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STUDIES OF SAP-TRANSMISSIBLE VIRUSES OF FLOWERING CHERRIES

A thesis presented in partial fulfilment of

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ABSTRACT

Six sap-transmitted viruses were identified during a study of 434 flowering cherry trees (*Prunus serrulata* Lindl. *sensu lato*) in the North Island of New Zealand. These included *Prunus* necrotic ringspot ilarvirus strain G (PNRSV-G), apple mosaic ilarvirus (ApMV), flowering cherry virus B (FCVB), strawberry latent ringspot virus (SLRV), prune dwarf ilarvirus (PDV) and flowering cherry virus I (FCVI). Of these, ApMV, FCVB, SLRV and FCVI were new records for this host. FCVB and FCVI are newly described viruses. The most common virus was PNRSV-G (30.6%); the other viruses ranged in incidence from 10.2% (FCVB) to 0.5% (PDV). A further nine viruses were also detected by mechanical transmission, but were not characterized in this study. Repeated sampling of 30 flowering cherry trees during late winter and early spring showed that ELISA was more sensitive for detecting PNRSV-G infection of flowering cherries than sap-transmission.

Three methods for purifying PNRSV-G isolates from flowering cherry were assessed and the best method was one that used ether as a clarification agent. Yields of 5.0 mg/100 g of tissue were obtained. An antiserum was produced to PNRSV-G in New Zealand white rabbits which had a titre in microprecipitin tests of 1/8192. A 338 nucleotide cDNA clone was made to PNRSV-G which hybridised to RNA-3 in Northern analysis.

FCVI had a narrow host range, quasi-isometric particles of *c.* 26 nm diam. morphologically similar to the particles of ilarviruses, some bullet shaped particles (also characteristic of ilarviruses), four RNA species of 3550, 2800, 2000 and 1050 nucleotides,

and a coat protein of M_r 30 000. These properties indicate that FCVI has affinities with the ilarvirus group, but it differs in host range and symptoms, physical characteristics and serological properties from other members of this group.

FCVB infected both monocotyledons and dicotyledons, but had a limited host range. FCVB has four RNA species of 3900, 2150, 1800 and 800 nucleotides (estimated from denatured dsRNA). Partially purified preparations contained isometric particles about 24nm in diameter. When purified at pH 7.5 FCVB sedimented in sucrose gradients as three UV absorbing components and virus particles appeared to be swollen. At low pH (5.0 or 6.0) or at pH 7.5 with the addition of magnesium ions, FCVB sedimented as a single predominant UV absorbing component and virus particles were not swollen. One major protein band (M_r 19 300) was extracted from partially purified preparations. Based on these features, it is proposed that FCVB is a new member of the bromovirus group. However, serological interrelationships were not detected with antisera to three bromoviruses, brome mosaic virus, broad bean mottle virus and cowpea chlorotic mottle virus.

SLRV was isolated from flowering cherry trees in close proximity to each other in Auckland, New Zealand. The virus was not isolated from any of 390 flowering cherry trees tested from four other regions in the North Island. The virus was identified by host range, particle morphology, RNA and protein content and by serology. This is the first record of SLRV in flowering cherry.

The nucleotide sequence of the 3'-terminal 2427 nucleotides of SLRV RNA-2 were

determined using cDNA clones. The sequence contains a single reading frame terminating at an ochre stop codon 552 nucleotides from a 3'-terminal poly(A) tract. The N-terminal sequences of the two SLRV coat proteins determined by Edman degradation indicated that the larger 43K protein had a N-terminal Gly and the smaller 27K protein was cleaved at a Ser/Gly bond. No homologies were found in amino acid sequences or nucleotide sequences to four comoviruses or six nepoviruses suggesting that SLRV should be placed in a separate plant virus group.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Botanical relationships of cherries.

Flowering cherry is an ornamental deciduous tree, in the family Rosaceae, which was first recorded as a distinct variety in Japan and China over a thousand years ago. Flowering cherry taxonomy is confused by many selections and cross-breeding over the intervening centuries producing a number of varieties. The species which occur in New Zealand, as recorded by commercial growers, include *Prunus campanulata* Maxim., *P. cerasifera* J.F. Ehrh., *P. sargentii* Rehd., *P. serrulata* Lindl. *sensu stricto*, *P. subhirtella* Miq., and *P. yedoensis* Matsum.. As species discrimination is difficult the broad species concept of Chadbund (1972) and Bean (1976) will be adopted and all flowering cherries referred to as *P. serrulata sensu lato.*

The genus *Prunus* also includes a number of common fruit trees e.g. almond (*P. dulcis* (Mill.) D.A. Webb), apricot (*P. armeniaca* L.), sweet cherry (*P. avium* L.), sour cherry (*P.

cerasus L.), peach (*P. persica* (L.) Batsch), plum (*P. domestica* L.) and nectarine (*P. persica* var. *nectarina* (Ait.) Maxim.). Other related genera of commercial importance include *Malus sylvestris* Mill. (apples), *Pyrus* spp. (pears) and *Rosa* spp. (roses).

1.2 Study aims and rationale.

Viruses within the Rosaceae are often found to infect a number of species within the group. However, knowledge of virus diseases of *P. serrulata* is comparatively limited. Virus infections of *P. serrula* and *P. cerasus* can be disfiguring, reduce flowering, be detrimental to graft take, and kill trees (Berkeley & Willison, 1948; Fulton, 1970a; Klos & Parker, 1960; Milbrath, 1950, 1957; Nyland *et al.*, 1976; Parker *et al.*, 1959). Symptoms of chlorotic patterns on leaves, death and die-back have been described in *P. serrulata* and attributed to infection with prune dwarf virus (PDV) (Ramaswamy & Posnette 1972) and *Prunus* necrotic ringspot virus (PNRSV) (Milbrath, 1957; Gella & Herrero, 1986; Zeller & Milbrath, 1942). The New Zealand Nursery Research Centre at Massey University was interested in developing an indexing scheme for flowering cherries to achieve a 'high health' propagating stock of these trees. Advantages of higher grafting success, improved tree quality and greater tree longevity were anticipated as benefits of such a programme. However, it is necessary to have an understanding of both the identity of virus diseases infecting flowering cherry and their relative importance. The results of overseas and New Zealand surveys of fruiting cherries gave an indication of the virus diseases to expect, but it was necessary to conduct a survey to determine the sap-transmissible virus diseases of *P. serrulata*.

The relationship of viruses within the ilarviruses is confused due to lack of information resulting from the difficulties of working with this virus group (Francki *et al.*, 1985). Sequencing information, particularly of the coat protein gene, may help to clarify this issue. Therefore a significant effort was made to obtain cDNA clones to the coat protein gene of PNRSV-G for subsequent sequencing. Information obtained from monoclonal antibody studies (Halk *et al.*, 1984) suggests that there are both epitopic differences and similarities between different strains of each of PNRSV and apple mosaic virus (ApMV), and between these two viruses. This would be reflected in the coat protein genome sequence. It is also apparent that Fulton's PNRSV-G antisera does not detect some isolates of PNRSV (Barbara *et al.*, 1978). Therefore knowledge of similarities of sequences between all strains of PNRSV and ApMV should enable the construction of a ubiquitous cDNA probe for survey work.

Sequencing information may enable more exact classification of ilarviruses such as flowering cherry virus I (this study), American plum line pattern virus, spinach latent virus, lilac ring mottle virus and hydrangea mosaic virus without the necessity for antiserum production, which has been shown to not always be a reliable determinant (Barbara *et al.*, 1978).

Strawberry latent ringspot virus is also ambiguous in its relationships with other viruses, and could equally be ascribed to either the Comovirus or the Nepovirus group. Once more, sequencing information of the coat protein gene could elucidate these relationships.

Two new viruses which did not react with available antisera were isolated from flowering

cherry. These were flowering cherry virus B (FCVB), a tentative bromovirus, and flowering cherry virus I (FCVI), an ilarvirus. Because of their uniqueness, and because of the limited amount known about these two viruses, both were studied further.

A review of the literature on viruses relevant to this study follows.

1.3 Incidence and importance of virus and virus-like diseases recorded in flowering cherries

A number of virus and virus-like agents have been recorded infecting flowering cherries. These include *Prunus* necrotic ringspot virus (PNRSV) (Zeller & Milbrath, 1942), prune dwarf virus (PDV) (Ramaswamy & Posnette, 1972), green ring mottle (CGRM) (Milbrath & Zeller, 1945), little cherry (Reeves et al., 1955), rusty mottle (Zeller & Milbrath, 1951; Lott & Keane, 1960), X disease (Gilmer & Blodgett, 1974) and tobacco ringspot virus (TobRSV) (Uyemoto *et al.*, 1977).

1.4 Incidence and importance of virus and virus-like diseases of other Rosaceae

Of more economic importance than *P. serrulata* is *P. avium* L., the sweet or fruiting cherry. Consequently more work has been done on this host and more viruses have been recorded infecting *P. avium* than flowering cherry. *P. cerasus* L. (sour cherry) is also an economically important species of cherry overseas. Because of the close relationship between these *Prunus* species, it is likely that viruses are able to infect cherries ubiquitously. Viruses which infect other members of the Rosaceae are also briefly

discussed where it is most relevant to the present study.

1.4.1 Virus-like disorders of cherry

A number of virus-like disorders which are not sap-transmissible to herbaceous indicators have been reported. These include albino virus, black canker, blossom anomaly, boron russet, cherry green ring mottle, twisted leaf, freckle fruit, little cherry, rough bark, rusty mottle, short stem, spur cherry, rough fruit, X-disease and xylem aberration (Table 1.1). As this study is concerned with sap-transmissible viruses only, these diseases will not be further discussed.

1.4.2 Viruses isolated from cherry

A number of viruses have been isolated from *P. avium* and *P. cerasus* overseas. These are apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), arabis mosaic virus (ArMV), cherry leaf roll virus (CLRV), cherry rasp leaf virus (CRLV), cucumber mosaic virus (CMV), PDV, PNRSV, raspberry ringspot virus (RRV), strawberry latent ringspot virus (SLRV), tobacco mosaic virus (TMV), tobacco ringspot virus (TobRSV), tomato black ring virus (TBRV), tomato bushy stunt virus (TomBSV) and tomato ring spot virus (TomRSV) (Table 1.2). The most important of these viruses are PNRSV, PDV, CLRV and TomRSV (Cropley, 1961; Mink & Parsons, 1965; Mink & Aichele, 1984; Mink & Howell, 1980).

Table 1.1: Virus-like diseases of cherry and their occurrence.

Disorder	Occurrence[1]	References
albino virus	rare	Zeller *et al.*, 1944
black canker	rare	Zeller *et al.*, 1947
blossom anomaly	rare	Cheney & Parish, 1969
boron russet	rare	Milbrath *et al.*, 1966
green ring mottle	common	Rasmussen *et al.*, 1951
twisted leaf	less common	Lott, 1943
freckle fruit	rare	Williams & Milbrath, 1955
little cherry	common	Foster & Lott, 1947
rusty mottle	common	Reeves, 1940
short stem	rare	Afanasiev & Mills, 1961
spur cherry	rare	Blodgett *et al.*, 1965
rough fruit	rare	Wadley, 1963
xylem aberration	rare	Lott *et al.*, 1962
X-disease	common	Rawlins & Thomas, 1951

[1] Information from Gilmer *et al.* (1974).

In New Zealand, the relative incidence of the main sap-transmissible viruses affecting *P. avium* have been determined (Wood & Fry, 1972) as follows: PNRSV 92%, PDV 78% and ACLSV 13%. SLRV was isolated from one cherry tree (Fry & Wood, 1973; Wood, 1979). CLRV and TomRSV have not been isolated from fruiting cherries in New Zealand, nor has ApMV although it is common in apples (Wood, 1979).

1.4.3 Incidence of PDV and PNRSV in *Prunus persica*

A number of surveys using ELISA have been undertaken. For example, an ELISA survey of 5833 *P. persica* trees in South Carolina for PNRSV-G found that overall 30% (range 7-74%) of *P. persica* cultivars were infected (Scott *et al.*, 1989). In some orchards 100% of trees were infected, but the authors ascribed this to use of infected propagation material. Barrat & Schwartz (1988) found 391 of 2113 *P. persica* trees in West Virginia (18.5%) were infected with PNRSV-G as assessed by ELISA testing. Jaffee *et al.* (1986) found 6% of 324 peach trees in Pennsylvania were infected with PNRSV-G by ELISA. About 30% of peach trees were infected with PDV and/or PNRSV in other surveys (Barrat & Otto, 1985; Uyemoto *et al.*, 1989; Wells *et al.*, 1986).

In Australia, a survey of peaches infected with PDV and PNRSV-G based on symptoms showed that infection resulted in yield reductions of 13.2% (Smith & Challen, 1977).

Table 1.2: Reported occurrence of viruses from sweet cherries

Virus	Group	Occurrence	Reference
ApMV	ilarvirus	rare	Barbara, 1980
ArMV	nepovirus	less common	Németh, 1986
ACLSV	closterovirus	less common	Fry & Wood, 1973
CLRV	nepovirus	common	Cropley, 1961
CMV	cucumovirus	rare	Tremaine, 1974
CRLV	nepovirus	less common	Hansen *et al.*, 1974
PDV	ilarvirus	common	Mink & Parsons, 1965
PNRSV	ilarvirus	common	Mink & Aichele, 1984
RRV	nepovirus	rare	Németh, 1986
SLRV	nepovirus	less common	Németh, 1986
TBRV	nepovirus	rare	von Bercks & Mischke, 1964
TBSV	tombusvirus	less common	Albrechtovà *et al.*, 1980
TomRSV	nepovirus	common	Mink & Howell, 1980
TobRSV	nepovirus	rare	Uyemoto *et al.*, 1977
TMV	tobamovirus	rare	Gilmer, 1974

Key:

ApMV apple mosaic virus
ArMV arabis mosaic virus
ACLSV apple chlorotic leafspot virus
CLRV cherry leaf roll virus
CMV cucumber mosaic virus
CRLV cherry rasp leaf virus
PDV prune dwarf virus
PNRSV prunus necrotic ringspot virus
RRV raspberry ringspot virus
SLRV strawberry latent ringspot virus
TBRV tomato black ring virus
TomRSV tomato ringspot virus
TobRSV tobacco ringspot virus
TMV tobacco mosaic virus

1.4.4 Incidence of ilarviruses in cherry

The incidence of ilarviruses as assessed by ELISA in *P. avium* is more variable than in peach. In California ELISA indexing revealed 2 of 90 *P. avium* trees infected with PNRSV-G (2%), and a further 2 infected with PDV (Uyemoto *et. al.*, 1989). Mink (1980) found 58% of 50 sweet cherry trees from commercial orchards were infected with PNRSV-G. However, suspected diseased trees were preferentially collected. Barbara (1980) found that 18.3% of 104 cherry trees were infected with PNRSV-G. One tree was infected with ApMV. Mink & Aichele (1984) surveyed 15000 *P. avium* trees for PNRSV and found 28% were infected. Mink & Parsons (1965) found that the incidence of PNRSV and/or PDV as determined by indexing to 'Shirofugen' flowering cherry was 93% in old trees (more than 20 years old) and 65% in young trees.

1.4.5 Incidence of PNRSV-G in rose in New Zealand

In New Zealand ELISA surveys of commercial roses for PNRSV-G in 1983, 1984 and 1985 showed infection rates between 5 and 11% (Anon, 1984; 1985; Everett, 1986; Gardner, 1983).

1.5 Characteristics of viruses and virus groups relevant to the current study

Although a large number of viruses have been isolated from cherry and other members of the Rosaceae, only a few of these were found in the present study. These were PDV, PNRSV-G, ApMV, SLRV, CMV, ACLSV, and two new viruses of which one is an

ilarvirus and the other a bromovirus. Thus the ilar- and bromo-virus groups will be discussed, as will the nepo- and como-viruses as SLRV is closely related to both these groups. CMV and ACLSV will also be briefly discussed.

1.5.1 Characteristics of the Ilarviruses

Three viruses (PNRSV-G, PDV and ApMV) that are reported to infect cherry are in the ilarvirus group. A fourth unknown ilarvirus was isolated in the current study and named flowering cherry virus I (FCVI). Ilarviruses are isometric labile ringspot viruses. They are characterised by having three or more types of quasi-isometric particle, *c.* 30nm diameter, which sediment as three components of 80-90, 89-98 and 101-114S. Each type of particle has approximately the same amount of RNA and protein and sedimentation differences are due to size not density. Some ilarviruses also form a small proportion of bacilliform particles which tend to sediment faster than the other particles and thus constitute the third peak. The nucleic acid is comprised of three genomic and one sub-genomic positive sense single-stranded RNA of M_r 1.1-1.3, 0.9-1.1, 0.7-0.9 and 0.3 x 10^6 (RNA-1 to RNA-4, respectively). RNA-1, RNA-2 and RNA-3 are encapsidated separately, and two molecules of RNA-4 are encapsidated together (Francki *et al.*, 1985).

Ilarviruses are unstable, and in the presence of oxidising agents in plant sap are quickly degraded. Longevity *in vitro* can be as short as 9-12h, up to about a week and the thermal inactivation point ranges from 42-65C (Fulton, 1983). Addition of antioxidants makes these viruses more stable, due to inhibition of the enzyme responsible for oxidizing

host polyphenols to compounds which destroy the virus (Hampton & Fulton, 1961). For example addition of sodium diethyldithiocarbamate (DIECA), which is a copper-chelating inhibitor of polyphenol oxidase, stabilised two isolates of PNRSV-G in plant sap. Competitive inhibitors of polyphenol oxidase, *p*-nitrophenol and *o*-benzaldehydeoxime, also stabilised PNRSV-G in plant sap. DIECA plus cysteine hydrochloride stabilised PDV (Hampton & Fulton, 1961).

Ilarviruses have a single coat protein species of M_r between 24 000 and 28 000 (Francki *et al.*, 1985). The coat protein is coded for on RNA-4 and on the 3' end of RNA-3. All RNA species are required for infectivity, but RNA-4 can be substituted for by coat protein. Coat protein of different ilarviruses can be used. For example, coat protein from tobacco streak virus (TSV) (the type member) and alfalfa mosaic virus (AMV) (a closely related virus) can activate non-infective preparations of each others RNA-1 to 3 (Van Vloten-Doting, 1975). The nucleoprotein components, however, were not interchangeable (Van Vloten-Doting, 1975). Gonsalves & Garnsey (1975) showed that non-infectious RNA of citrus leaf rugose (CLRV), citrus variegation (CVV) and AMV, all without RNA-4, were able to be activated by coat protein of CLRV, CVV, AMV and TSV. However, non-infectious RNA of AMV was unable to be activated by proteins extracted from cucumber mosaic cucumovirus or tobacco mosaic tobamovirus (Jaspers & Van Kammen, 1972; Bol *et al.*, 1971). Gonsalves & Fulton (1977) found that RNA-4 of related ilarviruses (rose mosaic virus (RMV), PNRSV, AMV and CLRV) could substitute for RMV RNA-4 to promote infectivity of preparations of RNA-1 to 3. They also reported that RNA from CLRV, RMV and CVV uncoated AMV particles.

Examination of sequences of the 5' end of RNA-4 and RNA-3 failed to reveal any homologies between TSV and AMV, and it was concluded that similarities in secondary structure brought about by a repeated AUGC codon (Koper-Zwarthoff & Bol, 1980; Zuidema & Jaspars, 1984) were responsible for interchangeability of coat proteins. No sequence homology exists between these two viruses as determined by competition hybridisation experiments (Bol *et al.*, 1975).

1.5.1.1 Serological relationships within the ilarvirus group

Ilarviruses are morphologically similar, and often the only differences distinguishing them are serological (Fulton, 1983; Francki *et al.*, 1985; Casper, 1973). Working with ilarviruses is notoriously difficult due to their low concentrations in plants, general instability and thus difficulties of purification (Fulton, 1981; Francki, 1985). However, despite this Francki (1985) has categorised the ilarviruses into eight serological sub-groups (Table 1.3). The first four of these consist of viruses which have been well-characterised and serologically examined (Table 1.3). Subgroups 5 to 8 consist of viruses about which relatively little is known (American plum line pattern virus (APLPV), spinach latent virus (SPLV), lilac ring mottle virus (LRMV) and hydrangea mosaic virus (HMV), and consequently each is ascribed to its own serological subgroup. Each of these serological subgroups will be discussed together with relationships within groups where they are known.

1.5.1.1.1 Subgroup 1

This serological subgroup contains the ilarvirus type member, tobacco streak virus (TSV). It has a wide host range and a number of strains which although serologically related have been shown to have some epitopic differences (Fulton, 1972; Ghabrial & Lister, 1974; Halk *et al.*, 1984), and some strains also differ in host range and symptoms (Fulton, 1985). Thus black raspberry latent virus (BRLV) and asparagus stunt virus (ASV) are considered as separate strains of TSV (Francki, 1985), with bean red node virus (BRNV) also probably a distinct strain (Fulton, 1985).

The BRLV strain of TSV is pollen and seed transmissible (Converse & Lister, 1969), a potato strain is seed transmissible (Salazar *et al.*, 1981) and a further isolate from white clover was seed transmitted and was able to be transmitted by two species of thrips (*Thrips tabaci* and *Frankliniella occidentalis*; Kaiser *et al.*, 1982).

1.5.1.1.2 Subgroup 2

The viruses in subgroup 2 (Tulare apple mosaic virus (TAMV), CLRV, CVV, citrus crinkly leaf virus (CCLV), elm mottle virus (EMV) and asparagus virus II (AVII)) are serologically related but are not identical. They all sediment as three components in density gradients, but at slightly different rates. The absorption spectra are also similar (Uyeda & Mink, 1983) and can be used to separate these viruses from TSV. There are also differences in host range and symptoms between viruses within this group.

Table 1.3: Serological subgroups of Ilarviruses (Francki *et al.*, 1985)

Subgroup 1[1]

Tobacco streak virus
 Black raspberry latent virus
 Asparagus stunt virus

Subgroup 2

Tulare apple mosaic virus
Citrus leaf rugose virus
Citrus variegation virus
Citrus crinkly leaf virus
Elm mottle virus
Asparagus virus II

Subgroup 3

Prunus necrotic ringspot virus
 Cherry rugose mosaic virus
 Rose mosaic virus (some isolates)
 Hop virus C
Apple mosaic virus
 Danish plum line pattern virus
 Rose mosaic virus (some isolates)
 Hop virus A

Subgroup 4

Prune dwarf virus

Subgroup 5

American plum line pattern virus

Subgroup 6

Spinach latent virus

Subgroup 7

Lilac ring mottle virus

Subgroup 8

Hydrangea mosaic virus

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[1]subgroups based on serological relationships (indented names refer to synonyms or virus strains)

The serological relationships within this subgroup have been determined by use of reciprocal SDI's published by Uyeda & Mink (1983). Strong serological relationships exist between EMV, AVII and CVV. Weak serological relationships exist between TAMV and AVII, and between CLRV and CVV. There is also a strong relationship between TAMV and CLRV, and between CLRV and AVII, and CLRV and EMV (Rybicki & Von Wechmar, 1985).

1.5.1.1.3 Subgroup 3

As can be seen from Table 1.3, PNRSV and ApMV are in the same serological subgroup, and can be considered to be different serotypes of the same virus (Barbara *et al.*, 1978) as they cross-react. Serotypes have been isolated which are intermediate between ApMV andPNRSV-G (Casper, 1973; Barbara et al., 1978; Skotland & Kaniewski, 1981; Hay, 1989). In order to clarify serological relationships between some of these viruses, Halk *et al.* (1984) produced a number of monoclonal antibodies (MAB). These differentiated three serotypes of PNRSV and five serotypes of ApMV. Seven hybridomas were selected for further study, of which two were specific to PNRSV, three were specific to ApMV and two reacted with both PNRSV and ApMV.

Of the seven hybridomas that reacted with PNRSV or ApMV, only three formed precipitates in gel diffusion with purified virus. Two of these three reacted with PNRSV only in ELISA, and the third with both PNRSV and ApMV in ELISA, but in gel diffusion only with PNRSV. Halk *et al.* (1984) conclude that the inability of hybridomas to precipitate virus is because antibodies are not able to bind to two, or more, virus particles

due to the position of the antigenic site. They suggest that the difficulties encountered in producing polyclonal antibodies to ilarviruses is due to a predominance of non-precipitate forming antibodies and a few precipitate forming ones. Reactions were obtained to these monoclonal antibodies in ELISA where antibodies binding to a single virus particle cause a positive reaction, but not in gel diffusion where only precipitating reactions are positive.

Using these MABs to differentiate different isolates of PNRSV and ApMV showed that some epitopes were common to some strains of both PNRSV and ApMV. Some MABs reacted only to ApMV or only to PNRSV, indicating specificity of some epitopes. One MAB reacted with all PNRSV isolates except danish plum line pattern virus (DPLV), confirming that DPLV lacks some epitopes common with other isolates of PNRSV (Fulton, 1968). Four distinct antigenic differences in PNRSV were recognized, and four or five in ApMV. Isolates of PNRSV-G and ApMV from hop reacted to MABs characteristic of ApMV. Thus PNRSV-hop represents an intermediate serotype as proposed by Barbara *et al.* (1978). Failure to react with MABs characteristic for PNRSV indicates that MABs to the epitopes characteristic to this strain of PNRSV were not produced. None of the MABs to PNRSV or ApMV reacted with TSV, PDV or AMV. Thus there is further experimental justification for prune dwarf, tobacco streak and alfalfa mosaic viruses to be assigned to serological subgroups separate from the PNRSV/ApMV subgroup.

Because rose mosaic disease has been found to be caused by both ApMV and PNRSV-G, rose mosaic virus (RMV) should not be considered a virus but rather a virus disease.

Cherry rugose mosaic disease is associated with chlorotic leaf spots or blotches, leaf twisting, enations on the abaxial leaf surface and delayed ripening of fruit (Nyland *et al.*, 1976). The strain of PNRSV which causes this disease was unable to be consistently serologically distinguished by ELISA from other isolates of PNRSV (Mink, 1980). Three distinct serotypes of PNRSV, as assessed by gel diffusion, were isolated from trees showing rugose mosaic (Mink *et al.*, 1987). One of these serotypes did not react with Fulton's PNRSV-G antisera in gel diffusion tests. However, this serotype reacted to antisera prepared against the other two serotypes which did react with PNRSV-G antisera.

Another strain, plum line pattern virus (PLPV) has been reported to affect 'Amanogawa', 'Shirotae', 'Naden', 'Kwanzan' and 'Temari' flowering cherries. Symptoms of faintly chlorotic to pronounced golden or white leaf borders are produced. The patterns are sometimes made up of large rings but more often form oakleaf patterns. One PLPV isolate from these flowering cherries has been serologically shown to be ApMV, whilst another was shown to be PNRSV-G (Kirkpatrick & Fulton, 1974).

Therefore it seems that plum line pattern and cherry rugose mosaic also should be used as disease descriptions rather than being associated with virus names.

1.5.1.1.4 Subgroup 4

Subgroup 4 contains prune dwarf virus which is serologically unrelated to PNRSV or ApMV. It does, however, produce similar symptoms in cherries and has other common characteristics to PNRSV and ApMV (Fulton & Hamilton, 1960). Its herbaceous host

range is slightly different from PNRSV and ApMV (Waterworth & Fulton, 1964; Kirkpatrick *et al.*, 1967), and PDV particles sediment in gradients according to a difference in morphology rather than size, unlike PNRSV and ApMV (Halk & Fulton, 1978; Fulton, 1982).

1.5.1.1.5 Subgroup 5

This subgroup contains American plum line pattern virus (APLPV) which was first described by Kirkpatrick *et al.* (1964) as causing a line pattern disease on plum. It is a quasi-isometric virus of *c.* 26-33nm diam. sedimenting as four components. It is stabilised by EDTA and is unstable in sap. It is serologically unrelated to PNRSV, ApMV and PDV (Paulsen & Fulton, 1969) which cause similar diseases on plum. It is also not serologically related to RBDV (Barnett & Murant, 1970).

1.5.1.1.6 Subgroup 6

Spinach latent virus (SPLV) was isolated from spinach by Štefanac (1978) and Bos *et al.* (1980). SPLV has quasi-isometric particles of *c.* 27nm diam. and some bacilliform particles. The virus sediments as three components and has five RNA species of M_r 1.30, 1.18, 0.91, 0.35 and 0.27 x 10^6. All RNA are required for infectivity, but RNA-4 and 5 can be substituted for by coat protein. The M_r of the coat protein was 28 000. Of the ilarviruses and related viruses, SPLV does not react with antisera to TSV, EMV, PNRSV-G, ApMV, LRMV, AMV and RBDV.

1.5.1.1.7 Subgroup 7

Lilac ring mottle virus (LRMV) was isolated from lilac by Van der Meer *et al.*(1976). LRMV is considered to be an ilarvirus because it is a spherical multicomponent virus of *c.* 27nm diam. which sediments as two components. It has three genomic RNA species and one sub-genomic. All four RNA species are needed for infectivity, but RNA-4 can be replaced by coat protein (Huttinga & Mosch, 1976). It was not serologically related to TSV, EMV, PNRSV or ApMV (Van der Meer & Huttinga, 1979).

1.5.1.1.8 Subgroup 8

Hydrangea mosaic virus (HMV) was isolated from hydrangea in which it caused chlorotic mosaic leaf symptoms (Thomas *et al.*, 1983). It is unstable in sap, and when purified separates into three components on density gradients. It has quasi-isometric particles of *c.* 29nm and a single coat protein of M_r 26 400. Five RNA species were extracted from purified preparations of HMV of M_r 1.25, 1.08, 0.83, 0.36 and 0.27 x 10^6. All five RNA were required for infectivity but RNA-4 and 5 could be replaced with coat protein. HMV was serologically unrelated to TSV, AVII, PNRSV-G, ApMV and SPLV.

1.5.1.2 Herbaceous indicator hosts

When inoculated to herbaceous indicator plants, ilarviruses of the same serological groupings (Table 1.3) except for the viruses of subgroup 2, generally infect similar hosts. Those ilarviruses which infect *Cucumis sativus* L. by producing prominent chlorotic

primary lesions on cotyledons and systemic invasion which causes chlorotic net patterns on leaves, stunting and, in some viruses, tip death are BRLV and ASV of subgroup 1, CCLV of subgroup 2, both viruses of subgroup 3 (PNRSV and ApMV), subgroup 4, and subgroup 5. SPLV (subgroup 6) causes local lesions only when inoculated to this host. Those that infect *Chenopodium quinoa* Willd. are all the viruses of subgroup 1, EMV, AVII and CCLV of subgroup 2, subgroup 6, subgroup 7 and subgroup 8. Viruses from all serological subgroups except 3 and 4 infect *Nicotiana* spp. (vis. all viruses of subgroup 1; all viruses of subgroup 2; subgroup 5; subgroup 6; subgroup 7 and subgroup 8). No information was available for CVV of subgroup 2.

1.5.1.3 Other viruses closely related to ilarviruses

1.5.1.3.1 Alfalfa mosaic virus (AMV)

Some virologists consider AMV to be closely related to the ilarvirus group. This is based on similarity of coat protein (M_r 24 300), RNA (four species of single-stranded, positive-sense RNA with M_r 1.04 to 0.28 x 10^6), particle morphology (quasi-spherical to bacilliform, about 18nm in diameter) and activation of RNA-1 to 3 by coat protein (Lister & Saksena, 1976; Gonsalves & Fulton, 1977; Halk & Fulton, 1978; Van Vloten-Doting *et al.*, 1981). AMV has been sequenced and the 4 RNA species consist of 3644, 2593, 2037 and 881 nucleotides (RNA-1 to 4) (Cornelissen *et al.*, 1983a; Cornelissen *et al.*, 1983b; Barker *et al.*, 1983; Brederode *et al.*, 1980). The coat protein is encoded for on RNA-4 and the 3' terminus of RNA-3. The four RNA species are capped, but are not

polyadenylated at the 3' end and cannot accept amino acids (Van Vloten-Doting & Jaspars, 1977).

AMV and ilarviruses differ from other tri-partite genome virus groups (bromoviruses and cucumoviruses) because they cannot be aminoacylated (Koper-Zwarthoff & Bol, 1980) and cannot be folded into a tRNA-like structure. Nor do they contain a 3'-terminal -CCA characteristic of tRNAs (Koper-Zwarthoff & Bol, 1980; Gunn & Symons, 1980).

1.5.1.3.2 Raspberry bushy dwarf virus (RBDV)

RBDV was at one stage considered to be an ilarvirus, but is no longer (Francki, 1985; Forster, pers. comm.). Properties that resemble ilarviruses are the coat protein size (M_r 30 000), easy dissociation with 0.01% SDS and morphological similarity (Barnett & Murant, 1970; Murant, 1976). However, RBDV has 3 not 4 RNA species (M_r 2, 0.8 and 0.3 x 10^6) (Murant, 1976) and RNA-3 is a messenger RNA for the coat protein of RBDV (Mayo *et al.,* 1991). There are no serological affinities with other ilarviruses (Francki, 1985). RNA-1, RNA-2 and RNA-3 of RBDV have been sequenced and have 5449, 2231, and 946 nucleotides, respectively (Mayo *et al.*, 1991; Natsuaki *et al.*, 1991; Ziegler *et al.*, 1992).

1.5.1.3.3 Pelargonium zonate spot virus (PZSV)

PZSV has also been considered to be a possible ilarvirus because it sediments as three components, is easily disrupted by neutral PTA, has unstable quasi-spherical particles, and has a coat protein of about M_r 23 000, is seed transmitted and may be pollen transmitted

(Gallitelli, 1982; Francki *et al.*, 1985). However, it is serologically unrelated to other ilarviruses. PZSV consists of only 2 RNA species which are infective as a mixture and infectivity is not enhanced by addition of coat protein (Francki, 1985).

1.5.1.4 Symptoms and characteristics of ilarviruses infecting cherry

1.5.1.4.1 *Prunus* necrotic ringspot virus (PNRSV)

PNRSV was first described as peach ring spot virus (Cochran and Hutchins, 1941). Synonyms for PNRSV are cherry (sour) necrotic ringspot virus, necrotic ringspot virus, peach ringspot virus and *Prunus* ringspot virus. Virus particles are *c.* 23nm in diameter. Both bacilliform and isometric particles can be seen in purified preparations with the electron microscope. The virus is extremely labile and in undiluted sap most infectivity is lost within a few minutes (Fulton, 1970a).

There is fairly conclusive evidence for pollen transmission of PNRSV in cherry (Cameron et al., 1973) and the virus can also infect seed (Kelley & Cameron, 1986). Swenson & Milbrath (1964) tested, without success, a large number of aphids, leafhoppers and other insect species for transmission of this virus. Proesler (1968) reported experimental transmission by the mite *Vasates fockeui*, and Fritzche & Kegler (1968) by the nematode, *Longidorous macrosoma.* However, Francki (1985) urges caution as neither of these reports have been further substantiated.

Identification of some strains of the virus is possible by using selected herbaceous hosts

(George, 1962; Nyland & Lowe, 1964; Waterworth & Fulton, 1964), although symptom expression can be dependent on environmental conditions.

In fruit trees, PNRSV causes poor bud and scion "take" and reduces growth (Milbrath, 1950; 1957), and yield (Klos & Parker, 1960; Milbrath, 1957; Parker *et al.*, 1959). Symptoms include chlorosis, necrosis, leaf deformity, stunting, chlorotic patterns of rings, lines, bands, spot, mottle and mosaic. Chlorotic spots on leaves can become necrotic and then drop out causing a shot hole or tatter leaf symptom. Buds, leaves, shoots, large branches and roots may become necrotic. Epinasty, twisting rugosity and enations are leaf symptoms sometimes associated with infection. Entire plants may be dwarfed or only portions of them may be stunted. Initial shock symptoms following first infection also occur (Nyland *et al.*, 1974). PNRSV is symptomless in some varieties of sweet and sour cherries (Berkeley & Willison, 1948).

Infection of the flowering cherry *P. serrulata* 'Shirofugen', used as an indicator for infection of fruiting cherry with PNRSV (Afanasiev, 1968; Gualacinni, 1957; Gilles & Bormans, 1986; Helton, 1962; Hampton *et al.*, 1966; Milbrath & Zeller, 1945; Schmid, 1986) is eventually fatal (Milbrath, 1957; Gella & Herrero, 1986). Infection of other varieties of flowering cherry by PNRSV is not fatal. Infected 'Amanogawa', 'Okochin' and 'Temari' showed a chlorotic leaf pattern (Zeller & Milbrath, 1942). Some dieback of old (16 years or more) 'Amanogawa' trees was seen and attributed to the effect of the virus. Symptoms could be transferred from infected flowering cherries to *P. avium* 'Mazzard' by grafting (Zeller & Milbrath, 1942).

Detection of PNRSV can be achieved serologically (Adams, 1978; Barbara, 1980; Barbara *et al.*, 1978; Cambra *et al.*, 1982; Clark *et al.*, 1976; Mink, 1980; Mink *et al.*, 1987; Mink & Aichele, 1984; Stein *et al.*, 1987; Torrance & Dolby, 1984), by sap transmission to *Cucumis sativus* L. (which it infects systemically and can cause tip death) and other herbaceous hosts e.g. *Momordica balsamina, Vinca rosea,* (Boyle *et al.*, 1954; George, 1962; Gilmer, 1961; Milbrath, 1953; 1956; Tremaine *et al.*, 1964; Williams *et al.*, 1962) or by grafting to *P. serrulata* 'Shirofugen', which induces a necrotic reaction in positives (Helton, 1962; Fulton, 1970a).

In cherry, best detection of PNRSV occurred following indexing flowers or young rapidly growing leaves in spring. Dormant buds were also good sources of plant tissue for indexing as were leaf samples stored frozen at -20C. Forcing dormant buds immediately or after storage at 4C was found to be a viable technique to extend the period for testing of cherry, apple and plum trees for PNRSV, PDV and cherry leaf roll virus using ELISA (Torrance, 1981). Virus was still detected after 1-2 months storage of bud, flower and leaf tissue.

Scott *et al.* (1988) found, using ELISA, that PNRSV-G is sometimes restricted to certain main branches of peach trees, can also infect systemically, and that a localised infection can spread to become a systemic infection. They also reported that if blossoms were found to be infected, then leaves from the same plant would generally also index positively.

1.5.1.4.2 Prune dwarf virus (PDV)

Although causing similar symptoms in natural and herbaceous indicator hosts, PDV has no serological cross-reaction with PNRSV or ApMV. It is thus classified by Francki *et al.* (1985) as a separate serological subgroup (Table 1.3, Section 1.5.1.1.4). Synonyms for PDV are sour cherry yellows virus, Muir peach dwarf virus, peach stunt virus, 'yellows' strain of ringspot virus, and virus B. The virus was first described by Thomas and Hildebrand (1936). Virus particles are *c*. 22nm in diameter. There is no known vector.

The virus is sap transmissible to species in 15 dicotyledonous families and transmits to *Cucumis sativus* systemically but causes no tip death (Fulton, 1970b). In undiluted cucumber sap about half the infectivity is lost in about 30 sec.

PDV is pollen and seed borne in cherry (George & Davidson, 1964).

Infection with PDV causes chlorotic ringspot, small etched necrotic spots, or yellow mosaic in *P. avium.* Symptoms in sour cherry (*P. cerasus*) are generally milder than those caused by PNRSV (Fulton, 1970b). In sweet cherries, in combination with other viruses, PDV causes 30% reduction of yield (Posnette *et al.*, 1968). In sour cherry, infection reduces yield by 50% or greater (Davidson & George, 1964; Klos & Parker, 1960).

Ramaswamy and Posnette (1972) reported chlorotic rings, lines and mottle on foliage of several cultivars of ornamental cherries ('Yae Murasaki Zakura', 'Tai Haku', 'Ukon', 'Amanogawa', 'Kwanzan', *P. avium* 'Plena,', *P. flontaesiana* (Spach) Schneid., Fuji

cherry (*P. incisa* Thunb.), *P. lannesiana* (Carr.) Wils.). They attribute symptoms to infection with a strain of PDV.

ELISA has been successfully used to detect PDV (Baumann *et al.*, 1984; von Casper, 1977). *P. serrulata* 'Shirofugen' is used as an indicator for infection with PDV (Gilles & Bormans, 1986; Hampton, 1963; Schmid, 1986) and similar symptoms to those produced by PNRSV are apparent. That is, severe bark necrosis and gummosis in 4-6 weeks (Schmid, 1986).

*P.*x *effusa* (Host) Schneid. 'Krassa severa' has also been used as a PDV index host (Hampton *et al.*, 1966). *Momordica balsamina* L. and *Cyamopsis tetragonoloba* (L.) Taub. are differential hosts (Gilmer *et al.*, 1974) used to separate PDV from PNRSV-G.

Stone fruits can be freed from infection with PDV relatively easily following thermotherapy at 36-37 C for 15 days or longer (Nyland, 1960).

1.5.1.5 Conclusion

The foregoing information reveals ilarvirus taxonomy to be confused and tentative. The relationship between ApMV and PNRSV is not clear due to the presence of intermediate serotypes. The inclusion of AMV in the ilarviruses is probably warranted, but more information is required before the group can be more definitively catalogued. Apart from TSV and AMV sequencing information is lacking. There is therefore a priority for this information to be obtained for other ilarviruses.

1.5.2 Bromoviruses

Bromoviruses have polyhedral virus particles about 26nm in diameter consisting of 180 identical polypeptides of M_r about 20 000 which encapsidate 4 RNA species of M_r 1.1, 1.0, 0.8 and 0.3 x 10^6. Bromovirus RNA are not polyadenylated at the 3' termini, and are capped at the 5' terminus (Francki, 1985). Brome mosaic virus (BMV; the type member) has a tripartite genome of positive sense RNA plus one sub-genomic RNA species (Lane, 1981). RNA-1 and 2 are encapsidated separately and RNA-3 and 4 together. This results in particles of similar weights, and bromoviruses sediment as a single component (85*S*). Unlike ilarviruses and alfalfa mosaic virus, RNA-4 is not necessary for infectivity.

Originally, the bromovirus group contained three members, BMV, broad bean mottle virus (BBMV) and cowpea chlorotic mottle virus (CCMV) (Lane, 1979). More recently, the membership has been expanded to include cassia yellow blotch virus (CYBV) (Dale *et al.*, 1984), Melandrium yellow fleck virus (MYFV) (Hollings & Horvàth, 1981) and spring beauty latent virus (SBLV) (Valverde, 1985) based on swelling characteristics, coat protein size, RNA size and particle morphology. Antigenically, BMV and CCMV are related distantly (Scott & Slack, 1971), and unrelated to BBMV (Tremaine *et al.*, 1977). Other serological studies using gel diffusion failed to demonstrate any relationship between SBLV, BBMV and MYFV (Valverde, 1985) or between MYFV or CYBV and BMV, CCMV or BBMV (Dale *et al.*, 1984; Hollings & Horvàth, 1981). A recent study (Valverde & Glascock, 1991) has shown that antisera to SBLV binds to coat protein of BMV, CCMV, BBMV and MYFV in Western blotting analysis. The greater sensitivity of this method is attributed to this difference. These results confirm the inclusion of SBLV

and MYFV in the bromovirus group. CYBV was not tested.

BMV, CCMV and BBMV are stable at pH 5.0 and swell at pH 7.5 and high salt concentration. Once swollen they dissociate into RNA and protein and become susceptible to enzymatic degradation. In the presence of $MgCl_2$, swelling of BMV is not achieved to the full extent at pH 7.5. Swelling is reversible in the presence of $MgCl_2$ (Chauvin *et al.*, 1978). Dale *et al.* (1984) have shown that CYBV also swells at pH 7.0 but not at pH 5.0.

Bromoviruses are quite similar to cucumoviruses, apart from their tendency to swell at high pH (Pfeiffer & Hirth, 1974; Francki, 1985). Both virus groups have a large amount of sequence homology at their 3' ends between the 4 RNA species. There are some regions of sequence homology between bromoviruses (brome mosaic virus, cowpea chlorotic mottle virus, broad bean mosaic virus) and cucumoviruses (cucumber mosaic virus, tomato aspermy virus) at the 3'-terminal 45 residues of RNA-4 (Symons, 1985). All have a 3' terminal -CCA characteristic of tRNAs indicating that they can be aminoacylated.

RNA-3 of brome mosaic virus has been sequenced and has 2114 bases. The 3' end of RNA-3 is homologous to RNA-4 and encodes for the coat protein. There is a poly A tract of about 16-22 nucleotides immediately prior to the coat protein cistron towards the 5' terminus on RNA-3. It is thought that this has the function of identifying the initiation of sequences encoding RNA-4 (Francki *et al.*, 1985).

1.5.3 Nepoviruses

Nepoviruses (nematode transmitted polyhedral viruses) are nematode transmitted polyhedral viruses of about 28-30nm diameter which separate into three sedimenting components (top, T; middle, M; and bottom, B). They have a bipartite genome of RNA of M_r about 2.1-2.8 x 10^6 (RNA-1) and 1.4-2.4 x 10^6 (RNA-2) which are encapsidated separately. Coat proteins have an apparent M_r of about 52 000-60 000.

1.5.4 Strawberry latent ringspot virus (SLRV)

SLRV has two RNA species of M_r 2.6 x 10^6 and 1.6 x 10^6 (Mayo *et al.*, 1974). It is nematode transmitted, has hexagonal virus particles of *c.* 30 nm diam. (Murant, 1974) and sediments in density gradients as three UV absorbance peaks. The first peak contains empty particles as evidenced by stain penetration and lack of RNA, and the bottom peak contains entire particles. Thus it is a nepovirus, but unlike this virus group SLRV has 2 coat protein species of M_r 44 000 and 29 000 (Mayo *et al.*, 1974). Francki *et al.* (1985) suggest that these are simply trimers and dimers of the true coat protein subunit, which in this case would be about 14 500, which is about the same size as that of the type member, tobacco ringspot virus (Chu & Francki, 1979). SLRV is polyadenylated at the 3' end of both RNA species (Mayo *et al.*, 1979). Some isolates have a third RNA species (Mayo *et al.*, 1974; Gallitelli *et al.*, 1982) of M_r 0.4 x 10^6. Because RNA-3 is not infective by itself and no sequence is shared with RNA-1 and 2 it is thought to be a satellite RNA (Mayo *et al.*, 1982).

Electron microscopic examination of infected *Chenopodium quinoa, Nicotiana rustica* L., *C. amaranticolor* Coste & Reyn, *Cucumis sativus* L. and *Apium graveolens* L. showed the presence of tubule-like structures in squash homogenates (Hicks, 1985; Walkey & Webb, 1970; Roberts & Harrison, 1970; Babini & Beraccini, 1982; Murant, 1974; Ikin & Frost, 1976). These structures are common in nepovirus infections, and are thought to facilitate cell to cell translocation by penetrating plasmodesmata (Francki *et al.*, 1985).

SLRV has been recorded from one sweet cherry tree in New Zealand (Fry & Wood, 1973; Wood, 1979) and overseas has been recorded from one Bing sweet cherry tree in Ontario, Canada (Allen *et al.*, 1970). It has been isolated from other Rosaceous plants, i.e. peach (Belli *et al.*, 1980) and rose (Ikin & Frost, 1976; Thomas, 1984), strawberry, raspberry, cherry, plum, black currant (Lister, 1964).

1.5.4.1 Genomic structure of four nepoviruses

Sequencing studies have shown that RNA-2 of nepoviruses is polyadenylated at the 3' end, followed by a short (about 300 nucleotides) non-coding section, then the coat protein genome (about 1500 nucleotides), then a middle section probably responsible for movement (590-800 nucleotides, TBRV protein), a 5' terminus section for which no function has been ascribed, followed by a non-coding region of about 200 nucleotides at the 5' end (Brault *et al.*, 1989).

The lengths of RNA-2 of those nepoviruses which have been sequenced are as follows: TBRV 4662 nucleotides (Meyer *et al.*, 1986), GCMV 4441 nucleotides (Brault *et al.*,

1989), GFLV 3774 nucleotides (Serghini *et al.*, 1990), RRV 3928 nucleotides (Blok *et al.*, 1992), TomRSV 7273 nucleotides (Rott *et al.*, 1991). There was about 60% sequence homology between the RNA-2 encoded polyproteins of four of these viruses. TBRV and GCMV are 57% identical and GFLV and ArMV are 69% identical. RRV was 22 to 24% identical and 46% to 49% similar to TBRV, GCMV, GFLV, TomRV and ArMV. The C-terminal coat protein region was less homologous (54%) which accounts for the serological difference between GCMV and TBRV. Homologies in the 215 residue 3' non-coding region of RNA-2 were found between three nepoviruses (GFLV, TBRV and GCMV) and a comovirus (cowpea mosaic virus) (Serghini *et al.*, 1990).

1.5.5 Comoviruses

SLRV is structurally related to comoviruses. This virus group has two RNA species (2.4 and 1.4 x 10^6) encapsidated in a polyhedral particle *c.* 30nm in diameter. Both RNA are polyadenylated at the 3' end. Particles consist of 60 subunits each of 2 protein species of about 37 000 and 23 000 which form a polyhedra. Comoviruses are predominantly vectored by leaf-feeding beetles.

1.5.5.1 Genomic structure of cowpea mosaic virus (CoMV)

CoMV has been completely sequenced, and consists of 5889 nucleotides RNA-1 (Francki *et al.*, 1985) and 3481 nucleotides of RNA-2 (van Wezenbeek *et al.*, 1983), excluding a poly A tail of 150-200 residues at the 3' termini. The sequences are quite similar adjacent to the poly A tails for about 65 bases (80% homology) (Davies *et al.*, 1979). The coat

protein consists of a 23K and a 37K tract of bases on the 3' end of RNA-2.

1.5.6 Apple chlorotic leafspot virus (ACLSV)

ACLSV is a closterovirus (Walkey, 1985). Other closteroviruses are citrus tristeza virus (CTV), beet yellows virus (BYV), carnation necrotic fleck virus (CNFV) (Bar-Joseph & Murant, 1982). ACLSV has very flexuous filamentous particles *c.* 720 x 12nm encapsidating a single-stranded positive sense RNA of M_r 2.5 x 10^6 (Lister & Bar-Joseph, 1981). There is one coat protein species of M_r 23 500 (Yoshikawa & Takahashi, 1988). ACLSV is found in woody rosaceous plants from which it is sap transmissible to a limited range of herbaceous species. It is not known how ACLSV spreads in nature. ACLSV degrades after one day in extracted sap at 20C. In *C. quinoa* ACLSV causes large (2mm diam.) etched primary lesions, becoming pale brown and necrotic. Later, systemic chlorotic spotting and mottling, with ring and line patterns, culminating in more general chlorosis with necrosis develops (Lister, 1970). In fruiting cherry ACLSV is symptomless (Hansen & Gilmer, 1974). Fry and Wood (1973) described a symptomless infection of sweet cherry with dark green sunken mottle virus, and characterised the causal organism as ACLSV. The virus was found in 13% of sweet cherry trees in New Zealand (Wood & Fry, 1972).

ACLSV RNA has recently been completely sequenced (German *et al.*, 1990). It is 7555 nucleotides in length with a 3' terminal poly A tail.

1.5.7 Cucumber mosaic virus (CMV)

CMV is a cucumovirus which has isometric particles of *c.* 28nm diameter (Tremaine, 1974). The virus was isolated with PNRSV from fruiting cherry showing symptoms of tatter leaf. This isolate reacted with antisera to CMV strain Y. The symptoms induced by CMV alone in *Prunus* hosts are not known (Tremaine, 1974), although it is unlikely that CMV is a serious pathogen of cherry.

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CHAPTER TWO

MATERIALS AND METHODS

2.1 Indicator plants

2.1.1 Indicator plants used in survey.

Indicator plants chosen for the survey of mechanically transmitted viruses were those known to be susceptible to most viruses isolated from sweet cherry. Meristematic leaves or flowers from all flowering cherry trees sampled were inoculated to *Cucumis sativus* L. and, in addition, most were inoculated to *Chenopodium quinoa* Willd. (Table 2.1).

Plants were grown in peat:pumice media (80:20) amended with fertilisers (Appendix I) in a temperature controlled glasshouse (20C ± 5C).

Table 2.1: Indicator plants, used in a survey, which react to mechanically transmitted viruses of *Prunus* spp.

Indicator hosts/virus infecting		reference
Cucumis sativus	*Chenopodium quinoa*	
	ApCLSV	Lister (1970)
ApMV	ApMV (some strains)	Fulton (1968)
ArMV	ArMV	Murant (1970a)
CLRV	CLRV	Jones (1985)
CMV	CMV	Tremaine (1974)
	CRLV	Nyland (1974)
PDV		Fulton (1970)
PNRSV	PNRSV (some strains)	Fulton (1968)
RRV	RRV	Cropley (1964)
SLRV	SLRV	Murant (1974)
TobRSV	TobRSV	Stace-Smith (1970)
	TomBSV	Martelli *et al.* (1971)
	TomBRV	Murant (1970b)
TomRSV	TomRSV	Stace-Smith (1970)

Key:
ApCLSV apple chlorotic leafspot virus
ApMV apple mosaic virus
ArMV arabis mosaic virus
CLRV cherry leaf roll virus
CMV cucumber mosaic virus
CRLV cherry rasp leaf virus
PDV prune dwarf virus
PNRSV *Prunus* necrotic ringspot virus
RRV raspberry ringspot virus
SLRV strawberry latent ringspot virus
TobRSV tobacco ringspot virus
TomBSV tomato bushy stunt virus
TomBRV tomato black ring virus
TomRSV tomato ringspot virus

Information from CMI/AAB virus disease descriptions unless otherwise stated.

2.1.2 Indicator plants used to determine host range.

Indicator plants which were used to determine host range were as follows:

Aizoaceae: *Tetragonia expansa* Murr..

Amaranthaceae: *Gomphrena globosa* L..

Chenopodiaceae: *Chenopodium amaranticolor* Coste & Reyn., *C. foetidum* Schrad., *C. quinoa.*

Compositae: *Tithonia speciosa* Hook..

Cruciferae: *Brassica pekinensis* (Lour.) Rupr. 'Chi Hi Li'.

Cucurbitaceae: *Citrullus lanatus* (Thunb.) Mansf. 'Charleston Grey', *Cucumis melo* L. var. *cantalupensis* Naud. 'Hales Best Jumbo', *C. sativus*, *Cucurbita maxima* Duch. 'Buttercup Squash', *C. maxima*'Golden Hubbard', *C. moschata* (Duch.) Duch. ex Poir 'Buttercup squash', *C. moschata* 'Whanga Crown', *C. pepo* L. 'Zucchini'

Fabaceae: *Dolichos lablab* L., *Phaseolus aureus* Roxb., *P. vulgaris* L., *Pisum sativum* L., *Vicia faba* L., *Vigna cylindrica* Skeels, *V. sinensis* Savi..

Gramineae: *Avena sativa* L., *Hordeum murinum* L., *Triticum aestivum* L..

Solanaceae: *Lycopersicon esculentum* Mill., *Momordica balsamina* L., *Nicotiana clevelandii* Gray, *N. glutinosa* L., *N. megalosiphon* Heurch & Muell., *N. tabacum* L. 'White Burley', *N. tabacum* 'Havana', *N. benthamniana* (L.) Domin..

2.2 Virus maintenance.

Viruses were maintained in *C. sativus* (SLRV, FCVB, FCVI, PNRSV, ApMV and uncharacterised viruses) and *C. quinoa* (SLRV, ACLSV). Viruses maintained in *C. sativus* were transferred every 2 weeks, and those in *C. quinoa* every 5-6 weeks. Viruses were transferred using transfer buffer (5% polyvinylpyrrolidone in 0.02 M sodium phosphate buffer, pH 8.0; Gardner, 1983) and 0.01g Celite (Section 2.6).

2.3 Biochemicals and reagents.

Unless otherwise stated, all biochemicals and reagents used were of analytical grade. All solutions were made up in double distilled water (DDW).

2.4 Serology.

2.4.1 Source of antisera.

The antisera and their sources are listed in Table 2.2.

2.4.2. Preparation of antisera.

Antisera was produced in 'New Zealand White' rabbits following intramuscular injection of rabbits at 2-3 day intervals for 2-3 weeks with 2 ml antigen (purified virus) emulsified in Freund's incomplete adjuvant (1:1). Rabbits were bled by heart puncture, 1 and 2

weeks after final injection. The blood was allowed to clot, and the serum collected following removal of corpuscles by centrifugation and stored at -18 C.

2.4.3 Gel diffusion.

Gel diffusion was carried out in 0.75% Oxoid ionagar No. 2 in 0.01M sodium phosphate buffer (pH 7.0) containing 0.85% NaCl and 0.02% sodium azide. A Camag Immunoelectrophoresis Immunodiffusion mini-well system was used.

2.4.4 ELISA (Enzyme-linked immunosorbent assay)

The double antibody sandwich method of Clark and Adams (1977) was used. For reagents and buffers used, refer to Appendix 2.1. The appropriate purifed antiserum (200 µl) was added to wells in microtitre plates and plates were then incubated in humid conditions for 6 h at 37 C. Following washing 3 times with PBS-Tween 20 wells were loaded with 200 µl sap extract which had been crushed in extraction buffer. Plates were incubated at 4 C overnight, washed as before and loaded with 200 µl of the appropriate enzyme-labelled γ-globulin. Following incubation as before at 37 C for 6h, plates were washed and 200 µl freshly prepared enzyme substrate added to each well. Plates were incubated at room temperature for 1 h and then at 4 C overnight if reactions were not visible. Absorbance at 405 nm was recorded by a Dynatech Miniplate Reader.

Reagent concentrations were optimised according to the factorial layout of Clark and Adams (1977).

Table 2.2: Antisera and their sources.

Virus group	Antisera	Source
Bromovirus	broad bean mosaic	P. Ahlquist, U. Wisc.
	brome mosaic	P. Ahlquist, U. Wisc.
	cowpea chlorotic mottle	P. Ahlquist, U. Wisc.
Cucumovirus	cucumber mosaic	
Ilarvirus	apple mosaic	R. Fulton, U. Wisc.
	asparagus virus II	G. Mink, U. Wash.
	hop virus A	J. Fletcher, Lincoln
	hop virus C	J. Fletcher, Lincoln
	plum line pattern	R. Fulton, U. Wisc.
	prune dwarf	R. Fulton, U. Wisc.
	prune dwarf	G. Wood, HortResearch, Auck.
	Prunus necrotic ringspot-G	R. Fulton, U. Wisc.
	Prunus necrotic ringspot-H	R. Fulton, U. Wisc.
	rose mosaic	R. Fulton, U. Wisc.
	tobacco streak	D. Maat, IPO, Wageningen
Nepovirus	arabis mosaic	Scottish Crop Research Institute
	cherry leaf roll	Scottish Crop Research Institute
	raspberry ringspot	Scottish Crop Research Institute
	strawberry latent ringspot	Scottish Crop Research Institute
	tobacco ringspot	J. Uyemoto, U.C., Davis
	tomato black ring	Scottish Crop Research Institute
	tomato ringspot	J. Uyemoto, U.C., Davis
Others	alfalfa mosaic	R. Francki, Adelaide
	raspberry bushy dwarf	Scottish Crop Research Institute

2.4.4.1 Purification of antiserum.

The γ-globulin fraction of rabbit antisera was purified as follows: 4.5 ml distilled water was added to 0.5 ml antiserum. Five ml saturated ammonium sulphate was added to the diluted antisera and the mixture incubated at room temperature for 60 min. to allow the γ-globulin to precipitate. The precipitate was collected by centrifugation (10 min., 6000 rpm) and then dissolved in 1 ml half-strength phosphate buffered saline (1/2 PBS). The redissolved pellet was dialysed overnight against 500 ml 1/2 PBS to remove ammonium sulphate. The cellulose for the column was prepared by addition of 2 g of DE 52 to 12 ml of 1/2 PBS. The pH was adjusted to 7.4 with KH_2PO_4 and the cellulose allowed to settle. The liquid was decanted off, cellulose resuspended in 1/2 PBS and the pH adjusted to 7.4 with KH_2PO_4. This was repeated 2-3 times, the final time the cellulose was left to settle overnight before decantation. Following resuspension, the cellulose slurry was poured into a glass column plugged with glass wool and washed with 1/2 PBS. Following dialysis, the γ-globulin was loaded onto the column and eluted through with 1/2 PBS. The eluant was collected in 1 ml fractions and absorbance at 280 nm was measured by spectrophotometry. Fractions containing γ-globulin as indicated by absorbance peaks were retained, and those fractions with the highest absorbance readings were used in ELISA. Purifed γ-globulin was stored in siliconised tubes either at -20 C or at 4 C.

2.4.4.2 Gamma-globulin conjugation.

Calf intestine alkaline phosphatase (Sigma, type VII-T) was conjugated with γ-globulin by dissolving 5 mg enzyme with 2 mg γ-globulin. Fresh glutaraldehyde solution (25%, EM grade) was added to 0.06% and the mixture was left at room temperature for 4 h. The conjugate was dialysed three times against 1/2 PBS to remove glutaraldehyde after which 5 mg/ml bovine serum albumin (RIA grade) was added to the dialysate. Conjugates were stored in siliconised tubes at 4 C.

2.5 Survey.

Flower and young expanding leaf tissues from flowering cherries were collected from Palmerston North, Gisborne, New Plymouth, Taupo, Auckland, and Hastings during spring (September to December). Following collection, plant material was kept in a polystyrene container at 4 C until mechanical inoculation to indicator hosts.

2.6 Mechanical inoculation.

Indicator plants were inoculated with a pestle used to macerate chilled young (leaf or

flower) flowering cherry or young leaf indicator plant material in a mortar containing a small volume of transfer buffer (5% polyvinylpyrrolidone in 0.02 M sodium phosphate buffer, pH 8.0; Gardner, 1983) and 0.1 g Celite. Inoculated leaves were immediately rinsed with tap water.

2.7 Electron microscopy.

Sap from young leaf tissue of flowering cherries was prepared for electron microscopy by crushing in a 1:1 mixture of 2% sodium phosphotungstate (PTA) (pH 7.0) and 2% ammonium molybdate (AM) (pH 7.0). Carbon coated 200 mesh copper electron microscope grids were dipped in this slurry, drained on clean filter paper and air dried. Grids were examined with a Philips 201C or a Jeol JEM1200EXII electron microscope.

Droplets of purified virus particles resuspended in buffers containing 10 mM Tris.HCl or 20 mM phosphate pH 7.5 were air dried onto a carbon coated grid as before. Grids were then stained with 1% uranyl acetate (UA) (pH 4.5), 2% AM or 2% PTA.

2.8 Virus purification.

Reagents and buffers used in virus purification are detailed in Appendix 2.2.

2.8.1 Flowering cherry virus B

A number of different purification methods were tried of which the optimum was as follows: *C. sativus* plants were harvested 3 weeks following inoculation and macerated in 2 volumes of cold 0.02 M Tris.HCl buffer (pH 6.0) with 0.01 M $MgSO_4$. All purification steps were at 4 C. Macerated tissue was filtered through muslin and the filtered solution clarified with diethyl ether and carbon tetrachloride (1 ml/g tissue). Plant tissue and solvent was removed by centrifugation for 10 min. at 10 000 rpm in a GSA rotor of a Sorvall RC5B centrifuge. PEG 8000 (10%, w/v) and NaCl (1%, w/v) were added to the supernatant which was shaken for 1 h at 4 C. The precipitate was recovered following centrifugation at 10 000 rpm for 10 min. The pellet was resuspended by shaking for 2 h in 0.02 M Tris.HCl buffer (pH 6.0) and 2% Triton X-100 (v/v) with 0.01 M $MgSO_4$. The resuspended pellet was concentrated by centrifugation at 47 000 rpm for 60 min. in a Sorvall TFT65.13 rotor, and the ensuing pellet resuspended overnight in 200 µl 0.02 M Tris.HCl buffer (pH 6.0) with 0.01 M $MgSO_4$. The virus containing pellet was further purified following addition to a 10-40% sucrose gradient prepared in 0.02 M Tris.HCl buffer (pH 6.0) with 0.01 M $MgSO_4$ and centrifuged for 4 h at 25 000 rpm in a Beckman^R SW28 rotor. The gradient was fractionated on an ISCO model D density gradient fractionator attached to an ISCO model UA-5 absorbance/fluorescence monitor and the fractions containing virus as ascertained by absorbance at 254 nm were pooled and concentrated by centrifugation for 2 h at 47 000 rpm in a Sorvall TFT65.13 rotor. The virus containing pellet was resuspended overnight in 1 ml 0.02 M Tris.HCl buffer (pH 6.0) with 0.01 M $MgSO_4$.

2.8.2 Strawberry latent ringspot virus.

Virus was purified using either the method of Lot *et al.* (1972) or by the following procedure: *C. sativus* plants were harvested 3 weeks following inoculation and macerated in 2 volumes of cold 0.2 M sodium phosphate buffer (pH 7.0) with 10 mM EDTA and 10 µl ß-mercaptoethanol. All purification steps were at 4 C. Macerated tissue was filtered through muslin and clarifed with 0.25 volumes chloroform (v/v) and 0.25 volumes (v/v) butanol added whilst homogenising. The suspension was centrifuged for 10 min. at 10 000 rpm in a GSA rotor of a Sorvall RC5B centrifuge. PEG 8000 (10%, w/v) and NaCl (1%, w/v) were added to the supernatant which was shaken for 1 h at 4 C. The precipitate was recovered following centrifugation at 10 000 rpm for 10 min. The pellet was resuspended overnight in 0.02 M sodium phosphate buffer (pH 7.0). The resuspended pellet was further purified following addition to a 10-40% sucrose gradient prepared in 0.02 M sodium phosphate buffer (pH 7.0) and centrifuged for 4 h at 25 000 rpm in a Beckman^R SW28 rotor. The gradient was fractionated on an ISCO model D density gradient fractionator attached to an ISCO model UA-5 absorbance/fluorescence monitor. The fractions containing virus were ascertained by absorbance at 254 nm, either pooled or kept separate and concentrated by centrifugation for 1 h at 56 000 rpm in a Sorvall TFT65.13 rotor. The virus containing pellet was resuspended overnight in 1 ml 0.02 M sodium phosphate buffer (pH 7.0).

2.8.3 Ilarviruses.

Virus was purified according to a method based on that of P. Revill [2](personal communication to K.S. Milne). *Cucumis sativus* plants were harvested 2 weeks following inoculation and macerated in 2 volumes of cold 0.01 M sodium phosphate buffer (pH 7.5) with 0.1 g/l ascorbic acid. All purification steps were at 4 C and all solutions were cooled to this temperature. Macerated tissue was filtered through muslin and the suspension was centrifuged for 20 min. at 15 000 **g**. The supernatant was clarified with an equal volume of ether and shaken for 1 min., followed by centrifugation for 20 min. at 15 000 **g**. The lower aqueous phase was carefully removed with a 20 ml pipette and all traces of ether removed by bubbling air through the solution for 1 h. Triton X-100 was added (20 ml/l) and the virus pelleted by centrifugation at 60 000 rpm for 38 min. The pellet was resuspended in 1 ml 0.01 M phosphate buffer (pH 7.5) and further purified following addition to a 10-40% sucrose gradient prepared in 0.01 M sodium phosphate buffer (pH 7.5) and centrifuged for 4 h at 25 000 rpm in a Beckman[R] SW28 rotor. The gradient was fractionated on an ISCO Model D Density Gradient Fractionator attached to an ISCO Model UA-5 Absorbance/Fluorescence Monitor and the fractions containing virus as ascertained by absorbance at 254 nm were pooled and concentrated by centrifugation for 1 h at 56 000 rpm in a Sorvall TFT65.13 rotor. The virus containing pellet was resuspended overnight in 1 ml 0.01 M sodium phosphate buffer (pH 7.5).

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[2] Dr Peter Revill, Plant Research Institute, Burnley, Victoria.

2.9 Viral coat protein extraction and size determination.

Protein was extracted from purified virus in 10 mM sodium phosphate buffer (pH 6.5) containing 8 M urea and 1% ß-mercaptoethanol (v/v) made up to 50 µl with sterile DDW followed by boiling for 2 min. (Forster & Jones, 1979). Extracted protein was then analysed by electrophoresis following addition of 10x BPB (0.25% bromophenol blue (w/v), 25% Ficoll type 400 (w/v)) on a discontinuous 11% SDS-polyacrylamide gel based on the method of Laemmli (1970) and size comparisons made with BioRad molecular weight markers (soybean trypsin inhibitor, 21,500 daltons; bovine carbonic anhydrase, 31,000 daltons; hen egg white ovalbumin, 45,000; bovine serum albumin, 66,200 daltons; rabbit muscle phosphorylase b, 97,400 daltons) following staining in Coomassie Brilliant Blue R250.

The gel was cast between the glass plates of a vertical Mini-Protean gel system, and the resolving mix (11% polyacrylamide, 1:40; bis:acrylamide) was poured from stock solutions following the addition of 0.1% TEMED (v/v) (N,N,N',N'-tetramethylethylenediamine, BRL) and 0.04% fresh ammonium persulphate (v/v of a 10% solution) leaving about 1.5 cm at the top of the plates. The polymerising solution was overlaid with about 1 ml of DDW to ensure a flat upper surface and to stop inhibition of polymerisation by oxygen. Following polymerisation the water was poured off, the surface rinsed with stacking gel solution, 3% stacking gel (3% polyacrylamide, 1:4) poured on top and a teflon gel comb inserted. Tank buffer (0.025 M Tris.HCl, 0.19 M glycine, 0.1% SDS; pH 8.3) was added once the stacking gel had polymerised, then

samples were added and electrophoresed at 80-100 V for 60-180 min.

Staining was achieved by removing the gel from glass casting plates and placing in a solution of 0.125% (w/v) Coomassie Brilliant Blue R250 in 40% methanol (v/v) and 7% glacial acetic acid (v/v) for 20 min. The gel was destained in a fresh solution of 40% methanol and 7% glacial acetic acid which was changed approximately every 30 min. until the gel stopped changing colour.

2.10 General nucleic acid methods.

2.10.1 Phenol:chloroform extraction of nucleic acids.

Nucleic acids were mixed with an equal volume of water saturated phenol and chloroform (1:1, v/v). Following mixing, the phases were separated by centrifugation (MSE microfuge, 13 000 rpm for 10 min), the aqueous phase removed and re-extracted in phenol:chloroform, followed by extraction in an equal volume of chloroform to remove traces of phenol.

Water saturated phenol was prepared following addition of 200 ml sterile DDW to 500 g phenol (May & Baker; A.R. grade) and incubating at 37 C until dissolved. Chloroform (May & Baker; A.R. grade) was stored separately and added when required.

2.10.2 Ethanol precipitation of nucleic acids.

The NaCl concentration of nucleic acid samples was adjusted to 150 mM, then two volumes of absolute ethanol were added, and the solution incubated at -18 C overnight. Nucleic acid was collected following centrifugation at 4 C for 15 min. at 13 000 rpm. Following removal of the supernatant, the pellet was washed by addition of 70% ethanol and centrifugation as before. The nucleic acid pellet was then vacuum dried and resuspended in an appropriate volume of sterile DDW. DNA was stored at -18C, and RNA was stored at -70 C.

2.10.3 Spin dialysis.

Unincorporated [α-^{32}P]-labelled nucleotides, excess salts in hexamer priming, and NaOH from denatured dsDNA during DNA sequencing were removed by spin dialysis through Sepharose CL-6B (Pharmacia) (Murphy & Kavanagh, 1988).

Autoclaved glass beads (50 µm diameter) were pipetted into a 650 µl microfuge tube which had been pierced with the tip of a needle. The tube was then filled with a slurry of autoclaved Sepharose CL-6B and placed within an empty pierced 1.5 ml microfuge tube. The Sepharose was dried by centrifuging at 2000 rpm for 3 min. in a MSE bench

centrifuge. The sample in a maximum volume of 100 μl was then centrifuged through the Sepharose (2000 rpm, 3 min.) and collected in an intact 1.5 ml microfuge tube.

Sepharose CL-6B (Pharmacia) was prepared by washing to remove fines and preservative. This was achieved by addition of five volumes TE (10 mM Tris.HCl pH 8.0, 1 mM EDTA), allowing to settle, and decanting off TE containing contaminants. This was repeated 2-3 times, and washed Sepharose was resuspended in 0.5 volumes of TE and autoclaved.

2.10.4 Restriction enzyme digestions.

DNA was digested with 5-10 units restriction endonuclease per μg DNA in the suppliers recommended buffers for 1-2 h at 37 C. Completed digestions were then electrophoresed on a 1% agarose gel (Chpt 2.10.5).

2.10.5 Non-denaturing gel electrophoresis.

Purity and size of nucleic acids was determined following electrophoresis in a horizontal slab gel in native conditions for 30 min. at 100 V. The gel was 1% agarose (w/v) (low EEO, BRL) in sterile TBE (0.089M Tris, 0.089M boric acid, 0.002M EDTA). Molten

agarose was cooled to 60 C before pouring into a gel casting slab and then a gel comb was placed at one end of the slab. Nucleic acids were diluted 1:10 with 10x BPB (0.25% bromophenol blue (w/v), 25% Ficoll type 400 (w/v)), added to wells and electrophoresed at 100 V in TBE. Following electrophoresis the gel was stained by soaking in a solution of 0.5 µl/ml ethidium bromide in DDW for 10 min. The gel was destained for 10 min. in sterile DDW and viewed by a UV transilluminator (Ultra-Violet Products Inc.). If a photograph was required, it was taken with a Polaroid MP-4 land camera with a red filter.

2.10.6 Alkaline agarose gels for cDNA.

To determine the products obtained from first strand cDNA synthesis, radioactively labelled product was run on an alkaline agarose gel prepared according to the method of Sambrook *et al.* (1989). Agarose (0.5g, low EEO, BRL) was dissolved in 50 ml buffer (50 mM NaCl, 1 mM EDTA), poured into a gel casting block and a gel comb inserted. After setting, the gel was submerged in electrophoresis buffer (30 mM NaOH, 1 mM EDTA) for 30 min. to allow buffer to soak into the gel. The sample in loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, 0.025% bromocresol green) was added to wells and was electrophoresed at 50V until loading dye had migrated approximately 2/3rds of the distance through the gel. Following electrophoresis behind a perspex screen, gels were dried for 3h on paper towels, then for a further 2h or until dried on a bed of paper towels on a vacuum gel drier (BioRad). Autoradiography was performed for 24 h to 72 h in a Kodak X-Omatic intensifying screen at room temperature.

2.10.7 Gel purification of DNA fragments.

Following electrophoresis in agarose gels and staining with ethidium bromide (0.5 µl/ml), DNA bands were removed with a sterile scalpel blade and placed in a sterile microfuge tube.

Extraction and purification of DNA from agarose gel was carried out using the Geneclean™ Kit (Bio 101 Inc.) as follows: sodium iodide (6 M, 250 µl) was added to the gel slice which was then melted by heating at 55 C for 5 min. TBE modifier™ (50 µl) and Glassmilk™ (silica matrix in water, 5 µl) were added to molten agarose and placed on ice for 5 min. to allow the DNA to bind to the Glassmilk™ which was then centrifuged at 13 000 rpm for 25 s and washed with 600 µl New Wash™. Centrifugation and washing was repeated two more times, and on the third wash centrifugation was for 45 s. The supernatant was aspirated carefully, the pellet resuspended in 20 µl sterile DDW, incubated at 55 C for 5 min. to remove DNA from Glassmilk™, then centrifuged for 45 s and supernatant containing purified DNA removed and placed in a fresh sterile microfuge tube. Gel purified DNA was stored at -20 C.

2.11 Viral RNA extraction.

2.11.1 Extraction of RNA from purified virus.

RNA was extracted by the addition of 150 mM NaCl and 1% SDS (sodium dodecyl sulphate, w/v) to freshly purified virus preparations. Immediately after the addition of SDS, 0.5 volumes each of chloroform and water saturated phenol were added. The mixture was incubated at 37 C for 15 min., and then centrifuged at 13 000 rpm for 10 min. in an Eppendorf 5414 microfuge at 4 C. The supernatant was re-extracted with phenol:chloroform and then precipitated following the addition of 2 volumes of absolute ethanol and overnight incubation at -20 C. The viral RNA was collected by centrifugation, then washed with 70% ethanol, vacuum dried and resuspended in 20 μl sterile double distilled water and stored at -70 C.

All manipulations of RNA were made using disposable sterile rubber gloves to avoid contamination with RNases. All glassware was baked overnight at 160 C. Solutions were used exclusively for RNA work and were sterilised by autoclaving at 121 C for 15 min. unless otherwise stated. Gel boxes were kept solely for RNA work and RNases removed either with 0.1% (v/v) diethylpyrocarbonate (DEP) or with alcoholic KOH (2 M KOH in 95% ethanol).

2.11.2 Size determination of RNA by formaldehyde denaturing electrophoresis.

The buffers and reagents for formaldehyde gels are detailed in Appendix 2.3.

Size of RNA was determined following analysis on a formaldehyde denaturing gel with BRL RNA molecular weight markers (0.24, 1.35, 2.47, 4.40, 7.46 and 9.49 kb), alfalfa mosaic virus (0.88, 2.04, 2.59, and 3.64 kb) and, on some occasions, white clover mosaic virus (5.85 kb).

In a fume hood, 16.7% formaldehyde (v/v) and 1.7% MOPS buffer (pH 7.0) (v/v) were added to molten 1% agarose, the mixture poured into a casting slab and a gel comb was inserted. Following setting, the gel was submerged in Tank buffer (10% MOPS buffer and 8% formaldehyde).

RNA was denatured by heating to 65 C for 2 min. in 3 volumes of denaturation buffer. Loading buffer was added to samples (1:10, v/v) which were then loaded into wells in the gel and electrophoresed at 100V for 1 h.

2.11.3 Double-stranded RNA extraction.

Replicative and intermediate form viral double-stranded RNA (dsRNA) was extracted from virus-infected plant tissue using the preferential adsorption of dsRNA to cellulose CF-11

in 18% ethanol (Morris & Dodds, 1979).

The buffers and reagents for dsRNA extraction are detailed in Appendix 2.4.

Plant tissue was ground in liquid nitrogen and phenol:chloroform extracted in the presence of glycine buffer (w/v) and 1% SDS. The extract was centrifuged for 20 min. at 10 000 rpm in a GSA rotor of a Sorvall RC-50 Superspeed centrifuge. The aqueous layer was adjusted to 18% ethanol and 0.25 g/ml cellulose CF11 was added. The mixture was shaken for 60 min. at 4 C, and then centrifuged for 10 min. at 10 000 rpm as before. The cellulose pellet was resuspended in a small volume of the supernatant and washed in a column with 120 ml STE/ethanol to remove single-stranded RNA (ssRNA). The dsRNA was then eluted off the column with four 1 ml aliquots of STE, 2.5 volumes of absolute ethanol added to the dsRNA/STE and stored at -18 C overnight. The dsRNA was collected by centrifugation (12 000 rpm, 30min), vacuum dried, resuspended in 50 µl TE (10 mM Tris.HCl (pH 7.0), 0.1 mM EDTA) and stored at -18 C.

2.12 Preparation of cDNA.

Primers used are specified in the appropriate experimental chapters.

2.12.1 First strand synthesis.

All reactions were carried out in sterile microfuge tubes and were based on the procedure of Gubler & Hoffman (1983). In order to prime RNA, 100 ng primers and 1 nM EDTA was added to 1 µg viral RNA. The mixture was heated at 65 C for 5 min. to remove secondary structure, left at room temperature for 10 min. and then placed on ice.

For first strand synthesis, 10x RT buffer (25 mM Tris.HCl pH 8.3, 4 mM $MgCl_2$, 50 mM KCl, 200 µM dithiothreitol) 500 µM dATP, dGTP, dCTP, dTTP and 20-30 units avian myeloblastosis virus reverse transcriptase (RT) (Life Sciences Inc.) were added to the above mixture and incubated at 42 C for 2 h.

2.12.2 Second strand cDNA synthesis.

Second strand cDNA synthesis was achieved by adding second strand buffer (100 mM Tris.HCl pH 8.1, 470 mM KCl, 20 mM $MgCl_2$), 200 µM dATP, dGTP, dCTP, dTTP, 1 unit of ribonuclease H (BRL), 20 units of *E. coli* DNA polymerase I (BRL) to the RNA/DNA hybrid obtained in the previous reaction and incubating for 2 h at 16 C. Following second strand synthesis, ends of cDNA were blunted in preparation for blunt cloning by addition of 10 units T4 DNA polymerase (Promega) and incubation for 10 min. at 37 C, or restriction enzyme digested as recommended by the supplier.

The dscDNA was extracted with phenol:chloroform and ethanol precipitated (Chpt. 2.12.1). The nucleic acids were collected by centrifugation for 10 min. at 13 000 rpm in an Eppendorf microfuge, washed in 70% ethanol and then the supernatant was carefully removed and the pellet vacuum dried. The pellet was then resuspended in sterile DDW.

When cDNA synthesis was traced by labelling with [α-^{32}P]dATP, 10% of each reaction (first strand and second strand synthesis) were aliquoted into a separate microfuge tube and 0.5 μl [α-^{32}P]dATP was added to the mixture in a parallel reaction. The products of first strand synthesis were analysed on alkaline agarose gels (Chpt. 2.10.6). The products of second strand synthesis were analysed on 1% agarose gels in TBE (Chpt. 2.10.5). Gels were dried and autoradiographed (Chpt. 2.10.6).

2.13 Transformation of *Escherichia coli*.

The following *E. coli* strain was used for plasmid preparation:

E. coli strain MC1022 *ara*D139 del(*ara leu*)7697 ϕ80d*lac*Z M15 *gal*U *gal*K *str*A (Casadaban & Cohen, 1980).

2.13.1 pUC19 cloning.

The buffers and reagents used in pUC19 cloning are detailed in Appendix 2.5.

Complementary DNA was cloned into the bacterial plasmid pUC19 and transformed into *E. coli* strain MC1022. The plasmid pUC19 has a polylinker region, which when cloned into interrupts the α-peptide of the ß-galactosidase gene, resulting in loss of the ability to metabolise 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (BCIG). When grown on agar containing BCIG, recombinant plasmid colonies appear colourless compared with non-recombinant plasmids with an intact ß-galactosidase gene which appear blue (lac+). Plasmid pUC19 also contains a β-lactamase gene conferring on the transformed bacteria the ability to metabolise ampicillin. Thus white colonies growing on media containing ampicillin usually represent colonies containing a cDNA insert in the genome of plasmid pUC19. cDNA was usually cloned into the *Sma*I polylinker site of pUC19.

2.13.1.1 Ligation of cDNA to pUC19.

Ligation of cDNA to plasmid pUC19 was accomplished by adding 1 μl of the cDNA produced in the preceding reaction (2.13) to 100 ng *Sma*I cut pUC19 in ligase buffer (25 mM Tris.HCl (pH 7.6), 5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, 2.5% (w/v) polyethylene glycol-8000; BRL) and 0.1-0.4 units T4 DNA ligase (BRL). The ligation was allowed to proceed 2-12 h at room temperature.

Ligation control reactions of vector only, vector and ligase, and competent cells only were carried out as well as the ligation reaction to assess the extent of background deletion ligations and contamination.

2.13.1.2 Transformation of *E. coli* strain MC1022.

Competent cells of *E. coli* strain MC1022 were produced by inoculating 10 ml of 2x TY (bacto-tryptone 16g/l, bacto-yeast extract 10g/l, NaCl 5g/l) with a loopful of a stock culture of MC1022. Following overnight incubation at 37 C with agitation at 225 rpm, 100 ml of 2x TY was inoculated with 1 ml of the overnight culture. This was grown for 1-2 h at 37 C with shaking until mid log phase (OD 660 nm of 0.4-0.7) after which the culture was placed on ice for 1 h. The *E. coli* cells were collected by centrifugation at 2000 rpm for 10 min. in the coldroom and resuspended in 10 ml ice-cold 50 mM $CaCl_2$. After 30 min. on ice, the cells were again centrifuged at 2000 rpm for 5 min. and resuspended in 4 ml ice-cold 50 mM $CaCl_2$ for 30 min. or until required.

Five µl of ligation reaction was added to 20 µl water in a microfuge tube, and chilled on ice for 5 min. Competent cells, 50 µl per tube, were added to chilled and diluted (5 µl ligation reaction in 20 µl water) ligated pUC19 and the mixture chilled on ice for 20 min. or longer. Cells were heat shocked for 1 min. at 42 C and then placed on ice for 1 min. Transformed cells were transferred to disposable test-tubes containing 0.5 ml 2x TY and incubated at 37 C for 1 h with shaking (225 rpm). Dividing cells were plated on two L

plates containing 100 μg/ml ampicillin and 40 μg/ml BCIG per treatment and incubated at 37 C overnight.

2.13.1.3 Small-scale Plasmid DNA Isolation from *E. coli* (Minipreps).

White colonies were picked off L plates containing 100 μg/ml ampicillin and 40 μg/ml BCIG with a sterile toothpick, concurrently subcultured and inoculated to 3 ml of 2x TY containing 100 μg/ml ampicillin. Liquid cultures were incubated on a shaker at 225 rpm at 37 C overnight. Following incubation, the inoculated broth was aliquoted into microfuge tubes and centrifuged at 6500 rpm for 2 min. The supernatant was removed by aspiration and the pellet resuspended in 200 μl STET (Appendix 2.5) buffer. Bacterial cell membranes were lysed by the addition of 5 μl lysozyme (40 mg/ml) and contaminating RNA removed by the addition of 1 μl RNase A (10 mg/ml) to the resuspended pellet and incubation at room temperature for 5 min. The mixture was boiled for 45 sec., then centrifuged at 13 000 rpm for 45 min. in a Heraeus microfuge to pellet cell debris and chromosomal DNA. The pellet was removed and discarded with a sterile toothpick, and 20 μl of the supernatant was added to 2 μl of 10x BPB (0.25% bromophenol blue (w/v), 25% Ficoll type 400 (w/v)) and electrophoresed (Chpt. 2.10.5). If inserts were larger than 400 bp, the remaining supernatant was further purified by precipitation of the DNA with 10 μl CTAB (cetyltrimethylammonium bromide; Del Sal *et al.*, 1988). The precipitate was collected following centrifugation at 13 000 rpm for 5 min. and the supernatant discarded. The pellet was resuspended in 300 μl 1.2 M NaCl, to which was added 750 μl absolute

ethanol. Following incubation at -18 C for 1 h or longer, the precipitated DNA was pelleted (centrifugation at 13 000 rpm for 15 min), washed with 70% ethanol, vacuum dried and resuspended in sterile DDW.

2.13.1.4 Large-Scale Plasmid DNA Isolation From *E. coli* (Maxipreps)

White colonies which had an insert of interest were prepared for dideoxy sequencing using an alkaline denaturation method which results in larger quantities of DNA of greater purity than miniprep DNA (Anonymous, 1989). Following denaturation, selective renaturation of plasmid DNA was achieved by neutralisation of the solution. Precipitation using PEG (polyethylene glycol) was included to remove contaminants which could interfere with restriction digests or sequencing procedures.

Terrific broth (Appendix 2.5) containing ampicillin (100 µg/ml) was inoculated with a loopful of bacteria from colonies of interest and incubated with shaking (225 rpm) overnight at 37 C. Cells were collected following centrifugation at 5000 **g** for 15 min. at 4 C and the pellet was resuspended in 6 ml fresh lysis buffer (25 mM Tris.HCl (pH 7.5), 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme). To the resuspended pellet, 12 ml of 0.2 M NaOH and 0.1% SDS was mixed by inversion and incubated on ice for 10 min. Sodium acetate (7.5 ml, 3 M, pH 4.6) was added and mixed by inversion and lysis completed following incubation on ice for 10 min. The lysed cells were removed by centrifugation for 10 min. at 13 000 rpm. To the supernatant, 500 µg of RNase A was

added and incubated at 37 C for 2 h, after which the solution was extracted twice with phenol and chloroform and once with chloroform followed by precipitation with ethanol. DNA was pelleted by centrifugation (13 000 rpm for 20 min.) and dissolved in 1.6 ml sterile DDW. To the resuspended pellet was added 0.32 ml 5M NaCl and 2 ml 13% PEG-8000 and the suspension was incubated at 4 C overnight. The purified DNA was pelleted by centrifugation at 10 000 rpm for 10 min., washed with 70% ethanol, vacuum dried and resuspended in sterile DDW.

2.14 Probing RNA immobilised on nitrocellulose.

2.14.1 Northern blotting.

The transfer of RNA from a gel to a nitrocellulose support (Northern blotting) was accomplished following electrophoresis on a formaldehyde denaturing gel (Chpt. 2.11.2). The gel was then placed upside down on a nitrocellulose membrane cut to size which had been pre-wetted in 2x SSC (300 mM NaCl, 30 mM sodium citrate). Two pieces of 3MM paper were placed on the nitrocellulose, and the exposed surface of the gel placed on the 3MM paper covering the glass plate of the gel transfer apparatus. Parafilm was placed around the gel so that the pile of tissues placed on top of the gel was not able to touch the 3MM paper of the gel transfer apparatus. The gel transfer apparatus consisted of a plastic container containing 2x SSC about 10 cm deep at the bottom, in which a raised glass plate was placed. A rectangular piece of 3MM paper acting as a wick was placed over the

glass plate so that two overhanging ends were inserted in the liquid. A weighted plastic lid was placed on top of the pile of tissues. The gel was left overnight to allow RNA to transfer to the nitrocellulose by capillary action as the 2x SSC in the bottom of the container was drawn up through the gel and into the pile of tissues. The nitrocellulose was then removed, oven-dried (80 C for 2 h) and stored in a plastic bag.

2.14.2 Dot-blotting.

To test cDNA for specificity to virus-infected plants or RNA the sample was immobilised on nitrocellulose supports. Nitrocellulose was prepared by soaking in 20x SSC (3 M NaCl, 0.3 M sodium citrate) for 5 min. Following air-drying, 1 µl of RNA or sap extracted in water was added to the nitrocellulose, which was then oven-dried for 2 h at 80 C to bind the nucleic acid to the nitrocellulose. The dot-blot was then stored at room temperature in a plastic bag.

2.14.3 Random hexamer-primed labelling of DNA.

Radioactively labelled probes were prepared according to the method of Feinberg & Vogelstein (1983).

Reagents and buffers used for hexamer priming are detailed in Appendix 2.6.

Complementary DNA was removed from vector DNA by restriction enzyme digest, gel purified (Chpt. 2.10.7) and 25-50 ng resuspended in 12.5 μl sterile DDW. The DNA was heated to 100 C for 2 min. to remove secondary structure, cooled on ice, followed by the addition of 11.5 μl A reaction mix, 2 μl BSA (10 mg/ml RNase free), 3 μl [α-^{32}P]dATP and 5 units of DNA polymerase I (Klenow fragment). The reactants were incubated at room temperature overnight to allow the synthesis of radioactively labelled primer extension products after which the reaction volume was made up to 50 μl with sterile DDW and spin dialysed (Chpt. 2.10.3) to remove unincorporated nucleotides and excess salts.

2.14.4 Hybridisation with nucleic acid immobilised on nitrocellulose.

Reagents and buffers used in hybridisation of labelled DNA to nucleic acid immobilised on nitrocellulose are detailed in Appendix 2.7.

Nitrocellulose prepared as described (Chpt. 2.14.1 & 2.14.2) was prehybridised for 1 h or more in hybridisation buffer, 1 mg denatured salmon sperm DNA boiled for 2 min. prior to addition, 10x Denhardts solution, 0.1% SDS (w/v), at 45 C.

Hexamer primed probe prepared as described (Chpt. 2.14.3) was boiled for 2 min. then added to the hybridisation buffer by injection into the plastic bag. Hybridisation was allowed to take place overnight at 45 C. Hybridised nitrocellulose was washed in 2x SSC, 0.2% SDS at room temperature for 15 min., then in this buffer again at 65 C for 15 min.,

then in 0.2x SSC, 0.2% SDS at 65 C for 5 min. (low stringency). The washed nitrocellulose was then placed on 2 layers of 3MM paper, placed in a sealed plastic bag and autoradiographed in a Kodak X-Omatic intensifying screen at room temperature for 1-3 days.

2.15 DNA sequencing.

The chain termination method of sequencing double-stranded plasmid pUC19 cloned DNA was used (Sanger *et al.*, 1977; Murphy & Kavanagh, 1988). In this method, pUC/M13 primers bind to the reverse and normal nucleotide sequences of pUC19 and the enzyme Sequenase^R (a modification of T7 DNA polymerase, purchased from United States Biochemical Corporation) catalysed the extension of copy DNA from these primers. Normal bases (dNTPs; 2'-deoxynucleoside 5'-triphosphates) and termination bases (ddNTPs; 2',3'-dideoxynucleoside 5'-triphosphates, which lack the 3'-OH group necessary for DNA chain elongation) were included in the reaction mix. Four separate reactions with each of the four ddNTPs (ddATP, ddGTP, ddTTP, ddCTP) provide total sequence information. Radioactively labelled [α-^{32}P]dATP was included in the reaction as well to enable tracing of the products. Plasmid DNA for sequencing was isolated by miniprepping (Chpt. 2.13.1.3) or maxiprepping (Chpt. 2.13.1.4). The procedures described in the Sequenase^R version 2.0 kit (United States Biochemical Corporation) were followed, and 8 μg DNA, 10 μM (1000-1500 Ci/mM) [α-^{32}P]dATP were routinely used.

The buffers and reagents used in sequencing reactions are detailed in Appendix 2.8.

2.15.1 Preparation of Sequencing gel.

Glass plates were prepared for use in electrophoresis of sequencing products by first scrubbing with detergent, then after thorough rinsing and drying with paper towels, were cleaned with 95% ethanol. The lower surface of the upper "eared" glass plate was then siliconised with dichlorodimethylsilane. The two plates (the bottom one without "ears") were then taped together at the sides after the insertion of wedge or 0.8 mm non-wedge spacers, followed by taping along the bottom. The upper edge was left free. A 6% acrylamide/7M urea gel in TBE was polymerised with 1% ammonium persulphate (w/v) and 0.1% TEMED (v/v), the gel was poured between the two taped plates and a sharkstooth comb inserted upside down at the top of the gel. The gel plates were held together with paper clamps. After polymerisation, un-polymerised acrylamide was washed off, the sharkstooth comb reversed, inserted so that it was just touching the gel/buffer interface and washed thoroughly. The bottom tape was removed, and the paper clamps used to attach the gel to a vertical sequencing gel apparatus. The gel was pre-electrophoresed for 30 min. in TBE at 35 mA.

2.15.2 Sequencing reactions.

DNA for sequencing was obtained using the CTAB miniprep method (Chpt. 2.13.1.3) or the maxiprep method (Chpt. 2.13.1.4). DNA (1 µg/µl) was denatured in 27 mM EDTA and 277 mM NaOH at room temperature for 15 min. The DNA was then spin dialysed to remove NaOH and added to 40 mM Tris.HCl (pH 7.5) and 0.5 pmol reverse or normal primer. The reactants were then incubated at 37 C for 15 min. to allow primers to anneal to DNA. All sequencing reactions were carried out using Sequenase™ kits.

The reaction mixture (Appendix 2.8) was then added to the primer/DNA solution and four aliquots were added to the side of each of four tubes containing 80 µM of each of three of dATP, dGTP, dCTP or dTTP, 80 µM of the appropriate dideoxy-nucleotide, either ddATP, ddGTP, ddCTP or ddTTP, and 50 mM NaCl (termination mixes, Appendix 2.8). Following mixing by centrifugation, the solutions were incubated for 5 min. at 37 C after which 4 µl STOP solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% xylene cyanol FF) was added to tube sides and mixed by centrifugation. Samples were then denatured by incubation at 100 C for 3 min., 2 µl denatured extension products loaded onto the acrylamide gel and electrophoresed at 35 mA for 90-180 min.

Following electrophoresis, the gel was fixed in a solution of 10% drum glacial acetic acid (v/v) and 12% drum methanol (v/v) for 20 min., then drained and transferred to a piece of Whatman 3MM paper cut to size, covered with cling wrap and vacuum dried at 80 C for 60-120 min. Autoradiography was performed for 24 h to 72 h between wooden boards at

room temperature.

2.16 Polymerase chain reaction (PCR) amplification.

2.16.1 PCR amplification.

PCR amplifies a segment of DNA between two primers by repeated polymerisation cycles catalysed by a thermally stable enzyme. Primers are both available commercially and can be made using a DNA synthesizer. Primers used in this study are discussed in detail in the relevant experimental chapters. The PCR reactions used routinely included 1 ng of template DNA (single or double stranded cDNA), 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP and 0.2 mM dTTP, 0.25 μM normal and reverse primers, 5 mM KCl, 2 mM Tris.HCl (pH 8.0), 0.25 mM $MgCl_2$ and 0.5 units Taq DNA polymerase in a total volume of 100 μl. To prevent evaporation of reagents during heating paraffin oil was layered on top of each reaction. Reactions in 500 μl microfuge tubes were then placed in a DNA thermal cycler (Perkin-Elmer Cetus) and secondary structure of DNA removed by heating to 94 C for 5 min., then 30-40 cycles of denaturing, annealing and extension were executed. Temperatures and times are specified in the relevant experimental chapters.

To remove paraffin oil after completion of cycling, 100 μl chloroform was added, the mixture vortexed, then centrifuged at 13 000 rpm for 5 min. The top aqueous layer was

retained and phenol:chloroform extracted, then ethanol precipitated (Chpt. 2.10.2).

2.16.2 Cleavage of synthetic oligonucleotides from a column.

Oligonucleotides were synthesised in a Milligen/Biosearch Cyclone™ Plus DNA synthesiser. To facilitate cleavage and deprotection from columns in which they had been synthesised, an empty 2 ml syringe was attached to one end, and a 2 ml syringe containing 3 ml 30% ammonium hydroxide (NH_4OH) was attached to the other end of the column. The NH_4OH was passed through the column 3-4 times by depressing the syringe plunger, and then left at room temperature for 45 min. This process was repeated 3-4 times, then finally all the solution was drawn into one of the syringes, removed from the column and about 600 µl aliquoted into 1.5 ml microfuge tubes. After incubation at room temperature for 16-24 h, the DNA was dried under a vacuum and stored dry at -18 C. When required oligonucleotides were resuspended in 100 µl water, concentration determined following spectrophotometric examination at 280 nm, and adjusted as required.

2.17 Computing.

The computer programmes of Devereux *et al.* (1984), comprising the University of Wisconsin GCG package version 6.1, installed on a MicroVAX 3400 computer were used for the analysis of sequence data and searching of sequence databanks.

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CHAPTER THREE

Sap-transmissible viruses in flowering cherry in New Zealand

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Abstract Six sap-transmitted viruses were identified during a study of 434 flowering cherry trees in the North Island of New Zealand. These included *Prunus* necrotic ringspot ilarvirus strain G (PNRSV-G), apple mosaic ilarvirus (ApMV), flowering cherry virus B (FCVB), strawberry latent ringspot virus (SLRV), prune dwarf ilarvirus (PDV) and

flowering cherry virus I (FCVI). Of these, ApMV, FCVB, SLRV and FCVI were new records for this host. FCVB and FCVI are newly described viruses with characteristics of members of the bromovirus and ilarvirus groups respectively. The most common virus was PNRSV-G (30.6%); the other viruses ranged in incidence from 10.2% (FCVB) to 0.5% (PDV). A further nine viruses were also detected by mechanical transmission, but were not characterized in this study. Repeated sampling of 30 flowering cherry trees during late winter and early spring showed that ELISA was more sensitive for detecting PNRSV-G infection of flowering cherry trees than sap-transmission.

Keywords flowering cherry; viruses; sap-transmission; *Prunus* necrotic ringspot virus.

INTRODUCTION

Flowering cherry is an ornamental deciduous tree in the family Rosaceae. The species which occur in New Zealand include *Prunus campanulata* Maxim., *P. cerasifera* J.F. Ehrh., *P. sargentii* Rehd., *P. serrulata* Lindl. *sensu stricto*, *P. subhirtella* Miq., and *P. yedoensis* Matsum.. Interspecific hybrids are common, and discrimination is difficult. Therefore the broad species concept of Chadbund (1972) and Bean (1976) is adopted in this study and all flowering cherries referred to as *P. serrulata sensu lato.*

A range of virus-like symptoms have been observed on both young and mature leaves of flowering cherry in New Zealand. These include chlorotic mottling, blotches, vein netting, stippling closely associated with the veins and necrotic shot-holes, and witches' brooms. In studies in the USA and the UK, symptoms of chlorotic patterns on leaves, death and

die-back have been described in *P. serrulata* and attributed to infection with prune dwarf ilarvirus (PDV) (Ramaswamy & Posnette 1972) and *Prunus* necrotic ringspot ilarvirus (PNRSV) (Milbrath 1957; Zeller & Milbrath 1942). One further virus, tobacco ringspot nepovirus (TobRSV), has been reported in flowering cherry in the USA (Uyemoto et al. 1977).

Although several studies have been conducted in New Zealand on other Rosaceous plants (e.g. *Prunus avium*: Fry & Wood 1973; *Rosa* spp.: Gardner 1983), studies have not been conducted on mechanically transmissible viruses of flowering cherry trees. This information is required before an indexing service for nursery stocks of flowering cherry similar to that available in New Zealand for PNRSV in roses, can be implemented.

This paper reports results of a study of sap-transmissible viruses from flowering cherry trees. Results show that a number of viruses are present in New Zealand flowering cherry trees, that more than 48% of flowering cherry trees in New Zealand which were tested are infected with one or more viruses, and that PNRSV-G is the most common of these.

MATERIALS AND METHODS

Incidence of sap-transmitted viruses in flowering cherry trees

Four hundred and thirty-four flowering cherry trees from five locations in the North Island of New Zealand (Auckland, Gisborne, New Plymouth, Palmerston North, and Taupo, Appendix VII) were surveyed in 1987 by mechanical inoculation to *Cucumis sativus* L. 'Heinz Pickling'; 306 of these were also mechanically inoculated to *Chenopodium quinoa* Willd.. Trees were sampled between spring bud break (September) and the time of

development of secondary leaves (December). Flowers, or young expanding leaf tissues were sampled, depending upon availability. A single tissue sample was collected from one branch of each tree. Tissues were macerated in buffer (5% polyvinylpyrrolidone M.W. 40000 in 20mM sodium phosphate buffer, pH 8.0; Gardner 1983) containing 0.01g Celite and rubbed onto cotyledons of *C. sativus* and leaves of *C. quinoa.* Virus isolates were maintained in cucumber or *C. quinoa* and transferred to fresh seedlings every 2-5 weeks.

ELISA

The double antibody sandwich ELISA method of Clark & Adams (1977) was used. ELISA plates were from Dynatech. In all ELISA tests a known isolate of the appropriate virus was included. Isolates of ApMV (from apple), PNRSV-G and PDV (both from peach) were obtained from G. A. Wood[3]. An isolate of SLRV was obtained from the Scottish Crop Research Institute. Antisera to ApMV, PNRSV-G and PDV were obtained from R. W. Fulton[4]. Antiserum to SLRV was obtained from the Scottish Crop Research Institute.

For ELISA tests with antisera to ApMV, PNRSV-G, PDV and SLRV, sap extracts were diluted 1/10 in PBS-Tween 20 containing 2% w/v polyvinylpropolidione (extraction buffer). The coating gamma-globulins of each antiserum were at a concentration of 1 µg/mL. Alkaline phosphatase-labelled gamma-globulins were diluted 1/200 in extraction

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buffer containing 0.2% w/v ovalbumin. Two wells were used for each sample, and positive reactions were taken as at least twice the absorbance of healthy samples.

Investigation of types of tissue and time of sampling.

Because of the difficulties encountered in re-isolating viruses from infected flowering cherry trees, a trial was undertaken to investigate the efficacy of types of tissue and time of sampling on virus detection. ELISA testing of dormant buds was compared with forced and fresh tissue. Forced tissue was young leaf and flower tissue produced from buds on twigs placed in tap water in a glasshouse. Sap-transmission from forced tissue was compared with sap-transmission from young expanding leaves from the same trees. Over a four-month period in which suitable tissues were available, 30 trees were sampled at monthly intervals. Four leaf/flower samples from each of north, south, west and east sectors were combined from each of the 30 flowering cherry trees and mechanically inoculated to cucumber and subsequently identified by ELISA using antisera to PNRSV-G, ApMV and SLRV.

RESULTS

Incidence of sap-transmitted viruses

Cucumber was used as the major indicator host, as most sap-transmissible viruses of

Prunus spp. can be transmitted to this plant. Virus-like symptoms were induced in cucumbers mechanically inoculated with extracts from 211 of the 434 flowering cherry trees tested from five locations in the North Island of New Zealand (Auckland, Gisborne, New Plymouth, Palmerston North, and Taupo; Appendix VII). One hundred and thirty-three cucumbers showing virus-like symptoms were infected with PNRSV-G as determined by ELISA. On this basis 30.6% of the flowering cherry trees tested were infected with PNRSV-G. The identity of six isolates of PNRSV-G chosen at random was confirmed by host range and symptoms, particle morphology in purified preparations, and the sizes of RNA and coat protein.

The rest of the viruses isolated to cucumbers were divided into 14 'classes' based on symptoms in cucumber, and where used, *C. quinoa* (Table 1, Appendix V). Virus isolates in 'class' I were identified as a tentative new member of the bromovirus group and named flowering cherry virus B (FCVB) based on morphology, sizes of the viral RNA and coat protein (Everett et al. 1993a). FCVB was initially found in 14 flowering cherry trees. However, results of mechanical transmission studies from cucumber to cucumber of viruses placed in other 'classes' indicates that the incidence of FCVB may be significantly higher.

In some instances transfer of ApMV and PRNSV-G from systemically infected tissue was unsuccessful. This process of 'running out' has been referred to in other studies with ilarviruses (Loesch & Fulton 1975; Tolin 1965). In 30 instances where 'running out' occurred during transfer of cucumber leaves systemically infected with ilarviruses, symptoms of FCVB were produced on the cotyledons of the inoculated cucumber, indicating that this virus was also present, but masked by the presence of the co-infecting ilarvirus.

Table 1: Classes of viruses other than PNRSV-G isolated from flowering cherry based on symptoms in cucumbers, *Chenopodium quinoa*, and serology.

Class	No.	Symptoms in cucumbers	Symptoms in *C. quinoa*	Causal virus	Notes
I	44	Necr. pinpoint LL, ss.	None	FCVB	Described in Everett et al. (1993a)
II	1	Chlor. LL, syst. chlor. mosaic	None	FCVI	Described in Everett et al. (1993b)
III	1	Chlor. & necr. LL, syst. necr. spots	Necr. LL, syst.fine spots	ACLSV	Tentatively identified by herbaceous host range and EM
IV	14	Chlor. LL, syst. chlor. blotches, syst. necr. spots	None	ApMV	Serologically identified
V	2	Chlor. LL, syst. chlor. flecks.	None	PDV	Serologically identified
VI	3	Chlor. LL, syst. chlor. ringspots.	Chlor. LL, syst. distortion, terminal necr.	SLRV	Identified by serology and physical characteristics (Everett et al. 1993c)
VII	19	Chlor. LL, 4-10mm.	None		Identity unknown
VIII	10	Necr. LL or chlor. LL & syst. necr. spots or syst. iv. chlor.	None		" "
IX	2	Necr. LL or chlor. LL & syst. necr. spots or syst. iv. chlor.	Syst. chlor. flecks or local chlor. ringspots		" "
X	1	Syst. rugosity of leaves	None		" "
XI	2	Syst. necr. centres of leaves.	None		" "
XII	5	Syst. tip necr.	None		" "
XIII	2	Syst. chlor. ringspots	None		" "
XIV	2	Chlor. ringspot LL	None		" "

Key: necr. = necrotic/necrosis; chlor. = chlorotic
LL = local lesions; iv. = interveinal
ss. = symptomless systemic infection; None = no symptoms
Syst. = systemic

A single virus isolate placed in 'class' II was tentatively identified as a new ilarvirus, and named flowering cherry virus I (FCVI), based on host range, morphology, serological tests, sizes of the viral RNA and coat protein (Everett et al. 1993b). A single virus isolatein 'class' III was tentatively identified as apple chlorotic leafspot virus (ACLSV) based on symptoms in a range of herbaceous indicator hosts (Everett, unpublished) and presence of 650-1100 nm flexuous filamentous rods in sap from an infected *C. quinoa*. Thirteen virus isolates placed in 'class' IV and two isolates in 'class' V were identified by ELISA as ApMV and PDV respectively. One ApMV and one PDV isolate were confirmed by host range and symptoms, particle morphology, Ouchterlony gel diffusion tests and RNA and coat protein size. The virus isolates in 'class' VI were identified as SLRV by ELISA, and further characterised as described in Everett et al. (1993c). The remaining 43 virus isolates of 'classes' VII-XIV were not identified.

Investigation of types of tissue and time of sampling

A comparison of ELISA and mechanical transmission was undertaken to determine the best methods for virus detection, and the best sampling times (Table 2).

The trial involved 30 flowering cherry trees, chosen at random in the Auckland area, which were sampled at monthly intervals. Fifteen trees were found to be uninfected and the remaining 15 were all infected with PNRSV-G. PNRSV-G was detected in all 15 trees by ELISA but in only six trees by mechanical transmission, indicating that ELISA is more sensitive for detecting this virus. However, the number of PNRSV-G infected trees detected by ELISA varied from as few as two to a maximum of nine in any one monthly

sample. None of the trees was consistently shown to be infected either by ELISA or by mechanical transmission (Table 2).

In ELISA tests, the highest number of PNRSV-G infections (nine) occurred in October and involved soft, rapidly expanding leaf tissue. A relatively high level of detection (eight) also occurred in September using buds at the onset of bud burst. Conversely, the lowest number of detections (two) occurred using dormant bud tissue collected in August. Floral tissue from buds collected in August forced in the glasshouse also gave low levels of detection (four infections).

Using mechanical transmission to cucumber, three of the 15 trees infected with PNRSV-G were also found to be infected with SLRV and one with ApMV. SLRV was mechanically transmitted from one of the infected trees on two occasions, and from both of the other two trees once (Table 2). ApMV was isolated once only, from forced tissues in August, and was not isolated from young tissues in the spring growth flush (Table 2). Detection of SLRV or ApMV by ELISA directly from the flowering cherry trees was not attempted.

DISCUSSION

In the present study, sap-transmission to cucumber showed 30.6% of 434 flowering cherry trees were infected with PNRSV-G. A further 78 (17.9%) flowering cherry trees were infected with sap-transmissible viruses other than PNRSV-G. Of the 78, one virus isolate was tentatively identified as ACLSV (0.2%), 14 were identified as ApMV (3.2%), one as FCVI (0.2%), two as PDV (0.5%) and three as SLRV (0.7%). FCVB was detected in a

Table 2: Monthly comparison of ELISA indexing in August to November, 1990, of dormant, forced and fresh flowering cherry tissue for PNRSV, as well as comparison of sap-transmission of viruses from both forced and fresh tissue for ApMV and SLRV.

	Dormant buds	Forced Buds[a]				Field tissue[b]			
	ELISA	ELISA	Mechanical transmission[c]			ELISA	Mechanical transmission		
MONTH	PNRSV	PNRSV	PNRSV	SLRV	ApMV	PNRSV	PNRSV	SLRV	ApMV
August	3/15[d]	4/12[f]	2/12[f]	0/12[f]	1/12[f]	NT[e]	NT	NT	NT
September	8/15	7/15	1/13[f]	0/13[f]	0/13[f]	NT	NT	NT	NT
October	NT	NT	NT	NT	NT	9/15	2/15	2/15	0/15
November	NT	NT	NT	NT	NT	6/15	2/15	2/15	0/15

[a]Leaf and floral tissue from buds forced under glass.
[b]Leaf and floral tissue.
[c]Mechanical transmission to cucumber ‘Heinz Pickling’ and virus isolates identified by ELISA.
[d]Number testing positive divided by number tested.
[e]Not tested, due to unavailability of tissue.
[f]lower numbers were due to buds not forcing.
Note: ELISA for ApMV and SLRV from cherry trees was not done.

total of 44 trees (10.1%). Based on symptom 'classes' in cucumber and *C. quinoa* the remaining 43 virus isolates appear to represent at least eight additional viruses.

Although comparatively common in flowering cherry in New Zealand, FCVB has not been found in other studies. It is possible that FCVB has not been detected in flowering cherry and in other *Prunus* spp. in New Zealand and overseas due to the masking of FCVB symptoms in indicator plants in the presence of ilarviruses. The frequent use of extraction buffers containing nicotine in other studies on mechanically transmissible viruses in *Prunus* spp. may explain the failure to detect FCVB, as this study has found that it cannot be transmitted in the presence of nicotine (unpublished data).

Some flowering cherry trees were infected with several viruses. For instance, PDV, PNRSV-G and ACLSV were isolated from one tree, three trees were infected with both SLRV and PNRSV-G and two others with both ApMV and PNRSV-G. FCVB was also detected in 30 trees infected with other viruses. The incidence of this virus may have been underestimated due to the difficulty experienced in identifying the virus in mixed infections.

Some trees from which viruses were not sap-transmitted, despite repeated sampling, also showed virus-like symptoms. Thus additional viruses or virus-like organisms may have been present in the sampled flowering cherry trees that were not mechanically transmissible to cucumber or *C. quinoa.* Lack of transmission from these trees, or from trees in which virus was detected only by ELISA, may be due to uneven distribution of virus within the tree (Scott et al. 1989), to variable virus concentrations in the infected trees, and to presence of non sap-transmissible viruses or virus-like organisms (Wood 1993).

In ELISA tests, the highest levels of detection of PNRSV-G from trees sampled monthly

during Spring, were from unburst buds immediately prior to bud burst, forced buds from twigs collected just prior to burst, and from flowers and the first emerging leaves in October. These results indicate that the best sampling time is close to the period of bud burst. However, in mechanical transmission tests, no pattern emerged as to the best time of sampling for PNRSV-G.

Results presented here suggest that repeated sampling is required to detect all viruses in flowering cherry trees when using either mechanical transmission or ELISA. These results indicate that although up to 14 distinct viruses were detected in flowering cherry trees in New Zealand, the incidence of these viruses is likely to have been significantly underestimated.

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CHAPTER FOUR

ILARVIRUS PURIFICATION, CHARACTERISATION AND cDNA CLONING

4.1 Introduction.

In the surveys undertaken, flowering cherries were found to be infected with a number of ilarviruses; prune dwarf virus (PDV), *Prunus* necrotic ringspot virus serotype G (PNRSV-G), apple mosaic virus (ApMV), and a tentative new ilarvirus designated flowering cherry virus I (FCVI) (Chapter 5).

As outlined in Chpt.1, ilarvirus taxonomy is tentative due to the unstable nature of these viruses and thus difficulties of purification and consequent studies. The aims of this study were to obtain cDNA to the coat protein gene of PNRSV-G and to sequence this gene. However, because of the difficulties of working with this virus attempts to do so were largely unsuccessful. A problem that was encountered was the contamination of viral RNA preparations with plant nucleic acid. The methods attempted and results are presented in this chapter.

4.2 Purification.

In this study three purification methods were evaluated: the methods of Gardner (1983), which was a modification after Fulton (1959), J. Uyemoto (pers. comm. to K.S. Milne) and a modification of P. Revill (pers. comm. to K.S. Milne).

4.2.1 Gardner's method.

Systemically infected *C. sativus* leaves were harvested and macerated at 4 C in 1.5 volumes of cold 0.02 M phosphate buffer (pH 8.0) containing 0.02 M sodium diethyldithiocarbamate (DIECA), 0.02 M β-mercaptoethanol and 5% (w/v) Dow Corning antifoam A. In order to increase yields, virus was purified from inoculated cotyledons and systemically infected leaves only, without including stems. The resultant extract was strained through muslin and separated from plant material by low speed centrifugation (20 min, 1500 *g*, Sorvall GSA rotor). The supernatant was clarified by addition of 0.8 ml hydrated calcium phosphate (HCP)/g tissue (refer Appendix 4.1 for preparation) followed by centrifugation as before. The clarified supernatant was then centrifuged at 13 000 *g* (GSA rotor) for 10 min to remove any remaining HCP and plant material. The virus was pelleted by high speed centrifugation (160 000 *g*, Sorvall TFT 80.13 rotor, for 1.5 h). The pellet was resuspended in 0.02 M phosphate buffer (pH 8.0) with a glass rod, then centrifuged at 13 000 *g* (GSA rotor) for 10 min. The supernatant was centrifuged at 160 000 *g* (Sorvall TFT 80.13 rotor) for 1.5 h then resuspended overnight in 0.6 ml 0.02 M phosphate (pH 8.0) buffer. The virus was further purified by centrifugation through a 10-40% sucrose gradient prepared in 0.02 M phosphate (pH 8.0) and fractionated on an ISCO

model D density gradient fractionator attached to an ISCO model UA-5 absorbance/fluorescence monitor. Virus containing fractions, as determined by absorbance at 254 nm, were pooled and concentrated by centrifugation (1.5 h for 160 000 *g*, Sorvall TFT 80.13 rotor). The resultant pellet was resuspended in 0.02 M phosphate buffer (pH 8.0).

4.2.2 Uyemoto's method.

Systemically infected *C. sativus* plants, including both leaves and stems, were harvested and macerated in 2.5 vol. (w/v) of tissue extraction buffer [0.06 M potassium phosphate buffer pH 8.0, containing 0.02 M DIECA and 0.02 M thioglycollic acid (Sigma, No. T-3758)] and an equal weight of crushed ice made from distilled water. The homogenate was squeezed through a double layer of cheese cloth. A few drops of chloroform were added to stop foaming, and the pH was immediately adjusted to 5.0 with glacial acetic acid to precipitate plant matter. The supernatant was clarified by centrifugation (5000 *g* for 5 min, Sorvall GSA rotor), strained through glass wool and the pH readjusted to 7.0 with saturated NaOH. The supernatant was centrifuged (5000 *g* for 5 min, GSA rotor) to remove any remaining plant matter and the virus was pelleted by centrifugation for 1.5 h at 160 000 *g* (Sorvall TFT 80.13 rotor). The pellet was resuspended in Tris acetate EDTA (TAE) buffer pH 7.8 (Appendix 4.2) using a glass rod and overnight shaking in the cold room (4 C). The virus was further purified by centrifugation through a 10-40% sucrose gradient made up in 0.5 x TAE, and virus containing fractions determined as before (Chpt. 4.2.1) were pooled and concentrated by centrifugation for 1.5 h at 160 000 *g* (Sorvall TFT 80.13 rotor).

4.2.3 Revill's method.

A modification of Revill's method as detailed in section 2.8.3 was used for the purification of PNRSV isolates.

4.3 Results.

4.3.1 Gardner's method.

Purification of PNRSV-G using Gardner's method resulted in virus preparations which were seen in the electron microscope to contain few contaminants (Fig. 4.1d). Spectrophotometric examination of fractionated sucrose gradients showed that virus profiles consisted of 2-3 peaks with minimal host contamination (Fig. 4.1c). However, yields were low (4-66 μg/100 g plant tissue, assuming an extinction coefficient of 5.0 at 260 nm ($A^{0.1\%}_{1cm}$); Loesch & Fulton, 1975). An increase in yield to 160 μg/100 g resulted from purification of cotyledons and systemically infected leaves only, instead of stems as well. However, yields were still low compared with those reported from the literature (Gardner, 1983, Patrakosol, 1985, Fulton, 1959, Revill, pers. comm.). Reduction in the amount of HCP added increased host contamination without improving yields. The same result occurred when one high/low speed centrifugation was used.

4.3.2 Uyemoto's method.

This method resulted in considerable host contamination. ELISA indexing of fractions

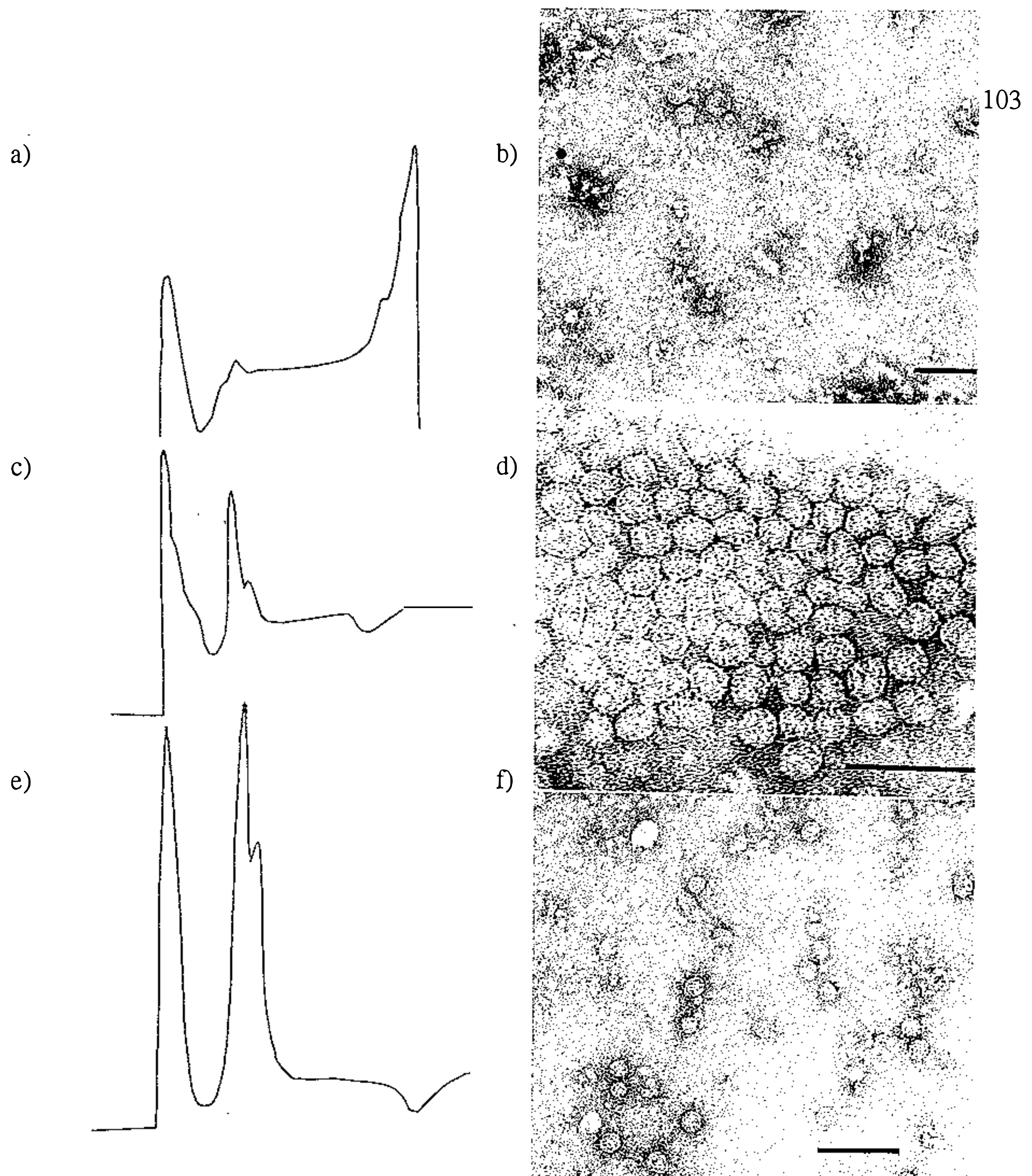

Fig. 4.1: Optical density profiles of sucrose density gradients of preparations of PNRSV-G, purified by the method of a) Uyemoto, c) Gardner and e) Revill. Sedimentation from left to right. Relative absorbance at 254 nm. Electron micrographs of purifed particles of PNRSV-G, purified by the method of b) Uyemoto, d) Gardner, and f) Revill. Bar = 100 nm.

from sucrose gradients confirmed the presence of PNRSV-G coat protein, and mechanical inoculation of these same fractions to *C. sativus* confirmed that purified virus was infectious.

Sucrose was removed from fractions following density gradient centrifugation by dialysis against 0.02 M EDTA or TAE with 0.001 M EDTA as an alternative to ultracentrifugation. However, virus was not seen in dialysed fractions following examination with the electron microscope. Centrifugation through a 20% sucrose cushion reduced host contamination, but again virus was not visible in the electron microscope. Addition of 0.03 M EDTA to tissue extraction buffer resulted in small virus peaks in sucrose density gradients, but virus particles were not visible following examination with the electron microscope.

The final high speed pellet was resuspended in 0.02 M phosphate buffer containing 0.75% glutaraldehyde. Virus particles were not visible following examination with the electron microscope. Extraction in the presence of 0.02 M EDTA did not improve yields. Centrifugation through a second sucrose density gradient resulted in complete loss of virus as evidenced by spectrophotometric examination of sucrose density gradients.

Several ultra-violet absorbing peaks were resolved by sucrose density gradient centrifugation of preparations subjected to two cycles of high speed and low speed centrifugation (Fig. 4.1a). Some virus was seen under the electron microscope following high speed centrifugation of the fractions of the sucrose gradient corresponding to these peaks, but host contamination was also observed (Fig. 4.1b) and yields were low.

This purification method was considered unsatisfactory due to low yields and to host contamination.

4.3.3. Revill's method.

Virus yields as determined by spectrophotometric methods were 3.2-8.9 mg virus/ 100 g plant material, at least 100 times greater than those obtained with either of the preceding two methods. Because of high yields, separate viral UV-absorbing peaks were not usually discernable, but for the purposes of illustration a virus preparation from a small amount of plant material is included (Fig. 4.1e). Electron microscope examination of virus preparations showed that virus was present, and appeared to be contaminated with phytoferritin (Fig. 4.1f). However, because of the high yields this method was adopted for PNRSV and for other ilarviruses in this study.

4.4 Antiserum production.

Because Revill's method was not available early in the study, Gardner's method was used for purification of virus for antiserum production. Sub-optimal amounts of virus were injected into rabbits due to low yields. However, Van Regenmortel (1982) has stated that the amounts of immunogen usually used for virus antibody production are excessive. He suggested that 50-100 μg is sufficient. Thus the amounts of virus used in the present study (4-66 μg) were enough to produce an antigenic reaction. Virus was glutaraldehyde fixed, which also increases antigenicity (Van Regenmortel, 1982). The procedure outlined in Chpt. 2.4.2. was followed.

Antigen from the first bleeding had a titre of 1/4 in gel diffusion when PNRSV-G was used as the antigen, and 1/8192 in the microprecipitin test, again with PNRSV-G antigen. Antigen reacted with healthy sap in the microprecipitin test, but did not react with healthy sap in gel diffusion. Following purification and conjugation for ELISA (Chpt. 2.4.4), reactions to virus were a maximum of 3.4 times the reactions to healthy controls at a sap dilution of 1:100, 10 μg/ml coating γ-globulin, and 1:3200 dilution of enzyme labelled conjugate. In comparison, when Fulton's antiserum to PNRSV-G was optimised, the difference between healthy and diseased reactions was about 95 times.

To reduce healthy reactions, antiserum was adsorbed with healthy sap (Noordam, 1973). This was accomplished by the addition of 10 volumes of healthy cucumber sap in 0.5% NaCl to one volume of antiserum. The mixture was kept at 37 C for 2 h, then at 4 C for a further 2 h. The adsorbed antiserum and healthy tissue was removed by centrifugation (20 000 **g** for 30 min) followed by freezing the supernatant. After thawing the antiserum was centrifuged again and antibodies in the supernatant precipitated by addition of 40% saturated ammonium sulphate. Following low speed centrifugation and resuspension of the pellet in 0.5% NaCl, the ammonium sulphate was removed by dialysis against 0.85% NaCl. Adsorbed antiserum from the first bleeding had a titre of 1/256 in the microprecipitin test. However, there was no improvement following purification and conjugation in ELISA reactions. The antiserum from the second bleeding had a titre of 1/128 in the microprecipitin test and thus was not tested further.

4.6 Purification of different isolates of ilarviruses.

A modification of Revills' purification method was successfully used for other ilarviruses. Flowering cherry isolate 30 was purified and was morphologically indistinguishable from isolates of PNRSV-G when examined by the electron microscope (Fig. 4.2). This isolate was serologically identified as PDV by ELISA.

Isolate 126 was also purified using this method, and will be further discussed in Chpt. 5.

4.7 Protein extraction.

Protein extracted from purified preparations of flowering cherry isolate 76 was electrophoresed under denaturing conditions (Chpt. 2.9) and isolate 76 of PNRSV-G was found to have a coat protein of M_r 28 000 (Fig. 4.3).

4.8 RNA extraction.

RNA extracted from purified preparations of PNRSV-G isolate 76 from flowering cherries was electrophoresed in formaldehyde denaturing gels (Chpt. 2.11.2), and sizes of 3.5, 2.9, 2.0 and 1.0 kbs estimated (Fig. 4.4).

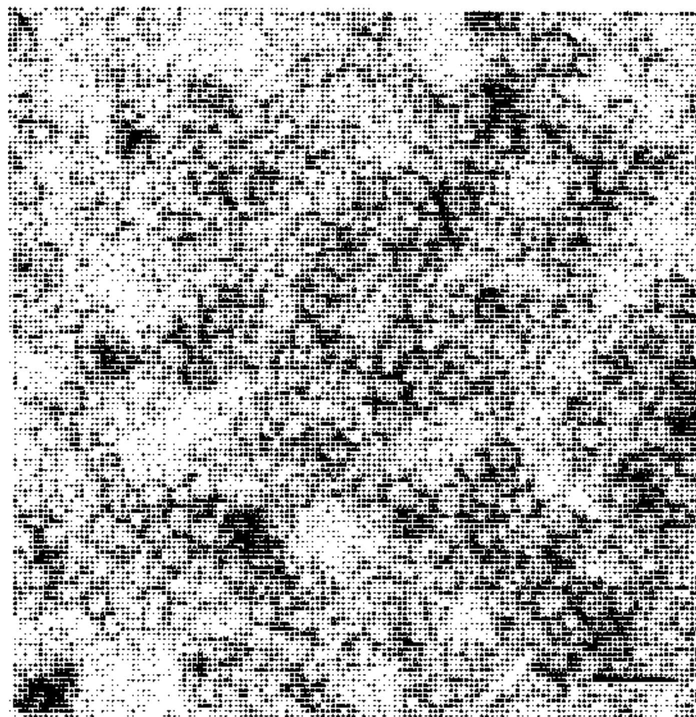

Fig. 4.2: Electron micrograph of purifed particles of PDV, isolate 30, purified by the method of Revill. Bar = 100 nm.

Fig. 4.3: SDS-polyacrylamide gel electrophoresis of, lane 1; BRL molecular weight markers (top to bottom; M_r 200 000, 97 400, 68 000, 43 000, 29 000, 18 400 and 14 300) lane 2 and 3; PNRSV-G.

4.9 cDNA cloning.

4.9.1 Primer design.

In view of the fact that only one ilarvirus, tobacco streak virus (TSV), and one closely related virus, alfalfa mosaic virus, have been sequenced, it is unclear whether conserved regions exist in the 3' ends of the genomic RNAs of these viruses (Koper-Zwarthoff & Bol, 1980; Zuidema & Jaspars, 1984). This necessitated the use of either random primers or polyadenylation of the viral RNA for cDNA synthesis. Preliminary trials using polyadenylated RNA (Drummond *et al.*, 1985) were without success. Therefore only the random primer method was pursued further. Random primers were prepared from salmon sperm DNA (BRL) as described in Sambrook *et al.* (1989).

4.9.2 First strand synthesis.

First strand synthesis was carried out with avian myeloblastosis virus reverse transcriptase (RT) as described in Chpt. 2.12.1. cDNA was synthesised to RNA extracted from purified preparations of flowering cherry isolate 198 of PNRSV (PNRSV-198).

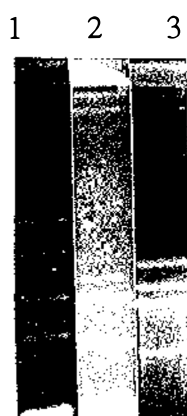

Fig. 4.4: BRL RNA size markers lane 1; (top to bottom; 9.49, 4.40, 2.37, 1.35 and 0.24 kb), lane 2; AMV, lane 3; PNRSV-G.

4.9.3 Second strand synthesis.

Second strand synthesis was catalysed with ribonuclease H and DNA polymerase I as described in Chpt. 2.12.2.

4.9.4 Cloning dscDNA into pUC19.

A non-radioactively labelled product of first and second strand synthesis was blunt-ended with T4 DNA polymerase as outlined in Chpt. 2.12.2. This double-stranded DNA was then ligated into *Sma*I digested pUC19 as described in Chpt. 2.13.1.1 and transformed into *E. coli* strain MC1022. Following BCIG/ampicillin selection, 139 white colonies and more than 500 blue colonies were obtained. Of these 139 white colonies, 128 were sub-cultured, and the DNA extracted by the minipreparation method (Chpt. 2.13.1.3). The DNA of some blue colonies was extracted as a control. DNA from control blue colonies and from white colonies were then electrophoresed on a 1% agarose gel (Chpt. 2.10.5) and nine isolates with inserts greater than 400bps as evidenced by retarded migration compared with control isolates were selected for further testing.

4.9.5 Dot-blotting for specificity.

The DNA from the nine white colonies with retarded migration in 1% agarose gels were further purified, and the cDNA insert removed following digestion with *Eco*RI and *Bam*HI (Chpt. 2.10.4). These inserts were then gel purified (Chpt. 2.10.7), hexamer primed and

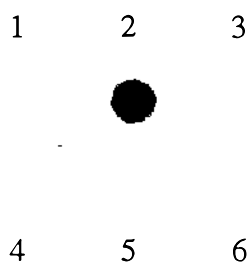

Fig. 4.5: Dot blot analysis of clone pB2 to PNRSV-G RNA-3. 1: FCVB RNA, 2: PNRSV-G RNA, 3: sap from *Chenopodium quinoa* infected with tobacco streak virus, 4: sap from cucumber infected with FCVB, 5: sap from cucumber infected with PNRSV-G, 6: healthy cucumber sap.

labelled with [α-^{32}P]-dATP (Chpt. 2.14.3). Healthy cucumber sap, sap from cucumbers infected with PNRSV and FCVB, and RNA from PNRSV, FCVB and TSV were hybridised to nitrocellulose as described in Chpt. 2.14.2.

Clone pB2 of PNRSV-198 hybridised specifically to PNRSV-198 infected cucumber sap and RNA in dot-blots (Fig. 4.5). This clone did not react with sap from cucumbers infected with FCVB, TSV or healthy cucumber sap. Seven other clones of PNRSV-198 hybridised with healthy cucumber sap as well as all other samples on dot-blots.

4.9.6 Northern analysis.

Clone pB2 was further analysed by Northern blotting. PNRSV RNA was electrophoresed under denaturing conditions as described in Chpt. 2.11.2, and transferred onto nitrocellulose (Chpt. 2.14.1). DNA from colony pB2 was purified using the maxi-preparation method (Chpt. 2.13.1.4) and hexamer labelled with [α-^{32}P]-dATP (Chpt. 2.14.3) following excision of the cDNA insert by digestion with *Eco*RI and *Bam*HI and gel purification (Chpt. 2.10.7). The RNA bound to nitrocellulose was then probed as described in Chpt. 2.14.4. and autoradiographed (Fig. 4.6). Comparison with ethidium bromide stained RNA following electrophoresis but immediately prior to transfer showed that this probe was specific to RNA-3 of PNRSV.

4.9.7 Sequencing.

This clone (pB2) was sequenced using the chain termination method (Chpt. 2.15), and the

Fig. 4.6: Northern hybridisation of clone pB2 to PNRSV-G RNA-3 electrophoresed in a formaldehyde denaturing gel before transfer to nitrocellulose.

```
        10                  30                  50                  70                  90                  110
CGAGAATATAGATGTCAAACGATTAGGGGATAATAGGAAGGTTTTGACTATTCAACCGAAAGCCCCGATCGTAGAGGAAATTAATGACGATGTTGAACCGTTAGGTTCGAATGGTGAAAA
       130                 150                 170                 190                 210                 230
TCATATGGAAGAGAAGACTGTGACCGTTAAGGTCGGTAGTTCTGGAAGTGCTTGAGTGACTATGTCCCAGAGCGTGCGCTTTGTGTGAGCGTTTTTCTTTTCTTTCTTCCGAACATCTCT
       250                 270                 290                 310                 330
TTCATTTGATAATGGTTTGCCGATTTTGCAATCATACCCACGCTGGTGGATCGCCACGTATCTTGCAAGAAGTAGACACATCCGAACGATGACTCTGG
```

Fig. 4.7: Nucleotide sequence of clone pB2 to PNRSV-G RNA-3.

cDNA insert was 338 nucleotides long (Fig. 4.7). No sequence homologies were found with RNA-3 of TSV, AMV or with RBDV. No recognisable reading frames were found.

4.10 Discussion.

Purified preparations of PNRSV-G obtained using Uyemoto's method contained host contamination as determined by electron microscopy. Modifications to reduce contamination were not successful. It was concluded that the initial acidification did not sufficiently clarify virus preparations and virus may have been subsequently lost in low speed centrifugations through absorption to plant components.
Clarification with hydrated calcium phosphate (HCP) produced purified virus with no visible host contamination, but yields were low. Virus has been reported to bind with HCP (Fulton, 1959), and is therefore lost in low speed centrifugations. Because yields were not increased in this study when the amount of HCP added was reduced, either the amount of HCP was still sufficient to cause virus loss by binding, or some other factor may be responsible for low virus yields (see later in this discussion).

Some improvement of yield (5 fold) was found when inoculated cotyledons and systemically infected leaves only were harvested. However, yields remained low.

A dramatic improvement in yield of virus purification was obtained using a modification of Revill's method. The addition of ascorbic acid and use of ether as a clarifying agent were the two steps that were unique to this method of purification. Ascorbic acid may act as a virus stabiliser, and ether may have been a more effective clarifying agent than was

HCP or acid precipitation resulting in less loss of virus.

Yields of PNRSV-G obtained using a modification of Revill's method (3.2-8.9 mg virus/100 g plant material) were higher than those recorded in the literature for this virus (Gardner, 1983, 1-3 mg/100 g; Patrakosol, 1985, 0.42 mg/100 g; Revill (pers. comm. to K.S. Milne), 5 mg/100 g). *Prunus* necrotic ringspot virus-G is reported to occur in low concentrations in infected plants (Loesch & Fulton, 1975; Fulton, 1981) because of low yields following purification. It appears that this may be a function of virus loss during the purification process rather than a true indication of virus concentration within an infected plant. For example, purification of tobacco ringspot virus, which is a relatively stable nepovirus, yields 5-10 mg/100 g (Stace-Smith, 1970) which is within the range of yields of PNRSV-G obtained in this study.

The molecular weight of the coat protein of PNRSV-G isolate 76 (M_r 28 000) in this study was within the range of those recorded for this virus in the literature (M_r 29 500, Thomas, 1981; M_r 25 000, Barnett & Fulton, 1969; M_r 25 000, Gonsalves & Fulton, 1977). Differences in molecular weight of RNA between these isolates were small, and may be ascribed to differences in concentration of polyacrylamide, and to experimental error, rather than representing genuine differences.

The RNA sizes obtained for flowering cherry isolates of PNRSV-G of 3.5, 2.9, 2.0 and 1.0 kbs show similarity to other multicomponent, icosahedral viruses which have been sequenced (Francki, 1985) including: tobacco streak virus (type member of the ilarvirus group; 2.9, 2.8, 2.2 and 0.9 kb), alfalfa mosaic virus (3.6, 2.6, 2.0 and 0.9 kb), CMV (type

member of the cucumovirus group; 3.4, 3.0, 2.1 and 1.0 kb) and brome mosaic virus (type member of the bromovirus group; 3.2, 2.9, 2.1 and 0.9 kb).

Particle morphology, serology, coat protein and RNA analysis of flowering cherry isolates of PNRSV-G all yielded results consistent with descriptions of other isolates of this virus (Fulton, 1970). Symptoms in herbaceous indicator hosts are discussed separately in Chpts. 3 and 5, but were also consistent with reports for this virus.

A tract of cDNA was produced which was complementary to RNA-3 of PNRSV. Analysis of this cDNA segment (pB2) by probing Northern transferred PNRSV RNA showed that pB2 was specific to RNA-3. Thus this clone was located upstream of the coat protein gene. Attempts to use primers to synthetically polyadenylated 3' terminus PNRSV RNA and to pB2 in the polymerase chain reaction failed to result in any product. Further work may produce additional cDNA that can be used to produce PCR primers at the 3' terminus of RNA-3 and RNA-4 and thus produce a PCR product complementary to the coat protein gene which can then be sequenced. In this study more promising avenues of research were instead followed (Chpt. 8).

Further testing is required to ascertain the specificity or otherwise of the 338 bp (pB2) clone within the ilarvirus group. Results of probing to a range of ilarviruses would provide further information about the relationships between the viruses in this group. If it is found that clone pB2 hybridises with other members of this virus group, it may be useful to use it as a probe in a commercial environment, because flowering cherries are infected with a number of these viruses.

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CHAPTER FIVE

Flowering cherry virus I, a possible new ilarvirus from flowering cherry

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Flowering cherry virus I (FCVI) is a labile virus from *Prunus serrulata sensu lato* Lindl. (flowering cherry). It has a narrow host range, as well as quasi-isometric and some bullet-shaped particles of ca. 26nm diam., four RNA species of 3550, 2800, 2000 and 1050 nucleotides, and a coat protein of M_r 30 000. These properties indicate that FCVI has affinities with the ilarvirus group, but it differs in host range and symptoms, and serological properties from other members of this group.

INTRODUCTION

Flowering cherries (*Prunus serrulata* Lindl. *sensu lato*) are ornamental woody trees in the

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Rosaceae. Viruses recorded in flowering cherry in New Zealand include strawberry latent ringspot virus (SLRV) (Everett *et al.*, 1993a), a tentative new bromovirus, flowering cherry virus B (FCVB) (Everett *et al.*, 1993b), and three ilarviruses, apple mosaic virus (ApMV), *Prunus* necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) (Everett *et al.*, 1993c). Another virus isolated in this study, called here flowering cherry virus I (FCVI), also has properties of the ilarvirus group.

Ilarviruses are characterised by having three or more types of quasi-isometric particle, c. 30 nm diameter, which sediment as three components of 80-90, 89-98 and 101-114 S. The nucleic acid is comprised of three genomic and one sub-genomic positive sense single-stranded RNA of M_r 1.1-1.3, 0.9-1.1, 0.7-0.9 and 0.3 x 10^6 (RNA-1 to RNA-4, respectively). RNA-1, RNA-2 and RNA-3 are encapsidated separately, and two molecules of RNA-4 are encapsidated together (Francki *et al.*, 1985).

Ilarviruses are morphologically similar, and frequently the most definitive method of distinction involves serology (Fulton, 1983; Francki *et al.*, 1985; Casper, 1973). Francki (1985) has categorised the ilarviruses into eight serological sub-groups. The first four of these consist of viruses which have been well-characterised and serologically examined. Tobacco streak virus (TSV), black raspberry latent virus (BRLV) and asparagus stunt virus (ASV) are considered as separate strains of TSV (Francki, 1985), with bean red node virus (BRNV) also probably a distinct strain (Fulton, 1983) (subgroup 1). The viruses of subgroup 2 [Tulare apple mosaic virus (TAMV), citrus leaf rugose virus (CiLRV), citrus variegation virus (CVV), citrus crinkly leaf virus (CCLV), elm mottle virus (EMV) and asparagus virus II (AVII)] are serologically related but are not identical (Rybicki & von Wechmar, 1985). PNRSV and ApMV are both in serological subgroup 3, and can be considered to be different serotypes of the same virus (Barbara *et al.*, 1978) as they cross-

react. Prune dwarf virus, in serological subgroup 4, does not react with antisera to any other ilarvirus. Subgroups 5 to 8 consist of viruses about which relatively little is known (American plum line pattern virus (APLPV), spinach latent virus (SPLV), lilac ring mottle virus (LRMV) and hydrangea mosaic virus (HMV), and consequently each is ascribed to its own serological subgroup.

This paper describes flowering cherry virus I (FCVI). The virus shares many of the characteristics of the ilarvirus group but, based on host range and symptoms, coat protein size and serology appears to be a distinct new member of this group.

MATERIALS AND METHODS

Virus isolation and propagation

Flowering cherry virus I was isolated from young flowering cherry leaf tissue macerated in cold 0.02M phosphate buffer pH 8.0 with 5% w/v polyvinylpyridone (transfer buffer) (Gardner, 1983) containing 0.01 g Celite and mechanically inoculated to young *Cucumis sativus* L. 'Heinz Pickling' cotyledons. FCVI was maintained in cucumber and mechanically transmitted using transfer buffer every two weeks.

Purification

Virus was purified according to a method of Dr. P. Revill (personal communication). All purification steps were done at 4°C. Cucumber plants were harvested 2 weeks following

inoculation and macerated in 2 vol (w/v) of 0.01 M sodium phosphate buffer (pH 7.5) with 0.0006 M ascorbic acid. The buffered extract was filtered though muslin and the aqueous phase centrifuged at 15 000 **g** for 20 min in a Sorvall GSA rotor. The supernatant was mixed with an equal volume of ether and shaken for 1 min, followed by centrifugation at 15 000 **g** for 20 min. The lower aqueous phase was retrieved with a pipette and all traces of ether removed by bubbling air through the extract for 1 h. Triton X-100 was added (20 ml/l) and the virus sedimented by centrifugation at 200 000 **g** for 1 h in a Sorvall TFT 80.13 rotor. The pellet was resuspended in 0.01 M phosphate buffer (pH 7.5) and further purified by layering on a 10-40% sucrose gradient prepared in 0.01 M sodium phosphate buffer (pH 7.5) and centrifuging for 4 h at 85 500 **g** in a Beckman SW28 rotor. Gradients were scanned at 254 nm with an ISCO model D density gradient fractionator attached to an ISCO model UA-5 absorbance monitor. Pooled virus containing fractions were concentrated by centrifugation at 200 000 **g** for 1 h in a Sorvall TFT 80.13 rotor and resuspended in 0.01 M sodium phosphate buffer (pH 7.5).

Serology

Antisera and sources are listed in Table 1. Gel diffusion tests were carried out in 0.75% Oxoid ionagar No. 2 containing 0.01M sodium phosphate buffer (pH 7.0), 0.85% NaCl, and 0.02% sodium azide.

ELISA tests were carried out according to Clark & Adams (1977) using Dynatech microtitre plates. Coating gamma globulins were used at a concentration of 1 µg/ml, healthy or infected cucumber sap extracts at a dilution of 1/10, and alkaline phosphatase

(Type VII, Sigma) conjugate antibodies at a dilution of 1/200. Two wells were used per sample.

Electron microscopy

Purified virus was analysed using the Philips 201C electron microscope after air drying onto a carbon coated grid, and staining with 1% uranyl acetate (pH 4.5).

Coat protein analysis

Coat protein was prepared from purified virus preparations according to Forster & Jones (1979), and electrophoresed in a discontinuous 12.5% polyacrylamide gel (Laemmli, 1970). Size comparisons were made with BioRad SDS PAGE low molecular weight markers.

RNA analysis

Purified virus preparations in 50 mM Tris-HCl pH 8.0 containing 150 mM NaCl, 10 mM EDTA and 1% SDS (w/v) were mixed with an equal volume of a 1:1 (v/v) mixture of phenol and chloroform, incubated at 37°C for 15 min. and centrifuged at 13 000 r.p.m. for 10 min. at 4°C. The supernatant was re-extracted once with phenol and chloroform and once with chloroform, then precipitated with two volumes of ethanol at -20°C overnight.

Table 1: Antisera used, sources and serological subgroup

Ilarviruses		
Serological Subgroup	Antisera	Source
1.	Tobacco streak virus	D. Maat, IPO, Wageningen
2.	Asparagus virus II	G. Mink, Washington State
3.a	PNRSV-G	R. Fulton, Wisconsin
	PLPV	R. Fulton
	PNRSV-H	R. Fulton
	Hop virus C	J. Fletcher, Lincoln, New Zealand
	Rose mosaic virus	R. Fulton
3.b	Apple mosaic virus	R. Fulton
	Hop virus A	J. Fletcher
4.	Prune dwarf virus	G. Wood, Auckland, New Zealand
Related viruses		
	RBDV	R. Goold, Invergowrie
	Alfalfa mosaic virus	R. Francki, Adelaide

Key: PNRSV-G/H *Prunus* necrotic ringspot virus serotype g/h
PLPV Plum line pattern virus
RBDV Raspberry bushy dwarf virus

The viral RNA was collected by centrifugation, the pellet washed with 70% ethanol, vacuum dried, resuspended in sterile double distilled water, and stored at -70°C. Viral RNA was analysed on a 1% agarose gel electrophoresed in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.3). RNA sizes were determined following analysis on formaldehyde denaturing gels (Sambrook *et al.*, 1989) with RNA size markers (BRL 0.24-9.5 kb RNA ladder).

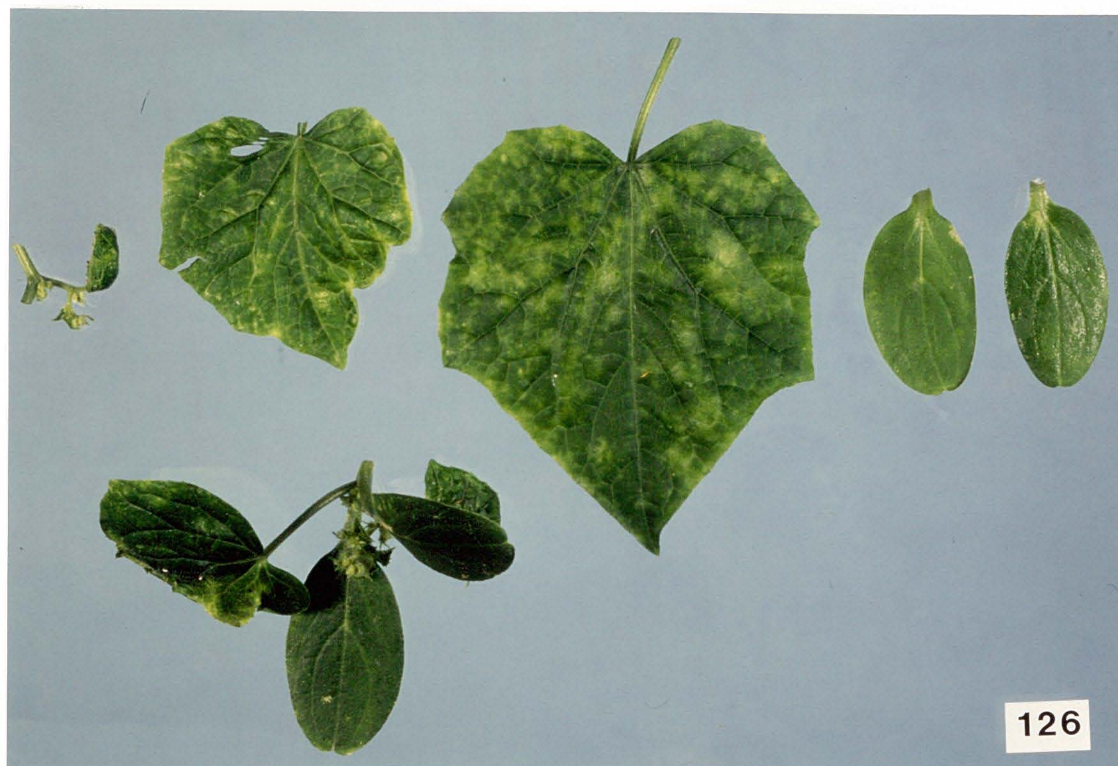
RESULTS

Host range and symptoms

FCVI infected members of the Cucurbitaceae only.

Cucumis sativus L. 'Heinz Pickling': local chlorotic lesions, systemic mosaic, interveinal chlorosis, and marginal distortion (Fig. 1a);
Citrullus lanatus (Thunb.) Mansf. 'Charleston Gray': pinprick necrotic lesions on cotyledons;
Cucurbita maxima Duch.'Golden Hubbard': systemic chlorosis (Fig. 1b);
Cucumis melo L. var. *cantalupensis* 'Hales Best Jumbo': pinprick necrotic local lesions, systemic necrotic spots and systemic interveinal yellowing;
C. moschata (Duch.) Duch. ex Poir 'Whanga Crown' local chlorotic spots, systemic chlorotic spots and terminal chlorosis.

a)



b)

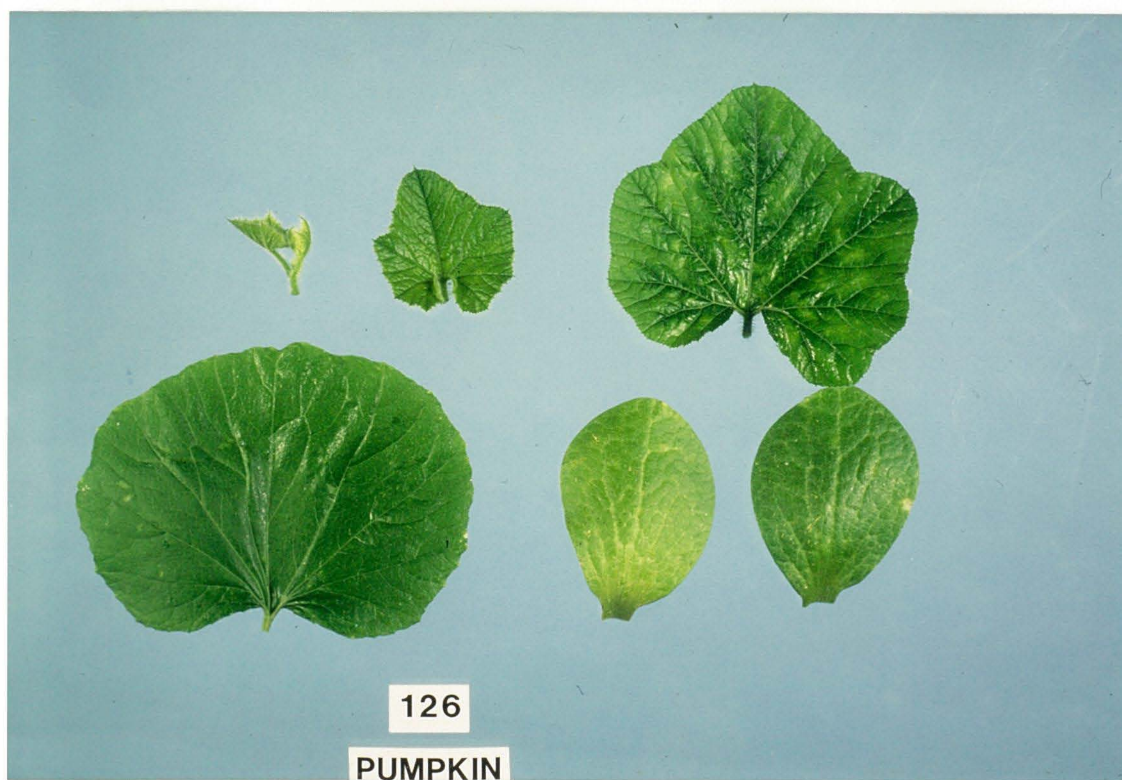


Fig. 1. Symptoms of flowering cherry virus I in herbaceous indicator hosts 14 days after inoculation. a) *Cucumis sativus* 'Heinz Pickling' and b) *Cucurbita maxima* 'Golden Hubbard'.

FCVI did not infect the following plant species as determined by back testing from uninoculated leaves 14 days after inoculation to cucumber seedlings: Curcurbitaceae, *Cucurbita maxima* 'Buttercup Squash', *C. pepo* L. 'Zucchini' and *Momordica balsamina* L.; Chenopodiaceae *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd.; Solanaceae *Nicotiana tabacum* L. 'Havana', *N. tabacum* 'White Burley', *N. clevelandii* Gray, *N. benthamniana* (L.) Domin., *N. megalosiphon* Heurch & Muell.; Fabaceae *Dolichos lablab* L., *Vigna cylindrica* Skeels, *V. sinensis*, *Phaseolus aureus* Roxb., *P. vulgaris* L. 'Top Crop' and *Vicia faba* L.; Compositae *Tithonia speciosa* Hook..

FCVI was distinguishable from the three other ilarviruses infecting flowering cherry based on host range and symptoms. Table 2 lists symptoms induced by isolates of ApMV, FCVI, PDV and PNRSV (isolates G and Q) from flowering cherry.

Longevity in vitro

FCVI was infectious after 4 h but not after 24 h in sap extracts in water or in 0.02 M sodium phosphate buffer, pH 8.0 containing 5% polyvinylpyrrolidone at c. 20°C.

Purification

Following sucrose density centrifugation, four virus containing peaks, and a peak near the meniscus containing plant proteins were detected (Fig. 2). Yields of 0.5-1.0 mg/ml/100 g were obtained. The A260/280 ratio was 1.6 and Amax/Amin was 1.068.

Table 2: Host range comparison between FCVI and other ilarviruses.

Virus	Symptoms in hosts				
	Cucumis sativus 'Heinz Pickling'	*Cucurbita maxima* 'Buttercup Squash'	*Chenopodium quinoa*	*Citrullus lanatus* 'Charleston Grey'	*Cucurbita pepo* 'Zucchini'
ApMV	chl. LL, syst. ringspots	n.s.	n.s.	n.s.	necr. LL syst. chl.
PDV	chl. LL, syst. mos. term. necr.	chl. LL, syst. mos.	n.s.	n.t.	n.t.
PNRSV-G	chl. LL, syst. mos. term. necr.	chl. LL, syst. mos.	n.s.	necr. LL	n.s.
PNRSV-Q	chl. LL, syst. mos.	necr. LL, syst. mos.	syst. chl. mottle	n.s.	n.s.
FCVI	chl. LL, syst. mos.	n.s.	n.s.	necr. LL	n.s.

Key:
necr. = necrotic/necrosis chl. = chlorotic
LL = local lesions n.t. = not tested
syst.= systemic n.s. = no symptoms
term. = terminal

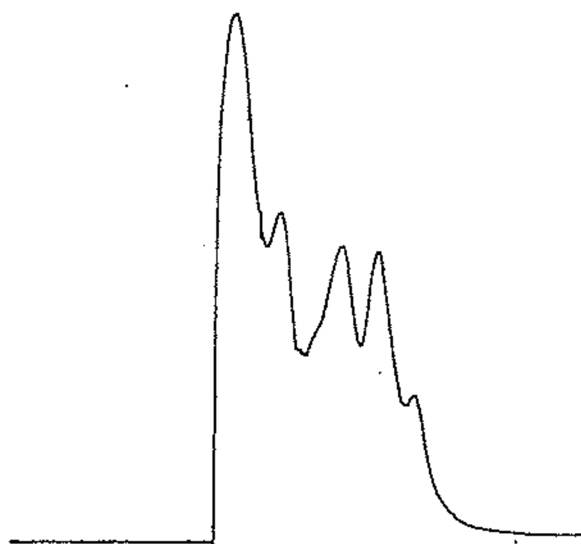


Fig. 2. Optical density profile of sucrose density gradients of a preparation of FCVI. Sedimentation from left to right. Relative absorbance at 254 nm.

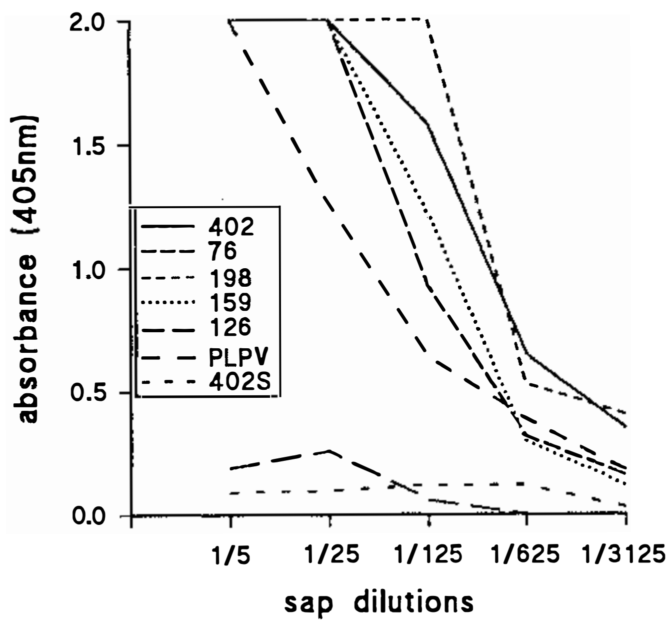
Serology

In Ouchterlony gel diffusion FCVI did not react with antisera tested to 10 ilarviruses in four serological subgroups (Table 1). Tests involved FCVI infected sap or purified virus preparations as antigen. Purified PNRSV-G reacted with the homologous antiserum in gel diffusion tests. Antisera to TSV, AVII, PNRSV-G, PLPV, ApMV and PDV, representing serological subgroups 1, 2, 3a, 3b and 4, reacted with homologous viruses in ELISA tests. In ELISA tests using infected sap in a series of five-fold dilutions, PNRSV-G antisera reacted with PNRSV-G from flowering cherry, and PLPV from plum, but did not react with PDV or with FCVI infected sap (Fig. 3a). Similarly antisera to ApMV reacted in ELISA tests with infected sap containing ApMV but not with FCVI, three PNRSV isolates or PDV (Fig. 3b).

Electron microscopy

Electron microscopy of purified FCVI showed quasi-isometric particles typical of ilarviruses of 26 nm diam (mean of 10 measurements; Fig. 4). Bacilliform particles of 40 nm were also present. Insets show bullet-shaped particles which are found in preparations of most ilarviruses.

a)



b)

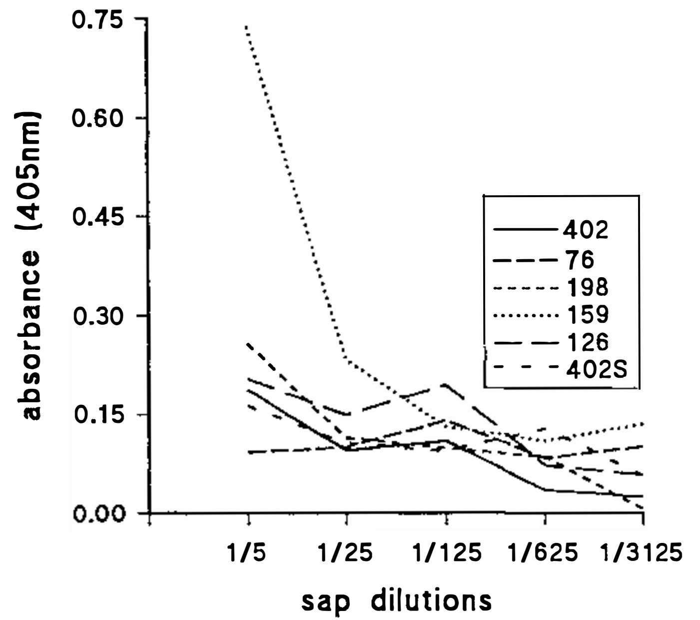


Fig. 3. ELISA dilution series of a) PNRSV-G antisera and b) ApMV antisera. Virus isolates 76, 198 and 402 are PNRSV-G; 159 is a mixed isolate of both ApMV and PNRSV-G; 402S is PDV; and 126 is FCVI.

Coat Protein

In polyacrylamide gels, a major band was observed of M_r 30 000 (mean of four determinations) (Fig. 5).

Nucleic acid

Four nucleic acid species with sizes in the range expected for ilarviruses were obtained from purified FCVI. Under denaturing conditions, sizes of 3900, 2500, 1900 and 1300 nucleotides (Fig. 6) and under non-denaturing conditions sizes of 3550, 2800, 2000 and 1050 nucleotides were obtained.

DISCUSSION

In possessing four RNA species of 3900, 2500, 1900 and 1300 nucleotides, FCVI is placed within the Tricornaviridae. The quasi-isometric and bullet shaped virus particles and the coat protein molecular weight (26 kD) are features of the ilarvirus group (Van Vloten-Doting *et al.*, 1981).

FCVI did not react with viral antisera of the serological subgroups 1, 2, 3a, 3b and 4 (Francki, 1985). Within serological subgroup 2, FCVI was tested with antiserum to AVII,

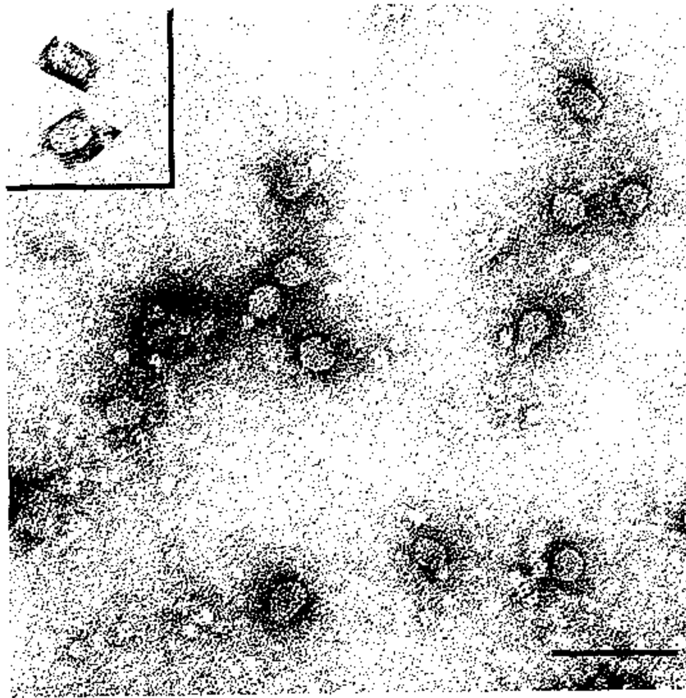


Fig. 4. Electron micrograph of purifed particles of FCVI. Bar = 100 nm. Insets show bullet-shaped particles.

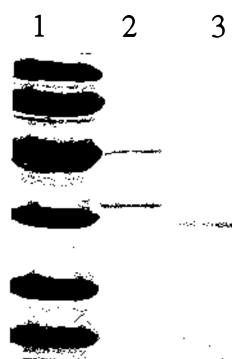


Fig. 5. SDS-polyacrylamide gel electrophoresis of, lane 1; Biorad molecular weight markers (top to bottom; M_r 97.4, 66.2, 45, 31, 21.5 and 14.4 x 10^3), lane 2, of strawberry latent ringspot virus (M_r 45 and 32 x 10^3) and lane 3, of FCVI. Both proteins from viruses were extracted from purifed preparations by the method of Forster and Jones (1979).

Fig. 6. RNA from BRL size markers (lane 1; top to bottom; 9.49, 4.40, 2.37, 1.35 and 0.24 kb), lane 2 and 3; FCVI.

but not with antiserum to TAMV, which has been reported to be serologically unreactive to AVII antiserum (Uyeda & Mink, 1983). However, FCVI can be distinguished from TAMV on the basis of host range and symptoms and size of its coat protein (Table 3). TAMV has a coat protein of M_r 19 000, compared to the value obtained in this study for FCVI (30 000).

Antisera to serotypes 5 to 8, viz. American plum line pattern virus (APLPV), spinach latent virus (SLV), lilac ring mottle virus (LRMV) and hydrangea mosaic virus (HyMV), were not tested. However, FCVI differs from these three viruses on the basis of host range, stability in sap, sizes of RNA species and coat protein molecular weights (Table 3).

On the basis of host range and symptoms, sizes of RNA and coat protein, flowering cherry virus I appears to be a new member of the ilarvirus group.

ACKNOWLEDGEMENTS

This study was funded by the New Zealand Nurserymens' Association and a Massey University post-graduate research fellowship. Many thanks for the provision of antisera to R. Fulton (University of Wisconsin), J. Fletcher (Lincoln, New Zealand), R. Goold (Scottish Horticulture), D. Maat (IPO, Wageningen), G. Wood (HortResearch, Mt Albert, New Zealand), G. Mink (Washington State University); and to P. Revill (Institute of Plant Sciences, Melbourne, Australia) for the purification method, and to D. Hopcroft and R. Bennett of the Electron Microscope Unit, HortResearch, Palmerston North.

Table 3: Comparison of FCVI with ilarviruses.

	TSV	TAMV	AVII	PNRSV	ApMV	PDV	APLPV	SPLV	LRMV	HydMV	FCVI
nucleic acid (M_r x 10^6)											
RNA-1	1.35	1.01	n.d.	1.3	1.23	1.26	n.d.	1.3	1.18	1.25	1.24
RNA-2	1.10	0.92	n.d.	0.89	1.00	0.95	n.d.	1.18	1.13	1.08	0.74
RNA-3	0.85	0.74	n.d.	0.69	0.69	0.76	n.d.	0.91	0.90	0.83	0.60
RNA-4	0.40			0.31	0.31			0.35	0.37	0.36	0.32
coat protein (M_r x 10^3)	30	19	n.d.	25	25	24	n.d.	28	28	26	30
LIV	<36h	n.d.	>2d	9-18h	a few min.	15-18h	2h	6-8d	3-5d	3-4d	4-24h
particle size	27-35	28-31	26-32	*c.*23	25-29	20-23	26-33	*c.*27	*c.*27	28-30 x 30-38	26
A260/A280	1.56	1.36	1.31-1.36	1.56	1.5	1.53	1.67	n.d.	1.43-1.46	*c.*1.32	1.6
N. tabaccum 'White Burley'	LL syst. necr.	LL syst.necr. & chlor.	n.t.	LL, some isolates syst.	not infected	syst. chlor.	symptomless infection	symptomless syst.	tip lf. wilting	not infected	not infected
N. megalosiphon	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	LL,sys. RS.	n.t.	LL, syst. mott.	n.t.	not infected
C. quinoa	LL tip necr.	n.t.	LL syst. mott. or necr.	LL, syst. chlor.	n.t.	not infected	not infected	LL, syst.	vein mott., tip necr., stunting	LL, syst. chlor.	not infected
Reference	Fulton (1971a) Salazar *et al.* (1981)	Fulton (1971b)	Uyeda & Mink (1981 & 1984)	Cropley *et al.* (1964)	Fulton (1952)	Cropley *et al.* (1964)	Kirkpatrick *et al.* (1967) Fulton, (1984)	Bos *et al.* (1980)	Van der Meer *et al.* (1976)	Thomas *et al.* (1983)	

Key: LL= local lesions
chlor.= chlorotic
necr. = necrotic/necrosis
syst. = systemic
n.t. = not tested
n.d. = not determined

NS = no symptoms
mott.= mottling
RS = ringspot
lf. = leaf

TSV= tobacco streak virus
TAMV= Tulare apple mosaic virus
AVII= asparagus virus II
PNRSV= prunus necrotic ringspot virus
ApMV= apple mosaic virus
PDV= prune dwarf virus

APLPV= American plum line pattern virus
SPLV= spinach latent virus
LRMV= lilac ring mottle virus
HydMV= hydrangea mosaic virus
FCVI= flowering cherry virus I

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CHAPTER SIX

Flowering cherry virus B, an isometric virus from flowering cherry with properties of bromoviruses

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Summary

A virus designated flowering cherry virus B (FCVB) was isolated from 44 of 434 of flowering cherry trees (*Prunus serrulata s.l.*) tested in the North Island of New Zealand. FCVB infected both monocotyledons and dicotyledons, but had a limited host range.

DsRNA preparations from infected leaves contained four RNA species of 3900, 2150, 1800 and 800 nucleotides (estimated from denatured dsRNA). Partially purified preparations contained isometric particles about 24nm in diameter. When purified at pH 7.5, FCVB sedimented in sucrose gradients as three UV absorbing components and virus particles frequently appeared to be swollen. At low pH (5.0 or 6.0) or at pH 7.5 with the addition of magnesium ions, FCVB sedimented as a single predominant UV absorbing component and virus particles were not swollen. One major protein band (molecular weight 19 300) was extracted from partially purified preparations. Based on these features, it is proposed that FCVB is a new member of the bromovirus group.

Key words: Flowering cherry virus B, bromovirus, *Prunus serrulata*

Introduction

A number of viruses were detected during a study of sap-transmissible viruses in flowering cherry (*Prunus serrulata sensu lato*) in the North Island of New Zealand (Everett, Milne & Forster, 1993). These included three ilarviruses; *Prunus* necrotic ringspot virus, apple mosaic virus, prune dwarf virus; a possible new ilarvirus, flowering cherry virus I; and strawberry latent ringspot virus. A further virus with similarities to bromoviruses was detected in 10% of flowering cherry trees (Everett *et al.*, 1993) and named flowering cherry virus B (FCVB).

Bromoviruses have polyhedral virus particles *c.* 26 nm in diameter, a coat protein of

molecular weight *c.* 20 000 daltons and four ssRNA species of *c.* 1.1, 1.0, 0.8 and 0.3 x 10^6 daltons. The bromovirus genome is contained within the three larger RNAs (RNAs 1-3) and the smallest species (RNA 4) is a subgenomic RNA for the coat protein (Lane, 1981). The RNAs are capped at their 5' termini and have a tRNA-like structure at their 3' termini (Francki, 1985).

BMV, broad bean mottle virus (BBMV), cowpea chlorotic mottle virus (CCMV) (Lane, 1979), cassia yellow blotch virus (CYBV) (Dale, Gibbs & Behncken, 1984), Melandrium yellow fleck virus (MYFV) (Hollings & Horvàth, 1981) and spring beauty latent virus (SBLV) (Valverde, 1985) are the viruses attributed to the bromovirus group based on particle morphology, coat protein and RNA size. Rybicki & Von Wechmar (1981) showed serological relationships between BMV, BBMV and CCMV in Ouchterlony double diffusion tests under neutral or alkaline conditions. In addition Valverde & Glascock (1991) reported serological relationships between SBLV and BMV, CCMV, BBMV and MYFV by Western blotting with SBLV antiserum. Serological relationships between CYBV and other bromoviruses have not been reported.

BMV, CCMV, BBMV and CYBV are stable at pH 3-6, but swell at pH 7.0 or 7.5 and high salt concentration (Bancroft *et al.*, 1968; Bancroft & Hiebert, 1967; Bockstahler & Kaesberg, 1962; Chiu & Sill, 1963). Once swollen they become susceptible to enzymatic degradation (Pfeiffer, 1980). In the presence of $MgCl_2$, the degree of swelling of BMV at pH 7.5 is decreased.

Bromoviruses are similar to cucumoviruses, apart from their tendency to swell at high pH (Pfeiffer & Hirth, 1974; Francki, 1985). There are some regions of sequence homology between bromoviruses (brome mosaic virus, cowpea chlorotic mottle virus, broad bean mosaic virus) and a cucumovirus (cucumber mosaic virus) in the 3'-terminal

45 residues of RNA-3/4 (Ahlquist, Dasgupta & Kaesberg, 1981).

The host range and symptoms, morphology, behaviour under different pH conditions, and molecular weights of the RNA and coat protein of FCVB are described in this paper. Similarities to the bromoviruses are discussed.

Materials and Methods

Virus detection and propagation

FCVB isolates were maintained in *Cucumis sativus* L. 'Heinz Pickling' and transferred from symptomless systemically infected tissue using 5% polyvinylpyrrolidone (PVP, molecular weight 40000) in 20 mM phosphate buffer, pH 8.0 containing 0.01 g/ml Celite. Brome mosaic virus was maintained in oats, and cowpea chlorotic mottle virus in cowpeas. Viruses were transferred to fresh seedlings every 2-4 weeks.

Purification

All purification steps were at 4°C. Systemically infected *C. sativus* plants, harvested 3 weeks after inoculation, were macerated in two volumes (w/v) of 20 mM Tris-HCl buffer (pH 6.0) with 10 mM $MgSO_4$, filtered through muslin and the buffer phase emulsified with diethyl ether and carbon tetrachloride (1 ml/g tissue). The emulsion was broken by low speed centrifugation (10 min at 16 300 *g* in a GSA rotor of a Sorvall RC5B centrifuge) then polyethylene glycol (PEG, molecular weight 8000) (10%) and NaCl (1%) were added to the supernatant and the mixture shaken for 1 h at 4°C. The precipitate was pelleted by low speed centrifugation (10 min at 16 300 *g*) and resuspended by shaking for

2 h in 20 mM Tris-HCl buffer (pH 6.0) and 2% Triton X-100 (v/v) with 10 mM $MgSO_4$. Virus particles were sedimented by high speed centrifugation (158 400 *g* for 1 h in a Sorvall TFT80.13 rotor). The pellets were resuspended overnight in 20 mM Tris-HCl buffer (pH 6.0) with 10 mM $MgSO_4$. The virus was further purified following centrifugation through 10-40% sucrose gradients prepared in 20 mM Tris-HCl buffer (pH 6.0) with 10 mM $MgSO_4$ (82 700 *g* for 4 h in a Beckman SW28 rotor). The banding pattern in the gradients was monitored using an ISCO Model D Density Gradient Fractionator equipped with an ISCO Model UA-5 Absorbance/Fluorescence Monitor. The fractions displaying absorbance at 254 nm were collected, pooled, and concentrated by centrifugation (2 h at 158 400 *g* in a Sorvall TFT80.13 rotor). The high-speed pellet was resuspended overnight in 20 mM Tris-HCl buffer (pH 6.0 or 7.5) with 10 mM $MgSO_4$.

Serology

The antisera and sources are listed in Table 1. Ouchterlony double diffusion tests were carried out using 0.75% Oxoid ionagar No. 2 containing 10 mM sodium phosphate buffer (pH 6.0, 7.0 and 7.5), 0.85% NaCl, and 0.02% sodium azide.

Electron microscopy

Purified virus was prepared for examination in a Philips 201C electron microscope by air drying onto a carbon coated grid followed by staining with 1 g/100 ml uranyl acetate.

a)

b)

Fig. 1. Symptoms of flowering cherry virus B in herbaceous indicator hosts. Except for the two leaves on the right in (c), all leaves show local lesions. *(a) Cucumis sativus* 'Heinz Pickling', *(b) Cucurbita maxima* 'Buttercup squash', *(c) Nicotiana benthamniana,* and *(d) Phaseolus aureus.*

c)

d)

Table 1: Antisera used, sources and serological subgroup

Ilarviruses		
Serological Subgroup	Antisera	Source
1.	Tobacco streak virus	D. Maat, IPO, Wageningen
2.	Asparagus virus II	G. Mink, Washington State
3.g	PNRSV-G*	R. Fulton, Wisconsin
	Plum line pattern virus	R. Fulton
	PNRSV-H*	R. Fulton
	Hop virus C	J. Fletcher, NZ
	rose mosaic virus	R. Fulton
3.a	Apple mosaic virus	R. Fulton
	Hop virus A	J. Fletcher
4.	Prune dwarf virus	G. Wood, NZ
Bromoviruses		
	Cowpea chlorotic mottle virus	P. Ahlquist, Wisconsin
	Broad bean mosaic virus	P. Ahlquist
	Brome mosaic virus	P. Ahlquist
Other viruses		
	Raspberry bushy dwarf virus	R. Goold, Invergowrie
	Alfalfa mosaic virus	R. Francki, Adelaide
	Cucumber mosaic virus	H. Neilson, NZ

*PNRSV-G/H, *Prunus* necrotic ringspot virus serotypes G and H.

Coat protein analysis

Protein was extracted from purified virus particles according to Forster & Jones (1979). The denatured protein preparation was electrophoresed on a discontinuous 12% acrylamide gel (Laemmli, 1970). Relative size comparisons were made with standard molecular weight markers (BioRad low molecular weight markers).

Nucleic acid analysis

RNA was extracted from purified virus preparations by incubation at 37°C for 15 min in 50 mM Tris-HCl pH 8.0 containing 150 mM NaCl, 10 mM EDTA and 1% SDS (w/v) and an equal volume of a 1:1 (v/v) mixture of phenol and chloroform. Following centrifugation at 13 000 r.p.m. for 10 min. at 4°C, the supernatant was re-extracted once with phenol and chloroform and once with chloroform, and precipitated with two volumes of ethanol at -20°C overnight. RNA was analysed on 1% agarose gels in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.3). RNA sizes were determined on formaldehyde denaturing gels (Sambrook, Fritsch & Maniatis, 1989) using RNA size markers (BRL 0.24-9.5 kb RNA ladder).

DsRNA was extracted from FCVB infected cucumber leaves by the method of Abou-Elnasr, Jones & Mayo (1985).

Results

Host range and symptoms

FCVB infected members of the Cucurbitaceae, Fabaceae, Solanaceae and Gramineae.

Symptoms of infection were as follows:

Cucumis sativus L., local necrotic pin-prick lesions on cotyledons, symptomless systemic infection (Fig. 1*a*);

Cucurbita maxima Duch. 'Buttercup Squash', local necrotic 2-5mm diameter lesions on cotyledons. No systemic symptoms (Fig. 1*b*);

Dolichos lablab L., local necrotic 2-5mm diameter lesions. No systemic symptoms;

Nicotiana benthamniana (L.) Domin., local necrotic lesions 2-5mm diameter, systemic vein clearing (Fig. 1*c*);

Phaseolus aureus Roxb., local reddish necrotic lesions 2-5mm diameter. No systemic symptoms (Fig. 1*d*);

Vigna cylindrica Skeels, local reddish necrotic lesions 2-5mm diameter. No systemic symptoms;

Vigna sinensis Savi., local reddish necrotic lesions 2-5mm diameter. No systemic symptoms.

The following hosts were systemically infected without symptoms, as determined by back inoculation to cucumber two weeks after inoculation: *Hordeum murinum* L., *Triticum aestivum* L. and *Pisum sativum* L..

FCVB failed to infect the following species, as determined by back inoculation to cowpeas from inoculated and uninoculated leaves two weeks after inoculation: Aizoaceae (*Tetragonia expansa* Murr.); Amaranthaceae (*Gomphrena globosa* L.); Chenopodiaceae (*Chenopodium amaranticolor* Coste & Reyn., *Chenopodium foetidum* Schrad.,

Chenopodium quinoa Willd.);

Compositae (*Tithonia speciosa* Hook.); Cruciferae (*Brassica pekinensis* (Lour.) Rupr.'Chi Hi Li'); Cucurbitaceae (*Cucurbita pepo* L. 'Zucchini'; Fabaceae (*Phaseolus vulgaris* L. and *Vicia faba* L.) and Solanaceae, (*Lycopersicon esculentum* Mill., *Momordica balsamina* L., *Nicotiana clevelandii* Gray, *Nicotiana glutinosa* L., *Nicotiana megalosiphon* Heurch & Muell., *Nicotiana tabacum* L. 'White Burley', *Nicotiana tabacum* 'Havana').

Persistence of infectivity in plant sap

FCVB was inactivated in cucumber sap by heating for 10 min at 50°C but not at 40°C. Infectivity of sap extracts was inactivated after 72 h but not after 24 h at room temperature. Sap extracts in 10 mM Tris-HCl pH 7.5 were highly infectious after 24 h (more than 40 local lesions/ cucumber cotyledon). Addition of 10 mM $MgSO_4$ retained high infectivity whereas addition of 10 mM EDTA significantly reduced or abolished all infectivity. Sap extracts in 10, 30 and 100 mM sodium acetate (pH 5.0), 30 mM sodium phosphate (pH 7.0) and 100 mM sodium phosphate (pH 6.0 and 7.0) were highly infectious (more than 40 local lesions per cucumber cotyledon) after 24 h at room temperature, whereas the infectivity of sap extracts in 100 mM sodium citrate (pH 6.0), 100 mM sodium borate (pH 8.0) and 100 mM sodium phosphate (pH 8.0) was reduced (5 - 10 local lesions/ cucumber cotyledon).

Purification

During purification of FCVB, large UV absorbing peaks, as measured by the least sensitive scale of the ISCO model UA-5 absorbance/fluorescence monitor, were consistently obtained from 40-50 g of infected cucumber tissue. However, relatively small

non-infectious pellets (< 1 mm diameter) were recovered after sucrose gradient centrifugation. No improvement in infectivity was obtained following autoclaving sucrose solutions. Yields, determined spectrophotometrically, were *c.* 5.0 mg/100g, A260/280 ratios were 1.7-1.9, Amax/Amin 1.3-1.5.

Virus preparations purified at pH 7.5 in 100 mM Tris-HCl consistently contained several peaks in sucrose gradients (Fig. 2*c*). Virus particles purified under these conditions appeared swollen and disrupted following negative staining with uranyl acetate. Addition of 10 mM $MgSO_4$ produced one predominant peak in sucrose gradients, sedimenting more slowly than the peaks observed without $MgSO_4$ (Fig. 2*b*). In the electron microscope decreased swelling was observed, although particles were usually partially degraded. FCVB was completely degraded, as judged by the complete lack of UV-absorbing peaks in sucrose gradients other than at the meniscus, when extracted in the presence of 10 mM EDTA. Partial degradation, as indicated by the appearance of virus particles, a reduction in the size of UV-absorbing peaks on sucrose gradients, and retarded migration in gradients, also occurred when high molarity Tris-HCl was used (100 mM, pH 7.0 or 7.5) or when $CaCl_2$ was added to buffers.

Virus preparations purified at pH 5.0 or pH 6.0 in 100 mM sodium acetate containing 10 mM $MgSO_4$ also contained a slow sedimenting predominant peak in sucrose gradients (Fig. 2*a*). In the electron microscope particles appeared relatively intact and unswollen.

All RNA preparations from purifed FCVB, irrespective of the purification conditions showed significant degradation as judged by the variable number of small products in agarose gels (Fig. 2). RNA extracted from preparations of FCVB purified at pH 7.5 was

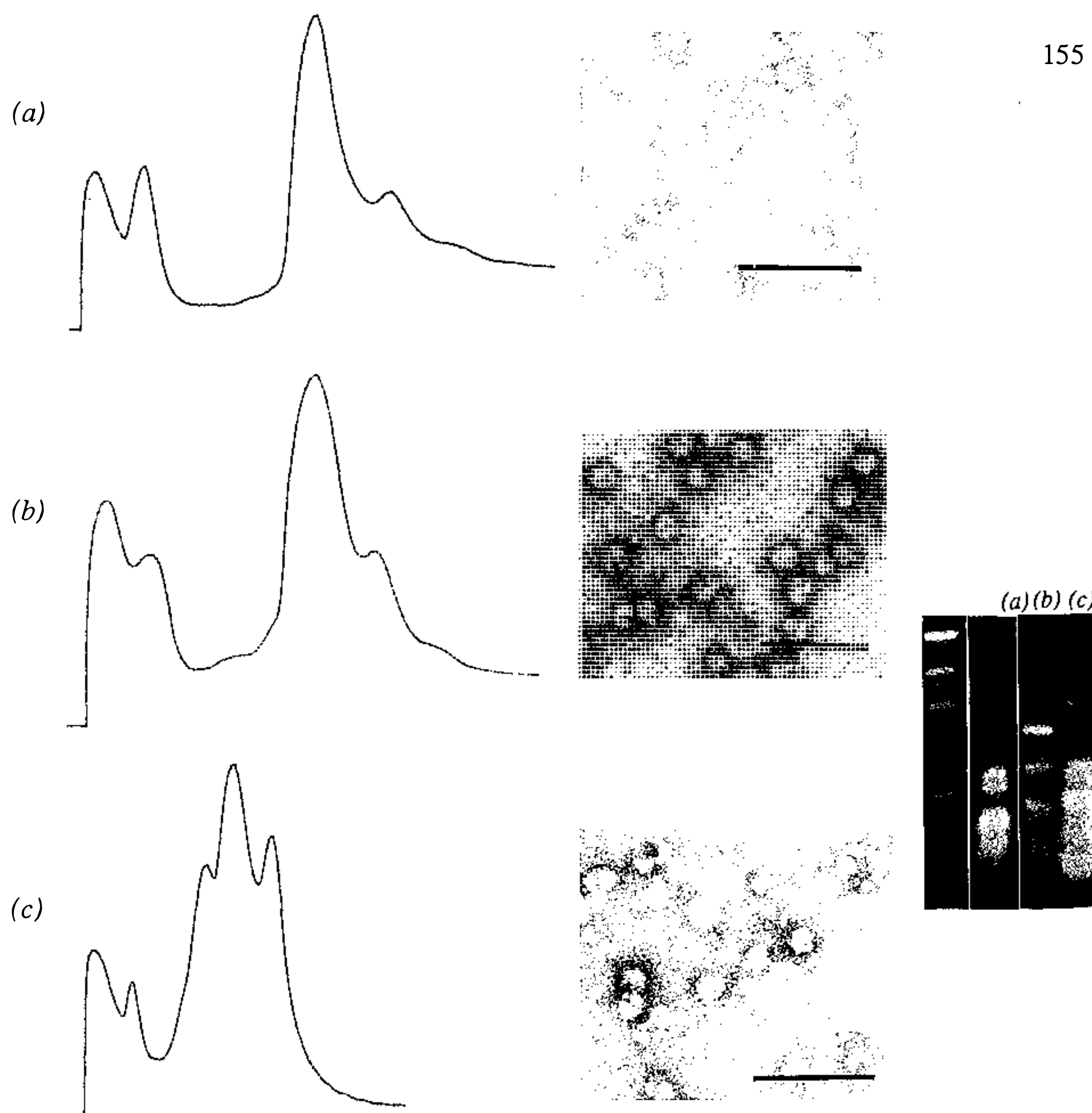

Fig. 2. Sedimentation profiles in sucrose density gradients, virion RNA profiles, and electron micrographs of particles of FCVB. FCVB preparations were purified in 100 mM acetate buffer (pH 5.0) containing 10 mM $MgSO_4$, resuspended overnight in, and added to sucrose gradients containing *(a)* 100 mM sodium acetate buffer (pH 5.0) containing 10 mM $MgSO_4$ *(b)* 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgSO_4$ and *(c)* 20 mM Tris-HCl buffer (pH 7.5). Bars on electron micrographs represent 100nm. The unmarked lane on the RNA gel is alfalfa mosaic virus, and RNA sizes correspond to (top to bottom) 3644, 2593, 2037 and 881 nucleotides.

less degraded than RNA from preparations made in low pH buffers (Fig. 2*b*).

Preparation of FCVB following clarification with 1% bentonite in 20 mM Tris-HCl pH 7.0 or 100 mM acetate pH 6.0, both containing 10 mM $MgSO_4$ (Lister, Bancroft, & Nadakavukaren, 1965), failed to yield ultraviolet absorbing peaks in sucrose gradients. In contrast, addition of 0.02% bentonite to the initial plant/buffer homogenate in either 100 mM acetate pH 6.0 or 20 mM Tris pH 7.5 yielded ultraviolet absorbing peaks. However, the bentonite failed to stabilise the virus, as indicated by retarded migration in sucrose gradients, particle morphology, and RNA integrity. Preparation of FCVB by clarification with hydrated calcium phosphate (Fulton, 1959), ether with ascorbic acid (P. Revill, pers. comm.), Celite (Francki & McLean, 1968), or by acidification at pH 4.8 (J.K. Uyemoto, pers. comm.) also failed to yield ultraviolet absorbing peaks in sucrose gradients.

Serology

In Ouchterlony tests involving both sap extracts in water and purified preparations in 20 mM Tris-HCl buffer at pH 7.0, FCVB failed to react with antisera to 10 ilarviruses (listed in Table 1) cucumber mosaic cucumovirus, alfalfa mosaic virus (AMV) and raspberry bushy dwarf virus.

Heterologous reactions among the bromoviruses are dependent on pH (Rybicki & Von Wechmar, 1981; Valverde, 1985). In the present study, serological reactions occurred between purified CCMV and BMV and CCMV and BMV antisera, respectively at pH 7.5. However, no reaction occurred between FCVB and antisera to BMV, BBMV or CCMV in Tris-HCl buffer at pH 7.5, 7.0 and 6.0 with or without 10 mM $MgSO_4$.

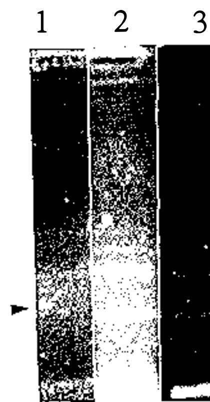

Fig. 3. DsRNA extracted from cucumber plants infected with FCVB and electrophoresed on formaldehyde denaturing gels. The fourth sub-genomic RNA species is not easily discernable, and its position has been marked with an arrow. Lane 1 = FCVB, lane 2 = *Prunus* necrotic ringspot virus, lane 3 = RNA markers (BRL). Markers correspond in size, top to bottom, to 9.49, 7.46, 4.40, 2.37, 1.35 and 0.24 kb.

Coat Protein

Protein from purified virus preparations contained a single major species of 19 300 daltons (mean of 4 determinations).

Nucleic acid

In view of the degree of degradation of RNA from purified virus preparations, all analyses of FCVB involved dsRNA. In denaturing gels, four RNA species were observed of 3900, 2150, 1800 and 800 bp (Fig. 3).

Discussion

Purification of FCVB under a range of pH, buffer, and ionic strength conditions resulted in non-infectious particles which appeared to be poorly defined and often partially disrupted in the electron microscope. Although purification at low pH in the presence of $MgSO_4$ appeared to prevent swelling, virion RNA was significantly degraded. This suggests shearing of the RNA within the virion, as proposed by Bancroft *et al.* (1968) for BMV, or the fact that the virion RNA is poorly protected from ribonucleases during purification. Attempts to purify FCVB in the presence of bentonite were unsuccessful, probably because of adsorption to the bentonite.

FCVB differs from most other bromoviruses in its lack of serological reactivity with antisera to BMV, BBMV and CCMV in gel diffusion tests. Rybicki & Von Wechmar (1981) reported that at pH 6.0 BMV, BBMV and CCMV did not cross react in

Table 2. Comparison of FCVB with bromoviruses.

	BMV	BBMV	CCMV	MYFV	CYBV	SBLV	FCVB
nucleic acid (x 10^6)							
RNA-1	1.1[a] (3234)[b]	1.1[a] (3158)	1.2[a] (3171)	1.2[a]	1.1[a]	1.27[c]	1.27[d]
RNA-2	0.97 (2865)	1.03 (2811)	1.07 (2774)	1.1	0.96	1.13	0.70
RNA-3	0.72 (2173)	0.90 (2293)	0.81 (2173)	1.0	0.78	0.84	0.59
RNA-4	0.30 (876)	0.36	0.28 (824)	0.3	0.31	0.25	
coat protein (daltons)	20 300	21 000	19 400	22 000	20 800	22 000	19 300
Thermal inactivation point(°C)	80	95	65-70	85	n.d.[e]	n.d.	40-50
Longevity *in vitro*	n.d.	20d	2-4d	3-5wk	n.d.	n.d.	1-3d
particle size(nm)	26	26	25	25	25-27	28	24
A260/A280	1.75	1.7	1.7	1.7	n.d.	n.d.	1.7-1.9
References	Lane (1977) Ahlquist *et al.* (1981 & 1984) Dasgupta & Kaesberg (1982)	Gibbs (1972) Dzianott & Bujarski (1991) Romero *et al.* (1992)	Bancroft (1971) Dzianott & Bujarski (1991) Allison *et al.* (1989) Dasgupta & Kaesberg (1982)	Hollings & Horváth (1981)	Dale (1988)	Valverde (1985)	

[a] RNA sizes estimated under non-denaturing conditions
[b] RNA sizes (in parentheses) determined by nucleotide sequencing
[c] RNA sizes estimated under denaturing conditions
[d] RNA sizes estimated under denaturing conditions using dsRNA
[e] n.d., not determined

BMV; brome mosaic virus
BBMV; broad bean mottle virus
CCMV; cowpea chlorotic mottle virus
MYFV; melandrium yellow fleck virus
CYBV; cassia yellow blotch virus
SBLV; spring beauty latent virus
FCVB; flowering cherry virus B

Ouchterlony gel diffusion tests, whereas at pH 7.0 or 7.5 strong cross reactivity occurred. Similarly, SBLV reacted with antisera to SBLV, CCMV and BMV at pH 7.0 but not at pH 6.0 (Valverde, 1985). Swelling under neutral or alkaline pH appears to expose conserved epitopes in the bromovirus group (Johnson & Argos, 1985).

In possessing four RNA species of 1800-3900 bps, FCVB is placed in the Tricornaviridae (Van Vloten-Doting *et al.*, 1981). Based on particle morphology, RNA and coat protein sizes, and swelling of particles at high pH in the absence of $MgSO_4$, FCVB most closely resembles the bromovirus group. A comparison of FCVB with other members of the bromovirus group is provided in Table 2. FCVB also differs from all other bromoviruses, however, in its instability in sap extracts and purified preparations.

Acknowledgements

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CHAPTER SEVEN

A new host record: strawberry latent ringspot virus isolated from flowering cherry

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Abstract

Strawberry latent ringspot virus (SLRV) was isolated from three flowering cherry (*Prunus serrulata* Lindl. *sensu lato*) trees in close proximity to each other in Auckland, New Zealand. The virus was not isolated from any of 390 flowering cherry trees tested from four other regions in the North Island. The virus was identified by host range, particle morphology, RNA and protein content and by serology. This is the first record of SLRV in flowering cherry.

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Introduction

Strawberry latent ringspot virus (SLRV), a tentative member of the nepovirus group, has been found to naturally infect numerous species and cultivars of several plant families. These include the Amaryllidaceae (narcissus), Apocynaceae (oleander), Caprifoliaceae (elderberry), Celastraceae (*Euonymus europaea* L.), Hippocastanaceae (*Aesculus carnea* Hayne), Liliaceae (asparagus), Oleaceae (olive), Papilionaceae (*Robinia pseudoacacia* L.), Polygonaceae (rhubarb), Rosaceae (blackberry, blackcurrant, cherry, peach, plum, raspberry, redcurrant, rose and strawberry), Umbelliferae (celery and parsnip) and Vitaceae (grapevine) (Betti and Canova 1989; Cammack 1966; Cooper 1981; Credi *et al.* 1981; Harrison 1967; Lister 1964; Marte and Gadani 1986; Murant 1974; Von Schmelzer 1969; Tomlinson and Walkey 1967; Vuittenez *et al.* 1970; Walkey and Mitchell 1969). In the Rosaceae, infection sometimes occurs without symptoms, but has been thought to be responsible for leaf mottle and growth decline of strawberry and raspberry, and stunting and chlorotic ringspots on leaves of rose (Murant 1974). Infection has also been shown to have a deleterious effect on grafting success, flower quantity and quality, and growth of young rose and peach seedlings (Thomas 1984; Németh 1980). In cherry, SLRV was first detected in 'Bing' sweet cherry (*Prunus avium* L.) in Ontario, Canada (Allen *et al.* 1970). It was later found in a sweet cherry tree of the same cultivar in New Zealand (Fry and Wood 1973).

During a survey of flowering cherry (*P. serrulata* Lindl. *sensu lato*) viruses in New Zealand, SLRV was detected by mechanical transmission from three flowering cherry trees growing in close proximity on a street in Auckland, but not from any of 390 trees from Palmerston North, Gisborne, New Plymouth or Taupo (Everett *et al.* 1993a; Appendix

VII). This is the first record of SLRV in this host.

Methods

Purification Virus was purified by the following procedure: Inoculated and systemically infected leaves of *Cucumis sativus* L. were harvested 3 weeks after inoculation and macerated in a mortar and pestle in 2 volumes of cold 0.2 M sodium phosphate buffer (pH 7.0) with 10 mM sodium EDTA and 0.01% mercaptoethanol. All purification steps were at 4°C. The extract was filtered through muslin, mixed with 0.25 volumes chloroform and 0.25 volumes butanol and centrifuged for 10 min at 16323 **g** in a Sorvall GSA rotor. Polyethylene glycol (Mr 8000, 10% w/v) and NaCl (1% w/v) were added to the supernatant which was shaken for 1 h at 4°C. The precipitate was recovered following centrifugation at 16323 **g** for 10 min and resuspended overnight in 0.02 M sodium phosphate buffer (pH 7.0). The resuspended preparation was further purified on 10-40% sucrose gradients containing 0.02 M sodium phosphate buffer (pH 7.0) by centrifugation for 4 h at 82700 **g** in a Beckman SW28 rotor. Fractions containing SLRV were concentrated by centrifugation for 1h at 158400 **g** in a TFT80.13 rotor and resuspended in 0.02 M sodium phosphate buffer (pH 7.0).

Serology Virus isolates were tested with antisera to SLRV (obtained from the Scottish Crop Research Institute) by DAS-ELISA (double antibody sandwich-enzyme-linked immunosorbent assay; Clark and Adams 1977) and by Ouchterlony double-diffusion. Gel diffusion was carried out in 0.75% Oxoid ionagar No. 2 in 0.01 M sodium phosphate

buffer (pH 7.0) containing 0.85% NaCl and 0.02% sodium azide.

Electron microscopy Purified virus preparations were stained with 1% sodium phosphotungstate pH 7.0, and examined using a Philips 201C electron microscope.

Gel electrophoresis of proteins and nucleic acids The virus coat proteins were extracted by the method of Forster and Jones (1979). Extracted proteins were analysed by electrophoresis on 12.5% polyacrylamide gels (Laemmli 1970) and size determinations made by comparison with molecular weight markers (Biorad).

Nucleic acids were extracted from SLRV by the method described in Everett *et al.* (1993b). Molecular weights were determined by comparison with RNA molecular weight markers (BRL) separated by formaldehyde denaturing gel electrophoresis (Maniatis *et al.* 1982).

Results

Herbaceous Host Range The isolate of SLRV obtained from flowering cherry infected species in the Chenopodiaceae, Solanaceae, Papilionaceae and the Compositae, and one species of the Cucurbitaceae.

Symptoms of infection on *Chenopodium quinoa* Willd. and *C. amaranticolor* Coste & Reyn., were systemic tip necrosis, local and systemic chlorosis, and systemic twisting and distortion. *Cucumis sativus* showed local chlorotic lesions and systemic vein netting. SLRV systemically infected *Nicotiana clevelandii* Gray, *N. megalasiphon* Heurch & Muell., *N. tabacum* L. 'White Burley', *Pisum sativum* L., and *Tithonia speciosa* Hook.

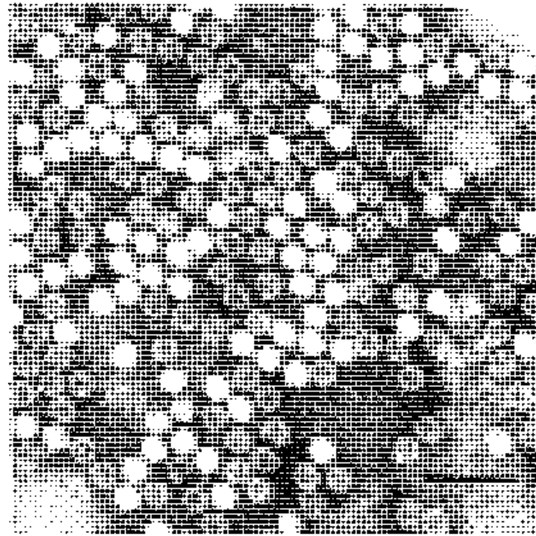

Figure 1 Electron micrograph of SLRV from flowering cherry, stained with sodium phosphotungstate. Bar = 100nm.

without visible symptoms, as determined by back inoculation to *Chenopodium quinoa* two weeks after inoculation.

On the basis of backtesting, SLRV did not infect members of the Amaranthaceae, other members of the Cucurbitaceae, the Gramineae and a member of the Papilionaceae that were tested, namely *Gomphrena globosa* L., *Momordica balsamina* L., *Cucurbita moschata* Duch., *Avena sativa* L., *Triticum aestivum* L., *Hordeum murinum* L. and *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*.

Properties of purified particles Virus preparations sedimented as two components following sucrose density gradient centrifugation, corresponding to the top and bottom components reported by Murant (1974). Hexagonal virus particles c.28 nm in size were detected in purified preparations (Figure 1). Particles from the top component were penetrated by sodium phosphotungstate as has been reported for nepoviruses (Harrison and Murant 1977).

Serology In Ouchterlony gel double diffusion tests, purified preparations reacted with antisera to SLRV but not with sap extracts from systemically infected leaves of *C. quinoa*. All three isolates, and the top and bottom components of purified SLRV from sucrose gradients, reacted positively in ELISA tests.

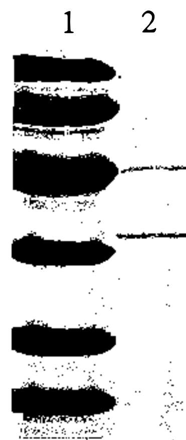

Figure 2 SDS-polyacrylamide gel electrophoresis of, lane 1, Biorad molecular weight markers (top to bottom; M_r 97.4, 66.2, 45, 31, 21.5 and 14.4 x 10^3) and lane 2, coat protein of strawberry latent ringspot virus extracted from flowering cherries by the method of Forster and Jones (1979) (M_r 45 and 32 x 10^3).

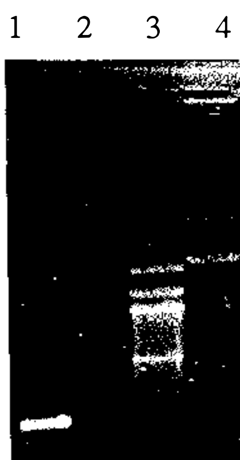

Figure 3 Denaturing gel with RNA size markers (BRL) (lane 1; top to bottom; 9.49, 7.46, 4.40, 2.37, 1.35 and 0.24kb, brome mosaic virus (lane 2; 2.9, 2.6, 2.1 and 0.79kb), alfalfa mosaic virus (lane 3; 3.6, 2.6, 2.0 and 0.88 kb) and strawberry latent ringspot virus (lane 4; 9.0 and 5.15 kb).

Properties of coat protein and nucleic acid Two coat protein species of Mr 45 and $32x10^3$ were identified from both the top and bottom components by polyacrylamide gel electrophoresis (Figure 2). These values are similar to the sizes of Mr 44 and 29 x 10^3 reported by Mayo *et al.* 1974 and Gallitelli *et al.* 1982.

Two RNA species of 9.0 kb (Mr $2.9x10^6$) and 5.15 kb (Mr $1.7x10^6$) were determined on denaturing gels (Figure 3). Molecular weights of Mr 2.6 and $1.6x10^6$ (Mayo *et al.* 1974; Gallitelli *et al.* 1982) on non-denaturing gels, and of Mr 2.9 and $1.4x10^6$ on denaturing gels have been reported elsewhere (Francki *et al.* 1985).

Discussion

A virus from *P. serrulata sensu lato* was identified as SLRV by serology, particle morphology, coat protein and RNA size, and symptoms on herbaceous indicator hosts. Symptoms produced by mechanical inoculation of SLRV isolates from *P. serrulata sensu lato* to *Chenopodium amaranticolor*, *C. quinoa* and *Cucumis sativus*, infections without symptoms (*Nicotiana clevelandii*, *N. tabacum*) and non-hosts (*Momordica balsamina*, *Vigna unguiculata*) were similar to those reported elsewhere (Betti and Canova 1989; Credi *et al.* 1981; Hicks *et al.* 1986; Lister 1964; Tomlinson and Walkey 1967; Walkey and Mitchell 1969). The flowering cherry isolate, in common with the isolates of Betti and Canova (1989) and Credi *et al.* (1981), did not infect *G. globosa* whereas most other isolates systemically infect this host.

This is the first record of the use of *N. megalasiphon* as a herbaceous host for SLRV. Although SLRV has been recorded in *Prunus avium* (Fry and Wood 1973; Allen *et al.*

1970), this is the first record in *P. serrulata sensu lato*.

As SLRV was not found in other geographical areas surveyed, the virus may have been introduced from a single restricted source. Several nematode vectors of nepoviruses occur in New Zealand (Mossop *et al.* 1983; Thomas and Procter 1972) but none were found (Wouts, W.M. pers. comm.) in soil samples obtained from under SLRV infected flowering cherries identified during this study. Alternatively, the commercial nursery practice used in past years of propagating both sweet and flowering cherries on rootstocks obtained from the same trees may have allowed SLRV to infect the flowering cherry trees from a sweet cherry source (Wood 1993).

Acknowledgements

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CHAPTER EIGHT

Nucleotide sequencing shows the coat proteins of strawberry latent ringspot virus are unrelated to the nepoviruses and comoviruses

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The nucleotide sequence of the 3'-terminal 2427 nucleotides of strawberry latent ringspot virus (SLRV) RNA-2 were determined using cDNA clones. The sequence contains a single reading frame terminating at an ochre stop codon 552 nucleotides from a 3'-terminal poly(A) tract. The N-terminal sequences of the two SLRV coat proteins determined by Edman degradation indicated that the larger 43K protein had a N-terminal Gly and the smaller 27K protein was cleaved at a Ser/Gly bond. No homologies were found in amino acid sequences or nucleotide sequences to four comoviruses or six nepoviruses suggesting that SLRV is unrelated to members of either virus group and should form a new group.

Strawberry latent ringspot virus (SLRV) has a wide host range, is seedborne and is spread by nematodes of the genus *Xiphenema*. Virus particles are sometimes visible in tubule-like structures in the cytoplasm (Hicks, 1985) which probably facilitate cell to cell translocation through plasmodesmata (Francki *et al.*, 1985). Purified preparations of SLRV contain 30 nm hexagonal particles which sediment in sucrose density gradients as two components of c. 55S and c. 130S (Murant, 1974). The 55S 'top' component is comprised of RNA-free coat protein shells which are penetrated by negative electron microscope stains. Virus peaks in the 130S 'bottom' component contains viral RNA and are not penetrated by negative stains. Some isolates also have a middle component of 95-99S (Gallitelli *et al.*, 1982). The genome of SLRV comprises two RNA species of M_r 2.6 x 10^6 and 1.6 x 10^6 (Mayo *et al.*, 1974) which are expressed as polyproteins (Hellen *et al.*, 1991). The genomic RNAs are polyadenylated at their 3' termini (Mayo *et al.*, 1979), and based on sensitivity to proteases, probably contain a protein covalently bound to their 5' termini (Mayo *et al.*, 1982). Some isolates have a satellite RNA species (Mayo *et al.*, 1974; Mayo *et al.*, 1982; Gallitelli *et al.*, 1982) of M_r 0.4 x 10^6.

These characteristics of SLRV are shared by the nepoviruses (Harrison & Murant, 1977), and for this reason SLRV has been tentatively placed in the nepovirus group. However, unlike all other nepoviruses, which have a single species of coat protein of *c.* 55K, SLRV has two coat protein species of 44K and 29K (Mayo *et al.*, 1974). In this respect, SLRV more closely resembles the comoviruses which have two coat proteins of 50K and 38K; bean pod mottle virus (BPMV) and cowpea severe mosaic virus (CPSMV) (MacFarlane *et al.*, 1991; Chen & Bruening, 1992), 41K and 24K; cowpea mosaic virus (CPMV) (van Wezenbeek *et al.*, 1983), and 37K and 23K; red clover mottle virus (RCMV) (Goldbach & van Kammen, 1985).

Fig. 1. *(a)* Northern hybridisation of clone pB6 to SLRV RNA-2 electrophoresed in a formaldehyde denaturing gel before transfer to nitrocellulose. *(b)* SLRV RNA electrophoresed in a formaldehyde denaturing gel and stained with ethidium bromide.

We have determined the nucleotide sequences of the 3' 2.4 kb of SLRV RNA-2 and shown that the deduced amino acid sequences of the two SLRV coat proteins are unlike those of the nepoviruses or the comoviruses. On this basis we propose that SLRV should be regarded as belonging to a distinct plant virus group.

The isolate of SLRV used in this study was from flowering cherry (Everett *et al.*, 1993*a*) and was maintained in cucumber and purified as described (Everett *et al.*, 1993*b*). RNA was extracted from purified virus according to Everett *et al.* (1993*b*). First-strand cDNA was synthesized from purified SLRV RNA using avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences Inc.) and oligo$(dT)_{12\text{-}18}$ as described by Sambrook *et al.* (1989). Second-strand synthesis of cDNA clones to the 3' region was as described by Gubler & Hoffman (1983). The cDNA was blunt-ended with T4 DNA polymerase and cloned into the *Sma*I site of pUC19. Clones corresponding to the 5' region of the 43K coat protein were obtained by PCR amplification of oligo(dT)-primed cDNA using the synthetic oligonucleotides
5'..AGCTGGATCCGGN(C/T)TNCA(C/T)GA(A/G)GA(C/T)(C/T)TNGTNCC..3, and
5'..ATAAACCAGACACCCTGCA..3' (corresponding to the 5' terminus of the 43K protein and to nucleotides 368-350 of the 43K protein respectively). PCR involved 1µl of cDNA heated for 5 min at 94°C in a 100µl reaction using Taq polymerase (Amersham), and 30 cycles of 94°C/1 min, 42°C/1 min, 50°C/1 min and 72°C/ 2 min.

The cDNA clone pB6 with an insert of 2281 nucleotides, and the cloned PCR product pPCR3 with an insert of 368 nucleotides, were selected for sequencing. Clone pB6 was shown to be specific to RNA-2 by Northern analysis (Fig. 1). DNA sequences were determined by dideoxynucleotide chain termination (Sanger *et al.*, 1977) using Sequenase^{TM} version 2. All regions were sequenced completely in both directions using

Fig. 2. Nucleotide sequence of the 3' terminal 2427 nucleotides of SLRV RNA-2 (the coat protein genes) and, in lines below the nucleotide sequence, the deduced amino acid sequence. The underlined amino acids correspond to the N terminus of the coat protein genes as determined from Edman degradation. ♦ indicates the presumed cleavage sites of the SLRV RNA-2 27K protein. Nucleotides in lower case are from a PCR primer containing redundancies.

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      10                  30                  50                  70                  90                  110
gggtttcatgaggatttggtccCTGCGGCATCTGGTGGTACTGAAGCCATTTTCTTTTCACCCAAGAGCATTCCTGTTCCAGGTAGTGCTAAGTTTGTTGGCTCTCACCCCTTTTCGTTT
G  L  H  E  D  L  V  P  A  A  S  G  G  T  E  A  I  F  F  S  P  K  S  I  P  V  P  G  S  A  K  F  V  G  S  H  P  F  S  F
      130                 150                 170                 190                 210                 230
CCTATAAATAGCAATGTTGGTACCACCGTCTACTCTCTACCTTTGATTAGTACCTCCTTAAAAGATACAGAGTGGGGAAGGTATTATAGGAGCTATACCTTCATGCGCTTTAAACCAACT
P  I  N  S  N  V  G  T  T  V  Y  S  L  P  L  I  S  T  S  L  K  D  T  E  W  G  R  Y  Y  R  S  Y  T  F  M  R  F  K  P  T
      250                 270                 290                 310                 330                 350
GTTAGATTGATTTCTTCTGCACCCATACAGGCCAAGGGGCTCCTGTGGCTATGTTATGATCCTTGTGAAACTCTTGCAAAGTATCCTAGTAGGGAGAGAGCTTTGATGCTGCAGGGTGTC
V  R  L  I  S  S  A  P  I  Q  A  K  G  L  L  W  L  C  Y  D  P  C  E  T  L  A  K  Y  P  S  R  E  R  A  L  M  L  Q  G  V
      370                 390                 410                 430                 450                 470
TGGTTTATGCCAGGTAGACATGATTCTGTCACGCTCACGATTGATGAACTTGCAACACCTTCTGGCTACTCTATAATGAATAGTGATCATAATGGAGCTTTCAAGGTGGTCATTATTAAG
W  F  M  P  G  R  H  D  S  V  T  L  T  I  D  E  L  A  T  P  S  G  Y  S  I  M  N  S  D  H  N  G  A  F  K  V  V  I  I  K
      490                 510                 530                 550                 570                 590
GATCTTGAAAATTTTGAGGTGGCTGACCTTGGGATGGAATTATCCCTTTTCCTAGATGTGCAAGATATTGGTATGGGTATCGGCCCTGAACTACCCCTCACGGATAGTTTTTTACCTCTT
D  L  E  N  F  E  V  A  D  L  G  M  E  L  S  L  F  L  D  V  Q  D  I  G  M  G  I  G  P  E  L  P  L  T  D  S  F  L  P  L
      610                 630                 650                 670                 690                 710
CGTCAGGTGGTTGTTGATTTCGATCTGTCAACCACAACTCCTAAGGGCAAGGCACTGGTGGTTCCTTTAAATCCCTTGCTTCCTGGTTTTGATGGAGCCCAGTGGTATCCTAGTTGCTCT
R  Q  V  V  V  D  F  D  L  S  T  T  T  P  K  G  K  A  L  V  V  P  L  N  P  L  L  P  G  F  D  G  A  Q  W  Y  P  S  C  S
      730                 750                 770                 790                 810                 830
TCTTCTATCCTGGAGAATCATCGTTACTGGAAAGGAACTCTTGTCCTAGAGGTTATTTTTAACCTTCCGGCCATGGGTGGTGGTACTGTGGAAATGGGCTTTGCAAATGACTCATACTCT
S  S  I  L  E  N  H  R  Y  W  K  G  T  L  V  L  E  V  I  F  N  L  P  A  M  G  G  G  T  V  E  M  G  F  A  N  D  S  Y  S
      850                 870                 890                 910                 930                 950
GGGTGGGAAAGCGATGCCTATCGTTACTTTGGTTCGACAGTTGTGGATCTGCGAGCGCATAGGTTGCTGCGTGCCAAGGTTCCCTTGTATGGTTATGGTGGGTATCTAATGGGGGGTAGT
G  W  E  S  D  A  Y  R  Y  F  G  S  T  V  V  D  L  R  A  H  R  L  L  R  A  K  V  P  L  Y  G  Y  G  G  Y  L  M  G  G  S
      970                 990                 1010                1030                1050                1070
GGATCTCTGTTTGCTGTACCGCCACTCACAGACTATGGGCAATCTTTGCGATTTGTCCTACTTTTCACAGCACCACTTCATATCAGTGACACTACTAAGAAGGGGTCTGTTATGATCCGT
G  S  L  F  A  V  P  P  L  T  D  Y  G  Q  S  L  R  F  V  L  L  F  T  A  P  L  H  I  S  D  T  T  K  K  G  S  V  M  I  R
      1090                1110                1130                1150                1170                1190
TATCTTGGCCTTGAGGACTGTGAGTATATTCAACCCACAACTTCTTTGGGGAGACTGAATCCAGCAACAACCCTAGTAGCATCTGGAGCGCCTGTCGTTCAGGTTGGAACATCTGACTGG
Y  L  G  L  E  D  C  E  Y  I  Q  P  T  T  S  L  G  R  L  N  P  A  T  T  L  V  A  S  G  A  P  V  V  Q  V  G  T  S  D  W
      1210                1230                1250                1270             ◆  1290                1310
ATGGAGCCACCACTTTTGCTGCCTTCCTCTGGGGCTTCTTCAGAAGAAGTTCCGCCTTCTTACTATTTCCAAGTGGCCCAAGAGTGGCTTCCTCTTTTTCCCAATGACACCAAGTTCCCA
M  E  P  P  L  L  L  P  S  S  G  A  S  S  E  E  V  P  P  S  Y  Y  F  Q  V  A  Q  E  W  L  P  L  F  P  N  D  T  K  F  P
      1330                1350                1370                1390                1410                1430
TATGCCAAAATTGGTTGGGTCACTTTTGAGGGTGAAGTTGAGCAACATAGCCCTTTGATGCATAGGAGCCAGGAGAATGCCCAATGGTGTGGTTCTCTGACGTATTATCTCTCCATTCGC
Y  A  K  I  G  W  V  T  F  E  G  E  V  E  Q  H  S  P  L  M  H  R  S  Q  E  N  A  Q  W  C  G  S  L  T  Y  Y  L  S  I  R
      1450                1470                1490                1510                1530                1550
TATTCGGGAGCTACACCTCAGGGAGTTCTTCCCATGAGGCCAGTTTGTTTTGGGGCCACAGTTCTGGATAATATACTGGATAAACCCTGTTTTGTGGAGAAGGACACGTTTATTCAGGTT
Y  S  G  A  T  P  Q  G  V  L  P  M  R  P  V  C  F  G  A  T  V  L  D  N  I  L  D  K  P  C  F  V  E  K  D  T  F  I  Q  V
      1570                1590                1610                1630                1650                1670
ATGCCAATGGCCGACCCTCGAGAGACTATTTATCTCCCGACCACTGAGGGTGTGGCTTTCTATGAGACACCACCAAGGCGTTGGGTCAACACCCATTTTGGGGCCACAGAATCATATGGA
M  P  M  A  D  P  R  E  T  I  Y  L  P  T  T  E  G  V  A  F  Y  E  T  P  P  R  R  W  V  N  T  H  F  G  A  T  E  S  Y  G
      1690                1710                1730                1750                1770                1790
GTTCGAACCTGTCCTGCATGGGTTCTCCTCCAATTTCCGAATGAAGAGGCTTCTCATTTAGGTGTTAGGGATGTCTCTTTGTGGGTGGAGCCAAATATCAGTTTCCGACATGCTGTCGGT
V  R  T  C  P  A  W  V  L  L  Q  F  P  N  E  E  A  S  H  L  G  V  R  D  V  S  L  W  V  E  P  N  I  S  F  R  H  A  V  G
      1810                1830                1850                1870                1890                1910
GGGTTCCCAATCTTGAAACCAACTCCTGCTCCTTCTATTGACTACTACTACGAGAATACTTTTCCGGTAGGTTAATCTCTGTTGGTGTTTTTCTTAGGCATTTCTCAAGGAGAATATCCC
G  F  P  I  L  K  P  T  P  A  P  S  I  D  Y  Y  Y  E  N  T  F  P  V  G  *
      1930                1950                1970                1990                2010                2030
TGGCCCAGATGGCGTTTTAAAAGGCAAGCCGGTGAACTTCGTGTGTCACTACCATGGAGCTACATGGTTTAGGGTCTTTAGTTAGTCCCTTGGTTACTTTTACCTCTTCATTGTCCATGT
      2050                2070                2090                2110                2130                2150
GTTGAGGCTGACTTTAATTAGTTGGATCTTTGGACCCCTCCTCGGGTTATTAGGTAAGCTGTAAGTAAGCAGCCGCTAGCGTTCTGGAATTCCAGGCATAGTGAGAATCAGTGGCAGATT
      2170                2190                2210                2230                2250                2270
AGCGACGCCACACGCTCGTAGACCGTACGATAGTCTACCCTTACGTGCTTTTGTAGTTTAGGGCCTTTTGATGTTATCTCTTTATTGAGTTGTACGTCACTTGTTACGCCACTCTGCTTT
      2290                2310                2330                2350                2370                2390
TGTGTGTTTTCTTGAGCCTTTCATGTCTTCCTTCATTGGATGTACGTGATTGTTATGCTCTTTGCTTTCTTTTGTGTTAAATAAAGGATTAACCAGGCATCCTCCCAGGTTTTACCGGGT
      2410
TTAGTTCTGGCACTTAGAATAAGCTTT
```

overlapping subclones generated by restriction digestion, and three oligonucleotides 5'..CCATCTGGGCCAGGGATA..3', 5'..TAGAAGGAGCAGGAGTTG..3', 5'..CAGTCCTCAAGGCCAAGA..3', complementary to nucleotides 1932-1915, 1123-1139, and 1101-1083, respectively. Sequences in the 3' region of the polyprotein and 3' untranslated region (UTR) were confirmed using five additional RNA-2 clones pA1, pB10, pC4, pD8, and pE5.

The nucleotide sequence and deduced amino acid sequence of SLRV coat protein genes are shown in Fig. 2. Because of the degenerative nature of the oligonucleotide used to clone the 5'-terminal 22 nucleotides of the coat protein gene, these nucleotides are in lower case. Clone pB6 was found to include the 3' UTR, and approximately 94% of the SLRV coat protein genes. Clone pPCR3 overlapped clone pB6 by 146 nucleotides and was identical in this region indicating that it was also derived from SLRV RNA-2. Excluding a 3'-terminal tract of 19 (A) residues in clone pB6, the cloned regions of pB6 and pPCR3 spans 2427 nucleotides of SLRV RNA-2.

The sequenced region of SLRV RNA-2 contains a single continuous reading frame beginning at a glycine codon and terminating at an ochre stop codon at nucleotide 1873. The N-terminal amino acid sequences of the two coat proteins (underlined in Fig. 2) were determined from purified virus by Edman degradation using an Applied Biosystems 470A automatic protein sequencer (Hewick *et al.*, 1981). Alignment of these amino acid sequences within the polyprotein shows that the smaller SLRV coat protein is located 3' of the larger coat protein. This is similar to the order of coat proteins of comoviruses. The larger coat protein of SLRV has 388 amino acids and a molecular weight of 43K, and the smaller has 236 amino acids and a molecular weight of 27K. This compares with reported sizes of M_r 44K and 29K (Murant, 1974) and 45K and 30K (Everett *et al.*,

1993*b*) determined on 12.5% and 15% polyacrylamide gels respectively. The N-terminal sequence of the 27K protein determined by Edman degradation is identical to the sequence deduced from the nucleotide sequence. The N-terminal sequence of the 43K protein determined by Edman degradation shown underlined in Fig. 2 differs at amino acid residue two from that deduced from the nucleotide sequence, presumably indicating that an inexact oligonucleotide primer molecule was incorporated in clone pPCR3. The C-terminal 13 residues of the 43K peptide determined by Edman degradation are identical to those predicted from the nucleotide sequence. The results of N-terminal amino acid sequencing indicate that the 27K protein is probably cleaved from the SLRV RNA-2 polyprotein at a Ser/Gly bond. Cleavage sites for the coat protein genes of related nepoviruses and comoviruses occur at Arg/Gly (grapevine fanleaf virus, GFLV, ArMV; Serghini *et al.*, 1990, Bertioli *et al.*, 1991), Arg/Ala (grapevine chrome mosaic virus, GCMV; Brault *et al.*, 1989), Lys/Ala (tobacco ringspot virus, TBRV; Demangeat *et al.*, 1991) Cys/Ala, (RRV; Blok *et al.*, 1992); Glu/Met and Gln/Gly (CPMV; van Wezenbeek *et al.*, 1983); Gln/Ser and Gln/Ser (CPSMV; Chen & Bruening, 1992). To our knowledge a Ser/Gly bond has not been reported previously as a cleavage site for plant viral polyproteins.

To date the nucleotide sequences of the coat proteins of six nepoviruses and four comoviruses have been determined. Rott *et al.* (1991) have aligned the amino acid sequences of four nepoviruses (TomRSV, TBRV, GCMV and GFLV) and shown that 38 invariant amino acids are shared by all four viruses. Alignment of the coat protein sequence of arabis mosaic nepovirus (ArMV) (Bertioli *et al.*, 1991) and raspberry ringspot virus (RRV) (Blok *et al.*, 1992) with the line-up of Rott *et al.* (1991) shows that 35 and 28, respectively, of the invariant amino acids are shared with these two viruses. Among

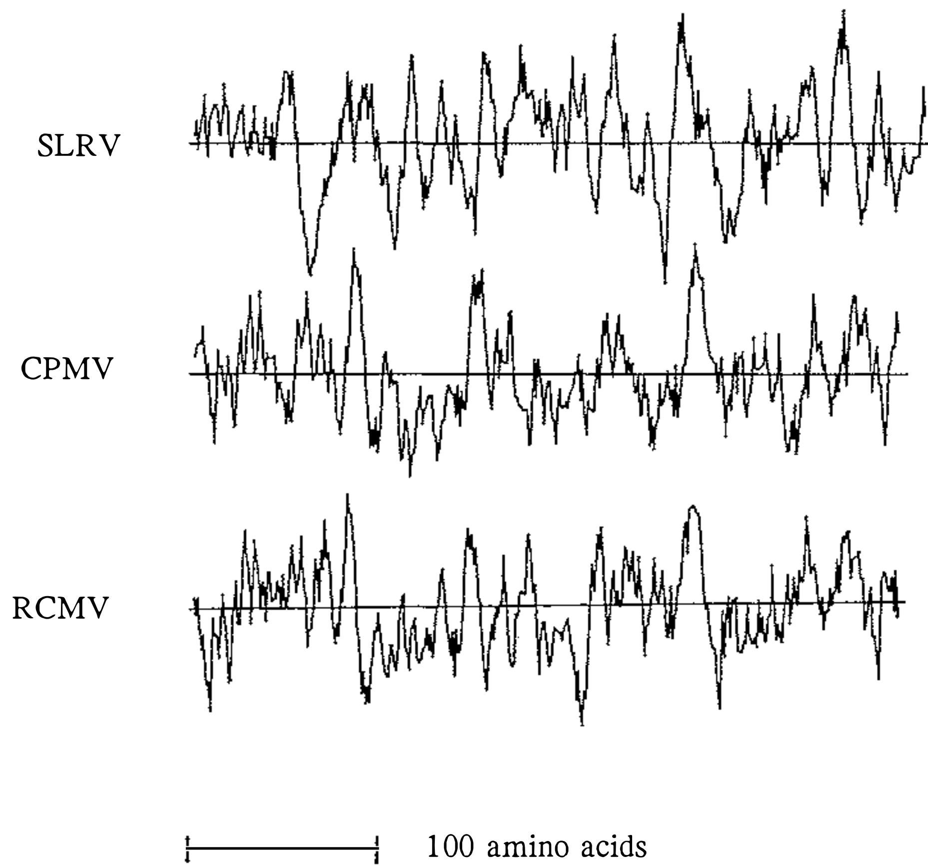

Fig. 3. Hydropathy profile of the deduced amino acid sequence of the 5' coat protein gene of SLRV and two comoviruses (CPMV and RCMV) computed by the algorithm of Kyte & Doolittle (1982) using the GCG program PEPPLOT. Hydrophobic regions appear above the abscissa.

the conserved amino acids are two motifs; (F/L)DA(Y/F)X(R/K)(I/Y) and (F/M)FYGX(S/T). In contrast to the nepoviruses, neither of the coat proteins of SLRV contains the invariant nepovirus amino acids and motifs. Similarly there is no homology between the SLRV coat protein and the comoviruses sequenced to date, which share with cowpea mosaic comovirus (CPMV) 67% identity in the 41K protein and 49% identity in the 24K protein. The hydropathy profiles of the larger coat protein of the comoviruses are very similar throughout the sequence, and are also similar, although to a lesser degree, in the smaller proteins (MacFarlane *et al.*, 1991). The hydropathy profile of the SLRV 43K protein shows little similarity to the 41K and 37K coat proteins of the comoviruses, CPMV and RCMV, respectively (Fig. 3). The 27K SLRV protein has no obvious similarities to the comovirus 23K (RCMV), 24K (CPMV), or 38K (CPSMV, BPMV) proteins (data not shown).

Excluding the poly(A) tail, the 3' UTR of SLRV RNA-2 is 552 nucleotides and contains a putative AAUAAA polyadenylation signal separated from the poly(A) tail by 62 nucleotides. Although most of the nepoviruses and comoviruses have shorter 3' UTRs, eg nepoviruses [TBRV; 301 nucleotides (Meyer *et al.*, 1986), GCMV; 251 nucleotides (Brault *et al.*, 1989), GFLV; 212 nucleotides (Serghini *et al.*, 1990), RRV; 397 nucleotides (Blok *et al.*, 1992)] and comoviruses [RCMV; 263 nucleotides (Shanks *et al.*, 1986), CPSMV; 471 nucleotides (Chen & Bruening, 1992), CPMV; 179 nucleotides (Van Wezenbeek *et al.*, 1983), BPMV; 455 nucleotides (MacFarlane *et al.*, 1991)], tomato ringspot nepovirus (TomRSV) (Rott *et al.*, 1991) has a significantly longer 3' UTR (1550 nucleotides). Cooper *et al.* (1992) reported that a United Kingdom isolate of SLRV has a 3' nontranslated region of 350 nucleotides.

Serghini *et al.* (1990) have reported four regions of nucleotide sequence homology in the

3' UTR of RNA-2 of the nepoviruses, GFLV, TBRV and GCMV, three of which are shared with CPMV. Within the first of these regions is a stretch of 10 nucleotides 5'..UUUUGUGUGU..3' found in GFLV that is also found in the 3' UTR of ArMV and of the comovirus RCMV and in the 3' UTR of SLRV RNA-2. However, this sequence is not found in the 3' UTR of other nepoviruses and comoviruses. The four regions of homology reported by Serghini *et al.* (1990) are not found in the 3' UTR of SLRV, and also are not found in the more recently sequenced nepoviruses and comoviruses, raising doubts about their significance.

In conclusion, we have identified the SLRV coat protein genes in the 3' region of SLRV RNA-2. SLRV shares numerous obvious similarities with both the nepoviruses and the comoviruses. However, the conservation of amino acids among the nepoviruses and among the comoviruses, and the lack of any homology between SLRV and these viruses, indicates that SLRV should be regarded as belonging to a distinct virus group.

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CHAPTER NINE

GENERAL DISCUSSION

The results of a survey conducted in this study showed that flowering cherry trees in the North Island of New Zealand are infected with at least six sap-transmissible viruses. Based on symptoms in indicator hosts, up to nine further viruses were also detected but were not characterised further in this study. Many centuries of cultivation involving grafting, clonally propagating rootstocks and scions, and cross-breeding, has probably resulted in the spread of viruses from a large geographical area within the flowering cherry species. The interchangeability of rootstocks between *Prunus avium* and flowering cherry in New Zealand has almost certainly assisted the spread of viruses between these species.

Because viral infection causes minimal damage to most flowering cherries, a decision needs to be made concerning the benefits compared to the costs of producing virus-free flowering cherries. Improvements of growth rate and fruit production, which have been shown to be detrimentally affected by viral infection (Klos & Parker, 1960; Milbrath, 1957; Parker *et al.*, 1959), are not necessarily relevant to an ornamental species such as flowering cherry. In some varieties a dwarfing capability is desirable. The possible benefits of a virus-free status may be more prolific flowering, an improvement of graft

take, improved longevity of trees, removal of yellow patterns from leaves, and decreased susceptibility to non-viral pathogens. Furthermore, the use of virus-free material should decrease the risk of viruses, particularly the pollen-borne ilarviruses, spreading into commercial fruit producing *Prunus* spp.

Because of the large number of sap-transmissible viruses found in flowering cherries, indexing to improve the virus status would be difficult. The number of viruses that would have to be tested would make the procedure extremely laborious and time consuming. Utilisation of the ELISA technique for screening flowering cherry mother stock may be feasible, if indirect ELISA was used, and thus only one antiserum would need to be conjugated. Even so, at least six antisera would need to be used for each flowering cherry sample making the costs of testing prohibitive. DsRNA extraction could be used for indexing flowering cherries, as all RNA viruses infecting flowering cherry would be detected using this method. Viroids, if present, may also be detected. However, further work is required to optimise the dsRNA technique to detect viruses and viroids infecting flowering cherry trees.

Further sequence analysis of the ilarviruses infecting flowering cherry may provide stretches of homology which could be utilised to synthesise group-specific cDNA probes for use in virus testing flowering cherries. However, this has not been achieved to date in this study, and sequences for these viruses have not been published elsewhere.

This study has shown that when using either sap-transmission to cucumbers, or ELISA, to detect PNRSV-G in flowering cherries, repeated temporally separated sampling is required

to ensure accuracy. ELISA was shown to be more reliable than sap-transmission. There was some indication that combining samples collected from the north, west, south and east quadrants of flowering cherry trees may dilute the virus below detectable levels, especially when using sap-transmission. However, further studies are required to confirm this. Separate testing, rather than combining, these four samples may result in greater accuracy of testing for viruses.

Based on testing single samples, combined samples, and temporally separated samples, approximately half the flowering cherries tested were free of viruses sap-transmissible to cucumbers. However, these trees cannot be considered healthy, because there are a number of virus-like agents that can only be graft-transmitted. These were not tested for in this study, but it is possible that they may infect a significant proportion of flowering cherry trees. In addition, there may have been mechanically transmissible viruses present which do not infect cucumbers or *Chenopodium quinoa.*

If virus-free flowering cherries were considered desirable, the most practicable method of producing these would be to heat treat a nucleus of mother plants. Propagation by cuttings, or possibly tissue culture, and then periodic testing for known viruses would then be required. This study has revealed that a field oriented testing programme for virus and virus-like agents would be difficult because of the prevalence of infections, the variety of infectious agents and sampling difficulties. Alternatively, trees found to be free of detectable viruses in the present study could be further tested using techniques such as grafting to *Prunus* spp. indicators, electron microscopy and dsRNA extraction.

Despite improvements in yield following purification of PNRSV-G, synthesis of cDNA was complicated by the presence of host contamination in the viral RNA preparations. The polymerase chain reaction could be used to produce further virus specific cDNA and overcome the problem of host contamination, but because only one virus-specific cDNA clone was synthesised in this study, this technique was not able to be effectively utilised. Taxonomic problems of the ilarviruses could be further illuminated using sequencing information, as in the instance of SLRV in this study.

Two new viruses were isolated, and tentatively identified as an ilarvirus (FCVI) and the other a bromovirus (FCVB). Development of an antisera to FCVI and subsequent ELISA indexing may show that this virus is more common than suggested in this study. Based on symptom types, it is possible that other new viruses are present in flowering cherry, due to the large number of sap-transmissible viruses that did not react with antisera to viruses known to be most common in other *Prunus* spp. (Wood, 1979).

The tentativly identified bromovirus, FCVB, defied every attempt to purify it without degradation. Purification conditions which prevented RNA and virion degradation were not found in the course of this study, although magnesium ions gave partial success.

Both FCVB and FCVI are members of the Tricornaviradae, with tri-partite, single-standed RNA genomes. The molecular weight of RNAs (M_r 1.26 x 10^6, 0.70 x 10^6, and 0.60 x 10^6 or 3900, 2150, 1800 nucleotides, FCVB; M_r 1.15 x 10^6, 0.90 x 10^6, 0.65 x 10^6 or 3550, 2800 and 2000 nucleotides, FCVI) and coat protein (M_r 19 300, FCVB; M_r 30 000, FCVI), are within the parameters proposed for the Tricornaviridae (Van Vloten-Doting *et al.*,

1981). Both FCVB and FCVI also have a fourth subgenomic RNA (M_r 0.26 x 10^6 or 800 nucleotides, FCVB; M_r 0.34 x 10^6 or 1050 nucleotides, FCVI), another characteristic of the Tricornaviridae. RNA-4 of FCVB is slightly smaller than that described by Van Vloten-Doting *et al.* (1981) for the Tricornaviridae. Because of the morphological similarity of FCVI particles to ilarviruses (quasi-isometric, 26 nm diameter, with some bullet-shaped particles) this virus is most likely to belong to the ilarvirus group. Particles of FCVB swell above pH 6.0, a characteristic of the bromoviruses, the group to which this virus is most likely to belong.

The information obtained in the current study showed that SLRV is dissimilar to the nepoviruses and comoviruses. Previously SLRV has been tentatively placed in the nepovirus group because it shares a number of properties with these viruses including nematode transmission, hexagonal *c.* 30 nm particles, two RNA species, particle stability, wide host range, and seed transmission. However, the tentative status of SLRV in this group is due to the presence of two coat protein species, compared to the other nepoviruses which have a single coat protein species. Sequencing information showed further differences between SLRV and the nepoviruses. SLRV does not share amino acid homologies or nucleotide homologies with those nepoviruses or comoviruses which have been sequenced. Based on this information, SLRV should be considered to belong to a separate viral group.

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APPENDICES

APPENDIX I

FERTILISERS FOR INDICATOR PLANT GROWTH

g/m³ peat/pumice 80:20	
1500	Osmocote 14-6.1-11.6
720	calcium ammonium nitrate
450	potassium nitrate
560	superphosphate
3000	dolomite
150	FTE 36

APPENDIX II

REAGENTS AND BUFFERS

A2.1 Reagents and buffers used in ELISA.

DE 52 cellulose: diethylaminoethyl cellulose (Whatman).

10x PBS: 0.01 M phosphate buffer (pH 7.4) + 0.14 M NaCl, 0.003 M KCl, 0.003 M NaN_3.

PBS-Tween 20: 1x PBS, 0.05% TWEEN 20 (Poly(oxyethylene)n-sorbitan-monolaurate).

Extraction buffer: 1x PBS, 2% polyvinylpyrollidone (w/v).

Coating buffer: 0.03 M carbonate buffer pH 9.6.

Substrate buffer: 97 ml diethanolamine, 800 ml DDW.

Enzyme substrate: ρ-nitrophenyl phosphate

Enzyme: Calf intestine alkaline phosphatase (Sigma, type VII-T).

A2.2 Reagents and buffers used in virus purification.

0.01 M Phosphate pH 7.5: 0.2 M Na_2HPO_4 adjusted to pH 7.5 with 0.2 M NaH_2PO_4 and diluted to 0.01 M as required.

0.01 M Tris.HCl pH 7.5: 0.1 M Tris (Tris[Hydroxymethyl]aminomethane) adjusted to pH 7.5 with 0.1 M HCl and diluted to 0.01 M as required.

0.02 M acetate pH 5.0: 0.2 M sodium acetate adjusted to pH 5.0 with 0.2 M acetic acid and diluted to 0.02 M as required.

Triton X-100: octylphenolpoly(ethyleneglycolether)n (Sigma).

PEG 8000: Polyethylene glycol, M.W. 8000 (Sigma).

10-40% sucrose density gradients: 25% sucrose solution dispensed into Beckman SW 28 centrifuge tubes, frozen for 12 h and thawed overnight at 4 C.

A2.3 Reagents and buffers for formaldehyde gels.

10x MOPS buffer: 0.04 M MOPS (3-[N-Morpnolino]propanesulphonic acid), 10 mM sodium acetate (CH_3COONa), 1 mM EDTA (ethylenediaminetetraacetic acid), adjusted to pH 7.0 with NaOH and sterilised by filtration.

Deionised formaldehyde: 10 g Biorad Amberlite mixed bed resin, Analytical Grade, /100 ml formaldehyde, stirred for 2 h at room temperature, followed by filtration.

Tank buffer: 100 ml 10x MOPS buffer and 80 ml formaldehyde/l DDW.

Denaturing buffer: 1.3x MOPS buffer, 22.6% formaldehyde (v/v), 64.5% formamide (v/v).

Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol.

A2.4 Reagents and buffers for dsRNA extraction.

Glycine buffer: 0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, pH adjusted to 9.5 with NaOH.

STE: 100 mM NaCl, 50 mM Tris.HCl (pH 7.0), 1 mM EDTA.

STE/ethanol: 18% ethanol in STE (v/v).

TE: 10 mM Tris.HCl (pH 7.0), 0.1 mM EDTA.

A2.5 Buffers and media used in pUC19 cloning.

Luria (L) broth: 1% (w/v) bacto-tryptone (Difco^R), 0.5% (w/v) yeast extract (Difco^R), 0.5% (w/v) NaCl, pH 7.0 (Luria & Burrows, 1957)

L agar: L broth containing 1.5% (w/v) Davis agar.

L plates containing BCIG and ampicillin: L agar containing 100 μg/ml ampicillin (BRL), 40 μg/ml BCIG, (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, BRL) added when agar cooled to 60C.

2x TY: bacto-tryptone 16g/l, bacto-yeast extract 10g/l, NaCl 5g/l.

STET buffer: 50 mM EDTA (pH 8.0), 55 mM Tris.HCl (pH 8.0), 8% (w/v) sucrose, 1% (v/v) Triton X-100.

Terrific broth (TB): bacto-tryptone 6 g, bacto-yeast extract 12 g, glycerol 2 ml, water to 450 ml sterilised by autoclaving, plus 50 ml terrific salts added after autoclaving.

Terrific salts: 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, sterilised by autoclaving.

RNase A: RNase A (Sigma) was dissolved in sterile DDW to a concentration of 10 mg/ml, then incubated at 100 C for 15 min to destroy any contaminating DNase I activity. The solution was stored in 1 ml aliquots at -20 C.

A2.6 Reagents and buffers used for hexamer priming.

DTM mix: 250 mM Tris.HCl pH 8.0, 25 mM $MgCl_2$, 50 mM ß-mercaptoethanol, 100 µM dCTP, 100 µM dGTP, 100 µM dTTP.

Hexamer mix: 100 µg/ml (Pharmacia 27-2166-01) in 1 mM Tris.HCl pH 8.0, 1 mM EDTA.

A reaction mix: 80 µl DTM mix, 11.4 µl Hexamer mix, 80 µl 1 M HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulphonic Acid) pH 7.0.

A2.7 Reagents and buffers used in hybridisations.

NaCl/PIPES/EDTA: 3 M NaCl, 100 mM PIPES (1,4-Piperazinediethanesulphonic acid), 20 mM EDTA; pH adjusted to 6.8 with NaOH.

Hybridisation buffer: 5 ml formamide (BRL nucleic acid grade stored frozen), 2 ml NaCl/PIPES/EDTA

100x Denhardts solution: 2% Ficoll (w/v), 2% gelatin (w/v), 2% Polyvinylpyrollidone (w/v), autoclaved.

A2.8 Reagents and buffers used in sequencing reactions.

40% acrylamide gel stock: Acrylogel (BDH; 19:1 premix of acrylamide and bisacrylamide) resuspended in sufficient sterile DDW to make a 40% solution.

Enzyme dilution buffer: 10 mM Tris.HCl pH 7.5, 5 mM dithiothreitol (DTT), 0.5 mg/ml bovine serum albumin.

Forward Primer: 5'-GTTTTCCCAGTCACGAC-3', 0.5 pmol/µl

Reverse Primer: 5'-AACAGCTATGACCATG-3', 0.5 pmol/µl

Dithiothreitol (DTT) 0.1 M

Labelling mix (dGTP) 5x concentrate: 7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP.

Reaction mixture: 1.6 mM DTT, 0.32 x labelling mix, 0.32 x enzyme dilution buffer, 10 µM (1000-1500 Ci/mM) [α-^{32}P]dATP, 16 units Sequenase® Version 2.0.

ddG termination mix: 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddGTP, 50 mM NaCl.

ddA termination mix: 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddATP, 50 mM NaCl.

ddT termination mix: 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddTTP, 50 mM NaCl.

ddC termination mix: 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddCTP, 50 mM NaCl.

Stop solution: 95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% xylene cyanol FF.

APPENDIX III

ABBREVIATIONS FOR VIRUS NAMES (following Francki *et al.*, 1985)

ACLSV	apple chlorotic leafspot virus
AMV	alfalfa mosaic virus
ApMV	apple mosaic virus
APLPV	American plum line pattern virus
ArMV	arabis mosaic virus
ASV	asparagus stunt virus
AVII	asparagus virus II
BBMV	broad bean mottle virus

BMV	brome mosaic virus
BRLV	black raspberry latent virus
BRNV	bean red node virus
BYV	beet yellows virus
CCLV	citrus crinkly leaf virus
CCMV	cowpea chlorotic mosaic virus
CGRM	cherry green ring mottle virus
CLRV	cherry leaf roll virus
CMV	cucumber mosaic virus
CNFV	carnation necrotic fleck virus
CoMV	cowpea mosaic virus
CRLV	cherry rasp leaf virus
CTV	citrus tristeza virus
CVV	citrus variegation virus
CYBV	cassia yellow blotch virus
DPLV	danish plum line pattern virus
EMV	elm mottle virus
FCVB	flowering cherry virus B
FCVI	flowering cherry virus I
GCMV	Hungarian chrome mosaic virus
HMV	hydrangea mosaic virus
LRMV	lilac ring mottle virus
MYFV	melandrium yellow fleck virus
PDV	prune dwarf virus

PLPV	plum line pattern virus
PNRSV	*Prunus* necrotic ringspot virus
PPV	plum pox virus
PZSV	pelargonium zonate spot virus
RBDV	raspberry bushy dwarf virus
RMV	rose mosaic virus
RRV	raspberry ringspot virus
SBLV	spring beauty latent virus
SLRV	strawberry latent ringspot virus
SPLV	spinach latent virus
TAMV	Tulare apple mosaic virus
TBSV	tomato bushy stunt virus
TMV	tobacco mosaic virus
TobBRV	tobacco black ring virus
TobRSV	tobacco ringspot virus
TBRV	tomato black ring virus
TMV	tobacco mosaic virus
TomBSV	tomato bushy stunt virus
TomRSV	tomato ringspot virus
TSV	tobacco streak virus

APPENDIX IV

A4.1 Preparation of hydrated calcium phosphate (HCP).

A slightly less than equal volume of 0.1 M $CaCl_2$ was added to 0.1 M Na_2HPO_4 and allowed to precipitate. After 4-6 h the supernatant was decanted off and discarded. The precipitate was washed overnight with reverse osmosis water then allowed to settle. The supernatant was decanted off and discarded, then the precipitate collected by low speed centrifugation (8000 *g*, 10 min) and stored at room temperature (a modification of the method of Fulton, 1959).

A4.2 Reagents and buffers.

Tissue extraction buffer: 0.06 M potassium phosphate pH 8.0, 0.02 M DIECA, 0.02 M thioglycollic acid liquid (Sigma, No. T-3758).

TAE: 0.04 M Tris, 0.04 M sodium acetate, 0.01 M EDTA, pH adjusted to 7.8 with glacial acetic acid.

APPENDIX V

Table A.1: Symptoms of sap-transmissible viruses from flowering cherries in *Cucumis sativus*, grouped according to symptom type and reaction to *Prunus* necrotic ringspot virus serotype g (PNRSV-G) antisera in ELISA. These include 211 viruses isolated from 434 flowering cherry trees.

type	tree	symptoms															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
I	31	+															
	34	+															
	56	+															
	97	+															
	101	+															
	102	+															
	106	+															
	139	+															
	192	+															
	195	+															
	199	+															
	214	+															
	220	+															
	230	+															
	231	+															
	232	+															
	233	+															
	271	+															
	300	+															
	315	+															
	352	+															
	421	+															
II	48		+														
	154		+														

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	339		+														
	152			+													
	188			+													
	205			+													
	58				+												
	169		+		+												
	172	+	+														
	88					+											
	161					+											
	168					+											
III	104	+							+								
	291	+							+								
	163	+								+	+						
	176				+				+								
	130	+							+	+							
	211	+									+		+				
	343	+									+		+				
	135												+				
	64												+				
	67	+								+							
	187	+								+							
	125	+								+		+					
	39	+								+		+					
	66	+								+		+					
	121	+											+				
	4			+									+				
	57		+	+								+				+	
	61	+										+				+	
	71	+		+	+				+	+		+				+	
	78	+							+				+				
	89	+		+						+	+		+			+	
	107			+	+					+							
	151	+								+	+				+		

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	156	+		+						+							
	157			+	+					+		+					
	160	+								+		+				+	
	162	+			+				+			+					
	184	+		+	+				+				+			+	
	2	+											+			+	
	6		+							+		+				+	
	84	+											+			+	
	87			+	+				+								
IV	80			+	+			+	+								
	183	+						+		+							
V	75			+	+					+			+		+		
	93		+							+		+			+		
	164	+		+					+	+					+		
	174	+								+		+			+		
	180	+	+							+					+		
	182	+				+			+	+					+		
	186	+				+			+	+					+	+	
	196	+		+					+	+					+		
	165					+				+		+					
	171	+				+											
	173	+				+			+	+			+			+	
	181			+		+			+								
	49		+			+			+			+					
	108	+											+		+		
	145			+	+	+				+						+	
	146	+				+			+	+		+			+	+	
	149	+				+				+					+		
	207	+							+						+		
VI	5		+	+		+	+	+								+	
	7			+		+	+					+				+	
	28					+	+			+						+	
	147					+	+			+							

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	166				+	+	+		+								
	41	+		+		+	+		+	+							
	167	+	+	+			+			+		+			+	+	
	10	+	+	+		+	+			+		+				+	
	170	+	+		+		+		+	+					+		
	179	+	+	+	+		+	+	+	+		+			+		
	3	+		+			+			+							
	177	+		+			+			+						+	
	95	+		+			+		+								
	193	+		+	+		+		+	+							
	60			+	+		+									+	
	153			+	+		+		+								
	284	+					+		+								
	420	+			+		+			+	+						
VII	418	+					+						+		+		
	240	+				+	+						+				
	155	+	+		+	+	+						+			+	
	235	+					+						+				
	236	+					+						+				
	237	+					+						+				
	283	+					+						+				
	287	+					+						+				
	338	+					+						+				
	286	+					+				+		+				
	238	+					+				+		+				
	270	+					+				+		+				
	282	+					+				+		+				
	289	+					+				+		+				
	295	+					+				+		+				
	301	+					+				+		+				
	326	+					+				+		+				
	433	+					+			+	+		+				
	442	+			+	+	+			+	+		+				

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	242					+	+						+	+		+	
	333	+					+						+	+			
	239	+					+						+	+			
	221	+					+						+	+			
	222	+					+						+	+			
	9	+					+		+				+			+	
	290	+					+		+				+				
	292	+					+		+				+				
	69	+			+		+	+	+			+	+			+	
	83	+		+			+						+				
VIII	40	+															
	44	+															
	50	+															
	59	+															
	73	+															
	103	+															
	120	+															
	123	+															
	134	+															
	137	+															
	178	+															
	200	+															
	202	+															
	224	+															
	225	+															
	226	+															
	258	+															
	265	+															
	296	+															
	308	+															
	310	+															
	311	+															
	318	+															

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	330	+															
	341	+															
	351	+															
	360	+															
	361	+															
	369	+															
	374	+															
	375	+															
	381	+															
	384	+															
	388	+															
	398	+															
	400	+															
	401	+															
	428	+															
	435	+															
	483	+															
IX	18		+														
	158		+														
	30		+	+													
	376		+	+													
	367		+	+	+												
	387		+	+	+												
	150		+	+	+												
	21				+												
	201				+												
	32				+												
	185			+													
	159	+			+												
X	11	+											+				
	26	+											+				
	189		+										+				
	105		+	+									+				

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	191								+								
	194	+							+								
	305	+							+								
	427	+								+	+						
	131	+							+			+					
	143								+				+				
XI	402	+		+	+				+								
	126	+											+			+	
XII	129								+			+			+		
XIII	55				+			+								+	
XIV	76							+	+								+
	118			+	+				+								+
XV	298	+					+				+		+				
	336	+					+				+		+				
	334	+					+										
	377	+					+							+			
	379	+			+	+	+										
XVI	68														+		
	198		+	+	+		+	+	+						+		
XVII	320					+	+		+								
	86					+											
	419	+	+			+	+						+				

Key:

Type

I. PNRSV serotype g (PNRSV-G), local lesions type 1.
II. PNRSV-G, local lesions type 2.
III. PNRSV-G, systemic symptoms type 1.
IV. PNRSV-G, systemic symptoms type 2.
V. PNRSV-G, systemic symptoms type 3.
VI. PNRSV-G, systemic symptoms type 4.
VII. PNRSV-G, systemic symptoms type 5.
VIII. PNRSV-G negative, local lesions type 1.
IX. PNRSV-G negative, local lesions type 2.
X. PNRSV-G negative, systemic symptoms type 1.
XI. PNRSV-G negative, systemic symptoms type 2.
XII. PNRSV-G negative, systemic symptoms type 3.
XIII. PNRSV-G negative, systemic symptoms type 4.
XIV. PNRSV-G negative, systemic symptoms type 5.
XV. PNRSV-G negative, systemic symptoms type 6.
XVI. PNRSV-G negative, systemic symptoms type 7.
XVII. PNRSV-G negative, systemic symptoms type 8.

Symptoms

1. chlorotic blotches on cotyledons
2. necrotic spots on cotyledons, <1mm diameter
3. necrotic spots on cotyledons, 1-3mm diameter
4. necrotic spots on cotyledons, >3mm diameter
5. ringspot on cotyledons
6. tip death
7. cotyledon death
8. systemic necrotic spots, 1-2mm diameter
9. systemic necrotic spots, 2-3mm diameter
10. systemic necrotic spots, >3mm diameter
11. coalescing necrotic spots
12. interveinal chlorosis on true leaves
13. distorted true leaves
14. ringspots on true leaves
15. rugose true leaves
16. necrotic centres of true leaves.

APPENDIX VI

Papers submitted or to be submitted to the following journals:

Everett, K.R., Milne, K.S. & Forster, R.L.S. (1993). Sap-transmissible viruses in flowering cherry in New Zealand. New Zealand Journal of Crop and Horticultural Science.

Everett, K.R., Milne, K.S. & Forster, R.L.S. (1993). Flowering cherry virus I, a possible new ilarvirus from flowering cherry. Plant Pathology.

Everett, K.R., Milne, K.S. & Forster, R.L.S. (1993). Flowering cherry virus B, an isometric virus from flowering cherry with properties of bromoviruses. Annals of Applied Biology.

Everett, K.R., Milne, K.S. & Forster, R.L.S. (1993). A new host record: strawberry latent ringspot virus isolated from flowering cherry. Australasian Plant Pathology.

Everett, K.R., Milne, K.S. & Forster, R.L.S. (1993). Nucleotide sequencing shows the coat proteins of strawberry latent ringspot virus are unrelated to the nepoviruses and comoviruses. Journal of General Virology.

APPENDIX VII

Table A.2: Geographic location of viruses mechanically transmissible from young flowering cherry tissue to *Cucumis sativus.*

Virus	Area sampled[a]					
	Palmerston North	New Plymouth	Auckland	Taupo	Gisborne	total
PNRSV-G	108	5	6	12	2	133
ApMV	8	3	2	1	-	14
PDV	2[b]	-	-	-	-	2
SLRV	-	-	3	-	-	3
FCVI	-	-	1[b]	-	-	1
FCVB	40[b]	-	4[b]	-	-	44
ACLSV	1[b]	-	-	-	-	1
unidentified virus	34	7	6	1	1	45
total	276 (322)	15 (38)	15 (44)	14 (20)	3 (10)	211(434)

Key:

[a] Samples were taken from September to December, 1987.
[b] Viruses present in combination with other viruses.
note: bracketed values are total numbers of flowering cherry trees sampled.

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LURIA, S.E. & BURROWS, J.N. (1957). Hybridization between *Escherichia coli* and *Shigella*. Journal of Bacteriology 74: 461-476.