is transmissible by sap inoculation. The aphid, *Myzus persicae*, sometimes transmitted only the vein distorting virus, sometimes the mottle virus and sometimes both viruses. However, aphids from plants infected only with the mottle virus did not transmit any disease. It was subsequently shown that aphid transmission of the mottle virus was dependent on the presence of the vein distorting virus in the infected plants (Smith, 1946). He further suggested that a synergistic effect leading to an increase in the concentration of the vein distorting virus to the point that it is readily picked up by the aphid was responsible for this behaviour.

Kassanis (1961) suggested that nucleic acid of one virus may be encapsidated into particles of another virus, and the phenomenon was referred to as transcapsidation (phenotypic mixing). Hull and Adams (1968) suggested that a dependent virus might rely on its helper virus coat protein for transmission by aphids. This mechanism was proposed to account for the dependence of groundnut rosette virus on its assistor virus for transmission by aphids.

Rochow (1970) presented serological evidence that transcapsidation occurred in mixed infections of the barley yellow dwarf virus (BYDV) isolates, RPV and MAV. Although both are referred to as BYDV, they are serologically unrelated. The aphid *Rhopalosiphum padi* transmitted the RPV isolate of BYDV but not the MAV isolate, which in turn was transmitted specifically by another aphid, *Macrosiphum avenae*. From leaves mixedly infected by both viruses, *M. avenae* transmitted only MAV. *R. padi*, however, often transmitted virus which was subsequently shown to be transmitted by both aphid species. Further tests by serology and symptoms on infected plants showed that both MAV and RPV had been transmitted. Using serological cross-absorption tests, Rochow (1970) showed that MAV nucleic acids had been encapsidated in RPV coat protein thus enabling *R. padi* to transmit MAV. There was no evidence that MAV coat protein encapsidated RPV RNAs.

Serological evidence for transcapsidation also exists for the beet western yellows virus (BWYV)/lettuce speckles mottle virus (LSMV) complex (Falk et al., 1979a) and the carrot red leaf virus (CRLV)/carrot mottle virus (CMotV) complex (Waterhouse and Murant, 1983). These however, differ from the RPV/MAV model in that one component relies entirely on the other for its aphid transmissibility. The virus complex consisting of CRLV and CMotV is transmitted in a persistent manner by the aphid *Cavariella aegopodii* (Stubbs, 1948; Watson et al., 1964). Aphid transmission of CMotV occurs only from plants which also contain CRLV; aphids that are allowed to feed first on a pure source of CRLV and then a pure source of CMotV do not acquire and transmit the latter though they transmit CRLV (Watson et al., 1964; Elnagar and Murant, 1978). Waterhouse and Murant (1983) subsequently showed that it is the encapsidation of CMotV RNA with CRLV protein that confers the aphid transmissibility.

The BWYV/LSMV and CRLV/CMotV complexes, together with tobacco mottle virus (Smith, 1945), tobacco yellow vein virus (Adams and Hull, 1972) and bean yellow vein banding virus (Cockbain, 1978) constitute a collection of apparently similar entities that spread in association with luteoviruses (Waterhouse and Murant, 1983).

Mossop and Francki (1977) constructed pseudorecombinants involving the *in vitro* exchange of RNAs 3 of aphid transmissible (QCMV) and aphid non-transmissible (MCMV) strains. Subsequent aphid transmission from plants inoculated with these pseudorecom-

binants alone showed that aphid transmissibility of QCMV had been confered on the pseudorecombinant by the exchange of their RNAs 3.

Transcapsidation is thus not only a mechanism for aphid transmission of one virus dependent on the presence of another in a source plant (Waterhouse and Murant, 1983; Kassanis, 1963), but may also be a means of isolating the products of pseudorecombination leading to the appearance of new viruses or virus strains.

In contrast with transcapsidation, aphid transmission of potyviruses and caulimoviruses is dependent on a virus encoded non-capsid viral protein (Pirone, 1977; Harrison, 1987). Purified virus particles of either group are not aphid transmissible (Pirone and Megahed, 1966; Govier and Kassanis, 1974; Govier et al., 1977). The protein has been described as a helper component (HC) for potyviruses (Harrison and Murant, 1984) and as the aphid transmission factor (ATF) for caulimoviruses (Lung and Pirone, 1974). A similar transmission strategy has been suggested for anthriscus yellows and parsnip fleck viruses (Elnager and Murant, 1976; Harrison, 1987).

1.4. Evolution of RNA viruses.

The evolution of RNA viruses has received a great deal of attention in the past several years. The subject is based on information very different from that considered in discussing the evolution of other organisms. This is because as extremely small obligate intracellular parasites, viruses leave no fossil records.

Until recently, viruses were grouped and classified by such parameters as the structure of the virion, host range, transmitting vector, and for closely related viruses antigenic cross-reaction and symptom induction (Bennet, 1953). Viral taxonomy has more recently been refined to include such features as genome composition, protein structure and differential stability in the presence of various chemical and physical agents (Francki, 1981; Gibbs, 1969; Matthews, 1982). By such methods most known viruses have been grouped into families, genera or groups.

Recent comparisons at the level of nucleotide sequences have led to suggested "super groupings" containing more than one family which were thought to reflect ancestral relationships among seemingly divergent groups (Gibbs, 1987; Goldbach, 1986; Goldbach

and Wellink, 1988; Strauss and Strauss, 1988). These comparisons have led to insights into RNA virus evolution, including the beginning of taxonomy based on the relatedness of the primary structure of their genomes and gene function (Goldbach, 1986; Goldbach and Wellink, 1988; Strauss and Strauss, 1988). These comparisons may also lead to a better understanding of the diversion of viruses to different hosts (Goldbach, 1986).

With the available complete nucleotide sequences of different viruses, it has become clear that there are long stretches of amino acid sequence similarities in the replicase proteins of certain groups of both plant and animal viruses (Ahlquist et al., 1985; Cornellisen and Bol, 1984; Franssen et al., 1984; Haseloff et al., 1985). Further evidence has come from X-ray diffraction studies at high resolution which has revealed that many icosahedral viruses previously considered totally unrelated have capsid proteins whose folding in three dimensions is very similar despite the absence of sequence similarities in the proteins (Rossman and Ruecket, 1987). On the basis of sequence and gene function similarities it has been speculated that all the positive-strand RNA viruses may have originated from a common ancestor (Goldbach, 1986; Goldbach and Wellink, 1988; Strauss and Strauss, 1988). Mutation, recombination and selection may have produced the present members of this group which infect many different hosts (plants, insects and higher animals) and have a variety of different morphologies.

Three different pathways have been proposed to account for the inter-viral relationships (Goldbach, 1986); common ancestry, convergent evolution and transduction of host genes. The homologies observed between diverse groups of viruses support the concept of common ancestry, and imply that plant and animal viruses, though at present separated ecologically by different host ranges, diverged from a common ancestor, with insect hosts as the most likely "bridge" between them (Goldbach, 1986).

On the other hand, viruses of very different origin may encode proteins with similar functions (eg. replicase enzyme) and interact with the same highly conserved host proteins. Therefore, it may be solely for this reason they may have evolved similar tertiary and hence primary structures.

The third proposed pathway suggests that similar viruses may have evolved independently from their host cells by adopting the same conserved genes from their host's

chromosomes to use for their own replication. The observed homologies in protein sequences would then be the result of a common gene transfer mechanism and a strong conservation of the genes so "captured".

Convergent evolution and transduction of host genes however do not explain the colinearities in the genetic maps of the various viruses considered (Goldbach, 1986; Goldbach and Wellink, 1988; Strauss and Strauss, 1988).

1.4.1. *Mutations and Evolution of RNA genomes*

Many early and more recent observations suggest that RNA genome populations consist of complex distributions of variants (Domingo et al., 1985). The evidence for this includes:-

(1) the presence of mutants in preparations of viruses, and of revertants in mutant stocks.

(2) the frequent occurrence of antigenic variants, detected with monoclonal antibodies.

(3) the genetic variations seen among independent natural isolates of one virus.

Genetic heterogeneity appears to be attained rapidly as a result of high mutability and large population sizes. The evolutionary consequences of these facts have been emphasized by Reanney (1982).

1.4.2. *Recombinations in Evolution of RNA virus genomes*

It has been suggested that recombination may be an important force in RNA virus evolution (Goldbach, 1986; Strauss and Strauss, 1988). RNA genome recombination and reassortment can greatly add to the potential variability of rapidly mutating genomes, not only by generating new variants but by bringing together mutations in one segment with sequences (and mutations) in another (Domingo and Holland, 1988). This should allow an otherwise unfit mutation to survive and even dominate in a competitive quasis-species population (Domingo et al., 1985).

The most common form of "recombination" among RNA viruses is the independent reassortment (or pseudorecombination) of different viral genome segments during a mixed infection to produce progeny with characteristics from both parents (Strauss and Strauss,

1988). Such reassortment has been demonstrated for most segmented RNA viruses of vertebrates. The best studied case of reassortment in nature, is influenza virus. It has been shown that new epidemic strains arise when the genes allowing the virus to replicate in humans (ie. replicase genes) are combined with genes from another host that encode new surface antigens. The recombinants (reassortants) can replicate in humans, but the human population has no immunological resistance to them (Desselberger et al., 1978; Webster et al., 1982). There is to date no documented evidence of plant virus reassortment or pseudo-recombination in the wild, even though *in vitro* pseudorecombinants have been constructed for many years(Van Vloten-Doting, 1983).

Recombination may occur within a segment of a non-segmented or segmented virus. In either case the progeny virus consists of covalently linked polynucleotides that were derived from more than one parent. This type of recombination is more difficult to demonstrate *in vivo* for RNA viruses (Strauss and Strauss, 1988). Recombination has occurred in cell cultures of picornaviruses (Cooper, 1977), coronaviruses (Makino et al., 1986) and by "forced selection" in brome mosaic virus (Bujarski and Kaesberg, 1986). In addition to cell cultures, recombination in poliovirus has also been demonstrated in humans (Kew and Nottay, 1984) who received simultaneous high doses of three different vaccine strains each of which had been impaired to some extent.

The importance of such recombination as a general mechanism for generating new successful virus strains has not been proven (Strauss and Strauss, 1988), although a clear cut case in FMDV has been described (Hahn et al., 1988).

1.5. Cucumoviruses.

The choice of two cucumoviruses as a model for this study was primarily based on the wide host range, variability and geographic distribution of members of the group. The cucumoviruses are named after the type and best known member of the group, cucumber mosaic virus (CMV). CMV causes numerous diseases in a wide variety of plants. Its extremely wide host range, numbering some 775 dicotyledon and monocotyledon plant species in 365 genera and 85 families (Douine et al., 1979) is probably responsible for it being considered as one of the most cosmopolitan viruses.

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). In addition, robinia mosaic virus (Schmelzer, 1971) and clover blotch virus (Musil et al., 1975) previously described as distinct viruses were later considered as strains of PSV (Richter et al., 1979).

The research described in this thesis initially involved mixed infections of two strains of CMV, the M strain and the K strain. The choice was based on differences in symptoms induced as well as results of *in vitro* pseudorecombination showing that all three genomic RNAs can be exchanged to produce viable, infectious viruses (Rao and Francki, 1982a). The analysis of these infections soon proved to be very complex due to the absence of clear serological and nucleotide sequence differences between the two viruses.

Subsequently the study was changed to involve CMV and TAV. The two viruses have tripartite genomes consisting of three single strand, positive sense RNAs (designated RNAs 1, 2 and 3; Habili and Francki, 1974b) encapsidated in icosahedral particles of about 30nm diameter. The RNAs 1 and 2 are individually encapsidated, while the RNA 3 is encapsidated together with a fourth subgenomic RNA, which is transcribed from the 3' terminus of RNA 3 and functions as the mRNA for coat protein (Schwinghammer and Symons, 1977). The RNAs 1 and 2 encode the genes for the viral replicase (Schwinghammer and Symons, 1977; Nitta et al., 1988). The 5' terminal gene of RNA 3 encodes a non-structural protein of molecular weight of approximately 30 kilodaltons to which a role in cell-to-cell movement of virus has been tentatively assigned, by analogy to a similar protein coded for by TMV (Atabekov and Dorokov, 1984). This protein is translated directly from the RNA 3.

The capsids consist of 180 identical protein sub-units (Finch et al., 1967; Habili and Francki, 1974a). The three particles are indistinguishable in sucrose density or isopycnic gradients, CMV cannot be distinguished from TAV by this method.

CMV is only remotely related, serologically, to TAV (Devergne and Cardin, 1975; Habili and Francki, 1975; Rao et al., 1982). The nature of this relationship has been the subject of some controversy only resolved by recognising the roles of the antiserum titre and the animal in which the antiserum is raised, as well as the particular strains used (Rao et al.,

1982). The RNAs of the two viruses have no significant base sequence homology as measured by molecular hybridization analysis (Gonda and Symons, 1978). MCMV, a "Golden" mosaic mutant derived from Price's No 6 strain of CMV, was selected because of its distinct chlorotic mosaic symptoms and aphid non-transmissibility by *Myzus persicae* (Mossop and Francki, 1977). VTAV on the other hand is transmitted by *M. persicae* . The two viruses must be purified by different methods.

To study the *in vivo* molecular interactions between two viruses, it is essential to find a pair which can replicate simultaneously without one "dominating" the other, and in similar sites and structures within the cell. It would seem that such interactions as transcapsidation, genomic masking , pseudorecombination and recombination are less likely to occur between widely different viruses. This may be due to incompatibilities between genomes and gene function of, for example, the type described by French and Ahlquist, (1987), French et al., (1986) and Kiberstis et al., (1981) for BMV. On the other hand, inoculation with two closely related viruses may result in the suppression or elimination of one virus by the other (Fulton, 1986; Palukaitis and Zaitlin, 1984). MCMV and VTAV represented a possible pair with which the two extremes, cross-protection or non-interaction, could be avoided.

1.6. Scope of this thesis.

The scope of this thesis was to

(1) Establish a mixed infection of two viruses sufficiently related as to enable molecular interactions of the types described, but not close enough to cross-protect against each other.

(2) Develop assay methods for the independent detection of either virus in coinfections of the two.

(3) Develop methods of identifying, isolating and characterising variants incorporating genetic material from both parental viruses.

(4) As far as possible, determine the genomic composition of any variants, and the effect of the genetic changes on biological properties.

Addendum :   In an extensive review, Dodds and Hamilton (1976) have described the conditions required for, and the range of structural interactions between viruses *in vitro* and *in vivo* , as a result of mixed infections (Adv. Virus Res. *20* , 34-86).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Virus Culture, Propagation and Storage.

All viruses were routinely propagated in *Nicotiana glutinosa* , or *N. clevelandii*, in the glasshouse. Plants were lightly dusted with caborundum and either extracts from infected leaves or purified virus were rubbed on to the leaves. Excess inoculum was washed off by overhead watering of the plants immediately after inoculation. When required for extraction and purification, *Nicotiana clevelandii* was the more suitable host because of its higher virus yields.

To maintain virus cultures, leaves from infected plants were stored over long periods by shredding infected leaves and drying them over calcium chloride *in vacuo*, and keeping them in McCartney bottles over calcium chloride at 4°C.

2.2 Virus Extraction and Purification.

2.2.1. *MCMV Extraction and Purification.*

MCMV was extracted as described by Mossop et al., (1976). Systemically infected *N. clevelandii* leaves were harvested 14 days after inoculation and extracted in two volumes (w/v) of 0.1M phosphate buffer, pH 8.0, containing 0.1% thioglycollic acid and 0.1% sodium diethyldithiocarbamate (DIECA). The extract was strained through cheese cloth, and then clarified by centrifuging at 10,000 g for 15 min. Triton X-100 was added to the recovered supernatent to a final concentration of 2% (w/v) and stirred for 15 min at 4°C. Virus was then pelleted by centrifugation at 78,000 g for 2 hr. The pellets were resuspended in 1/10 the original volume of extraction buffer, and clarified by centrifugation at 5,000 g for 5 min. The supernatent containing the virus was then layered over a 10% sucrose (w/v in 50 mM phosphate buffer, pH 7.6) cushion and centrifuged for 60 min at 144,000 g. The pellets were resuspended in 0.1M phosphate buffer, pH 7.6, and again clarified by low speed centrifugation. Such preparations are henceforth referred to as "purified".

2.2.2. *VTAV extraction and purification.*

VTAV was extracted and purified by a modification of the method described by Peden and Symons (1973) for the purification of the Q strain of CMV. Systemically infected *N. clevelandii* leaves harvested 14 days after inoculation were extracted in two volumes (w/v) of 0.5M citrate buffer, pH 6.5, containing 0.5% (v/v) thioglycollic acid. The extract was emulsified with an equal volume of chloroform, and the emulsion broken by centrifugation at 15,000 g for 15 min. Polyethylene glycol (PEG) 6000 was added to the recovered buffer phase to a final concentration of 10% (w/v) and stirred for one hour at 4°C. The precipitated virus was sedimented by centrifugation at 15,000 g for 15 min, resuspended in 5mM sodium borate buffer, pH 9.0, and Triton X 100 was added to a final concentration of 2% (v/v). After stirring for 30 min at 4°C, the preparation was subjected to two cycles of high (144,000 g for 90 min) and low speed centrifugation (5,000 g for 5 min). The pellets from the second high speed centrifugation were resuspended in 5mM borate buffer, pH 9. Such preparations are henceforth referred to as "purified".

2.2.3. *Virus Yields.*

Virus yields were estimated using $E_{260nm, 10mm} = 5.0$. MCMV and VTAV infected *N. clevelandii* yielded 350-500mg/kg and 500-700mg/kg leaves of purified virus, respectively.

2.2.4. *Sucrose Density-Gradient Purification of Virus.*

Purified virus preparations required to be used for antiserum production, or for preparing RNAs as templates for cDNA synthesis were further purified by sucrose density-gradient centrifugation. Virus (0.5-3.0mg per gradient, depending on rotor used) was layered on 5-25% (w/v) sucrose gradients (in 20mM phosphate buffer, pH 7.6) and centrifuged at 25,000 rpm for 150 min in a Beckman SW28 rotor or at 37,000 rpm for 90 min in a Beckman SW 41 rotor. The virus bands detected with an ISCO model UA-5 absorbance monitor were recovered with the aid of an ISCO model 640 density gradient fractionator. The virus was concentrated by centrifugation at 144,000 g for 90 min, and the

pellets were resuspended in 20 mM phosphate buffer, pH 7.6. Such preparations are referred to henceforth as "highly purified".

2.3. Serology.

2.3.1. *Glutaraldehyde Fixation of Virus.*

Highly purified virus required for use as immunogen was glutaraldehyde-fixed by a modification of the method described by Francki and Habili, (1972). Glutaraldehyde was added, to a final concentration of 0.2% (w/v), to preparations containing 2.0-5.0mg/ml of virus. The preparations were dialysed overnight against 20mM phosphate buffer, pH 7.6, containing 0.2% glutaraldehyde. Excess glutaraldehyde was removed by dialysis for a further 24 hr against buffer alone. The fixed virus was then adjusted to a concentration of 1.0mg/ml and stored at 4°C until required.

2.3.2. *Immunization of rabbits..*

Rabbits were each given an initial intravenous injection of 1.0mg of fixed virus after bleeding for pre-immune sera. Two weeks later, a first test bleed was taken, and the rabbits given a subcutaneous booster injection of 1.0mg of virus in an equal volume of Freund's incomplete adjuvant. At weekly intervals after this, the rabbits were bled and if required, after two weeks, a second intravenous booster injection of 1.0 mg virus was given.

Blood samples were left at room temperature for 2 hr, and then overnight at 4°C for the serum to separate from the other blood components. The serum collected was then clarified by low speed centrifugation at 3,000 g for 10 min and stored at -20°C in 5-10ml aliquots. Alternatively, the serum was mixed with an equal volume of glycerol and stored at -20°C.

2.3.3. *Antiserum Titre and Specificity.*

The titres and specificity of the antisera were determined by Ouchterlony tests in 0.75% agar in 20mM phosphate buffer, pH 7.5, containing 0.2% sodium azide (w/v) (Habili and Francki, 1975).

2.3.4. *Purification of Gamma Globullin (Ig-G).*

Ig-G from selected antisera were prepared as described by Clark and Adams (1977). An equal volume of saturated ammonium sulphate was added to a 1:10 dilution of antiserum in distilled water and left at room temperature for 30-60 minutes. After centrifugation at 6,000 g for 15 min the pellets were dissolved in a small volume (0.5-1.0ml) of 5X PBS (1 litre of 10X PBS contained 80gm NaCl, 2gm KH_2PO_4, 14.4gm Na_2HPO_4, 2gm KCl, and 2gm NaN_3, pH 7.4) and dialysed with three buffer changes against 1X PBS for 24 hr. The Ig-G was purified by cellulose DE 22 column chromatography. The crude Ig-G (1.0ml) was layered over a 5ml DE 22 column in 5X PBS and eluted with the same buffer. Twenty 1.0ml fractions were collected and the 3 to 4 peak fractions with absorbance at 280nm greater than 1.5 were pooled and adjusted to a concentration of 1.0mg/ml using an $E_{280nm,10mm}$ of 1.8=1.0mg/ml. The Ig-G thus prepared was stored at 4°C, or glycerol added and stored at -20°C, and used at one in one thousand dilution in coating buffer.

2.3.5. *Conjugation of Ig-G with Alkaline Phosphatase.*

Conjugation of Ig-G to alkaline phosphatase was done as described by Clark and Adams (1977). Two mg of alkaline phosphatase was pelleted by centrifugation at 5,000 rpm for 5 min, and the pellet directly dissolved in 5 ml of purified Ig-G solution at a concentration of 1.0mg/ml. The solution was dialysed overnight against 1X PBS. Fresh glutaraldehyde was added to 0.06% (v/v), and left at room temperature for 3 hr. Excess glutaraldehyde was then removed by overnight dialysis against 1X PBS with three changes of buffer. Bovine serum albumin (BSA) was added to a final concentration of 5mg/ml and the preparation stored at 4°C. Conjugate thus prepared was used at one in one thousand dilution in conjugate buffer [1X PBS containing 0.5% (w/v) polyvinyl pyrollidone (PVP) 40,000 and 1% (w/v) BSA).

2.3.6. *Enzyme Linked Immunosorbent Assay (ELISA).*

The procedure used was the double antibody sandwich method (DAS-ELISA) described by Clark and Adams (1977). Ig-G (1.0mg/ml) was diluted 1/1000 in coating

buffer (1.59gm Na2CO3, 2.93gm NaHCO3, 0.2gm NaN3, in 1 litre, pH 9.6). Into each well of a microtitre plate, 200 μl of the diluted Ig-G was placed and incubated for 3 hr at 25C. The plates were then washed 3 times, each time for 3 min, with wash buffer (1XPBS, 0.005% (v/v) Tween 20). Test antigens in appropriate buffers were then applied in duplicate or triplicate (200μl per well) and incubated overnight at 4°C. Unbound antigen was removed by three washes of the plate with wash buffer, each time for 3 min, and Ig-G- enzyme conjugate diluted 1/1000 in conjugate buffer [1XPBS, 0.5%PVP (w/v), 1%BSA (w/v)], was added to each well. After incubating for 3 hr at room temperature, the plates were washed 3 times with wash buffer to remove any unbound conjugate. The plates were dried and 200 μl enzyme substrate [1.0mg/ml of p-nitrophenyl phosphate disodium hexahydrate in substrate buffer, containing 10% (w/v) diethanolamine, 0.01% (w/v) NaN3] placed in each well. The virus antigen present was expressed by the concentration of the substrate breakdown products as measured at 405nm in a BIO-RAD EIA reader.

2.3.7. *Preparation of Infected Tissue for Serological Analysis.*

Leaf samples, from 40-50μg to about 0.5 gm depending on the experiment, were placed in small plastic bags and 1.0 ml of antigen buffer [1X PBS-Tween 20 containing 0.1% (w/v) PVP] added. The sample was gently pounded, and a small piece of cheese cloth placed in the bag as an *in situ* strainer. Where leaves were sampled repeatedly, a pasteur pipette tip was used as a borer to take 1mm diameter discs. Otherwise, 0.1-0.5 gm of leaf tissue was used. Successive sampling of leaves was done as shown in Figure 2.1.

2.4. Extraction and Purification of Viral RNA.

2.4.1. *Extraction of RNA from Purified Virus..*

Viral RNA was extracted by the phenol-SDS method described by Peden and Symons (1973). About 5mg of virus at 1.0-2.0mg/ml was emulsified with equal volumes of water-saturated phenol containing 0.1% (w/v) 8-hydroxyquinolline, and RNA buffer [0.6M sodium acetate containing 0.6% (w/v) SDS, and 20mM EDTA, pH 8.0). After shaking for 10-15 min, the buffer and phenolic phases were separated by centrifugation at 7,000 g for 10 min and the buffer phase transferred into a fresh sterile tube. One half the original volume of
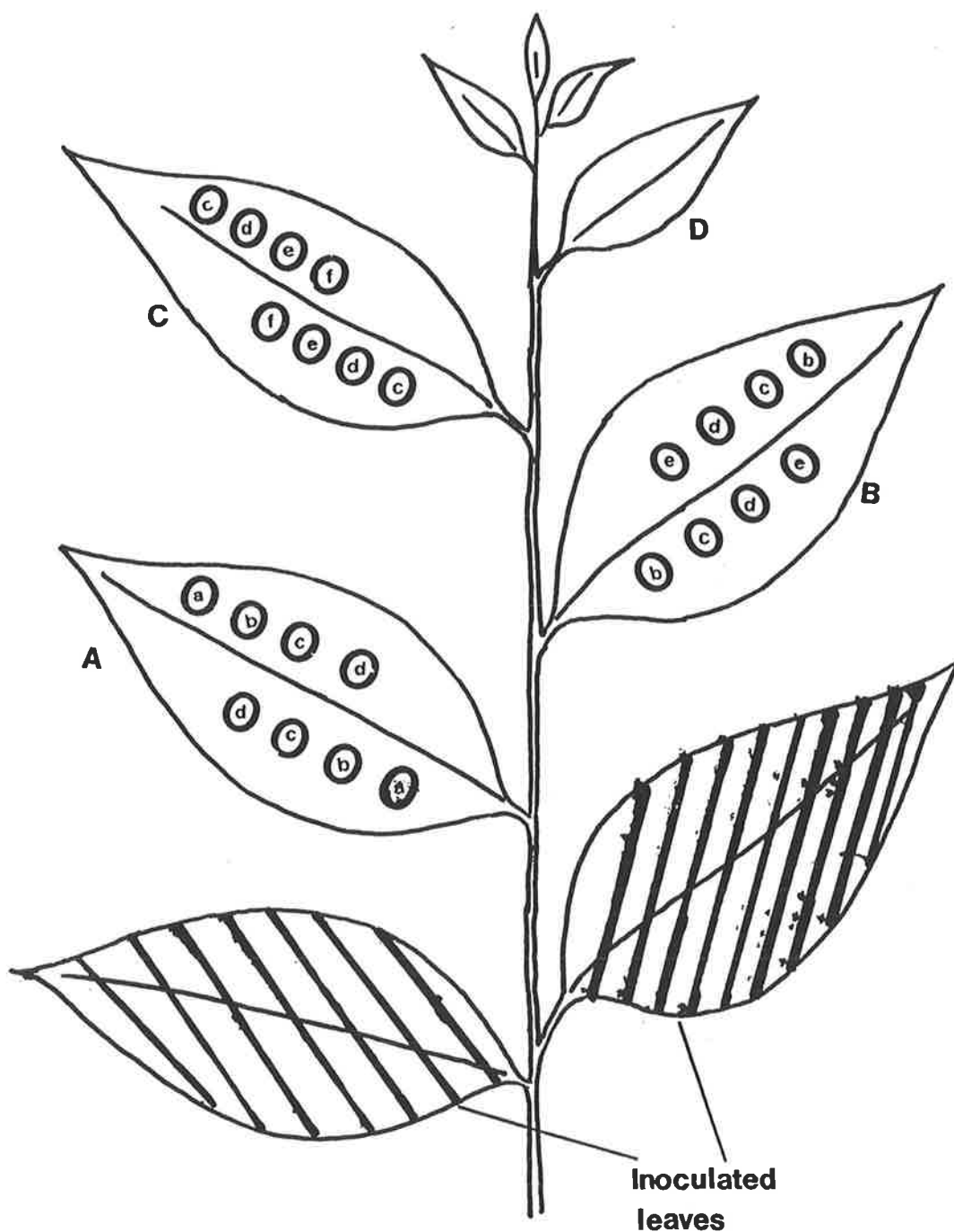
Fig 2.1

Procedure for non-destructive sampling of leaf tissue for ELISA.

Pasteur pipette tips were used as borers to remove two small leaf discs (a-d) from systemic leaves (A-D). Sampling commenced with leaf A, with leaf discs (a) taken from both ends of the leaf. Sample (b) of leaf A was taken on the same day as the first sample (b) on leaf B, and so on.

phenol was added, and the extraction repeated. Traces of phenol were removed by washing the buffer phase with two volumes of ether, and the nucleic acid was precipitated by adding 2.5-3.0 volumes of re-distilled ethanol. After at least 1 hr at -70°C or 3 hr at -20°C, the RNA was pelleted by centrifugation at 5,000 g for 10 min. The pellet was washed once with 80% ethanol, dried *in vacuo*, resuspended in sterile double distilled water (SDDW) and stored at -20°C.

2.4.2. *Extraction of RNA Directly from Leaf Tissue..*

The procedure described by Palukaitis et al., (1984) was used for obtaining nucleic acid from small leaf samples for dot blot hybridization analysis. Each tissue sample (0.2gm or less) and 0.3ml of AMESS buffer [0.5M sodium acetate, pH 6.0, containing 10mM MgCl$_2$, 20% (v/v) ethanol, 3% (w/v) SDS, 1.0M NaCl and 0.05% (w/v) bromophenol blue] were mixed in a 1.5ml Eppendorf centrifuge tube with a trace of acid washed sand and ground for 1-2 min with a stainless steel device designed to fit into the tube. The slurry was vortexed for 30 sec and incubated at 37°C for 5 min. After addition of two volumes of chloroform (v/v) and vortexing for 30 sec the samples were left on ice while other samples were being prepared, and were then centrifuged for 5 min at 15,000 g at 4°C. The upper bluish aqueous phase was used for spotting onto nitrocellulose, or kept frozen until required.

When groups of experimental plants were being individually sampled for analysis, three leaf discs of diameter 0.5mm were taken using the wide end of a 1.0ml disposable pipette tip as a borer, giving uniform amounts of tissue.

Alternatively, a modification of the method described by Loening and Ingle (1967) was used. Whole leaves of 0.5-1.5gm were extracted, at 1.0ml per gm of tissue, with 1X TBE (10X TBE contained 108gm Tris-HCl, 55gm boric acid, and 9.3gm EDTA in 1 litre of distilled water, pH 8.3) containing 0.1 M NaCl. SDS was added to a final concentration of 2% (w/v), and the mixture was extracted first with an equal volume of phenol-chloroform (1:1), and then half volume of water-saturated phenol. The buffer and phenol phases were separated by centrifugation at 7,000 g for 10 min. Sodium acetate (3M) was added to the buffer phase to a final concentration of 0.3M, and the RNA was precipitated with 2-3

volumes of redistilled ethanol. The RNA pellets were obtained by centrifugation at 5,000 rpm for 10 min and resuspended in 200 µl of SDDW per gm of leaf tissue.

2.5. Agarose Gel Electrophoresis.

2.5.1. *Analytical Gel Electrophoresis.*

Routinely, viral RNAs were analysed on 1.5-2.0% agarose gels prepared in TAE buffer [4.84gm Tris-HCl, 1.64gm anhydrous sodium acetate, 0.745gm EDTA and 1.35ml (v/v) glacial acetic acid per litre, usually prepared as a 10X stock solution]. The autoclaved agarose (50ml) was dispensed into a slab tray of 11cm (width) by 14cm (length) of a BRL submerged gel apparatus. Aliquots of one to 3µg of RNA in up to 5µl of SDDW were added to 10µl of sample buffer [50% (v/v) glycerol in 0.1X TAE containing 0.05% (w/v) Bromophenol blue] and heated for 3-5 min at 55°C. Samples were loaded into the wells, relying on the higher density of the sample buffer to keep the RNA submerged in the gel below the surface of the running buffer which also contained 0.0002mg/ml of ethidium bromide stain. Electrophoresis was at 100V for 2 hr and the gel was examined in UV light.

If required, RNAs were also denatured, with glyoxal, for electrophoresis. To 9µl of glyoxal stock mix [1.11M glyoxal, 77.8% (v/v) formamide, 11.1mM sodium phosphate in SDDW, pH 7.0) was added 1.0µl (2-6µg) of RNA. The mixture was heated at 55°C for 15 min and then placed on ice. One to 2µl of tracking buffer [20% (w/v) Ficoll, and 1% (w/v) Orange G, in 5mM EDTA, pH 8.0) was added and the samples loaded as before on to agarose slab gels and electrophoresed for 2 hr at 100V. If gels were to be photographed, they were stained for a further 15 min in 0.005µg/ml ethidium bromide, washed in distilled water and viewed in UV light.

2.5.2. *Preparative Agarose Gel Electrophoresis.*

When segments of viral RNA were required for constructing pseudorecombinants or as templates for cDNA synthesis, they were purified by electrophoresis in 2% agarose gels. Gels were poured into sterile glass tubes, 100mm long and 10mm in diameter, and sealed at one end with dialysis tubing. A flat loading surface of the gel was ensured by trimming with a sterile razor blade, and the tubes were placed in the apparatus as shown in Figure 2.2. An

Fig 2.2

An apparatus for preparative agarose or polyacrylamide gel electrophoresis.

Fig 2.2

Apparatus for preparative agarose and polyacrylamide gel electrophoresis

equal volume of sample buffer [50% (w/v) glycerol, and 0.02% (w/v) bromophenol blue in 0.1X TAE] was added to the RNA sample (20-100 μg RNA in 20-50μl of sample buffer per tube), heated at 90°C for 10 min, and then chilled on ice for 10 min. The samples were pre-electrophoresed at 2.4mA per tube until they had entered the gel, and then at 12mA per tube for 4-5 hr. The gels were stained for about 1 min with 0.05% (w/v) toluidine blue in 20mM sodium acetate, and then repeatedly washed with sterile distilled water.

2.5.3. *Recovery of Nucleic Acids.*

The lightly stained RNA bands were cut out and placed in glass digesters adapted for use with an MSE mini atomix overhead driven blender, and a small volume of RNA extraction buffer added. An equal volume of water-saturated phenol was added and after mixing the buffer and phenolic phases were separated by centrifugation at 6,000 g for 10 min. The RNA was then precipitated with ethanol as previously described.

2.6. Polyacrylamide Gel Electrophoresis.

2.6.1. *Electrophoresis of Viral Coat Proteins.*

The electrophoretic mobilities of virus coat proteins were compared in polyacrylamide gels as described by Laemli, (1970). Twelve per cent polyacrylamide gels were prepared by mixing while stirring, 13.9ml of SDDW, 10ml of "lower" Tris buffer [18.17gm Tris-HCl, 2.5ml 6N HCl, and 4.0ml of 10% (w/v) SDS in a total volume of 100ml], and 16ml of acrylamide/bis-acrylamide [30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide]. Freshly prepared 10% (w/v) ammonium persulphate (120μl) and 20μl of N,N,N'N'- tetramethyl ethylenediamine (TEMED) were added, stirred briefly, and poured between the plates of a Biorad model 220 vertical slab electrophoresis apparatus, leaving room for the stacking gel and comb. One ml of water-saturated butanol was layered over the gel to ensure a level surface, and the gel was allowed to polymerize. After polymerization, the butanol layer was washed off with SDDW. The stacking (upper) gel was prepared by mixing, while stirring, 3.85ml SDDW, 1.7ml of "upper" Tris buffer [6.06gm Tris HCl, 6N HCl to pH 6.8, and 4.0ml of 10% (w/v) SDS in 100ml], 1.15ml of acrylamide/bis-acrylamide. Fresh

ammonium per sulphate (40μl) and 10μl of TEMED were added, stirred briefly, and the gel poured. The comb was then inserted, and the stacking gel allowed to polymerize.

2.6.2. *Sample Preparation and Loading.*

A small volume of highly purified virus (1-2μl, containing 0.5-2.0μg of virus), was placed in an Eppendorf tube, and an equal volume of SDS-PAGE sample buffer [consisting of 10ml of glycerol, 5ml of 2-mercaptoethanol, 30ml of 10% (w/v) SDS, 12.5ml of 4 X 'upper' Tris buffer, made up to 100ml with sterile distilled water] was added, thoroughly mixed and heated at 90°C for 5-10 min. Bromophenol blue [0.1% (w/v) in 0.1 X TAE] was added to 1/10 volume, and the sample loaded into the gel. The samples were pre-electrophoresed for 5-10 min at 80 mA to pass through the stacking gel, and then at 180mA until the marker dye ran out of the gel. The plates were removed, and the gel placed in a container for silver staining.

2.6.3. *Silver Staining of Polyacrylamide Gels.*

This was done by the method described by Wray et al., (1981). Immediately following electrophoresis, the gel was washed in three changes of about 200ml of 50% (v/v) methanol overnight on a rocking platform. Silver staining solution was prepared by mixing 21.0ml of 0.36% (w/v) NaOH and 1.4ml of freshly prepared 14.8M ammonium hydroxide. To this was added, with vigorous stirring, drops of 0.8gm of silver nitrate dissolved in 4ml of SDDW. The volume was made up to 100ml with SDDW and the solution used immediately. The gel was quickly washed for 1-2 min in deionised water. After decanting the water, the silver staining solution was added and staining allowed to take place at room temperature with gentle agitation for 15 minutes. Excess stain was washed off twice with distilled water, gently agitating each time for 5 min. Freshly prepared developing solution [2.5ml of 1.0% (w/v) citric acid solution, 0.25ml of 38% formaldehyde made up to 500ml with distilled water] was added and gently rocked for 5-15 min until the bands appeared. The gel was then washed several times with distilled water, incubated with Kodak Rapid Fix to remove background staining and/or to reduce over stained bands, and was then washed with several changes of water while agitating. After incubating in a Kodak hypoclearing

agent for 30 min with agitation, the gel was further rinsed with distilled water and left in 10% (v/v) methanol containing 5%(w/v) glycerol till photographed.

2.7. Hybridization Analysis.

2.7.1. *Synthesis of α-^{32}P-Labeled Complementary DNA (cDNA).*

This was done by the method described by Gould and Symons (1977) involving random priming. Two to 3μg viral RNA purified as previously described was used as template in a reaction mixture as follows:

5μl S-2 buffer (200mM Tris-HCl, pH 8.3, containing 700mM KCl, and 100mM MgCl$_2$)

5μl of Primer, P'

5μl of reducing agent, R (200mM dithiothreitol)

3μl of unlabelled triphosphates (TTP+ATP+GTP, 8.3 mM each)

2-3μl reverse transcriptase, (200units/μl).

2-3 μl α–^{32}P-labeled dCTP (1-1.5mM)

5μl 40mM pyrophosphate (added last)

SDDW was added to give a final reaction volume of 50μl.

The mixture was incubated at 42°C for 1.5-2 hr. To stop the reaction, 5μl each of 5% (w/v) SDS and 0.4M EDTA, 15μl of 4M NaOH, and 125μl of SDDW were added, and the mixture was left overnight to undergo hydrolysis.

The cDNA thus synthesized was purified under sterile conditions by column filtration. Autoclaved Sephadex G50 in 10mM Tris-HCl, pH 8.0, containing 1mM EDTA, was packed into a 5ml pipette, and washed repeatedly with freshly prepared 0.1M ammonium bicarbonate. The cDNA was loaded on to the column, and eluted with the bicarbonate solution. Twenty fractions were collected, the first void volume of 1.0 ml, and subsequent *fractions* of 0.5ml . The fractions were counted by Cerenkov counting in a Packard model 3320 Tri-Carb Liquid Scintillating Spectrometer. The cDNA peak fractions (coming immediately after the void volume) were retained. To each retained fraction, triethylamine was added to 10% (v/v), then frozen and freeze dried in a Dynavac model CDI Centrifugal Freeze Drying Unit.

The dried cDNA fractions were resuspended in SDDW, pooled to a total vol of 500-1000µl and stored at -20°C.

2.7.2. Blotting Procedure.

Nitrocellulose membranes of 0.2µm pore from Schleicher and Schuell were used for dot and Northern blots. The membranes were soaked first in SDDW and then in 20X SSC (175.3gm NaCl, 93.3gm tri-sodium citrate, and 200µl of 0.2N HCl, per litre). For dot-blots, a Schleicher and Schuell minifold was used, utilizing vacuum pressure to draw the RNA samples into the nitrocellulose membrane.

RNA from agarose gels were transferred to nitrocellulose as described by Palukaitis et al., (1985). The gels were trimmed to the right size after examination under ultraviolet light and placed between the sheets of nitrocellulose. Three sheets each of Whatman 3MM chromatography paper also presoaked in 20X SSC were placed below and above the sandwiched gel. This sandwich was then placed between a stack of paper towels and two glass sheets with a weight on top. Transfer of the RNAs into the nitrocellulose was allowed to take place overnight.

2.7.3. Baking, Prehybridization and Hybridization of Blots.

Nitrocellulose membranes prepared as described above were baked at 80°C for 2hr in a vacuum oven. The blots were then placed in plastic bags and hybridization buffer added to 500µl/cm^2 of nitrocellulose. The buffer used was as described by Maule et al., (1983) and consisted of 3 X SSC, containing 0.08% (w/v) BSA, 0.08% (w/v) Ficoll, 0.08% (w/v) PVP 40,000, 1mM EDTA and 250µg/ml phenol extracted yeast RNA. After ensuring complete evacuation of air bubbles, the membranes were sealed and prehybridised for 24 hr on a shaking water bath at 60°C.

After pre-hybridization, the cDNA probe was added at the rate of 50,000-200,000 cpm/ml of buffer using up to 1X 10^6 cpm per blot, and hybridization was allowed to proceed at 60°C for 24 hr in a shaking water bath.

2.7.4. *Washing and Autoradiography of Membranes.*

After hybridization, the membranes were washed as follows:

(1) 2 times in 2X SSC containing 0.5% (w/v) SDS, 5 min per wash at room temperature.

(2) 2 times in 2X SSC containing 0.5% (w/v) SDS, 5 min per wash at 55°C.

(3) 2 times in 0.1X SSC containing 0.5% (w/v) SDS, 15 min per wash at 55°C.

The membranes were placed between two sheets of Gladwrap, and excess liquid and bubbles removed, and then exposed to X-ray film, placed in cassettes with X-ray intensifier screens at -70°C. The films were developed, after an appropriate exposure time, as specified by the manufacturer.

2.7.5. *Liquid Hybridization Analysis.*

Liquid hybridization was done as described by Gonda and Symons, (1978). The hybridization was performed in 1.5ml Eppendorf tubes. Reaction mixtures, total volume 40μl, consisted of 100cpm/μl of ^{32}P-labeled probe in hybridization buffer [10mM Tris-HCl, pH 7.0, containing 1mM EDTA, 0.05% (w/v) SDS and 0.18M or 0.54M NaCl] and dilutions of test viral RNA ranging from 320pg/ml to 25μg/ml, in duplicate. Stringency of hybridization conditions was determined by the NaCl concentration; the lower concentration being more stringent. After overlaying each sample with a drop of sterile liquid paraffin, they were first pre-heated at 100°C for 3 min, and hybridization allowed to proceed at 65°C for 18 hr. For each cDNA probe, there was a blank reaction mixture, also in duplicate, in which there was no test RNA.

2.7.6. *S1 Nuclease Assay for Extent of Hybrid Formation.*

To each reaction mixture was added 400μl of S1 nuclease buffer [30mM sodium acetate, pH 4.6, containing 50mM NaCl, 1.0mM ZnSO4, and 5% (v/v) glycerol] containing 40μg/ml of denatured calf thymus DNA. After gently mixing to avoid emulsifying with the paraffin, two aliquots of 200 μl were taken from each reaction mixture and transferred into culture tubes. To one of each pair, 10μl of S1 nuclease buffer containing 2 units of S1

nuclease was added. All tubes were incubated for a further 45 min at 45°C. The S1 nuclease reactions were terminated by the addition of 1.0 ml of 10% (w/v) trichloroacetic acid (TCA) and 75μl of 1.0μg/ml BSA. The reaction mixtures were chilled for 20 min, and the TCA-insoluble RNA/DNA hybrids precipitated were collected on Whatman GF/A glass microfilters. The filters were washed with 5% (w/v) TCA followed by 80% (v/v) ethanol, dried, and the radiation determined in the presence of scintillant (toluene containing 0.4% PPO).

The fraction of each hybrid resistant to S1 nuclease was determined by dividing the cpm obtained from the S1 nuclease treated sample, by the cpm of the untreated sample. It was necessary to correct for hybridization percentages, using the relationship:

$$\text{corrected \% hybridization} = \frac{100 \times (X - Y)}{100 - Y}$$

where X = calculated % hybridization

Y = % S1 nuclease resistance of cDNA of the blanks.

The log of R_{ot}, given by the relationship

$$R_{ot} = \frac{\text{conc of RNA } (\mu g/ml \times \text{hybridization time (sec)}}{320,000}$$

was plotted against the percentage hybridization of the probe to the test nucleic acid.

2.8. CHEMICALS AND REAGENTS.

Chemicals and reagents used were laboratory or analytical grade, as required, and obtained from Sigma, BDH, M&B, AJAX or UNIVAR as the case may be.

$\alpha-^{32}P$-labeled dCTP or dATP was obtained from Bresatec, Adelaide University, or Amersham.

Reverse transcriptase was obtained from Bethesda Research Laboratories.

Unlabeled deoxy nucleotides were from Boehringer Mannheim GmbH.

The choice of chemicals used was based entirely on their availability as general laboratory requirements of the Department of Plant Pathology, Waite Institute.

CHAPTER THREE

COMPARATIVE STUDIES OF MCMV AND VTAV.

3.0. Introduction

Even though it is generally accepted that CMV and TAV are distinct viruses in the cucumovirus group, there has been some controversy about their relationship. In particular, there has been a lack of agreement on whether they are serologically related. Both viruses also appear to be distantly related to the third member of the group, peanut stunt virus (PSV) (Devergne and Cardin, 1975). Contradictory results have been obtained in attempts to determine serological relationships between CMV and TAV (Devergne and Cardin, 1975; Francki and Habili 1975). Rao et al., (1982), however, showed that the relationships deduced depend on the antiserum titre as well as the vertebrate host in which the antiserum was raised.

Gonda and Symons (1978) showed that there is no significant RNA sequence homology (less than 5%) between the RNAs of CMV and TAV. In this section, biophysical and biological comparisons of MCMV and VTAV are made in some detail. The purpose of this was to determine whether there are any physical and biological properties in which the two viruses differ and which can be used to distinguish between them routinely.

3.1. Symptomatology and Host Range.

The results of inoculation of MCMV and VTAV to a range of plant species is presented in Table 3.1. All the fourteen species tested were susceptible to MCMV, and all but two, *Gomphrena globosa* and *Cucumis sativus*, were also infected by VTAV. However, the symptoms produced on all the common hosts were quite different (Fig 3.1), with MCMV inducing severe yellow mosaic symptoms in most hosts compared to the relatively mild mosaic symptoms and leaf distortion associated with VTAV infection. A search for plant species susceptible to VTAV but not to MCMV, was unsuccessful. Of particular interest, however, were the morphologically different lesions induced in *B. vulgaris* by the two

Fig 3.1

Comparison of symptoms induced by MCMV and VTAV in (a) *Nicotiana tabacum* cv White Burley , (b) *N. glutinosa*, (c) *Zinnia elegans* cv. Golden Queen (d) *Gomphrena globosa*, (e) *Petunia hybrida* cv. Dazzler

a

b

c

d

e

MCMV   healthy   VTAV
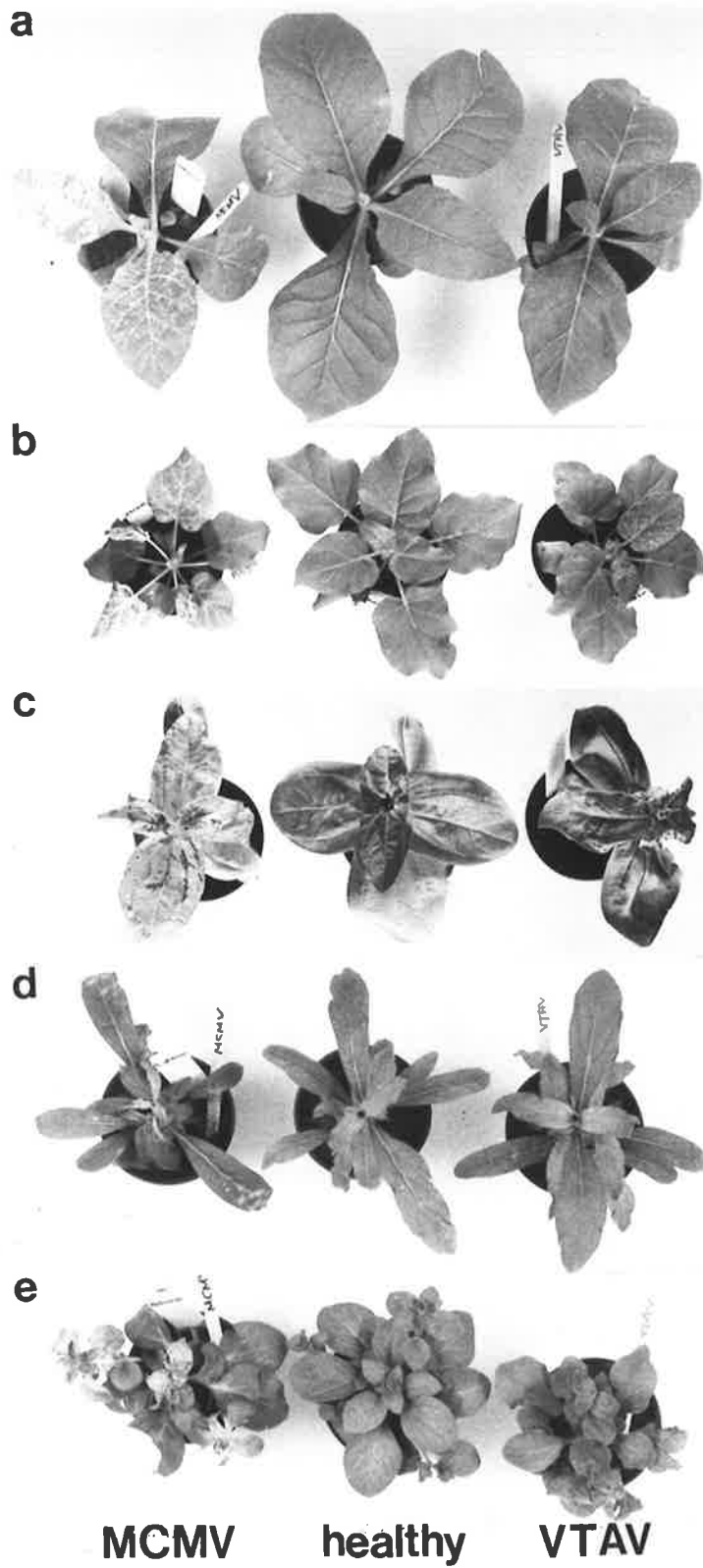
Fig 3.1

viruses (Fig 3.2), which suggested that this species could be used to biologically separate them.

Table 3.1
Host range and symptomatology of MCMV and VTAV.

| Plant tested | Symptoms induced by | |
	MCMV	VTAV
Nicotiana glutinosa	Systemic yellow mosaic and leaf distortion.	Systemic mosaic and severe leaf distortion.
Nicotiana tabacum cv. White Burley	Systemic yellow mosaic.	Systemic mosaic.
Nicotiana edwardsonii	Systemic yellow mosaic.	Systemic mosaic.
Nicotiana clevelandii	Systemic yellow mosaic and leaf distortion.	Systemic mosaic and leaf distortion.
Nicotiana benthamiana	Systemic yellow mosaic and leaf distortion.	Systemic mosaic.
Gomphrena globosa	Chlorotic local lesions, systemic yellow mosaic and severe leaf distortion.	Not infected.[1]
Solanum melongena	Chlorotic local lesions and systemic yellow mosaic.	Mild systemic mosaic.
Cucumis sativus cv. Polaris	Chlorotic local lesions and systemic mosaic.	Not infected[1].
Spinacea hybrid cv. English	Chlorotic local lesions, systemic yellow mosaic and leaf distortion.	Mild systemic mosaic.
Zinnia elegans cv. Golden Queen	Chlorotic local lesions, systemic yellow mosaic and leaf distortion.	Mild systemic mosaic.
Petunia hybrida cv . Dazzler	Systemic yellow mosaic.	Mild systemic mosaic.
Physalis floridana	Chlorotic local lesions and severe systemic yellow mosaic.	Chlorotic local lesions and systemic mosaic.
Beta vulgaris	Purple lesions with chlorotic rings, 2-5mm in diameter, no systemic spread.	Purple local lesions, 1-3mm in diameter, no systemic spread.

Lycopersicon esculentum cv. Rutgers.	Systemic yellow mosaic.	Systemic mosaic.
Chenopodium amaranticolor	Chlorotic local lesions,no systemic spread.	Chlorotic local lesions, no systemic spread.
Chenopodium quinoa	Chlorotic local lesions, no systemic spread.	Chlorotic local lesions, no systemic spread.

[1] Both systemic and inoculated leaves were tested for presence of virus antigen and none was detected

3.2. Serological Analysis.

Antisera to glutaraldehyde-fixed MCMV and VTAV had titres ranging from 1/64 to 1/256. For use in ELISA, early bleeding antisera of titres 1/128 for both MCMV and VTAV were selected. Gel diffusion tests revealed that the antisera were specific in their reaction to their respective antigens, with no cross-reaction (Fig 3.3). However, as in a previous study (Habili and Francki, 1975), VTAV produced two precipitin lines in the homologous reaction. In this study the absence of serological cross-reactivity was a desirable characteristic of the antisera.

Virus concentrations well below 0.1μg/ml were detected in double antibody sandwich ELISA (Fig 3.4a and b). There was no cross-reaction even when heterologous antigen at concentration of 50μg per ml was applied. It was thus concluded that the methods were suitable for the independent detection of the two viruses.

The sensitivity and specificity of detecting virus by ELISA in the presence of leaf extracts from uninfected plants, or equal amounts of heterologous antigen were tested. As shown in Fig 3.5a, the sensitivity of detection of MCMV was reduced in the presence of dilute leaf extracts from uninfected plants. However, the presence of equal amounts of heterologous antigen did not affect the sensitivity of the reaction (Fig 3.5a). Similarly, the presence of heterologous antigen did not affect the sensitivity of VTAV detection, nor did the presence of leaf extracts from uninfected plants, using the VTAV antiserum (Fig 3.5b). Unlike the anti-MCMV serum, however, there was some reaction with extracts from uninfected plant leaves. However, it was concluded that the antibody and conjugate systems used were satisfactory for the detection of the two viruses in single or mixed infections directly from leaf extracts.

Fig 3.2

Differences in appearance of local lesions induced by MCMV and VTAV in *Beta vulgaris* plants.
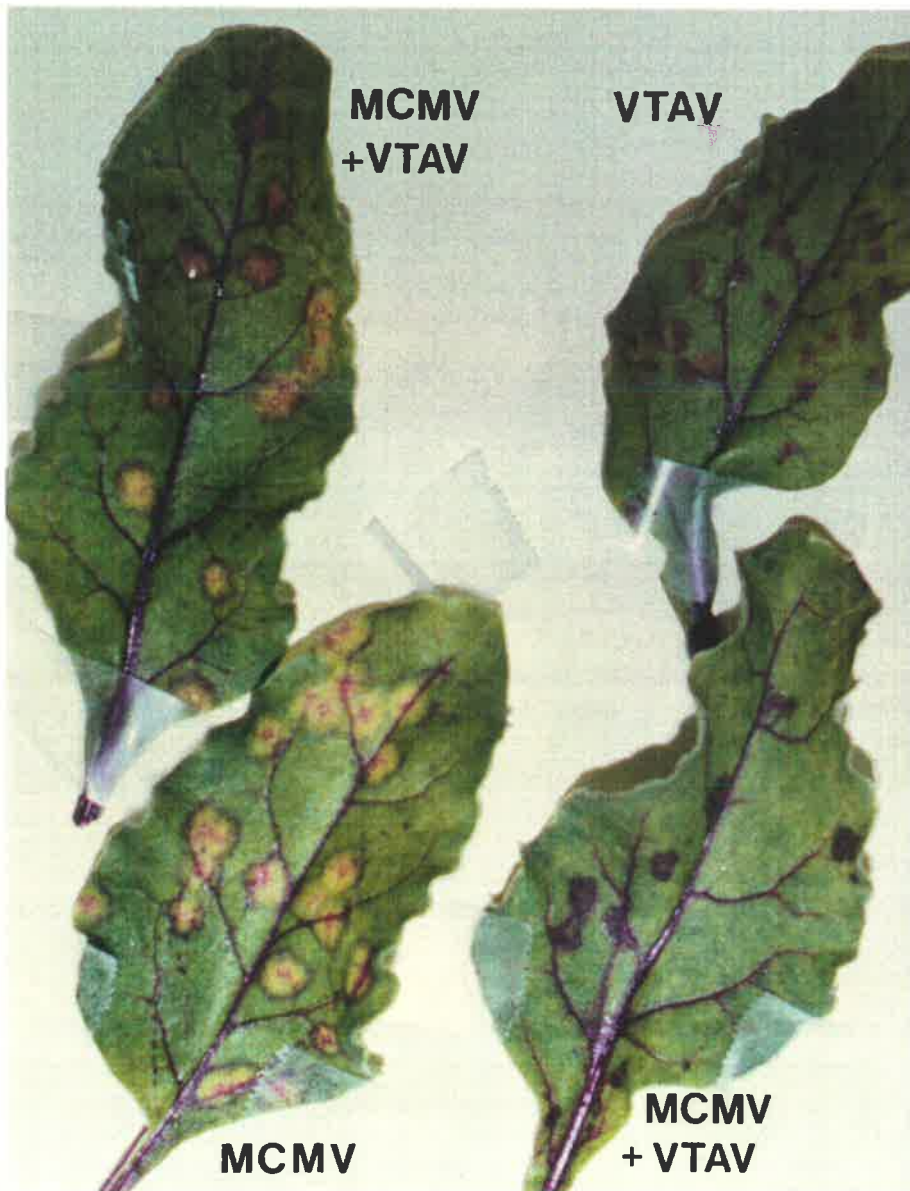
Fig 3.3

Serological properties of MCMV and VTAV.

Determination of the specificity of MCMV and VTAV antisera by gel immuno-diffusion analysis. VTAV antiserum produced two precipitin bands with unfixed VTAV preparation.
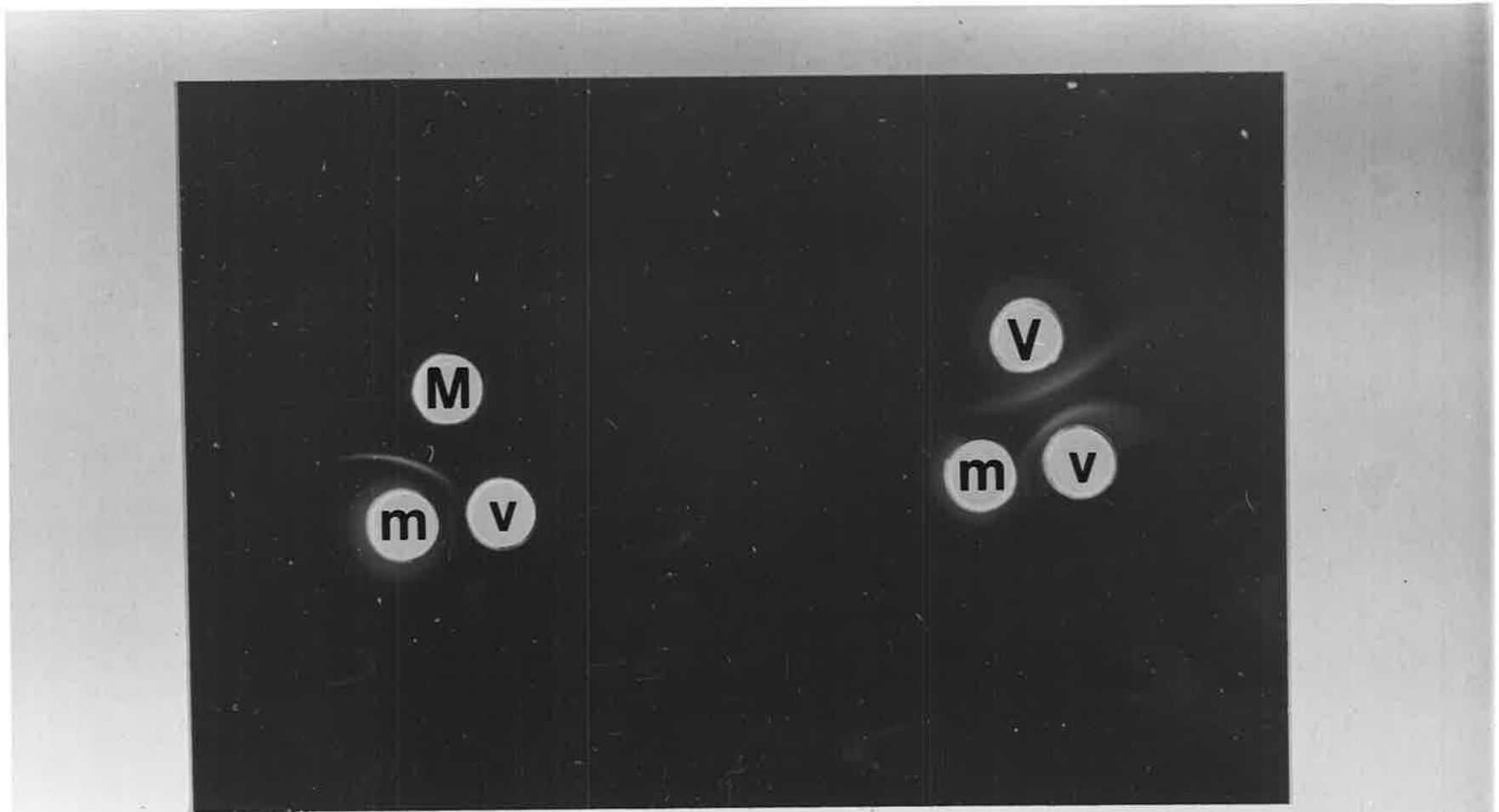
Fig 3.3
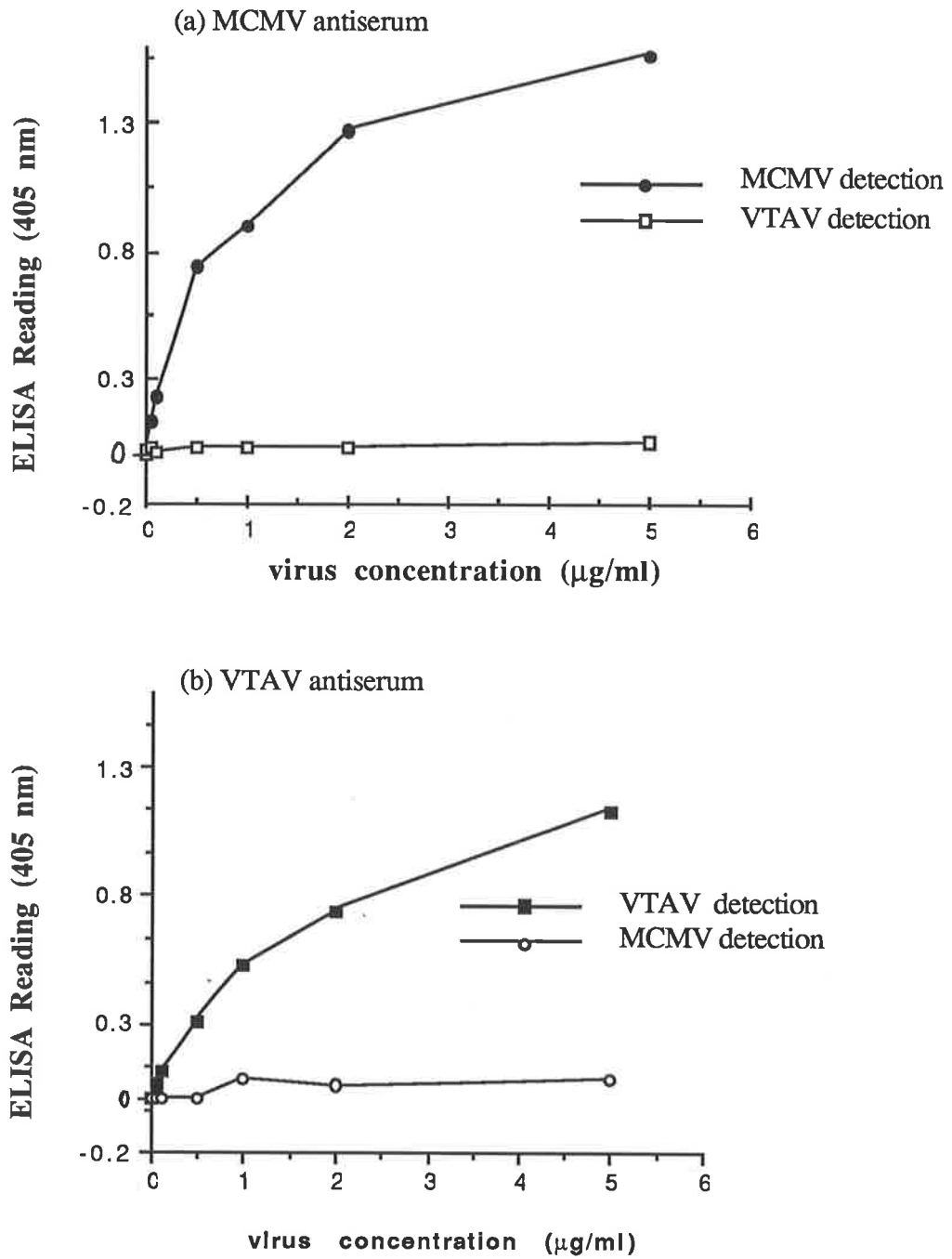
Fig 3.4 Specificity and sensitivity of ELISA for detection of MCMV and VTAV antigens.

(a) MCMV antiserum and conjugate.

- ■ MCMV purified virus
- ▣ MCMV +diluted sap(1/10 of stock extract)
- □ Equal amounts of MCMV and VTAV
- ▲ Uninfected N. glutinosa (serial dilution from 1 gm/ml stock)

(b) VTAV antiserum and conjugate

- ● VTAV purified virus
- □ Equal amounts of VTAV and MCMV
- ▣ VTAV + dilute uninfected leaf extract
- ▲ Uninfected N.glutinosa extract (serial dilution from 1 gm/ml stock)
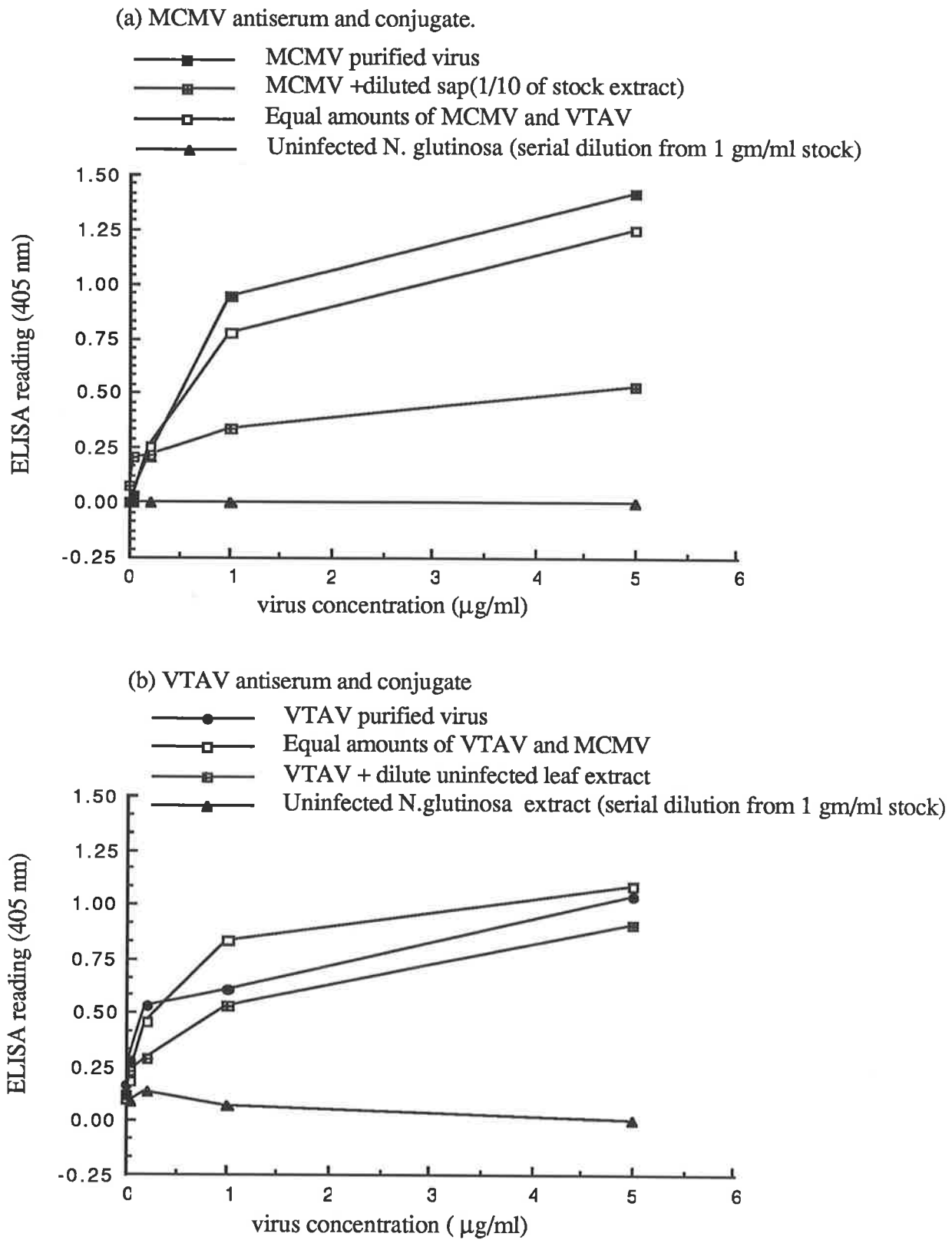
Fig 3.5 Effect of heterologous antigen and uninfected leaf extracts on detection of MCMV and VTAV by ELISA.

3.3. Effect of Purification Procedures on Recovery of MCMV and VTAV

MCMV and VTAV have to be purified by different methods (Mossop et al., 1976). The effect of each extraction and purification method on the other virus was investigated to determine if this was a suitable means of isolating one from the other when the two viruses are present in the same tissue. Two groups of *N. clevelandii* plants were infected, each with one of the two viruses. Fourteen days after inoculation, infected leaves from each group of plants were harvested. The leaves infected with MCMV were divided into two batches; one was extracted and purified by the method described for the purification of MCMV (method M), and the other by the method for VTAV (method V). Similarly, leaves from plants infected by VTAV were divided into two portions and virus extracted and purified by the two methods.

Sucrose density-gradient (5 to 25%, w/v) sedimentation profiles of the preparations presented in Fig 3.6 show that the VTAV purification method completely disrupts the MCMV particles (Fig 3.6a). The MCMV purification method also disrupts some but not all VTAV particles (Fig 3.6b). When *Nicotiana glutinosa, N. tabacum* cv. White Burley, *N. clevelandii* and *N. edwardsonii* plants were inoculated with the four virus preparations, all except the MCMV preparation purified by method V were infectious. Even though most of the VTAV particles were disrupted by method M (Fig 3.6b), there was enough intact virus recovered to infect the host plants tested.

In conclusion, method V was totally unsuitable for purifying MCMV. In contrast, even though much of the virus was lost, some intact and infectious VTAV was recovered when method M was used to purify virus from plants infected with VTAV.

3.4. Polyacrylamide Gel Electrophoretic Analysis of MCMV and VTAV Coat Proteins.

Fig 3.7 shows comparative electrophoretic mobilities of MCMV and VTAV coat protein subunits. When applied in the same well, (Fig 3.7) there was no detectable difference in the migration of the two coat proteins.
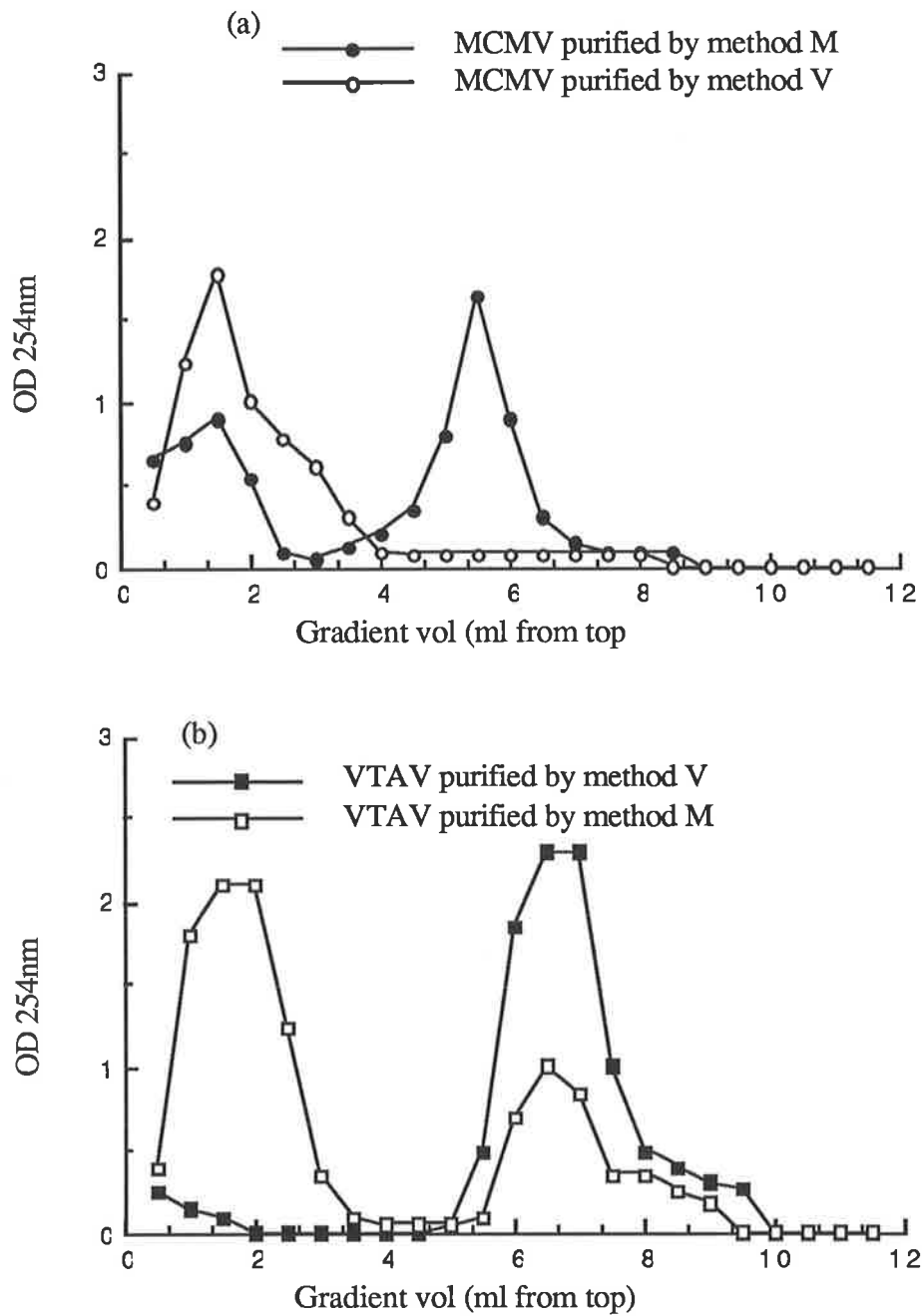
Fig 3.6 Differential effects of virus purification methods on recovery of MCMV and VTAV from sucrose density-gradients.

Fig 3.7

Comparison of MCMV and VTAV coat protein subunits by polyacrylamide gel electrophoresis under denaturing conditions.

Protein markers and their molecular weights were (a) phosphorylase **b**, Mr, 94,000; (b) BSA, 67,000; (c) ovalbumin, 43,000; (d) carbonic anhydrase, 30,000;

(e) soybean trypsin inhibitor, 20,000 and (f) ∂-lactalbumin, 14,000.

Fig 3.8

Comparison of MCMV and VTAV RNAs by 2% agarose gel electrophoresis under non-denaturing conditions.

The RNAs 1, 3 and 4 of MCMV and VTAV were resolved in these conditions but not the RNA 2 (lane MCMV+VTAV).
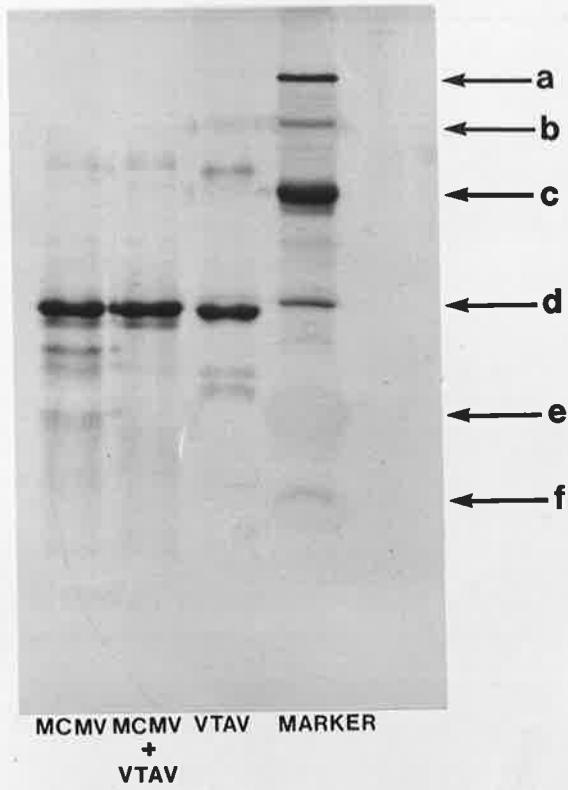
Fig 3.7
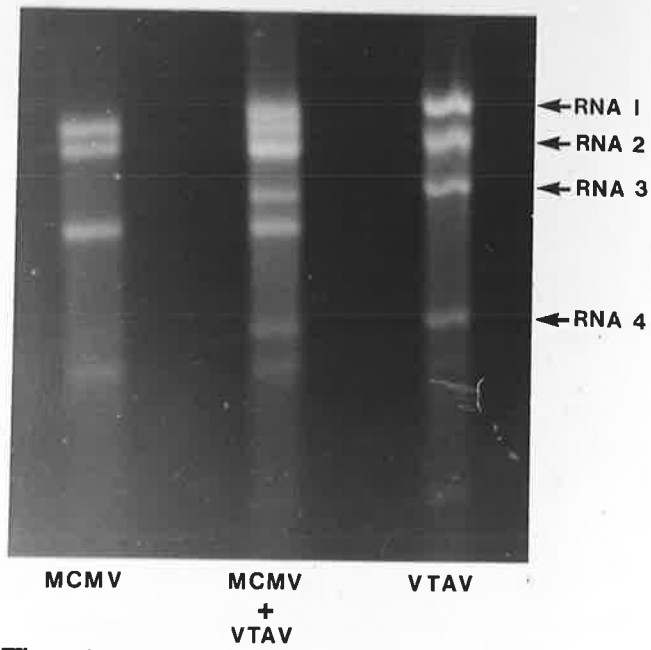
Fig 3.8

3.5. RNA Composition of MCMV and VTAV.

The RNAs extracted from highly purified preparations of MCMV and VTAV were compared by electrophoresis in a 2% horizontal agarose slab gel. Results presented in Fig. 3.8 show that the RNAs 1,3 and 4 of MCMV and VTAV have readily distinguishable mobilities, whereas the RNAs 2 could not be resolved.

The extent of base sequence homology between MCMV and VTAV RNAs were compared by dot blot, Northern blot and liquid hybridization analysis using ^{32}P-labeled cDNA probes synthesized from the total RNAs of the two viruses as templates. The results of the Northern blot hybridization analysis presented in Fig 3.9a and b show that there was no cross-hybridization with the cDNA probes of the two viruses. However, in both dot-blot hybridization analysis (Fig 3.10 a and b) and liquid hybridization analysis (Fig 3.11a and b) there was some cross-hybridization, indicating less than 5% homology between the RNA sequences of the two viruses. These results are consistent with those obtained in comparisons of homology between VTAV and other CMV strains including MCMV (Gonda and Symons 1978). It was concluded that differences in the RNA sequences of the two viruses can be used to distinguish between them.

3.6. Pseudorecombinants of MCMV and VTAV.

Genomic RNAs of MCMV and VTAV were isolated by two cycles of preparative agarose gel electrophoresis. The second cycle was designed to reduce any contamination of RNAs 1 and 2 by aggregates of RNA 3. Rao and Francki (1981) showed that viable pseudorecombinants between MCMV and VTAV could be constructed involving only the exchange of their RNAs 3. Consequently, only the pseudorecombinant resulting from combining MCMV RNAs 1 and 2 with VTAV RNA 3 ($M_1M_2T_3$) and that from VTAV RNAs 1 and 2 with MCMV RNA 3 ($T_1T_2M_3$) were constructed for use in these studies.

The pseudorecombinants were tested for their authenticity by examining their serological specificity to indicate the source of the RNA 3 present, and by Northern blot hybridization analysis for their RNA composition. Ouchterlony test results presented in Fig 3.12a and b show MCMV and $T_1T_2M_3$ both reacted with anti-MCMV serum but not with anti-VTAV serum. Similarly, VTAV and $M_1M_2T_3$ reacted with anti-VTAV serum but not

Fig 3.9

Comparison of MCMV and VTAV RNAs for sequence homology by Northern blot hybridization analysis.

Fig 3.10

Comparison of MCMV and VTAV RNAs for sequence homology by dot-blot hybridization analysis.

Viral RNAs were used at the amounts indicated in the central panel, ie, 50ng, 20ng, 5ng, 0.5ng and 0.05ng. Uninfected leaf total RNA extract was used as a negative control and were at twice the amounts of viral RNA applied per spot.

a.
MCMVcDNA

b.
VTAVcDNA

M   M   V   M   M   V
    +   +
    V   V

Fig 3.9

a. MCMV cDNA

b. VTAV cDNA

0.05ng
0.5ng
5ng
20ng
50ng

MCMV Healthy VTAV

M   H   V   M   H   V

Fig 3.10

(a)

Y-axis: % hybrid formation with MCMV cDNA
X-axis: ln(x) RNA concentration (μg/ml)

Legend:
● MCMV RNA
□ VTAV RNA

(b)

Y-axis: % hybrid formation with VTAV cDNA
X-axis: ln(x) of RNA concentration (μg/ml)

Legend:
■ VTAV RNA
○ MCMV RNA
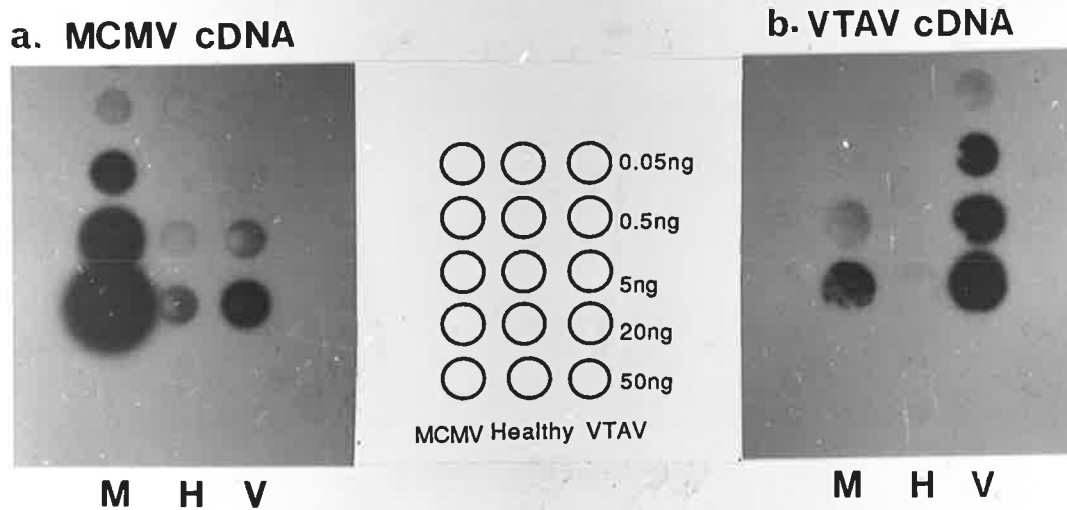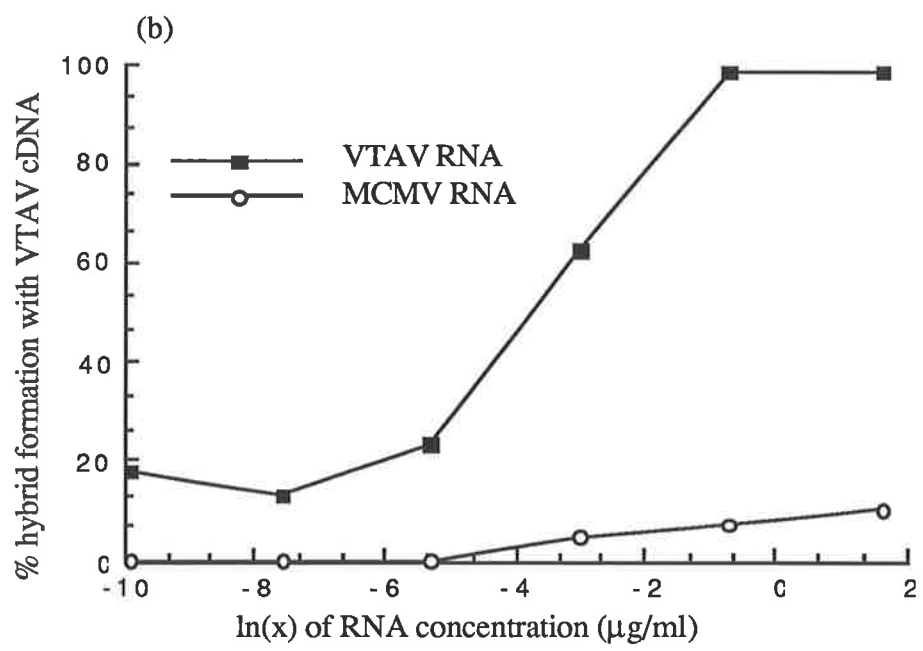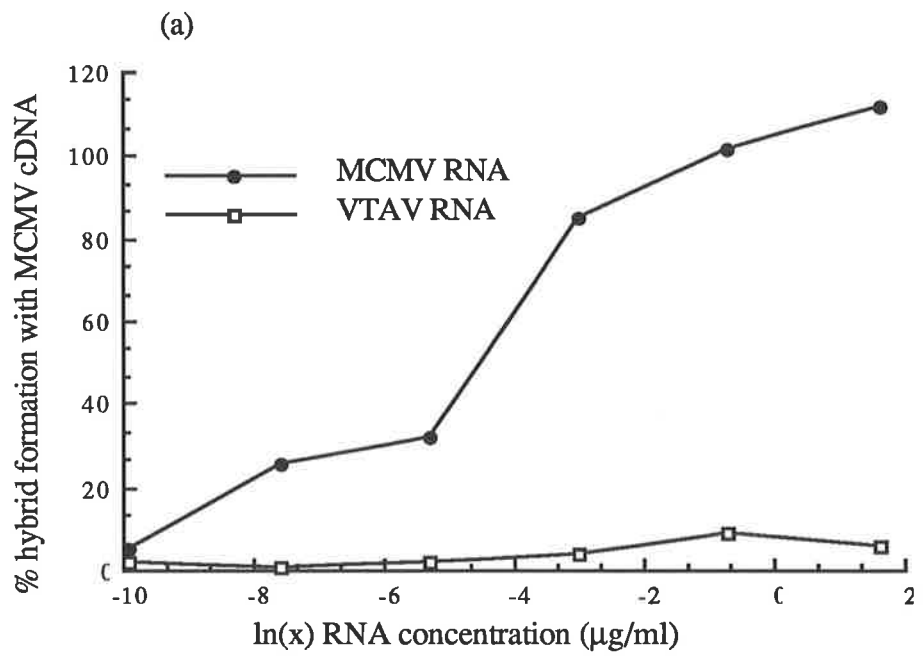
Fig 3.11 Determination of the extent of RNA sequence homology between MCMV and VTAV by liquid hybridization analysis.

Fig 3.12

Authenticity of the RNA composition of pseudorecombinants $M_1M_2T_3$ and $T_1T_2M_3$.

Determination of the sources of coat protein (hence RNAs 3) by serological analysis using MCMV (M) and VTAV (V) antisera. MCMV (m) and $T_1T_2M_3$ (b) purified virus preparations formed immunoprecipitin lines with MCMV (panel **a**) but not VTAV (panel **b**) antiserum . VTAV (V) and $M_1M_2T_3$ (a) formed immunoprecipitin lines with VTAV (panel **b**) but not MCMV (panel **a**) antiserum.

Fig 3.13

Determination of RNA composition of pseudorecombinants $M_1M_2T_3$ and $T_1T_2M_3$ by northern hybridization analysis using MCMV (panel a) and VTAV (panel b) cDNA probes.
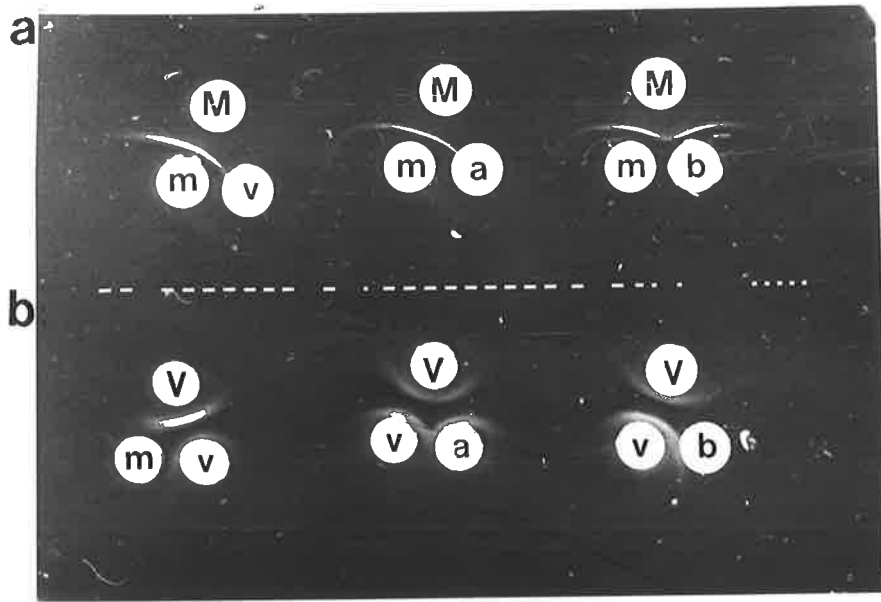
Fig 3.12
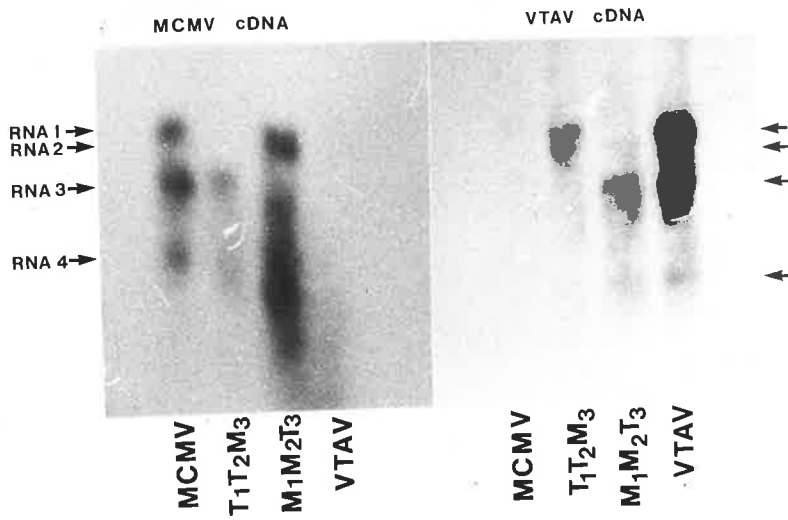
Fig 3.13

with anti-MCMV serum. Thus, $T_1T_2M_3$ had MCMV coat protein (hence RNA 3), and $M_1M_2T_3$ had VTAV coat protein (hence RNA 3).

Results of Northern hybridization analysis presented in Fig 3.13a and b confirmed the RNA composition of the two pseudorecombinants.

3.7. Symptomatology and Host Range of Pseudorecombinants $M_1M_2T_3$ and $T_1T_2M_3$.

The infectivity and host range of the pseudorecombinants, compared with the parental viruses MCMV and VTAV, are presented in Table 3.2. In general, $M_1M_2T_3$ and $T_1T_2M_3$ infected most of the plants susceptible to MCMV and VTAV. Their effects in most cases were milder than those of the parental viruses, $T_1T_2M_3$ more so than $M_1M_2T_3$. However, whereas $M_1M_2T_3$ induced VTAV-like symptoms in most hosts tested, $T_1T_2M_3$ was characterized by yellow vein banding and mosaic symptoms and patchy yellow chlorosis, particularly in *Nicotiana* species. These symptoms were quite different from those induced by both parental viruses. The effects of the two pseudorecombinants in *G. globosa* and *C. sativus* were of the most interest. Neither of these plants are infected by VTAV. $M_1M_2T_3$ induced local lesions on inoculated leaves of *C. sativus* but without systemic movement. In contrast, $T_1T_2M_3$ induced neither local lesions nor systemic infection of *C. sativus*. In *G. globosa*, however, $T_1T_2M_3$ induces only local lesion on the inoculated leaves, whilst $M_1M_2T_3$ was not infectious. These properties of the two pseudorecombinants made them useful for studying the roles of, and interactions between, the genomic RNAs of MCMV and VTAV.

3.8. CONCLUSIONS.

MCMV and VTAV have a wide range of common hosts. In the species tested, the common symptom produced by the viruses was systemic mosaic with varying degrees of severity from species to species. MCMV was characterized by severe bright yellow mosaic symptoms in systemically infected leaves, and could thus be readily distinguished from VTAV. The immunity of *Cucumis sativus* and *Gomphrena globosa* to VTAV was particularly useful for distinguishing between the two viruses. Unfortunately, I was unable

Table 3.2

Host range and symptomatology of pseudorecombinants $M_1M_2T_3$ and $T_1T_2M_3$.

Plant tested	Symptoms induced by[1]	
	$M_1M_2T_3$	$T_1T_2M_3$
Nicotiana glutinosa	Systemic mosaic (=VTAV, less severe)	Systemic mosaic and yellow vein banding (≠MCMV,≠VTAV).
Nicotiana tabacum cv.White Burley	Systemic mosaic (=VTAV,less severe leaf distortion)	Systemic mosaic with patchy chlorosis (≠MCMV,≠VTAV)
Nicotiana clevelandii.	Systemic mosaic (=VTAV)	Systemic mosaic and yellow vein banding (≠MCMV,≠VTAV)
Nicotiana benthamian	Systemic mosaic (=VTAV)	Systemic mosaic (=VTAV)
Gomphrena globosa	Not infected (=VTAV)[1] on inoculated leaves (≠MCMV,≠VTAV)	Chlorotic local lesions with no systemic spread
Cucumis sativus	Chlorotic local lesions on inoculated leaves	Not infected (=VTAV)[1] (≠MCMV,≠VTAV).
Spinacea hybrid cv.English	Mild systemic mosaic (=VTAV)	Mild systemic mosaic (=VTAV)
Zinnia elegans cv.Golden Queen	Mild systemic mosaic (=VTAV)	Mild systemic yellow mosaic (=MCMV, mild)
Petunia hybrida cv.Dazzler(=VTAV)	Mild systemic mosaic (=MCMV,mild)	Mild systemic yellow
Physalis floridana	Chlorotic local lesions on inoculated leaves andsystemicmosaic (=VTAV)	Chlorotic local lesions on inoculated leaves andsystemic yellow mosaic(=MCMV,mild)
Chenopodium amaranticolor	Chlorotic local lesions no systemic spread (=MCMV=VTAV)	Chlorotic local lesions no systemic spread (=MCMV=VTAV).
Chenopodium quinoa	Chlorotic local lesions no systemic spread. (=MCMV,=VTAV)	Chlorotic local lesions no systemic spread. (=MCMV,=VTAV)

[1] (=) Indicates similar to and (≠) indicates different from the parental virus symptoms.
[2] Both systemic and inoculated leaves were tested for the presence of antigens and none was found.

to find a host which was susceptible to VTAV but not MCMV. The distinct lesions produced by *B. vulgaris* inoculated with the two viruses was another useful host property particularly for obtaining local lesion isolates of the viruses. Of some interest, also, was the observation that *B .vulgaris* was less sensitive to VTAV than to MCMV as suggested by the fact that the former induced significantly fewer lesions than the latter.

The ELISA system developed was shown to be capable of detecting the presence of either virus in mixed infections. Even though leaf extracts from uninfected plants lowered the sensitivity, it did not affect the specificity of detection. The presence of the second virus, however, affected neither the sensitivity, nor the specificity of the assays.

MCMV and VTAV have similar physical properties, as previously reported by Mossop and Francki, (1976). However, they also have significant differences in a number of other features. These include the absence of serological-cross reactivity, differences in the electrophoretic mobilities of their RNA components, particularly of their RNAs 3 and 4, as well as lack of homology between the nucleotide sequences of their RNAs. While VTAV is aphid transmissible, MCMV is not (Mossop and Francki, 1977). Together with the immunity of *C. sativus* and *G. globosa* to VTAV, this provides two biological methods for distinguishing between, and possibly separating them. The conclusion by Rao and Francki (1981) that production of viable, infectious pseudorecombinants between CMV and TAV was limited to an exchange of their genomic RNAs 3 made MCMV and VTAV a suitable model for studying virus-virus and virus-host interactions in which the range of exchange of genetic material was presumed to be limited by their genetic compatibility.

Pseudorecombinants from the two viruses infected most of the host plants infected by the parental viruses. The differences observed provide further tools for characterizing the gene functions of the RNAs of MCMV and VTAV, as well as any recombinants, pseudorecombinants and transcapsidants arising from mixed infections of the two viruses.

CHAPTER FOUR
MIXED INFECTIONS OF MCMV AND VTAV, AND THEIR TWO PSEUDORECOMBINANTS.

4.0. Introduction

In order to study the interaction of two viruses in plants it is necessary to find a pair of viruses which can multiply simultaneously. In this chapter, the conditions required for coinfecting a number of plant species by MCMV and VTAV, and their pseudorecombinants were investigated. The responses of the hosts to such infections were examined, as were the RNA composition of the infecting species.

4.1. Symptom Induction And Antigen Detection in *Nicotiana glutinosa* Inoculated With MCMV and VTAV.

In preliminary experiments, one *N. glutinosa* leaf infected with MCMV only, and one leaf infected with VTAV only, were extracted together in water. The extract was used to inoculate 20 *N. glutinosa* plants. Twelve days after inoculation the plants were examined for symptoms, and leaf samples were extracted and analysed by ELISA to determine which antigens were present. Results presented in Fig 4.1 (and frontispiece) show the spectrum of symptoms ranging from MCMV-like to VTAV-like induced in the plants. Twelve of the 20 plants showed MCMV-like symptoms, three plants showed some chlorotic patches in a green mosaic, and five plants showed VTAV-like symptoms. When individually tested by ELISA, 13 of the plants contained both MCMV and VTAV antigens, five contained only MCMV antigens, and two contained only VTAV antigens.

4.2. Effect of Varying Relative Concentrations of MCMV and VTAV in Mixed Inoculum on Virus Multiplication and Symptomatology.

Purified preparations of MCMV and VTAV each containing 100µg/ml of virus were mixed in the following ratios of MCMV to VTAV (v:v): 100:0; 80:20; 60:40; 50:50; 40:60;

Fig 4.1 (and Frontispiece)

A "Spectrum" of symptoms induced in *N. glutinosa* plants coinoculated with MCMV and VTAV.

Most of the plants were shown by ELISA to contain both MCMV and VTAV antigens while a few contained only MCMV or VTAV antigens.

Fig 4.1

20:80 and 0: 100. Each inoculum was used to infect four *N. glutinosa* plants at the 4-5 leaf stage. Four days after inoculation, sampling commenced at daily intervals starting with the leaves immediately above those inoculated as described in Fig 2.1. Four leaves from each plant were sampled at daily intervals for five days giving 20 samples for each plant. The leaf samples were extracted and analysed by ELISA.

Table 4.1 shows that regardless of the relative amounts of the two viruses in the inoculum (except for 100%), both (antigens) were detected although at different times after inoculation. Generally, the antigen of that virus present in the higher concentration in the inoculum was detected first. MCMV-like symptoms were the most frequently expressed, even in treatments in which VTAV was the major component of the inoculum.

4.3. Test for Cross-protection Between MCMV and VTAV in *Nicotiana glutinosa*.

Results of symptoms and antigens detected in plants after primary and challenge inoculations of the two viruses are presented in Table 4.2. Four *N.glutinosa* plants were used for each treatment. The challenge inoculations were applied 24, 48, and 72 hr after the primary inoculations. Leaf samples were taken as previously described from day five after the primary inoculation, commencing with the leaves immediately above those inoculated. Four leaves were sampled per plant, and each leaf was sampled four times at daily intervals, and the extracts analysed by ELISA.

Both antigens were detected in all the plants inoculated with both viruses except those inoculated with VTAV 72 hr before MCMV (Table 4.2, treatment d). In general, the symptom expressed was that for the virus used in the primary inoculation. However, when VTAV was inoculated 24 hr before MCMV (Table 4.2, treatment b) not only was MCMV antigen detected first, but the symptoms expressed were MCMV-like.

Together with the results presented in the preceding sections, it appears that the chlorosis associated with MCMV infection was more likely to be expressed than the VTAV-like symptoms in coinfections of the two viruses. However, some plants containing both antigens exhibited VTAV-like symptoms.

Table 4.1.

The effect of varying relative amounts of MCMV and VTAV in mixed inocula on antigen detection and symptoms on *N. glutinosa*.

Ratio of MCMV:VTAV (μg/ml) in inoculum		Antigen first detected[1] (daysafter inoculation)		Symptoms induced[2]
MCMV	VTAV	MCMV	VTAV	
100	0	5	-	MCMV-like
80	20	5	12-14	MCMV-like
60	40	6	12-13	MCMV-like
50	50	7	10-11	MCMV-like
40	60	8	8	MCMV-like
20	80	8	7	VTAV-like
0	100	-	6	VTAV-like

[1]Antigens present were detected by analysis of leaf extracts from infected plants by ELISA

[2]The symptoms recorded were those prevailing in the test plants 21 days after inoculation.

Table 4.2

Test for cross protection between MCMV and VTAV in *N.glutinosa* .

Primary inoculum[2]	Challenge inoculum[2]	Challenge time (days)	Antigen detected (days after primary inoculation)[1]		Symptom Induced[3]
			MCMV	VTAV	
(a)buffer[4]	VTAV	-	none	6-7	VTAV-like
(b)VTAV	MCMV	1	10-15	6-8	MCMV-like
(c)VTAV	MCMV	2	12-15	6-7	VTAV-like
(d)VTAV	MCMV	3	none after 19	6-7	VTAV-like
(e)buffer[4]	MCMV	-	5-6	none	MCMV-like
(f)MCMV	VTAV	1	5-6	10-14	MCMV-like
(g)MCMV	VTAV	2	5-6	12-14	MCMV-like
(h)MCMV	VTAV	3	5-6	15-16	MCMV-like

[1] Antigens present in leaf extracts detected by ELISA.

[2] Primary and challenge inoculations were applied to the same leaves.

[3] Symptoms induced were recorded 19 days after primary inoculations

[4] Phosphate buffer (50mM, pH 7.6) as a control inoculum was also used for diluting purified virus preparations to concentration of 100µg/ml.

4.4. Time Course of Virus Multiplication in Single And Mixed Infections of MCMV and VTAV in *Nicotiana glutinosa*.

The rate of virus multiplication and systemic transport in single and mixed infections of MCMV and VTAV were compared in *N. glutinosa*. Equal volumes of each purified virus at 100μg/ml were mixed and used to inoculate the two largest leaves of each of five plants at the 4-5 leaf stage. As controls, MCMV and VTAV at concentration of 50μg/ml were each similarly inoculated to groups of test plants. The three leaves emerging above those inoculated were sampled at daily intervals starting from day four after inoculation. Each leaf was sampled five times. Data presented in Fig 4.2, are those obtained for the leaf in each treatment in which antigen was first detected. The results show that there was a significant delay in the detection of both viruses when they occur in mixed infections compared to single infections. This delay was more pronounced with VTAV than with MCMV.

4.5. Persistence of Mixed Infections of MCMV and VTAV.

The persistence of mixed infections of the two viruses in a number of common host species was tested. Plants were inoculated with a mixture of equal volumes of MCMV and VTAV at 100μg/ml. Twelve to 18 days after inoculation depending on the species used, leaf samples were taken from each plant and analysed by ELISA. Extracts from plants in which both antigens were detected was used as inoculum to infect further plants. Inoculum was taken 14-20 days after inoculation of the test plants.

Results presented in Table 4.3 show that through at least two passages except for *P. floridana* (Table 4.3, treatment c), antigens of both viruses were present in most of the test plants. With successive passages however more and more plants contained only MCMV antigen, and eventually, VTAV antigens were no longer detected in them. In *N. glutinosa*, however, both antigens were still present in all seven plants inoculated after four passages (Table 4.3, treatment f).
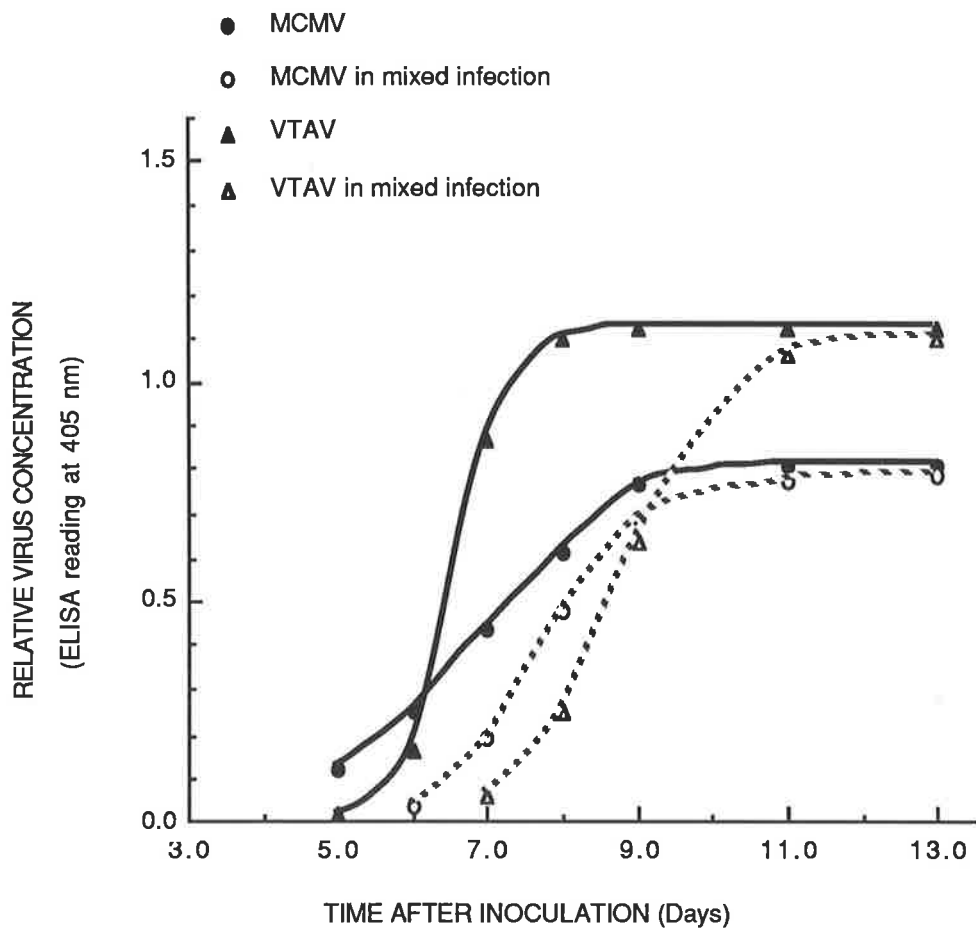
Figure 4.2

Time course of virus multiplication in single and mixed

infections of MCMV and VTAV in *N. glutinosa*

Table 4.3

Persistence of mixed infections of MCMV and VTAV in a variety of common plant species.

ANTIGENS DETECTED AFTER EACH PASSAGE[1]

HOST PLANT	PASSAGE 1[2]			PASSAGE 2			PASSAGE 3			PASSAGE 4		
	M	V	M+V	M	V	M+V	M	V	M+V	M	V	M+V
Petunia hybrida	0/5	0/5	5/5[3]	4/6	0/6	2/6		-			-	
Nicotiana benthamiana	3/4	0/4	1/4	1/6	0/6	5/6	3/5	0/5	2/5	6/6	0/6	0/6
Physalis floridana	1/5	0/5	4/5	6/6	0/6	0/6		-			-	
Spinacea hybrida	0/9	6/9	3/9	0/5	0/5	5/5	4/6	0/6	2/6		-	
Nicotiana clevelandii	4/14	0/14	10/14	0/7	0/7	7/7	4/6	0/6	2/6		-	
Nicotiana glutinosa	2/26	1/26	21/26	2/12	0/12	10/12	3/10	0/10	7/10	0/7	0/7	7/7
Lycopersicon esculentum cv. Rutgers	0/5	0/5	5/5	7/12	0/12	5/12		-			-	

[1] Leaf discs from test plants were taken for analysis by ELISA.
[2] Extracts from leaf samples from plants testing positive for the presence of both MCMV and VTAV antigens were used as inoculum for infecting further plants. ELISA tests were done 12-18 days, and inoculum 14 to 20 days after inoculation.
[3] Numerator denotes numbers of plants containing the antigen, and denominator the numbers of plants tested.

4.6. *Beta vulgaris* and *Datura stramonium* as Local Lesion Hosts.

To determine whether the interactions between the two viruses in systemic hosts also occur in their common local lesion hosts, *B. vulgaris* plants were coinoculated with the two viruses as detailed in Table 4.4. The lesions produced were excised and extracts tested by ELISA to determine their antigenic content. The results presented in Table 4.4 show that the two viruses multiply in close enough proximity as to occur together in some of the lesions. However, MCMV antigen alone was detected more often than VTAV alone. Similar results were obtained when lesions produced by *D. stramonium* inoculated with the two viruses were similarly analysed (Table 4.4b).

4.7. Survival of Heterologous RNAs 3 in Plants coinfected with MCMV or VTAV and Their Pseudorecombinants.

Purified virus preparations of MCMV, VTAV and the two pseudorecombinants, $M_1M_2T_3$ and $T_1T_2M_3$, each at concentrations of 100µg/ml, were used to coinfect *Nicotiana glutinosa, N. clevelandii,* and *N. edwardsonii* plants. MCMV was coinoculated with $M_1M_2T_3$, and VTAV with $T_1T_2M_3$, using equal amounts of each virus. Leaf extracts were analysed by ELISA, 12 and 14 days after inoculation. Results presented in Table 4.5 show that in all three species of plants used, the MCMV coat protein gene (and presumably, MCMV RNA 3) survived in all plants inoculated with the VTAV/$T_1T_2M_3$ mixture. Moreover, it appears to have displaced the VTAV coat protein gene in about one third of the plants. In contrast, only MCMV antigens were detected in plants inoculated with the MCMV/$M_1M_2T_3$ mixture in all three species of plants. These results indicate that whereas MCMV RNA 3 survived and sometimes replaced the VTAV RNA 3 (Table 4.5a), the RNA 3 of VTAV is unable to survive in the presence of only MCMV RNAs 1 and 2.

The effect of varying the relative amounts of the components of the mixed inocula was tested to determine if there were any conditions under which the VTAV RNA 3 could survive. In other treatments the inoculations were staggered, with one virus being inoculated 24 hr before the other.

Table 4.4.

Occurence of mixedly infected lesions in *Beta vulgaris* and *Datura stramonium* co-inoculated with MCMV and VTAV.

	ANTIGENS DETECTED[2]		
Inoculum[1]	MCMV alone	VTAV alone	MCMV and VTAV
a)*B. vulgaris*			
1)Mixture of equal volumes of MCMV and VTAV purified virus, each at 100µg/ml.	17/40	14/40	10/40
2)Aqueous extract from *Nicotiana clevelandii* co-infected with MCMV and VTAV.	11/15	0/15	4/15
3)Aqueous extract from coinfection of MCMV and VTAV passaged once in *N. clevelandii*	28/32	0/32	4/32
b)*D. stramonium* mixture of equal volumes of MCMV and VTAV purified virus each at 100µg/ml	10/13	0/13	3/13

[1] Extracts from plants used as inoculum was pre-tested by ELISA for presence of both MCMV and VTAV antigens.

[2] Lesions were excised from test plants 8-10 days after inoculation, and the extracts tested by ELISA. Numerator denotes numbers of lesions containing the antigen, and denominator the total number of lesions tested.

Table 4.5 Persistence of MCMV and VTAV RNAs 3 in mixed infections of pseudorecombinants and parental viruses in *Nicotiana glutinosa N. clevelandii.* and *N. edwardsonii.*

Inoculum[2]	Host plant	Antigens detected in plants[1]			Symptoms Induced[3]
		MCMV only	VTAV only	MCMV+VTAV	
(a)$T_1T_2M_3$+VTAV	*N.glutinosa*	3/8[4]	0/8	5/8	$T_1T_2M_3$-like (3/8) VTAV-like(5/8)
	N. clevelandii	2/7	0/7	5/7	$T_1T_2M_3$-like(2/7) VTAV-like (5/7)
	N. edwardsonii	2/6	1/6	1/6	$T_1T_2M_3$-like(2/6) VTAV-like(2/6)
(b)$M_1M_2T_3$+MCMV	*N.glutinosa*	8/8	0/8	0/8	MCMV-like
	N. clevelandii	8/8	0/8	0/8	MCMV-like
	N. edwardsonii	5/5	0/5	0/5	MCMV-like

[1]Antigens present in leaf extracts were determined by ELISA.
[2]Purified virus diluted in 0.1 M phosphate buffer pH 7.6 to 100μg/ml were used. Equal amounts of the two viruses were mixed and used to inoculate the test plants.
[3]Figures in parenthesis represent numbers of plant testing positive for that antigen out of total number tested.
[4]Numerator indicates number of plants in which the antigen(s) detected were present, and the denominator indicates the number of plants inoculated

Results presented in Table 4.6 show that when inocula contained equal amounts of MCMV and $M_1M_2T_3$, all the infected plants contained MCMV antigen only. However, as the proportion of MCMV to $M_1M_2T_3$ decreased in the inoculum, some plants still contained only MCMV antigen but others contained VTAV antigen only, and one plant contained both antigens (Table 4.6, treatment b). All the plants containing MCMV antigen alone, or both MCMV and VTAV antigens, showed MCMV-like symptoms. The plant in which only VTAV antigen was detected showed $M_1M_2T_3$-like symptoms.

When equal amounts of VTAV and $T_1T_2M_3$ were used to inoculate *N. glutinosa* plants, however, two of the three plants contained both antigens, and one plant contained only MCMV antigen (Table 4.6 treatment d). As the relative amount of VTAV in the inocula decreased, only MCMV antigen was detected in all the plants (Table 4.6 treatments e and f). Those plants containing both antigens, however, showed VTAV-like symptoms.

When the inoculations were staggered, MCMV-like symptoms were expressed by those plants inoculated with $M_1M_2T_3$ 24 hr before MCMV, and no VTAV antigens were detected (Table 4.6, treatment g). In contrast, $T_1T_2M_3$-like symptoms were expressed when that virus was inoculated first, and no VTAV antigens were detected (Table 4.6 treatment h). When VTAV was inoculated 24 hr before $T_1T_2M_3$, all the plants expressed VTAV-like symptoms. However, two of the three plants contained both MCMV and VTAV antigens (Table 4.6, treatment i)

It was concluded that the MCMV RNA 3 was more competitive than the VTAV RNA 3. This property appears to be independent of the genomic composition of the mixed infection, ie whether or not other MCMV RNAs were present.

4.8. Does MCMV or VTAV coat protein present with mixture of MCMV and VTAV RNAs 1 And 2 Preferentially Encapsidate some RNA species?

In the preceding sections, it was observed that even though MCMV and VTAV coinfected a variety of plants, a larger proportion of the plants developed MCMV-like symptoms and in many cases no VTAV antigen could be detected. It was unclear whether this was due to suppression of replication of all the VTAV RNAs (1, 2 and 3), or only the exclusion of the

Table 4.6

Effect of varying conditions of inoculation on survival of pseudorecombinants in mixed infections with parental viruses in *Nicotiana glutinosa*

Inoculum composition	Antigens detected in plants[1]			Symptoms Induced
	MCMVonly	VTAVonly	MCMV+VTAV	
(i) MCMV plus $M_1M_2T_3$				
(c) 100μg/ml 100μg/ml	3/3[2]	0/3	0/3	MCMV-like (3/3)[3]
(b) 10μg/ml 100μg/ml	1/3	1/3	1/3	$M_1M_2T_3$-like(1/3) MCMV-like(2/3)
(a) 5μg/ml 100μg/ml	1/3	2/3	0/3	$M_1M_2T_3$-like(2/3) MCMV-like(1/3)
(ii) VTAV plus $T_1T_2M_3$				
(a) 100μg/ml 100μg/ml	1/3	0/3	2/3	$T_1T_2M_3$-like(1/3)[2] VTAV-like(2/3)
(b) 10μg/ml 100μg/ml	3/3	0/3	0/3	$T_1T_2M_3$-like
(c) 5μg/ml 100μg/ml	3/3	0/3	0/3	$T_1T_2M_3$-like
(iii) Staggered inoculations[4]				
(a)$T_1T_2M_3$ 24hr before VTAV	3/3	0/3	0/3	$T_1T_2M_3$-like
(b)VTAV 24 hr before $T_1T_2M_3$	0/3	1/3	2/3	VTAV-like
(c)$M_1M_2T_3$ 24hr before MCMV	3/3	0/3	0/3	MCMV-like

[1] Antigens in leaf extracts were detected by ELISA 12-14 days after inoculation
[2] Numerator denotes numbers of plants containing the antigen(s) and denominator numbers of plants tested.
[3] Figures in brackets represent numbers of plants showing that symptom out of total numbers of plants inoculated.
[4] Virus used was at 100μg/ml and primary and challenge inoculations were applied to the same leaves.

RNA 3 and the expression of its coat protein gene. The latter possibility would suggest that VTAV RNAs may be encapsidated by MCMV coat protein. Consequently, even though the antigens were not detected, the VTAV RNAs 1 and 2 may be present in the infection together with the RNAs 1, 2 and 3 of MCMV. Similarly, MCMV RNAs 1 and 2 may be present in infections consisting of VTAV RNAs 1, 2 and 3, but not MCMV RNA 3. Indeed, Dodds et al., (1985) pointed out that some definitions of cross-protection do not address the question of whether the challenge virus accumulates in the "protected" plant without being able to express its ability to cause symptoms.

To test these possibilities, mixed inocula of MCMV and $T_1T_2M_3$, and of VTAV and $M_1M_2T_3$ were prepared with the two viruses in each pair present in different relative concentrations. *N. glutinosa* test plants were inoculated with each mixture. In other treatments, the inoculation of the two viruses was staggered at time intervals ranging from 6-72 hr.

Total RNA extracts were prepared from small leaf samples of the test plants as previously described, and analysed by dot-blot hybridization with MCMV and VTAV cDNA probes. The remaining leaf material from the plants of each treatment was harvested, pooled and the virus purified. Virus was purified from the plants inoculated with the MCMV and $T_1T_2M_3$ mixtures by method M, and from the VTAV/$M_1M_2T_3$ mixtures by method V. RNA extracts from the purified virus preparations were analysed by agarose gel electrophoresis and Northern blot hybridization.

Results of the MCMV/$T_1T_2M_3$ experiment presented in Table 4.7 and Fig 4.3 show that in all but one treatment (Table 4.7, treatment a), both MCMV and VTAV RNA sequences were detected. In treatment 4.7a, however, only MCMV RNA sequences were detected (Fig 4.3 lane 1) and the symptoms induced in all the plants were MCMV-like. This shows that the VTAV RNAs 1 and 2 had been excluded from the infection. In two treatments (Table 4.7, treatments c and e), 2 of the 3 plants showed $T_1T_2M_3$-like symptoms, suggesting that the MCMV RNAs 1 and 2 were excluded from those infections. This is because in most of the infected plants, even those in which both MCMV and VTAV RNA sequences were detected, the symptoms induced were MCMV-like. This is consistent with the observations that MCMV is the more aggressive of the two viruses. These conclusions

were confirmed by Northern hybridization analysis. As shown in Fig 4.3a and b, lane

5, the MCMV RNAs 1 and 2 appear to be absent. In treatments e, f and g in Table 4.7, involving staggered primary and challenge inoculations, it appears that increasing the time between inoculations from 24 to 72 hr did not result in exclusion of MCMV RNAs 1 and 2 (Fig 4.3 lanes 6 and 7). However, the inoculation of $T_1T_2M_3$ 4 hr earlier than MCMV resulted in the exclusion of MCMV RNAs 1 and 2 from two of the three plants (Table 4.7 treatment e, and Fig 4.3 lane 5). When the two viruses were inoculated on adjacent leaves, both MCMV and VTAV RNAs 1 and 2 species survived (Table 4.7, treatment d and Fig 4.3 lane 4)

In the mixed infections of VTAV and $M_1M_2T_3$, results similar to those above were obtained. Dot-blot hybridization results summarized in Table 4.8 and Northern hybridization results show that in one treatment, (Table 4.8, treatment c and Fig 4.4a and b lane 3), only VTAV RNA sequences were detected. Even though MCMV RNAs had been detected by dot-blot hybridization analysis as shown in Table 4.8, treatment d, none were detected by Northern blot hybridization analysis (Fig 4.4a and b lane 4). In all the other treatments, both MCMV and VTAV sequences were detected in most of the plants by dot-blot hybridization analysis, and subsequently confirmed by Northern blot hybridization analysis (Fig 4.4a and b lanes 1, 2,.5, 6 and 7) Unlike the mixed infections of MCMV, however, there is no significant difference between the symptoms induced in *N. glutinosa* by VTAV and $M_1M_2T_3$, and therefore it was not possible to determine by symptomatology or dot-blot analysis the numbers of plants in these treatments in which the VTAV RNAs 1 and 2 may have been excluded from the infection. Northern hybridization analysis showed that plants in treatments a, b, e, f and g contained MCMV RNAs 1 and 2 (Fig 4.5 lanes 1, 2, 5, 6 and 7). In plants in Table 4.8 treatments a and b the VTAV RNAs 1 and 2 were also detected by Northern hybridization analysis, indicating that those infections consisted of MCMV RNAs 1 and 2 and VTAV RNAs 1, 2 and 3.

Table 4.7

Replication and encapsidation of MCMV and VTAV RNAs 1 and 2 in the presence of MCMV coat protein in Mixed infections of MCMV and $T_1T_2M_3$.

Inoculum composition[1]	Symptoms expressed[2]	RNA Sequences detected[3]
(a) MCMV(1):$T_1T_2M_3$(1)	3/3 MCMV-like	MCMV only
(b) MCMV(1):$T_1T_2M_3$(10)	3/3 MCMV-like	1/3 M+V 2/3 M only
(c) MCMV(1):$T_1T_2M_3$(20)	2/3 $T_1T_2M_3$-like 1/3 MCMV-like	2/3 M+V 1/3 M only
(d) MCMV adjacent $T_1T_2M_3$	3/3 MCMV-like	2/3 M+V 1/3 M only
(e) MCMV 4hr after $T_1T_2M_3$	2/3 $T_1T_2M_3$-like 1/3 MCMV-like	3/3 M+V
(f) MCMV 24hr after $T_1T_2M_3$	3/3 MCMV-like	1/3 M+V 2/3 M only
(g) MCMV 72h after $T_1T_2M_3$	3/3 MCMV-like	1/3 M+V 2/3 M only

[1] Purified virus at 100 µg/ml was used in all inoculations. Mixtures (ratios of relative amounts given in brackets) were such as to ensure the lowest final concentration of any component was higher than the dilution end point. When inoculations were staggered, the second virus was applied to the same leaves on to which the first virus had been inoculated. In adjacent inoculations, two adjacent leaves were inoculated, one with each virus.

[2] Symptoms expressed in *N. glutinosa* were recorded 14 days after inoculations. The numerator in the figures given denotes the numbers of plants showing that symptom, and denominator numbers of plants inoculated

[3] Viral RNA sequences present were detected by dot-blot hybridization analysis. The numerator in the figures given denotes numbers of samples (plants) containing those RNA sequences, and the denominator the numbers of samples tested.

Fig 4.3

Persistence of VTAV RNAs 1 and 2 in mixed infections of MCMV and the pseudorecombinant $T_1T_2M_3$ in $N.$ $glutinosa$.

Infected leaves of plants inoculated as follows were pooled: (1) equal vol of MCMV and $T_1T_2M_3$; (2) 10 vol of $T_1T_2M_3$ to 1 vol of MCMV; (3) 20 vol of $T_1T_2M_3$ to 1 vol of MCMV; (4) MCMV and $T_1T_2M_3$ on adjacent leaves; (5) MCMV inoculated 4 hr after $T_1T_2M_3$; (6) MCMV inoculated 24 hr after $T_1T_2M_3$; and (7) MCMV inoculated 72 hr after $T_1T_2M_3$. From each pool of infected leaves purified virus preparations 1-7 were obtained, and RNA extracts from these (RNA preparations 1-7) were analysed by Northern hybridization analysis with MCMV (panel a) and VTAV (panel b) cDNA probes.

Fig 4.4

Persistence of MCMV RNAs 1 and 2 in mixed infections of VTAV and the pseudorecombinant $M_1M_2T_3$ in $N.$ $glutinosa$ plants.

Infected leaves from plants inoculated as follows were pooled: (1) one vol of VTAV to 20 vol of $M_1M_2T_3$; (2) 1 vol of VTAV to 10 vol of $M_1M_2T_3$; (3) equal volumes of VTAV and $M_1M_2T_3$; (4) VTAV and $M_1M_2T_3$ on adjacent leaves; (5) VTAV 24 hr after $M_1M_2T_3$; (6) VTAV 48 hr after $M_1M_2T_3$; and (7) VTAV 72 hr after $M_1M_2T_3$. From each pool of infected leaves purified virus preparations 1-7 were obtained, and RNA extracts from these (RNA preparations 1-7) were analysed by Northern hybridization analysis with MCMV (panel a) and VTAV (panel b) cDNA probes.
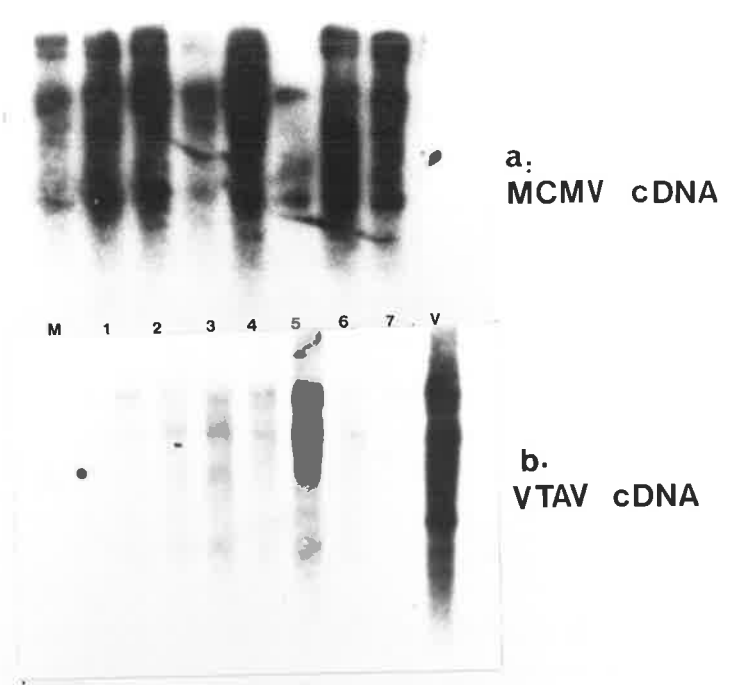
a.
MCMV cDNA

M 1 2 3 4 5 6 7 V

b.
VTAV cDNA

Fig 4.3

a.
MCMV cDNA

V

M 1 2 3 4 5 6 7

b.
VTAV cDNA
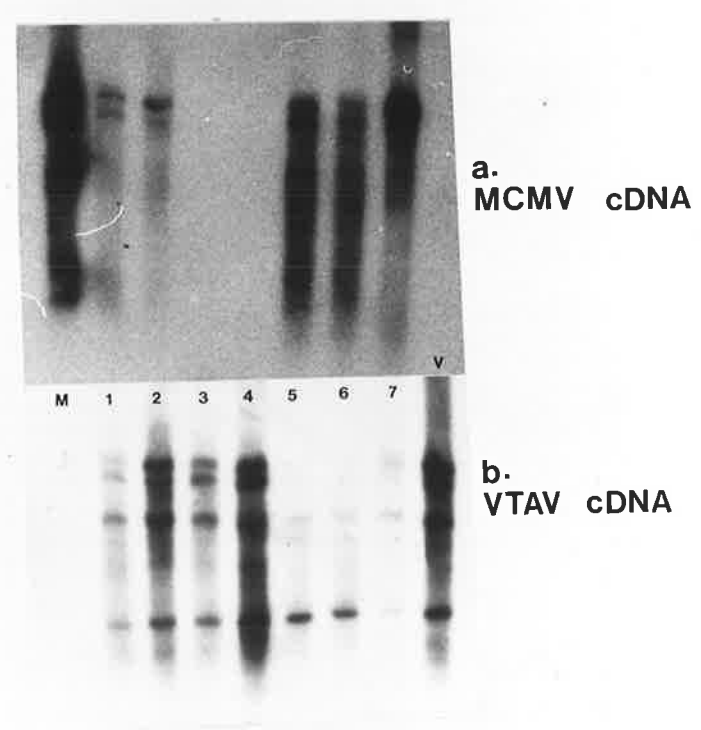
Fig 4.4

Table 4.8

Replication and encapsidation of MCMV and VTAV RNAs 1 and 2 in the presence of VTAV coat protein in Mixed Infections of VTAV and $M_1M_2T_3$,

Inoculum composition[1]	Symptoms expressed[2]	RNA Sequences detected[3]
(a)VTAV(1):$M_1M_2T_3$(20)	3/3 VTAV-like[4]	3/3 M+V
(b)VTAV(1):$M_1M_2T_3$(10)	3/3 VTAV-like	1/3M+V 2/3 V only
(c) VTAV(1):$M_1M_2T_3$(1)	3/3 VTAV-like	3/3 V only
(d)VTAV adjacent $M_1M_2T_3$	3/3 VTAV-like	2/3 M+V 1/3 V only
(e) VTAV 24 h after $M_1M_2T_3$	3/3 VTAV-like	3/3 M+V
(f) VTAV 48 h after $M_1M_2T_3$	3/3 VTAV-like	3/3 M+V
(g) VTAV 72 h after $M_1M_2T_3$	3/3 VTAV-like	3/3 M+V

[1] Purified virus at 100 µg/ml was used in all inoculations. Mixtures (ratios of relative amounts given in brackets) were such as to ensure the lowest final concentration of any component was higher than the dilution end point. When inoculations were staggered, the second virus was applied to the same leaves on to which the first virus had been inoculated. In adjacent inoculations, two adjacent leaves were inoculated, one with each virus.

[2] Symptoms expressed in *N. glutinosa* were recorded 14 days after inoculations. The numerator in the figures given denotes the numbers of plants showing that symptom, and denominator numbers of plants inoculated

[3] Viral RNA sequences present were detected by dot-blot hybridization analysis. The numerator in the figures given denotes numbers of samples (plants) containing those RNA sequences, and the denominator the numbers of samples tested.

[5] There were no significant differences between symptoms induced by VTAV and the pseudorecombinant $M_1M_2T_3$ in *N. glutinosa*.

4.9. Test For Cross Protection Against MCMV Infection In *Gomphrena globosa* and *Cucumis sativus* Using VTAV and Pseudorecombinants as Protecting Strains

As determined previously, MCMV infects both *C. sativus* and *G. globosa*, but VTAV does not. The pseudorecombinant $T_1T_2M_3$ induces necrotic lesions on the inoculated leaves of *G. globosa* but does not infect *C. sativus*, while $M_1M_2T_3$ induces chlorotic local lesions on *C. sativus* but does not infect *G. globosa*. These two plant hosts therefore represented a system for testing the roles of either the RNAs 3 alone on the one hand, or the RNAs 1 and 2 on the other, in the infection of *C. sativus* and *G. globosa*, and possibly in cross-protection from MCMV infection.

Purified preparations of MCMV, VTAV, and the two pseudorecombinants at concentrations of $100\mu g/ml$ were used. Ten *G. globosa* plants and 20 *C. sativus* plants were used for each treatment. VTAV, $T_1T_2M_3$ and $M_1M_2T_3$ were used to inoculate the plants, and three days after the primary inoculation, MCMV was inoculated on to the leaves to which the primary inocula had been applied. As controls, each virus alone was used to inoculate similar numbers of each species of plants.

Results presented in Table 4.9 show that *C. sativus* plants were not protected from MCMV infection by prior inoculation with VTAV, $T_1T_2M_3$ or $M_1M_2T_3$. Only MCMV antigen was detected in all the systemically infected tissues, and no significant differences were observed in the numbers of plants infected with the MCMV controls as compared to the "protected plants".

Similar results were obtained using *G. globosa* as shown in Table 4.10. All the systemically infected plants contained only MCMV antigens. VTAV and the two pseudo-recombinants failed to infect any plants systemically. However, $T_1T_2M_3$ infected the inoculated leaves to produce chlorotic local lesions. Thus, none of the viruses used protected *Cucumis sativus* or *Gomphrena globosa* from infection by MCMV.

Table 4.9.

Test for protection of *C. sativus* from MCMV infection by primary inoculation of VTAV, $M_1M_2T_3$ or $T_1T_2M_3$.

Primary inoculum[1]	Secondary inoculum[2]	No of Plants systemically infected	Symptoms induced	Antigens (systemic)[3]
(a)$M_1M_2T_3$	MCMV	16/20[4]	Chlorotic lesions and systemic mosaic.	MCMV only
(b)$T_1T_2M_3$	MCMV	15/19	Chlorotic lesions and systemic mosaic.	MCMV only
(c)VTAV	MCMV	13/17	Chlorotic lesions and systemic mosaic.	MCMV only
(d) $M_1M_2T_3$	--	0/20	Chlorotic local lesions only.	None
(e) $T_1T_2M_3$	--	0/20	Not infected	None
(f) VTAV	--	0/18	Not infected	None
(g)Buffer	MCMV	17/20	Chlorotic lesions and systemic mosaic.	MCMV

[1] Purified virus preparations adjusted to concentration of 100 μg/ml were used in all inoculations.

[2] Secondary inoculations were applied 3 days later,to the same leaves previously inoculated.

[3] Extracts from systemically infected leaves were analysed by ELISA to determine the antigens present.

[4] The numerator denotes numbers of plants systemically infected and denominator the total number infected

MCMV is more aggressive than the VTAV RNA 3, irrespective of whether it was replicating in the presence of MCMV or VTAV RNAs 1 and 2. The second was that MCMV and VTAV RNAs 1 and 2 were capable of replicating together, and in the presence of a single species of coat protein (ie MCMV or VTAV coat protein) were both encapsidated. The exclusion of either species from the infection, for example in Fig 4.3 lane 3 depended on the relative amounts in the inocula or the timing of the application inocula. These comparisons were thought to have an added significance in host determinance, particularly in the infections of $C.\ sativus$ and $G.\ globosa$. While $M_1M_2T_3$ induces local lesion but no systemic infection in $C.\ sativus$, it does not infect $G.\ globosa$; conversely, $T_1T_2M_3$ induces local lesions in $G.\ globosa$ but does not infect $C.\ sativus.$. Neither species of plants is infected by VTAV.

Based on these observations, an attempt was made in this section to further characterize the roles of the RNAs 1 and 2 on one hand, and the RNAs 3 on the other, in the infection of the two plant species, $N.\ glutinosa$ and $C.\ sativus$, using mixtures of the two pseudorecombinants. Both pseudorecombinants infect $N.\ glutinosa$, systemically, but neither infects $C.\ sativus$ systemically.

Results presented in Table 4.11 show that in $N.\ glutinosa$, MCMV antigens were more frequently detected. In Table 4.11, treatment a which is similar to a mixture of MCMV and VTAV, all the plants showed MCMV-like symptoms. When analysed by ELISA, half the plants were found to contain only MCMV antigens, and the other half contained both MCMV and VTAV antigens. As the amount of $T_1T_2M_3$ (and hence MCMV RNA 3) was decreased (Table 4.11, treatments a and b) in the inoculum, results similar to those in Table 4.11, treatment c were obtained. However, as the MCMV RNA 3 concentration increased relative to VTAV RNA 3 (Table 4.11, treatments d and e), the symptoms became $T_1T_2M_3$-like, and no VTAV antigens were detected. Thus, as MCMV RNAs 1 and 2 concentrations decreased relative to VTAV RNAs 1 and 2, and MCMV RNA 3 increased relative to VTAV RNA 3, the plants became $T_1T_2M_3$-like.

Table 4.11

Interactions of the pseudorecombinants $M_1M_2T_3$ and $T_1T_2M_3$ in *N. glutinosa*.

| | Inoculum composition[1] | | Symptoms | Antigens Detected[3] | | |
	$M_1M_2T_3$	$T_1T_2M_3$	induced[2]	MCMV	VTAV	MCMV+VTAV
(a)	100	5	MCMV-like	4/8	0/8	4/8
(b)	100	10	MCMV-like	2/4	0/4	2/4
(c)	100	100	MCMV-like	2/4	0/4	2/4
(d)	10	100	$T_1T_2M_3$-like	4/4	0/4	0/4
(e)	5	100	$T_1T_2M_3$-like	6/8	0/8	0/8[4]

[1] The two viruses were diluted with phosphate buffer pH 7.4 to concentrations of 100 µg/ml, and these stock preparations used in the ratios (by volume) indicated.

[2] Symptoms induced were recorded 14 to 16 days after inoculation.

[3] Extracts from systemically infected leaves were analysed by ELISA for the antigens present.

[4] Two of the plants in this treatment showed no symptoms of infection and no viral antigens were detected.

Results presented in Table 4.12, treatments a to e show that *C. sativus* plants were infected systemically by all the inocula used. Whereas both MCMV and VTAV antigens were detected in lesions excised from the inoculated leaves, only MCMV antigens and RNA sequences were detected in systemically infected leaves. When $T_1T_2M_3$ was inoculated 6 hr after $M_1M_2T_3$, there was no systemic infection of the plants and only VTAV antigens were detected in extracts of the local lesions induced in the inoculated cotyledons (Table 4.12, treatment f). This confirmed that MCMV RNA 3 is required for systemic infection. However, when $M_1M_2T_3$ was inoculated 6 hr after $T_1T_2M_3$, both MCMV and VTAV antigens were detected in local lesion extracts of some plants, and these became systemically infected.

Table 4.12

Interactions of the pseudorecombinats $M_1M_2T_3$ and $T_1T_2M_3$ in *Cucumis sativus*.

Inoculum composition[1]		Numbers of plants systemically infected	Antigens detected in local lesions[2]	Antigens in systemic leaves[3]	RNA Sequences detected in systemic leaves[4].
$M_1M_2T_3$	$T_1T_2M_3$				
(a) 100	5	8/12[5]	MCMV and VTAV	MCMV only	MCMV only
(b) 100	10	7/7	MCMV and VTAV	MCMV only	MCMV only
(c) 100	100	13/14	MCMV and VTAV	MCMV only	MCMV only
(d) 10	100	14/14	MCMV and VTAV	MCMV only	MCMV only
(e) 5	100	4/12	MCMV and VTAV	MCMV only	MCMV only
staggered inoculations[6]					
(f) $M_1M_2T_3$ 6hr before $M_1M_2T_3$		0/15	VTAV only	none	none
(g) $T_1T_2M_3$ 6hr before $M_1M_2T_3$		4/20	MCMV and VTAV	MCMV only	MCMV only

[1] Purified virus preparations of the two pseudorecombinants were diluted to concentrations of 100μg/ml in phosphate buffer pH 7.4, and combined in the ratios indicated

[2] Twenty local lesions were excised from the inoculated cotyledons from each group of plants and extracts from each lesion was analysed by ELISA.

[3] Extracts of systemic leaves of each test plant were analysed by ELISA.

[4] Total leaf RNA extracts from systemically infected leaves were analysed by dot-blot hybridization analysis using MCMV and VTAV cDNA probes.

[5] The numbers of plants showing symptoms of infection after 7-14 days in systemic leaves. The numerator denotes the numbers of plants infected, and denominator the numbers of plants inoculated.

[6] Virus preparations at concentration of 100μg/ml were used in these experiments, with the primary and secondary inoculations applied on to same leaves.

4.11. Conclusions.

It was demonstrated that MCMV and VTAV not only coinfected all their common host species, but that the resulting mixed infections can persist with successive passaging in many of the species tested. There was evidence that MCMV was very competitive in these mixed infections. Not only were MCMV antigens detected first, and often alone, but the symptoms induced were most often MCMV-like.

These "dominance" characteristics of MCMV were also expressed by the pseudo-recombinant $T_1T_2M_3$, which was found to be very competitive and persistent in mixed infections with VTAV. In contrast, the VTAV RNA 3 in $M_1M_2T_3$ was easily eliminated from mixed infections by MCMV. There were, however, some conditions under which the RNA 3 from $M_1M_2T_3$ could survive, and occasionally exclude MCMV RNA 3 from the infection.

Mixtures of the two pseudorecombinants behaved differently from coinfections of the parental viruses. While coinoculation of MCMV and VTAV resulted in coinfection, similar inoculations of the pseudorecombinants resulted in single infections of MCMV-like, or $T_1T_2M_3$-like character, with both MCMV and VTAV antigens detected in only a few of the plants coinoculated with the two pseudorecombinants. It was concluded that whereas their relative concentrations determined which of MCMV and VTAV RNAs 1 and 2 survived in these mixed infections, the relatively more aggressive nature of the MCMV RNA 3 was the major factor in determining which antigens were present.

CHAPTER FIVE

ATTEMPTS TO ISOLATE VIRUS VARIANTS FROM MIXEDLY INFECTED LEAF TISSUE USING THE DIFFERENTIAL EFFECTS OF THEIR PURIFICATION METHODS.

5.0. Introduction.

When the MCMV method of virus purification (method M) was used to extract and purify virus from leaf tissue from plants infected with VTAV, it was shown that even though much of the virus was degraded, there were enough intact particles to infect test plants, and no changes were observed in their antigenic or sedimentation properties (Section 3.4). In contrast, when virus was purified from MCMV infected leaves by the VTAV method (method V), there was complete degradation of the capsids. No MCMV was recovered from sucrose density-gradients, and when used to inoculate *N. glutinosa* plants, the preparations were found to be non-infectious.

In experiments reported in this section, attempts were made to utilize the differential effects of the purification methods as a means of isolating products of transcapsidation arising from mixed infections of MCMV and VTAV.

5.1. Virus purification from plants co-infected with MCMV and VTAV.

Each *N. clevelandii* plant inoculated with both MCMV and VTAV was tested by ELISA to determine their antigen content. Leaves from plants in which both antigens had been detected were harvested and divided into two portions. One portion was extracted and purified by method M (and virus preparation designated preparation M) and the other portion was purified by method V (preparation V). As controls, leaves from plants infected with MCMV only, were harvested and divided into two portions. To each portion, an equal weight of leaves from plants infected with VTAV only was added. Virus was extracted and purified from one mixture by method M, and from the other by method V. Virus preparations obtained by the two methods were designated control preparation M and control preparation V, respectively.

Table 5.1
Properties of Virus Preparations Recovered From Plants Coinfected With MCMV and VTAV and purified by M and V Methods.

Leaf material Used	Virus Preparation	Method of Purification	Antigens Detected[1]	Properties of *N. glutinosa* plants inoculated with Virus Preparati	
				Symptoms Expressed	Antigens Detected[1]
From Plants Coinfected	M	(a) Method M	MCMV and VTAV	MCMV-like	MCMV and VTAV
With MCMV and VTAV		(b) Method M, including Density-Gradient step.	MCMV and VTAV	VTAV-like	MCMV and VTAV
	V	(c) Method V	VTAV	VTAV-like	VTAV only
		(d) Method V, including Density-gradient Step	VTAV	VTAV-like	VTAV only
From equal weights of leaves infected with MCMV alone and VTAV alone	Control M	(e) Method M	MCMV and VTAV	MCMV-like	MCMV and VTAV
		(f) Method M, including Density-gradient Step	MCMV and VTAV	VTAV-like	MCMV and VTAV
	Control V	(g) Method V	MCMV and VTAV	MCMV-like (2/4)[2] VTAV-like (2/4)[2]	MCMV and VTAV
		(h) Method V, including Density-gradient step	VTAV	VTAV-like	VTAV only

[1] Antigens present in purified virus preparations and leaf extracts were determined by ELISA.

[2] Numerator indicates numbers of plants showing those symptoms, and denominator indicates total numbers of plants inoculated.

5.1.1. *Sucrose density-gradient sedimentation profiles of virus preparations.*

The four virus preparations were each subjected to sucrose density-gradient centrifugation and the gradients were scanned with an absorbance monitor (Fig 5.1 a, b, e and f), and the 1.0ml fractions collected were analysed for the antigens present by ELISA (Fig 5.1 c,d, g and h) after ten fold dilution with sample buffer.

Results presented in Fig 5.1a and b show that particles in virus preparations M and V sedimented with profiles similar to those obtained for MCMV purified by method M (presented in Fig 3.6a), and VTAV purified by method V (presented in Fig 3.6b). Results of analysis by ELISA presented in Fig 5.1c show that both MCMV and VTAV antigens were present in the peak fractions of preparation M, but with VTAV in a lower concentration than MCMV. In contrast, as shown in Fig 5.1d, preparation V contained very few MCMV particles (absorbance of 0.2 at the peak), with VTAV being the major component of that preparation (absorbance of 2.0 of the peak fraction). Control preparation M also sedimented like MCMV purified by method M (Fig 5.1e). Serological analysis of the gradient fractions showed that both MCMV and VTAV were present but at significantly lower concentrations (Fig 5.1g). The sedimentation profile of control preparation V presented in Fig 5.1f shows two peaks, one at the top of the gradient, and the other in the region associated with intact virus particles. When analysed by ELISA, the amounts of virus detected were significantly lower than in the other treatments, and a correspondingly smaller amount of virus was recovered. However, both MCMV and VTAV antigens were detected in the gradient fractions.

5.1.2. *Infectivity of Virus Preparations.*

Portions of the purified virus preparations and virus recovered from sucrose density-gradients were each diluted to concentrations of 100µg/ml and used in infectivity tests.

Results presented in Table 5.1 treatment a show that *N. glutinosa* plants inoculated with preparation M induced MCMV-like symptoms, but both MCMV and VTAV antigens were detected in leaf extracts. However, when virus recovered from the sucrose density-gradient was used, all the infected plants induced VTAV-like symptoms, though both
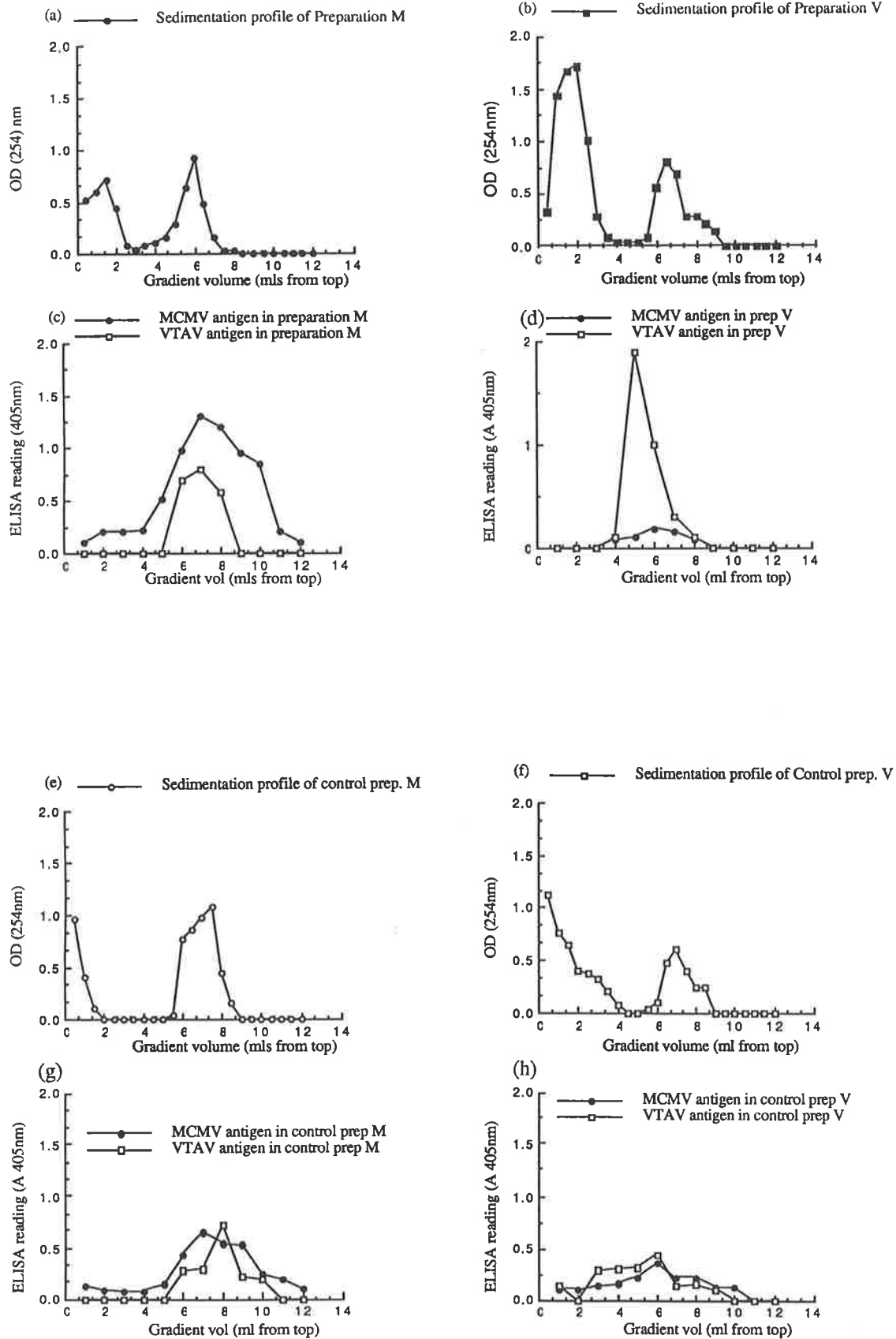
Fig 5.1 Differential effects of virus purification methods on composition of virus purified from leaf tissue coinfected by MCMV and VTAV.

Table 5.2.

The Use of Specific Antiserum Precipitation As a Method of Separating MCMV and VTAV in Mixed Virus Preparations.

Virus preparation Used	Antigens detected[1] in virus preparation[1]	Antiserum used in precipitation[2]	Antigens detected after immunoprecipitation[1]	Sequences detected[3]
Preparation M	MCMV and VTAV	(a) anti-MCMV	VTAV only	None
		(b) anti-VTAV	MCMV only	MCMV
Control Preparation M	MCMV and VTAV	(c) anti-MCMV	VTAV only	VTAV
		(d) anti-VTAV	MCMV only	MCMV
Control Preparation V	MCMV and VTAV	(e) anti-MCMV	VTAV only	VTAV
		(f) anti-VTAV	None	None
Preparation V	VTAV only	(g) anti-MCMV	VTAV only	VTAV
		(h) anti-MCMV	None	None

[1] Antigens present were detected by ELISA.
[2] Antiserum to MCMV and to VTAV with immunodiffussion titre of 1/128 were used.
[3] Viral RNA purified from supernatents and the RNA sequnces present were determined by dot-blot hybridization analysis using MCMV and VTAV cDNA probes

MCMV and VTAV antigens were detected (Table 5.1, treatment b). In Chapter 4.2, Table 4.1, it was shown that the symptoms induced by plants coinfected with MCMV and VTAV depend on which virus is the major component of the inoculum. It appears that during sucrose density-gradient centrifugation some MCMV was lost or inactivated. This apparently leads to a change in the relative amounts of the two viruses, and hence the symptoms induced when these preparations are used to inoculate plants.

When preparation V was used to inoculate *N. glutinosa* plants VTAV-like symptoms were induced and only VTAV antigens were detected in leaf extracts (Table 5.1, treatment c). Similar results were obtained when virus recovered from sucrose density-gradient was used as inoculum (Table 5.1, treatment d).

When inoculated with control preparation M, all the plants showed MCMV-like symptoms, but both MCMV and VTAV antigens were detected in infected leaf extracts (Table 5.1, treatment e). When virus recovered from a sucrose density-gradient was used, all the plants showed VTAV-like symptoms, but again both MCMV and VTAV antigens were detected in infected leaf extracts (Table 5.1, treatment f). These results are similar to those obtained with virus preparation M, and indicate that method M was not suitable for separating MCMV from VTAV.

When control virus preparation V was used as inoculum (Table 5.1, treatment g), two out of four plants showed MCMV-like symptoms, and two out of four showed VTAV-like symptoms. When leaf extracts were analysed by ELISA, each plant was found to contain both MCMV and VTAV antigens (Table 5.1, treatment g). However, when virus recovered from the sucrose density-gradient was used, all the plants showed VTAV-like symptoms, and only VTAV antigens were detected (Table 5.1, treatment h). These results also confirmed that during sucrose density-gradient centrifugation some MCMV was inactivated. As MCMV was detected serologically in the gradient fractions (Fig 5.1h), it seemed that the particles become non-infectious after sucrose density-gradient centrifugation.

5.1.3. *RNA Sequences Present in Virus Preparations.*

RNA purified from the virus preparations M and V were analysed by Northern blot hybridization using MCMV and VTAV cDNA probes. The results presented in Fig 5.2a

Fig 5.2

Differential effects of virus purification methods on nucleoproteins present in recovered virus.

The composition of RNA extracts from virus preparation M (lane 1), control virus preparation M (lane 2), virus preparation V (lane 3) and control virus preparation V (lane 4) were determined by Northern hybridization using MCMV (panel a) and VTAV (panel b) cDNA probes.

Fig 5.3

Effectiveness of specific antisera in separating MCMV and VTAV in virus preparations from coinfected leaf tissues.

RNA extracts obtained from supernatents obtained after immunoprecipitation were analysed by dot-blot hybridization analysis with MCMV and VTAV cDNA probes. MCMV and VTAV RNA controls were at 50, 20, 5 and 0.5ng per spot . RNA extracts were obtained from the supernatents of virus preparations as follows: virus preparation M cross-absorbed with MCMV antiserum (a) and VTAV antiserum (b); control preparation M cross-absorbed with MCMV antiserum (c), and VTAV antiserum (d); control preparation V cross-absorbed with MCMV antiserum (e) and VTAV antiserum (f); preparation V cross-absorbed with MCMV antiserum (g) and VTAV antiserum (h). The RNA preparations were each applied at approximately 20ng per spot, and analysed by dot-blot hybridization using MCMV and VTAV cDNA probes..
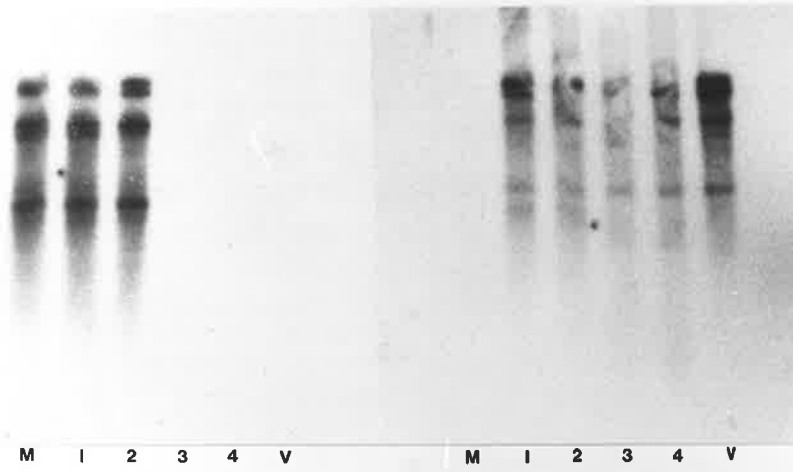
a. MCMV cDNA   b. VTAV cDNA

M I 2 3 4 V   M I 2 3 4 V

Fig 5.2

i. MCMV cDNA

M a b c d e f g h V
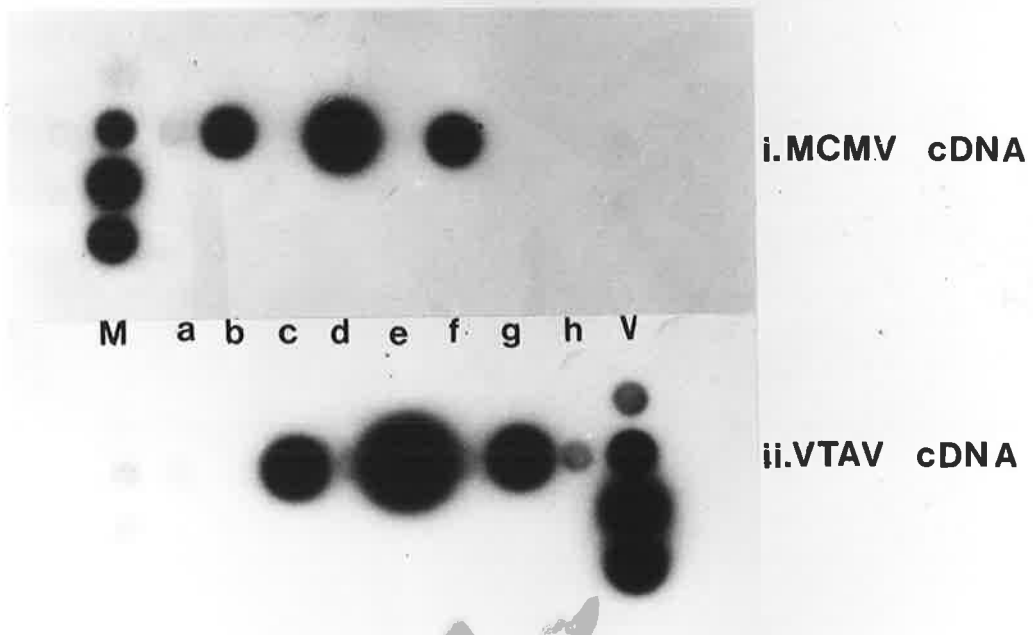
ii. VTAV cDNA

Fig 5.3

show that the MCMV cDNA probe hybridized only with RNAs from preparation M, and control preparation M (Fig 5.2a lanes 1 and 2) but not with those from preparation V and control preparation V (Fig 5.2a lanes 3 and 4). The VTAV probe, however, hybridized with RNA from all four virus preparations (Fig 5.2b lanes 1 to 4).

5.2. Immunoprecipitation as a Method of Separating MCMV and VTAV From Each Other in Virus Preparations Containing Both.

The results in section 5.1 showed that extraction and purification of virus from coinfected tissue by method M was not suitable for separating particles with MCMV coat protein from those with VTAV coat protein. However, method V appears to be suitable for separating particles with VTAV coat protein from those with MCMV coat protein although the latter were not always completely removed. Consideration was therefore given to the possibility of precipitation with specific antisera as a means of further purifying each of the viruses. Two lots of 1.0mg of virus from each of the four virus preparations (Table 5.1, a, c, e, and f) were taken, and to one of each pair, 1.0ml of MCMV antiserum, (titre 1/128) was added, and to the other a similar volume of VTAV antiserum, (titre 1/128) was added. The volume of antisera used was based on what was determined to be required in preliminary tests to precipitate 1.0mg of each virus.

The samples were incubated at 25°C for 3 hr, and the tubes containing the reaction mixtures were then centrifuged at 10,000 g for 10 min. The supernatants were recovered and after taking 50μl aliquots for serological analysis, RNA was extracted from the remainder with phenol and SDS. The RNA preparations were subsequently analysed by dot-blot hybridization with MCMV and VTAV cDNA probes.

The results presented in Fig 5.3 and in Table 5.2 show that when preparation M was precipitated with anti-MCMV serum, only VTAV antigens were detected in the supernatant, but no RNA sequences were detected in dot-blot hybridization analysis (Table 5.2, treatment a and Fig 5.3i and ii spots a). When VTAV antiserum was used for the precipitation, only MCMV antigens and MCMV RNA sequences were detected (Table 5.2, treatment b, and Fig 5.3i and ii spots b). When the control preparation M was used (Table 5.2, treatments c and d, and Fig 5.3i and ii spots c and d) only VTAV antigens and VTAV RNA sequences were

detected after immunoprecipitation with MCMV antiserum, and only MCMV antigens and MCMV RNA sequences were detected after immunoprecipitation with VTAV antiserum. It was therefore concluded that both MCMV and VTAV were present in preparation M and control preparation M, and that either could be removed by precipitation with the appropriate antiserum.

When preparation V was similarly analysed, only VTAV antigens and VTAV RNA sequences were detected when MCMV antiserum was used in the precipitation (Table 5.2, treatment g, and Fig 5.3i and ii spots g). However, when preparation V was precipitated with the VTAV antiserum no antigens or RNA were detected (Table 5.2, treatment h and Fig 5.3i and ii spots h), suggesting that all the virus present was encapsidated in VTAV coat protein and had been removed. When the control preparation V was precipitated with MCMV antiserum, only VTAV antigens and VTAV RNA sequences were detected, and similarly, only MCMV antigens and MCMV RNA sequences were detected when that preparation was precipitated with VTAV antiserum (Table 5.2, treatments e and f and Fig 5.3i and ii spots e and f).

It was therefore possible by immunoprecipitation with specific antisera to separate MCMV and VTAV from each other in preparations containing both viruses. However, RNA extracts obtained from some of the treatments were not infectious. Consequently, no further attempts were made to characterize these immuno-purified preparations.

5.3. Isolation of "Variants" from Preparation M

Virus from preparation M (see Table 5.1, treatment a) was used to inoculate *Beta vulgaris*. Local lesions which were morphologically different from those produced by MCMV (see Fig 3.2) were excised and purified by three further local lesion passages. Extracts from twelve lesions thus purified were each used to infect *Nicotiana glutinosa*. Eight of the local lesion isolates induced MCMV-like symptoms and were designated MVLA1-8. Four of the isolates induced VTAV-like symptoms and were designated MVLB1-4. Two isolates from each group (MVLA1 and 2, and MVLB1 and 2) were selected for further study. In Fig 5.4, the symptoms induced by the four isolates were compared with those induced by MCMV and VTAV in *N. glutinosa*. Isolates MVLA1 and

Fig 5.4

Symptoms induced by virus isolates MVLA1, MVLA2, MVLB1 and MVLB2 in

N. glutinosa plants.

Isolates MVLA1 and MVLA2 induced MCMV-like symptoms, while isolates

MVLB1 and MVLB2 induced VTAV-like symptoms.

Fig 5.5

Determination of RNA composition of virus isolates MVLA1 MVLA2, MVLB1

and MVLB2 by Northern hybridization analysis with MCMV and VTAV cDNA

probes.

All four isolates hybridized with MCMV cDNA probe (panel a) but only MVLB1

(lane LB1) and MVLB2 (lane LB2) RNAs 1 or 2 appeared to hybridize with

VTAV cDNA probe (panel b). However, this hybridization was detected only at

long exposures of autoradiographs, at which stage the MCMV control (lane M)

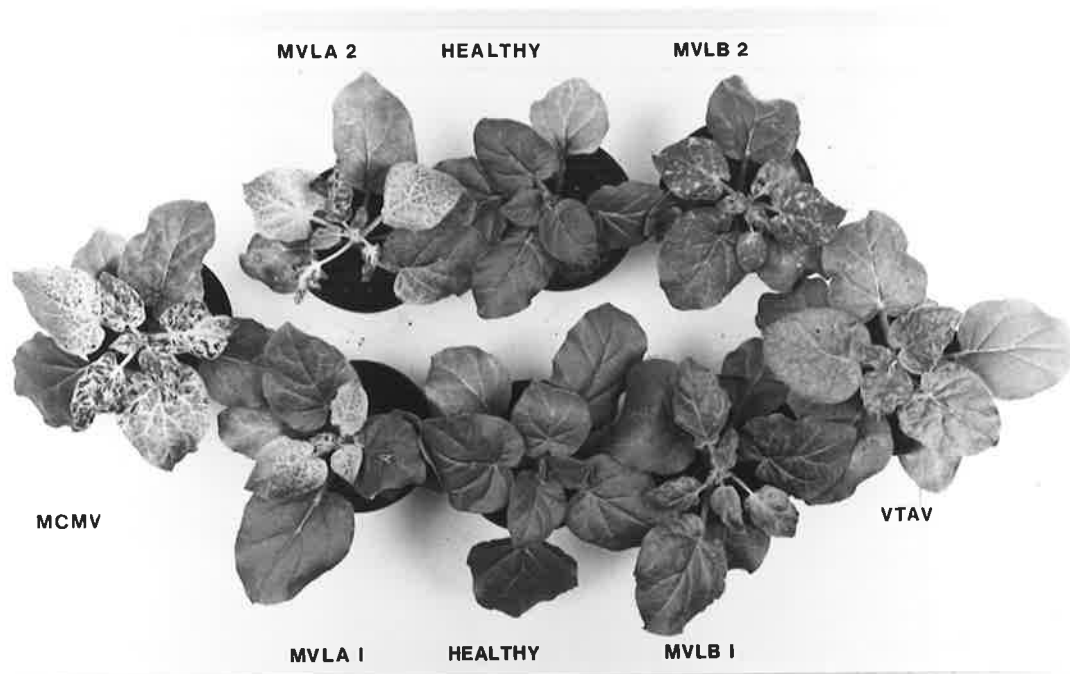also appeared to have hybridized with the VTAV cDNA probe.

Fig 5.4

Fig 5.5

A2 induced MCMV-like symptoms, MVLB1 VTAV-like symptoms and MVLB2 symptoms intermediate between those induced by VTAV and the pseudorecombinant $T_1T_2M_3$.

5.3.1. *Serological characterization of isolates MVLA1 and 2 and MVLB1 and 2.*

The serological properties of the four virus isolates were compared with MCMV and VTAV by ELISA. All four isolates reacted with MCMV but not VTAV antiserum. On the basis of these results it was concluded that method M can be used to purify all the four isolates, but not method V.

5.3.2. *RNA composition of isolates.*

Since MVLB1 and B2 induced VTAV-like symptoms, it was thought that the isolates may contain RNA sequences of VTAV origin responsible for the VTAV-like symptoms. Results of preliminary dot-blot hybridization analysis of RNA extracts from the isolates showed that all the four isolates hybridized with the MCMV cDNA probe (data not shown). However, isolates MVLB1 and B2 hybridized with the VTAV probe as well as with the MCMV probe.

Results of Northern hybridization analysis of RNA from purifed virus preparations presented in Fig 5.5a show that the MCMV cDNA probe hybridized with all the RNAs of all the four isolates. The VTAV probe, however, hybridized with only the RNA 1 of isolates MVLB1 and MVLB2 (Fig 5.5b lanes 3 and 4), but not with isolates MVLA1 and MVLA2 (Fig 5.5b lanes 1 and 2). Rather unexpectedly, the VTAV probe also hybridized with the MCMV RNA (Fig 5 5b lane M). Not withstanding these latter results, two possible genome structures were proposed for the two isolates inducing VTAV-like symptoms, MVLB1 and B2:

(a) MCMV RNAs 2 and 3 plus VTAV RNA 1

(b) MCMV RNAs 2 and 3, and a recombinant RNA 1 consisting of MCMV and VTAV sequences.

To test the possibility that MVLB1 and B2 consisted of a mixture consisting of MCMV RNAs 1 and 2 plus VTAV RNAs 1 and/or 2, *Cucumis sativus* was used as a filter host to remove VTAV RNA segments or sequences present. Results presented in Fig 5.6 show that

Fig 5.6

Symptoms induced by virus isolates MVLA1, MVLA2, MVLB1 and MVLB2 in

N. glutinosa after two successive passages through *Cucumis sativus*.

The filtration resulted in the loss of the VTAV-like symptoms induced by isolates

MVLB1 and MVLB2 which became MCMV-like.

Fig 5.7

Northern blot hybridization analysis to determine the RNA composition of virus

isolates MVLA1, MVLA2, MVLB1 and MVLB2 after two successive passages

through *C. sativus*.

All the RNAs of the four isolates hybridized with MCMV (panel a) but not VTAV

(panel b) cDNA probes.

Fig 5.6

a. MCMV cDNA   **b. VTAV cDNA**

M   LA I LA2 LB I LB2 V   M   LA I LA2 LB I LB2 V
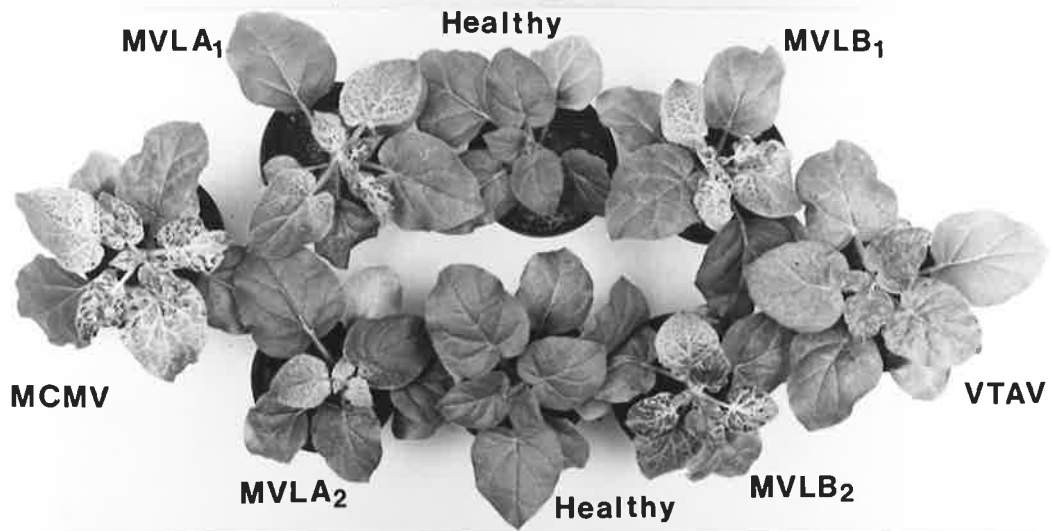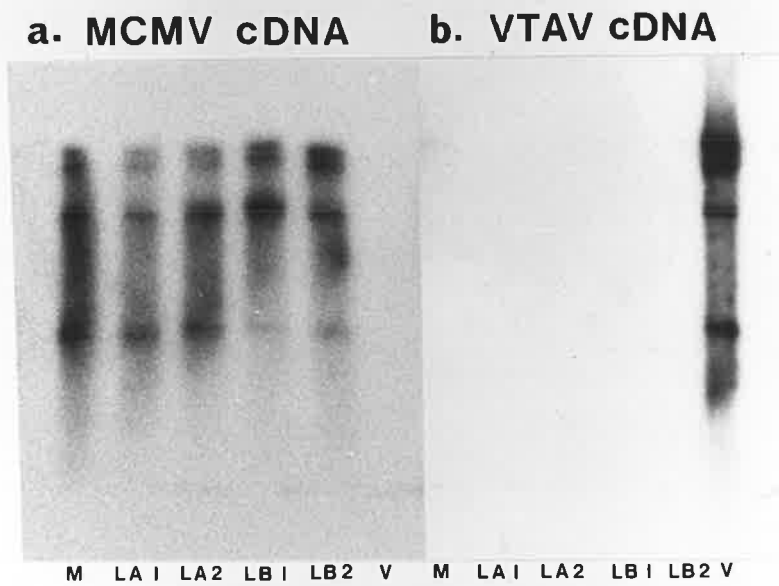
Fig 5.7

Fig 5.8

The use of MVLB1 (a) and MVLB2 (b) RNAs as templates for cDNA probes to determine origin of VTAV-like character in the isolates.

The probes were made from RNA obtained from virus preparations before filtration through *C. sativus*, and used to analyse MCMV and VTAV RNAs, as well as RNA preparations from all four isolates before and after the filtration. VTAV RNAs failed to hybridize with either cDNA probe.

a. MVLB₁ cDNA

M I 2 3 4 5 6 7 8 V

original isolates after 2 filtrations

b. MVLB₂ cDNA

M I 2 3 4 5 6 7 8 V

original isolates after 2 filtrations
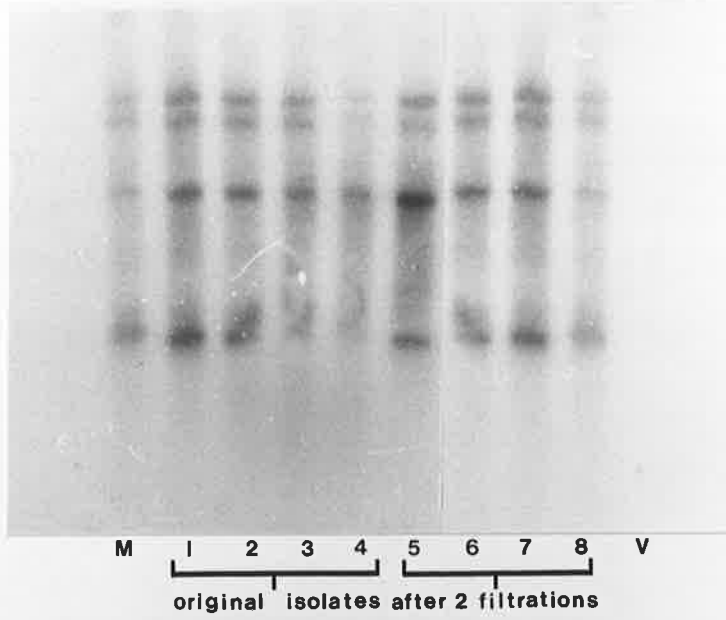
Fig 5.8

after two passages through *C. sativus* the isolates MVLB1 and B2 induced MCMV-like symptoms in *Nicotiana glutinosa*.

Northern blot hybridization results presented in Fig 5.7a show that the MCMV cDNA probe hybridized with all the four isolates as before. However, after they had been passaged twice through *Cucumis sativus* , none of the isolates hybridized with the VTAV cDNA probe (Fig 5.7b lanes 1-4). The RNA sequences originally present and responsible for the VTAV-like symptoms induced were apparently eliminated by passaging through *C. sativus* and no longer detected.

In further attempts to determine which of the genomic RNAs were of VTAV origin, total RNAs from purified preparations of MVLB1 and MVLB2 (before passaging through *C. sativus*) were used as templates for cDNA synthesis. These probes were used in Northern hybridization analysis of the four isolates before and after passaging through *C. sativus*, and MCMV and VTAV. Results presented in Fig 5.8a show that when MVLB1 cDNA was used, it hybridized with all the four isolates, before (Fig 5.8a lanes 1 to 4) and after two passages through *C sativus* (lanes 5 to 8), as well as MCMV (lane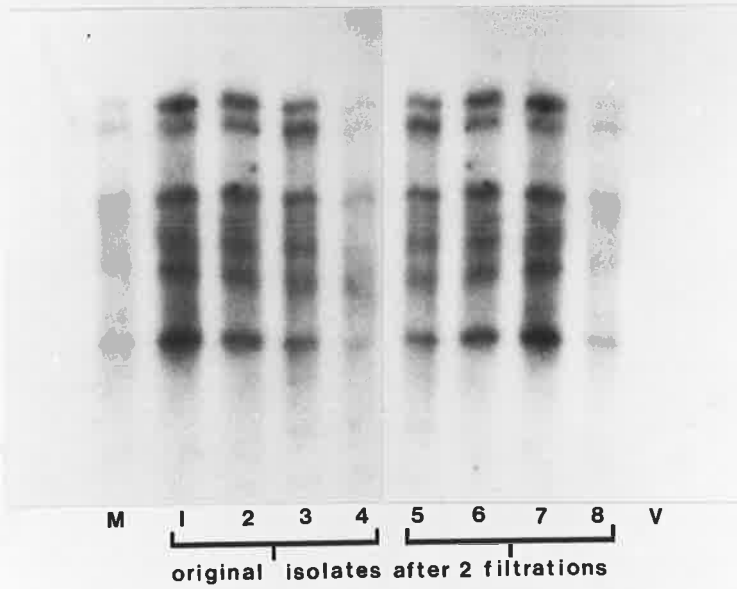 M) but not with VTAV (lane V). Similarly, the MVLB2 probe did not hybridize with the VTAV RNAs (Fig 5.8b lane V), but hybridized with MCMV and the four isolates (Fig 5.8b lanes M, and 1-8).

The RNA sequences responsible for the VTAV-like symptoms induced by isolates MVLB1 and B2 could therefore not be determined by the methods used. The removal of the VTAV-like sequences by filtration through *C. sativus* left both isolates MVLB1 and B2 with MCMV-like characteristics, suggesting that the complete MCMV genome must have been present in addition to whichever RNA segments or sequences were conferring the VTAV-like character on those isolates.

5.4. Local Lesion Isolates from *Beta vulgaris* Inoculated With Virus in Preparation V.

Purified virus (100 μg/ml) from preparation V (Table 5.1, treatment c) was used to inoculate *Beta vulgaris* plants. Two types of lesions were formed, one chlorotic with a purple ring (MCMV-like) and the other purple (VTAV-like). Both types of lesions were regular and of about 1.5mm in diameter. Fifty of the MCMV-like lesions were excised and

extracts from each used to inoculate *N clevelandii* plants. Five out of 50 plants inoculated were found to be infected.

Four of the five infected plants showed MCMV-like symptoms, while the fifth showed symptoms similar to that induced by the pseudorecombinant $T_1T_2M_3$. The four isolates inducing MCMV-like symptoms were designated VMLA1-4, and the one exhibiting $T_1T_2M_3$-like symptoms, VMLB1. When leaf extracts were analysed by ELISA all the isolates reacted to MCMV but not VTAV antiserum.

Leaf extracts were used to infect further *N. clevelandii* plants, from which virus was purified by method M. RNA was purified from these virus preparations and used in the characterization of the genomes of the five isolates.

5.4.1. *RNA Composition of Isolates VMLA1-4, and VMLB1.*

RNAs purified from the virus preparations were analysed by dot-blot and Northern blot hybridization. Dot-blot hybridization results presented in Fig 5.10a and b show that isolates VMLA3, and B1 hybridized with both MCMV and VTAV cDNA probes (Fig 5.9a and b, spots 3 and 5), but isolates VMLA1, A2 and A4 hybridized with only the MCMV cDNA probe (Fig 5.9a and b, spots 1, 2 and 4).

Northern blot hybridization results presented show that the RNAs 3 and 4 of all the five isolates (Fig 5.10a, lanes 1-5) as well as the RNAs 1 and 2 of isolates VMLA1, A2, A3, and A4 (Fig 5.10a, lanes 1-4) hybridized with the MCMV cDNA probe. The VTAV probe hybridized with RNA 1 of isolate VMLB1 but only weakly to its RNA 2 (Fig 5.10b, lane 5), which was also found to stain weakly in agarose gels. It is interesting to note that RNA from isolate VMLA3 which hybridized strongly with cDNA to VTAV in dot-blots (Fig 5.9b spot 3) failed to do so in Northern hybridization analysis (Fig 10b, lane 3).

5.4.2. *Host range and symptomatology of VMLA1-4 and VMLB1*

The effects of the five isolates were tested on a variety of host plants. When *C. sativus* was inoculated with purified preparations of the 5 isolates, all except isolate VMLB1 induced both chlorotic local lesions and systemic infection. Isolate VMLB1 did not infect *C sativus* and was in this respect like the pseudorecombinant $T_1T_2M_3$. In *N. clevelandii*, isolate

Fig 5.9

Preliminary analysis of RNA composition of isolates VMLA1-4 and VMLB1 by dot-blot hybridization analysis.

20ng of viral RNA preparations of local lesion isolates VMLA1 (spot 1), VMLA2 (spot 2) VMLA3 (spot 3), VMLA4 (spot 4) and VMLB1 (spot 5) was used per spot and hybridized with MCMV (panel a) and VTAV (panel b) cDNA probes. MCMV and VTAV positive controls, a dilution series of 50 to.05ng of RNA was applied as shown. Uninfected host total RNAs were used as negative controls at twice the concentration of MCMV and VTAV RNAs.

Fig 5.10

RNA composition of virus isolates VMLA1-4.andVMLB1 by Northern hybridization analysis.

RNAs 1 and 2 of isolates VMLA1 (lane A1), VMLA2 (lane A2), VMLA3 (lane A3) and VMLA4 (lane A4) hybridized with only the MCMV cDNA probe (panel a), while the RNAs 1 and 2 of isolate VMLB1 (lane B1) hybridized with only the VTAV cDNA (panel b). Isolate VMLA3 (lane A3) which hybridized strongly with VTAV cDNA probe in dot-blot hybridization analysis failed to hybridize with that probe in Northern blot analysis.

Panel a
MCMV cDNA

M   VML   H   V

Panel b
VTAV cDNA

M   VML   H   V

Fig 5.9

Panel a
MCMV cDNA

RNA 1→
RNA 2→
RNA 3→
RNA 4→

M   A₁   A₂   A₃   A₄   B₁   V

Panel b
VTAV cDNA

M   A₁   A₂   A₃   A₄   B₁   V
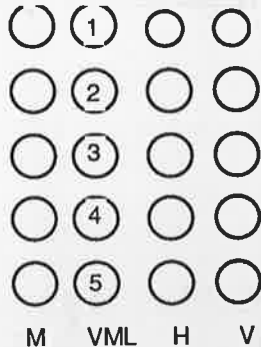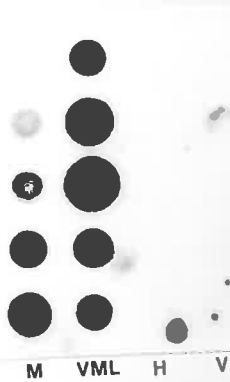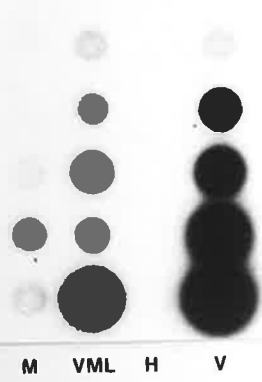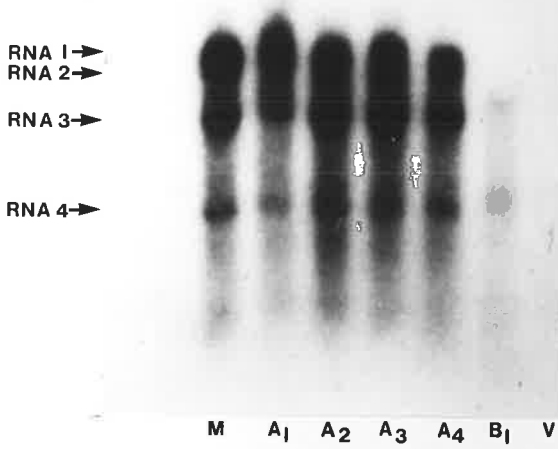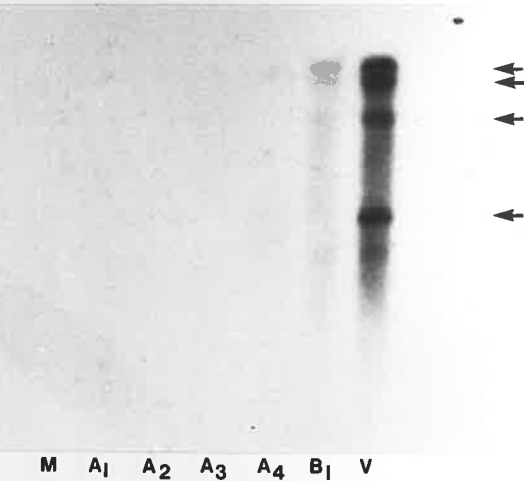
Fig 5.10

Fig 5.11

Symptomatology of isolates VMLA1-4 and VMLB1.

The symptoms induced by MCMV and VTAV were compared with those induced by isolates VMLA1, VMLA2, VMLA3, VMLA4 and VMLB1 in (a) *Nicotiana glutinosa*, (b) *N. clevelandii*, and (c) *Gomphrena globosa.*. Unlike VTAV and $T_1T_2M_3$, isolate VMLB1 induced systemic infection of *G. globosa*.

a

b

VMLA₁   VMLA₃   HEALTHY

MCMV   VMLA₂   VMLA₄   VMLB₁   VTAV
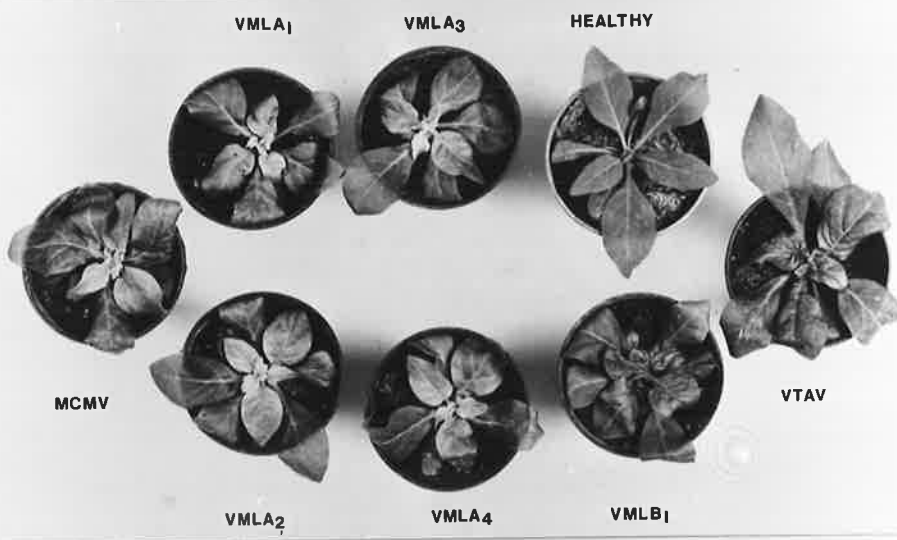
c
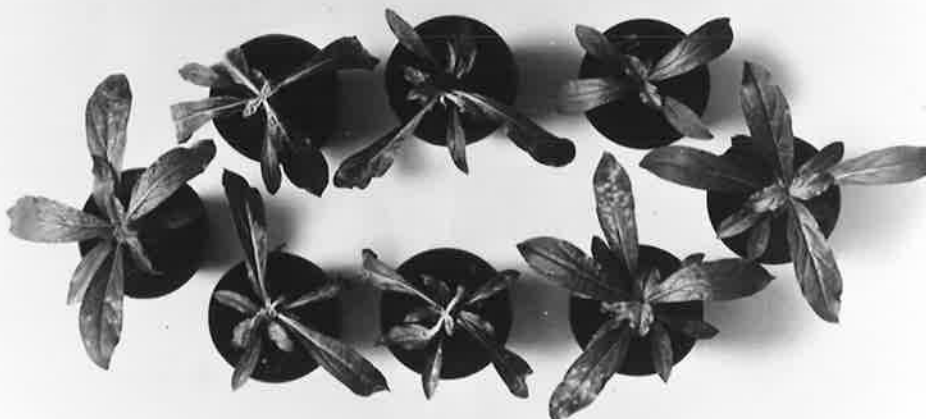
Fig 5.11

VMLB1 induced symptoms similar to those induced by $T_1T_2M_3$, but was poorly infectious in *N. glutinosa* in which symptoms which were VTAV-like appeared after about 2-3 weeks (compared to a few days for the other isolates and the parental viruses). Symptoms induced by isolates VMLA1 to A4 were MCMV-like in *G. globosa, N. glutinosa* and *N. clevelandii* (Fig 5.11a, b and c respectively). VMLB1 induced $T_1T_2M_3$-like symptoms in *N. glutinosa* and *N. clevelandii* Unlike the pseudorecombinant $T_1T_2M_3$, however, VMLB1 induced mild systemic symptoms of infection of *G. globosa* in addition to the local lesions induced on the inoculated leaves. Systemic infection of *G. globosa* was confirmed by back inoculation to *N. clevelandii..*

5.4.3. *Is the RNA 2 of isolate VMLB1 a "hybrid"?*

Based on the observation that it was poorly represented in the genome composition, it was thought the RNA 2 of isolate VMLB1 may be a "hybrid" consisting of MCMV and VTAV RNA sequences. The presence of the MCMV sequences, it was thought, may also account for the ability of this isolate to systemically infect *G. globosa*. To test this possibility, RNA extracts from purified virus preparation of isolate VMLB1 was used as template for cDNA synthesis. This probe was used in hybridization analysis of MCMV, VTAV and the five isolates.

Results presented in Fig 5.12 show that the probe hybridized with the RNAs 3 and 4 of MCMV and isolates VMLA1-4, but not their RNAs 1 and 2 (Fig 5.12, lanes M, and 1-4). It, however, hybridized with both VTAV RNAs 1 and 2 (Fig 5.12, lane V), as well as with the homologous RNA (Fig 5.12, lane 5). The isolate was characterized by a large amount of degradation product which hybridized with the probe (Fig 5.12, lane 5); no similar hybridization with RNAs from the other isolates was detected.

Isolate VMLB1 was also compared with the pseudorecombinant $T_1T_2M_3$ by Northern hybridization analysis. Results presented in Fig 5.13a show that the RNAs 3 and 4 of both VMLB1 and $T_1T_2M_3$ hybridized with the MCMV cDNA probe (Fig 5.13a lanes $T_1T_2M_3$ and VMLB1). The VTAV probe hybridized with the RNAs 1 and 2 of both VMLB1 and $T_1T_2M_3$ (Fig 5.13b). Further comparison showed that the representation of the RNAs 1 and 2 differed in the two virus isolates. Isolate VMLB1 was poorly represented in RNA 2 and this

Fig 5.12

Analysis of VMLB1 to determine whether there were any MCMV RNA sequences in its RNA 2.

VMLB1 RNA was used as template for cDNA probe used in Northern hybridization analysis of homologous RNA (lane 5), MCMV (lane M), VMLA1 (lane 1), VMLA2 (lane 2), VMLA3 (lane 3), VMLA4 (lane 4) and VTAV (lane V). The probe hybridized with only the RNAs 3 and 4 of MCMV, and the other four isolates, and the RNAs 1 and 2 of VTAV. There was also substantial hybridization with what may have been degradation products of its RNAs and unique to that virus isolate

Fig 5.13

Comparison of $T_1T_2M_3$ and VMLB1 by Northern hybridization analysis with MCMV and VTAV cDNA probes.

The two viruses appear to differ in the relative amounts of their RNAs 1 and 2; while VMLB1 contains a relatively low concentration of RNA 2, compared with its RNA 1, $T_1T_2M_3$ is characterised by a relatively low concentration of RNA 1 compared with its RNA 2.

VMLB₁ cDNA

M l 2 3 4 5 V

Fig 5.12

a.
MCMV cDNA

b.
VTAV cDNA

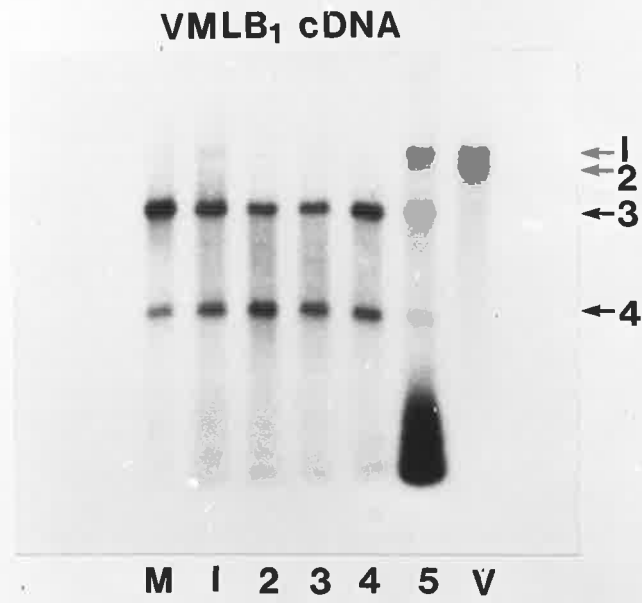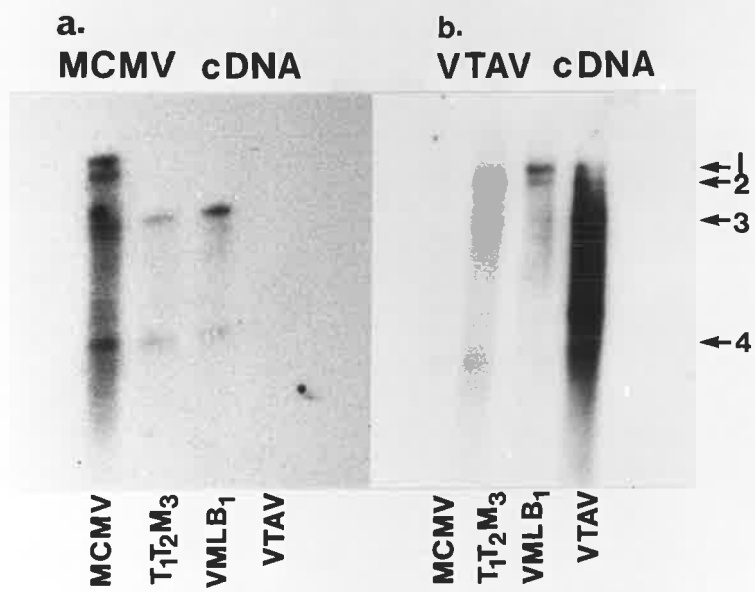**Fig 5.13**

was reflected in the hybridization with the VTAV cDNA probe (Fig 5.13 b) and stained weakly in agarose gels (results not presented). In contrast, $T_1T_2M_3$ was poorly represented in its RNA 1 (Fig 5.13b) and also stained weakly with ethidium bromide (results not shown)

It was concluded that isolate VMLB1 was $T_1T_2M_3$-like, but it remains obscure why VMLB1 was capable of infecting *G. globosa* while $T_1T_2M_3$ was not.

5.5. Conclusions.

The MCMV method of virus purification when used to purify tissue coinfected with MCMV and VTAV resulted in a virus preparation containing both viruses. While the purified virus induced MCMV-like symptoms, after sucrose density-gradient centrifugation the virus induced VTAV-like symptoms. As deduced in Chapter 4, the symptoms expressed are associated with the virus present at a higher relative concentration in the mixed inoculum. It appears therefore that some MCMV was lost or was inactivated during sucrose density-gradient centrifugation. In contrast, the VTAV method appears to remove all particles with MCMV coat protein, as shown by both ELISA and infectivity tests.

The use of specific antisera was effective in completing the separation of the two viruses. Hybridization analysis of the virus preparations, however, failed to provide evidence of transcapsidation. This may have been because the amounts of transcapsidated nucleic acids were too low to be detected by the assay methods used.

Using preparation M as inoculum, four virus isolates were obtained which induced VTAV-like symptoms, were serologically indistinguishable from MCMV, and also infected *C. sativus*. and *G globosa*. Passage of isolates MVLB1 and B2 through *C. sativus* resulted in the virus isolates assuming symptomatological properties indistinguishable from those of MCMV. Northern hybridization analysis of the isolates before and after passaging through *C. sativus* with a variety of cDNA probes failed to provide an insight into any changes in the genome composition.

When preparation V was used as inoculum, most of the lesions were VTAV-like. Extracts from each of fifty local lesions which differed in morphology from the lesions induced by the parental viruses were used to inoculate *N. clevelandii* plants, but only 5 caused infections. Four of the five infectious local lesion isolates were shown to be

indistinguishable from MCMV. These isolates may have been transcapsidated in VTAV coat protein and therefore protected from the effect of virus purification method V. A fifth isolate, VMLB1, showed $T_1T_2M_3$-like symptoms, was serologically indistinguishable from MCMV, and Northern hybridization analysis showed that it consisted of VTAV RNA 1 and MCMV RNA 3, and an RNA 2 which may have been a variant. This isolate differed from $T_1T_2M_3$ by its ability to infect *G. globosa* systemically, and a particularly slow rate of multiplication and symptom induction in *N. glutinosa* in which species symptoms were first detected 16-20 days after inoculation.

CHAPTER SIX

ISOLATION AND CHARACTERISATION OF A VARIANT ISOLATE
CONSISTING OF MCMV AND VTAV RNAS FROM MIXED
INFECTIONS OF MCMV AND VTAV.

6.0. Introduction.

In Chapter five, the differential methods of virus purification were combined with differences in morphology of local lesions induced by MCMV and VTAV to isolate virus variants inducing host symptoms intermediate to those associated with the parental viruses. The subject of this Chapter is the use of local lesions alone as a method of isolating variants.

6.1. *Beta vulgaris* as a Local Lesion Host for Biological Purification.

Beta vulgaris plants were inoculated with extracts from plants which had been tested serologically and shown to contain both MCMV and VTAV antigens during three successive passages in *N. clevelandii*.. From these inoculations, lesions which were morphologically different from those produced by either MCMV or VTAV were excised and passaged by successive local lesion transfers. After four passages through *B. vulgaris*, the selected lesions were inoculated on to *N. glutinosa* to test their effect on that host, and their viral antigen content was analysed by ELISA.

Most of the variants lesions proved non-infectious. Of those from which infectious virus was recovered when inoculated to *N. glutinosa,* most of the plants developed MCMV-like symptoms, contained only MCMV antigens, and infected *C. sativus* systemically. From these data it was concluded that these isolates were not significantly different from MCMV and were therefore not retained for further study. However, two local lesion isolates differed from the others. One contained MCMV and the other VTAV antigen only, and were designated isolates Ra and Rb respectively. In *N. glutinosa*, both induced vein banding, mosaic symptoms different from those induced by MCMV and VTAV (Fig 6.1) but neither

Fig 6.1

Comparison of symptoms induced in *N. glutinosa* by MCMV, VTAV, and local lesion isolates Ra and Rb.

The two isolates induced systemic veinbanding symptoms uncharacteristic of either MCMV or VTAV.

MCMV

Ra

Rb

VTAV
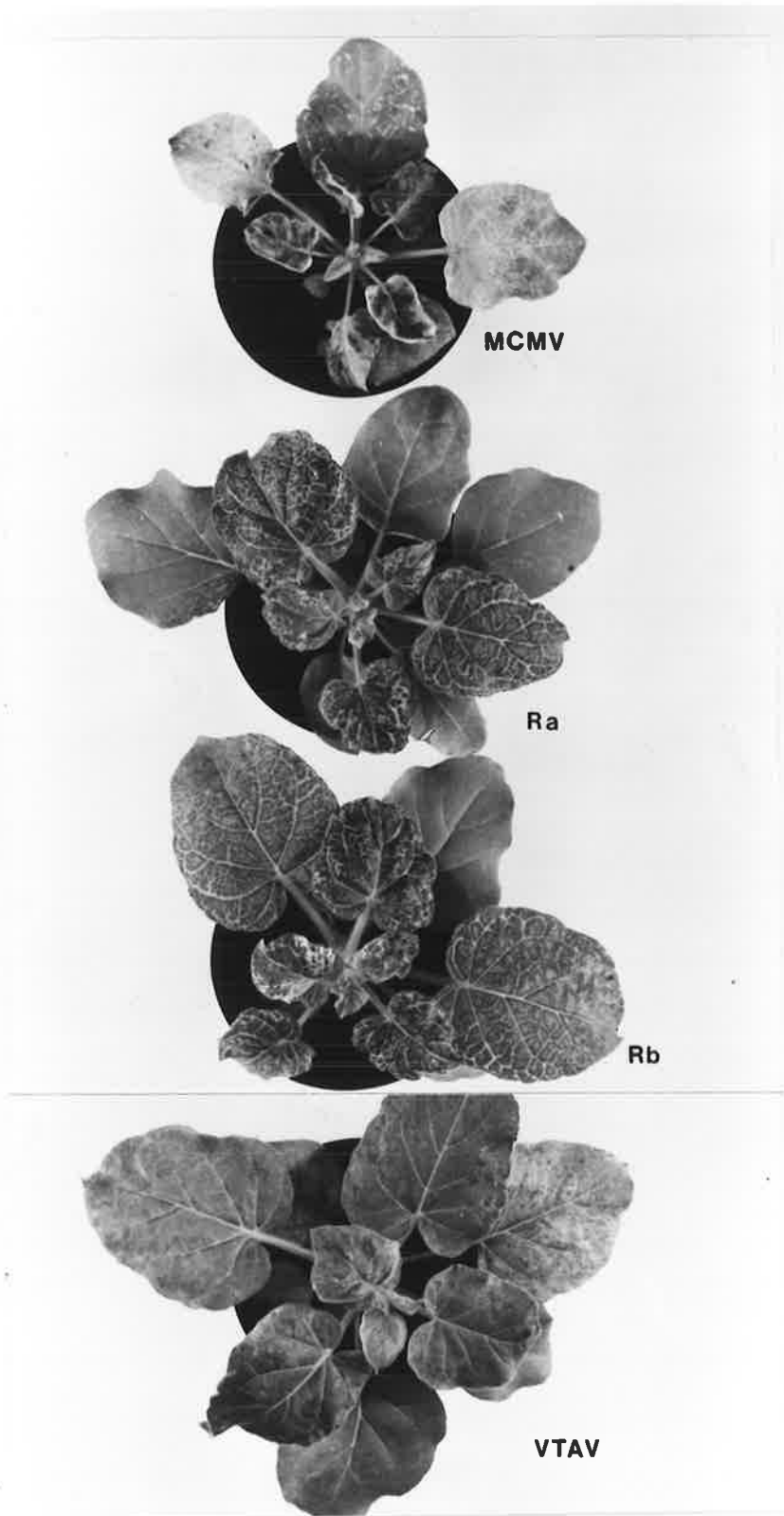
Fig 6.1

isolate induced local lesions or systemic infection in *C. sativus*. These were selected for further study.

6.2. Purification of virus variants Ra and Rb.

The differential effects of virus purification procedures demonstrated previously was utilized as the first physical means of determining whether the isolates were homogeneous. *N. clevelandii* plants inoculated with Ra alone and Rb alone were each divided into two groups. Virus from one of each group was purified by method M and the other by method V. The purified virus preparations were analysed by sucrose density-gradient centrifugation, and the virus recovered was used for infectivity tests in *N. glutinosa*.

Sucrose density-gradient profiles presented in Fig 6.2 show that when purified by Method M, intact virus of Ra was recovered (Fig 6.2a). However, when purified by method V, there was complete disruption of Ra capsid, and no virus was recovered (Fig 6.2b). When isolate Rb was purified by either method, two peaks were produced, one at the top of the gradient and containing no intact virus as determined by infectivity, and a second peak from which virus was recovered (Fig 6.2c and d). Preparations of isolate Rb by either method infected *N. glutinosa* to produce symptoms indistinguishable from those induced by VTAV. In view of these changes in infectivity and host response, Rb was considered unsuitable for further study.

6.3. Serological Properties of Ra.

A highly purified preparation of isolate Ra was glutaraldehyde-fixed as described previously and used for immunization of a rabbit. Antiserum with an immuno-diffusion titre of 1/128 was used in a comparative serological analysis with MCMV and VTAV. Ouchterlony test results presented in Fig 6.3 show that MCMV and Ra are serologically indistinguishable, reacting without spur formation to both MCMV (Fig 6.3a) and Ra (Fig 6.3b) antisera. Neither MCMV nor isolate Ra reacted with VTAV antiserum (Fig 6.3c). When tested by ELISA with MCMV antiserum and conjugate, however, Ra reacted less strongly than MCMV (Fig 6.4)
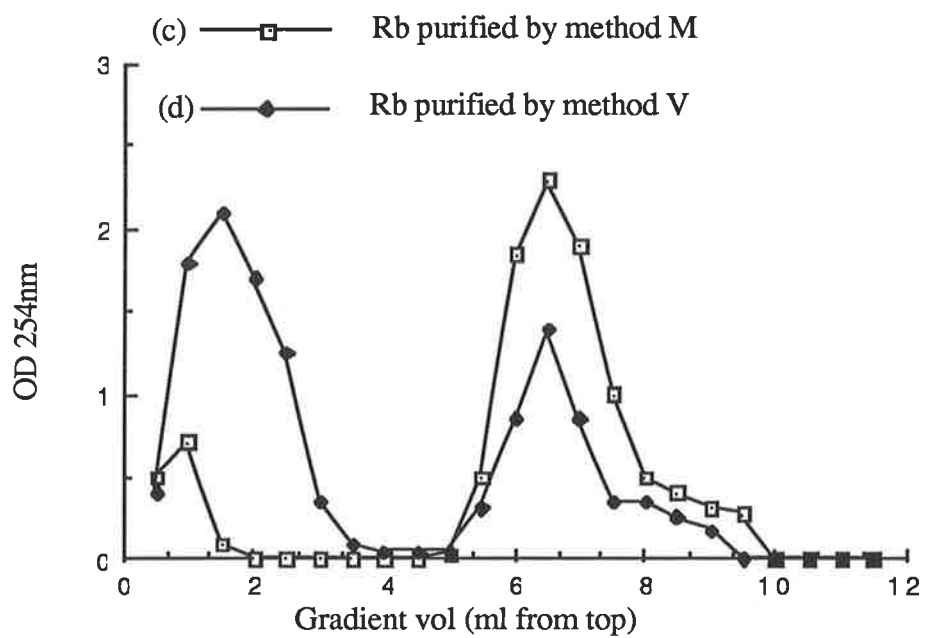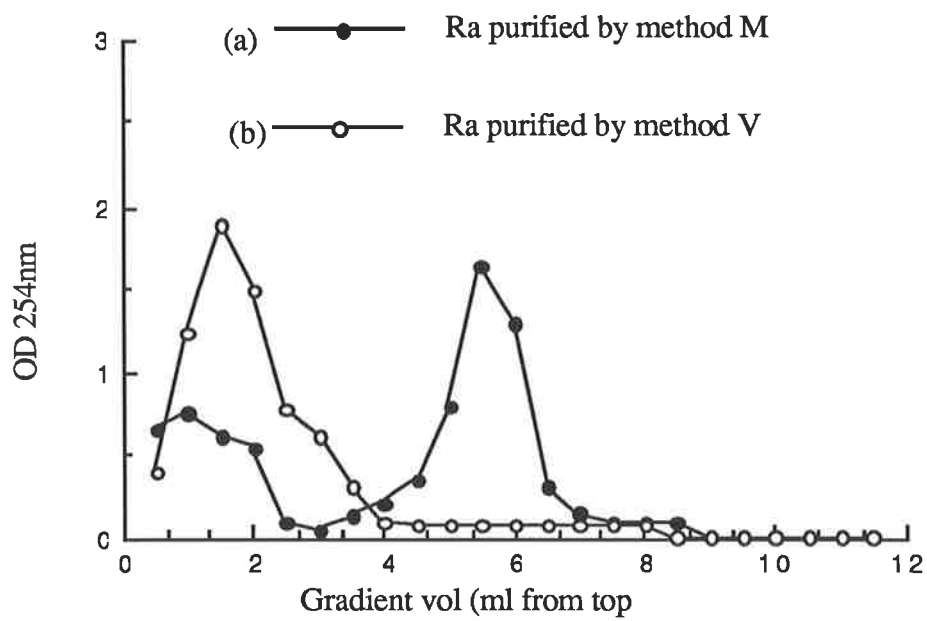
Fig 6.2 Effects of MCMV and VTAV purification methods on recovery of local lesion virus isolates Ra and Rb from sucrose density gradients

Fig 6.3

Serological properties of isolate Ra.

Gel diffusion analysis using (panel a) MCMV antiserum, M; (panel b) Ra antiserum, R and (panel c) VTAV antiserum V against purified virus preparations of MCMV (m), Ra (r), VTAV (v).
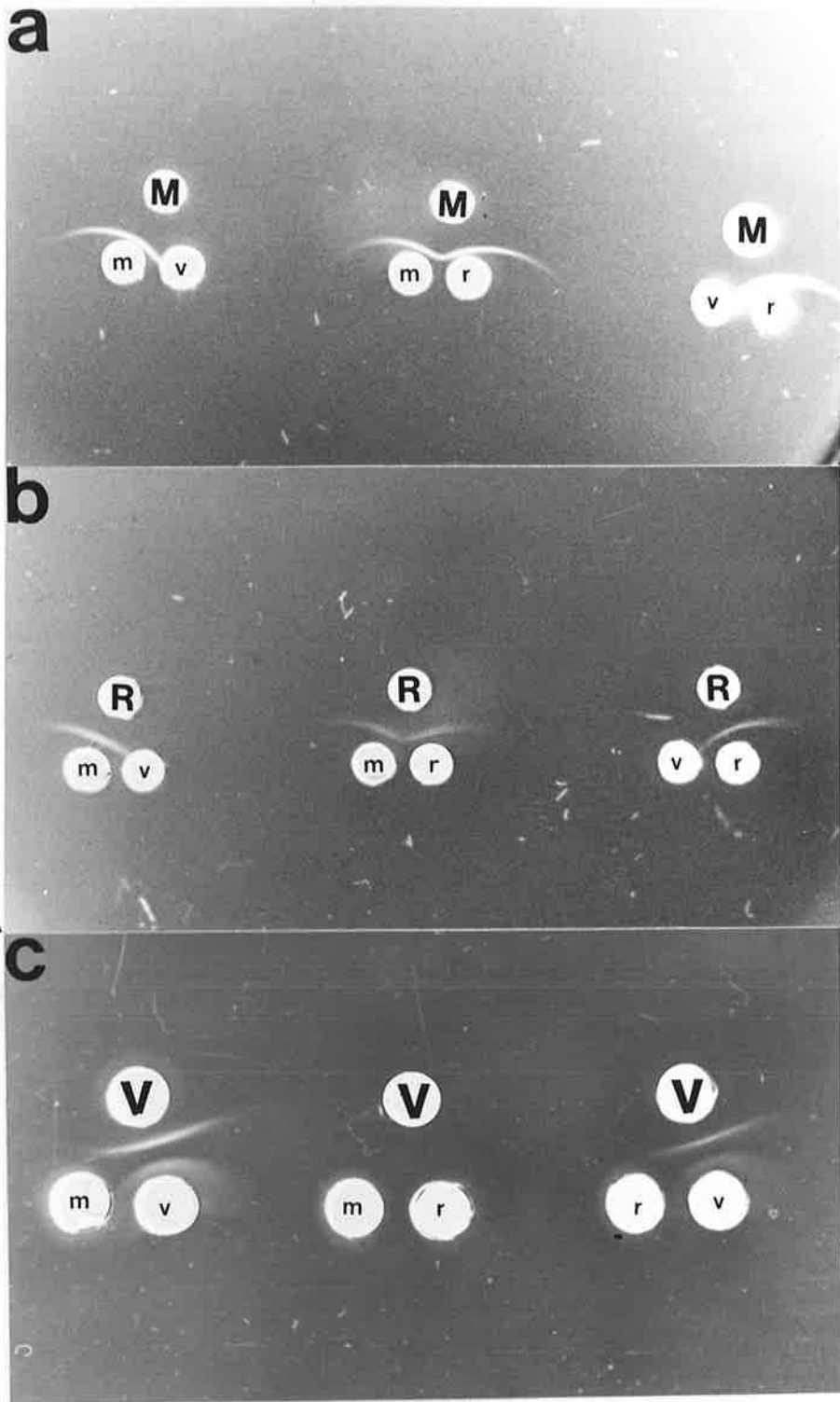
Fig 6.3
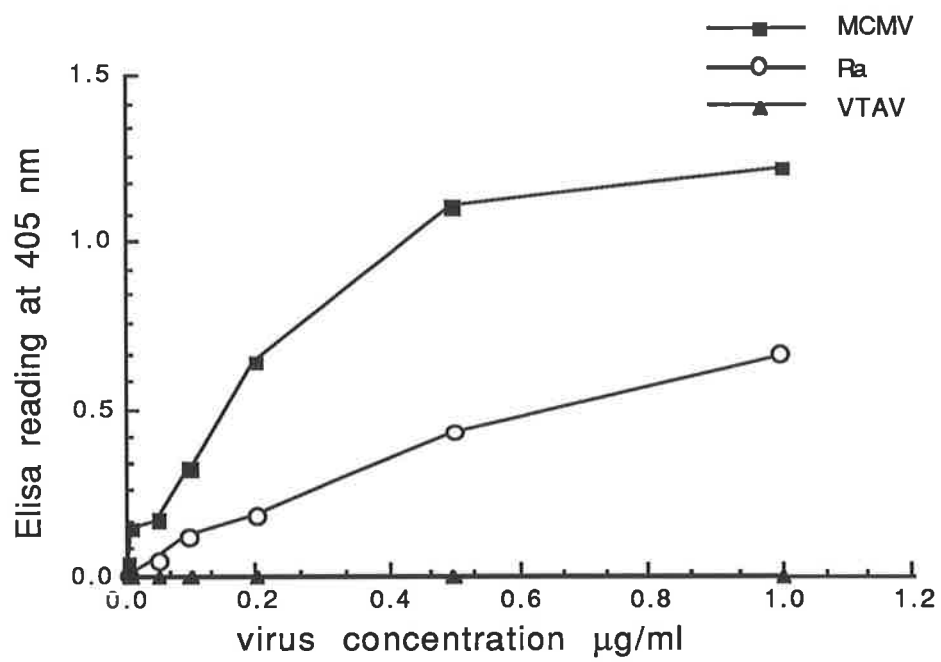
Fig 6.4   Sensitivity and specificity of MCMV antiserum and conjugate in detection of isolate Ra by ELISA.

6.4. Host Range and Symptomatology of Isolate Ra

Results of comparisons of the effects of isolate Ra with those of MCMV and VTAV on a range of host plants are presented in Table 6.1 and some of the differences are illustrated in Fig 6.5. Isolate Ra did not infect *C. sativus,* thus differing from MCMV which induces both local lesions and systemic infection. Ra was in this respect similar to $T_1T_2M_3$,.which does not infect *C. sativus,* but was different from $M_1M_2T_3$, which induces local lesions but no systemic infection. Ra induced local lesions on the inoculated leaves of *G. globosa* but the virus did not move systemically. This host species is not infected by VTAV or $M_1M_2T_3$, but is infected by MCMV which induces local lesions and systemic chlorosis and leaf distortion (Fig 6.5c). In this respect, Ra was like $T_1T_2M_3$ which also induces local lesions but no systemic infection. In general, the symptoms induced by Ra on the hosts tested tended to be less severe than those induced by MCMV or VTAV, with the exception of *Spinacea hybrida* (Fig 6.6b), *Zinnia elegans* and *Petunia hybrida* (Fig 6.5d) in which it produced visible symptoms compared to the almost symptomless infection of those species by VTAV.

When compared with the pseudorecombinant $T_1T_2M_3$, there was no difference in the range of host plants infected, even though isolate Ra often appeared to be more virulent (Fig 6.6a and b). In general, isolate Ra infected the species tested more readily than did $T_1T_2M_3$ with symptoms appearing 3 to 4 days earlier, though the final appearance of the infected plants was similar.

6.5. RNA Composition of Isolate Ra Particles.

The RNA composition of Ra was compared to those of MCMV and VTAV by agarose gel electrophoresis, dot-blot, and Northern blot hybridization analysis.

6.5.1. *Dot-blot hybridization analysis.*

Viral RNAs of MCMV, VTAV and Ra ranging from 0.05-50ng were used in dot blot hybridization analysis using MCMV and VTAV cDNA probes. Results presented in Fig 6.7b show that the VTAV cDNA hybridized with the RNA of isolate Ra to nearly the same extent as with that of homologous RNA. However, the RNA of Ra also hybridized significantly with the cDNA of MCMV (Fig 6.7a).

Table 6.1.

Host range and symptomatology of isolate Ra

Plant tested	Symptoms induced[1]
Nicotiana glutinosa	Systemic mosaic and yellow vein banding ($\neq$MCMV,$\neq$VTAV).
Nicotiana tabacum cv. White Burley	Systemic mosaic with patchy chlorosis ($\neq$MCMV,$\neq$VTAV)
Nicotiana clevelandii.	Systemic mosaic and yellow vein banding ($\neq$MCMV,$\neq$VTAV)
Nicotiana benthamian	Systemic mosaic (=VTAV)
Gomphrena globosa	Chlorotic local lesions on inoculated leaves with no systemic spread ($\neq$MCMV,$\neq$VTAV)
Cucumis sativus	Not infected (=VTAV)[2]
Spinacea hybrid cv. English	Mild systemic mosaic (=VTAV)
Zinnia elegans cv. Golden Queen	Mild systemic yellow mosaic (=MCMV, mild)
Petunia hybrida cv. Dazzler	Mild systemic yellow mosaic (=MCMV,mild)
Physalis floridana	Chlorotic local lesions on inoculated leaves and systemic yellow mosaic (=MCMV,mild)
Chenopodium amaranticolor	Chlorotic local lesions no systemic spread (=MCMV=VTAV).
Chenopodium quinoa	Chlorotic local lesions no systemic spread. (=MCMV,=VTAV)

[1]Symptoms induced were compared with those by MCMV and VTAV; = denotes like, and $\neq$ denotes unlike

[2]Both systemic and inoculated leaves were tested for the presence of antigens and none was found.Symptoms induced are compared with those by MCMV and VTAV; =(like), and $\neq$ (unlike).

Fig 6.5

Comparison of symptoms induced by MCMV, VTAV and isolate Ra in (a) *Nicotiana tabacum* cv White Burley; (b) *N. glutinosa*; (c) *Gomphrena globosa* and (d) *Petunia hybrida* cv Dazzler.
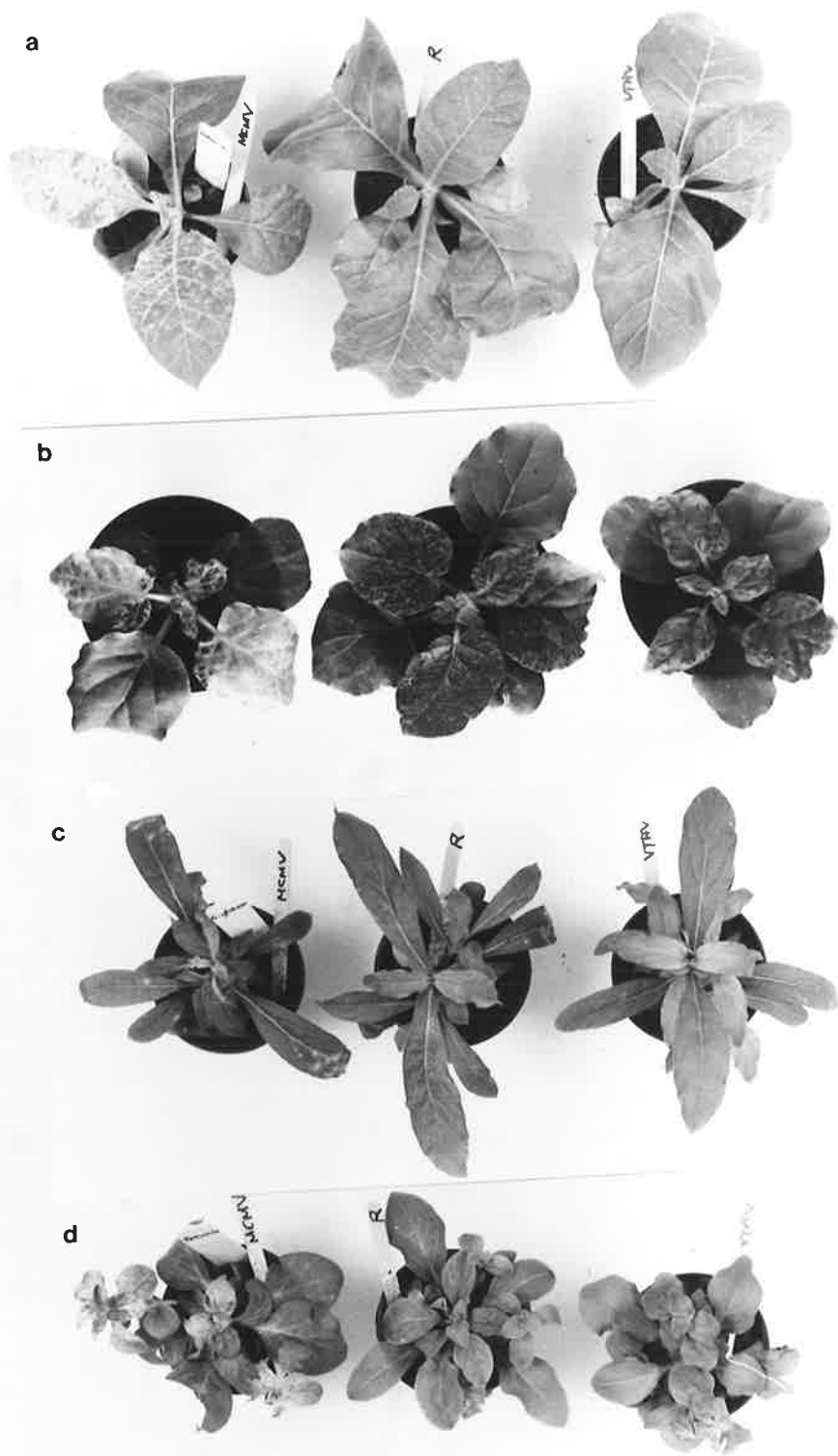
Fig 6.5

Fig 6.6

Comparison of symptoms induced by MCMV, VTAV, isolate Ra, and pseudorecombinants $M_1M_2T_3$ and $T_1T_2M_3$ in (a) *Nicotiana tabacum* cv. White Burley and (b)*Spinacea* hybrid cv. English.

Isolate Ra appeared to be more readily infectious and induced more severe symptoms than $T_1T_2M_3$ in some of the plant hosts tested.

a

VTAV

Ra

$M_1M_2T_3$

MCMV

$T_1T_2M_3$

b

VTAV

Ra

$M_1M_2T_3$

MCMV

$T_1T_2M_3$
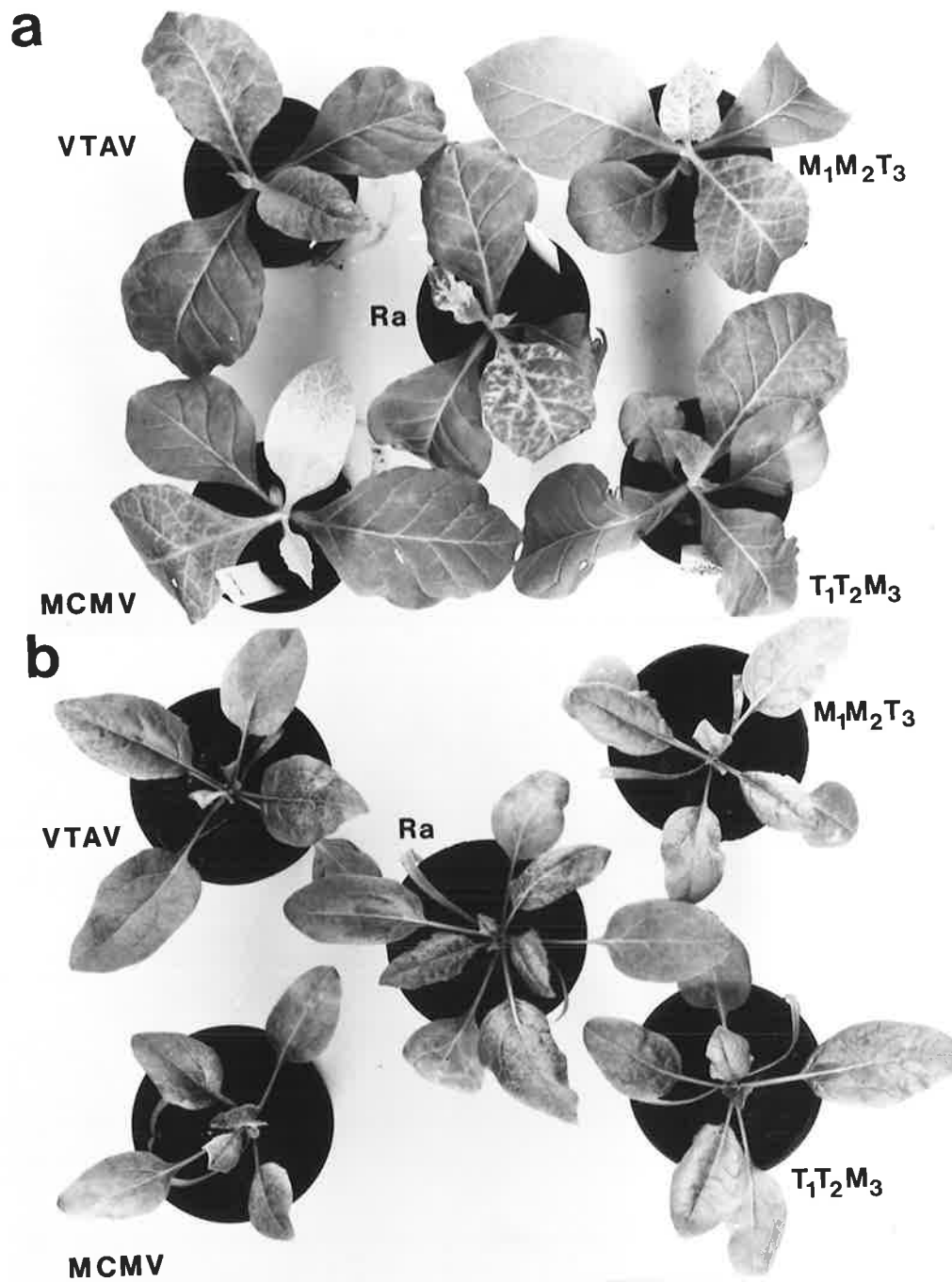
Fig 6.6

6.5.2. *Agarose gel electrophoresis and Northern hybridization analysis*

The RNA composition of Ra was compared with that of MCMV and VTAV by electrophoresis in 2% (w/v) agarose gels, and by Northern hybridization analysis. Results presented in Fig 6.8a show that Ra consists of RNAs 1 and 2 with electrophoretic mobilities similar to those of RNAs 1 and 2 of VTAV (Fig 6.8a lane Ra). The RNA 3 of Ra had electrophoretic mobility more similar to that of VTAV than MCMV RNA 3, but the subgenomic RNA 4 had a mobility similar to that of MCMV RNA 4 (Fig 6.8a and b). Compared to the MCMV and VTAV, Ra RNAs 1 and 3, tended to fragment, leading to multiple bands (Fig 6.8b).

In Fig 6.9a, b, and c, the northern blots were probed with MCMV, isolate Ra and VTAV cDNA probes, respectively. The MCMV probe hybridized with its homologous RNAs (Fig 6.9a, lane M) as well as the RNAs 3 and 4 of Ra, but not the RNAs 1 and 2 (Fig 6.9, lane Ra). The Ra probe hybridized with MCMV RNAs 3 and 4 (Fig 6.9b, lane M), VTAV RNAs 1, 2 and 3 (Fig 6.9b, lane V) as well as its homologous (Fig 6.9b, lane Ra). The VTAV probe hybridized with the homologous RNAs (Fig 6.9c, lane V) as well as the RNAs 1, 2 and 3 of Ra, (Fig 6.9c, lane Ra) but not RNA 4 (Fig 6.9c, lane Ra).

Isolate Ra was further analysed under denaturing conditions using glyoxal as the denaturant. Hybridization results presented in Fig 6.10a and b with MCMV and VTAV cDNA probes respectively were similar to those obtained under non-denaturing conditions, with the RNA 3 of Ra hybridizing with both cDNA probes. From the results presented in Figs 6.9 and 6.10, it appeared that Ra consisted of RNAs 1 and 2 similar in sequence to those of VTAV but not MCMV. The RNA 3 of isolate Ra was different in electrophoretic migration (and presumably, size) from those of MCMV or VTAV, but appeared to contain sequences of both. The RNA 4 of Ra which was MCMV-like in electrophoretic migration and hybridized with only the MCMV cDNA probe, was poorly represented and often not detected by ethidium bromide staining.

It was considered that these results may be due to RNAs 3 of both MCMV and VTAV being present in the isolate. However, if an intact VTAV RNA 3 was present, transcription of its coat protein mRNA would have occurred and should have been detected by agarose gel

Fig 6.7

Preliminary analysis of RNA composition of isolate Ra by dot-blot hybridization.

Purified viral RNA preparations of Ra, MCMV and VTAV were used and the amounts indicated in the central panel were applied per spot. Total uninfected leaf RNA extracts were used at twice the amounts of the viral RNAs at each spot.

Fig 6.8

Agarose gel electrophoresis of isolate Ra RNA.

Two RNA preparations of Ra were compared in 2% agarose. In both preparations, the RNAs 1 and 2 of Ra and VTAV on the one hand, and the RNAs 4 of Ra and MCMV on the other, have similar electrophoretic migration. Whilst intact Ra RNA 3 (panel a) is more VTAV-like in its eletrophoretic migration, degradation (panel b) results in two or three RNA segments which are clearly resolved in agarose.

a. MCMV cDNA

b. VTAV cDNA

0.05ng
0.5ng
5ng
20ng
50ng

M   Ra   H   V

M   Ra   H   V

M   Ra   H   V

Fig 6·7

a. intact RNA 3

b. degraded RNA 3

M   Ra   V

M   Ra   V
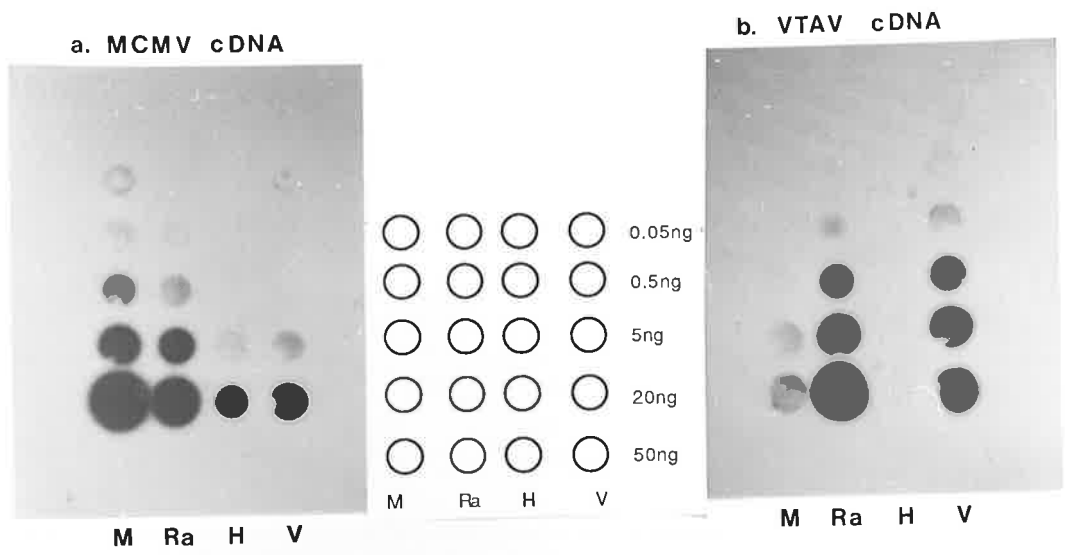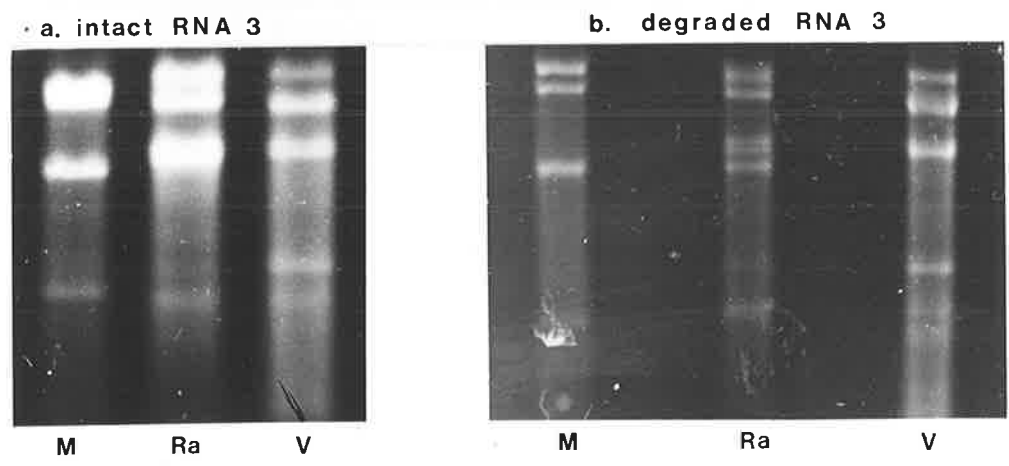
Fig   6·8

electrophoresis, and the coat protein (antigen) encoded should have been detected by ELISA. Indeed, the persistence of both antigens in mixed infections has been demonstrated in coinfections of MCMV and VTAV and of $T_1T_2M_3$ and VTAV (Chapter 4). However, no VTAV antigens were detected in plants infected with isolate Ra.

6.5.3. *Comparison of Ra and $T_1T_2M_3$ RNAs.*

From the results obtained above, the *in vitro* constructed pseudorecombinant $T_1T_2M_3$ appears to have an RNA composition closest to the proposed structure of Ra. This was tested by comparing the RNAs from Ra with $T_1T_2M_3$ by Northern hybridization analysis under denaturing conditions. Results of hybridization analysis presented in Fig 6.11a show that the MCMV probe hybridized with the RNAs 3 and 4 of both Ra and $T_1T_2M_3$ (Fig 6.11a lanes Ra and $T_1T_2M_3$), but not their RNAs 1 and 2. The VTAV probe hybridized with the RNAs 1 and 2 of Ra and $T_1T_2M_3$ (Fig 6.11b, lanes Ra and $T_1T_2M_3$) as well as their RNAs 3 though more strongly with the former than the latter.

On the basis of the results of hybridization analysis and the serological results, a preliminary genomic composition of Ra, presented in Fig 6.12 was proposed, suggesting that Ra RNA 3 consisted of the coat protein gene from MCMV and the 3a gene from VTAV, although the exact point of their cross-over could not be predicted.

6.5.4. *Use of purified RNAs 3 and 4 as templates for cDNA probes.*

It was considered possible that the sequences in isolate Ra RNA 3 which hybridized with the VTAV probe may be due to contaminating nucleotide sequences from RNAs 1 and/or 2 co-migrating with Ra RNA 3 during electrophoresis. Purified RNAs 3 and 4 of MCMV and VTAV were therefore used as templates for the synthesis of cDNA probes. Results presented in Fig 6.13 were similar to those obtained using total viral RNAs as templates for the probes. The Ra RNA 4 hybridized with both the MCMV probes (Fig 6.13a and b), but neither of the VTAV probes (Fig 6.13c and d). The Ra RNA 3 hybridized with both MCMV and VTAV RNA 3 cDNA probes (Fig 6.13b and d). However, the extent of hybridization of these probes with their respective RNAs 1 and 2 were inconsistent with the estimated homology between the RNAs 1, 2 and 3 of these viruses located at their 3' leader

Fig 6.9

Determination of RNA composition of isolate Ra by Northern hybridization analysis using cDNA probes to (a) MCMV (b) Ra and (c) VTAV.

Fig 6.10

Comparison by Northern blot hybridization analysis of two RNA preparations of Ra spanning a 12 month period and more than 10 passages by mechanical inoculation.

No changes were observed in the hybridization properties of the two RNA preparations of the virus isolate when hybridized with MCMV (panel a) and VTAV (panel b) cDNA probes.

a.
MCMV cDNA

b.
Ra cDNA

C.
VTAV cDNA

M   Ra   V   M   Ra   V   M   Ra   V

Fig 6.9

a.
MCMV cDNA

b.
VTAV cDNA

M   Ra Ra   V   M   Ra Ra   V
    '86 '87   '86 '87
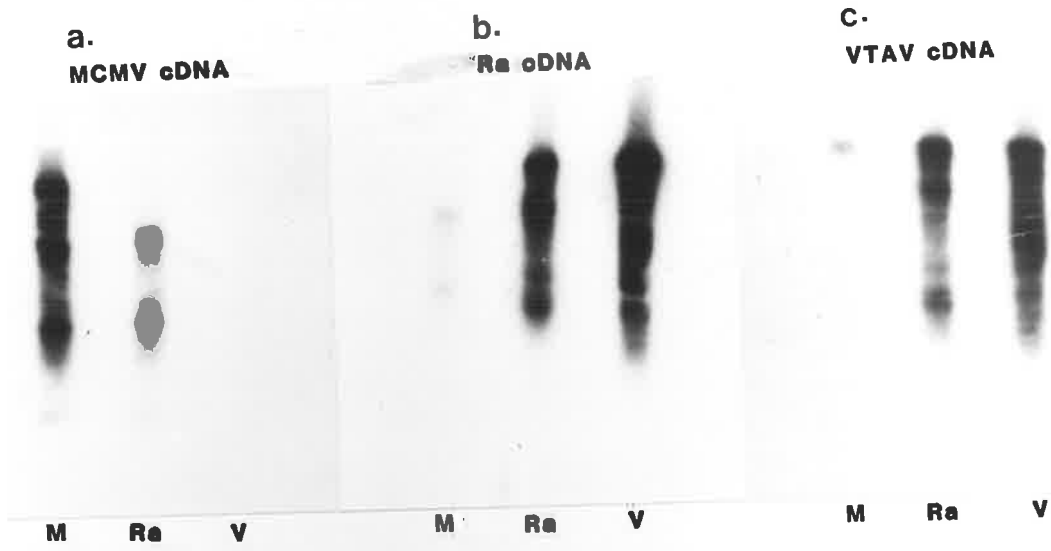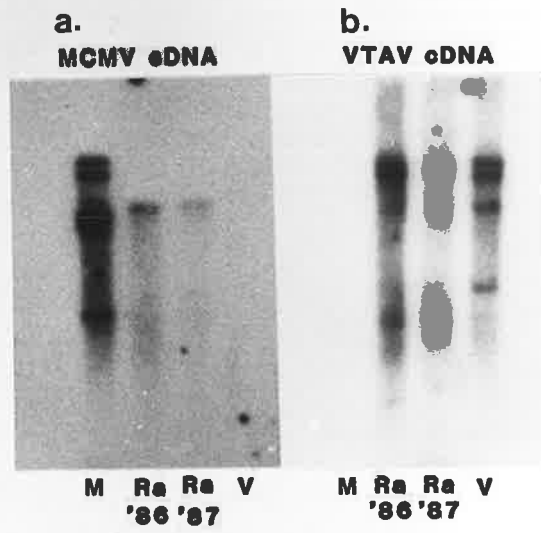
Fig 6.10

Fig 6.11

Comparison of isolate Ra and $T_1T_2M_3$ by Northern hybridization analysis under denaturing conditions.

RNAs were denatured with glyoxal, and Northern blots hybridized with MCMV (panel a) and VTAV (panel b) cDNA probes.

Fig 6.12

Proposed RNA composition of isolate Ra based on Northern hybridization and serological properties of the virus.

Based on the hybridization results, the RNAs 1 and 2 were concluded to be VTAV-like. The RNA 4 hybridized with only the MCMV probe, and together with the serological properties showed that the coat protein gene was of MCMV origin. It was proposed that since there was no loss of cell to cell or systemic movement of the isolate, then the VTAV sequences present may have been from the 3a gene (5' terminus).

a. MCMV cDNA   b. VTAV cDNA

1 →
2 →
3 →
4 →

M ↑ Ra V   M ↑ Ra V
   T1T2M3   T1T2M3

Fig 6.11

MCMV   Isolate Ra   VTAV

RNA1

RNA 1

RNA 2   RNA 2

                 3a   CP   RNA 3

RNA 3

                                        RNA 4

RNA 4
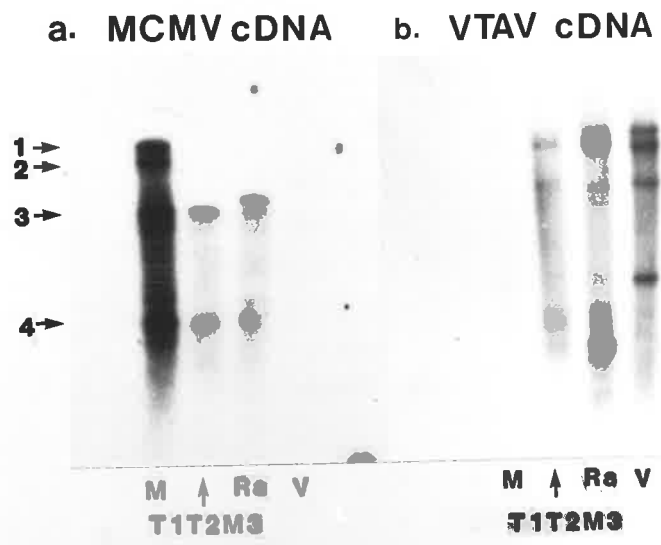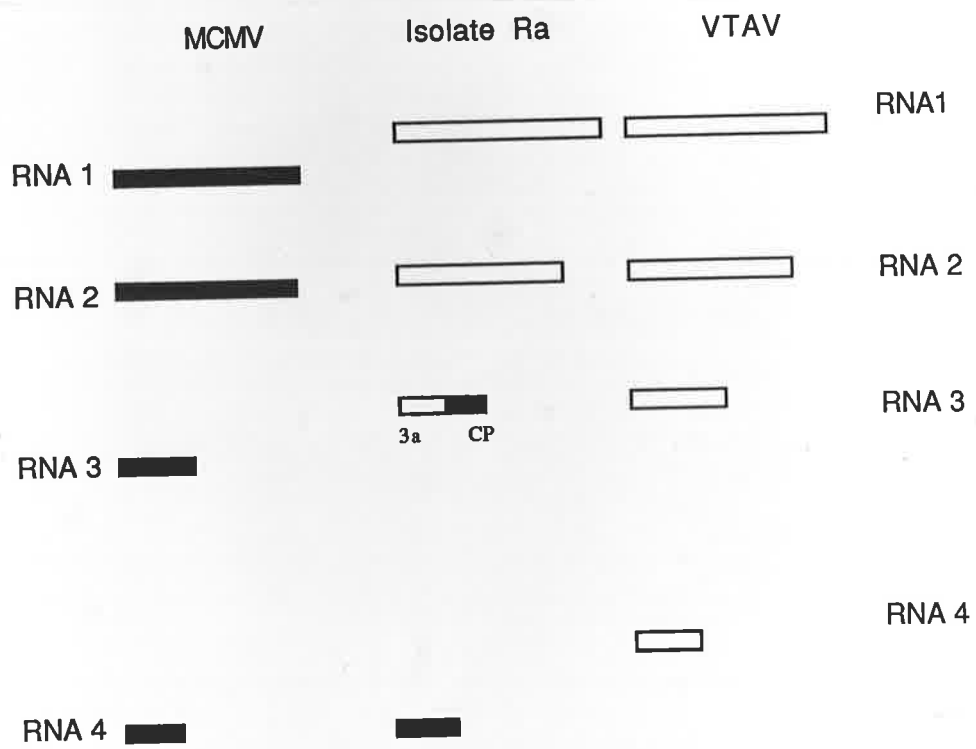
Fig 6.12

Fig 6.13

Analysis of Ra RNAs 3 and 4 by Northern hybridization.

Purified RNAs 3 and 4 of MCMV and VTAV were used as templates for cDNA synthesis for Northern blot hybridization analysis of isolate Ra RNA 3. High background hybridization with the homologous RNAs 1 and 2, however, suggested that these may have contaminated the RNAs 3 and 4 preparations.

Fig 6.14

Hybridization analysis of isolate Ra using RNA 3 preparations from $T_1T_2M_3$ and $M_1M_2T_3$ as templates for cDNA synthesis.

By using these as templates for RNAs 3 and 4 sequences, it was expected that any contaminating RNAs 1 and 2 sequences would hybridize with the MCMV or VTAV RNAs from which they originated and thus their presence be verified. The results confirmed that the RNA 3 preparations were contaminated by RNAs 1 and 2. The failure of $M_1M_2T_3$ RNA 3 cDNA to hybridize with Ra RNA 3 meant that there were no detectable amounts of VTAV RNA 3 (3a gene) sequences present in that RNA segment as proposed.
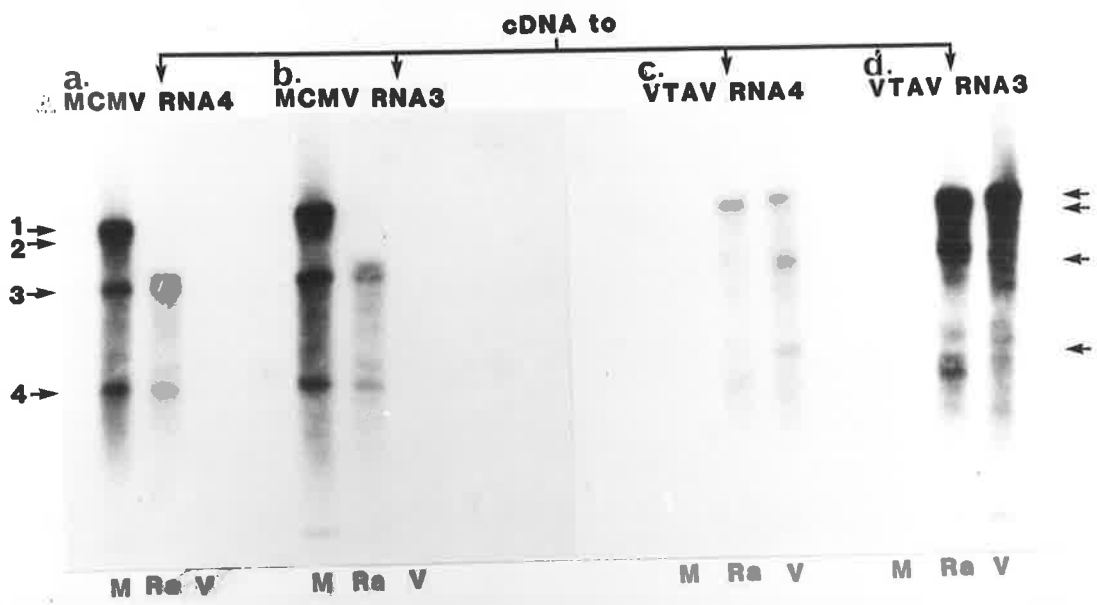
Fig 6.13

Fig 6.14

sequences (Rezaian et al.,1985). It was concluded that fragmentation products from the RNAs 1 and 2 may have contaminated the templates. Consequently, the possibility of Ra RNA 3 being contaminated with VTAV RNAs 1 and 2 sequences during electrophoresis could not be overlooked.

This conclusion was subsequently supported by experiments in which the RNAs 3 were derived from the pseudorecombinants, $M_1M_2T_3$ and $T_1T_2M_3$. Results of Northern blot hybridization analysis using RNA 3 from $M_1M_2T_3$ as template for the cDNA is presented in Fig 6.14b. The probe hybridized with VTAV RNAs 3 and 4 (Fig 6.14b, lane V), MCMV RNAs 1 and 2 (Fig 6.14b, lane M) but not with Ra (Fig 6.14b lane Ra). When RNA 3 from $T_1T_2M_3$ was used, it hybridized with MCMV RNAs 3 and 4 (Fig 6.14a, lane M), VTAV RNAs 1 and 2 (Fig 6.14a, lane V) and all the RNAs of Ra (Fig 6.14a, lane Ra).

The previous conclusion that Ra RNA 3 consisted of MCMV and VTAV RNAs 3 sequences and the proposed genomic structure could therefore not be validated. It was, however, necessary to determine the origin of the additional sequences responsible for the fact that under both denaturing and non-denaturing conditions Ra RNA 3 migrated slower in agarose gels than MCMV RNA 3.

6.6. RNA Protection Assay With Fny-CMV cDNA clones.

Owen and Palukaitis (1988), found extensive sequence homology between the RNAs 3 of several CMV isolates including Fny-CMV, but not with VTAV. These results suggested that cDNA clones of Fny-CMV RNA 3 may be useful in further characterization of Ra RNA 3, in particular, to determine the point of heterogeneity, if any. It was hoped that the RNA protection assays may show the presence of any extra nucleotides sequences responsible for that isolate's RNA 3 being slower migrating than that of MCMV. The sequence structures of the cDNA clones which were used are presented in Fig 6.15. Clone JO 103 was 1,800 nucleotides long, starting from the 3' terminus and hence encode the coat protein gene and all but 301 nucleotides of the 3a gene. Clone JO 104 was 1,600 long and JO 108 was 1,200 nucleotides long, both from the 3' terminus.
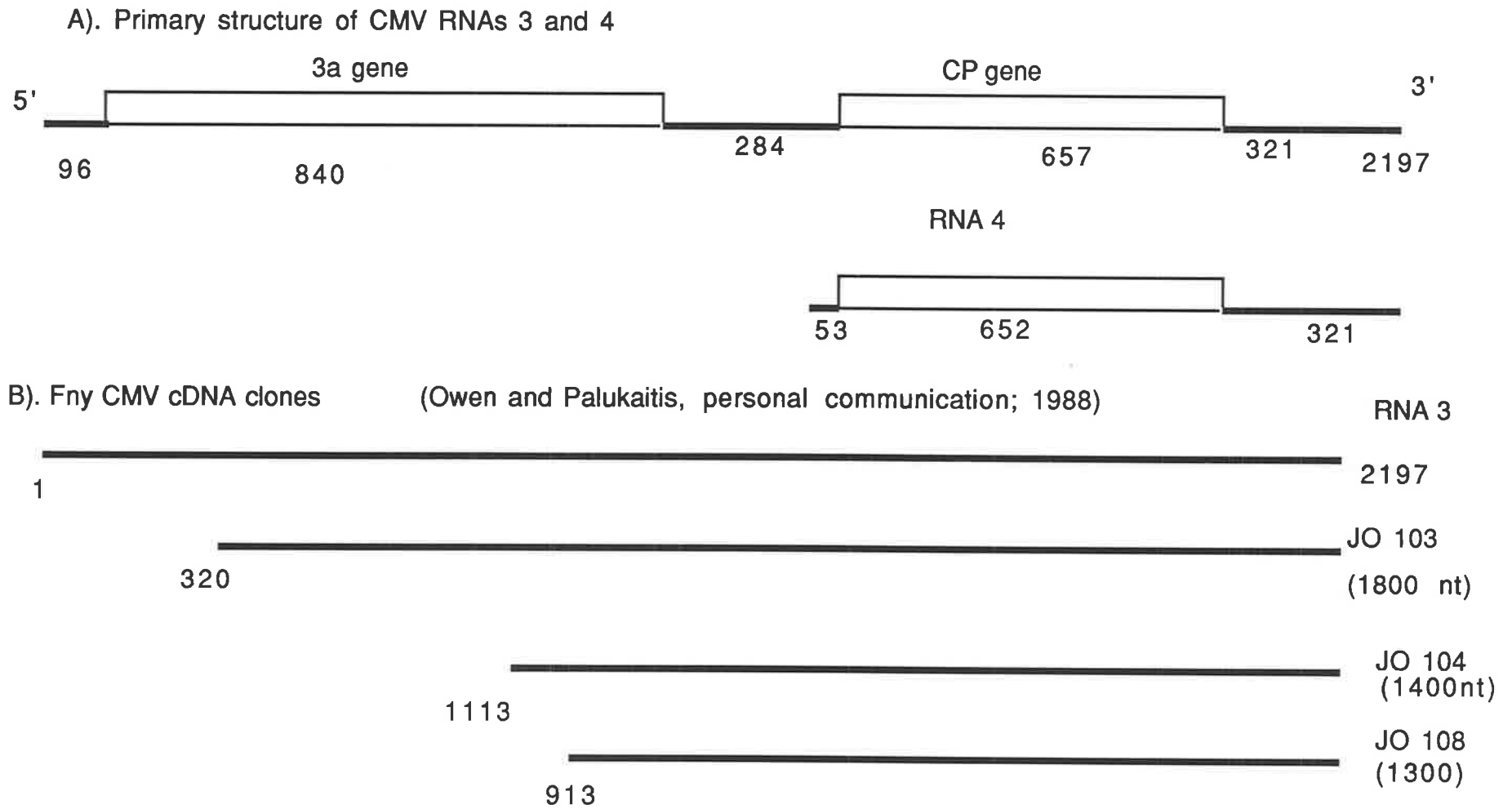
Fig 6.15 Primary Structure of Fny-CMV cDNA Clones.

6.6.1. *RNA Protection assay of Ra RNA 3*

RNA protection assay was done as described by Winter et al., (1985) and modified by Owen and Palukaitis, (1988). Results presented in Fig 6.16 were obtained by Dr R.I.B Francki and Dr Judith Owen and show a complex pattern of protected RNAs. Fig 6.16a shows that minus sense transcripts of clone JO 103 hybridized with the homologous RNA of Fny-CMV (Fig 6.16a lane 1), with MCMV (Fig 6.16a lane 2) and isolate Ra (Fig 6.16a lane 4) but not with VTAV (Fig 6.16a lane 3). Isolate Ra and Fny-CMV produced similar protected fragments which differed significantly from those of MCMV. Similar results were obtained when minus sense transcript of clone JO 104 (Fig 6.16b) and JO 108 (Fig 6.16c) were used. The MCMV culture used in these assays had been sent to the Dept of Plant Pathology, Cornell University from our laboratory two years previously. It is conceivable that this culture may have changed through mutation, and thus differ so significantly from isolate Ra which was derived from it.

From these results it was deduced that the 1800 nucleotides from the 3' terminus of Ra did not contain sequences homologous to VTAV and of significant size to affect the production of protected fragments similar to those by the homologous Fny-CMV. It was therefore concluded that both the MCMV coat protein gene and at least 60% of the 3a gene were encoded by the RNA 3 of isolate Ra.

6.7. CONCLUSION.

The variant Ra isolated by infecting *B. vulgaris* with extracts from plants coinfected with MCMV and VTAV was shown to consist of VTAV RNAs 1 and 2, and an RNA 3 which was initially believed to be a recombinant consisting of the coat protein gene of MCMV and the 3a gene of VTAV. Hybridization with a cDNA probe using the RNA 3 from the pseudorecombinant $M_1M_2T_3$ as template failed to confirm the earlier conclusion. The electrophoretic mobility of the RNA 3, when compared to those of MCMV and VTAV, however, suggests that it is of a higher molecular weight than MCMV. The RNA protection assay was considered a suitable test for heterogeneity between MCMV, VTAV and the isolate Ra. Using these tests, it was concluded that there were significant differences

Fig 6.16

Heterogeneity assay of Ra RNA 3.

RNA protection assay comparing homologous Fny-CMV RNA (lane 1), MCMV RNA (lane 2), VTAV RNA (lane 3) and Ra RNA (lane 4) were done using α–^{32}P-labeled transcripts of cDNA clones JO 103, 104 and 108 of Fny-CMV. Isolate Ra (lane 4) showed greater homology with Fny-CMV (lane 1) than with MCMV (lane 2)

a. JO 103   b. JO 104   c. JO 108
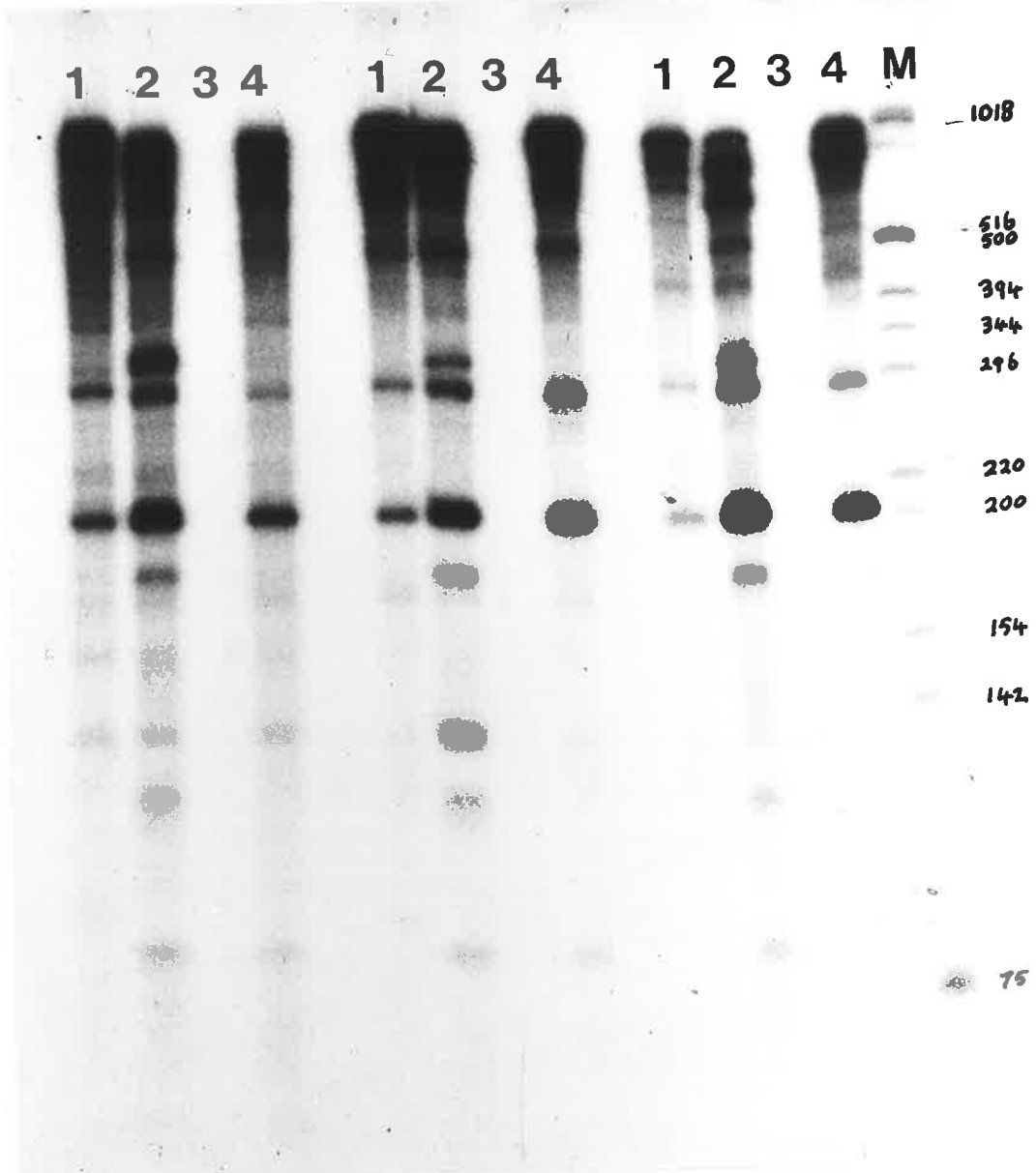
Fig 6.16

between MCMV and Ra which cannot be accounted for by point mutations. The origin of the extra nucleotide sequences responsible for the differences in size and electrophoretic mobility can only be determined by sequencing the RNA to determine its primary structure.

CHAPTER SEVEN

APHID TRANSMISSIBILITY AS A MECHANISM FOR ISOLATING
VARIANTS FROM MIXED INFECTIONS OF MCMV AND VTAV

7.0. Introduction.

In this section, the aphid transmissibility of VTAV but not MCMV was used as a
biological method of isolating variants based on their coat protein characteristics. The
method also tested the incidence of transcapsidation in mixed infections of the two viruses.

7.1. Preliminary Experiments on Aphid transmission from leaf tissues coinfected with MCMV and VTAV.

VTAV was passaged over several generations by aphids to enhance its aphid
transmissibility by Mr B. Chen, Dept of Plant Pathology, Waite Agricultural Research
Institute. With due regard to the relative virulence of MCMV and VTAV described in
Chapter 4, two leaves from *N. clevelandii* plants infected with VTAV were combined with
one similar sized leaf from a plant infected with MCMV. The leaves were extracted in water
and used to mechanically inoculate *N. clevelandii* plants. Twelve days after inoculation leaf
samples were taken from the plants and analysed by ELISA for the antigens present. Three
of the six inoculated plants were found to contain both antigens, and each was used as a
source of virus for aphid transmission. *N. clevelandii* plants were inoculated, using five
aphids per plant, and eight plants were inoculated using aphids from each of three plants
infected by both viruses. Controls involved aphid transmissions from plants infected with
MCMV only, and with VTAV only.

Four out of the 24 plants inoculated by aphids allowed to probe mixedly infected plants
became infected and virus isolates from these were designated isolates MVapA1-4. The
remaining plants showed no symptoms of infection after 16 days and were discarded. Leaf
samples from the four infected plants were analysed by ELISA for the antigens present.
Total RNA extracts from the leaf samples were prepared for analysis by dot-blot
hybridization. Leaf extracts from the infected plants were used to inoculate further plants

from which virus was purified by method V. RNA extracts from the purified virus preparations were analysed by dot-blot and Northern hybridization.

Results presented in Table 7.1 show that all the four isolates contained only VTAV antigens, and induced VTAV-like symptoms in *N. clevelandii*. Results of dot-blot hybridization analysis presented in Fig 7.1 and summarized in Table 7.1 show that total RNA preparations (F1) from two of the four infected plants, isolates A3 and A4 (Fig 7.1a and b, spots 3 and 4) hybridized with both MCMV and VTAV cDNA probes. After one passage through *N. clevelandii* (F2), however, none of the isolates hybridized significantly with the MCMV cDNA probe (Fig 7.1a, F2), but all four hybridized with the VTAV cDNA probe (Fig 7.1b F2).

Results of Northern blot hybridization analysis presented in Fig 7.2 show that the RNA 3 of one of the isolates, MVapA3, hybridized with both the MCMV and VTAV probes (Fig 7.2a and b lane A3). It was observed that there was no RNA 4 present, and as no MCMV antigen was present, the origin of the MCMV sequences i.e. whether from RNA1, or 2 (degradation products) or RNA 3 remained obscure. Isolate MVapA3 was also characterised by poor infectivity and low virus yields. Isolates MVapA1, A2 and A4 hybridized with only the VTAV cDNA probe and were therefore concluded to be VTAV isolates.

Of the 24 plants inoculated with aphids from plants infected by VTAV alone, 3 became infected. However, none of the 24 plants inoculated with aphids from plants infected with MCMV alone were infected. To confirm that *M persicae* was unable to transmit MCMV, a further 56 plants were each inoculated with 15 aphids. None of them became infected.

In a second similar experiment also with 5 aphids per plant, three out of 100 inoculated *N. clevelandii* became infected. Results presented in Table 7.2 show that when leaf extracts from the three isolates designated MVap B1, B2 and B3 were analysed by ELISA, only VTAV antigens were detected. All the three isolates induced VTAV-like symptoms.

Total leaf RNA extracts were analysed by dot-blot hybridization analysis. Results presented in Fig 7.3a and b and summarized in Table 7.2 show that isolates MVapB1 and B2 hybridized with both MCMV and VTAV cDNA probes (Fig 7.3a and b, spots 1 and 2), but that isolate MVapB3 hybridized only with the VTAV cDNA probe (Fig 7.3a and b, spot 3).

Table 7.1.
Aphid Transmission Experiment A: Biological Characterization of Isolates MVapA1, A2, A3 and A4

Isolate MVapA	Symptoms induced	Antigens detected[1]	RNA sequences detected[2]
1	VTAV-like	VTAV only	VTAV only
2	VTAV-like	VTAV only	VTAV only
3	VTAV-like	VTAV only	VTAV+MCMV
4	VTAV-like	VTAV only	VTAV+MCMV

[1]Antigens present were detected by ELISA

[2]RNA sequences present in total leaf RNAs were detected by dot-blot hybridization analysis.

Table 7.2

Aphid transmission experiment B: biological characterization of isolates MVapB1, 2 and 3.

Isolate MVapB	Symptoms induced	Antigens detected[1]	RNA sequences detected[2]
1	VTAV-like	VTAV only	MCMV+VTAV
2	VTAV-like	VTAV only	MCMV+VTAV
3	VTAV-like	VTAV only	VTAV only

[1]Antigens present were detected by ELISA
[2]RNA sequences present were determined by dot blot hybridization analysis.

Fig 7.1

Virus-specific RNA composition of aphid transmitted isolates MVapA1-4.

Total infected leaf RNA extracts (F1) and viral RNAs obtained after a single passage by mechanical inoculation of *N. clevelandii* plants (F2) were analysed by dot-blot hybridization. Total leaf RNA extracts were used at 50ng per spot and RNA from purified virus was at 20ng per spot. MCMV and VTAV positive controls were used at the concentrations shown in the central panel. Isolates MVap A1 (spot 1) and A2 (spot 2) hybridized with only the VTAV probe. Isolate MVapA3 and A4 (spots 3 and 4) hybridized with both MCMV(panel a) and VTAV (panel b) probes when the total leaf RNAs were used (F1) but not when the viral RNAs were used (F2).

Fig 7.2

Northern hybridization analysis of aphid transmitted isolates MVapA1-4.

RNA extracts of virus purified from *N. clevelandii* plants inoculated with leaf extracts from aphid transmitted plants were used. Isolates MVapA1, A2, and A4 (lanes A1, A2 and A4) hybridized with only the VTAV cDNA probe (panel b). All the RNAs of isolate MVapA3 (lane A3) hybridized with the VTAV cDNA probe. However, the MCMV probe also hybridized with the RNA 3 of isolate MVapA3 (lane A3, panel a) The origin and possible significance of the uncharacteristic hybridization between RNAs 3 and 4 remained uncertain.

a. MCMV cDNA

	1	1		0.02ng
	2	2		0.2ng
	3	3		2ng
	4	4		20ng
MCMV	F1	F2	VTAV	

MVapA

M   F1   F2   V

b. VTAV cDNA

M   F1   F2   V

Fig 7.1

a. MCMV cDNA   b. VTAV cDNA

M A1 A2 A3 A4 V   M A1 A2 A3 A4 V

Fig 7.2

Leaf extracts from infected plants were used to inoculate further plants. Virus was purified from these by method V, and RNA extracts from the purified virus preparations were analysed by dot-blot and Northern hybridization.

Results of dot-blot hybridization analysis of RNA from purified virus presented in Fig 7.3c show that the MCMV cDNA probe did not hybridize with any of the isolates. The three isolates, however, hybridized with the VTAV probe (Fig 7.3d). Results of Northern hybridization analysis of isolates MVapB1-3 presented in Fig 7.4 also show that the MCMV cDNA probe did not hybridize any of the three isolates (Fig 7.4 a lanes B1 to B3). The VTAV probe on the other hand, hybridized with all the RNAs of the three isolates (Fig 7.4b, lanes B1-B3).

It appears from the results that MCMV RNA sequences were transcapsidated in VTAV coat protein and thus transmitted by the aphids. However, after one passage by mechanical inoculation, the MCMV sequences in all but one of the seven isolates (isolate MVapA3) were lost. Further work on the MCMV RNA sequences apparently present in plants infected by the aphids required RNA from purified virus preparations. This was, however, precluded because the MCMV sequences were eliminated during a single passage of mechanical transmission. This suggests that the aphids transmitted the complete VTAV genome in addition to particles containing MCMV RNA segments or parts thereof.

7.2. Single aphid transmissions from Plants Infected With MCMV and VTAV.

Based on the above results, it was considered that the use of single aphids might reduce the extent of heterogeneity of the virus populations transmitted. By using single aphids and presumably reducing the number of particles initiating infection, it was expected that more homogeneous virus isolates would be obtained.

The experimental procedure was as previously described, except that one aphid was used to inoculate each plant. Three such independent experiments were done (experiments MVapC, D and E). Method M was used to purify those isolates in which MCMV antigens only, or both MCMV and VTAV antigens were detected, and Method V for those in which

only VTAV antigens were detected. RNA extracted from purified virus was further analyzed by Northern blot hybridization.

7.2.1. *Characterization of isolates MVap C1-9.*

In experiment MVap C, nine of the 100 plants inoculated became infected. Results of analysis of leaf extracts of the nine isolates are presented in Table 7.3. Eight of the nine isolates (designated virus isolates MVap C1-8) showed VTAV-like symptoms, and only VTAV antigens were detected when extracts of leaf tissues from these were analysed by ELISA. One plant showed MCMV-like symptoms, and only MCMV antigens were detected.

Results of dot-blot hybridization analysis of total leaf RNA extracts presented in Fig 7.5a and b and summarized in Table 7.3 show that 4 of the nine isolates, MVApC2,C3 C6 and C9 contained both MCMV and VTAV RNA sequences (Fig 7.5a and b spots 2, 3, 6 and.9). Five isolates, MVap C1, C4, C5, C7 and C8 contained only VTAV RNA sequences.

Isolate MVapC6 which hybridized with both MCMV and VTAV cDNA probes was lost during the attempt to passage it for virus purification. Results of Northern hybridization analysis of RNA from virus preparations obtained after one mechanical passage of the remaining eight isolates are presented in Fig 7.6. The MCMV cDNA probe hybridized with all the four RNAs of isolate MVapC9 (Fig 7.6a lane C9) but to none of the RNA segments of the other isolates. When VTAV cDNA was used to analyse the isolates, the RNAs of all except isolate MVapC9 hybridized with that probe (Fig 7.6b, lanes C1-5, C7 and C8). These were thought to be VTAV isolates. However, it was concluded that isolate MVapC9 was a variant of MCMV which, unlike the parental strain, was aphid transmissible. The aphid transmissibility of isolate MVapC9 was confirmed by three subsequent aphid transmission passages using single aphids, resulting in 1/20, 1/20 and 1/25 infected out of total numbers of plants inoculated.

7.2.2. *Characterization of Isolates MVapD1-3.*

In experiment MVapD, three of the 100 plants became infected. All three isolates, designated MVapD1-3 showed MCMV-like symptoms, and results of serological tests

Table 7.3.
Aphid transmission experiment C: characterization of isolates MVapC1-9

Isolate MVapC	Symptoms induced	Antigens detected[1]	RNA sequences detected[2]
1	VTAV-like	VTAV only	VTAV only
2	VTAV-like	VTAV only	MCMV+VTAV
3	VTAV-like	VTAV only	MCMV + VTAV
4	VTAV-like	VTAV only	VTAV only
5	VTAV-like	VTAV only	VTAV only
6	VTAV-like	VTAV only	MCMV + VTAV
7	VTAV-like	VTAV only	VTAV only
8	VTAV-like	VTAV only	VTAV only
9	MCMV-like	MCMV only	MCMV+VTAV

[1]Antigens present were detected by ELISA.

[2]RNA sequences present were detected by dot blot hybridization analysis.

Fig 7.3

Virus specific RNA composition of aphid transmitted isolates MVapB1-3.

Total leaf RNA extracts (F1) and viral RNA extracts obtained after a single passage by mechanical inoculation of *N. clevelandii* plants (F2) were analysed by dot-blot hybridization using MCMV and VTAV cDNA probes. Isolates MVapB1 and B2 total leaf extracts hybridized with both MCMV (a) and VTAV (b) cDNA probes (spots B1 and B2 panels a and b) while MVapB3 hybridized with only the VTAV probe (spot B3 panel b). After a single passage through *N. clevelandii*, all three isolates hybridized with VTAV (spots B1, B2 and B3 panel d) but not the MCMV probe (panel c). MCMV and VTAV positive controls were at 20ng, 2ng 0.2 ng and 0.02ng per spot as shown on central panel.

Fig 7.4

RNA composition of isolates MVapB1-3

RNA extracts of virus purified from *N. clevelandii* plants inoculated with leaf extracts from plants inoculated with virus transmitted by aphids were used in Northern blot hybridization analysis. All three isolates (lanes B1, B2 and B3) hybridized with the VTAV probe (panel b) but not the MCMV probe (panel a).
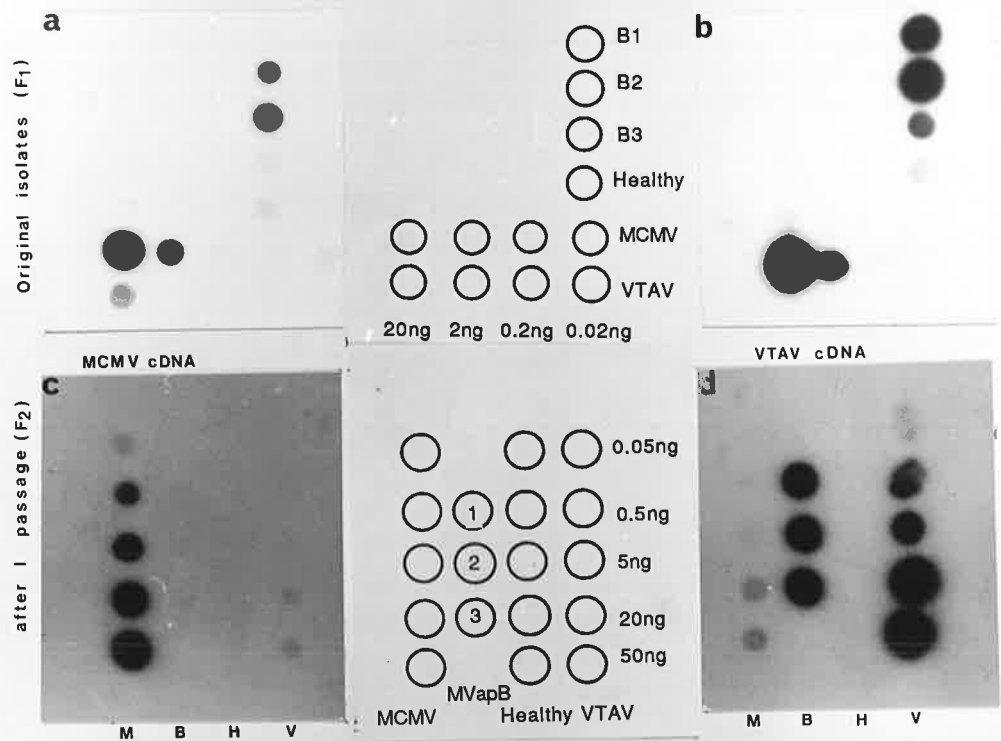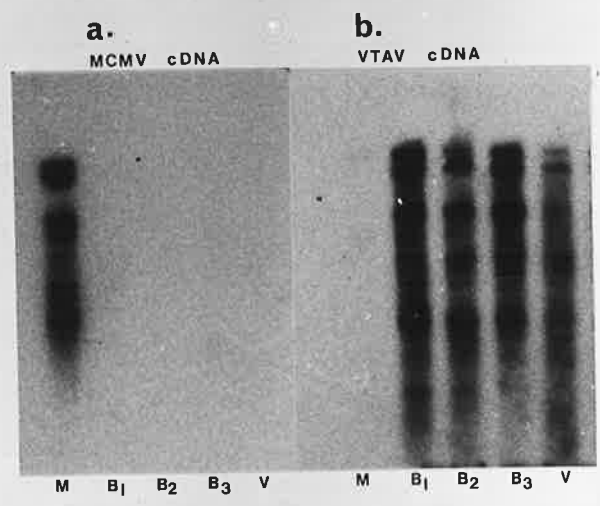
Fig 7.3

Fig 7.4

presented in Table 7.4 show that only MCMV antigens were detected in plants infected by all three isolates . Dot-blot hybridization results presented in Fig 7.7, and summarized in Table 7.4 show that isolate MVapD1 hybridized with both MCMV and VTAV cDNA probes, though more strongly with the former than the latter. Isolate MVapD2 and D3 hybridized with only the MCMV cDNA probe. However, unlike isolate MVapC9, I was able to transmit only one of these isolates (MVapD1) by aphids, as shown in Table 7.5.

Results of Northern hybridization analysis of RNA from virus preparations obtained after one mechanical passage of the isolates presented in Fig 7.8a show that all the three isolates hybridized with the MCMV cDNA probe (Fig 7.8a lanes D1-D3), but not with the VTAV probe (Fig 7.8b).

The symptoms induced by isolates MVapC9, D1, D2 and D3 were compared in different host species. Results presented in Table 7.6 show that all the four isolates induced MCMV-like symptoms in all the host species tested. They also infected *Cucumis sativus* systemically. Thus, the aphid transmissibilty of isolates MVapC9 and D1 did not result in changes in the biological properties tested.

7.2.3. *Characterization of isolates MVapE1-10.*

In experiment MVapE, 10 out of 119 plants inoculated, each with a single aphid, became infected. Results presented in Table 7.7 show that nine of the isolates, designated MVapE1-9, induced VTAV-like symptoms, and one (isolate MVapE10) induced MCMV-like symptoms. Serological analysis of leaf extracts showed two isolates (MVapE7 and E10) contained only MCMV antigens, and the rest, VTAV antigens. Results of dot-blot hybridization analysis of total leaf RNA presented in Fig 7.9 and summarized in Table 7.7 show seven isolates hybridized to only VTAV cDNA probe and 3 isolates (MVap E1, E8 and E10), hybridized with both MCMV and VTAV probes.

One of the aphid transmitted isolates in this experiment, isolate MVapE10 was lost in the attempt to passage it for virus purification. This isolate had only MCMV antigen and induced MCMV-like symptoms in *N. clevelandii*. The other nine isolates were successfully passaged and RNA obtained from purified virus preparations used for further analysis.

Fig 7.5

Virus specific RNA composition of aphid transmitted virus isolates MVapC1-9.

Total leaf RNA extracts were analysed by dot-blot hybridization. Isolates MVapC2, C3, C6 and C9 (spots 2, 3, 6, and 9) hybridised with both MCMV (panel a) and VTAV (panel b) cDNA probes. Isolates MVapC1, C4, C5, C7 and C8 (spots 1, 4, 5, 7 and 8) hybridized with only the VTAV probe (panel b). MCMV and VTAV positive controls were at the amounts indicated in the central panel.

Fig 7.6

RNA composition of aphid transmitted isolates MVapC1-5 and C7-9.

RNA extracts of virus purified from *N. clevelandii* plants inoculated with leaf extracts from plants inoculated with virus transmitted by aphids were used in Northern blot hybridization analysis. All the isolates MVapC1-5, C7 and C8 (lanes C1-5, C7 and C8) hybridized with only the VTAV cDNA probe (panel b). Isolate MVapC9 (lane C9) hybridized with only the MCMV probe (panel b). Isolate MVapC6 was lost during the attempt to propagate it by mechanical inoculation of *N. clevelandii*. The origin and possible significance of the uncharacteristic hybridization between RNAs 3 and 4 of isolates MVapC1-5, C7 and C8, but not C9 remained uncertain.
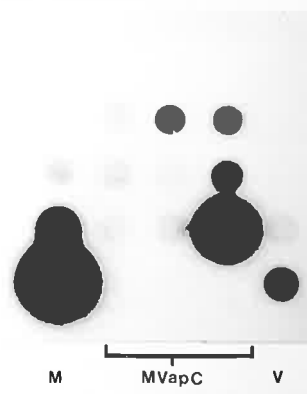
a. MCMV cDNA

b. VTAV cDNA
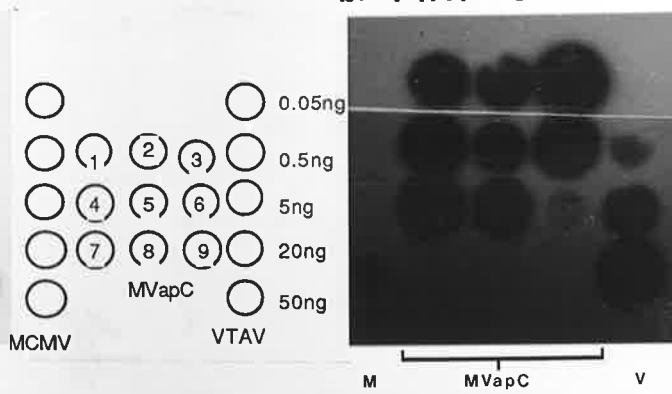
Fig 7.5

a.
MCMV cDNA

b.
VTAV cDNA

Fig 7.6

Table 7.4.
Aphid Transmission Experiment D: Characterization of Isolates MVapD1-3.

Isolate MVapD	Symptoms induced	Antigens detected[1]	RNA sequences detected[2]
1	MCMV-like	MCMV only	MCMV+VTAV
2	MCMV-like	MCMV only	MCMV+VTAV
3	MCMV-like	MCMV only	MCMV (+VTAV?)

[1]Antigens present were detected by ELISA
[2]RNA sequences present were detected by dot blot hybrydization analysis using MCMV and VTAV cDNA probes.

Table 7.5.
Aphid Transmission Characteristics of Isolates MVapD1, D2 and D3.

Isolate MVapD	Infection Rate[1]	
	Experiment 1[2]	Experiment 2[3]
1	0/10	1/56
2	0/10	0/52
3	0/10	0/54

[1]Infection rate defined as the number of plants infected (numerator) out of total inoculated (denominator).

[2]In experiment 1, each plant was inoculated with 15 aphids.

[3]In experiment 2, each plant was inoculated with 4 aphids.

Fig 7.7

Virus specific RNA composition of aphid transmitted isolates MVapD1-3.

Total leaf RNA extracts were analysed by dot-blot hybridization. Isolates MVapD1 (spot 1) hybridized with both MCMV (panel a) and VTAV (panel b) cDNA probes. However, isolates MVapD2 and D3 (spots 2 and 3) hybridized with only the MCMV probe (panel a). MCMV and VTAV positive controls were at the amounts indicated per spot.

Fig 7.8

RNA composition of virus isolates MVapD1-3.

RNA extracts of virus purified from *N. clevelandii* plants inoculated with leaf extracts from plants inoculated with virus transmitted by aphids were used in Northern blot hybridization analysis. All three isolates (lanes D1, D2 and D3) consisted of RNAs with electrophoretic migration similar to MCMV and hybridised with the MCMV (panel a) but not the VTAV (panel b) cDNA probe.

a. MCMV cDNA

h. VTAV cDNA

◯		◯	◯ 0.05ng
◯	①	◯	◯ 0.5ng
◯	②	◯	◯ 5ng
◯	③	◯	◯ 20ng
◯	MVapD	◯	◯ 50ng

MCMV   Healthy   VTAV

M   D   H   V

M   D   H   V

Fig 7.7

a. MCMV cDNA   **b.** VTAV cDNA

M   D₁   D₂   D₃   V   M   D₁   D₂   D₃   V
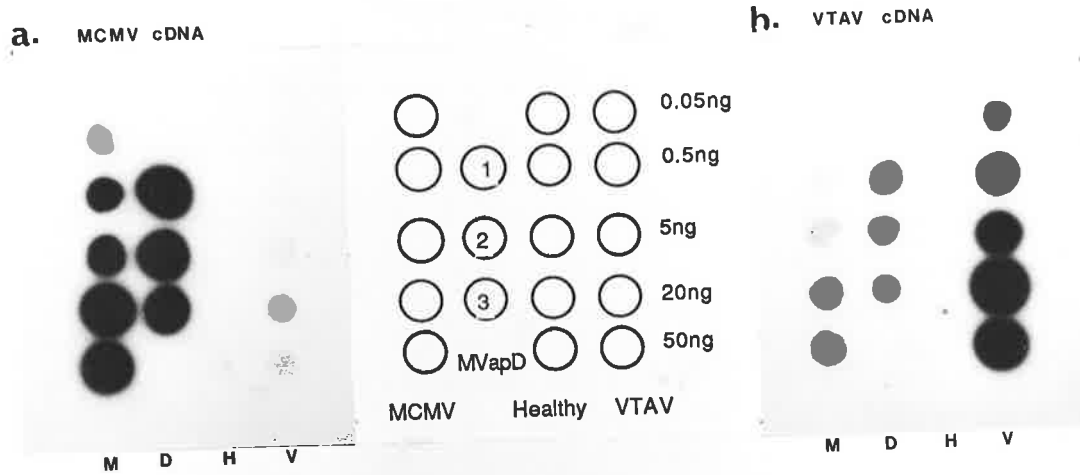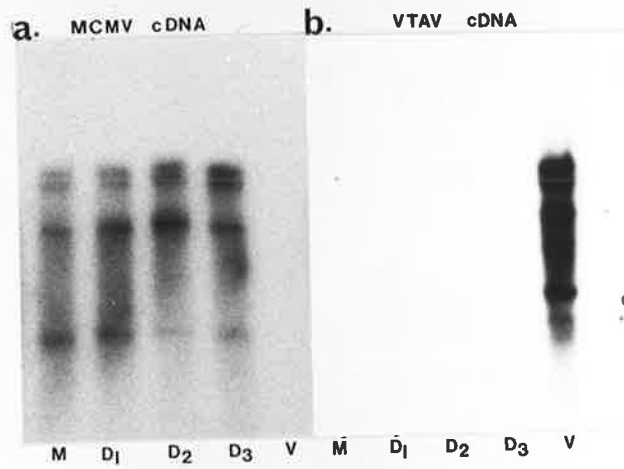
Fig 7.8

Table 7.6.

Effect of isolates MVapC9, D1, D2 and D3 on a number of plant hosts.

Plant species tested	MCMV	Symptoms induced[1] by VTAV	MVapC1	MVapD1	MVapD2	MVapD3
N. glutinosa	Systemic yellow mosaic	Systemic mosaic and leaf distortion	MCMV-like	MCMV-like	MCMV-like	MCMV-like
N. clevelandii	Systemic yellow mosaic	Syatemic mosaic and leaf distortion	MCMV-like	MCMV-like	MCMV-like	MCMV-like
C. sativus	Chlorotic local lesions on inoculated leaves and systemic mosaic	Not infected[2]	MCMV-like	MCMV-like	MCMV-like	MCMV-like
G. globosa	Chlorotic local lesions on inoculated leaves and severe systemic yellow mosaic	Not infected[2]	MCMV-like	MCMV-like	MCMV-like	MCMV-like
B. vulgaris	Chlorotic local no systemic invasion.	Purple local no systemic invasion.	MCMV-like	MCMV-like	MCMV-like	MCMV-like

[1] Symptoms induced were recorded 12-14 days after inoculation.
[2] Systemic leaf extracts were analysed by ELISA to determine if there were any antigens present.

Table 7.7.

Aphid transmission experiment E: characterization   of isolates MVapE1-9.

Isolate MVapE	Symptoms induced	Antigens detected[1]	RNA sequences detected[2]
1	VTAV-like	VTAV only	MCMV+VTAV
2	VTAV-like	VTAV only	VTAV only
3	VTAV-like	VTAV only	VTAV only
4	VTAV-like	VTAV only	VTAV only
5	VTAV-like	VTAV only	VTAV only
6	VTAV-like	VTAV only	VTAV only
7	VTAV-like	MCMV(+VTAV?)[3]	MCMV+VTAV
8	VTAV-like	VTAV only	VTAV only
9	VTAV-like	VTAV only	VTAV only
10	MCMV-like	MCMV only	MCMV+VTAV

[1]Antigens present were detected by ELISA.

[2]RNA sequences present were detected by dot blot hybridization analysis using MCMV and VTAV cDNA probes.

[3]Even though there was no VTAV antigen was detected in extracts of the plant infected by the aphid, subsequent passaging revealed the presence of VTAV antigen. After a single passage by mechanical inoculation, no MCMV antigen was detected, and when the RNA extract was analysed, no MCMV RNA 3 was present even when method M was used to purify the virus isolate.

Fig 7.9

Virus specific RNA compositon of aphid transmitted isolates MVapE1-10.

Total leaf RNA extracts were analysed by dot-blot hybridization. Isolates MVapE1, E8 and E10 (spots 1, 8 and 10) hybridized with both MCMV (panel a) and VTAV (panel b) cDNA probes. The remaining seven isolates hybridized with only the VTAV probe (spots 2, 3, 4, 5, 6, 7 and 9, panel b). Uninfected leaf total RNA extracts were at 100, 40, 10, 1 and 0.1 ng, while MCMV and VTAV positive controls were at 50, 20, 5, 0.5 and 0.05ng per spot.

Fig 7.10

RNA composition of aphid transmitted virus isolates MVapE1-9.

RNA extracts of virus purified from *N. clevelandii* plants inoculated with leaf extracts from plants inoculated with virus transmitted by aphids were used in Northern blot hybridization analysis. Isolate MVapE10 was lost during the attempt to propagate it in *N. clevelandii* - after using all the available leaf material no infection of the inoculated plants occured. The remaining nine isolates all hybridized with the VTAV (panel b) but not the MCMV (panel a) probe.The origin and possible significance of the uncharacteristic hybridi-zation between RNAs 3 and 4 remained unknown.

a. MCMV cDNA

b. VTAV cDNA

MCMV   MVapE   Healthy VTAV

M   MVapE   H   V   M   MVapE   H   V

Fig 7.9

a.
MCMV cDNA

M E_1 E_2 E_3 E_4 E_5 E_6 E_7 E_8 E_9 V

b.
VTAV cDNA

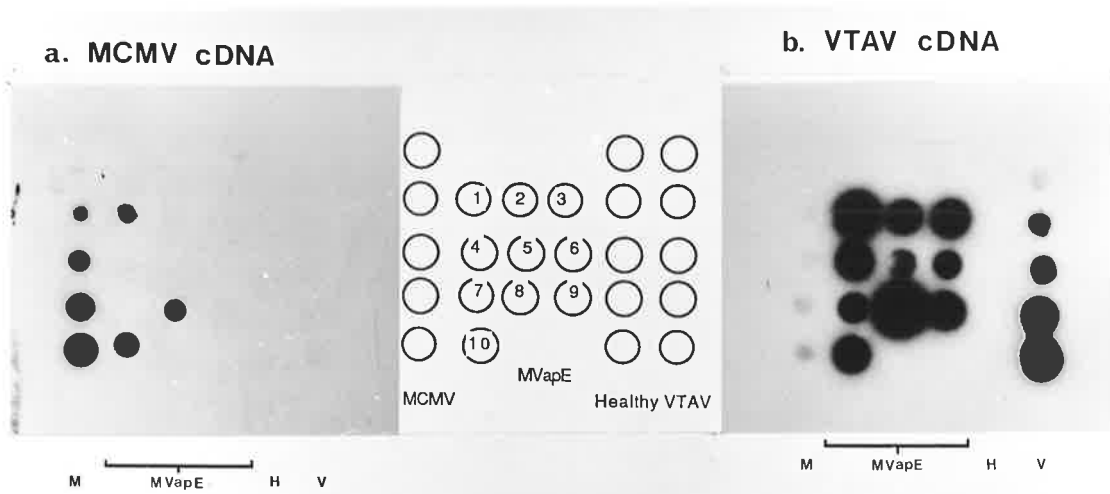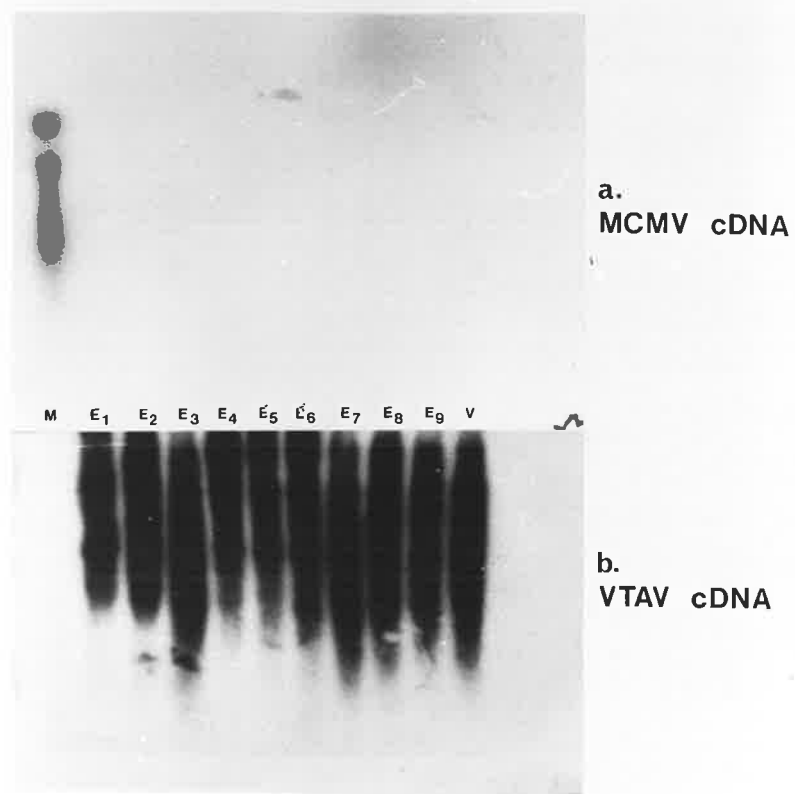**Fig 7.10**

Results of Northern hybridization analysis presented in Fig 7.10a and b for MCMV and VTAV cDNA probes respectively show that all the isolates hybridized with VTAV cDNA probe, but not the MCMV probe. It was concluded that 9 of the 10 isolates obtained in this experiment were VTAV isolates.

7.4. Conclusions.

In the first two experiments described, four out of the seven infected plants contained both MCMV and VTAV RNA sequences. The attempt to passage these isolates so that RNA obtained from purified virus could be further characterized resulted in the loss of the MCMV sequences in most of them. In one isolate, MVapA1, the MCMV RNA persisted but though its migration was like that of the RNA 3 of MCMV, the absence of an RNA 4 made its characterization inconclusive since it could very well have been fragmentation product of MCMV RNA 1 or 2.

Thus, it seems that both MCMV and VTAV RNA sequences were transmitted by the aphids. However, the MCMV RNAs were lost during passaging. It is possible that the dot-blot hybridization analysis on which these conclusions are based may be less specific than expected, and that some of the reactions may only have been high background activities. It is worth noting, however, that neither of the cDNA probes hybridized with the total RNAs from uninfected host plants, and that heterologous activity was only at the level of 50ng of RNA per spot, if any. The confirmed presence of MCMV RNA sequences by Northern blot might suggest that the results of the dot-blots could very well be a true representation of the viral RNAs present in the infected plants.

In the last three experiments, ten out of 22 isolates obtained contained both MCMV and VTAV RNA sequences. In most of these isolates the MCMV RNAs were subsequently eliminated during passaging by mechanical inoculation. Two isolates, MVapC9 and MVapD1 appear to be mutant strains of MCMV which are aphid transmissible. The aphid transmissibility of these two isolates did not appear to have changed any of the other biological or biophysical properties tested. Two other isolates, MVapD2 and D3 appear to have been the result of transcapsidation of the complete MCMV genome.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS.

8.1. MCMV and VTAV as a Model for Studying Interactions Between RNA Plant Viruses

The choice of MCMV and VTAV for studying mixed infections of RNA plant viruses was based primarily on the differences in the symptoms induced by the two viruses and the aphid transmission of VTAV but not MCMV. A third consideration was the finding by Rao and Francki (1981) that only the RNA 3 of CMV and TAV can be exchanged to produce viable pseudorecombinants. This limited compatibility, it was thought, would reduce the number of different viable reassortants and recombinants, and allow for ease of characterization.

Results of experiments reported in this thesis show that MCMV and VTAV do not cross-protect against each other if the primary and challenge inoculations are within three days. The two viruses not only coinfect a variety of common host species, but such infections persist during passage by mechanical inoculation. These results thus represent the first report of coinfections of CMV and TAV. It was also shown that MCMV appeared to replicate, multiply and migrate systemically slightly faster than VTAV. However, the impression that MCMV also dominated symptom expression may be due to the more dramatic nature of the yellow systemic mosaic symptoms induced by that virus.

My experiments also showed that in mixed infections of MCMV and VTAV and their pseudorecombinants, i.e. MCMV + $T_1T_2M_3$ and VTAV + $M_1M_2T_3$, the heterologous RNAs 1 and 2 replicated, multiplied and were encapsidated along with the homologous RNAs 1 and 2. These results support the note of caution sounded by Dodds et al., (1985) that many studies of cross-protection do not address the question of latent replication and multiplication of the challenge virus.

The biophysical and biological differences between MCMV and VTAV were used as the basis for methods developed for their independent detection and analysis in mixed

infections. Specific antisera to the two viruses were used in ELISA to independently detect each antigen in mixed infections. Differences in symptomatology were used in limited characterization of virus isolates derived from mixed infections. The absence of significant levels of nucleotide sequence homology together with the differences in electrophoretic mobility of their RNA segments were utilised in dot-blot and Northern blot hybridization analysis. By these methods, it was possible to determine the virus-specific RNA compositon of mixed infections of the two viruses. During attempts to determine the finer details of the RNA structure of various isolates it became obvious that these methods were inadequate. It is possible that the use of cDNA clones of MCMV and VTAV RNAs as templates for the synthesis of cDNA probes would have provided better tools for more detailed analysis. Such probes with known sequences could have been used in RNA protection assays which would not only have provided information on the extent of homology but also the point of heterogeneity, if any. Some of the variants, however, possessed characteristics which could only have been resolved conclusively by nucleotide sequencing of their RNAs.

8.2. Isolation of variants.

Based on the observed differences between MCMV and VTAV, three methods were developed for use in attempts to isolate variants from mixed infections.

The first method was based on the differential effects of the virus purification methods. The VTAV method of purification was effective in removing practically all virus particles encapsidated in MCMV coat protein. The MCMV method on the other hand was not effective in removing all particles encapsidated in VTAV coat protein. These methods were therefore supplemented with precipitation with specific antisera which successfully removed all traces of the contaminating virus.

In general, the method was considered to be of limited value. However, using virus preparations from mixedly infected leaf tissue, a virus isolate was obtained which by its RNA compositon provided evidence that transcapsidation may have occurred in these infections.

The second method devised for isolating variants was based on the morphological differences observed in the lesions induced by MCMV and VTAV in *Beta vulgaris*. The main disadvantage of this method was that the plant species used was more susceptible to infection by MCMV than by VTAV. Consequently, VTAV induced fewer local lesions and infectious virus could not be recovered from most of them. Rao (1982) and Marchoux et al., (1974a, 1974b) showed that lesion types produced by cucumoviruses in several plants were controlled by the RNA 3 alone or in combination with the RNA 2. It is possible that the greater susceptibility of this host plant to MCMV may have been responsible for the fact that all the virus isolates obtained from local lesions in *B. vulgaris* possessed MCMV RNA 3. However, the attempts to find host species equally susceptible to both viruses, or more susceptible to VTAV than to MCMV were unsuccessful.

The third method developed was based on the the aphid transmissibility of VTAV but not MCMV. Using the aphid *M. persicae*, attempts were made to selectively transmit virus and virus variants encapsidated in VTAV coat protein from coinfected tissue. Of the three methods devised, this was the one of most relevance to the behaviour of the viruses as in nature their transmission is dependent on insect vectors.

8.3. Variants obtained from coinfected leaf tissues.

Using a combination of the differential effects of virus purification methods and differences in the appearance of lesions induced, five virus isolates were obtained, three of which were characterised in some detail. Isolates MVLB1 and B2 contained only MCMV antigens, and all of their RNAs hybridised with MCMV cDNA probe, and like MCMV, systemically infected *C. sativus*. However, both isolates induced VTAV-like symptoms in *Nicotiana* species. It was further shown that after two passages by mechanical inoculation through *C. sativus*, both isolates became MCMV-like in the symptoms induced in *Nicotiana* species, even though no such changes had occurred through more than three years of mechanical passaging in that plant. Northern blot hybridization analysis using both denatured and non-denatured RNAs, however, failed to detect any VTAV RNA segments in the two isolates, and VTAV RNA sequences were detected only by dot-blot hybridization analysis prior to passage through *C. sativus*. The results suggested that in addition to

whichever sequences were responsible for the VTAV-like symptoms induced, the complete MCMV genome was present, and that passaging through *C. sativus* resulted in the filtering-out of the "VTAV-like" RNA sequences and loss of the uncharacteristic symptoms in *N. glutinosa.*. Alternately, these isolates may represent mutants which when passaged through *C. sativus* reverted to typical MCMV. Further analysis of these isolates by more refined methods may show the location of the RNA sequences responsible for the severe yellow chlorosis induced by MCMV in *Nicotiana* species. RNA protection assays with cDNA clones of MCMV and VTAV RNAs 1 and 2 will give information as to the location of the site(s) of heterogeneity, the primary base sequence of which can then be determined.

Another isolate obtained by these methods, isolate VMLB1, was shown to consist of VTAV RNAs 1 and 2 and MCMV RNA 3. This isolate represents the first report of an *in vivo* pseudorecombinant between two members of a virus group which showed less than 5% RNA sequence homology (Gonda and Symons, 1978), and very remote serological relationship (Rao et al., 1982). It is suggested that isolate VMLB1 arose from transcapsidated MCMV RNA 3 present in such low concentration that it was not detected in virus preparation V in the experiments described in Chapter 5. When *B. vulgaris* plants were inoculated with this virus preparation, the MCMV RNA 3, more competitive that its VTAV counterpart, replicated and multiplied and competitively encapsided VTAV RNAs 1 and 2. The fact that the plant species used was more susceptible to MCMV than VTAV, would have placed the MCMV RNA 3 at a further advantage. Isolate VMLB1 was further characterised by its ability to systemically infect *Gomphrena globosa*, a plant species which is not susceptible to VTAV or the pseudorecombinant $T_1T_2M_3$. Using more refined methods including nucleotide sequencing, it may be possible to determine why VMLB1 RNA 2 is unstable, and whether this has any bearing on its ability to infect *G. globosa*.

One isolate , VMLA3, which hybridized strongly with VTAV cDNA probes in dot-blot analysis, failed to hybridize with that probe in Northern blot analysis. Hybridization artifacts such as this have been encountered by other workers (eg P. Palukaitis, personal communication). It is suggested that such artifacts may be associated with terminal loop structures of the test RNAs which by steric hindrance, prevent the probe from hybridizing with them.

Using the local lesion method alone, a virus isolate, Ra, was obtained whose genome was composed of VTAV RNAs 1 and 2 and an RNA 3 which consisted of the coat protein gene and at least 60% of the 3a gene of MCMV. Isolate Ra was of particular interest not only because it is a pseudorecombinant, but also because it possessed an RNA 3 which was shown to be slower migrating in agarose gels than MCMV RNA 3 and yet faster than VTAV RNA 3. It was at first thought that Ra RNA 3 was a recombinant consisting of VTAV 3a gene and MCMV coat protein gene. However, rigorous tests failed to show the presence of the expected VTAV RNA sequences. It was concluded that the VTAV sequences detected were probably degradation products of its RNAs 1 and 2 comigrating with the RNA 3. Indeed, Gould and Symons (1977) reported contamination of RNA 3 preparations by degradation products of RNAs 1 and 2 comigrating with the RNA 3.

The RNA 3 of isolate Ra could also be a mutant MCMV RNA 3 present with other RNA 3 populations in in a mixture analogous to the "extremely heterogeneous mixture" described by Domingo et al., (1985) and which may have been selected for in the local lesion host. Garcia-Arenal et al., (1984) described the presence of genetically heterogeneous mutants in "pure" cultures of TMV. The presence of these mutants was thought to have been due, among other considerations, to the high rate of transcriptional infidelity known to occur during RNA synthesis and which results in a heterogeneous viral RNA population but with one predominant genotype. Whether Ra RNA 3 is a mutant or a recombinant, the origin and/or nature of the extra nucleotides accounting for its being larger than MCMV RNA 3 will be of some interest. This can only be determined by nucleotide sequencing of the RNA.

Using the selective transmission by *M. persicae*, 12 out of the 29 virus isolates obtained appeared to have contained both MCMV and VTAV RNA sequences. Most of these isolates were subsequently shown to consist of the complete VTAV genome together with some MCMV RNA sequences, which were eliminated during a single mechanical passage through *N. clevelandii*.

One isolate, MVapA3, (Fig 7.2) was obtained whose RNAs 1 to 4 hybridized with a VTAV cDNA probe and its coat protein was identified as that of VTAV. However, its RNA 3 but not RNA 4, also hybridized with MCMV cDNA probe, but no MCMV antigen could

be detected in leaf extracts or virus preparations. It seems unlikely that the isolate contained both MCMV and VTAV RNAs 3. This is because as shown in Chapter 4, MCMV RNA 3 is very competitive and, alone, will not only survive but sometimes exclude the VTAV RNA 3 in mixed infections of VTAV and $T_1T_2M_3$. Further characterization of this isolate would require cDNA clones of MCMV RNAs 1, 2 and 3, for use in RNA protection assays which would determine the origin of the MCMV RNA sequences detected in Northern blot analysis. Analysis with VTAV RNA 3 clones would show whether intact VTAV RNA 3 was present.

Two isolates, MVapD2 and D3, which were subsequently shown to be aphid non-transmissible, provide evidence of transcapsidation occurring in mixed infections of MCMV and VTAV. These isolates which contained the complete genome of MCMV, must have been encapsidated in VTAV coat protein which enabled their transmission by the aphids. In contrast, isolates MVapC9 and MVapD1 were able to be further passaged by aphid transmission. It was concluded that since these do not appear to contain any VTAV sequences, their aphid transmissibility may have been due to mutation which conferred that property on them. MCMV is a mutant isolate from Price's No 6 strain of CMV (Mossop et al., 1976). and consequently, there appears to have been a back-mutation leading to the virus re-acquiring the previously lost aphid transmission property. Badami (1958) described the loss of transmissibility by *Myzus persicae*, but not *M. ascalonicus* or *Aphis gossypii*, of an isolate of CMV from spinach. The loss occurred during propagation in conditions in which other strains remained aphid transmissible. It is therefore possible that such a mutation may have occurred to produce isolates MVapC9 and D1.

Aphid transmissibility has been associated with the RNA 3 of the cucumoviruses (Mossop and Francki, 1977). B. Chen and R.I.B. Francki (personal communication) have demonstrated by *in vitro* transcapsidation that when the coat protein of aphid transmissible TAV was used to encapsidate MCMV or TMV RNAs, these could subsequently be transmitted by the aphid *Myzus persicae* probing through a membrane. Isolates MVapC9 and D1, which are aphid transmissible may therefore provide information on the location of the amino acid sequences in the coat protein which may   affect aphid transmissibility of the cucumoviruses. Comparison of MCMV and the aphid non-transmissible isolates MVapD2

and D3 with isolates MVapC9 and D1 by RNA protection assay may locate the site of heterogeneity, if any. The exact sequence changes responsible for the aphid transmisibility can then be determined by direct RNA sequncing across the points of heterogeneity using appropriate oligo-nucleotide primers.

8.4. The Changing World of Cucumber Mosaic Virus.

CMV has been described as being one of the most cosmopolitan of plant viruses (Francki et al., 1979; Douine et al., 1979). The reason for this stems from the extremely wide range of plant species susceptible to the many strains of CMV. Price (1940) reported that CMV had been detected in 200 plant species in 40 families. Thornberry (1966) drew up a list of 307 susceptible plant species in 174 genera and 52 families. Douine et al., (1979) found in a world wide survey that CMV infect 750 plant species in 365 genera and 85 families. More significant, however, is the fact that every host range survey over the last 40 years has found increasing numbers of plant species susceptible to cucumovirus infection. It would seem that CMV is being detected in plant species previously unknown, and in new geographic locations. For example, CMV was extremely rare in South Australia twenty years ago, and yet today the virus has assumed increasing economic importance in lupins (Alberts et al., 1985) and appears to be moving into other crops as well (R.I.B. Francki, personal communication).

These "increases" in host range, geographic distribution and variability of the virus may be attributed to several reasons. Firstly, there has been an increasing interest in plant virus research, involving more workers in different parts of the world than ever before. The recognition of the economic inportance of plant virus diseases is the main reason for this interest. Secondly, improved methods of diagnosis have made it possible to detect viruses in small amounts of every type of plant tissue. These methods also make it possible to characterize virus isolates and strains quickly and accurately. Thirdly, improved and rapid means of travel has compounded disease spread, through the exchange of infected planting stocks and seed, thereby introducing new, susceptible plant species and/or viruses to new geographic locations. Notwithstanding these factors, the question has been asked: Do molecular interactions in mixed infections of plant viruses result in genomic variability , and

therefore, host range? The cucumoviruses by their very description as one of the most cosmopolitan RNA plant viruses, were a very suitable and relevant model for the study. However, though pseudorecombination and transcapsidation have been demonstrated in these studies with these two cucumoviruses, the fundamental question remains: what is the role of these mechanisms in the evolution of the virus?

Bibliography

Adams, A.N., and Hull, R. (1972). Tobacco yellow vein, a virus dependent on assistor viruses for its transmission by aphids. Ann. of appl. Biol. **71**, 135.

Ahlquist, P., Dasgupta, R., and Kaesberg, P. (1984). Nucleotide sequence of the brome mosaic genome and its implications for virus replication. J. Mol. Biol. **172**, 369.

Ahlquist, P., Strauss, E.G., Rice, C.M., Strauss, J.H., Haseloff, J. and Zimmern, D. (1985). Sindbis virus proteins nsP1 and nsP2 contain homology to non-structural proteins from several RNA plant viruses. J. gen Virol. **35**, 536.

Alberts, E., Hannay, J., and Randles, J.W. (1985). An epidemic of cucumber mosaic virus in South Australian lupins. Aust. J. Agric. Res. **36**, 267.

Allison, R.F., Janda, M., and Ahlquist, P. (1988). Infectious *in vitro* transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. J. virol. **62**, 3581.

Anonymous. (1951). Report of the West African Cacao Research Institute, Tafo, Gold Coast.

Appiano, A., Dennazio, S. and Redolfi, P. (1978). Cytological alterations in tissues of *Gomphrena globosa* plants systematically infected with tomato bushy stunt virus. J. Gen. Virol. **40**, 277.

Assink, A.M., Swaans, H., and van Kammen, A. (1973). The localization of virus specific double stranded RNA of cowpea mosaic virus in subcellular fractions of infected *Vigna* leaves. Virology **53**, 354.

Atabekov, J.G. and Dorokov, Y.L. (1984). Plant virus specific transport functions and resistance of plants to viruses. Adv. Vir. Res. **29**, 313.

Badami, R.S., and Kassanis, B.(1959). Some properties of three virus isolates from a diseased plant of *Solanum jasmnoides* Paxt. from India. Ann. appl. Biol. **41**, 397.

Badami, R.S. (1958). Changes in the transmissibility by aphids of a strain of cucumber mosaic virus. Ann. appl. Biol. **46**, 554.

Bagnall, R.H. (1956). Three viruses isolated from Irish Cobbler potatoes affected with interveinal mosaic disease. Diss. Abstr. **16**, 1761.

Bancroft, J.B. (1971). The significance of the multicomponent nature of cowpea chlorotic mottle virus RNA. Virology **45**, 830.

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

Bancroft, J.B., Hiebert, E., Rees, M.W. and Markham, R.M. (1968). Properties of Cowpea chlorotic mottle virus, its protein and nucleic acid. Virology **34**, 224.

Bar-Joseph, M. (1978). Cross-protection incompleteness: a possible cause for natural spread of tristeza virus after a prolonged lag period in Israel. Phytopathology **68**, 1111.

Bauldcombe, D.C., Saunders, G.R., Bevan, M.W., Mayo, M.A. and Harrison, B.D. (1986). Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature (London) **321**, 446.

Bawden, F.C. and Kassanis, B. (1945). The suppression of one plant virus by another. Ann. appl. Biol. **32**, 52.

Beachy, R.N., Powell, A.P., Olivier, M.J., De, B., Fraley, R.T., Rogers, S.G.and Horsch, R.B. (1985). Potential for applying genetic transformation to studies of viral pathogenesis and cross protection. In: *Biotechnology and Plant Sciences*, Zaitlin, M., Day, P.and Hollander, J.A. (eds). Academic Press, Orlando, pp 265-275.

Bennet, C.W. (1949). Recovery of plants from dodder latent mosaic. Phytopathology **39**, 637.

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

Best, R.J. (1961). Recombination experiments with strains A and E of tomato spotted wilt virus. Virology **15**, 327.

Bevan, M.W. and Chilton, M-D. (1982). T-DNA of the *Agrobacterium* TI and RI plasmids. Ann. Rev. Genet. **16**, 357.

Bevan, M.W., Mason, S.E. and Goelet, P. (1985). Expression of the tobacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plants transformed by Agrobacterium. EMBO. J. **4**, 1921.

Broadbent, L. (1976). Epidemiology and control of tomato mosaic virus. Ann Rev Phytopathology **14**, 75.

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

Cassels, A.C. and Herrick, C.C. (1977). Cross-protection between mild and severe strains of tobacco mosaic virus in doubly inoculated tomato plants. Virology **78**, 253.

Cees, M., van Dun, P., van Vloten-Doting, L. and Bol, J. (1988). Expression of alfalfa mosaic virus cDNA 1 and 2 in transformed tobacco plants. Virology **163**, 572.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. **34**, 475.

Cockbain, A.J. (1978). Bean yellow vein banding virus. Report of the Rothamsted Experimental Station for 1977, pp 221-222.

Cockbain, A.J. and Jones, P. (1981). Bean yellow vein banding virus. Report of the Rothamsted experimental station for 1980, p 189.

Cockbain, A.J., Jones, P. and Woods, R.D. (1986). Transmission characteristics and some other properties of bean yellow vein-banding virus and its association with pea enation virus. Ann. appl. Biol. **108**, 59.

Cooper, P.D. (1968). A genetic map of poliovirus temperature sensitive mutants. Virology **35**, 584.

Cooper, P.D. (1977). Genetics of picornaviruses. In: *Comprehensive Virology*. H. Fraenkel-Conrat and R.R. Wagner (eds). vol. 9. Plenum Publishing Corporation, N.Y. pp 133-207.

Cornelissen, B.J.C. and Bol, J. (1984). Homology between proteins encoded by tobacco mosaic virus and tricornaviruses. Plant. Mol. Biol. **3**, 379.

Costa, A.S. (1969). Conditioning of the plant by one virus necessary for systemic invasion of another. Phytopathol. Z. **65**, 219.

Costa, A.S. and Muller, G.W. (1980). Tristeza control by cross-protection: a US-Brazilian cooperative success. Plant. Dis. **64**, 538.

Crowdy, S.H. and Posnette A.F. (1947). Virus diseases of cacao in W. Africa.II Cross-immunity experiments with viruses IA, IB & IC. Ann. appl. Biol. **34**, 403.

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

De Jaeger, C.P. and van Kammen, A. (1970). The relationship between the components of cowpea mosaic virs III. Location of genetic information for two biological functions in the middle component of CPMV. Virology 41, 281.

De Zoeten, G.A., Gaard, G. and Diez, F.B. (1972). Nuclear vesiculation associated with pea enation mosaic virus-infected plant tissue. Virology **48**,638.

De Zoeten, G.A. and Fulton, R.W. (1975). Understanding generates possibilities. Phytopathology **65**, 221.

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

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

Desselberger, U., Nakajima, K., Alfino, P., Pederson, F.S., Haseltine, W.A., Hannoun, C. and Palese, P. (1978). Biochemical evidence that "new" influenza virus strains in nature may arise by recombination (reassortment). Proc. Natl. Acad. Sci. (USA) **75**, 3341.

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

Dingjan-Verstegh, A., van Vloten-Doting, L. and Jaspars, E.M.J. (1972). Alfafa mosaic virus hybrids constructed by exchanging nucleoprotein components. Virology **49**, 716.

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

Domingo, E. and Holland, J.J. (1988). High error rates, population equilibrium and Evolution of RNA Replication systems. In: *RNA Genetics*. Domingo, E., Holland, J.J. and Ahlquist, P. (eds). CRC Press Inc, Boca Raton, Florida. vol 1, pp 1-36.

Domingo, E., Martinez-Salas, E., Sobrino, F., de la Torre, J.C., Portela, A., Ortin, J., Lopez-Galindez, C., Perez-Brena, P., Villanueva, N., Najera, R., Van de Pol, S., Steinhauer, D., DePolo, N. and Holland, J.J. (1985). The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance - a review. Gene **40**, 1.

Douine, L, Quoit, J.B., Marchoux, G. and Archange, P. (1979). Recensement des especies vegetales sensibles au Virus de la Mosaique du Concombre (CMV) Etude bibliographique. Ann. Phytopath. **11**, 439

Elnagar, S. and Murrant, A.F., (1978). Relations of carrot red leaf and carrot mottle viruses with their aphid vector, *Cavariella aegopodii..* Ann. appl. Biol. **89**, 237.

Elnagar, S. and Murant A.F. (1976). The role of the helper virus, Anthriscus yellows, in the transmisson of parsnip yellow fleck virus by the aphid *Cavariella aegopodii..* Annals. appl. Biol. **84**, 169.

Emini, E.A., Leibowitz, J., Diamond, D., Bonin, J. and Wimmer, E. (1983). Recombinants of Mahoney and Sabin Strain Poliovirus Type 1: Analysis of in vitro Phenotypic Markers and Evidence that Resistance to Guanidine Maps in the Nonstructural Proteins. Virology 137, 74.

Falk, B.W., Duffus, J.E. and Morris T.J. (1979a). Transmission, host range and serological properties of the viruses that cause lettuce speckles disease. Phytopathology **69**, 612.

Falk, B.W., Morris, T.J. and Duffus, D.E. (1979b). Unstable infectivity and sedimentable ds-RNA associated with lettuce necrotic speckles mottle virus. Virology **96**, 239.

Fawcett, H.S. (1930). The importance of investigations on the affects of known mixtures of microorganisms. Phytopathology **21**, 545.

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

Fletcher, J.T. and Rowe, J.M. (1975). Observations and experiments on the use of avirulent mutant strain of tobacco mosaic virus as a means of controlling tomato mosaic. Ann. appl. biol. **81**, 171.

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

Francki, R.I.B. (1981). Plant virus taxonomy. In: *Handbook of Plant Virus Infections and Diagnosis*. E Kurstak (ed). Elsevier/North Holland Biomedical Press p 3.

Francki, R.I.B. and Habili, N. (1972). Stabilization of capsid structure and enhancement of immunogenecity of cucumber mosaic virus (Q strain) by formaldehyde. Virology **48**, 309.

Francki, R.I.B., Milne, R.G. and Hatta, T. (1985). Atlas of Plant Viruses, Vols I and II, Boca Raton, CRC Press.

Franssen, H., Lenmssen, J., Goldbach, R., Lomonossoff, G. and Zimmern, D. (1984). Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. EMBO. J. **3**, 855.

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

French, R. and Ahlquist, P. (1987). Intercistronic as well as terminal sequences are required for effecient amplification of brome mosaic virus RNA 3. J. Virol. **61**, 1457.

Fujisawa, I., Hayashi, T. and Matsui, C. (1967). Electron microscopy of mixed infections with two plant viruses I. Intracellular interactions between TMV and Tobacco etch virus. Virology **33**, 70.

Fulton, R.W. (1986). Practices and precautions in the Use of Cross-protection for plant virus disease control. Ann. rev. Phytopathol. **24**, 67.

Garcia-Arenal, F., Palukaitis, P. and Zaitlin, M. (1984). Strains and mutants of Tobacco mosaic virus are both found in virus derived from single-lesion-passaged inoculum. Virology **132**,131.

Gerlach, W.L., Llewellyn, D. and Haseloff, J. (1987). Construction of a plant resistance gene from the satellite RNA of tobacco ringspot virus. Nature **328**, 802.

Gibbs, A. (1969). Plant Virus classification. Adv. Virus. Res. **14**, 263.

Gibbs, A. (1987). Molecular Evolution of Viruses. "Trees", "Clocks" and "Modules". J. Cell Sci. sup. **7**, 319.

Goldbach, R.W. and Wellink, J. (1988). Evolution of Plus-strand RNA viruses. Intervirology **29**, 260.

Goldbach, R.W. (1986). Molecular evolution of Plant viruses. Ann. rev. Phytopathol. 24, 289.

Gonda, T.J. and Symons, R.H. (1978). The use of hybridization analysis with complementary DNA to determine the RNA sequence homology between strains of plant viruses: its application to several cucumoviruses. Virology **88**, 361.

Gould, A.R. and Symons, R.H. (1977). Determination of the sequence homology between the four RNA species of cucumber mosaic virus by hybridization analysis with complementary DNA. Nucl. acids Res. **4**, 3787.

Govier, D.A. and Kassanis, B. (1974). A virus induced component of plant sap needed when aphids acquire potato virus Y from purified preparations. Virology **61**, 420.

Govier, D.A., Kassanis, B. and Pirone, T.P. (1977). Partial purification and characterization of the potato virus Y helper component. Virology **78**, 306.

Grant, T.J. and Costa, A.S. (1951). A mild strain of the tristeza virus of citrus. Phytopathology **41**, 114.

Habili, N. and Francki, R.I.B. (1976). Some aspects of cucumovirus genetics. Ann. Microbiol. **127A**, 163.

Habili, N. and Francki, R.I.B. (1974a). Comparative studies of tomato aspermy and cucumber mosaic viruses. Virology **60**, 29.

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

Habili, N. and Francki R.I.B. (1975). Comparative studies on tomato aspermy virus and cucumber mosaic viruses VI. Immunogenic and serological properties. Virology **64**, 421.

Hahn, C.S., Lustig, S., Strauss, E.G. and Strauss, J.H. (1988). Western equine encephalitis virus is a recombinant virus. Proc. Natl Acad. Sci. (USA). **85**, 5997.

Hamilton, R.I. (1980). Defences triggered by previous invaders: viruses. In: *Plant Disease*. J.G. Horstall and E.B. Cowling (eds). Vol 5 Academic Press, NY pp 279-303.

Hanada, K. and Tochihara, H. (1975). Genetic analysis of cucumber mosaic, peanut stunt and chrysanthemum mild mottle viruses. Ann. Phytopath. Soc. Japan **46**, 159.

Harrison, B.D. (1987). Plant virus transmission by vectors: mechanisms and consequences. In: *Molecular basis of virus disease*. Russell, W.C. and Almond, J.W. (eds). Cambridge University Press pp 319-344.

Harrison, B.D., Mayo, M.A. and Baulcombe, D.C. (1987). Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature **328**, 799.

Harrison, B.D., Finch, J.T., Gibbs, A.J., Hollings, M., Shepherd, R.J. and Wetter, C. (1971). Sixteen groups of plant viruses. Virology **34**, 492.

Harrison, B.D. and Murant, A.F. (1984). Involvement of virus coded proteins in transmission of plant viruses by vectors. In: *Vectors in Virus Biology*. Mayo, M.A. and Harrap, K.A. (eds). N.Y. and London

Hasseloff,J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R. and Kaesberg, P. (1984). Striking similarities among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organisation. Proc. Natl. Acad. Sci. (USA). **81**, 4358.

Hatta, T. and Francki, R.I.B. (1978). Enzyme cytochemical identification of single stranded and double stranded RNAs in virus infected plant and insect cells. Virology **88**, 105.

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

Hatta, T. and Matthews, R.E.F. (1974). The sequence of early cytological changes in chinese cabbage leaf cells following systemic infection with turnip yellow mosaic virus. Virology **59**, 383.

Hatta, T. and Matthews, R.E.F. (1976). Sites of coat protein accumulation in turnip yellow mosaic virus infected cells. Virology **73**, 1.

Hatta, T. and Ushiyama, R. (1973). Mitochondrial vesiculation associated with cucumber green mottle mosaic virus. J. gen. virol. **21**, 9.

Hatta, T., Bullivant, S. and Matthews, R.E.F. (1973). Fine structure of vesicles induced in chloroplasts of chinese cabbage leaves by infection with turnip yellow mosaic virus. J. gen. virol. **20**, 37.

Hatta, T., Nakamoto, T., Takagi, Y. and Ushiyama, R. (1971). Cytological abnormalities of mitochondria induced by infection with cucumber green mottle mosaic virus. Virology **45**, 292.

Hemenway, C., Fang, R.X., Kaniewski, W.K., Chua, N.H. and Tumer, N.E. (1988). Analysis of protection in transgenic plants expressing potato virus X coat protein or its antisense RNA. EMBO. J. **7**, 1273.

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

Hirst, G.K. (1962). Genetic recombination with Newcastle disease virus, poliovirus and influenza. Cold Spring Harbor Symposium Quant. Biol. **27**, 303.

Hoekema, A., van Haaren, M.J.J., Hiile, J., Hoge, J.H.C., Hooykaas, P.J.J., Krens, F.A., Wullems, G.J. and Schilperoot, R.A. (1983). *Agrobacterium tumefaciens* and its TI plasmid as tools in transformation of plant cells. In: *Plant Molecular Biology*. A.R. Liss, N.Y. pp 3-22.

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

Horikoshi, M., Nakayama, M., Yamaoka, N.I. and Shishiyama, J. (1987). Brome mosaic virus coat protein inhibits viral RNA synthesis *in vitro*. Virology **158**, 15.

Houwing, C.J. and Jaspers, E.M.J. (1986). Coat protein blocks in vitro transcapsidation of virion RNAs of alfalfa mosaic virus. FEBS. lett. **209**, 284.

Hull, R. and Adams, A.N. (1968). Groundnut rosette and its assistor virus. Ann. appl. Biol. **62**, 139.

Jacquemond, M., Amselem, J. and Tepfer, M. (1988). A gene coding for a monomeric form of cucumber mosaic virus satellite RNA confers tolerance to CMV. Mol.Plant - Microbe Interact. **8**, 311.

Jennings, P.A., Finch, J.T., Winter, G. and Robertson, J.S. (1983). Does the higher order structure of the influenza virus nucleoprotein guide sequence rearrangements in influenza viral RNAs? Cell **34**, 619.

Kassanis, B. and Nixon, H.L. (1960). Activation of one plant virus by another. Nature **187**, 713.

Kassanis, B. and Govier, D.A. (1971). The role of the helper virus in aphid transmission of potato aucuba virus and potato virus C. J. gen. virol. **13**, 221.

Kassanis, B. (1961). The transmission of potato aucuba mosaic virus by aphids from plants also infected by potato viruses A or Y. Virology **13**, 83.

Kassanis, B. (1962). Properties and behaviour of a virus depending for its multiplication on another. J. gen. Microbiol. **27**, 477.

Kassanis, B. (1963). Interaction of viruses in plants. Adv. Virus. Res. **10**, 219.

Kavanau, J.L. (1949). On correlation of the phenomena associated with chromosomes, foreign proteins and viruses III. Virus associated phenomena. Amer. Nature **83**, 113.

Kew, M. and Nottay, B.K. (1984). Evolution of the oral polio vaccine strains in humans occur by both mutations and intermolecular recombination. In: *Modern approaches to vaccines: Molecular and Chemical Basis of virus virulence*. R.M. Chanock and R. Lerner, (eds) Cold Spring Harbor Lab. NY. pp 357-362.

Kiberstis, P.A., Loesch-Fries, L.S. and Hall, T.C. (1981). Viral protein synthesis in barley protoplasts inoculated with native and fractionated brome mosaic virus RNA. Virology **112**, 804.

King, A.M.Q. (1988). Genetic Recombination in positive strand RNA viruses. In: *RNA genetics*. Domingo, E., Holland, J.J. and Ahlquist, P. (eds). CRC Press Inc., Boca Raton, Florida. vol II , p 149.

King, A.M.Q., McCahon, D., Slade, W.R. and Newman, J.W. (1982). Biochemical evidence of Recombination within the unsegmented RNA genome of Aphthovirus. J. virol. **41** 66.

Köhler, E. and Hauschild, I. (1974). Betrachtung und versuche zum Proble der "erwarbenen Immunnität" gegen Virusfektionen bei Pflanzen. Züchter **17/18**, 97

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

Lai, M.M.C., Baric, R.S., Makino, S., Keck, J.G., Egbert, J., Leibowitz, J.L. and Stohlman, S.A. (1985). Recombination between nonsegmented genomes of murine coronaviruses. J. virol. **56**, 449.

Lane, L.C. and Kaesberg, P. (1971). Multiple genetic components in brome grass mosaic. Nat. New. Biol. **232** (28), 40.

Lazzarini, R.A., Keene, J.D., Schubert, M. (1981). The origins of defection interfering particles of the negative strand RNA virus. Cell **26**, 145.

Ledinko, N. (1963). Genetic recombination with poliovirus type I: studies between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain. Virology **20**, 107.

Leseman, D.E. (1977). Virus group-specific and viral specific cytopathological alterations induced by members of the Tymovirus group. Phytopathology **2**, 315.

Lister, R.M. (1968). Functional relationship between virus specific products of infection by viruses of the tobacco rattle virus type. J. gen. Virol. **2**, 43.

Lister, R.M. (1969). Tobacco rattle virus, NETU, viruses in relation to functional heterogeneity in plant viruses. Federation Proceedings **28**, 1875.

Loening, U.E. and Ingle, J. (1967). Diversity of RNA components in green plant tissue. Nature **215**, 363.

Loesch-Fries, L.S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Jarvis, N., Nelson, S. and Halk, E. (1987). Expression of alfafa mosaic virus RNA 4 in transgenic plants confers virus resistance. EMBO J. **6**, 1845.

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

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

Lung, M.C.Y. and Pirrone, T.P. (1974). Acquisition factor required for aphid transmission of purified cauliflower mosaic virus. Virology **60**, 260.

Makino, S. Keck, J.G., Stohlman, S.T. and Lai, M.M.C. (1986). High frequency RNA recombination of murine coronaviruses. J. gen. Virol. **57**, 729.

Marchoux, G., Marrou, J., and Quiot, J.-B. (1974a). Complementation entre ARN de differentes souches du virus de la mosaique cocombre. Mise evidence d'une interaction entre deux ARN pour determiner un type de symptome. Compte rendus Acad. Sci. Paris Serie D. **278**, 1943.

Marchoux, G., Devergne, J.-C., Marrou, J., Douine, L. and Lot, H. (1974b). Complementation entre ARN de differentes souches du virus de la mosaique du cocombre. Localisation sur l'ARN 3, de plusiers propreite, dont certaines liees a la nature de la capside. Compte rendus Acad. Sci. Paris Serie D. **279**, 2165

Martelli, G.P. and Russo, M. (1984). Use of thin sectioning for visualization and identification of plant viruses. Meth. Virol. **8**, 143.

Martelli, G., Di Franco, A. and Russo, M. (1984). The origin of multi-vesicular bodies in tomato bushy stunt virus infected *G. globosa* plants. J. Ultrastruct. Res. **88**, 275.

Martelli, G.P. and Russo, M. (1985). Virus host relationships: symptomatological and ultra structural aspects. In: *The Plant Viruses: Polyhedral Virions with Tripartite genomes.* Francki, R.I.B., (ed). Plenum Press, NY p 163.

Matthews, R.E.F. (1979). Classification and nomenclature of viruses. Intervirology **12**, 132.

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

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

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

McKinney, H.H. (1941). Virus antagonism, natural host resistance and the acquired-immunity concept with reference to plants. Phytopathology **31**, 1059.

McWhorter, F.P. (1938). The antithetic virus theory of tulip breaking. Ann. appl. Biol. **25**, 254.

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

Monroe, S.S. and Schlesinger, S. (1984). Common and distinct regions of defective interfering RNAs of Sindbis virus. J. virol. **49**,865.

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

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

Murant, A.F. and Goold, R.A. (1968). Purification, properties and transmission of parsnip yellow fleck, a semi-persistent aphid-borne virus. Ann. appl. Biol. **62**, 123.

Murant, A.F., Goold, R.A., Roberts, I.M. and Cathro, J. (1969). Carrot mottle - a persistent aphid-borne virus with unusual properties and particles. J. gen. virol. **4**, 329.

Murant, A.F., Roberts, I.M. and Goold, R.A. (1973). Cytopathological changes and extractable infectivity in *N clevelandii* leaves infected with carrot mottle virus. J. gen. virol. **21**, 269.

Musil, M., Marcinka, K. and Mucha, V. (1975). Some properties of clover blotch virus. Acta Virol. **19**, 437-440.

Nelson, R.N., Abel, P.P. and Beachy, R.N. (1987). Lesions and virus accumulation in tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virology **158**, 126.

Nitta, Naoto, Yochi Takanami, Shigeru Kuwata and Susumu Kuto (1988). Inoculation with RNAs 1 and 2 of CMV induces RNA replicase in tobacco mesophyll protoplasts. J. gen. virol. **69**, 2695.

Owen, J. and Palukaitis, P. (1988). Characterization of Cucumber mosaic virus II. Molecular Heterogeneity mapping of RNA 3 in eight CMV strains. Virology 166, 495

Palukaitis, P., Cotts, S. and Zaitlin, M. (1984). Detection and Identification of viroids and viral Nucleic Acids by "Dot-blot" hybridization. Acta Horticult. **164**, 109.

Palukaitis, P. and Zaitlin, M. (1984). A model to explain "cross-protection" phenomena shown by plant viruses and viroids. In: *Plant Microbe interactions: Molecular and genetic aspects*. Kosuge, T. and Nester, E.W. (eds). MacMillan, NY/London, pp 420-429.

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

Phatak, M.C., Diaz-Ruiz, J.R. and Hull, R. (1976). Cowpea ringspot virus: a seed transmitted cucumovirus. Phytopath. Z. **87**, 132.

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

Pirrone, T.P. (1977). Accessory factors in non-persistent virus transmission . In: *Aphids as Virus Vectors*. Harris, K.F. and Maramosch, K. (eds). Academic Press, NY and Lond. pp 221-235.

Posnette, A.F. and Todd, J.M. (1951). Virus diseases of cacao in West Africa VIII. The search for virus resistant cacao. Ann. appl. Biol. **43**, 433.

Pound, G.S. and Walker, J.C. (1945). Effect of air temperature on the concentration of certain viruses in cabbage. J. Agric. Res. **71**, 471.

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

Powell-Abel, P., Nelson, R.S., De, B., Hoffman, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science **232**, 738.

Price, W.C. (1940). Comparative host ranges of six plant viruses. Amer. J. Bot. **27**, 530.

Pringle, C.R. (1965). Evidence for genetic recombination in foot and mouth disease virus. Virology **25**, 48.

Rao, A.L.N. and Francki, R.I.B. (1981). Comparative studies on tomato aspermy and cucumber mosaic viruses VI. Partial compatibility of genome segments from two viruses. Virology **114**, 573.

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

Rao, A.L.N., Hatta, T. and Francki, R.I.B. (1982). Comparative studies on tomato aspermy and cucumber mosaic viruses VII. Serological relationships re-investigated. Virology **116**, 318.

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

Rast, A.T.B. (1972). M II-16, an artificial symptomless mutant of tobacco mosaic virus for seedling inoculation of tomato crops. Netherlands J. Plant. Path. **78**, 110.

Reanney, D.C. (1982). The evolution of RNA viruses. Ann. rev. Microbiol. **36**, 47.

Rezaian, M.A., Williams, R.H.V. and Symons, R.H. (1985). Nucleotide sequence of cucumber mosaic virus RNA 1. Presence of a sequence complementary to part of a viral satellite RNA and homologous with other viral RNAs. Eur. J. Biochem. **150** (2), 331

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

Robinson, D.J., Hamilton, W.D.D., Harrison, B.D. and Baulcombe, D.C. (1987). Two anomalous Tobravirus isolates: Evidence for RNA recombination in nature. J. gen. virol. **68**, 2551.

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

Rochow, W.F. (1972). The role of mixed infections in the transmission of plant viruses by aphids. Ann. Rev. Phytopathol. **10**, 101.

Rochow, W.F. (1977). Dependent virus transmission from mixed virus infections. In: *Aphids as Virus Vectors*. K.F. Harris and K. Maramorosch (eds). Academic Press, N.Y. pp 253-273.

Rochow, W.F. and Jedlinski, H. (1970). Variants of barley yellow dwarf virus collected in New York and Illinois. Phytopathology **60**, 1030.

Rochow, W.F. and Ross, A.F. (1954). Relative concentration of potato virus X in double and single infections. Phytopathology **44**, 504.

Ross, A.F. (1950). Local lesion formation and virus production following simultaneous inoculation with potato viruses X and Y. Phytopathology **40**, 24 (abstr)

Ross. A.F. (1974). Interaction of viruses in the host. Acta Horticul. **36**, 247.

Rossmann, M.G. and Ruecket, R.R. (1987). What does the molecular structure of viruses tell us about viral functions? Microbiol. Sci. 4, 206.

Russo, M. and Martelli, G.P. (1972). Ultrastructural observations on tomato bushy stunt virus in plant cells. Virology **49**, 122.

Russo, M., Di Franco, A. and Martelli, G. (1983). The fine structure of cymbidium ringspot virus infections in host tissues III. Role of peroxisomes in the genesis of multivesicular bodies. J. Ultra. Struct. Res. **82**, 52.

Salaman, R.N. (1933). Protective inoculation against a plant virus. Nature **131**, 468.

Salaman, R.N. (1937). Acquired immunity against the Y potato virus. Nature **139**, 924.

Sandford, J.C. and Johnson, S.A. (1985). The concept of parasite derived resistance: deriving resistance from the parasites' own genes. J. Theor. Biol. **113**, 395.

Sanger, H.L. (1968). Characteristics of tobacco rattle virus I. Evidence that its two particles are functionally defective and mutually complementing. Mol. and Gen. Genet. **101**, 346.

Schmelzer, K. (1971). Robinia mosaic virus. CMI/AAB Descriptions of plant viruses, No 65.

Schneider, I.R. (1977). Defective plant viruses. In: Bestville Symposium on Agricultural Research. Romberger, J.A., Anderson, J.D. and Powell, R.L. (eds). Montclair NJ: Allanheld. Osmun. Vol 1, pp 201-219.

Schwinghammer, M.W. and Symons, R.H. (1977). Translation of the four major RNA species of cucumber mosaic virus in plant cell free systems and in toad oocytes. Virology **79**, 88.

Sequira, L. (1984). Cross-protection and induced resistance. Their potential for plant disease control. Trends in Biotechnology **2**, 25.

Sherwood, J.C. and Fulton, R.W. (1982). The specific involvement of coat protein in tobacco mosaic virus cross-protection. Virology **119**, 150.

Simmons, J.H. (1959). Mild strain protection as a means of reducing losses from the Queensland woodiness virus in the passion vine. Queensland J. Agric Sci. **16**, 371.

Smith, K.M. (1946). The transmission of a plant virus complex by aphids. Parasitology **37**, 131.

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

Stark, C. and Kennedy, S.I.T. (1978). The generation of defective-interfering particles of semlinki Forest virus in different cell types. Virology **89**, 285.

Strauss J.H. and Strauss, E.G. (1988). Evolution of RNA viruses. Ann. rev. Microbiol. **42**, 657.

Stubbs, L.L. (1948). A new virus disease of carrots: its transmission, host range and control. Aust. J. Sci. Res. **31**, 303.

Sugimura, Y. and Ushiyama, R. (1975). Cucumber green mottle mosaic virus infection and its bearing on cytopathological alterations in tobacco mesophyll protoplasts. J. gen. Virol. **29**, 93.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments transfered to nitrocellulose. Proc. Natl. Acad. Sci. (USA). **77**, 5201.

Thompson, A.D. (1961). Interaction between plant viruses I. Appearance of new strains after mixed infection with potato virus X strains. Virology **13**, 507.

Thung, T.H. (1931). Epidemiologie de quelques malaiés de tabac. C.R. & Comm. 2nd Congr. Int. Path. Compar., Paris. vol 2, pp 482-484.

Tolskaya, E.A., Romanova, L.A., Kolesnikova, M.S. and Agol, V. (1983). Intertypic recombination in poliovirus: Genetic and biochemical studies. Virology **124**, 121.

Tumer N.E., O'Connel, K.M., Nelson, R.S., Sanders, P.R., Beachy, R.N., Fraley, R.T., Shah, D.M. (1987). Expression of alfafa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. EMBO. J. **6**, 1181.

Ushiyama, R. and Matthews, R.E.F. (1970). The significance of chloroplast abnormalities associated with infection with turnip yellow mosaic virus. Virology **42**, 293.

Van Kammen, A. (1967). Purification and properties of the components of cowpea mosaic virus. Virology **31**, 633.

Van Kammen, A. (1968). The relationship between the components of cowpea mosaic virus I. Two ribonucleoprotein particles necessary for the infectivity of CPMV. Virology **34**, 312.

Van Kammen, A. (1984). Expression of functions encoded on genomic RNAs of multipartite plant viruses. In: *Control of Virus Diseases*. Kurstak, E., (ed.) N.Y., Marcel Dekker, p 301.

van Vloten-Doting, L. (1983). Advantages of multipartite genomes of single-stranded RNA plant viruses in nature, for research and for genetic engineering. Plant. Mol. Biol. Reporter **1:2**, 55.

van Vloten-Doting, L, Dingjan-Versteech, A.M. and Jaspars, E.M.J. (1968). Three nucleoprotein components of alfafa mosaic virus necessary for infectivity. Virology **40**, 419.

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

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

Watson, M.A. (1960). Evidence for interaction or genetic recombination between potato viruses Y and C in infected plants. Virology **10**, 211.

Webster, R.G., Laver, W.G., Air, G.M.and Schild, G.C. (1982). Molecular mechanisms of variation in influenza viruses. Nature **296**, 115.

Wilson, T.M.A. and Watkins, P.A.C. (1986). Influence of exogenous viral coat protein on the cotranslational dissassembly of tobacco mosaic virus (TMV) particles in vitro. Virology **149**, 132.

Winter, E., Yamamoto, F., Almoguera, C. and Perucho, M. (1985). A method to detect and characterise point mutations in transcribed genes: amplification and over-expression of the mutant c-Ki-*ras* allele in human tumor cells. Proc. Natl. Acad. Sci. (USA). **82**, 7575.

Wray, W.T., Boulikas, T., Wray, W.P. and Hancock, R. (1981). Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**, 197.

Yarwood, C.E. (1951). Rust infection increases invasiveness of tobacco mosaic virus in bean. Science **114**, 127.

Yeh, S.D. and Gonsalves, D. (1984). Evaluation of induced mutants of papaya ringspot virus for control by cross-protection. Phytopathology **74**, 1086.

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