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UNIVERSITY OF ALBERTA

SEROLOGICAL AND GENETIC ANALYSES OF SWEET CLOVER NECROTIC MOSAIC VIRUS

BY

HANUMANTHA RAO PAPPU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

PLANT PATHOLOGY

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ABSTRACT

A new serotype of sweet clover necrotic mosaic virus (SCNMV) isolated from alfalfa (*Medicago sativa* L.) was characterized on the basis of serological and physical properties, host range and symptomatology. The antiserum of the new isolate (SCNMV-59) distinguished SCNMV-59 from the type strain (SCNMV-38) by homologous spur formation, whereas the antiserum of SCNMV-38 gave a reaction of identity with both antigens. Intra-gel cross-absorption tests between the two serotypes demonstrated the presence of heterospecific antibodies in the anti-SCNMV-38 serum which specifically reacted with the SCNMV-59 antigen.

During the infection of protoplasts from cowpea with SCNMV, replication of viral RNA was detected by Northern hybridization using ^{32}P-labeled complementary DNA (cDNA) probe. Inoculation of protoplasts with the separated RNA species showed replication and subsequent accumulation of RNA-1 during the entire period of incubation. In contrast, RNA-2 failed to replicate in the absence of RNA-1.

Using two electrophoretically distinct strains of SCNMV, separated, individual RNA species (RNA-1 or RNA-2) from each strain were not infectious, whereas both homologous and heterologous mixtures of RNAs were infectious to indicator plants. The hybrid nature of each of the genetic reassortants was identified by comparing their electrophoretic mobilities with the parent strains. Each genetic reassortant retained the characteristic migration pattern of the parent from which the RNA-1 was derived.

When seven distinct members of the dianthovirus group were analysed by virion electrophoresis, it was found that each virus possessed a single electrophoretic form. Two strains of SCNMV were electrophoretically distinct. Carnation ringspot virus (CRSV) strain A co-migrated with SCNMV-38, whereas CRSV-N had the slowest mobility of all the seven virus isolates tested. Red clover necrotic mosaic virus (RCNMV)-TpM 34 and RCNMV-TpM 48 had similar mobilities, whereas RCNMV-Aus was faster. The isoelectric points of all the strains were in the pH range 4.75-5.1 except that of CRSV-N which was between 6.0-6.2.

cDNA copies of the SCNMV genomic RNAs were prepared and cloned into Lambda gt10. A cDNA clone representing 80% of RNA-2 was further sub-cloned into M13 vectors and sequenced. Analysis of the sequence revealed the presence of one major open reading frame with the potential to code for at least a 35 kDa protein. Homology searches did not identify any other plant viral sequences with significant homology.

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LIST OF ABBREVIATIONS

BMV	Brome mosaic virus
CCMV	cowpea chlorotic mottle virus
CMV	cucumber mosaic virus
CRSV	carnation ringspot virus
EDTA	Ethylene diaminetetraacetic acid, disodium salt
EPPS	N-[2-hydroxyethyl] piperazine-N'-3-propanesulfonic acid
HCRV	Hibiscus chlorotic ringspot virus
IPTG	Isopropyl thio-ß-D-galactoside
ISEM	Immunosorbent electron microscopy
Oligo(dT)	Oligo-thymidylic acid
ORF	Open reading frame
PEG	Poly ethyleneglycol
PNRSV	Prunus necrotic ringspot virus
RCNMV	Red clover necrotic mosaic virus
RNasin	Ribonuclease inhibitor
SCNMV	Sweet clover necrotic mosaic virus
SDS	Sodium dodecylsulfate
TMV	Tobacco mosaic virus
Tris	Tris (hydroxymethyl) aminomethane

Chapter I

INTRODUCTION

Sweet clover necrotic mosaic virus

Sweet clover necrotic mosaic virus (SCNMV) is a member of the dianthovirus group, one of the recently established groups of plant viruses (Matthews, 1982; Hiruki, 1986; Hiruki, 1987b). The other two members of the group are carnation ringspot virus (CRSV), the type member, and red clover necrotic mosaic virus (RCNMV). They share the following general properties: two species of single-stranded RNAs of positive sense encapsidated in isometric particles 30-35 nm in diameter with a single species of coat protein having a molecular mass of about 39,000 daltons (Hiruki, 1987b).

SCNMV was first isolated in 1979 from sweet clover (*Melilotus officinalis* (L.) Lam.) during a field survey in the Athabasca area of Alberta (Hiruki et al., 1981). The virus causes chlorotic ringspot symptoms five days after inoculation on mechanically inoculated sweet clover plants. Subsequent symptoms may include systemic mottle, severe mosaic, leaf malformation and veinal necrosis . The virus has a wide experimental host range that includes both leguminous and non-leguminous plants (Hiruki et al., 1984b).

The virus is propagated in *Phaseolus vulgaris* L. 'Red Kidney', in which ringspot lesions were produced in the inoculated primary

leaves followed by systemic mosaic and veinal necrosis. Virus yields typically range from 100 to 200 mg per kg of infected leaf tissue. Upon analytical ultracentrifugation, the virus particles sediment as a single component with a sedimentation coefficient of 126S. The particles are isometric and 30-35 nm in diameter when negatively stained specimens were observed by electron microscopy (Hiruki et al., 1984b).

Serology

Polyclonal antibodies

The virus is a good immunogen with an average titer of 1:1024. Gel diffusion, ring interface and enzyme-linked immunosorbent assay (ELISA) tests revealed distant relationships between SCNMV and other RCNMV strains, RCNMV-SW, RCNMV-E and RCNMV-C. CRSV failed to show any positive reaction against SCNMV antiserum (Hiruki et al., 1984b).

Immunosorbent electron microscopy (ISEM) of dianthoviruses revealed varying degrees of antigenic relationships between SCNMV and other dianthoviruses. Heterologous reaction between SCNMV antiserum and RCNMV-SW showed a distant relationship with a titer of only 1:128, whereas no serological reaction was detected between SCNMV antiserum and CRSV antigen. The ISEM reactions between pseudorecombinants from SCNMV and RCNMV-SW provided additional evidence that RNA-1 determines the serological specificity (Chen et al., 1984).

Monoclonal antibodies

Twenty one clones of hybridoma cells capable of producing monoclonal antibodies specific to SCNMV were characterized by Hiruki et al. (1984a). They failed to react with RCNMV-SW, RCNMV-C or CRSV when tested by reverse passive haemagglutination inhibition test. However when tested by indirect ELISA, three of twenty one cell-lines were able to react with CRSV thus showing a degree of serological relationship between SCNMV and CRSV for the first time (Hiruki et al. 1984c). Moreover, indirect ELISA revealed serological relatedness between several RCNMV strains and SCNMV (Hiruki and Figueiredo, 1985).

Genetic reassortment* studies

Virion RNA can be easily by the standard SDS-disruption followed by phenol:chloroform extraction and ethanol precipitation (Okuno et al., 1983). Okuno et al. (1983) constructed a series of genetic reassortants between the two genomic RNAs of SCNMV, RCNMV-SW and RCNMV-C. Both homologous and heterologous combinations of RNA-1 and RNA-2 of all three viruses were infectious while individual RNA species were not. Serological specificity was shown to be determined by RNA-1 of the parent strain. It was also shown that RNA-1 of SCNMV determines the systemic infection of sweet clover at 26 C while RNA-2 of SCNMV complemented RNA-1 of RCNMV in causing local infection in sweet clover at 26 C.

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* The term 'genetic reassortant', discussed by Lane (1979) has been used through out this study.

Similar genetic reassortment studies between two RCNMV strains (RCNMV-E and RCNMV-TpM 34) demonstrated that RNA-2 determines the lesion morphology and systemic invasion of cowpea (*Vigna unguiculata*) (Osman et al., 1987). However, results of genetic reassortants between RCNMV-TpM 34 and RCNMV-TpM 48 showed only unilateral compatibility between their two genomic RNAs. A combination of RNA-1 of TpM 34 and RNA-2 of TpM 48 was infectious, whereas the reciprocal was not (Rao and Hiruki, 1987). In a recent report describing the properties of three spontaneous symptom mutants of RCNMV, genetic reassortment studies among the mutants mapped mutations on both RNA-1 and RNA-2 (Osman and Buck, 1989).

Epidemiology and transmission of SCNMV

The known distribution of SCNMV has been limited to Alberta only. During the field surveys for the last several years, SCNMV was found to be fairly widespread in the central and northern areas of Alberta (Hiruki, 1987a). The virus was not detected in alsike clover (*Trifolium hybridum*), red clover (*T. pratens*), white clover (*T. repens*) and crown vetch (*Coronilla varion*). Attempts to study the possible role of several vectors including zoospores of *Olpidium brassicae* and sweet clover weevil *Sitonia cylindricullis*, a major pest of sweet clover in Alberta, were unsuccessful (Hiruki, 1987a).

In a recent report, Hiruki et al. (1989) described the results of studies investigating the role of western flower thrips, *Frankliniella occidentalis*, in transmission of SCNMV. These thrips are found constantly associated with pollen grains in the florets of

sweet clover plants growing in the field. Extracts of pollen samples from virus-infected sweet clover plants, when assayed by ELISA, showed high concentrations of SCNMV antigen. Similar results were obtained when extracts were assayed for infectivity on an indicator plant. Furthermore, SCNMV was detected in washings of pollen grains collected from the flowers of sweet clover plants that were either experimentally inoculated or naturally infected in the field. However, experiments using thrips to transmit SCNMV from infected sweet clover plants to healthy ones did not show any evidence of virus transmission by thrips (Hiruki et al. 1989).

SCNMV Part 2: A new isolate from alfalfa

During field surveys for incidence of SCNMV in sweet clover, a new variant of SCNMV was isolated from naturally infected alfalfa in Northern Alberta (Inouye and Hiruki, 1985). The new isolate, designated as SCNMV-59 is the subject of the current investigation.

Outline of this thesis

The thesis was organized into several chapters, each dealing with a separate, individual study. Chapter II details the results on characterization of the new serotype of SCNMV. Chapter III describes the replication of SCNMV and its genomic RNAs in cowpea protoplasts. Chapter IV demonstrates the use of virion mobility as a useful phenotypic marker in characterizing genetic reassortants between the two strains of SCNMV. Chapter V describes the distinct differences in the virion mobilities among the three dianthoviruses and their various strains and discusses the

possible reasons for the observed differences. The last chapter deals with the complementary DNA cloning of the genomic RNAs of SCNMV and sequencing of clones specific to RNA-2, and attempts to discuss the results of computer-based homology searches with other plant viral RNA sequences.

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CHAPTER II

Characterization of a new serotype of sweet clover necrotic mosaic virus*

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* A version of this chapter has been published. Pappu, H.R., C. Hiruki and N. Inouye. 1988. *Phytopathology* 78: 1343-1348.

INTRODUCTION

While the type strain of SCNMV was first isolated from sweet clover growing in the Athabasca area of Alberta (Hiruki et al., 1984), a new isolate of SCNMV was found infecting alfalfa growing in the same area (Inouye and Hiruki, 1985). The present study describes its characterization on the basis of biological, physico-chemical and serological properties.

MATERIALS AND METHODS

Viruses

The type strain (SCNMV-38) and the alfalfa isolate (SCNMV-59) were multiplied in *Phaseolus vulgaris* L. 'Red Kidney' following several single-lesion transfers in the same host. A Czechoslovakian strain of red clover necrotic mosaic virus (RCNMV) TpM-34 (Rao et al. 1987) and carnation ringspot virus (CRSV) (Hiruki et al. 1984) were two additional dianthoviruses used as standards.

For purification, the method described by Gould et al (1981) was followed during this study. All viruses were maintained in Red Kidney bean plants grown in a greenhouse at 25 $\pm$ 2 C. Infected leaves were harvested 5-7 days after inoculation, homogenized in 0.1 M phosphate buffer (Na_2HPO_4-NaH_2PO_4) pH 7.0 containing 0.1% thioglycolic acid (Fisher Scientific). Plant debris was removed by filtering the homogenate through several layers of cheese cloth. The resulting sap was emulsified with equal volume of 1:1 mixture of chloroform and butanol and clarified by centrifuging at 10,000 rpm for 10 min. The supernatant was

subjected to two alternating cycles of low speed (10,000 rpm for 10 min. in a Sorvall SS34 rotor) and high speed (29,000 rpm for 120 min. using a Beckman T-30 rotor) centrifugations. The resulting pellet was suspended in 10 mM phosphate buffer and layered onto a 10-40% sucrose gradient and centrifuged for 27,000 rpm for 120 min. using a Beckman SW 27 rotor. Virus bands were collected and pelleted through another cycle of high speed centrifugation (29,000 rpm for 120 min. using a Beckman T-30 rotor). Virus pellets were finally suspended either in distilled water or 50% glycerol (UltraPure, BRL) and stored frozen at -20 C. For production of antisera, the virus pellet was subjected to another cycle of sucrose density gradient centrifugation.

Growth conditions

All plants were grown in 12-cm-diameter pots containing an autoclaved mixture of loam, sand and peat (1:1:1, v/v/v) in a glasshouse at 25 $\pm$ 2 C.

Inoculation and virus assay

Crude juice was obtained by grinding infected leaves with a mortar and pestle in the presence of 0.025 M phosphate buffer (Na_2HPO_4-NaH_2PO_4), pH 7.0 at a ratio of 1 g fresh leaf tissue/5 ml of buffer. The extract was rubbed on leaves that had been dusted with Carborundum. They were rinsed with water immediately after inoculation. To judge the susceptibility of a given plant species, virus recovery tests (back inoculations) were performed on young plants of *Chenopodium amaranticolor* Coste & Reyn. In some tests,

virus was detected by enzyme-linked immunosorbent assay (ELISA) as described by Okuno et al. (1983).

Serology

Virus preparations used for producing antisera were subjected to two cycles of sucrose density gradient centrifugation (Gould et al. 1981). Each New Zealand male rabbit was given two intravenous injections, followed by two intramuscular injections one week apart with an immunogen containing 1 mg of virus emulsified in Freund's incomplete adjuvant (van Regenmortel, 1982). Animals were bled two weeks after the final injection. Each antiserum was cross-absorbed with acetone-extracted powder from healthy Red Kidney bean leaves (da Rocha et al. 1986). Immunodiffusion tests and intra-gel cross-absorption tests were done in 100x15 mm sterile, disposable plastic petri plates (Fisher Scientific) containing 0.8% agarose (ICN Biomedicals), 0.02% sodium azide and 0.85% saline as described by van Regenmortel (1982). Antigen was used at a concentration of 1 mg/ml in all immunodiffusion tests. Antisera titers were determined by testing a series of two-fold dilutions of antiserum against its homologous and heterologous antigen. Precipitin patterns were recorded 24 hrs after incubation at room temperature. Each gel diffusion test was repeated at least three times using virus antigens purified from infected plants during different seasons of the year.

Estimation of molecular weight of coat protein

Purified virus preparations were heat-dissociated in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol

and were analyzed on 10% SDS-polyacrylamide gel (SDS-PAGE) using the discontinuous buffer system (Laemmli 1970). Bands were visualized by staining with Coomassie Brilliant Blue R250 followed by destaining in methanol:acetic acid:water (10:10:80 v/v/v). Molecular weight markers were obtained from Bio-Rad Laboratories (Richmond, CA).

Isoelectric focusing

Isoelectric focusing of purified virus preparations was performed using Bio-Rad's Mini-IEF cell (Pappu et al. 1988). Virus samples containing 1 μg of nucleoprotein in 20% glycerol were loaded on a 1% agarose gel (Isogel, FMC Corporation, Rockland, Maine), containing 5% sorbitol, 10% glycerol and 2% Bio-Lyte 4-7 ampholytes (Bio-Rad). Focusing and detection of proteins were done according to the protocol supplied by the manufacturer. Focusing was done with the voltage being gradually increased, initially at 100 V for 15 min., 200 V for 15 min. followed by 450 V for 1 hr. After focusing was complete, the gel was fixed for 15 min in a fixative solution (30% methanol, 5% trichloroacetic acid and 3.5% sulfosalicylic acid) followed by a brief rinse in 95% ethanol. The gel was stained for 30 min. with 0.2% Coomassie Brilliant Blue R250 made in 28% ethanol and 14% acetic acid followed by destaining in 28% ethanol and 14% acetic acid. Mobilities were compared to that of RCNMV-TpM 34. The pH gradient was determined by cutting a 1 cm wide portion of the gel into 5 mm sections. Each section was placed in a tube and the ampholytes were eluted by addition of 1 ml of distilled water. After an equilibration period of several hours, the pH was read using a

Radiometer pH meter. The isoelectric points of the virus isolates were determined by measuring the position of each band and using that value to derive the corresponding pH value by solving the equation resulting from a linear regression of values obtained for the pH gradient.

Agarose gel electrophoresis of virions

Agarose gel electrophoresis of nucleoproteins was done as described by Serwer et al. (1984) with some modifications. Agarose gels (Seakem LE, FMC Corporation) (0.8%) were made in 10 mM phosphate buffer pH 7.2. Purified virus preparation containing 3 μg/sample was mixed with an equal volume of sample buffer (0.05% bromophenol blue and 20% sucrose in electrophoresis buffer) and was electrophoresed in the same buffer using a horizontal slab gel apparatus (Tyler Research Instruments, Edmonton, AB) at 3V/cm constant voltage at 4 C with buffer recirculation (100 ml/min.) to avoid the development of a pH gradient. Gels were stained with ethidium bromide (1 μg/ml in distilled water containing 1 mM EDTA) for 1 hr. After a brief rinse with distilled water, the same gel was re-stained with Coomassie Brilliant Blue R250 for 30 min. and destained overnight in methanol:acetic acid:water (10:10:80 v/v/v) to locate the coat protein. Gels were photographed using Polaroid instant film (Type 57) using a red filter (for ethidium bromide-stained gels) or an orange filter (for Coomassie Blue-stained gels).

RESULTS

Symptoms of alfalfa infected by SCNMV-59

The original symptoms on naturally infected alfalfa were very mild chlorosis with mild stunting. No necrotic lesions were observed on the infected plant.

Host range and symptomatology

SCNMV-59 infected 28 species in 9 plant families out of 36 species in 11 families tested, indicating a relatively wide host range. The virus caused necrotic local lesions in most of the host plants. Systemic symptoms were produced in seven plant species. *Catharanthus roseus* (L.) Dan., *M. officinalis*, *M. alba* L., *Gomphrena globosa* L., *Nicotiana clevelandii* Gray., *Vigna unguiculata* (L.) Walp. and *P. vulgaris* showed necrotic local lesions on inoculated leaves and chlorotic spots and veinal necrosis on newly-emerged leaves. *N. clevelandii* developed necrotic local lesions on inoculated leaves 3-4 days after inoculation, and the upper leaves soon developed various degrees of mosaic, often accompanied by necrosis.

Another 18 species developed necrotic local lesions on inoculated leaves without systemic infection. These were *C. amaranticolor*, *C. quinoa* L., *Cucumis sativus* L. 'Long Green', *C. melo* L., *Cucurbita maxima* Duch., *C. pepo* L., *Glycine max* L., *Lathyrus odoratus* L., *M. sativa* L. 'Anchor', 'Beaver' clone #4, 'Pacer' and 'Roamer', *N. glutinosa* L., *N. rustica* L., *N. tabacum* L. 'Bright Yellow', *Sesamum indicum* L., *Tetragonia expansa* Murr., *Trifolium repens* L., *T. hybridum* L., *Vicia faba* L., and *Zinnia elegans* Jacq. Three

plant species were infected systemically without showing visible symptoms after developing necrotic local lesions. They were *S. indicum* L., *M. sativa* 'Algonquin', Beaver clone #46', 'Angus', 'Saranac' and *T. pratense* L. The virus failed to infect *Brassica rapa* L., *Datura stramonium* L., *Lycopersicon esculentum* Mill. 'Earliana', *Physalis ixocarpa* Brot., *P. peruviana* L., *P. pubescens* L., *Raphanus sativus* L. and *Zea mays* L.

Differences in host range and symptomatology between SCNMV-38 and SCNMV-59 were as follows: *L. esculentum* 'Earliana' was locally susceptible, without producing symptoms, to SCNMV-38 but was resistant to SCNMV-59. Compared to those caused by SCNMV-38, SCNMV-59 generally produced milder symptoms on *M. officinalis* (Fig. II.1A, B). The size of local lesions produced by SCNMV-59 on certain host species was discernibly smaller than those by SCNMV-38 (Table II.1 and Fig. II.2).

Serology

In gel diffusion tests, the homologous titers of anti-SCNMV-38 and anti-SCNMV-59 sera were 1:1024 and 1:512 respectively and their heterologous titers differed by only one two-fold dilution. When the two antigens were compared using anti-SCNMV-38 serum, the precipitin lines fused with each other (Ouchterlony, 1968) (Fig. II.3a), whereas the reaction of the same antigens with anti-SCNMV-59 serum resulted in a homologous spur which extended beyond the heterologous precipitin line (Fig. II.3b). To verify this serological behavior, both antigens and their respective antisera were examined in a four-membered pattern in homologous

and heterologous combinations (Campbell, 1964). Both homologous and heterologous precipitin lines crossed each other (Fig. II.3c, d). The homologous precipitin line between SCNMV-38 and its serum deflected towards the homologous precipitin line between SCNMV-59 and its serum, which was similar to that of a reaction of identity (Fig. II.3c). However, the homologous precipitin line between SCNMV-59 and its serum did not show any such deflection and formed a spur. When both antigens and their sera were used in heterologous combination, the precipitin lines gave an identical pattern (Fig. II.3d).

To further investigate the nature of their antigenic determinants, intra-gel cross-absorption tests were done in a six-well circular arrangement. When anti-SCNMV-38 serum was cross-absorbed with its homologous antigen, distinct precipitin lines were seen with SCNMV-59 antigen (Fig. II.4a). When the same serum was cross-absorbed with its heterologous antigen (SCNMV-59), all the antibodies were completely precipitated by the cross-absorbing antigen (Fig. II.4b). However, when anti-SCNMV-59 serum was cross-absorbed with its heterologous antigen, distinct precipitin lines towards SCNMV-59 appeared in addition to those precipitated by the cross-absorbing antigen, thus indicating the presence of type-specific antibodies in anti-SCNMV-59 serum (Fig. II.4c). When the same antiserum was cross-absorbed with its homologous antigen, all the antibodies were completely precipitated (Fig. II.4d). To confirm the presence of antibodies in anti-SCNMV-38 serum that were specific to its heterologous antigen, both antisera were separately cross-absorbed with a

mixture of two antigens. Anti-SCNMV-38 serum was completely precipitated around the central well, due to the presence of SCNMV-59 in the cross-absorbing antigen mixture. This indicated the heterospecific nature of the antibodies present in the anti-SCNMV-38 serum (Fig. II.4e). Anti-SCNMV-59 serum gave a pattern similar to the one in which the antiserum was cross-absorbed with its homologous antigen (Fig. II.4f). Neither of the antisera reacted with sap from healthy leaves, and neither antigen reacted with the pre-immune serum from the rabbits in which the antisera were produced.

Molecular weight of coat protein

The coat protein of the new serotype was found to contain one major polypeptide with a molecular weight of 39,000, when analyzed on SDS-PAGE. In addition, a minor component that migrated faster than the major component was seen in some purified preparations. No mobility differences could be seen between the type strain and the new serotype (Fig. II.5).

Isoelectric points of the serotypes

Using the pH gradient produced by the ampholytes (Fig. II.6), the isoelectric points of SCNMV-38 and SCNMV-59 were found to be 4.92 and 4.87 respectively. Some preparations showed a minor band in addition to the major one. Both serotypes could be distinguished from RCNMV-TpM 34 by isoelectric focusing (Fig. II.7).

Agarose gel electrophoresis

Both SCNMV-38 and SCNMV-59 migrated as a single nucleoprotein component when stained with Coomassie Blue (Fig. II.8). The identity of the nucleoprotein was confirmed by staining the same gel with ethidium bromide, a nucleic acid-specific stain (Fig. 8a). SCNMV-59 migrated slower than SCNMV-38 and CRSV (Fig. II.8a, b). This difference was consistent among different batches of virus preparations. CRSV, the type member of the group showed similar mobility to that of SCNMV-38.

DISCUSSION

The alfalfa isolate (SCNMV-59) and the type strain (SCNMV-38), like other dianthoviruses have a relatively wide host range (Hiruki, 1986; 1987b). The new isolate can easily be distinguished from the type strain on the basis of some differences in their host ranges and symptomatology on certain selected hosts (Table II.1).

There is a great degree of cross-reactivity between the two viral antigens and their respective antisera. The serological differentiation index differed by only a value of one which was expected for closely related strains of a virus. Spur formation in gel diffusion tests has been used as a reliable criterion to distinguish serotypes of several viruses (van Regenmortel, 1982). Anti-SCNMV-38 serum gave a reaction of complete identity (Crowle, 1972; Ouchterlony, 1968) when the two antigens were compared, whereas the two viral antigens were distinguishable by homologous spur formation when anti-SCNMV-59 serum was used (Fig. II.3a, b). Apparently SCNMV-38 shares all antigenic determinants with

SCNMV-59, whereas the latter seems to have additional epitopes that must have elicited the production of specific antibodies resulting in homologous spur formation when the two antigens were compared. The serological reactions obtained from homologous and heterologous combinations of both antigens and their sera in a four-membered pattern seem to support this observation. The reaction of identity by anti-SCNMV-38 serum could also be attributed to the presence of cross-reacting antibodies specific to SCNMV-59 antigen as demonstrated in intra-gel cross absorption tests. This phenomenon of unilateral cross reactivity between SCNMV-38 and SCNMV-59 is similar to earlier findings with other plant viruses (Gould et al., 1981; Rao et al., 1987; van Regenmortel, 1967). The presence of heterospecific antibodies was previously unique to tobacco mosaic virus (TMV) (Van Regenmortel, 1967) but a recent finding that antisera of certain strains of RCNMV also contain heterospecific antibodies (Rao et al., 1987) suggests that this may be common in other members of the dianthovirus group as well.

The origin of the antigenic variation between SCNMV-38 and SCNMV-59 may result from a mutation under field conditions or from the adaptation of the virus to a particular host. The type strain of SCNMV was originally isolated from sweet clover while the isolate of the new serotype was found occurring naturally in alfalfa. All isolates of SCNMV found exclusively in sweet clover over eight years of annual field surveys were similar to the original strain (SCNMV-38) (C. Hiruki, 1987a). Therefore, the new serotype must have been adapted to alfalfa long before it was detected. Both

SCNMV-38 and SCNMV-59 have been repeatedly passed through their propagating host, *P. vulgaris* 'Red Kidney' over a period of five years and their respective antisera have been produced in different rabbits during the same period. The serological reactions determined using their respective antisera have been quite consistent with time, indicating the individual identity and stability of the isolates.

Alternatively a bipartite genome system assures increased frequency of genetic reassortment. In the case of selected members of the dianthovirus group, RNA-1 determines the serological specificity of reassortant viruses derived from heterologous combinations of RNA-1 and RNA-2 as tested by ELISA (Okuno et al. 1983) and by immunosorbent electron microscopy (Chen et al.1984). The present results suggest that different sets of genes are available for genetic reassortment under field conditions. Furthermore, in a recent report on tobacco rattle virus, another bipartite genome virus, the terminal homology of the genomic RNA species was shown to be maintained by substitution of RNA-1 sequences into RNA-2 (Robinson et al. 1987).

The isoelectric points of the SCNMV type strain and the new isolate are similar to those of RCNMV strains (Gallo and Musil, 1984) but can be distinguished from RCNMV-TpM 34. The appearance of a minor band in some preparations shows charge heterogeneity from the major coat protein polypeptide. This phenomenon of charge heterogeneity in an otherwise homogeneous virus preparation seems to be widely prevalent in various kinds of protein preparations (Righetti, 1983). The minor polypeptide seen

on SDS-PAGE suggests proteolytic degradation during purification and this could be a cause of the charge heterogeneity.

Use of agarose gel electrophoresis to compare plant viruses is a relatively new technique, although it has been used extensively for comparative studies of bacteriophages (Serwer 1987). Recently this method has been used to characterize electrophorotypes of hibiscus chlorotic ring spot virus (Hurtt 1987), panicum mosaic virus and its strains (Serwer et al. 1984), and TMV (Asselin and Grenier, 1985) and the nucleoprotein components of prunus necrotic rinspot virus (Ong and Mink, 1989). In this study, in addition to their demonstrated serological differences, SCNMV-38 and SCNMV-59 were also electrophoretically distinct.

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TABLE II.1 Comparative symptomatology of SCNMV-38 and SCNMV-59 on selected host plants.

Plant species	Local symptoms/systemic symptoms	
	SCNMV-38	SCNMV-59
Melilotus officinalis	cs, nrs/cs, lc, vn, sv-st[a]	cs, nrs/cs, vn,m-st
Phaseolus vulgaris 'Red Kidney'	b- ns, vn/vn	s- b- ns, vn/vn
Chenopodium quinoa	s- LL/O, (-)[b]	vs- LL/O, (-)
C. amaranticolor	s- LL/O, (-)	vs- LL/O, (-)
Sesamum indicum	l- LL/O, (+)	s- LL/O, (+)
Glycine max	s- LL/O, (-)	vs- LL/O, (-)

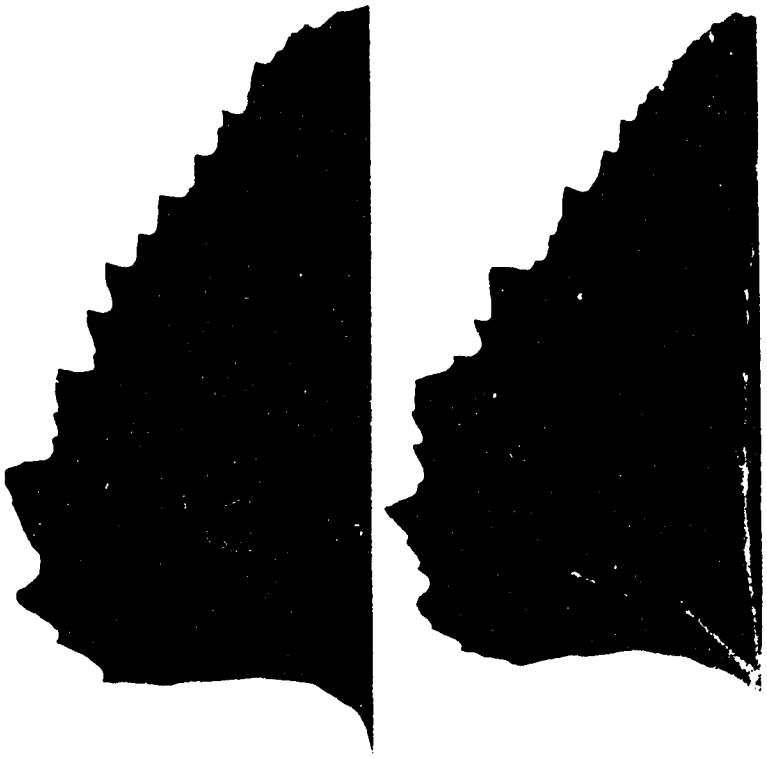
[a]Coded symptom descriptions: b=brown, cs=chlorotic spots, l=large, lc=leaf curl, LL=local lesions, m=mild, nrs=necrotic ringspots, ns=necrotic spots, O=no symptoms, r=reddish, s=small, st=stunting, sv=severe, vn=veinal necrosis, vs=very small.

[b]Results obtained by ELISA: (-), virus infection negative; (+), virus infection positive.

Fig. II.1A. Symptoms in sweet clover (*Melilotus officinalis*) incited by the type strain (SCNMV-38) of sweet clover necrotic mosaic virus.

Fig. II.1B. Symtoms in sweet clover (*Melilotus officinalis*) incited by the alfalfa isolate (SCNMV-59) of sweet clover necrotic mosaic virus.

Fig. II.2 (A,B) Half-leaves of *Chenopodium amaranticolor* showing local lesions incited by the type strain (SCNMV-38, A) and the alfalfa isolate (SCNMV-59, B) of sweet clover necrotic mosaic virus.



A B

Fig. II.3 Serological comparison of sweet clover necrotic mosaic virus (SCNMV) serotypes by double immunodiffusion in agar gel. A, SCNMV-38. B, SCNMV-59. 1, anti-SCNMV-38 serum. 2, anti-SCNMV-59 serum. Both antisera were used at 1/2 dilution. Letters a,b,c and d are referred to in the results section

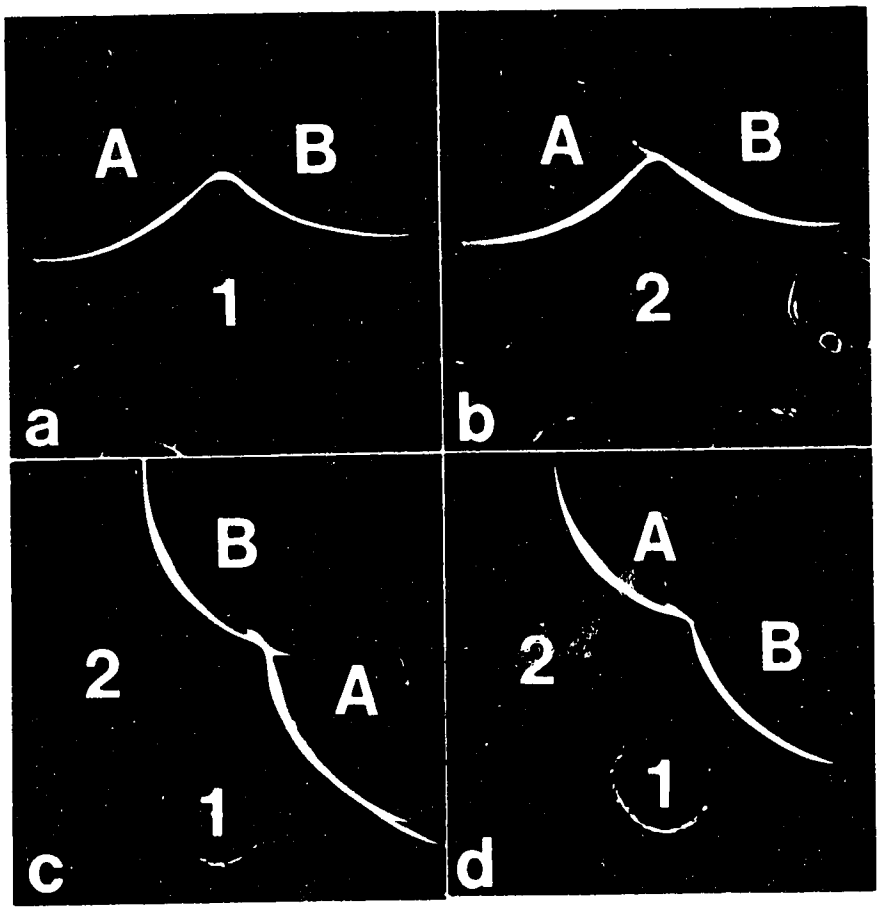

A
B
1
a
A
B
2
b
B
2
A
1
c
A
2
B
1
d

Fig. II.4 Intra-gel cross-absorption tests of sweet clover necrotic mosaic virus (SCNMV) serotypes. A, SCNMV-38, B, SCNMV-59. a, center well initially filled with SCNMV-38 antigen, 24 hr later with anti-SCNMV-38 serum. b, center well initially filled with SCNMV-59 antigen, 24 hr later with anti-SCNMV-38 serum. c, center well initially filled with SCNMV-38 antigen, 24 hr later with anti-SCNMV-59 serum. d, center well initially filled with SCNMV-59 antigen, 24 hr later with anti-SCNMV-59 serum. e, center well initially filled with a mixture of SCNMV-38 and SCNMV-59 antigens, 24 hr later with anti-SCNMV-38 serum. f, center well initially filled as in e, 24 hr later with anti-SCNMV-59 serum. Both antisera were used at 1/2 dilution.

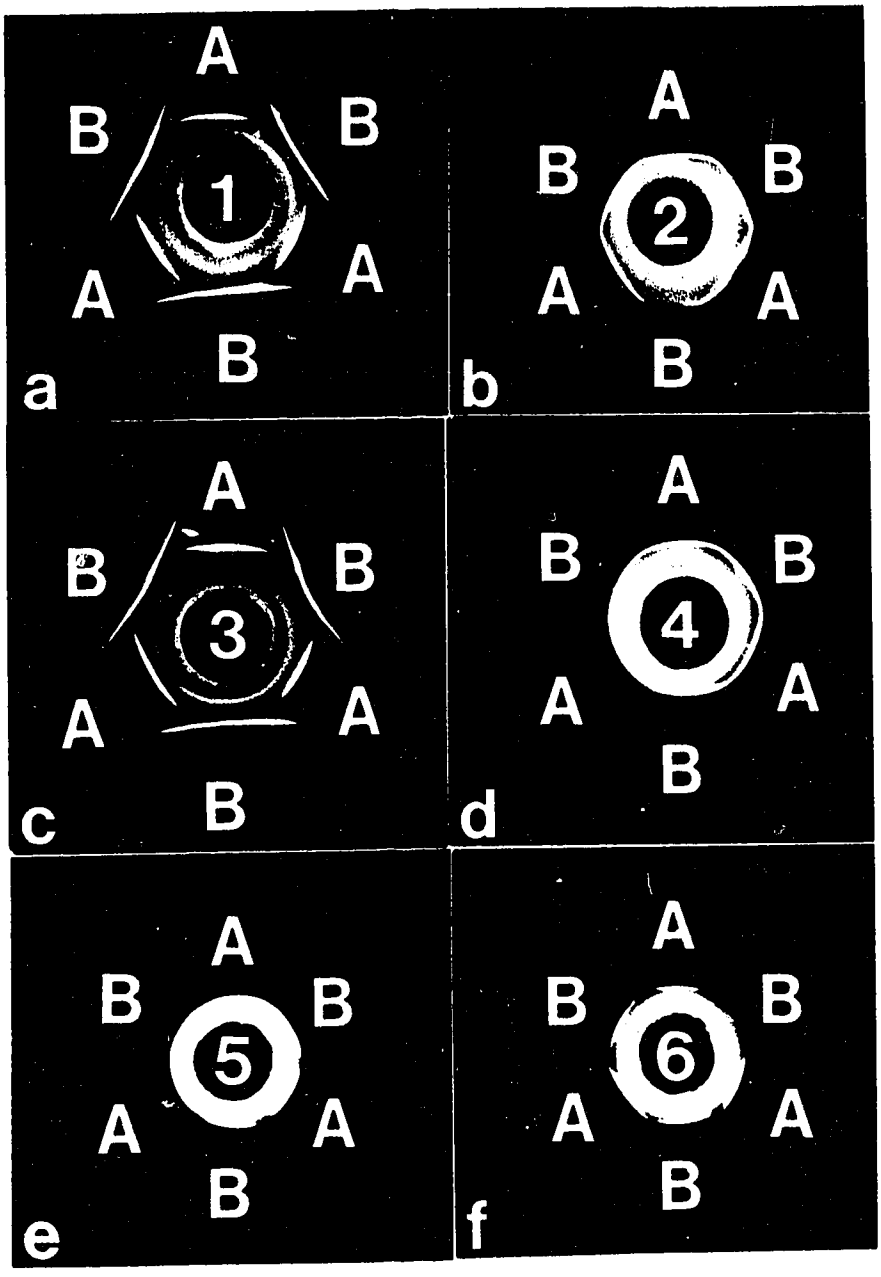

A
B
B
1
A
A
B
a
A
B
B
2
A
A
B
b
A
B
B
3
A
A
B
c
A
B
B
4
A
A
B
d
A
B
B
5
A
A
B
e
A
B
B
6
A
A
B
f

Fig. II.5 SDS-10% polyacrylamide gel electrophoresis of dissociated coat proteins of sweet clover necrotic mosaic virus (SCNMV) serotypes. SCNMV-38 (left), SCNMV-59 (right). Molecular weight markers (from top) phosphorylase B (98 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) (center). Direction of migration was from negative (top) to positive (bottom).

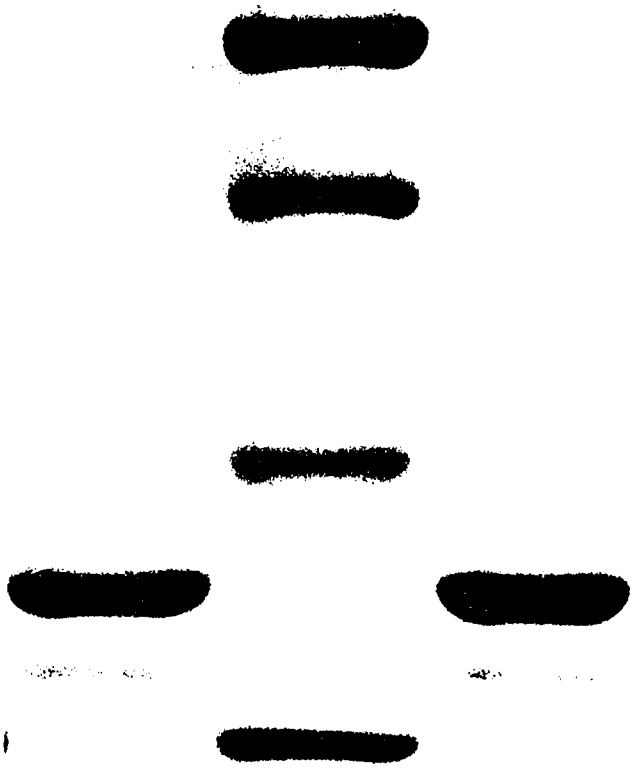


Fig. II.6 pH gradient of Biolyte ampholytes 4-7 following isoelectric focusing of sweet clover necrotic mosaic virus (SCNMV) serotypes.

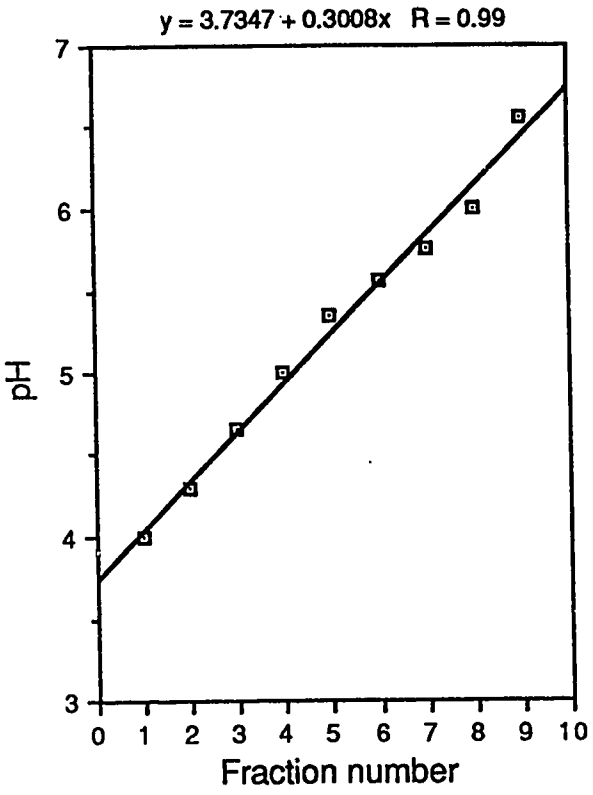

y = 3.7347 + 0.3008x R = 0.99
pH
7
6
5
4
3
0 1 2 3 4 5 6 7 8 9 10
Fraction number

Fig. II.7 Isoelectric focusing of sweet clover necrotic mosaic virus (SCNMV) serotypes. SCNMV-38 (lane 1), SCNMV-59 (lane 2), red clover necrotic mosaic virus (RCNMV)-TpM 34 (lane 3). PH gradient was generated by using Bio-Lyte ampholytes 4-7. Direction of migration was from positive (top) to negative (bottom).

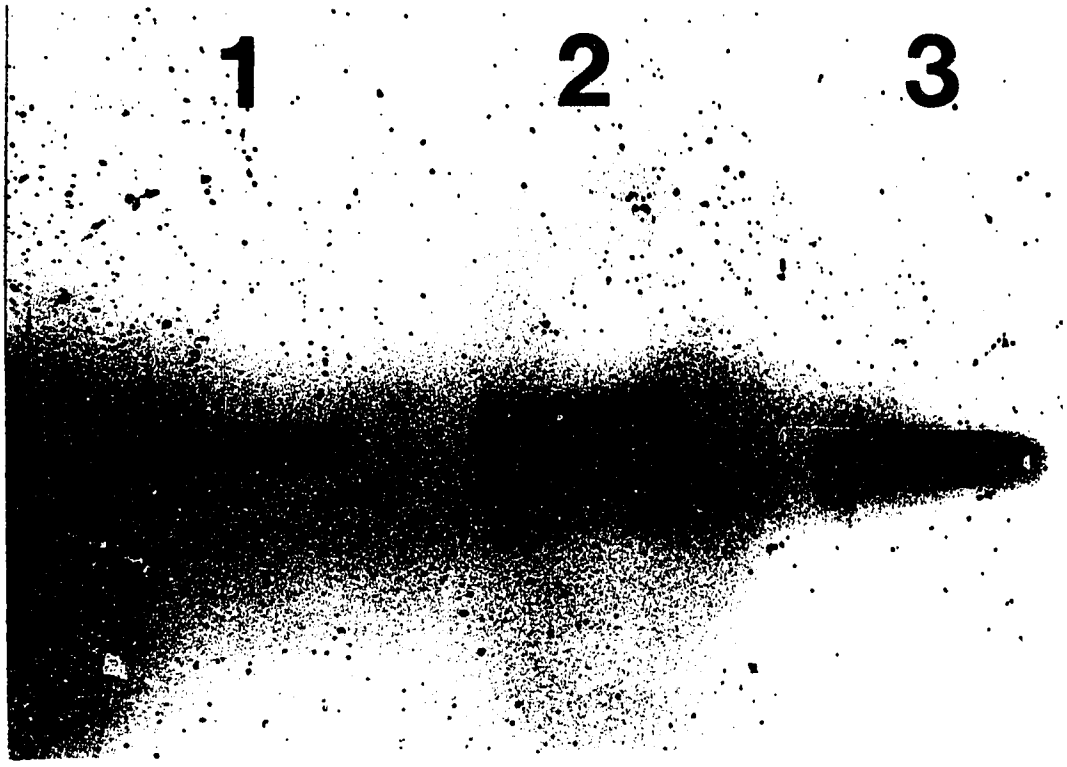
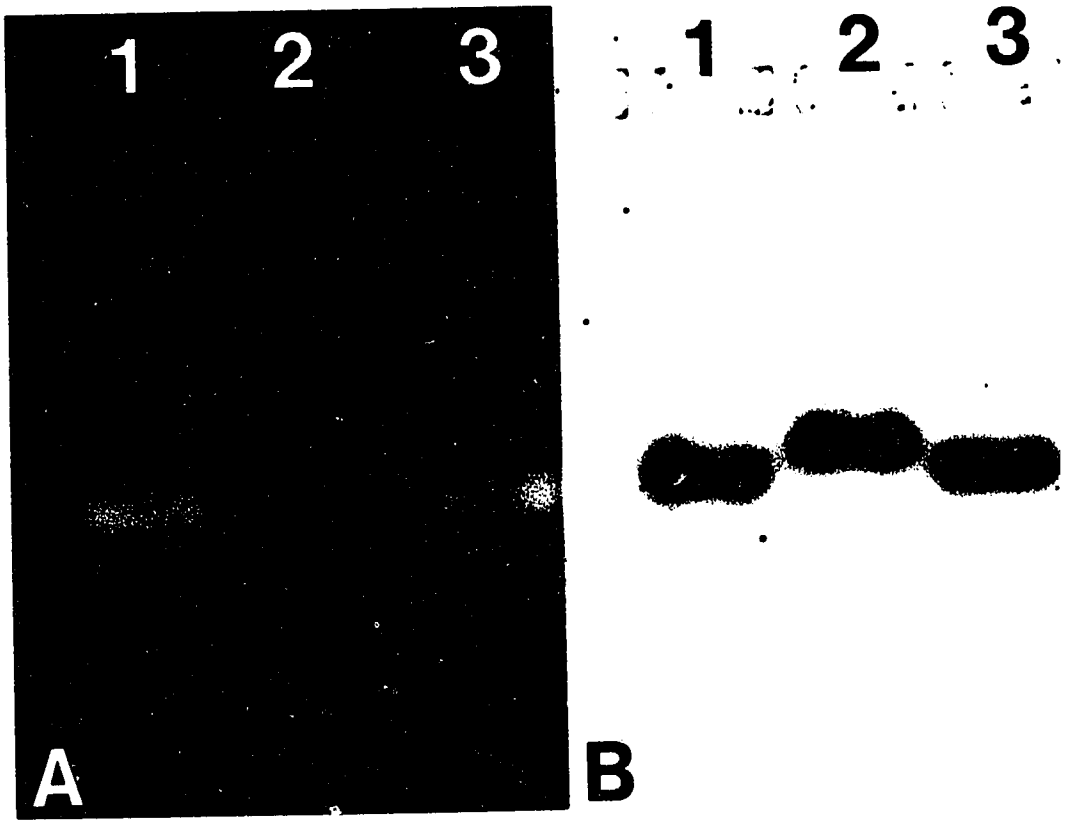

1
2
3

Fig. II.8 Agarose gel electrophoresis of sweet clover necrotic mosaic virus (SCNMV) serotypes. A, gel stained with ethidium bromide. SCNMV-38 (lane 1), SCNMV-59 (lane 2), and carnation ringspot virus (CRSV) (lane 3). B, same gel stained with Coomassie Brilliant Blue R250. Direction of migration was from negative (top) to positive (bottom).


1
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3
A
1
2
3
B

Chapter III

Replication of sweet clover necrotic mosaic virus in cowpea protoplasts*

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*A version of this chapter has been published. Pappu, H.R., and C. Hiruki. 1988. *Can. J. Plant Pathol.* 10:110-115.

INTRODUCTION

Isolated protoplasts have been successfully used to achieve synchronous infection and multiplication of many plant viruses (Takebe, 1983). They are particularly amenable to the study of the replicative strategies of divided genome viruses and in locating genes that control the replication of the virus. Detailed information on factors that affect the infection in a given virus-protoplast combination is important in effectively utilizing a protoplast system for such genetic studies. In this investigation, several factors were examined that affect the infection of cowpea protoplasts with purified preparations of SCNMV. This chapter also describes the replication of RNA-1 of SCNMV independent of RNA-2 in cowpea protoplasts.

MATERIALS AND METHODS

Purification of virus and RNA

SCNMV was purified from 12 day-old systemically infected *Phaseolus vulgaris* L. cv. Red Kidney bean plants as described by Gould et al. (1981, described in chapter II).

Total genomic RNA was extracted from purified virus preparation according to Okuno et al. (1983). Purified RNA was suspended in sterile distilled water and stored at -80 C. The purity of each RNA preparation was routinely checked on 1% agarose gel prepared in Tris-acetate buffer (Maniatis et al. 1982) using a horizontal mini-slab gel system (Tyler Research Instruments, Edmonton, AB). Total genomic RNA was fractionated on a 2%

agarose gel (low melting point agarose, Bethesda Research Laboratories, Gaithersburg, MD) made in Tris-acetate buffer. Duplicate wells were stained with ethidum bromide to locate RNA bands and the corresponding parts of the gel were remelted, phenol extracted and ethanol precipitated (Maniatis et al. 1982). Each RNA species thus separated was examined for its purity by Northern hybridization using ^{32}P-labeled cDNA probe prepared using total genomic RNA as template.

Protoplast isolation

Mesophyll protoplasts from 10 to 12 day-old cowpea plants grown under conditions described by Rao and Hiruki (1978) were isolated by a procedure similar to that of Hibi et al. (1975). Epidermis-peeled leaves were placed in a petriplate containing an enzyme solution of 1% Cellulysin (Calbiochem), 0.1% Pectolyase (Kinki Yakult Co.,Tokyo) and 0.5% potassium dextran sulfate (Meito Sangyo Co. Ltd., Tokyo) in 0.6M mannitol pH 5.5-5.7 and incubated at 30 C for 2.5 hrs with gentle shaking. The resulting digest was filtered through 85 μm mesh (Tetco, Switzerland) into a 15 ml conical bottom test tube and centrifuged at 600 rpm for 3 min. The protoplast pellet was washed three times with 0.6M mannitol in 10 mM calcium chloride and the protoplast concentration was determined with a hemacytometer before finally suspending in 0.6 M mannitol for subsequent infection studies.

Inoculation of protoplasts with SCNMV virions

Protoplasts were inoculated with a purified preparation of SCNMV in the presence of Poly-L-Ornithine (PLO), essentially as described by Rao and Hiruki (1978). Purified virus suspended in 10 mM phosphate buffered mannitol (PBM) is mixed with 0.8 µg/ml of PLO prepared in 10 mM PBM, held at room temperature for 30 min. and then poured onto a freshly pelleted protoplasts (1 x 10^6 per ml). The protoplast suspension was then held at room temperature (23 C) for 20 min. Protoplasts were pelleted by centrifugation, washed three times with 0.6 M mannitol in 10 mM calcium chloride and finally suspended in the incubation medium of Otsuki and Takebe (1969) at a concentration of 1 x 10^6 protoplasts/ml.

Inoculation of protoplasts with total and fractionated genomic RNA of SCNMV

Protoplasts were inoculated with the total gemonic RNA of SCNMV in the presence of polyethylene glycol (PEG) (Maule et al. 1980). A pellet of protoplasts (2 x 10^6) was suspended in 60 µl of 30% PEG containing 30 µg of total RNA or 20 µg of either RNA-1 or RNA-2 and after ten seconds, was slowly diluted with 0.6 M mannitol to make the total volume to one ml. The suspension was held at room temperature for 20 min. and then washed three times with 0.6 M mannitol before suspending the protoplasts in the incubation medium. After appropriate periods of incubation, protoplasts were collected by centrifugation and stored at -20 C until required.

Fluorescent antibody technique and infectivity assay

The percentage of infection of protoplasts inoculated either with virions or RNA was estimated by indirect fluorescent antibody staining as described by Nishiguchi et al. (1985) using anti-SCNMV serum as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody. Infectivity assay of samples of protplast extracts was done as described by Rao and Hiruki (1978) using *Chenopodium amaranticolor* as the local lesion host.

Northern hybridization

After thawing the protoplast samples, total nucleic acids were extracted with water-saturated phenol (BRL, Ultra-Pure) containing 0.1% 8-hydroxy quinoline (Sigma), followed by extraction with phenol:chloroform (1:1) and twice with water-saturated ether (Maniatis et al. 1982). Nucleic acids were precipitated overnight at -20 C by adding 2.5 volumes of cold ethnanol. For dot blotting, nucleic acids were denatured by heating at 65 C for 2 min. and spotted directly onto GeneScreen Plus (NEN Research products) and baked at 80 C for one hr. before probing with labeled cDNA. For Northern hybridization analysis, nucleic acid preparations were heat denatured as above in the presence of 50% deionized formamide (BRL, Ultra Pure), 6% (w/v) formaldehyde (Fisher Scientific) and electrophoresis buffer (20 mM N-[2-hydroxyethyl] piperazine-N'-3-propane-sulfonic acid (Sigma), 10 mM sodium acetate and 0.1 mM ethylene diamine tetra acetic acid, pH 8.0).

Samples were electrophoresed in 1.5% agarose-formaldehyde gel (Gietz and Hodgetts, 1985; Pappu and Hiruki, 1989) prepared in electrophoresis buffer. The samples were then transferred to GeneScreen Plus membrane by capillary blotting (Thomas 1980).

The membrane was baked at 80 C for 60 min. and pre-hybridized at 42 C using a shaker-waterbath for 4-6 hrs in plastic bags containing 50% formamide (v/v), 5x SSPE (1xSSPE=0.12 M NaCl, 0.015 M sodium citrate, 0.013 M Na_2HPO_4 pH 6.5, and 2 mM EDTA), 4xDenhardt's solution (1x Denhardt's=0.02% each of bovine serum albumin, Ficol 400, and Polyvinylpyrrolidone 40), 100 µg/ml of denatured calf thymus DNA and 0.1% SDS (Maniatis et al. 1982). Hybridization was carried out at 55 C for 24 hrs in the same but fresh solution containing 10% dextran sulfate and a ^{32}P-labeled cDNA probe specific to SCNMV genomic RNAs made by random priming using AMV reverse transcriptase (Pharmacia) (Taylor et al. 1976). The probe was used at a concentration of 10^6 cpm/ml of hybridization solution. Following hybridization, the membrane was washed three times for 15 min. each at room temperature in 2x SSC (1x SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and 0.1% SDS, and twice for 30 min. each at 55 C in 1x SSC and 0.5% SDS, and two times for 30 min. each at 55 C in 0.1x SSC and 0.1% SDS (Klssing and Berry, 1983) . The membrane was air-dried and exposed to Kodak X-OMAT X-Ray film at -20 C overnight and developed according to the manufacturer's recommendations.

RESULTS

Factors affecting the infection of cowpea protoplasts with SCNMV

The effect of several factors that influence the infection of cowpea protoplasts has been investigated in this study. Under optimum conditions, a maximum of 68% of protoplasts were infected by a purified preparation of SCNMV. The proportion of infected protoplasts increased with increasing amounts of virus in the inoculum, up to a maximum of 8 µg/ml. Thereafter, no significant increase in infection was obtained (Fig. III.1). Presence of PLO at a concentration of 0.8 µg/ml in the inoculation buffer facilitated a high rate of infection, whereas in its absence only 3% of protoplasts were infected(Fig. III.2). The kind of buffer used for inoculation had considerable effect on the infection rate. Phosphate buffer at a pH value of 6.2 gave better infection than citrate buffer (Fig. III.3) and the number of infected protoplasts decreased sharply at higher pH values. In a series of experiments in which the protoplasts were incubated for different periods of time (0 to 60 min.) with virus-PLO mixture that had been pre-incubated for 20 min., an incubation period of 20 min. gave a relatively high infection rate and prolonging incubation further did not improve the rate significantly.

Multiplication of SCNMV in cowpea protoplasts

The extent of multiplication of SCNMV in the protoplasts was assayed by indirect fluorescent antibody staining and infectivity assay. The time-course study of multiplication of the virus

determined by these methods showed a one-step growth of virus infection (Fig. III.4). Fluorescing protoplasts first appeared 12 hr after inoculation and the number increased rapidly for the remaining period of incubation (up to 72 hr), suggesting the active multiplication of the virus (Fig. III.5). No fluorescence was observed in uninoculated controls. The replication of SCNMV was also analysed by detecting viral RNA in protoplast extracts by Northern hybridization using cDNA probe that is specific to total genomic RNA of the virus. Synthesis of SCNMV-genomic RNA was detected 12 hr after inoculation which continued thereafter (Fig. III.6A). Mock-inoculated protoplasts did not show any virus-specific RNA.

Replication of total and fractionated RNAs of SCNMV

PEG-mediated infection of protoplasts with purified genomic RNA resulted in a maximum of 52% infection of the surviving protoplasts as judged by indirect fluorescent antibody staining. In experiments using fractionated RNA-1 and RNA-2, the purity of each RNA species was verified by Northern hybridization using a cDNA probe made from total genomic RNA. Only those RNA preparations which were free of cross contamination with the other RNA species were used in infection studies (Fig. III.6B). Protoplasts inoculated with RNA-1 alone, and samples analysed after various periods of incubation, showed that RNA-1 alone was capable of replication. RNA-1 was detected from samples as early as 12 hrs after inoculation (Fig. III.6C). Mock-inoculated protoplasts did not show any hybridization with the probe. In case

of inoculation with RNA-2, neither the synthesis nor the accumulation of progeny RNA could be detected when the same cDNA probe was used. However, a small amount of RNA-2 was seen in protoplast extracts sampled immediately after inoculation which could be attributed to the presence of residual inoculum. In subsequent sampling of protoplasts at various time intervals, it was evident that RNA-2 failed to replicate independently (Fig. III.6D).

DISCUSSION

In this study, under optimum conditions for the infection of cowpea protoplasts with SCNMV, 68% of surviving protoplasts got infected with a purified preparation in the presence of PLO. The fact that the virus could be detected 12 hrs after inoculation and the subsequent increase in infection suggested that the protoplasts supported active virus multiplication upon infection. The result of a time-course study of virus multiplicaiton was similar to those from several other virus-protoplast combinations (Takebe, 1983). Of the several factors that influence the infection of protoplasts, the presence of PLO in the inoculum was found to be the most critical factor for obtaining a high rate of infection, which seems to be common for many other virus-protoplast systems (Takebe,1983; Rao and Hiruki, 1978; Barker and Harrison, 1982). The need for the presence of PLO in inoculation buffer generally depends on the nature of the surface charge of the virus at a given pH. The iso-electric point of SCNMV was found to be in the range of 4.85-5.00 (chapter II). Hence, at higher pH values, the virus carries a

negative charge, resulting in a low infection rate since the protoplast membrane is also negatively charged. The relatively low rate of infection when purified RNA was used as inoculum, could be partly due to the low survival rate of protoplasts following the treatment with PEG. In case of inoculation of pea prtoplasts with CYMV-RNA, lower infection levels were obtained compared to those with virion preparations (Bains et al., 1988). Using the same method, infection of cowpea protoplasts with red clover necrotic mosaic virus RNA yielded about 75-85% infection (Osman & Buck, 1987). The use of PLO resulted in very poor infection, hence PEG-mediated inoculation was the method of choice in this study.

The replication of RNA-1 independent of RNA-2 suggests that the genes required for replication of the virus are located on RNA-1. This confirms the recently reported results with RCNMV, another member of the dianthovirus group (Osman & Buck, 1987; Paje-manalo and Lommel, 1989). However, RNA-1 alone is not able to initiate infection in intact leaves indicating its inablility to move from cell to cell in the absence of RNA-2. This lends support to the possibility of RNA-2 coding for the protein that enables the virus to become systemic in a susceptible host plant. In *in vitro* translation studies, RNA-2 of SCNMV codes for a 34K protein (Gietz et al. 1987), and it is possible that it is involved in cell-to-cell movement. For example, the 30k protein of TMV was reportedly involved in the systemic movement of the virus (Deom et al. 1987; Meshi et al. 1987). Independent replication of larger RNA species in divided genome viruses was also shown for tobacco rattle virus (Lister and Bracker, 1969; Sanger, 1960), tomato black ring virus (Robinson et

al. 1980) cowpea chlorotic mottle virus (Goldbach et al. 1980). Also the larger RNA species of narcissus mosaic virus was found to contain the information for all viral functions (Mackie and Bancroft, 1986). In the case of multipartite plant viruses, the large RNAs of alfalfa mosaic virus (Nassuth and Bol, 1983) and brome mosaic virus (Kiberstis et al. 1981) have been shown to replicate independently of their respective RNAs having the coat protein cistron. Of all these divided genome viruses, dianthoviruses may be unique since the larger RNA, besides having the ability to replicate independently, also carries the coat protein gene, whereas the putative transport protein, responsible for the cell-to-cell movement of the virus is coded by RNA-2.

The use of cloned DNA fragments of the virus in detecting viral specific RNA in combination with further improvements in infection efficiency by using recent methods like electroporation (Watts et al. 1987; Hibi et al. 1987) for introducing foreign genes should be valuable in elucidating the replicative strategy of the virus in a greater detail.

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Fig. III.1 Effect of concentration of sweet clover necrotic mosaic virus on percent infection of cowpea protoplasts.

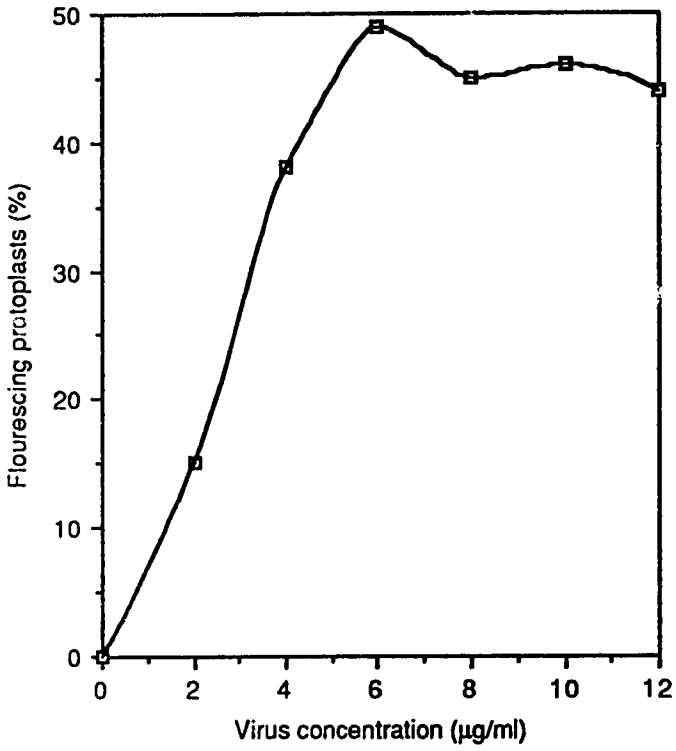

50
40
30
20
10
0
Flourescing protoplasts (%)
0
2
4
6
8
10
12
Virus concentration (µg/ml)

Fig. III.2 Effect of concentration of Poly-L-Ornithine on survival of cowpea protoplasts and their infection with sweet clover necrotic mosaic virus strain 59. (⊡--⊡), flourescing protoplasts; (■---■), intact protoplasts.

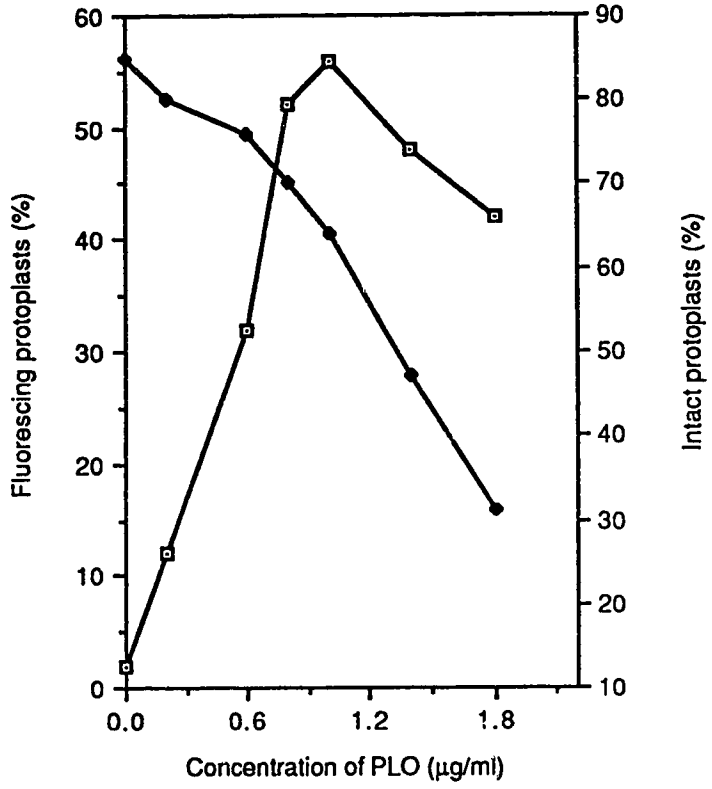

60
50
40
30
20
10
0
90
80
70
60
50
40
30
20
10
0.0
0.6
1.2
1.8
Fluorescing protoplasts (%)
Intact protoplasts (%)
Concentration of PLO (µg/ml)

Fig. III.3 Effect of pH of phosphate buffer (□--□) and citrate buffer (■--■) on infection of cowpea protoplasts with sweet clover necrotic mosaic virus strain 59.

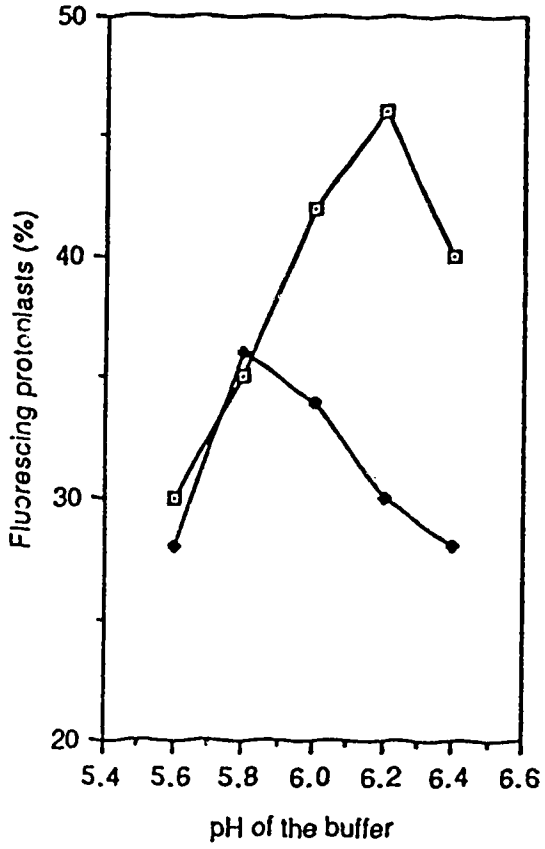

50
40
30
20
Fluorescing protoplasts (%)
5.4
5.6
5.8
6.0
6.2
6.4
6.6
pH of the buffer

Fig. III.4 Time-course study of multiplication of sweet clover necrotic mosaic virus during various periods of incubation as judged by infectivity assay (⊡---⊡, left ordinate) and fluorescent antibody staining (■--■, right ordinate).

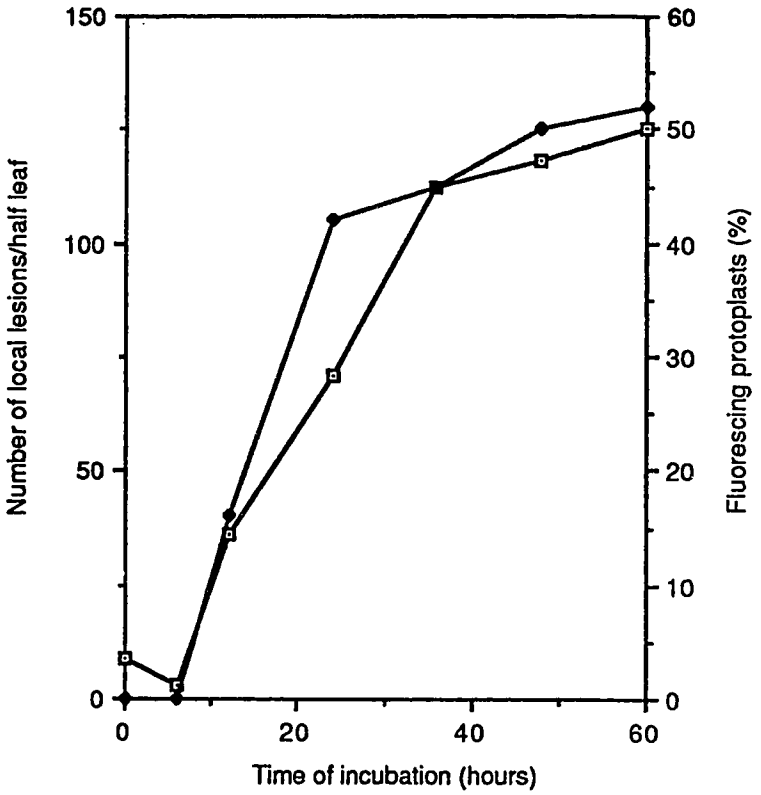

150
100
50
0
60
50
40
30
20
10
0
0
20
40
60
Number of local lesions/half leaf
Fluorescing protoplasts (%)
Time of incubation (hours)

Fig. III.5 Protoplasts from mesophyll cells of cowpea sampled after 48 hours of incubation. Inset shows flourescing protoplasts that were inoculated with sweet clover necrotic mosaic virus using indirect fluorescent antibody staining.

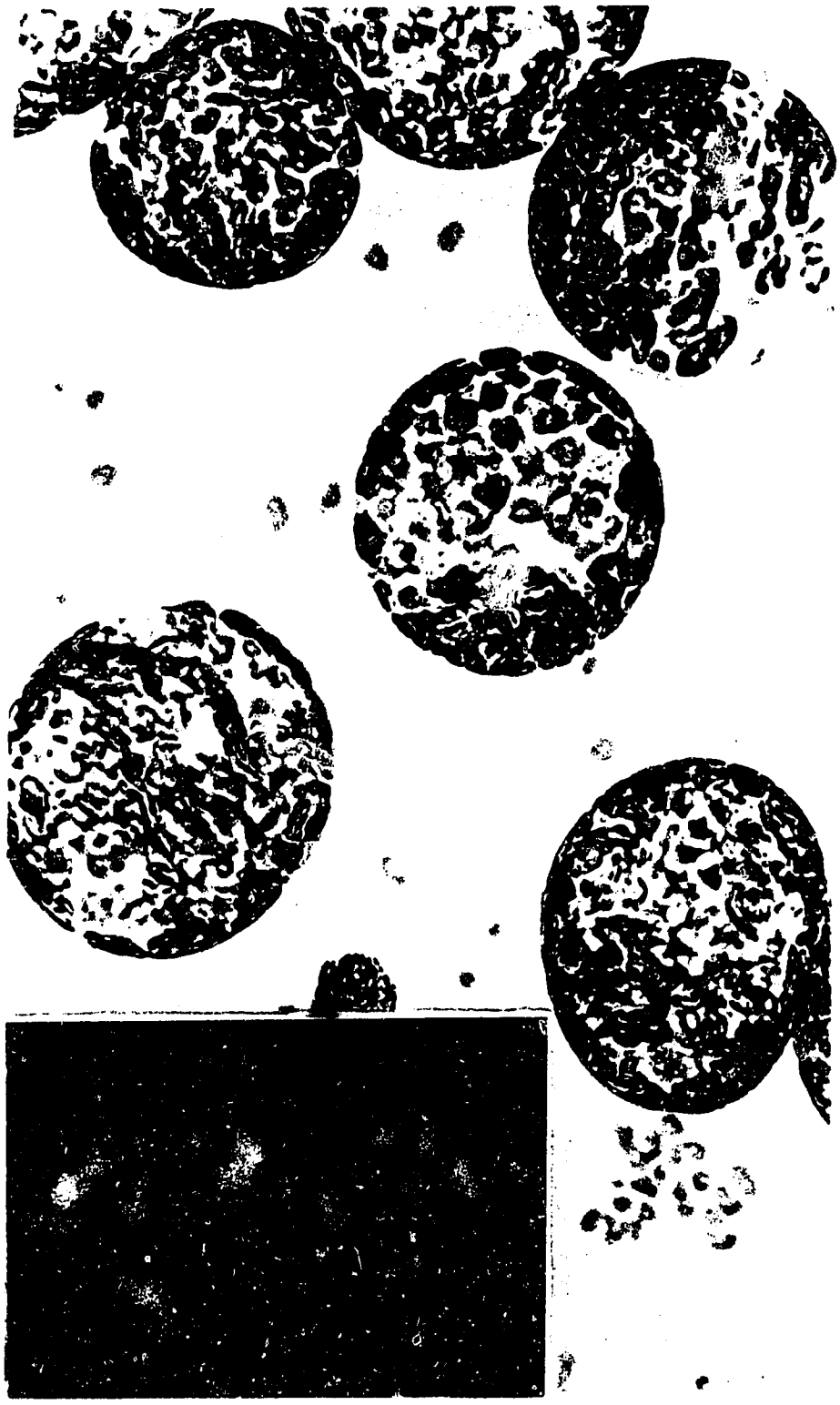


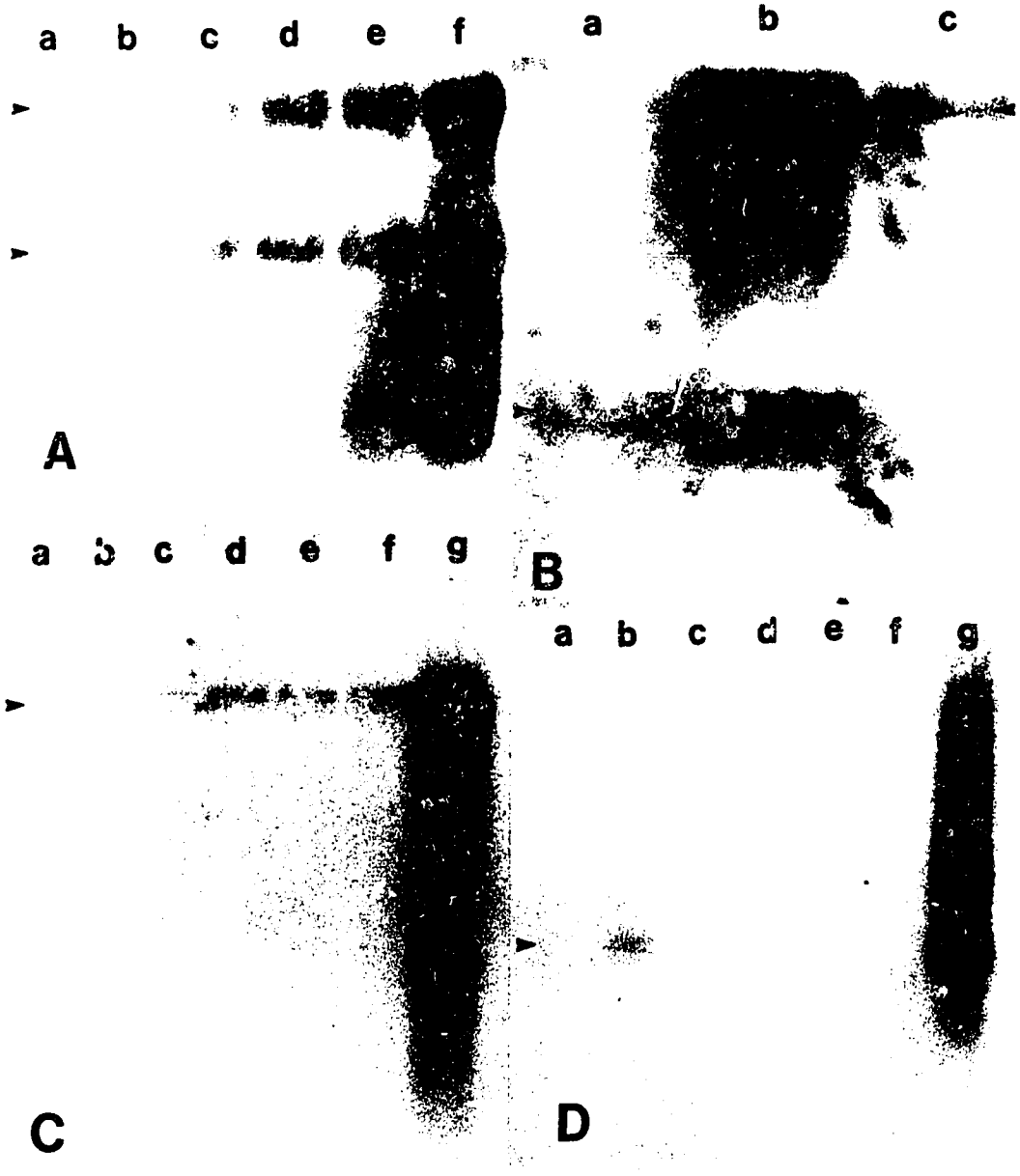
Fig. III.6 Northern blots of sweet clover necrotic mosaic virus (SCNMV-59) RNAs synthesized in protoplasts inoculated with total or fractionated SCNMV RNA.

(A) Replication of SCNMV in protoplasts inoculated with total virion RNA. (a) mock inoculated protoplasts after 48 hr of incubation. (b) to (f) Protoplasts sampled after 0, 12, 24, 36 and48 hr of incubation.

(B) Northern blot of separated and total genomic RNA probed by ^{32}P-labeled cDNA probe. (a) RNA-2; (b) Total RNA; (c) RNA-1.

(C) Northern blot of SCNMV synthesized in protoplasts inoculated with purified RNA-1 alone. (a) to (f) as in Fig. A; (g) SCNMV total RNA.

(D) Northern blot of protoplast extracts inoculated with purified RNA-2 alone. (a) to (f) as in Fig. A; (g) SCNMV total RNA.


a b c d e f
a b c
A
a b c d e f g
B
a b c d e f g
C
D

Chapter IV

Characterization of genetic reassortants of sweet clover necrotic mosaic virus by virion electrophoresis*

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*A version of this chapter has been accepted for publication.

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INTRODUCTION

Studies on distribution of genetic determinants among divided genome viruses are often made possible by the availability of virus strains with some defined properties that are characteristic of each strain. Constructing genetic reassortants between such strains often gives the nature and distribution of genes that specify the properties of that particular virus. In such cases, characterization of the hybrid nature of the resulting genetic reassortants is confirmed using methods such as infectivity tests, serological specificity of the reassortants and, more recently, Northern hybridization analysis. In this chapter, the migration pattern of purified virus preparations was shown to be a useful phenotypic marker in characterizing the genetic reassortants between two electrophoretically distinct strains of SCNMV.

MATERIALS AND METHODS

Viruses and fractionation of virion RNA

The type strain of SCNMV (SCNMV-38) and its new serotype (SCNMV-59) were used in this study. Both strains were separately multiplied in *Phaseolus vulgaris* L. 'Red Kidney' and purified according to a previously published procedure (Gould et al. 1981, Chapter II). Total virion RNA was extracted (Okuno et al. 1983) and stored frozen in distilled water at -80 C. Fractionation of the total virion RNA into RNA-1 and RNA-2 was done either by two cycles of sucrose density gradient centrifugation under denaturing conditions using formamide (Rao and Hiruki, 1987) or by

preparative gels using low melting point agarose (Maniatis et al. 1982). The purity of each separated RNA species was confirmed by the absence of infection on inoculated leaves of *Chenopodium amaranticolor* Coste & Reyn. and *Vigna unguiculata* L. (Walp) 'California Black Eye', and by Northern hybridization analysis.

Construction of genetic reassortants

Genetic reassortants were constructed by inoculating heterologous mixtures of RNAs, RNA-1 of SCNMV-38 and RNA-2 of SCNMV-59 (S_1s_2) and RNA-1 of SCNMV-59 and RNA-2 of SCNMV-38 (s_1S_2) on leaves of *V. unguiculata* and *C. amaranticolor*. Individual lesions were collected and each progeny virus was separately multiplied on *V. unguiculata*. The viruses were finally propagated on a large scale on *P. vulgaris* and purified. Infectivity tests of the separated RNAs and their mixtures were done on *C. amaranticolor* and *C. quinoa*.

Northern hybridization analysis

The identity of each virion RNA of the genetic reassortants was established using a ^{32}P-labeled complementary DNA (cDNA) probe made by random priming the purified virion RNA of the respective parental strains. For such analyses, RNAs were heat-denatured in the presence of formamide and electrophoresed in agarose gels using formaldehyde as the denaturant (Gietz & Hodgetts 1985; Davies et al. 1986; Chapter III) and blotted to Gene Screen Plus membrane (NEN Research Products) by capillary transfer. Prehybridization, hybridization and washings of the

membrane were done as previously described (Klessig and Berry, 1983; Chapter III).

Serology

Double-immunodiffusion tests in agarose gels were done using anti-SCNMV-59 serum (Pappu et al. 1988, chapter II).

Agarose gel electrophoresis of virions

Purified virus preparations (5 µg each) were subjected to denaturing agarose gel electrophoresis as described previously (Pappu et al. 1988; Chapter II). Following electrophoresis, the encapsidated RNA and the viral capsid were detected by differential staining of the same gel with ethidium bromide and Coomassie Brilliant Blue R250 respectively (Serwer, 1986).

RESULTS

Genetic complementation between two distinct strains of SCNMV

Neither each separated RNA species from the parent strains nor their genetic reassortants were infectious, whereas their homologous and heterologous mixtures produced infection upon testing on the two species of indicator plants (Table IV.1).

Characterization of genetic reassortants by serology

In gel immunodiffusion tests, anti-SCNMV-59 serum distinguished SCNMV-38 from SCNMV-59 by homologous spur

formation (Fig. IV.1a). Both genetic reassortants (S_1s_2 and s_1S_2), when tested against anti-SCNMV-59 serum, showed a similar serological reaction, the formation of a homologous spur, to that of the parental strains which donated the RNA-1 (Fig. IV.1b).

Characterization of genetic reassortants by Northern hybridization

The hybrid nature of each of the genetic reasssortants was also confirmed by Northern analysis using specific labeled cDNA probes. In such an analysis, cDNA to SCNMV-38 (S_1S_2) hybridized to RNA-1 of S_1s_2 and RNA-2 of s_1S_2 while cDNA to SCNMV-59 (s_1s_2) detected RNA-1 of s_1S_2 and RNA-2 of S_1s_2, thus confirming the hybrid nature of each of the genetic reassortants (Fig. IV. 2a, b). Hybridizations and subsequent washings at 42 C showed some cross hybridization, whereas under high stringency conditions at 60 C, both cDNAs hybridized to their respective RNAs only.

Agarose gel electrophoresis

When purified virus preparations of SCNMV-38 and SCNMV-59 were electrophoresed in agarose gels under non-denaturing conditions, each preparation migrated as a single electrophoretic form. SCNMV-59 had slightly slower mobility than SCNMV-38 (Fig. IV. 3a, b). The distinct electrophoretic profile of each parent strain was used to further confirm the hybrid nature of their genetic reassortants. Purified preparations of S_1s_2 and s_1S_2 were subjected to non-denaturing agarose gel electrophoresis followed by differential staining. S_1s_2 migrated to the same position as SCNMV-38 and s_1S_2 had the same mobility to that of SCNMV-59

(Fig. IV.4a, b). The differences in electrophoretic mobilities of the parent strains and their genetic reassortants were consistent and highly reproducible among different batches of virus preparations during a period of one year.

DISCUSSION

Using the two electrophoretically distinct strains of SCNMV as a model system, the above results demonstrate the feasibility of using the differences in virion mobility as a criterion in determining the distribution of the genetic determinant for the coat protein gene. The two genomic RNAs of SCNMV-38 and SCNMV-59 were interchangeable and complemented each other as demonstrated by the viability of their genetic reassortants upon inoculation of indicator plants. Studies on genetic reassortants among members of the dianthovirus group showed that the majority of strains form viable hybrids (Dodds et al. 1977; Okuno et al. 1983; Osman et al. 1986; Rao and Hiruki, 1987). Characterization of such genetic reassortants was mostly based on serological specificity, host range, symptomatology on selected host plants, and Northern hybridization analysis. In this report, we utilized the differences in the electrophoretic mobilities of the virions in order to study the phenotypic expression of genetic reassortants. Each genetic reassortant retained the distinct mobility pattern of the parental strain which supplied the RNA-1. This was supported by the fact that RNA-1 of SCNMV carries the coat protein gene (Okuno et al. 1983). Infectivity tests demonstrated the viability of reassortants

between genomic RNAs of the two SCNMV strains (Table IV.1). Using agar gel immunodiffusion tests, the serological identity of the genetic reassortants was established (Fig. IV.1a, b). Northern hybridization analysis was used to confirm the genomic composition of each reassortant (Fig. IV.2a, b).

Among other divided genome viruses, electrophoretically distinct strains have been described for CMV (Dodds, 1982), RCNMV (Musil and Gallo, 1982) and CRSV (Pappu and Hiruki, 1988). The genetic reassortants which arise among these strains may be identified by this method since the location of the coat protein gene for these viruses is known. Therefore virion electrophoresis offers another simple method, in addition to agar gel immunodiffusion test, for virion characterization although genetic composition of reassortants may have to be established by Northern analysis. Using this method, electrophoretically distinct strains have been reported in the case of TMV in both purified virus preparations and crude extracts from infected plants (Asselin and Grenier, 1985), which suggests the possibility of using this approach for rod shaped viruses as well.

Virion electrophoresis is simple and rapid to perform and requires only small amounts of purified virus preparations for analytical purposes (Serwer, 1986; Orban and Chrambach, 1988). Following electrophoresis, the differential staining of the same gel allows rapid visualization of the virion capsid as well as the encapsidated RNAs. Moreover, the observed differences in mobility of both strains and their genetic reassortants were consistent over a period of time. Thus the electrophoretic variablity

between virions may serve as a useful marker when used in conjunction with other methods like serological criteria.

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Table IV.1. Infectivity tests of individual RNAs of SCNMV-38 (S_1S_2) and SCNMV-59 (s_1s_2) and their genetic reassortants.

RNA species in inoculum*	Average number of lesions¶ *C. amaranticolor*	*C. quinoa*
S_1+S_2	42.5	38
s_1+s_2	40.0	36
S_1	0.3	0
S_2	0	0.5
s_1	0.6	0.3
s_2	0	1.0
S_1+s_2	48.0	42.5
s_1+S_2	56.0	38.0

* Each RNA species was present at 5 µg/ml.

¶Average number of lesions from six inoculated leaves for each inoculum.

Figure IV.1 Serological characterization of genetic reassortants between SCNMV-38 (S_1S_2) and SCNMV-59 (s_1s_2). Gel immunodiffusion tests in 0.8% agarose. Lower well in (a) and central well in (b) contained 1/4 dilution of anti-SCNMV-59 serum. A, SCNMV-38; B, SCNMV-59; C, genetic reassortant S_1s_2; D, genetic reassortant s_1S_2.

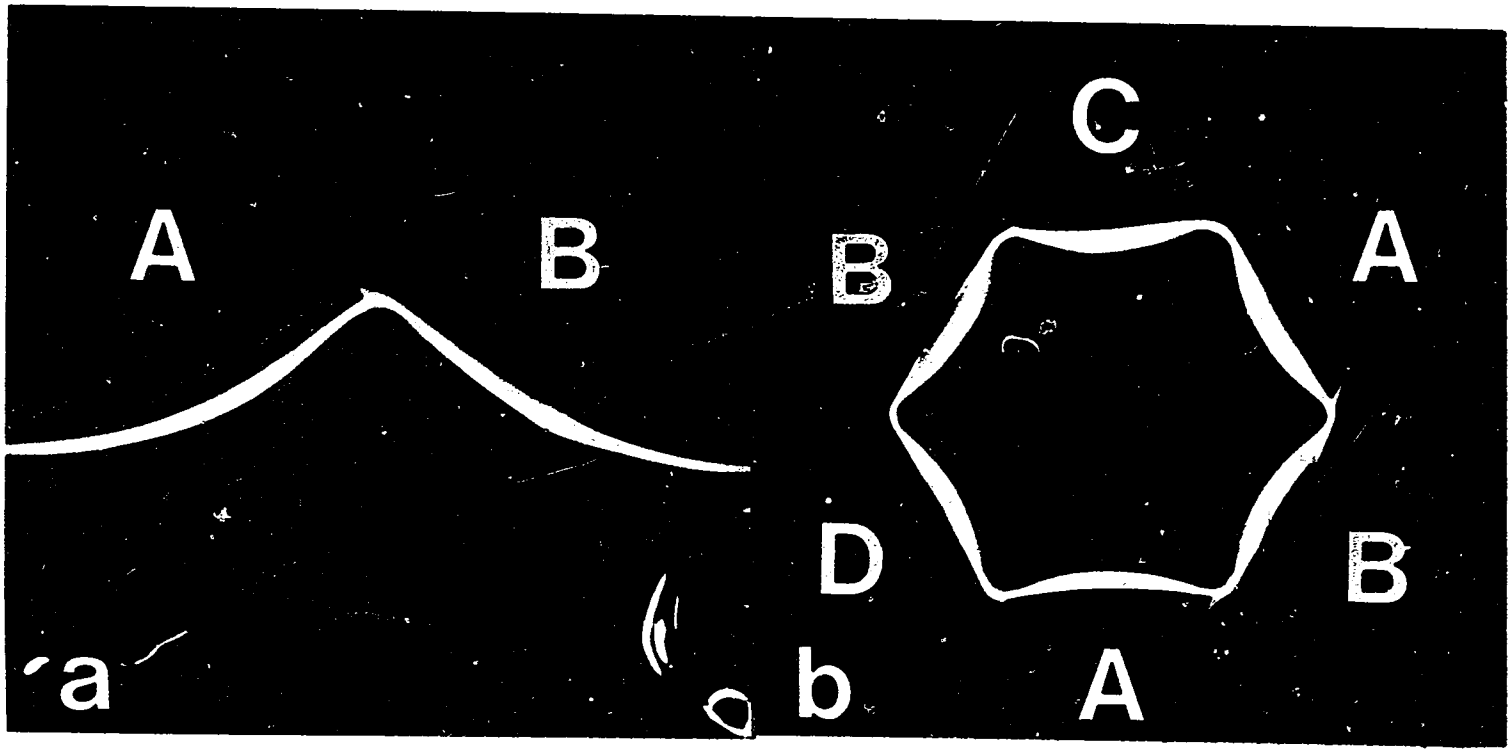

A
B
a
C
B
A
D
B
A
b

Figure IV.2 Northern hybridization analysis of SCNMV seroytpes and their genetic reassortants. Lane 1, SCNMV-38 (S_1S_2); Lane 2, RNA-1 of SCNMV-38 and RNA-2 of SCNMV-59 (S_1s_2); Lane 3, RNA-1 of SCNMV-59 and RNA-2 of SCNMV-38 (s_1S_2); Lane 4, SCNMV-59 (s_1s_2). Gene Screen Plus membrane hybridized with ^{32}P-labeled cDNA to (a) total virion RNAs of SCNMV-38 and (b) total virion RNAs of SCNMV-59.

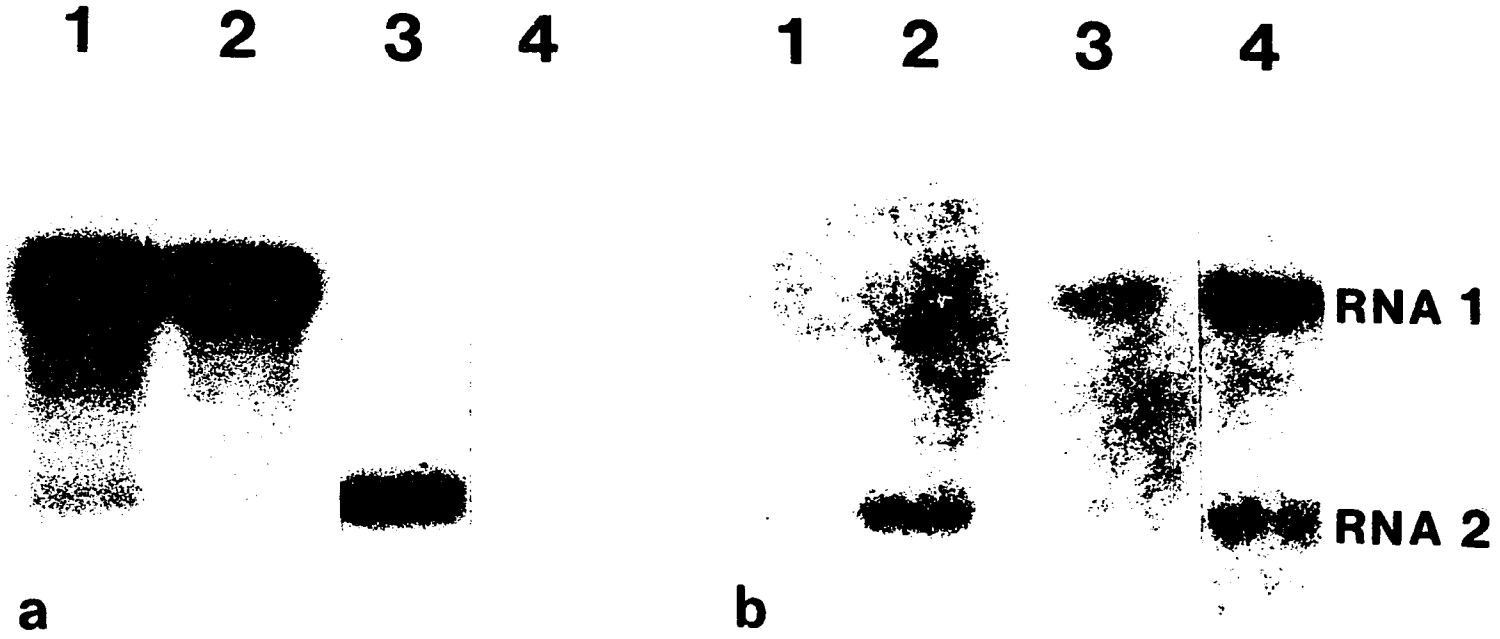

1 2 3 4
1 2 3 4
RNA 1
RNA 2
a
b

Figure IV.3 Non-denaturing agarose gel electrophoresis of purified virus preparations of SCNMV serotypes. Lane 1, SCNMV-38; Lane 2, SCNMV-59. (a) Gel stained with ethidium bromide (0.5 μg/ml) in distilled water containing 1 mM EDTA. (b) the same gel stained with Coomassie Brilliant Blue followed by destaining overnight.

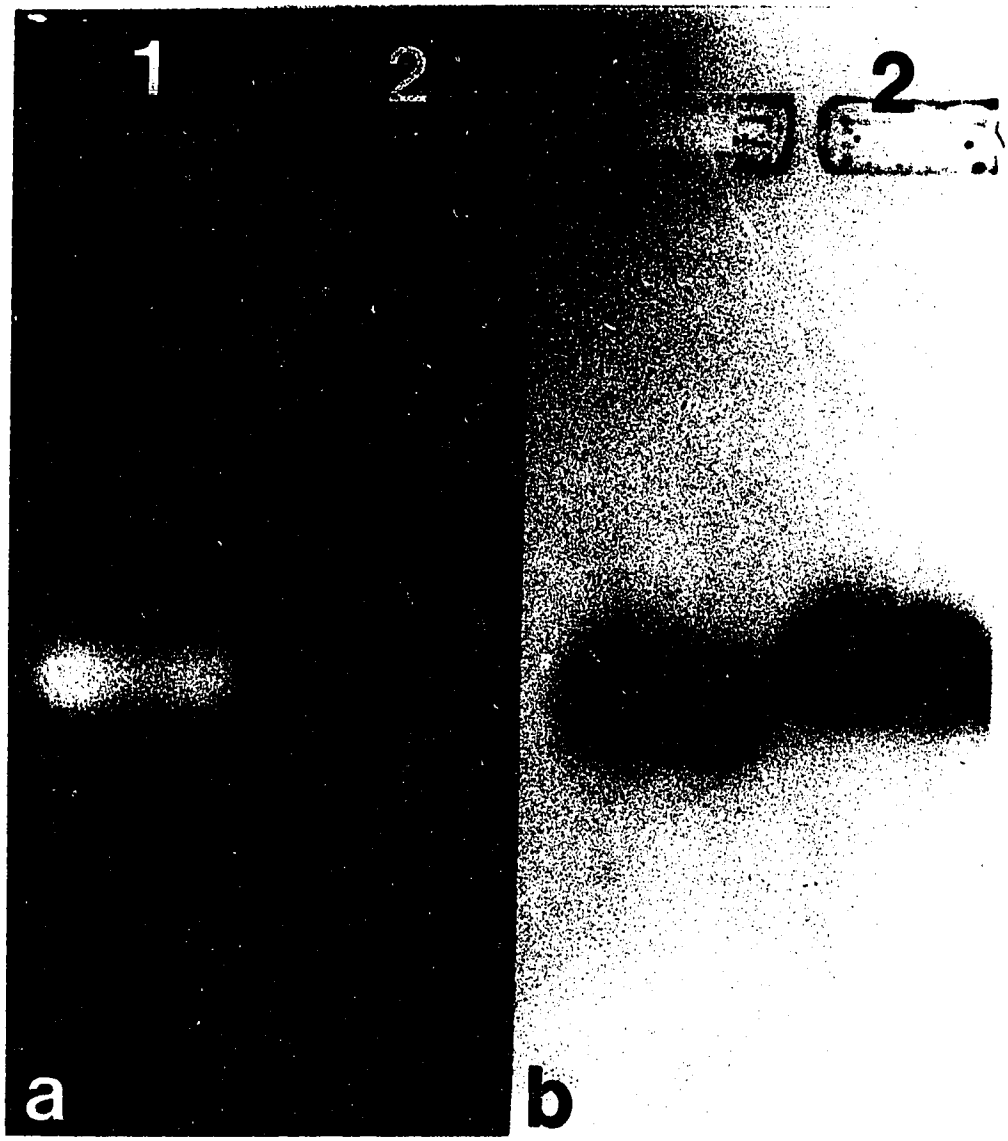
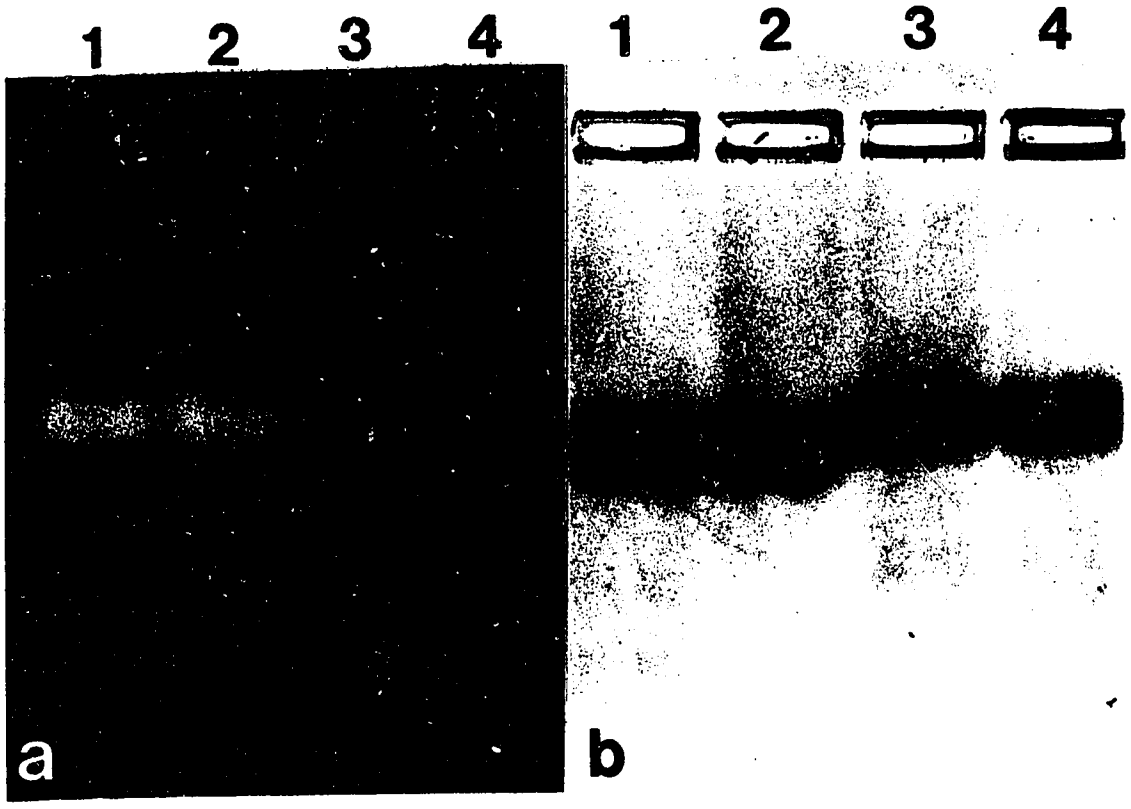

1
2
2
a
b

Figure IV.4 Non-denaturing agarose gel electrophoresis of purified preparations of SCNMV serotypes and their genetic reassortants. Lane 1, SCNMV-38; Lane 2, S_1s_2; Lane 3, s_1S_2; Lane 4, SCNMV-59. (a) Gel stained with eithidium bromide (b) the same gel re-stained with Coomassie Brilliant Blue followed by destaining overnight.


1
2
3
4
1
2
3
4
a
b

Chapter V

Electrophoretic variability among dianthoviruses*

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* A version of this chapter has been published. Pappu, H.R., and C. Hiruki. 1989. *Phytopathology* 79:1253-1257.

INTRODUCTION

Among the three members of the dianthovirus group, a considerable amount of antigenic variation is known to occur (Rao et al., 1987). Several strains of each virus have been characterized based on biological and serological properties. Serological relationships among the members and the strains revealed varying degrees of relationship as assessed by monoclonal (Hiruki et al. 1984b; Hiruki and Figueiredo, 1985) and polyclonal antibodies (Gould et al., 1981; Hiruki et al., 1984a; Hiruki et al., 1984b; Musil and Gallo, 1982; Musil et al., 1982; Okuno et al., 1983; Pappu et al., 1988a; Rao et al., 1987). In the preceding chapter, purified preparations of two strains of SCNMV were shown to be electrophoretically distinct. The present study examines several other strains of RCNMV and CRSV, and describes the occurrence of distinct differences in virion electrophoretic mobilities among dianthoviruses as determined by virion electrophoresis.

MATERIALS AND METHODS

Viruses

The various dianthoviruses used in this study were listed in Table V.1. Each virus was multiplied separately in *Phaseolus vulgaris* L. 'Red Kidney' and purified according to a previously published procedure (Gould et al. 1981 and chapter II). Final virus preparations were suspended in 50% glycerol and stored at -20 C.

Coat protein analysis

Purified virus preparations were dissociated at 100 C in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system (Laemmli 1970). Bands were located by staining with Coomassie Brilliant Blue R250 and destaining overnight in a destaining solution (methanol:acetic acid:water, 10:10:80 v/v/v). Protein molecular weight standards were obtained from Bio-Rad Laboratories, Richmond, CA.

Isoelectric focusing

Isoelectric focusing of purified virus preparations was done as described (Pappu et al. 1988b; Chapter II). Each virus preparation (2-5 μg) was subjected to focusing in 1% agarose gel (Isogel agarose, FMC Corporation) containing 10% glycerol and 2% Bio-Lyte ampholytes with pH ranges of 3-10 or 5-7 (Bio-Rad Laboratories, Richmond, CA) using Bio-Rad's Mini IEF cell (model 111).

Agarose gel electrophoresis of virions

Non-denaturing agarose gel electrophoresis of purified virus preparations (virion electrophoresis) was done as previously described (Pappu et al., 1988a and Chapter II).

RESULTS

Coat protein profiles

A single polypeptide species of about 39,000 daltons was detected for each virus (Fig.V.1). No significant mobility differences could be seen among the viral proteins.

Isoelectric focusing

The isoelectric points of SCNMV-38, SCNMV-59, CRSV-A, RCNMV-TpM 34, RCNMV-TpM 48 and RCNMV-Aus were in the pH range of 4.75-5.1 (average of three experiments), whereas that of CRSV-N was between 6.0-6.2 as determined by the regression analyses of the pH gradients of 3-10 or 5-7 following focusing (Fig. V.2A,B). Each virus preparation showed a major polypeptide band and occasionally a minor band (Fig. V.3B, Lane 2). The differences in the focusing pattern were consistent among the strains and the most discernible differences were found between CRSV strains, A and N and, RCNMV strains, TpM 48 and Aus (Fig.V.3A,B).

Agarose gel electrophoresis

When purified virus preparations were electrophoresed in agarose gels under non-denaturing conditions, a single electrophoretic form was detected (Fig. V.4). The migrating component is presumed to be a virion, based on the observation that it was stained by ethidium bromide (viral RNA) and Coomassie Brilliant Blue (viral protein). Significant mobility differences were noticed among the strains of all three viruses (Fig.V.4A,B). All

virions migrated from cathode to anode indicating a net negative charge for the virions under the electrophoretic conditions used. SCNMV-38 and SCNMV-59 were electrophoretically distinct as reported earlier (Pappu et al. 1988a; Chapters II and III) and CRSV-A showed the same mobility as that of SCNMV-38. CRSV-N had the slowest mobility of the strains tested in this study. RCNMV-TpM 34 and RCNMV-TpM 48 migrated identically, whereas RCNMV-Aus had a greater mobility and was similar to those of SCNMV-38 and CRSV-A (Fig.V.4A,B). The electrophoretic profile of each strain and the relative mobility differences among all the strains were consistent and highly reproducible when the experiment was run several times during a period of one year.

In order to confirm whether the mobility pattern displayed by each strain is a characteristic feature, the electrophoretically distinct strains were mixed in various combinations and then subjected to virion electrophoresis. Following differential staining as described above, each individual virus preparation in a mixture retained its characteristic mobility pattern and migrated as a single but distinct electrophoretic form (Fig.V.5A,B).

DISCUSSION

Results of the physical characterization of the dianthoviruses by virion electrophoresis and isoelectric focusing provide evidence for differences in electrophoretic mobilities among their various strains. Similar results have been obtained for other groups of plant viruses (Ball, 1966; Bawden, 1958; Dodds, 1982; Magdoff-Fairchild,

1967; Semancik, 1966). Taxonomic grouping of the dianthoviruses is based mainly on the serological relationships and the differences in electrophoretic mobility support this taxonomy to some extent.

The two electrophoretically distinct strains of SCNMV were also serologically distinguishable by immunodiffusion tests (Pappu et al. 1988a; chapter II). Similarly, two closely related strains of CRSV, CRSV-A and CRSV-N, which showed striking mobility differences in this study, were serologically distinguishable (Tremaine et al. 1975).

The reason for conflicting data on RCNMV-TpM 34 and TpM 48 is unknown. Previous reports stated that RCNMV-TpM 34 and TpM 48 were serologically and electrophoretically distinct (Galio and Musil, 1984a; Gallo and Musil, 1984b; Musil and Gallo, 1984; Musil et al., 1982). However, we found that the migration patterns of these two strains were similar, and consistent and reproducible among different batches of virus preparations which were made over a period of one year. Also, in this study, the isoelectric points of RCNMV-TpM 34 and TpM 48 were very similar. A possible explanation for this apparent dicrepancy might be that one or both of the strains used in this study could be mutants of the RCNMV strains originally described (Musil, 1969), since the symptomatology produced by our strains on selected host plants was different from that of the original strains (Rao and Hiruki, 1987). This discrepancy must be resolved by further studies using the original strains, since RCNMV-TpM 34 and TpM 48 are grouped into two different serotypes (serotype A and serotype B respectively) (Rao et al., 1987). RCNMV-Aus, belonging to serotype

D, has a different mobility pattern and a significantly different isoelectric point from those of RCNMV-TpM 34 and TpM 48.

There are several possible reasons for the origin of charge heterogeneity among dianthoviruses used in this study. The host passage effect may have occured, leading to an adaptive mutation in a particular virus strain (Yarwood, 1979). In the case of SCNMV, the type strain (SCNMV-38) was originally isolated from sweet clover (Hiruki et al. 1984a), whereas its new serotype was isolated from alfalfa (Inouye and Hiruki, 1985). Of the two CRSV strains, CRSV-A forms stable 12-particle aggregates at acidic pH whereas CRSV-N is a non-aggregating strain. They are also serologically distinguishable as determined by reactions of partial identity in gel diffusion tests (Tremaine et al. 1975). The striking differences in virion mobilities and isoelectric points of CRSV-A and CRSV-N are probably caused by differences in the amino acid composition of their respective coat proteins (Tremaine et al. 1984). Mobility differences have been correlated with differences in amino acid composition for strains of hibiscus chlorotic ringspot virus (HCRV) (Hurtt, 1987), thus the observed differences among the three strains of RCNMV may be explained similarly. The three strains were originally reported from Czechoslovakia (RCNMV-TpM 34 and TpM 48) (Musil, 1969) and Australia (RCNMV-Aus) (Gould et al., 1981). This geographical isolation may have allowed for some genetic drift in their coat proteins.

Upon analytical ultracentrifugation, dianthoviruses sediment as a single centrifugal component indicating the presence of only one nucleoprotein component (Hiruki, 1987). This is further

supported by the fact that, following electrophoresis, each virus preparation migrated as a single electrophoretic component as indicated by the differential staining of the same gel with ethidium bromide and Coomassie Brilliant Blue (Fig. V.4). In the case of some other viruses with segmented genomes, individual RNA species of the virus are known to be packaged in separate particles (Dougherty and Hiebert, 1987).

Current data on the electrophoretic profiles of dianthoviruses suggest the possibility of a simple packaging of both RNAs into a single particle instead of any other complex process. Virion electrophoresis may offer a simple alternative to an otherwise expensive gradient centrifugation methods in identifying nucleoprotein components (Ong and Mink, 1989). Several strains of prunus necrotic ringspot virus, which were otherwise serologically indistinguishable were distinguished on the basis of their virion mobilities (Ong and Mink, 1989).

As demonstrated in the previous chapter, virion electrophoresis should be useful in charcterizing genetic reassortants between electrophoretically distinct strains and/or viruses with divided genomes.

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Table V.1 Sources of viruses used in this study

VIRUS	STRAIN	REFERENCE
Carnation ringspot virus	strain A	Tremaine et al. 1975
	strain N	Tremaine et al. 1975
Red clover necrotic mosaic virus	TpM 34	Musil and Gallo, 1982
	TpM 48	Musil and Gallo, 1982
	Australia	Gould et al. 1981
Sweet clover necrotic mosaic virus	strain 38	Hiruki et al. 1984
	strain 59	Inouye and Hiruki 1985
		This study

Fig. V.1. SDS-PAGE (12.5%) of dissociated coat proteins of dianthoviruses. Lanes 1 and 9, Protein molecular weight standards: (from top to bottom) phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.6 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lane 2, sweet clover necrotic mosaic virus (SCNMV)-38; Lane 3, SCNMV-59; Lane 4, carnation ringspot virus (CRSV)-A; Lane 5, CRSV-N; Lane 6, red clover necrotic mosaic virus (RCNMV)-TpM 34; Lane 7, RCNMV-TpM 48; Lane 8, RCNMV-Aus. Gel stained with Coomassie Brilliant Blue and destained overnight. Migration was from top (negative) to bottom (positive).

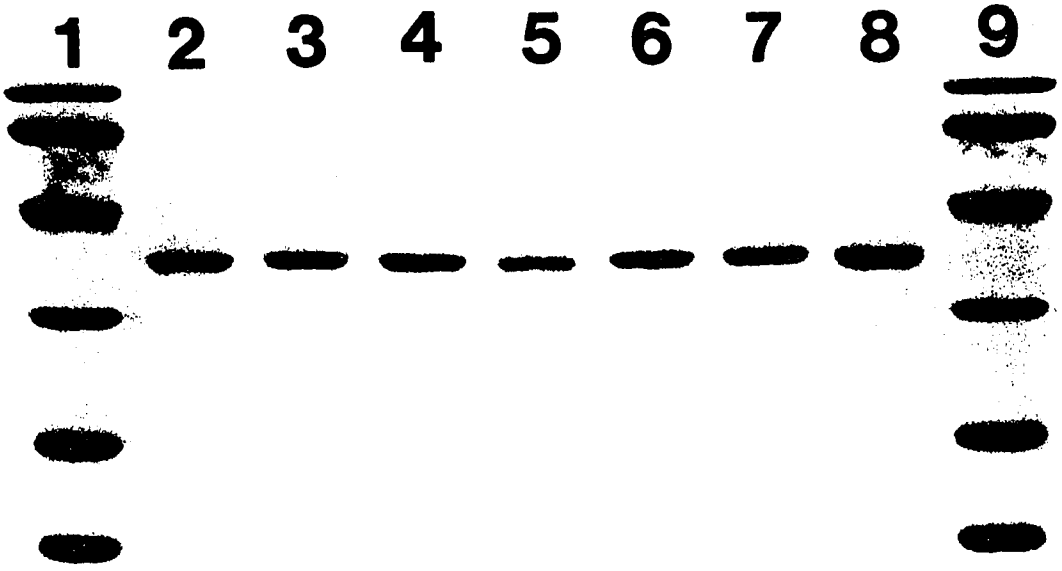
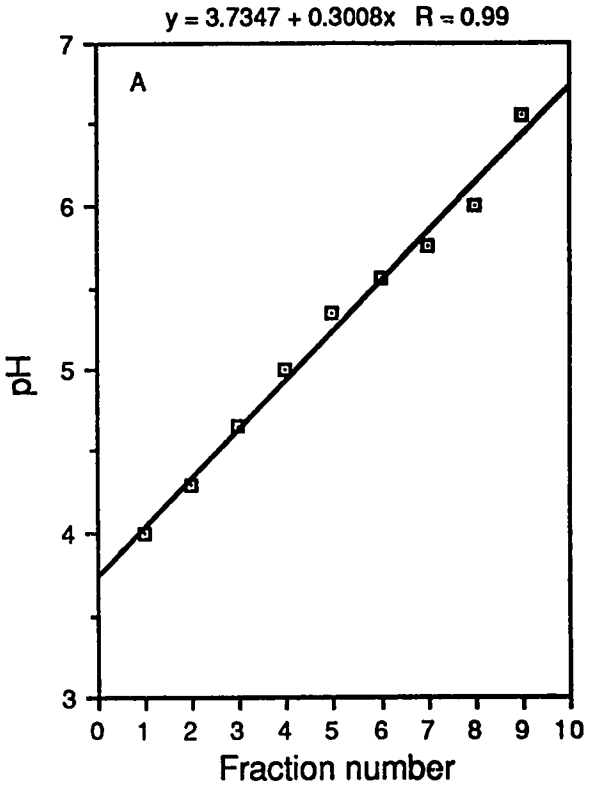

1
2
3
4
5
6
7
8
9

Fig. V.2. pH gradients generated following isoelectric focusing of dianthoviruses.

A. pH gradient generated from Bio-lyte ampholytes 4-7 following isoelectric focusing of carnation ringspot virus (CRSV)-N and CRSV-A.

B. pH gradient generated from Bio-lyte ampholytes 3-10 during isoelectric focusing of sweet clover necrotic mosaic virus (SCNMV)-38, SCNMV-59, CRSV-A, red clover necrotic mosaic virus (RCNMV)-TpM 34, RCNMV-TpM 48, and RCNMV-Aus.


y = 3.7347 + 0.3008x R = 0.99
A
pH
Fraction number
7
6
5
4
3
0 1 2 3 4 5 6 7 8 9 10

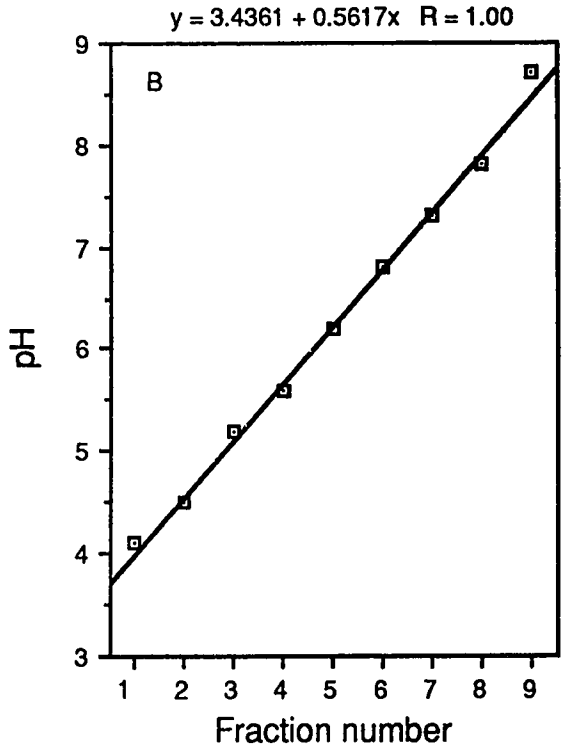
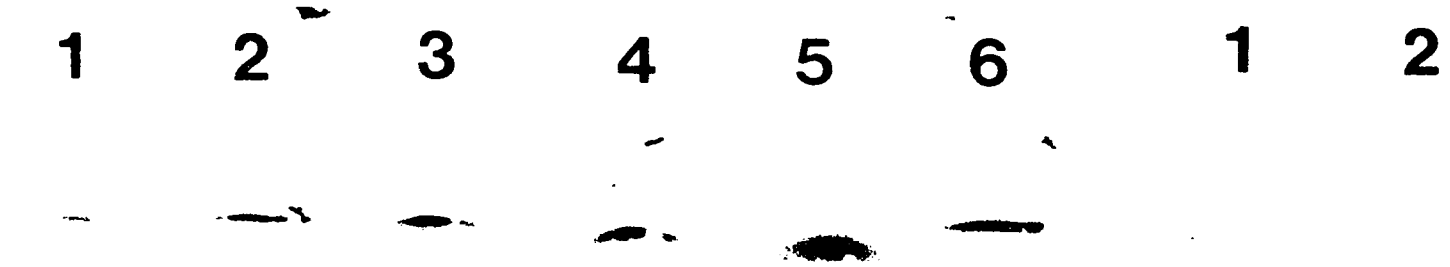

y = 3.4361 + 0.5617x R = 1.00
B
pH
Fraction number
9
8
7
6
5
4
3
1 2 3 4 5 6 7 8 9

Fig. V.3. Isoelectric focusing of purified preparations of dianthoviruses.

A. Lane 1, sweet clover necrotic mosaic virus (SCNMV)-38; Lane 2, SCNMV-59; Lane 3, carnation ringspot virus (CRSV)-A; Lane 4, red clover necrotic mosaic virus (RCNMV)-TpM 34; Lane 5, RCNMV-TpM 48; Lane 6, RCNMV-Aus. The pH gradient was generated using Bio-Lyte 4-7 ampholytes.

B. Lane 1, carnation ringspot virus (CRSV)-A; Lane 2, CRSV-N. The pH gradient was generated using Bio-Lyte 3-10 ampholytes. Both gels were stained with Coomassie Brilliant Blue and destained overnight. Migration was from top (positive) to bottom (negative).



A

B

Fig. V.4. Agarose gel (0.8%) electrophoresis of purified preparations of dianthoviruses. Lane 1, sweet clover necrotic mosaic virus (SCNMV)-38; Lane 2, SCNMV-59; Lane 3, carnation ringspot virus (CRSV)-A; Lane 4, CRSV-N; Lane 5, red clover necrotic mosaic virus (RCNMV)-TpM 34; Lane 6, RCNMV-TpM 48; Lane 7, RCNMV-Aus.

A. Gel stained with ethidium bromide (0.5 μg/ml in water containing 1 mM disodium EDTA).

B. The same gel re-stained with Coomassie Brilliant Blue and destained overnight. Migration was from top (negative) to bottom (positive).

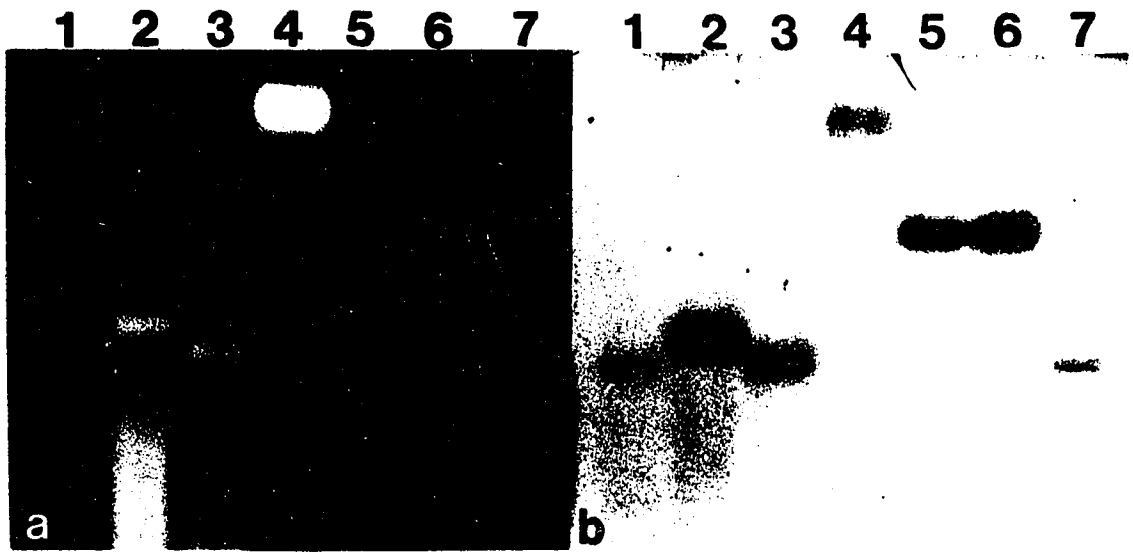
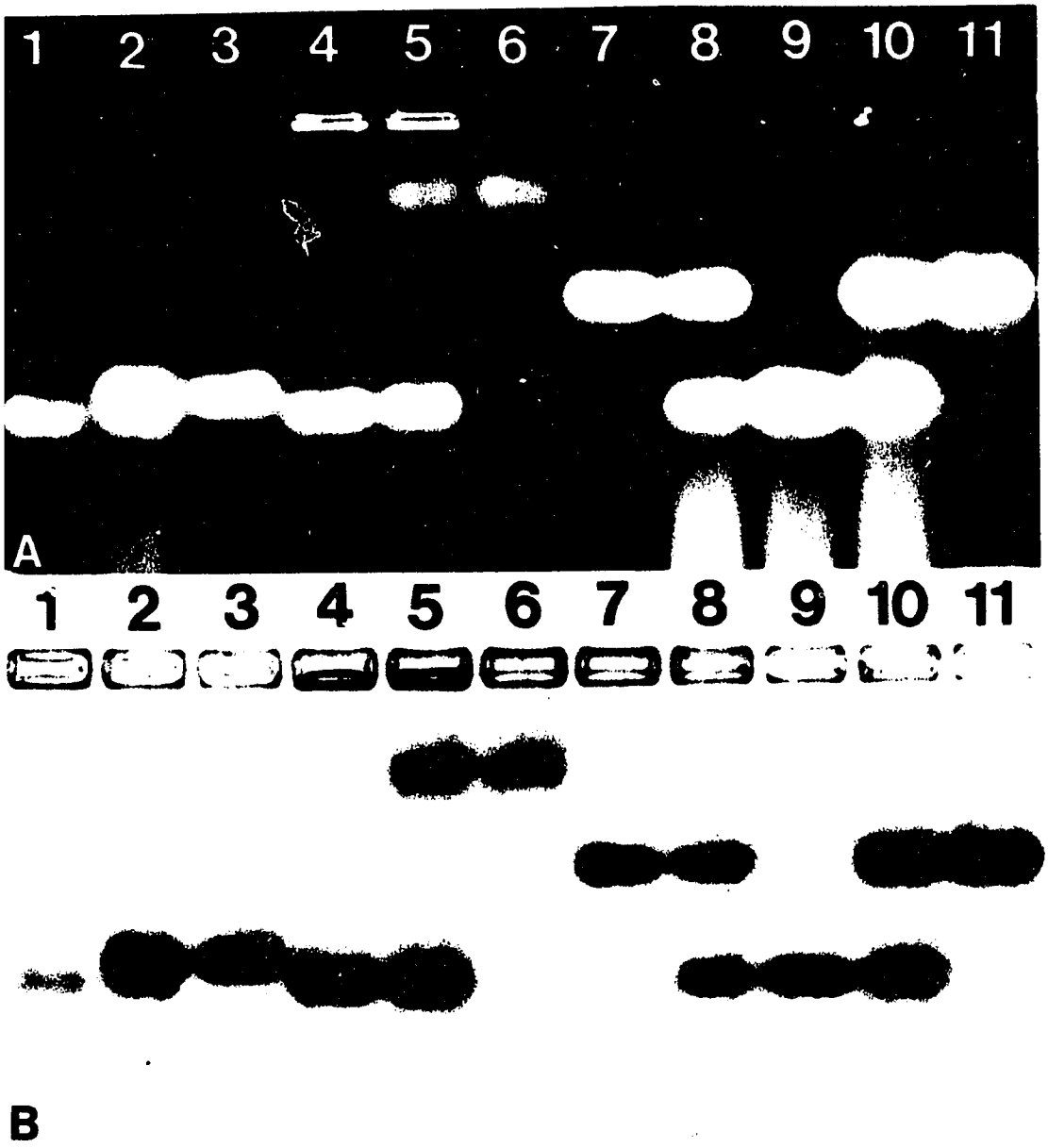

1 2 3 4 5 6 7
1 2 3 4 5 6 7
a
b

Fig. V.5. Agarose gel (0.8%) electrophoresis of mixtures of purified dianthoviruses. Lane 1, sweet clover necrotic mosaic virus(SCNMV)-38; Lane 2, SCNMV-38+SCNMV-59; Lane 3, SCNMV-59; Lane 4, carnation ringspot virus (CRSV)-A; Lane 5, CRSV-A+CRSV-N; Lane 6, CRSV-N; Lane 7, red clover necrotic mosaic virus (RCNMV)-TpM 34; Lane 8, RCNMV-TpM 34+RCNMV-Aus; Lane 9, RCNMV-Aus; Lane 10, RCNMV-TpM 48+RCNMV-Aus; Lane 11, RCNMV-TpM 48.

A. Gel stained with ethidium bromide (0.5 μg/ml in water containing 1 mm disodium EDTA).

B. The same gel re-stained with Coomassie Brilliant Blue and destained overnight. Migration was from top (negative) to bottom (positive).


1 2 3 4 5 6 7 8 9 10 11
A
1 2 3 4 5 6 7 8 9 10 11
B

Chapter VI

Molecular cloning and characterization of genomic RNAs of sweet clover necrotic mosaic virus (SCNMV-59)

INTRODUCTION

The ability to synthesize complementary DNA copies and clone these copies into plasmid or phage vectors has greatly facilitated the study of genome structure and organization of several RNA viruses. Of 28 recognized plant virus groups, the complete nucleotide sequences of at least one member of 15 virus groups have been published (Rochon, 1988). The advantages of cDNAs are manifold. Full-length cDNAs are increasingly being used to deduce the amino acid sequence of the putative protein product(s). Shorter cDNAs have found wide application in large scale screening, detection, and disease diagnosis (Pesic and Hiruki, 1988; Roy et al. 1988; Varveri et al. 1988). This has become more evident in cases where serological procedures are unsatisfactory (Lakshman et al. 1986; Owens and Diener, 1981; Robinson, 1988).

Among the members of the dianthovirus group, the nucleotide sequences of the RNAs of RCNMV has been recently published (Lommel et al. 1988; Xiong and Lommel, 1989). In an attempt to determine the nucleotide sequence of the SCNMV genomic RNAs and to study the possible homology with those of RCNMV and other plant viruses, cDNA copies of both RNAs of SCNMV were cloned into Lambda gt10 and cDNA clones representing 75% of RNA-2 were sequenced. This chapter describes the cDNA cloning strategy, isolation of specific clones, sub-cloning into suitable vectors and determination of a partial nucleotide sequence of RNA-2.

MATERIALS AND METHODS

Bacterial strains

Source: Dr. J.B. Bell, Professor, Dept. of Genetics, University of Alberta, Edmonton, Alberta.

E. coli C600 HflA (hsdR$^-$, hsdM$^+$, supE44, leuB6, lacY1, ton A21,thr) was used as a host for Lambda gt10.

E. coli JM83 (λ^- *ara* Δ(*pro-lac*)*rpsL thi* ø80 *dlacZ*ΔM15) was used as a host for Bluescribe (Stratagene).

E. coli JM105 (*thi rpsL endA sbc*B15 *hsp*R4 Δ(*lac-pro*AB) [F' *tra*D36*pro*AB *lacI*^{q}ZΔM15] was used as a host for M13mp18 and M13mp19.

Media

LB: The LB broth contained 10 g Tryptone, 10 g sodium chloride and 5 g of yeast extract per liter of distilled water; the pH was adjusted to 7.5 with 10 N NaOH. Bacto-agar (Difco) (12 g per liter) was added to solidify media. The media were sterilized by autoclaving.

Soft agar (top agar): soft agar consisted of LB broth containing 7 g of Bacto-agar (for colony lifts) or agarose (Ultrapure, BRL) for plaque lifts, per liter of distilled water.

McConkey agar: 40 g of McConkey agar (Difco), 12 g of lactose were dissolved in 1 liter of water and sterilized by autoclaving. Before pouring the plates, ampicillin (International Biotechnologies Inc.) was added to a final concentration of 100 μg/ml.

M9: The M9 medium contained 6.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g NaCl, 1.0 g of NH_4Cl, and 12.0 g of bacto-agar per liter of distilled water. The pH was adjusted to 7.4 with NaOH before adding agar. The medium was autoclaved and the following were added: 2.0 ml of 1M $MgSO_4$, 10 ml of 20% glucose, 0.1 ml of 1M $CaCl_2$ which were separately sterilized by autoclaving. 10 ml of filter-sterilized thiamine-HCl (Sigma, 10 mg/ml stock) was added per liter of medium.

SM: SM buffer contained 5.8 g of NaCl, 2.0 g of $MgSO_4.7H_2O$, 50 ml of 1 M Tris-HCl, pH 7.5, and 1.0 ml of 2% gelatin in 1 liter of water.

Purification of virus and genomic RNA

SCNMV-59 was purified from systemically infected Red kidney bean plants and genomic RNA was extracted as described in Chapters II and III.

Polyadenylation of SCNMV virion RNA

In a total reaction volume of 100 µl, the following solutions were mixed in the order as given below. Solutions of Tris-HCl, $MgCl_2$, NaCl, $MnCl_2$ and distilled water were sterilized by autoclaving for 20 min.

SCNMV-59 genomic RNA	25 µl (1 µg/µl)
1M Tris-HCl pH 7.9	5 µl
0.2 M $MgCl_2$	5 µl
2.5 M NaCl	10 µl
25 mM $MnCl_2$	10 µl
Bovine serum albumin	1 µl (10mg/ml)

10 mM ATP	2.5 µl
RNasin (Promega)	1 µl (2 units)
Poly(A) polymerase	6 µl (6 units, Pharmacia)
distilled water	34.5 µl

The contents were gently mixed, briefly centrifuged in a microcentrifuge (Microfuge 12, Beckman) and incubated at 37 C for 1 hr.

Oligo(dT) chromotagraphy

The oligo-dT chromotography was performed as described by Gietz and Hodgetts (1982) which was a modification of the method of Aviv and Leder (1972). The oligo-dT cellulose (type 7, Pharmacia) was equilibrated in 10 mM Tris-HCl pH 7.5, 400 mM NaCl, and 0.5% (w/v) SDS (application buffer). A small column was prepared in a sterile pasteur pipette. The oligo-dT in application buffer was packed and equilibrated at 10 ml/hr using the same buffer. The reaction mixture containing the poly(A)-tailed RNA was applied to the column and washed with application buffer. The eluant was passed through an absorbance monitor (LKB). When the absorbance value reached the baseline, the poly(A) RNA was eluted by applying the elution buffer (10 mM Tris-HCl, pH 7.5, and 0.05% SDS) to the column. Fractions of eluate were collected, pooled in a sterile 15 ml Corex tube and mixed with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice-cold 95% ethaanol. The mixture was kept at -70 C for 30 min. The RNA was pelleted by centrifuging the tube at 10,000 rpm for 10 min. in a Sorvall RC-5B with an SS-34 rotor. The resulting pellet was washed twice with

70% ethanol and dried in a freeze drier. The final pellet was dissolved in sterile distilled water and stored at -70 C.

cDNA synthesis

The method of Gubler and Hoffman (1983) was used to synthesize cDNA using the poly(A)-tailed virion RNA as a template.

Synthesis of first strand

To a sterile, 1.5 ml Eppendorf tube, the following were added in the following order:

poly(A)RNA	5 µl (5 µg)
OligodT_{12-18}	2 µl
distilled water	3 µl

	10.0 µl

The contents were mixed gently, briefly centrifuged and heated for 5 min. at 65 C and allowed to cool to room temperature. To the primer-template mixture (10 µl), the following were added: 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 4 mM sodium pyrophosphate, 1.25 mM each of dATP, dGTP, dTTP and 0.5 mM dCTP, 10 µCi of ^{32}P-dCTP (3000 Ci/mmol, NEN Research products) and 2 units of RNasin (Promega Biotech). The contents were mixed, and centrifuged briefly. 2 µl of AMV reverse transcriptase (15 units/µl, Pharmacia) were added and the tube was incubated at 42 C for 3 hrs.

Synthesis of second strand cDNA

To the first strand reaction mix, the following were added to give a total reaction volume of 100 µl: Tris-HCl, pH 7.5 to 20 mM, $MgCl_2$ to 5 mM, ammonium sulfate to 10 mM, KCl to 100 mM,

bovine serum albumin (BRL) to 50 μg/ml, 40 μM dNTPs, 8.5 units/ml of RNase H (Amersham) and 230 units/ml of DNA polymerase I (Amersham).

The tube was incubated at 12 C for 1 hour, 22 C for 1 hour and then 70 C for 10 min. T4 DNA polymerase (2.0 units in 1 μl) was added to the tube and incubation was continued at 37 C for 10 min. The reaction mixture was extracted once with equal volumes of phenol and chloroform and once with chloroform alone. The aqueous phase was passed through a Sephadex G-50 column prepared in a sterile 1 ml syringe as desribed by Maniatis et al. (1982).

From the reaction mixture, 1 μl of aliquot was taken into 2 ml of Aquasol 2 (NEN) and counted in a scintillation counter (Beckman, Model LS7500). An aliquot containing 25,000 cpm was loaded onto a 1% agarose gel prepared in 1xTAE buffer (Maniatis et al. 1982) . The sample was subjected to electrophoresis at a constant voltage of 50V for 3 hours at room temperature. The gel was then dried using a gel drier (Hoefer Scientific Instruments) and exposed overnight to a Kodak X-Ray film at room temperature and developed according to the manufacturer's recommendations.

Methylation of cDNA

The double-stranded cDNA was methylated by adding 10 μl of 1 M Tris-HCl, pH 8.0, 4 μl of 0.25 M EDTA, 1.2 μl of S-adenosyl methionine (100 mM stock, Sigma) and 1 μl of *Eco*RI methylase (10 units, New England Biolabs). The tube was incubated at 37 C for 1 hr. and extracted once with phenol:chloroform and once with

chloroform. The aqueous phase was carefully removed and passed through another freshly prepared Sephadex G-50 column. The cDNA was completely dried in a Speedvac (Savant Instruments) and resuspended in 10 µl of TE, pH 8.0.

Kinasing the linkers

Non-phosphorylated *Eco*R1 linkers were purchased from Pharmacia and dissolved in 100 µl of 50 mM Tris-HCl, pH 7.5 and 1 mM EDTA. The following were added to a sterile Eppendorf tube: 2 µl of the EcoR1 linkers, 2 µl of 0.5 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$, 1 µl of 0.2 M DTT, 0.2 µl of 100 mM ATP (Pharmacia) and 0.2 µl of polynucleotide kinase (Pharmacia, 10 units/µl). The tube was incubated at 37 C for 1 hr. The kinased linkers were then added to the tube containing cDNA. After adding 1 unit of T4 DNA ligase (BRL), the tube was incubated overnight at 14 C.

Following the incubation, the tube was heated to 70 C for 10 min. and sterile NaCl solution was added to a final concentration of 100 mM followed by addtion of 1 µl of *Eco*R1 (25 units/µl, BRL). The contents of the tube were gently mixed and incubated at 37 C for 4 hours. At the end of the restriction digestion, the total volume of the reaction mixture was increased to 100 ul with TE, and extracted once with phenol:chloroform and once with chloroform. The aqueous phase was carefully transferred into another Eppendorf tube and reduced to about 100 µl using a Speedvac concentrator.

Size fractionation of cDNA by agarose gel electrophoresis

Following digestion of linker-ligated cDNA with *Eco*R1, total cDNA was subjected to electrophoresis at 50V for 2 hours along with Lambda DNA digested with *Hind* III as size standard, in 1% agarose gel using 1xTAE as the electrophoresis buffer. The gel was stained with ethidium bromide, and cDNA in the range of 1.0 kb and above was electro-eluted using a Whatman 3MM filter paper backed by a dialysis membrane as described by Maniatis et al. (1982). The eluted cDNA was extracted once with phenol:chloroform, once with chloroform and ethanol precipitated.

Ligations

To the Eppendorf tube containing the size-selected cDNA, 3 µl of Lambda gt10 (imm^{434}, b^{527}) previously digested with *Eco*R1, 0.5 µl of ligation buffer (Maniatis et al. 1982), 0.5 µl of 0.1 M DTT, and 1 µl of T4 DNA ligase (1 unit/µl, BRL) were added, and incubated at 14 C for a period of 14-16 hours. At the end of incubation, the enzyme was inactivated by heating the tube to 60 C for 10 min.

Packaging

Lambda packaging extracts (Stratagene) were removed from the -70 C freezer and were slowly thawed on ice. The tube containing the ligation reaction mixture was supplemented with 15 µl each of freeze-thaw extract and sonicated extract (Maniatis et al. 1982). The contents were mixed thoroughly using a micropipette. The tube was left at room temperature for 2 hrs. One ml of SM

buffer was added to the tube and mixed well. One ml of this mixture was transferred to a sterile 5 ml glass test tube with cap and 0.2 ml of a fresh overnight culture of *E. coli* C600*HflA* grown to saturation was added. The contents were mixed thoroughly and incubated at 37 C for 30 min. Five ml of soft agar, previously equilibrated to 42 C, were added to the tube containing the packaged Lambda, the contents vortexed briefly and poured onto LB-glucose plates pre-warmed to 37 C. The agar was allowed to solidify for about 30 min. and the plates were incubated at 37 C overnight.

Isolation of cDNA clones from the cDNA library by plaque hybridization

Following packaging the recombinant Lambda DNA and plating, the resulting cDNA-Lambda library was screened using specific cDNA probes to isolate clones containing inserts. For this purpose, two cDNA probes were prepared. Total virion RNA of SCNMV-59 was fractionated on a 2% agaraose gel as described in chapter III. Such separated RNA-1 and RNA-2 species were used to prepare ^{32}P-labeled cDNA probes by reverse-transcription. The probes were prepared the same day that hybridizations were done using ^{32}P-dCTP not older than one week.

Plaque lifts

Plates containing the cDNA library were cooled for 1 hour at 4 C. Bio-Trans membrane (previously known as Biodyne, ICN Biomedicals), cut to the size of the petri diah (100 x 15 mm, Fisher Scientific) was layered carefully on top of the medium. After

leaving the plates with the membrane at 4 C for 5 min., a 1 ml syringe (Becton and Dickinson) containing India Ink was used to make holes through the membrane and the underlying agar medium, making sure the markings were asymmetric. The membrane was then removed and transferred to a glass dish containing a Whatman 3MM filter paper wetted with denaturing solution(0.5 M NaOH and 1.5 M NaCl). After 5 min, the membrane was transferred to another glass dish with a Whatman 3MM filter paper wetted with neutralizing solution (3 M ammonium acetate, pH 5.5). Following neutralization of the membrane for 5 min. at room temperature, the membrane was air-dried by keeping it on a dry Whatman 3MM filter paper. It was then baked at 80 C for 1 hour. Another sheet of membrane was used to lift some of the recombinant phage off the plaques and was subjected to the above treatments.

Prewashing of the Bio-Trans membrane

Following the 'plaque-lifts', the membranes were washed in a pre-wash solution (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, and 0.1% SDS) using a glass dish placed on an orbital shaker (New Brunswick Instruments) as described by Maniatis et al. (1982).

Pre-hybridization

The pre-wash solution was discarded and the membrane was placed in a plastic bag containing 10 ml of pre-hybridization solution (50% de-ionized formamide, BRL; 5x Denhardt's (described in Chapter III), 5x SSPE, 0.1% SDS, and 100 μg/ml of

heat-denatured salmon sperm DNA). Pre-hybridization was carried out for 4 hrs at 42 C with gentle shaking.

Hybridization

The pre-hybridization solution was discarded from the bag containing the membrane and the same, but fresh solution containing the appropriate ^{32}P-labeled cDNA probe ($\sim 10^6$ cpm per ml of solution) was added and incubated at 42 C for 12-16 hours. Following hybridization, the membrane was washed to remove the excess probe following the washing conditions described in Chapter III. The membrane was then exposed to a Konica X-Ray film at -20 C overnight and developed according to the manufacturer's instructions. The developed film was aligned on the petri dish containing the cDNA library, and plaques were picked with a sterile pasteur pipette, using the signals on the film. Each plaque was separately stored in an Eppendorf tube containing 1 ml of SM buffer with two drops of chloroform. The tubes were kept at room temperature for 4 hours and then transferred to 4 C.

Secondary screening

To ensure the purity of plaques identified as positives after the 'primary' screening, they were separately plated and subjected to a second cycle of plaque hybridization using the same ^{32}P-labeled cDNA probes as described above. Two well-isolated plaques from each plate containing clones specific to RNA-1 or RNA-2 were selected, and stored in 1.5 ml Eppendorf tubes containing 1 ml of SM buffer and two drops of chloroform.

Mini-preparation of DNA from recombinant Lambda gt10

An overnight culture of plating cells (*E. coli* C600*HflA*) was grown to saturation in 50ml of LB containing 0.2% maltose. The cells were sedimented by centrifugation at 4,000 rpm for 10 min. at room temperature and were resuspended in 0.4 volumes of 0.01 M $MgSO_4$ (Maniatis et al. 1982). In sterile Eppendorf tubes containing 100 µl of the plating cells, 1 µl and 10 µl of the appropriate phage stock was added and incubated at 37 C for 30 min. and transferred to sterile capped test tubes containing 5 ml of LB broth with 0.2% maltose and 10 mM $MgSO_4$. The tubes were incubated at 37 C with vigorous shaking until the cells lysed (3-5 hrs). Once the host cells were completely lysed, 100 µl of chloroform were added to each tube and the tubes were kept on ice for 30 min. The cultures, transferred to sterile centrifuge tubes, were centrifuged in an SS-34 rotor at 8000 rpm for 10 min. at 4 C. The recovered supernatant was treated at 37 C for 1 hour with RNase A and DNase I, each at a concentration of 10 µg/ml. An equal volume of a solution of 20% polyethylene glycol 8000 and 2 M NaCl in SM buffer was added, mixed well, and left overnight at 4 C. The phage were collected by centrifugation at 10,000 rpm for 20 min. at 4 C. The phage were resuspended in 0.5 ml of SM bufer and transferred to a fresh Eppendorf tube. Following a centrifugation at 8000 rpm for 2 min., the supernatant was transferred to another Eppendorf tube and treated with 10 ul of Proteinase K (3 mg/ml stock) and 10 µl of 10% SDS at 37 C for 30-60 min. It was then extracted once with phenol, once with phenol:chloroform and once with chloroform alone. The DNA was

ethanol precipitated and the DNA pellet was washed twice with cold 70% ethanol, and dissolved in 400 µl of TE, pH 8.0. The DNA was then treated with 10 µl of RNase A (10 mg/ml) followed by phenol, chloroform extractions and ethanol precipitation as above. The final DNA pellet was resuspended in 20 µl of TE, pH 8.0.

Restriction endonuclease digestion(s) of phage/plasmid DNA

All restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Restriction endonuclease digestions were carried out as described by Maniatis et al. (1982). In a final volume of 50 µl made up with sterile, distilled water, 1-2 µg of appropriate DNA to be digested, 5 µl of 10 x buffer, 2 µl of 0.1 M spermidine (Sigma) were mixed in an Eppendorf tube and kept on ice for 10 min. followed by addition of 1-2 units of the enzyme. The digestion was done at 37 C or as required for 2 hrs followed by addition of another aliquot of the enzyme. After another 2 hrs of digestion, the enzyme was inactivated by heating to 70 C for 10 min. The digested DNA was then subjected to agarose gel electrophoresis for further analysis.

Radio-labeling of cDNA inserts

cDNA inserts in Lambda gt10 were used to further confirm the specificity of each cDNA clone with respect to the genomic RNAs of SCNMV. Following restriction endonuclease digestion by *Eco*RI, the insert DNA was electroeluted by using a combination of Whatman 3 MM filter paper and dialysis membrane (Maniatis et al.

1982). The cDNAs were later labeled with ^{32}P by 'oligo-labeling' method of Feinberg and Vogelstein (1983 and 1984).

Northern hybridization

Electrophoretic separation of SCNMV RNAs under denaturing conditions, their transfer to GeneScreen Plus and subsequent hybridization were done as described in Chapter III.

Sub-cloning of cDNA clones from Lambda gt10 into Bluescribe

The cDNA clone of Lambda gt10 containing the 1.1 kb insert specific to RNA-2 of SCNMV-59 was sub-cloned into the Bluescribe plasmid vector (Vector Cloning Systems). Bluescribe DNA (2-3 μg) was digested with *Eco*RI and an aliquot of the digestion mixture was checked for completeness of digestion by agarose gel electrophoresis. The 1.1 kb cDNA of RNA-2, previously separated electrophoretically and electro-eluted, was ligated to *Eco*RI-digested Bluescribe DNA, in a reaction volume typically containing 1.0 μl of 10 x ligation buffer (Maniatis et al. 1982), 1.0 μl of 0.1 M DTT and 0.3 units of T4 DNA ligase. The contents were incubated overnight at 14 C before transforming the competent host cells.

Transformation of *E. coli* JM83

(a) Preparation of competent cells

An overnight culture of JM83 was grown to saturation in 10 ml of LB broth with vigorous shaking at 37 C. The following morning, 200 μl of the overnight culture was inoculated into 10 ml of fresh LB and grown at 37 C with vigorous shaking for 2-3 hours.

The cells were kept on ice for 30 min. and sedimented at 5,000 rpm for 10 min. in a pre-cooled centrifuge tube. The cells were gently resuspended in half the volume (5 ml) of 50 mM ice-cold, sterile $CaCl_2$. The tube was kept on ice for 30 min. and the cells were sedimented (5000 rpm for 5 min.) at 4 C. The cells were resuspended in 1/10 vol (1 ml) of cold 50 mM $CaCl_2$ and left on ice until required.

(b) Transformations

2.5 µl of ligation mixture was added to 200 µl of competent cells in a sterile, capped 5 ml glass tube and kept on ice for 30 min. then transferred to a water-bath at 42 C for 90 sec. Following the heat-shock, 1 ml of fresh LB broth was added to the tube, which was incubated for 30-60 min. at 37 C. The cells were sedimented in a Beckman microfuge, resuspended in 200 µl of LB and spread on a McConkey agar plate containing 100 µg/ml of ampicillin. The plate was incubated overnight at 37 C.

Mini-preparations of DNA from recombinant Bluescribe

Plasmid DNA from the resulting 'colorless' colonies on McConkey agar was prepared by the method of Birnboim and Doly (1979). The presence of the cDNA insert in the DNA thus prepared was verified by performing a restriction endonuclease digestion with *Eco*RI and subsequent analysis of the digest by agarose gel electrophoresis.

Sub-cloning the 1.1 kb cDNA insert into M13mp18 and M13mp19

Replicative forms of M13mp18 and M13mp19 (Messing, 1983; Yanisch-Perron et al. 1985) digested with *Eco*RI were separately ligated to the 1.1 kb *Eco*RI fragment isolated from the Bluescribe recombinant. In a separate set of experiments, 0.6kb and 0.5kb *Eco*RI-*Pst*I fragments resulting from restriction endonuclease digestions, were separately ligated to suitably digested M13mp18 and M13mp19 RF DNA. Conditions for ligations were essentially the same as those for sub-cloning into Bluescribe.

(a) Preparation of competent cells of *E. coli* JM105

The protocol used to prepare competent cells of JM105 was kindly provided by Dr. F. E. Nargang, Department of Genetics, University of Alberta, Edmonton. An overnight culture of JM105 was grown in 10 ml of M9-thiamine medium at 37 C with vigorous shaking. One hundred microliters of the culture were inoculated into 10 ml of LB broth and grown at 37 C in an incubator shaker for 2-3 hours. The cells were sedimented by centrifugation at 8000 rpm for 10 min. in an SS34 rotor and the cells were gently resuspended in 5 ml of transformation mix #1 (100 mM NaCl, 5 mM $MgCl_2$ and 5 mM Tris-HCl pH 7.6). The resulting cell suspension was centrifuged as above and resuspended in transformation mix #2 (100 mM $CaCl_2$, 250 mM KCl, 5 mM $MgCl_2$ and 5 mM Tris-HCl, pH 7.6). The tube was kept on ice for at least 1 hr. The cells were sedimented by centrifugation as above, gently resuspended in 1 ml of transformation mix #2 and kept on ice until further use.

(b) Transformation and plating

Ligation mixtures containing the appropriate vector and insert were added to 200 µl of competent cells of JM105 in a sterile, capped 5 ml glass tube and were kept on ice for 30 min. followed by heat shock at 42 C for 90 sec. The following were then added in the order given below:

10 µl of IPTG (25 mg/ml in distilled water, BRL)

50 µl of Bluo-gal (halogenated indolyl-β-D-glactoside, BRL; 25 mg/ml in N'N' dimethyl formamide)

200 µl of JM105 host cells in log phase

3.0 ml of soft agar (liquified, then equilibrated at 42 C)

The contents were vortexed and poured onto an LB plate pre-warmed to 37 C. After 30 min. at room temperature, the plate was incubated at 37 C for 12-16 hours.

Minipreparation of single-stranded DNA (ssDNA) from M13 recombinant clones

ssDNA form M13mp18 and M13mp19 recombinant clones was prepared according to the protocol provided by Dr. [illegible] E. Nargang of Department of Genetics, University of Alberta, Edmonton. Single, well-isolated, colorless plaque resulting from transformation, was collected using sterile pasteur pipettes and inoculated into 20 ml of LB broth containing 100 µl of a fresh overnight culture of JM105 grown in M9-thiamine. Phage were grown for 5-6 hours at 37 C with vigorous shaking. Cells were sedimented by centrifugation at 18,000 rpm for 30 min. in an SS-34 rotor. The supernatant was transferred to a fresh centrifuge

tube and 8.0 ml of 10% polyethylene glycol 8000 and 2.5 M NaCl solution (sterilized by filteration) was added, mixed by inversion and kept at 4 C for 12-16 hours. Phage were sedimented by centrifuging at 10,000 rpm for 15 min. The cells were resuspended in 700 µl of phenol extraction buffer (0.3 M NaCl, 0.1 M Tris-HCl pH 7.8 and 1 mM EDTA) and transferred to a fresh Eppendorf tube. 10 µl of 10% SDS and 10 µl of Proteinase K (10 mg/ml) were added and the tube was incubated at 37 for 60 min. DNA was isolated by phenol extraction and ethanol precipitation. The DNA pellet was washed three times with 70% ethanol before finally redissolving it in sterile distilled water. Selection of positives (recombinants with inserts) was done by the slower migration of DNA samples in agarose gel electrophoresis when compared to that of ssDNA prepared from parent M13mp18 and M13mp19 without such inserts.

Nucleotide sequencing of the cDNA clones of SCNMV RNA-2

The dideoxy chain termination method of Sanger et al (1977; 1980) was used to sequence cDNA clones of RNA-2, sub-cloned into M13mp18 and M13mp19. Besides the universal primer, sequencing was also done by using synthetic oligodeoxyribonucleotide primers wherever necessary. Sequenase version 2.0 purchased from U.S. Biochemical Corporation was used in all the sequencing reactions.

Annealing reaction

In a 1.5 ml Eppendorf tube, the following were added in a total volume of 10 µl:

ssDNA template	2-3 µg
5 x annealing buffer*	2 µl
Primer**	1 µl (5 ng/µl)
distilled water	X µl

*5 x annealing buffer contained 200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl.

** Sequencing of ssDNA M13 templates was initially done using the universal primer. Sequences farther from the priming site were obtained by using synthetic oligodeoxyribonucleotide primers provided by Dr. K.L. Roy, Professor, Department of Microbiology, University of Alberta, Edmonton.

The contents of the tube were mixed, centrifuged briefly, heated to 65 C for 2 min. and allowed to cool to room temperature over a period of 30-60 min.

Sequencing reactions

The protocol for sequencing ssDNA templates using Sequenase was provided by its supplier, US Biochemical Corporation, Cleveland, OH.

Labeling reaction

For standard reactions, the labeling mix (5x contains 7.5 µM each of dGTP dCTP and dTTP) was diluted five-fold with distilled water. Sequenase version 2.0 was diluted 1:8 in ice-cold enzyme dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM DTT and 0.5 mg/ml BSA). To the annealed primer-template the following were added in the order given below:

primer-template	10.0 µl
0.1 M DTT	1.0 µl

Diluted labeling mix	2.0 µl
[α-^{35}S]dATP	0.5 µl
diluted Sequenase	2.0 µl

The contents were mixed, briefly centrifuged and incubated at 37 C for 3 min.

Termination reactions

Before setting up the labeling reaction, four Eppendorf tubes, labeled A, C, G and T containing 2.5 µl of each of ddATP, ddCTP, ddGTP and ddTTP termination mixes were pre-warmed to 37 C in a waterbath.

The ddA termination mix contained 80 µM each of dATP, dCTP, dGTP and dTTP and 8 µM ddATP and 50 mM NaCl

The ddC termination mix contained 80 µM each of dATP, dCTP, dGTP and dTTP and 8 µM ddCTP and 50 mM NaCl

The ddG termination mix contained 80 µM each of dATP, dCTP, dGTP and dTTP and 8 µM ddGTP and 50 mM NaCl

The ddT termination mix contained 80 µM each of dATP, dCTP, dGTP and dTTP and 8 µM ddTTP and 50 mM NaCl

At the end of the labeling reaction, 3.5 µl of the reaction mixture was transferred to each of the above four tubes containing the termination mix. All four tubes were gently mixed, briefly centrifuged and incubated at 37 C for 5 min. The reaction was terminated by adding 5 µl of a 'stop' solution (95% de-ionized formamide, 20 mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF). The contents of each tube were mixed thoroughly and stored at -20 C until required.

Gel electrophoresis of sequencing reactions

The sequencing reactions were analysed by electrophoresis on a 6% polyacrylamide gel (38:2 acrylamide:bis acrylamide) containing 8.3 M urea in 1 x TBE (89 mM Tris, 89 mM Boric acid and 2 mM disodium salt of EDTA). Gels were run using a vertical gel apparatus (Tyler Research Instruments, Edmonton). Gels were pre-run at 35 W constant power for 30-45 min. Samples were heated to 90 C for 5 min. just before loading on the gel. Electrophoresis was done at 35 W until the Bromophenol Blue reached the end of the gel. In order to read more than 200 bases from the primer, gels were run for longer time (up to 8 hrs). Following electrophoresis, the gel was carefully lifted off on a Whatman 3 MM filter paper cut to size, and the gel was dried for 3 hours at 80 C using a slab gel drier (Bio-Rad). The dried gel was exposed to a Konica X-Ray film in a cassette containing intensifying screen (Dupont) for 24-36 hours, and developed according to the manufacturer's recommendations. The sequence was read manually and entered into a computer through a keyboard.

Analysis of nucloetide sequence of 1.1 kb cDNA of SCNMV RNA-2

The nucleotide sequence was manually entered into a computer (Apple Macintosh Plus or IBM PC) and analyzed by using DNA Strider (Marck, 1988) or SEQAID II (D.D. Rhoads and D.J. Roufa, 1986, Division of Biology, Kansas State University, Manhattan, KS.). A nucleotide sequence homology search was done with sequences deposited in GenBank and EMBL databases by accessing them through BIONET (Intelligenetics Inc, CA) using a

VT100 terminal equipped with a 9600 baud modem provided by the University of Alberta Computing systems.

RESULTS AND DISCUSSION

A general outline of the cDNA cloning strategy was shown in Fig. VI.1. Following poly (A)-tailing of the total virion RNA of SCNMV, the first strand was synthesized by reverse transcriptase using oligo-dT as a primer. The second strand was synthesized from the cDNA-RNA hybrid in a 'nick-translation-repair' reaction by using RNaseH and DNA polymerase I (holoenzyme). While RNaseH creates nicks at intervals along the RNA molecule in the hybrid, DNA polymerase I, by virtue of its 5'-3' exonuclease and polymerase activities, removes and replaces the RNA strand with DNA. This procedure forms the basis for the synthesis of double-stranded cDNA (Gubler and Hoffman, 1983; Gubler 1987).

The resulting double-stranded cDNA, with subsequent methylation, linker-ligation and *Eco*RI digestion, was cloned into *Eco*RI-digested Lambda gt10. Lamda gt10 contains a single *Eco*RI site within the phage repressor gene (Fig. VI.3). The insertion of a foreign DNA fragment into the repressor gene (cI) inactivates the gene, generating a cI^- phage which forms a 'clear' plaque (Murray et al. 1977) whereas the non-recombinant (parent) cI^+ phage forms a turbid plaque thus facilitating visual screening for recombinants. To further reduce the 'background' parent phage, Lambda gt10 was plated onto an *E. coli* strain carrying a high frequency lysogeny mutation (*hflA*150). When Lambda gt10 infects such a strain, the

cI^+ phage is repressed very efficiently resulting in no plaque formation at all, whereas the recombinant, cI^- phage forms clear plaques (Huynh et al. 1985).

Following screening of the cDNA library by virion RNA-specific cDNA probes (RNA-1 or RNA-2), several 'postives' were selected and DNA prepared from such plaques was analyzed for the presence of cDNA inserts by restriction endonuclease digestion with *Eco*RI. Fig. VI.2A shows two such clones one with a cDNA insert specific to RNA-1 and the other specific to RNA-2. The specificity of each clone was further confirmed by Northern hybridization analysis. Each cDNA insert was separately labeled by the 'oligo-labeling' method and hybridized to blots containing SCNMV-59 genomic RNAs that were previously electrophoresed under denaturing conditions. Fig.VI.2B demonstrates that each cDNA insert shown in Fig.VI.2A was specific to its respective RNA. The Lambda gt10 clone containing the 1.1 kb cDNA insert of RNA-2 was the subject of further studies (Fig. VI. 3).

Mapping of restriction endonuclease sites in cloned cDNA of RNA-2

In order to perform restriction endonuclease mapping, the cDNA insert in Lambda gt10 was sub-cloned into the unique *Eco*RI site of Bluescribe plasmid vector. Using the restriction endonuclease sites present in the cloning cassette in Bluescribe, one *Pst*I site was found in the cDNA (Fig.VI.4).

Subcloning into M13mp18 and M13mp19

The cDNA insert of RNA-2 in Bluescribe was further sub-cloned into the unique *Eco*RI site of M13mp18 (Fig. VI.5). Recombinant M13 were selected and the orientation of each clone was confirmed by sequencing either A or T tracks by dideoxy chain termination method, using the universal primer. Following restriction endonuclease digestion of the 1.1 kb cDNA with *Eco*RI and *Pst*I (double digestion), the resulting 0.5 kb and 0.6 kb fragments were separately sub-cloned into similarly digested M13mp18 and M13mp19 replicative form DNA. Single-stranded DNA prepared from the resulting recombinant clones was used to completely sequence the cDNA in both directions.

Nucleotide sequence of RNA-2 cDNA clone

The sequencing strategy of the cDNA clones is schematically shown in Fig.VI.6. In order to obtain overlapping sequence in both orientations of the cDNA, synthetic oligodeoxyribonucleotides were used where necessary. The complete nucleotide sequence of the 1.1 kb cDNA clone is shown Fig.VI.7. It is interesting to note the loss of poly(A) tail in the nucleotide sequence obtained, even though the cDNA was synthesized using polyadenylated virion RNAs. Judging by the published sequence of RCNMV RNA-2 (Lommel et al. 1988), it is assumed that the stop codon of the major open reading frame (ORF) of the putative 35 kDa polypeptide lies towards the 3' end of the RNA, which, however, is not represented in the cDNA clone in the present study.

The possible reason for the loss of the poly(A) tail could be due to the presence of an *Eco*RI site in 3' region of the cDNA that might not have been completely methylated during the methylation reaction. The subsequent digestion of the cDNA with *Eco*RI, ligation, and packaging into Lambda might have resulted in the loss of that region.

Computer translation of the nucleotide sequence in all three reading frames showed the presence of one major ORF starting at base 119 which remained open to the end of the cDNA clone (Figs. VI.7 and VI.8). Computer-aided analysis of codon usage revealed the presence of ten ATG (met) codons in the major ORF (Table. VI.1).

Interviral homologies

A computer-based search of nucleotide sequence data bases (GenBank and EMBL) did not identify any other plant viral sequences that have significant homology with that of SCNMV RNA-2. No significant homology could be seen when it was compared with that of RCNMV RNA-2 (Fig.VI.10). The complete nucleotide sequence of RCNMV RNA-2 revealed the presence of a major ORF coding for a 35 KDa protein. This protein is thought to be involved in potentiating the cell-to-cell movement of the virus, since RNA-1 is capable of replicating independently of RNA-2 in isolated protoplasts (Osman and Buck, 1987; Pappu and Hiruki, 1988; Paje-Manalo and Lommel, 1989). Among other multipartite genome viruses, similarly sized, viral-encoded proteins are considered to facilitate the cell-to-cell movement (Dougherty and Hiebert, 1985).

The RNA-2 sequence of SCNMV was compared with such viral RNAs carrying the putative cell-to-cell movement genes. No continuous stretches of homology were seen except for a few blocks of matched bases interspersed with mismatches, when compared either with that of RNA3a protein of BMV (Fig.VI.11) (Ahlquist et al. 1981), or with RNA3 of AlMV which codes for a 32 kDa P3 polypeptide (Fig.VI.12), or with that of 30 kDa gene of TMV (Fig.VI.13). This observation is supported by the fact that there is a general lack of significant homology among genes coding for the putative transport proteins by viruses with similar genome organization (Meshi et al. 1987).

It is interesting to observe the lack of significant nucleotide homology between RNA-2 of RCNMV and the sequenced region of RNA-2 of SCNMV. The RCNMV strain was originally isolated in Australia (Gould et al. 1981), whereas the known distribution of SCNMV is confined to Alberta only (Hiruki, 1987). This geographic delineation might have contributed to the divergence in the nucleotide sequences of these two viruses. Moreover, the two viruses were serologically distinguishable by gel immunodiffusion tests and ELISA (C. Hiruki, unpublished results). These observations suggest the possible evolution of the two viruses along independent pathways.

Isolation of cDNA clones that contain either the complete copy of the RNA-2, or the portion of the remaining part of the 3' region, would allow one to conduct a more detailed homology studies among the putative transport proteins of various plant viruses.

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Table VI.1 Codon-usage in the major open reading frame of RNA-2 cDNA

TTT	phe	F	13	TCT	ser	S	4	TAT	tyr	Y	4	TGT	cys	C	2
TTC	phe	F	6	TCC	ser	S	-	TAC	tyr	Y	2	TGC	cys	C	4
TTA	leu	L	5	TCA	ser	S	8	TAA	OCH	Z	-	TGA	OPA	Z	-
TTG	leu	L	4	TCG	ser	S	1	TAG	AMB	Z	-	TGG	trp	W	4
CTT	leu	L	9	CCT	pro	P	7	CAT	his	H	3	CGT	arg	R	5
CTC	leu	L	3	CCC	pro	P	1	CAC	his	H	4	CGC	arg	R	1
CTA	leu	L	2	CCA	pro	P	3	CAA	gln	Q	4	CGA	arg	R	4
CTG	leu	L	8	CCG	pro	P	4	CAG	gln	Q	5	CGG	arg	R	2
ATT	ile	I	17	ACT	thr	T	1	AAT	asn	N	15	AGT	ser	S	1
ATC	ile	I	8	ACC	thr	T	2	AAC	asn	N	8	AGC	ser	S	1
ATA	ile	I	2	ACA	thr	T	6	AAA	lys	K	17	AGA	arg	R	7
ATG	met	M	10	ACG	thr	T	4	AAG	lys	K	5	AGG	arg	R	3
GTT	val	V	4	GCT	ala	A	9	GAT	asp	D	13	GGT	gly	G	7
GTC	val	V	2	GCC	ala	A	3	GAC	asp	D	1	GGC	gly	G	2
GTA	val	V	4	GCA	ala	A	12	GAA	glu	E	17	GGA	gly	G	2
GTG	val	V	3	GCG	ala	A	-	GAG	glu	E	5	GGG	gly	G	4

Fig VI.1 Schematic illustration showing the cDNA cloning strategy adopted in this study.

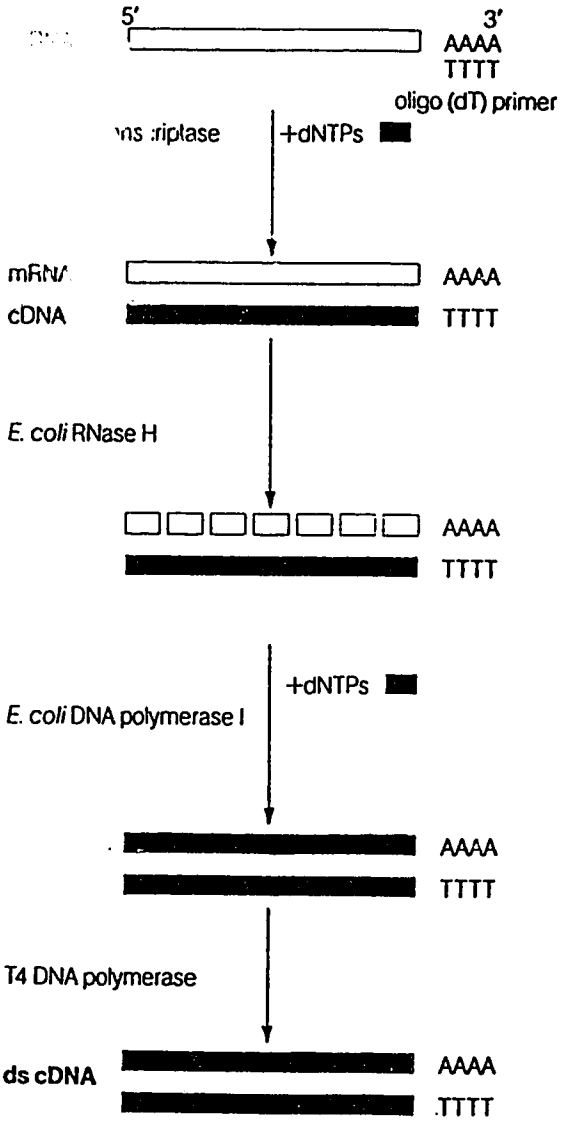

5'
3'
AAAA
TTTT
oligo (dT) primer
+dNTPs
mRNA
cDNA
AAAA
TTTT
E. coli RNase H
AAAA
TTTT
+dNTPs
E. coli DNA polymerase I
AAAA
TTTT
T4 DNA polymerase
ds cDNA
AAAA
TTTT

Fig.VI.2 Characterization of cDNA inserts specific to RNA-1 or RNA-2 of SCNMV obtained from cloned Lambda gt10.

(a) Agarose gel electrophoretic analysis of insert cDNAs following digestion with *Eco*RI. Lanes 1 and 6, Lambda DNA digested with *Hin*dIII; Lane 2, Lambda gt10 clone specific to RNA-1 of SCNMV-59, digested with *Eco*RI; Lanes 3 and 4, uncut Lambda gt10; Lane 5, Lambda gt10 clone specific to RNA-2 of SCNMV-59 digested with *Eco*RI.

(b) Northern blot showing the specificity of the above clones hybridized to SCNMV genomic RNAs. Lane 1, genomic RNAs of SCNMV-59 hybridized to labeled cDNA clone specific to RNA-1 (lane 2 in Fig.VI.2a); Lane 2, genomic RNAs of SCNMV-59 hybridized to labeled cDNA clone specific to RNA-2 (lane 5 in Fig.VI.2a).

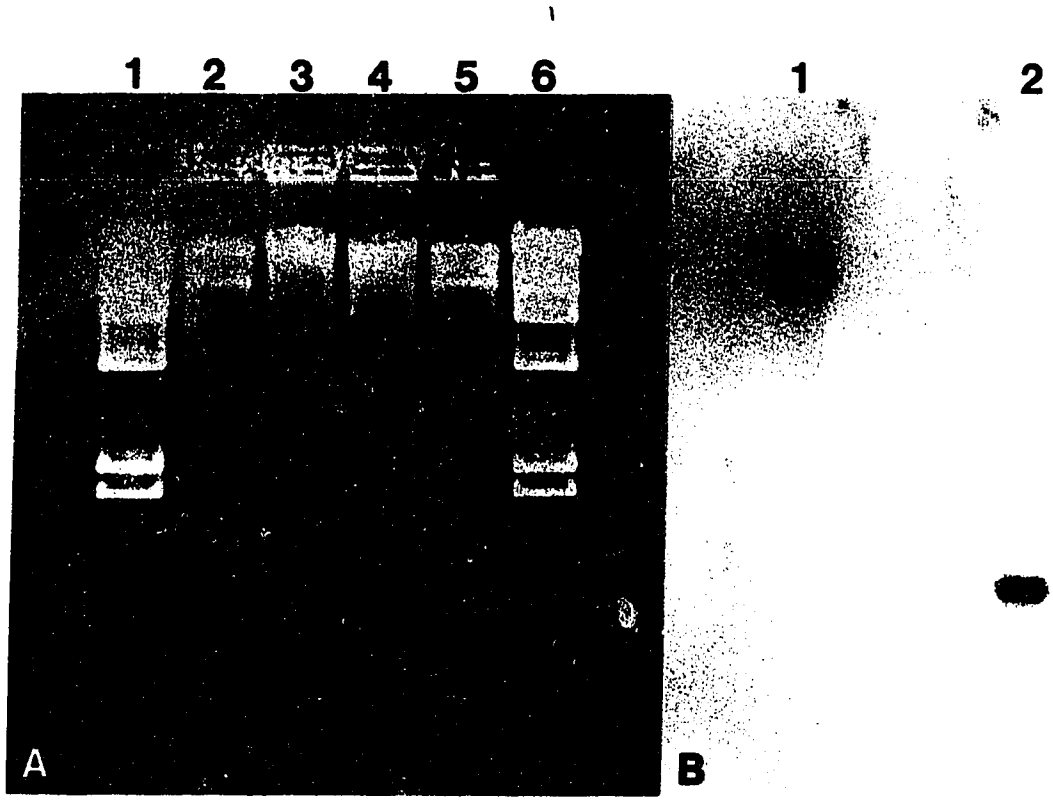
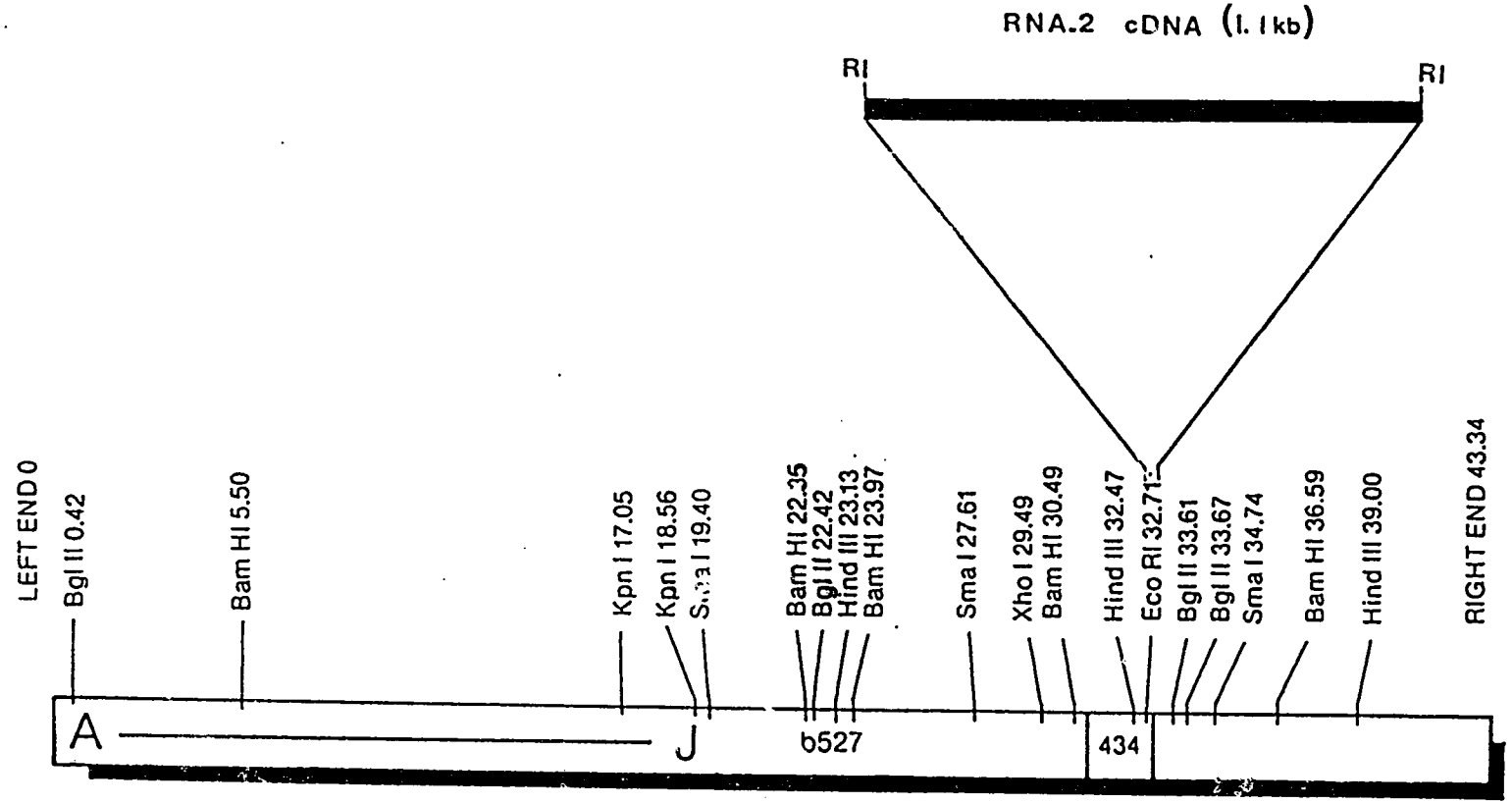

1 2 3 4 5 6
1 2
A
B

Fig.VI.3 Schematic diagram showing the Lambda gt10 clone containing the cDNA insert of RNA-2 (not to scale)


RNA.2 cDNA (1.1kb)
RI
RI
LEFT END 0
Bgl II 0.42
Bam HI 5.50
Kpn I 17.05
Kpn I 18.56
Sma I 19.40
Bam HI 22.35
Bgl II 22.42
Hind III 23.13
Bam HI 23.97
Sma I 27.61
Xho I 29.49
Bam HI 30.49
Hind III 32.47
Eco RI 32.71
Bgl II 33.61
Bgl II 33.67
Sma I 34.74
Bam HI 36.59
Hind III 39.00
RIGHT END 43.34
A
J
b527
434

Lambda gt10

Fig.VI.4 Schematic diagram showing the Bluescribe (Stratagene Cloning Systems, CA) clone containing the 1.1 kb cDNA insert of RNA-2.

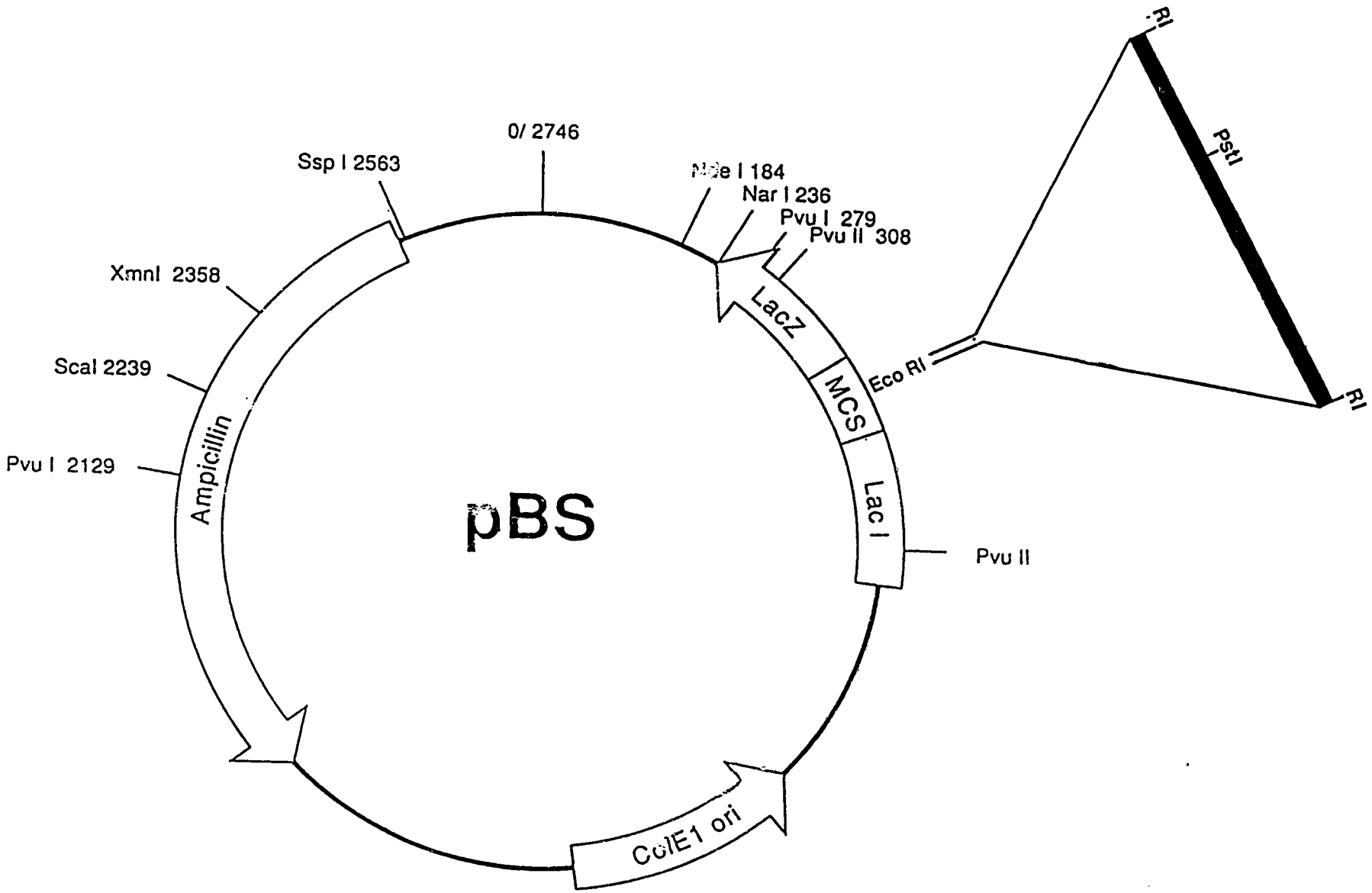

pBS
0/ 2746
Ssp I 2563
Nde I 184
Nar I 236
Pvu I 279
Pvu II 308
Xmn I 2358
Scal 2239
Pvu I 2129
Ampicillin
LacZ
MCS
Lac I
Eco RI
Pvu II
Col E1 ori
PstI
RI
RI

Fig.VI.5 Schematic diagram showing M13mp18 and M13mp19 (BRL, Gaithersburg, MD) clones containing the 1.1 kb cDNA insert of RNA-2 (not to scale).

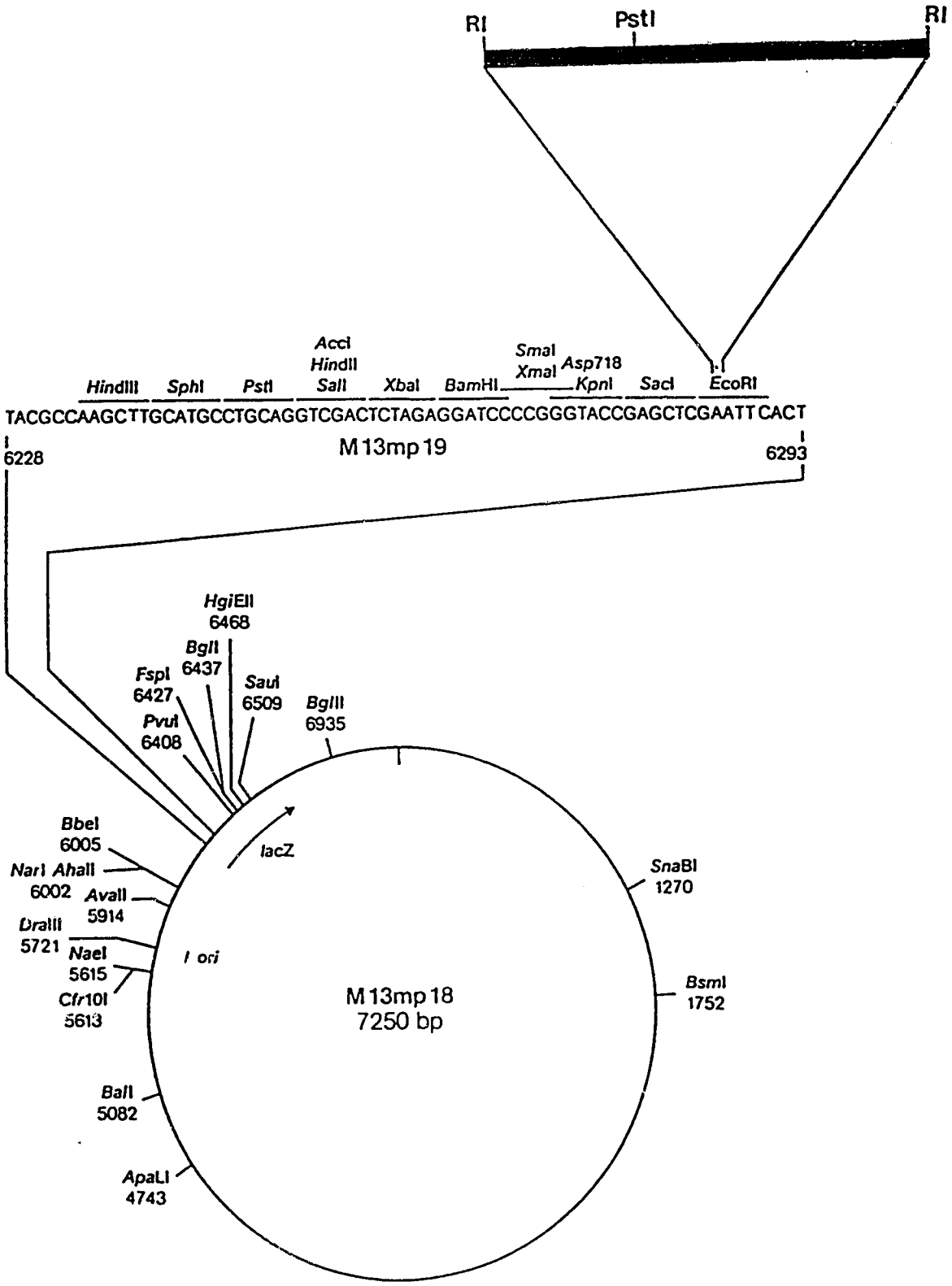

RI
PstI
RI
AccI
HindII
SmaI
XmaI
Asp718
HindIII
SphI
PstI
SalI
XbaI
BamHI
KpnI
SacI
EcoRI
TACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACT
6228
M13mp19
6293
HgiEII
6468
BglI
6437
FspI
6427
SauI
6509
BglII
6935
PvuI
6408
BbeI
6005
NarI AhaII
6002
AvaII
5914
DraIII
5721
NaeI
5615
Cfr10I
5613
lacZ
f ori
SnaBI
1270
BsmI
1752
M13mp18
7250 bp
BalI
5082
ApaLI
4743

Fig.VI.6 Sequencing strategy used to sequence the 1.1 kb cDNA of RNA-2.

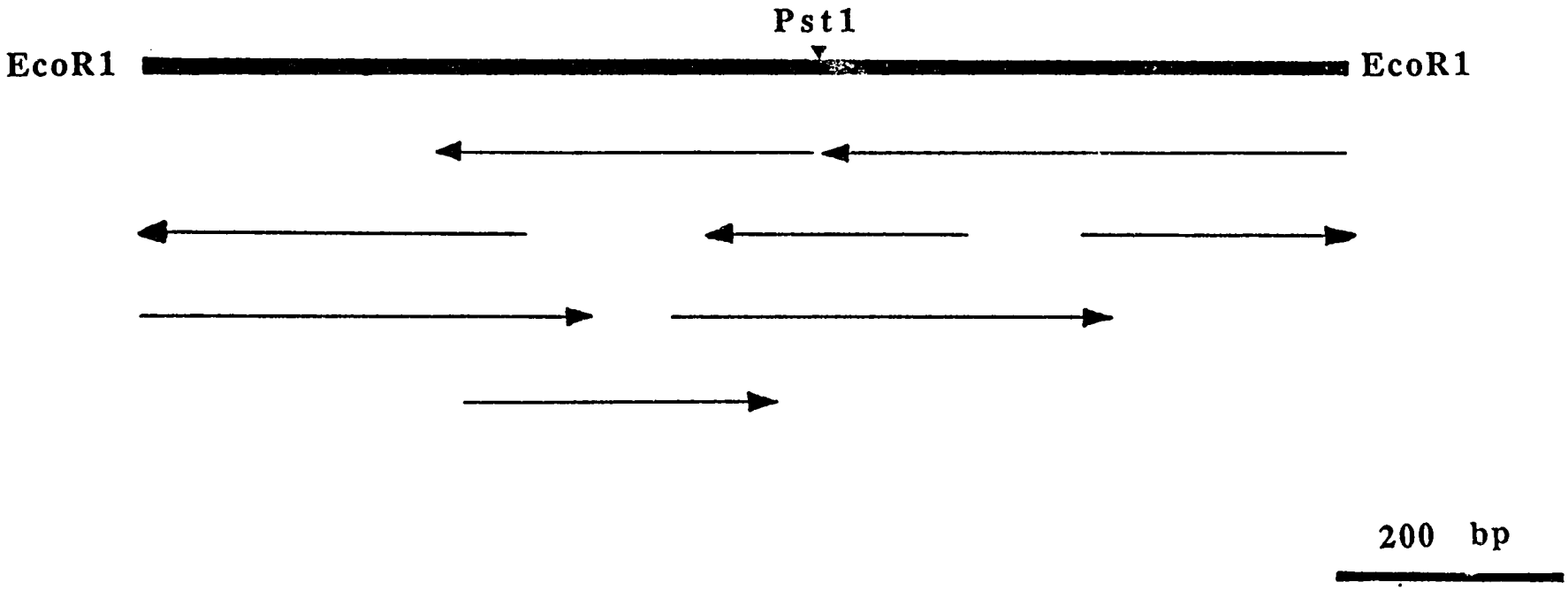

Pst1
EcoR1
EcoR1
200 bp

Fig.VI.7 Partial nucleotide sequence of RNA-2 shown as DNA. The nucleotides are numbered with the first base of the cDNA. The first initiation codon is underlined.

ATACTCAATT GCTGTCATCG GAGTGTAAAC ATCCTGCTAT TTCCGCCATT AATAGCGGAA 60

TAGCCATATT TGCTAAACAA TTTCTTAATT TAATTAAATA AGAGGCATTG CATGTTCAAT 120

GGTCGTCCTT TCCCTGTAGA TGCATTTCCT AAAATTATCA GGAATGCAAT TTATGAAGTG 180

GAACAGCATA CGCAGGCCCC TCAAGGTTTG ATTGCTGCTT CTGCTCTTGG GGTAATTTCT 240

CTTGCCTGTC AGAACCGGAT TGATGTTTGC CGATTGAATA ATCTACGTGG CCCGGTATCA 300

CTTTTCTTAA TGACTCTGGC TGAATCAGGT GAACGTAAGA GTACGGTTGA TAAACTGCTG 360

ATGAAGCCAT TATATCAACT GGAAGAGGAT TTATTTGAAA AATACACCCA CGATCTTACC 420

GCATGGAGAA ATGATGAAGC AATTTTTAAT ATTGAAAAAA AAGCACTGAT GTCAAAACTT 480

AAATCAGATA TTCGACGTAA CAAAGATCAC TTGGCAACAA ATGAAAGACT TAAAGAACTA 540

CTTACGACAA ACCCGAAAGC TCCAGTGAGA TTCAAATTTT TATTTAACGA TGCCACACCT 600

GCAGCTATTA AAGCTCATCT CTGTGGGCAC TGGCGATCAG TCGGCATCAT GTCTGATGAA 660

GCTGGGATCA TTTTTAATGG TTACACACTT AACGAGCTGC CGTTTATCAA TAAGATGTGG 720

GATGGTTCAA TATTTACGGT GGAAAGGAAA AACGAGCCCG AGAAATTAAT TAGAGATGCA 780

AGAATAACAC TGTCGCTGAT GGTCCAGCCT AATGTTTTTA AGGGTTATAT CGACAGGAAA 840

GGAGATATGG CAAAGGGGAT TGGATTTTTT GCACGGTGCC TCATGTGCCA GCCTGCTTCA 900

ACACAAGGTA ACAGAAAAAT TTTCAACCCA ATTTTTTCAA ATGAATATTT GCCGGTATTT 960

CACCAACGTC TTATGGAAAT TGTTAATGAG AGCATCATTA AAATTAATGA AAATAATCGC 1020

ATCTGCCTCC GATTCTCTGC AGAAGCAGAA AGACATTGGA TC 1062

Fig.VI.8 Positions and lengths of ORFs in all three possible reading frames in the cDNA of RNA-2.

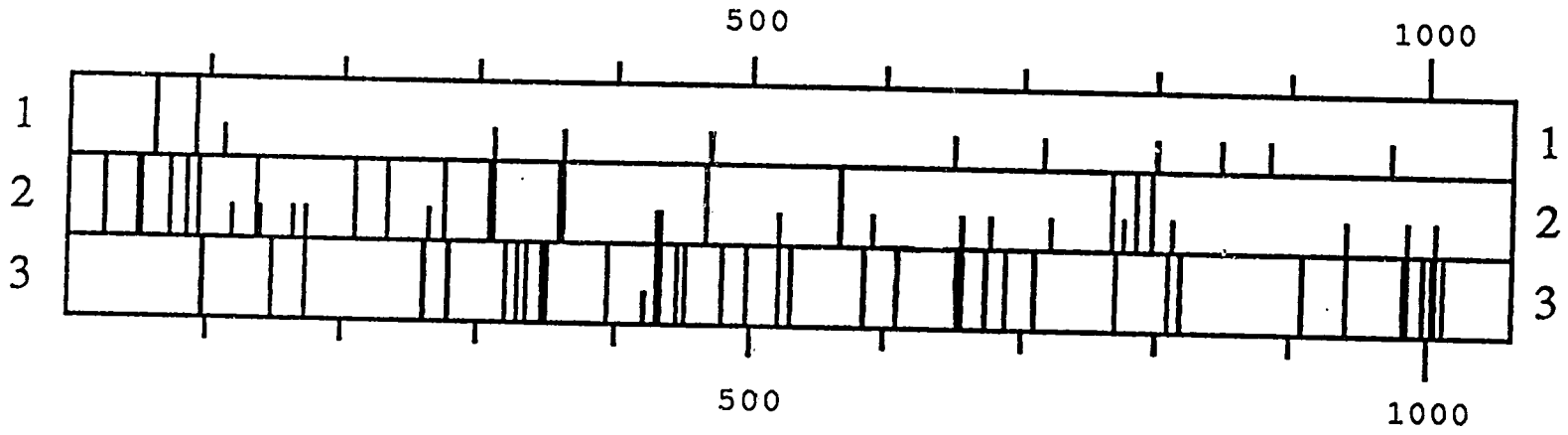

500
1000
1
2
3
1
2
3
500
1000

Fig.VI.9 Amino acid sequence of the putative polypeptide encoded by RNA-2 assuming that the first met codon at nucleotide position 119-121 is the first amino acid of the ORF.

```
1    /    1                                                  31   /   11
ATG TTC AAT GGT CGT CCT TTC CCT GTA GAT GCA TTT CCT AAA ATT ATC AGG AAT GCA ATT
met phe asn gly arg pro phe pro val asp ala phe pro lys ile ile arg asn ala ile
61   /   21                                                 91   /   31
TAT GAA GTG GAA CAG CAT ACG CAG GCC CCT CAA GGT TTG ATT GCT GCT TCT GCT CTT GGG
tyr glu val glu gln his thr gln ala pro gln gly leu ile ala ala ser ala leu gly
121  /   41                                                 151  /   51
GTA ATT TCT CTT GCC TGT CAG AAC CGG ATT GAT GTT TGC CGA TTG AAT AAT CTA CGT GGC
val ile ser leu ala cys gln asn arg ile asp val cys arg leu asn asn leu arg gly
181  /   61                                                 211  /   71
CCG GTA TCA CTT TTC TTA ATG ACT CTG GCT GAA TCA GGT GAA CGT AAG AGT ACG GTT GAT
pro val ser leu phe leu met thr leu ala glu ser gly glu arg lys ser thr val asp
241  /   81                                                 271  /   91
AAA CTG CTG ATG AAG CCA TTA TAT CAA CTG GAA GAG GAT TTA TTT GAA AAA TAC ACC CAC
lys leu leu met lys pro leu tyr gln leu glu glu asp leu phe glu lys tyr thr his
301  /  101                                                331  /  111
GAT CTT ACC GCA TGG AGA AAT GAT GAA GCA ATT TTT AAT ATT GAA AAA AAA GCA CTG ATG
asp leu thr ala trp arg asn asp glu ala ile phe asn ile glu lys lys ala leu met
361  /  121                                                391  /  131
TCA AAA CTT AAA TCA GAT ATT CGA CGT AAC AAA GAT CAC TTG GCA ACA AAT GAA AGA CTT
ser lys leu lys ser asp ile arg arg asn lys asp his leu ala thr asn glu arg leu
421  /  141                                                451  /  151
AAA GAA CTA CTT ACG ACA AAC CCG AAA GCT CCA GTG AGA TTC AAA TTT TTA TTT AAC GAT
lys glu leu leu thr thr asn pro lys ala pro val arg phe lys phe leu phe asn asp
481  /  161                                                511  /  171
GCC ACA CCT GCA GCT ATT AAA GCT CAT CTC TGT GGG CAC TGG CGA TCA GTC GGC ATC ATG
ala thr pro ala ala ile lys ala his leu cys gly his trp arg ser val gly ile met
541  /  181                                                571  /  191
TCT GAT GAA GCT GGG ATC ATT TTT AAT GGT TAC ACA CTT AAC GAG CTG CCG TTT ATC AAT
ser asp glu ala gly ile ile phe asn gly tyr thr leu asn glu leu pro phe ile asn
601  /  201                                                631  /  211
AAG ATG TGG GAT GGT TCA ATA TTT ACG GTG GAA AGG AAA AAC GAG CCC GAG AAA TTA ATT
lys met trp asp gly ser ile phe thr val glu arg lys asn glu pro glu lys leu ile
661  /  221                                                691  /  231
AGA GAT GCA AGA ATA ACA CTG TCG CTG ATG GTC CAG CCT AAT GTT TTT AAG GGT TAT ATC
arg asp ala arg ile thr leu ser leu met val gln pro asn val phe lys gly tyr ile
721  /  241                                                751  /  251
GAC AGG AAA GGA GAT ATG GCA AAG GGG ATT GGA TTT TTT GCA CGG TGC CTC ATG TGC CAG
asp arg lys gly asp met ala lys gly ile gly phe phe ala arg cys leu met cys gln
781  /  261                                                811  /  271
CCT GCT TCA ACA CAA GGT AAC AGA AAA ATT TTC AAC CCA ATT TTT TCA AAT GAA TAT TTG
pro ala ser thr gln gly asn arg lys ile phe asn pro ile phe ser asn glu tyr leu
841  /  281                                                871  /  291
CCG GTA TTT CAC CAA CGT CTT ATG GAA ATT GTT AAT GAG AGC ATC ATT AAA ATT AAT GAA
pro val phe his gln arg leu met glu ile val asn glu ser ile ile lys ile asn glu
901  /  301                                                931  /  311
AAT AAT CGC ATC TGC CTC CGA TTC TCT GCA GAA GCA GAA AGA CAT TGG ATC
asn asn arg ile cys leu arg phe ser ala glu ala glu arg his trp ile
```

Fig.VI.10 Computer-based homology search showing possible homologous regions between RNA-2 of SCNMV (upper strand) and that of RCNMV (lower strand).

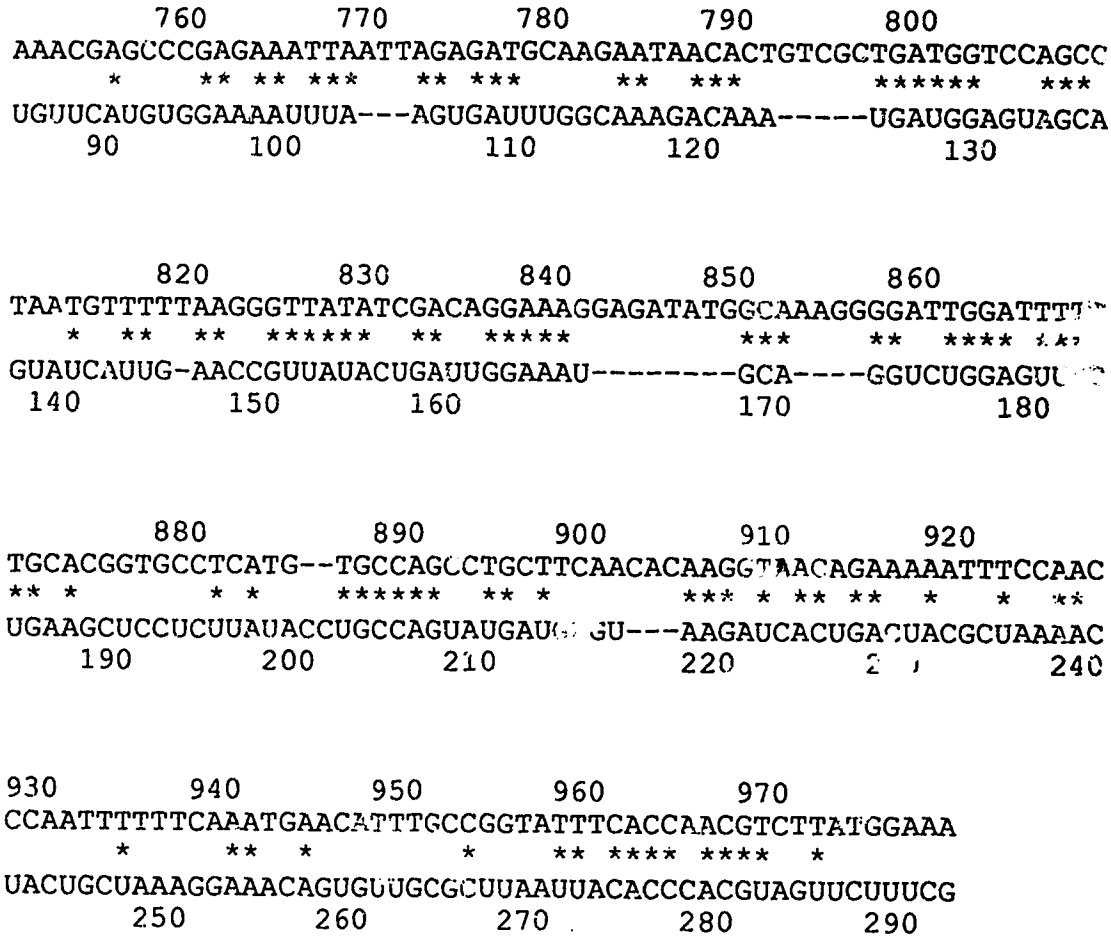

760 770 780 790 800
AAACGAGCCCGAGAAATTAATTAGAGATGCAAGAATAACACTGTCGCTGATGGTCCAGCC
UGUUCAUGUGGAAAAUUUA---AGUGAUUUGGCAAAGACAAA-----UGAUGGAGUAGCA
90 100 110 120 130
820 830 840 850 860
TAATGTTTTTAAGGGTTATATCGACAGGAAAGGAGATATGGCAAAGGGGATTGGATTTT
GUAUCAUUG-AACCGUUAUACUGAUUGGAAAU--------GCA----GGUCUGGAGUU
140 150 160 170 180
880 890 900 910 920
TGCACGGTGCCTCATG--TGCCAGCCTGCTTCAACACAAGGTAACAGAAAAATTTCCAAC
UGAAGCUCCUCUUAUACCUGCCAGUAUGAU GU---AAGAUCACUGACUACGCUAAAAC
190 200 210 220 240
930 940 950 960 970
CCAATTTTTTCAAATGAACATTTGCCGGTATTTCACCAACGTCTTATGGAAA
UACUGCUAAAGGAAACAGUGUUGCGCUUAAUUACACCCACGUAGUUCUUUCG
250 260 270 280 290

Fig.VI.11 Computer-based homology search showing possible homologous regions between RNA-2 of SCNMV (upper strand) and that of brome mosaic virus RNA-3 (lower strand).

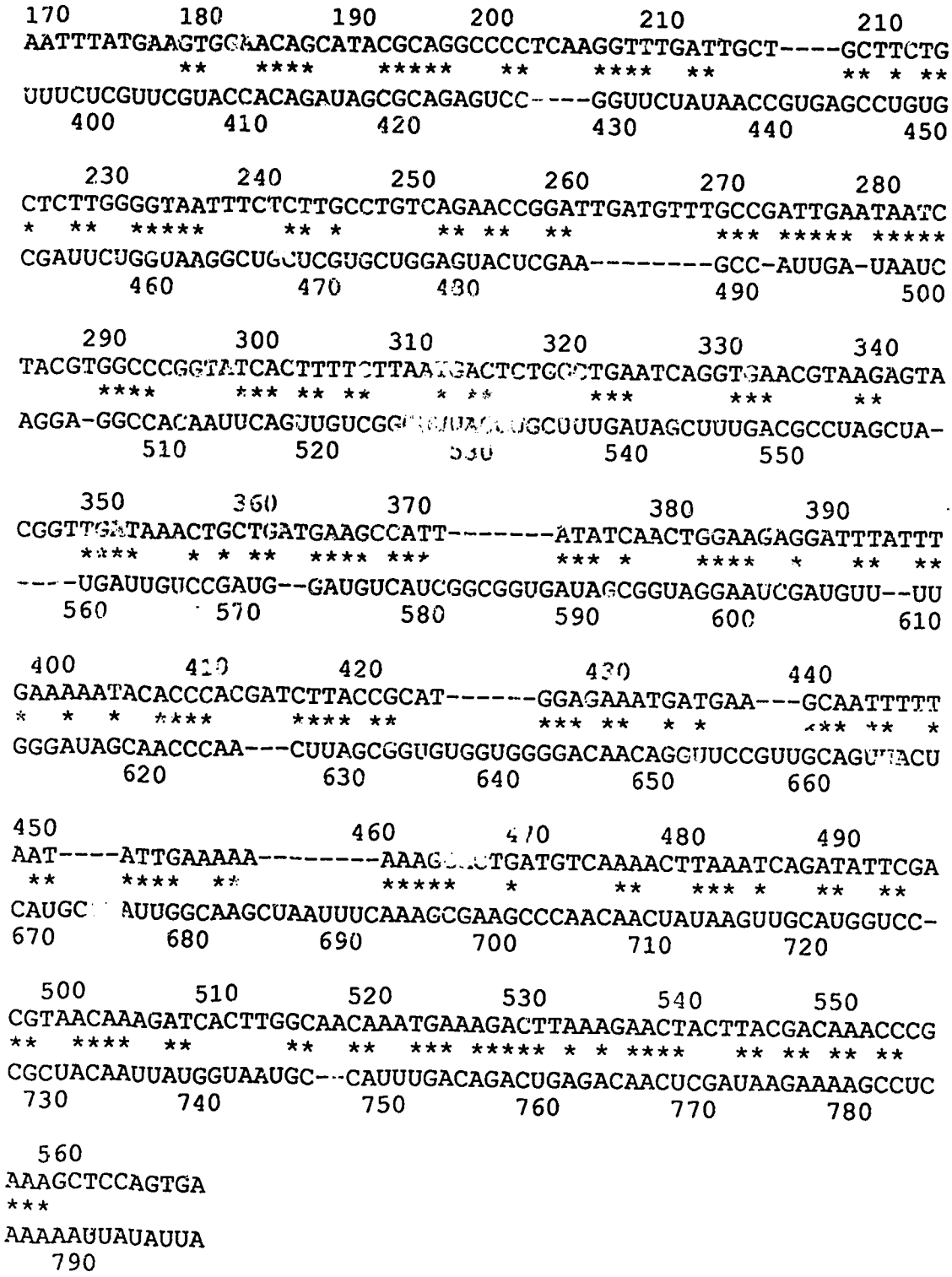


Fig.VI.12 Computer-based homology search showing possible homologous regions between RNA-2 of SCNMV (upper strand) and that of alfalfa mosaic virus RNA-3 (lower strand).

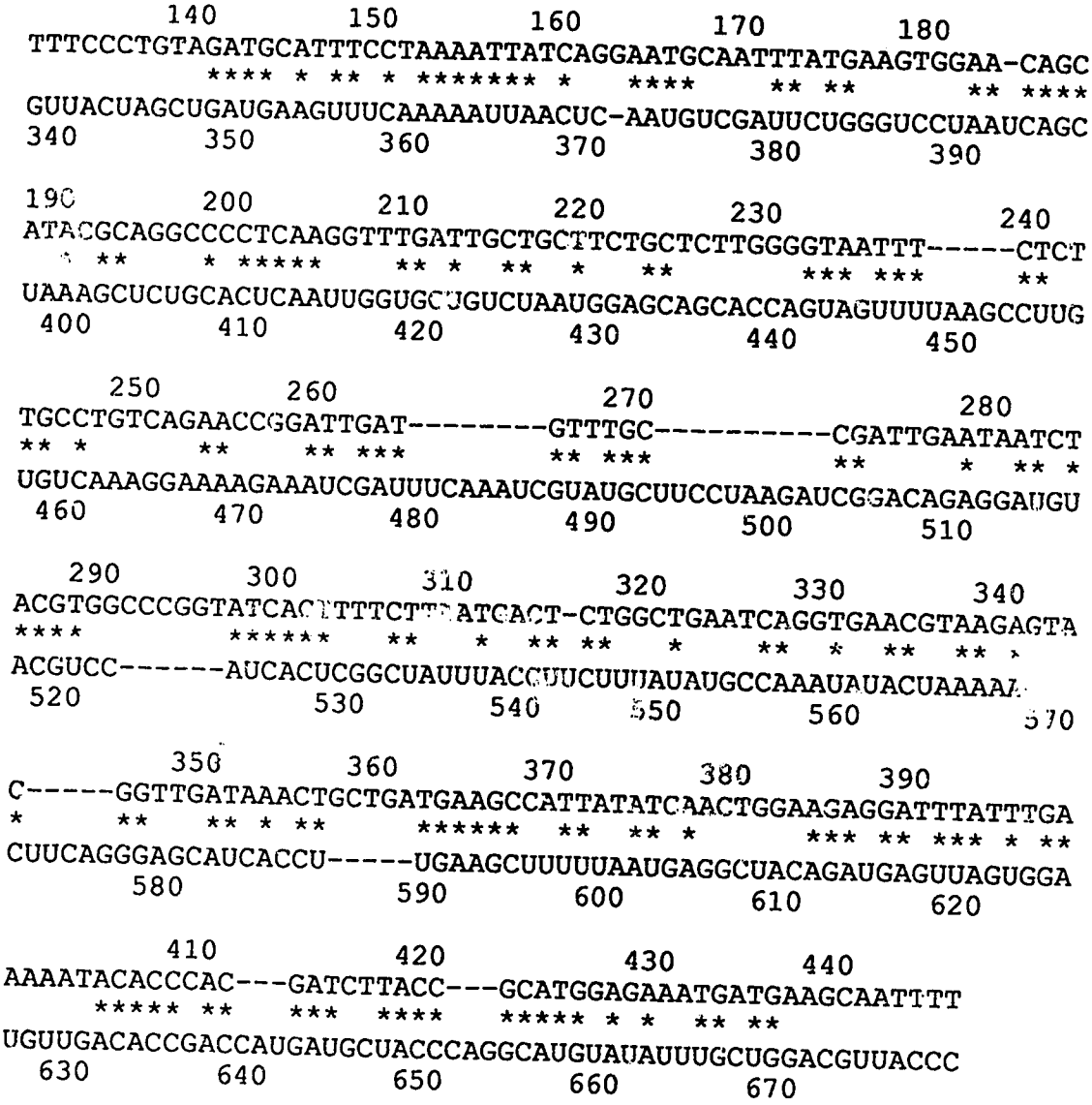
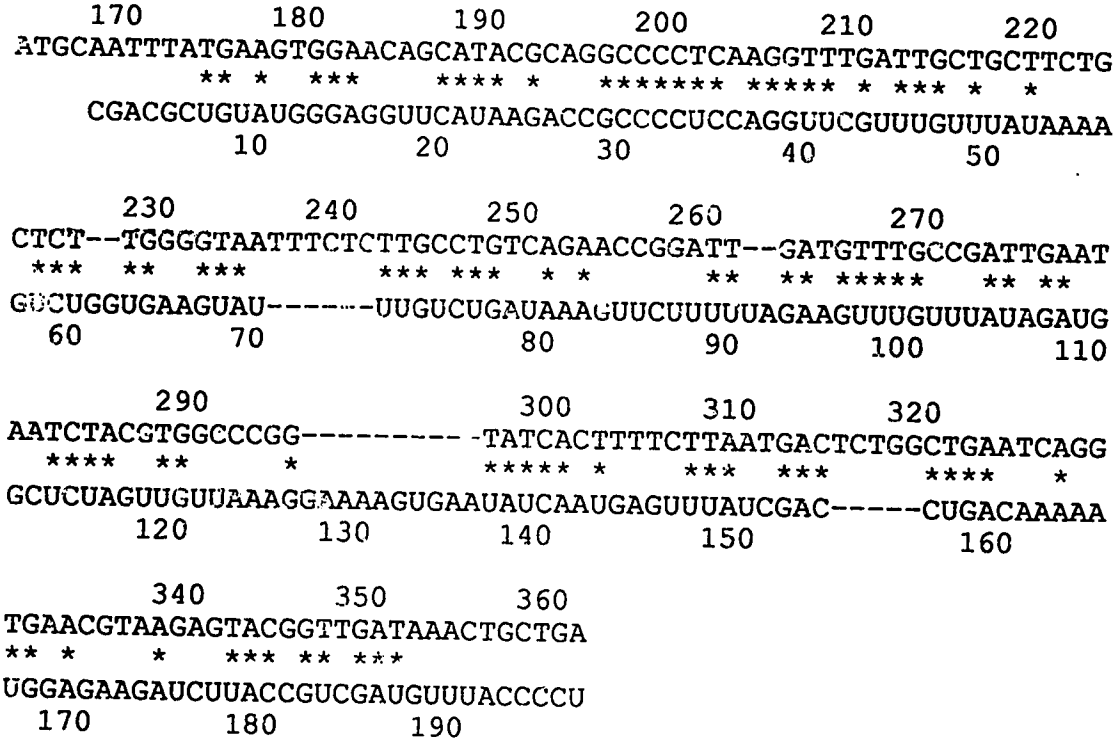

140 150 160 170 180
TTTCCCTGTAGATGCATTTCCTAAAATTATCAGGAATGCAATTTATGAAGTGGAA-CAGC
GUUACUAGCUGAUGAAGUUUCAAAAAUUAACUC-AAUGUCGAUUCUGGGUCCUAAUCAGC
340 350 360 370 380 390
190 200 210 220 230 240
ATACGCAGGCCCCTCAAGGTTTGATTGCTGCTTCTGCTCTTGGGGTAATTT-----CTCT
UAAAGCUCUGCACUCAAUUGGUGCUGUCUAAUGGAGCAGCACCAGUAGUUUUAAGCCUUG
400 410 420 430 440 450
250 260 270 280
TGCCTGTCAGAACCGGATTGAT--------GTTTGC----------CGATTGAATAATCT
UGUCAAAGGAAAAGAAAUCGAUUUCAAAUCGUAUGCUUCCUAAGAUCGGACAGAGGAUGU
460 470 480 490 500 510
290 300 310 320 330 340
ACGTGGCCCGGTATCAC TTTCTT ATCACT-CTGGCTGAATCAGGTGAACGTAAGAGTA
ACGUCC------AUCACUCGGCUAUUUACCUUCUUUAUAUGCCAAAUAUACUAAAAA
520 530 540 550 560 570
350 360 370 380 390
C-----GGTTGATAAACTGCTGATGAAGCCATTATATCAACTGGAAGAGGATTTATTTGA
CUUCAGGGAGCAUCACCU-----UGAAGCUUUUUAAUGAGGCUACAGAUGAGUUAGUGGA
580 590 600 610 620
410 420 430 440
AAAATACACCCAC---GATCTTACC---GCATGGAGAAATGATGAAGCAATTTT
UGUUGACACCGACCAUGAUGCUACCCAGGCAUGUAUAUUUGCUGGACGUUACCC
630 640 650 660 670

Fig.VI.13 Computer-based homology search showing possible homologous regions between RNA-2 of SCMV (upper strand) and that of tobacco mosaic virus 30 kDa gene (lower strand).


170 180 190 200 210 220
ATGCAATTTATGAAGTGGAACAGCATACGCAGGCCCCTCAAGGTTTGATTGCTGCTTCTG
** * *** **** * ******* ***** * *** * *
CGACGCUGUAUGGGAGGUUCAUAAGACCGCCCCUCCAGGUUCGUUUGUUUAUAAAA
10 20 30 40 50
230 240 250 260 270
CTCT--TGGGGTAATTTCTCTTGCCTGTCAGAACCGGATT--GATGTTTGCCGATTGAAT
*** ** *** *** *** * * ** ** ***** ** **
GUCUGGUGAAGUAU------UUGUCUGAUAAAGUUCUUUUUAGAAGUUUGUUUAUAGAUG
60 70 80 90 100 110
290 300 310 320
AATCTACGTGGCCCGG---------TATCACTTTTCTTAATGACTCTGGCTGAATCAGG
**** ** * ***** * *** *** **** *
GCUCUAGUUGUUAAAGGAAAAGUGAAUAUCAAUGAGUUUAUCGAC-----CUGACAAAAA
120 130 140 150 160
340 350 360
TGAACGTAAGAGTACGGTTGATAAACTGCTGA
** * * *** ** ***
UGGAGAAGAUCUUACCGUCGAUGUUUACCCCU
170 180 190

VII. GENERAL DISCUSSION AND CONCLUSIONS

The focus of this study was a new strain of sweet clover necrotic mosaic virus, isolated from alfalfa. Investigation into its biological, physical and serological properties established its identity as a new serotype of SCNMV. In comparative analyses with the type strain (SCNMV-38), the new serotype is distinguishable by the symptoms it produces on selected host plants, by serological reactions in gel diffusion tests and the electrophoretic mobility of its virions. The new serotype has a relatively wide experimental host range, as have the other dianthoviruses, and certain indicator plants are able to distinguish the new serotype from the type strain.

The observation that an antiserum to SCNMV-38 contains cross-reacting antibodies (heterospecific antibodies) to SCNMV-59 when analysed by intra-gel cross absorption tests, indicates the close serological relationship between the two viruses. Apparently, SCNMV-38 shares a majority of its antigenic determinants SCNMV-59. However, the latter seems to possess additional epitopes that were able to elicit the production of type-specific antibodies. This phenomenon of unilateral cross-reactivity due to the presence of heterospecific antibodies has been reported for a few other viruses (Gould et al. 1981; Rao et al. 1987; van Regenmortel, 1967). Moreover, when purified preparations of the two viruses were analysed by virion electrophoresis, distinct mobility differences were observed between SCNMV-38 and SCNMV-59. This might reflect the difference(s) in the coat proteins of the two viruses.

The fact that the type strain was isolated from sweet clover several years before the identification of the new isolate from alfalfa grown in the same area may indicate the emergence of field variants in a specific geographical area. Serologically distinct isolates of a virus have similarly been reported for RCNMV in Czechoslovakia (Musil, 1969; Musil and Gallo, 1982; Musil et al. 1982). The bipartite genome of RCNMV and SCNMV might have contributed to the observed variations among the various isolates. The advantages in virus epedemiology of a virus having a divided genome could be manifold. It offers an increased frequency of reassortment and/or recombination between related strains (Hiruki and Teakle, 1987). This might create a selective advantage in terms of an altered host range, improved vector transmissibility, changes in virulence (increased or decreased ability to invade the host tissue), and symptom variants. Such genetic flexibility may finally lead to a more stable and tolerant host-virus interaction with the virus being able to multiply to a reasonably high concentration (Fulton, 1980).

Regular field surveys in Alberta for the presence of SCNMV in forage crops yielded several isolates (C. Hiruki, unpublished). Detailed investigations into the antigenic relationships among these new isolates would provide more information on the incidence, geographic distribution, and emergence of new variants of the virus.

Chapter 3 dealt with the replication of the bipartite genome of SCNMV in isolated cowpea protoplasts. The fact the RNA-1 was able to replicate independently of RNA-2 indicates that the replicase gene is located on RNA-1. Larger RNAs of other bipartite

genome viruses have also been shown to replicate independently of their respective smaller RNAs (Dougherty and Hiebert, 1985). Besides, RNA-1 of SCNMV, like other dianthoviruses, determines the serological specificity (Okuno et al. 1983; this study: Chapter IV). Paje-Manalo and Lommel (1989) have recently shown that RCNMV RNA-1 was able to replicate independent of RNA-2 in electroporated protoplasts of *N. tabacum* BY-2 cell suspension cultures. However, the host plant is not a systemic host for the virus. The ability to cause subliminal infection by RNA-1 further supports the hypothesis of the involvement of RNA-2 in cell-to-cell movement. Besides, by constructing genetic reassortments between two strains of RCNMV, Osman and Buck (1986) showed that RNA-2 determines the lesion morphology and systemic invasion in cowpea. All these studies using various strains of SCNMV and RCNMV demonstrate the feasibility of using this bipartite genome virus as a model system to investigate in molecular terms, the mechanism by which the putative transport protein coded by RNA-2 potentiates the virus movement.

Current advances in plant transformation methodologies combined with the development of versatile vectors to introduce foreign genes into plants (Fraley et al. 1986) should prove useful in transforming suitable hosts with RNA-2 coded transport function of SCNMV. This might also pave the way to determine the *in situ* location of the putative transport protein in SCNMV-infected plants by highly sensitive cytological detection methods like immuno-gold labeling (Pesic et al., 1988; Stussi-Garaud et al. 1987; Tomenicus et al. 1987). In the case of replication of RCNMV, a

subgenomic RNA transcribed from RNA-1, although in low concentration, was shown to code for the coat protein (Marriott and Buck, 1988). Our studies, however, did not detect the subgenomic RNA, probably due to the relatively lower infection levels that were achieved when cowpea protoplasts were inoculated with SCNMV. Similarly, in another report on RCNMV replication in protoplasts (Paje-Manalo and Lommel, 1989), no subgenomic RNA was detected.

The SCNMV-cowpea protoplast system may be useful for studying the possible interaction of SCNMV and/or RCNMV genomic RNAs and their genetic reassortants. The availability of cDNA clones of SCNMV (this study: Chapter VI) should provide *in vitro* RNA transcripts which can be functionally mapped to study the replicative strategy in protoplasts (Ahlquist and French, 1988). Improvements in efficiency of infection protoplasts, combined with the use of cloned cDNAs of specific regions of SCNMV, would be useful in identifying and studying the mechanism of subgenomic RNA synthesis (Gargouri et al. 1989; French and Ahlquist, 1988). Since RNA-2 of SCNMV appears to be monocistronic, coding for a 35 KDa protein (this study: Chapter VI), it provides an excellent model system to introduce site-specific changes in its cDNA clones in order to study their effect on the function of protein products (Ahlquist et al. 1987).

Results of genetic reassortment studies were described in Chapter IV. The two strains of SCNMV form viable genetic reassortants. Similar results have been obtained between strains of RCNMV (Okuno et al. 1983; Osman and Buck, 1986; 1989), CRSV

(Dodds et al. 1977) and between viruses (Lommel and Morris, 1982; Okuno et al. 1983). However, genetic reassortants constructed between two strains of RCNMV indicated only unilateral compatibility (Rao and Hiruki 1987). The present study also confirms, from the genetic reassorants between SCNMV-38 and SCNMV-59, that RNA-1 determines the serological specificity. The observation that both strains are electrophoretically distinct (Chapter II) facilitated the use of this criterion for analysing the genetic reassortants. Virion electrophoresis thus offers a simple and reliable method to characterize genetic reassortants by utilizing such existing differences in virion electrophoretic mobilities.

Chapter V described a comprehensive study of the electorphoretic variability among dianthoviruses. The differences in virion mobilities reflect the variation in the nature of the coat protein of the respective viruses. Such variability correlates well with antigenic variation that exists among the various strains of dianthoviruses (Hiruki, 1987; Rao et al. 1987)). The origin of this variation could be due to several reasons. In the case of SCNMV, while the type strain was isolated form sweet clover, the new serotype was found in alfalfa. Host adaptation might have lead to the generation of variation in the nature of antigenic determinants of the new serotype (Yarwood, 1979; MacNeill and Boxall, 1974). In case of CRSV and RCNMV strains, the observed differences in their virion mobilities could be due to the geographic separation which might have led to the accumulation of variants in a natural population of each region. Variations in coat proteins of these viruses reflect the differences in their coat protein genes.

The major mechanisms of variation in RNA genomes are point mutation and recombination. Mutation rates are relatively high in RNA genomes, mainly due to errors in RNA polymerase activity combined with the absence of any proof-reading mechanism. Such point mutations may create a new pool of variants with differences in their coat protein genes. Studies on evolutionary relationships among RNA viruses seem to support the hypothesis that viruses have high tolerance levels with respect to variation in structural genes. This ability to 'accommodate' such mutations in coat protein genes might make the new variants prevail in the natural population. This eventually might be advantageous to the virus since it may provide a selective advantage to the variant (Zimmern, 1988).

The electrophoretic variability among dianthoviruses was utilized as a measurable phenotype to infer a genetic difference in their respective coat protein genes. In a recent report, three spontaneous, symptom mutants of RCNMV, which were otherwise serologically indistinguishable from one another, have been described (Osman and Buck, 1989). It would be interesting to identify variation in their virion mobilities, if any, since genetic reassortment studies among the mutants identified some mutations on RNA-1, which carries the coat protein gene.

The final chapter described the results of the complementary DNA cloning of the two genomic RNAs of SCNMV. The RNA-2 cDNA clone in Lambda gt10 was sequenced by sub-cloning in M13 vectors. The nucleotide sequence of the cDNA clone revealed the presence of one major ORF with a potential to code for atleast a 35

kDa protein. The complete nucleotide sequence of RNA-2 of RCNMV strain Aus (Lommel et al. 1988) seems to support this observation. However, it is interesting to note the lack of significant homology between RNA-2 of RCNMV and the sequenced region of SCNMV RNA-2. Since RNA-2 is considered to be coding for the putative transport protein, variation among sequences coding for such non-structural proteins may be under less stringent selection pressure, compared to those of replicase and protease genes (Mesk et al. 1987). The latter tend to be relatively well-conserved even among viruses with dissimilar genome organizations (Haseloff et al. 1984; Ahlquist et al. 1985; Franssen et al. 1984; Argos et al. 1984).

While it is prudent to expect that more sequence data on dianthoviruses would accumulate in due course, further studies on the nucleotide sequence of RNA-1 and RNA-2 of SCNMV would facilitate functional analysis of coding and non-coding sequences among these viruses. Such comparisons among dianthoviruses may reveal the regions that are more conserved than others. In conclusion, such molecular analysis would a better understanding of the evolutionary relationships of the members of this group and help delineate some of the current difficulties in plant virus classification.

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