Etiology

A New Serotype of Sweet Clover Necrotic Mosaic Virus

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ABSTRACT

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A new serotype of sweet clover necrotic mosaic virus (SCNMV) isolated from alfalfa (*Medicago sativa* L.) was characterized on the basis of serology, physicochemical properties, host range, and symptomatology. The antiserum of the new isolate (SCNMV-59) distinguished the type strain (SCNMV-38) from SCNMV-59 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 anti-SCNMV-38 serum that specifically reacted with SCNMV-59 antigen. The molecular mass of the coat protein of the new isolate was about 39,000 daltons when estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric points of the two serotypes were in the range of 4.85–5.00. Agarose gel electrophoresis of virions showed distinct mobility differences between the two serotypes. The new serotype could also be distinguished from the type strain on the basis of certain differences in host range and symptomatology on selected host plants.

Additional keywords: dianthoviruses, isoelectric focusing.

Sweet clover necrotic mosaic virus (SCNMV), a member of the dianthovirus group, is an isometric virus with a single-stranded, bipartite genomic RNA (7,14). The virus was originally isolated from sweet clover (*Melilotus officinalis* (L.) Lam.) in Alberta, Canada (10), and its known distribution is confined to Alberta (8). We report here the occurrence of a serologically distinguishable isolate of SCNMV from alfalfa grown in the same area. A preliminary report has been published (12).

MATERIALS AND METHODS

Viruses. The type strain (SCNMV-38) was originally isolated from sweet clover in 1979 (10) and the alfalfa isolate (SCNMV-59) from alfalfa in 1981 (7). Both isolates have been multiplied in *Phaseolus vulgaris* L. 'Red Kidney' after several single-lesion transfers in the same host. A Czechoslovakian strain of red clover necrotic mosaic virus (RCNMV) TpM-34 (17) and carnation ringspot virus (CRSV) (10) were two additional dianthoviruses used as standards. All viruses were purified following a previously published procedure (6).

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 ± 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 of fresh leaf tissue per 5 ml of buffer. The extract was rubbed on leaves dusted with Carborundum, which 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 previously (15).

Serology. Virus preparations used for producing antisera were subjected to two cycles of sucrose density gradient centrifugation (6). Each New Zealand male rabbit was given two intravenous injections, followed by two intramuscular injections 1 wk apart with an immunogen containing 1 mg of virus emulsified in Freund's incomplete adjuvant. Animals were bled 2 wk after the final injection. Each antiserum was cross-absorbed with acetone-extracted powder from healthy Red Kidney bean leaves. They did not react with healthy sap after the treatment. Immunodiffusion tests and intra-gel cross-absorption tests were done in 0.8% agarose (ICN Biomedicals), 0.02% sodium azide, and 0.85% saline as described by Van Regenmortel (22). Antigen was used at a concentration of 1 mg/ml in all immunodiffusion tests. Antisera titers were determined by testing a series of twofold dilutions of antiserum against its homologous and heterologous antigen. Precipitin patterns were recorded 24 hr 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 preparations of both isolates were heat-dissociated in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol and were analyzed on 10% SDS-polyacrylamide gel (SDS-PAGE) by using the discontinuous buffer system of Laemmli (13). Bands were visualized by staining with Coomassie Brilliant Blue R250 followed by destaining in methanol:acetic acid:water. Molecular weight markers were obtained from Bio-Rad (Richmond, CA).

Isoelectric focusing. Isoelectric focusing of purified virus preparations was performed by using Bio-Rad's Mini IEF cell (Model 111). Virus samples containing 1 μg of nucleoprotein in 20% glycerol were loaded on a 1% agarose gel (Isogel, FMC Corporation, Rockland, ME), containing 5% sorbitol, 10% glycerol, and 2% Bio-Lyte ampholytes (Bio-Rad). Focusing and detection of proteins were done according to the protocol supplied by the manufacturer. Mobilities were compared to RCNMV-TpM 34 (5). 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 recorded 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 essentially as described by Serwer et al (21) 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 per 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, Raleigh, NC) at 3V/cm constant voltage at 4 C with buffer recirculation (100 ml/min). Gels were stained with ethidium bromide (1 μg/ml in distilled water containing 1 mM EDTA) for 2 hr in the dark. The same gels were restained with Coomassie Brilliant Blue R250 and bands were located by destaining in methanol:acetic acid:water.

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 nine 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 or 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 with those caused by SCNMV-38, SCNMV-59 generally produced milder symptoms on *M. officinalis* (Fig. 1A and B). The size of local lesions produced by SCNMV-38 on certain host species was discernibly larger than those by SCNMV-59 (Table 1, Fig. 2).

Serology. In gel diffusion tests, the homologous titers of anti-SCNMV-38 and anti-SCNMV-59 sera were 1,024 and 512, respectively, and their heterologous titers differed by only one twofold dilution. When the two antigens were compared using anti-SCNMV-38 serum, the precipitin lines fused with each other (16) (Fig. 3A), whereas the reaction of the same antigens with anti-SCNMV-59 serum resulted in a homologous spur that extended beyond the heterologous precipitin line (Fig. 3B). To verify this serological behavior, both antigens and their respective antisera were examined in a four-membered pattern in

TABLE 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/0, (−)[b]	vs- LL/0, (−)
C. amaranticolor	s- LL/0, (−)	vs- LL/0, (−)
Sesamum indicum	l- LL/0, (+)	s- LL/0, (+)
Glycine max	s- LL/0, (−)	vs- LL/0, (−)

[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, 0 = no symptoms, r = reddish, s = small, st = stunting, sv = severe, vn = veinal necrosis, vs = very small.

[b] Results obtained by enzyme-linked immunosorbent assay − = virus infection negative; + = virus infection positive.

Fig. 1. Comparison of symptoms in sweet clover (*Melilotus officinalis*) incited by **A,** the type strain (SCNMV-38) and, **B,** the alfalfa isolate (SCNMV-59) of sweet clover necrotic mosaic virus.

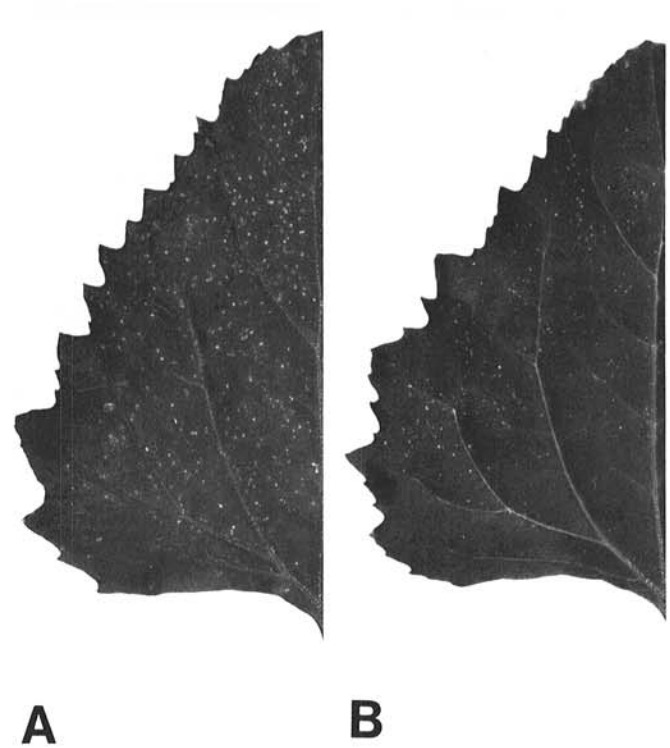

Fig. 2. Half-leaves of *Chenopodium amaranticolor* showing local lesions incited by **A,** the type strain (SCNMV-38) and, **B,** the alfalfa isolate (SCNMV-59) of sweet clover necrotic mosaic virus.

homologous and heterologous combinations (2). Both homologous and heterologous precipitin lines crossed each other (Fig. 3C and D). The homologous precipitin line between SCNMV-38 and its serum deflected toward the homologous precipitin line between SCNMV-59 and its serum, which was similar to that of a reaction of identity (Fig. 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. 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. 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. 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. 4C). When the same antiserum was cross-absorbed with its homologous antigen, all the antibodies were completely precipitated (Fig. 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. 4E). Anti-SCNMV-59 serum gave a pattern similar to the one in which the antiserum was cross-absorbed with its homologous antigen (Fig. 4F). Neither of the antisera reacted with sap from healthy leaves, and neither antigen reacted with the preimmune 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 mass of 39,000 daltons, 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. 5).

Isoelectric points of the serotypes. 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. 6).

Agarose gel electrophoresis. Both SCNMV-38 and SCNMV-59 migrated as a single protein component when stained with Coomassie Blue (Fig. 7B). The identity of the nucleoprotein was confirmed by staining the same gel with ethidium bromide, a nucleic acid-specific stain (Fig. 7A). SCNMV-59 migrated more slowly than SCNMV-38 and CRSV (Fig. 7A and B). This difference was consistent among different batches of virus preparations and buffer systems (data not shown). 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 (9). 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.

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 (22). Spur formation in gel diffusion tests has been used as a reliable criterion to distinguish between serotypes of several viruses (22). Anti-SCNMV-38 serum gave a reaction of complete identity (4,16) 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. 3A and 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 (6,17,23). The presence of heterospecific antibodies was previously unique to tobacco mosaic virus (TMV) (23), but a recent finding that antisera of certain strains of RCNMV also contain heterospecific antibodies (17) 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 to 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 8 yr of annual field surveys were similar to the original strain (SCNMV-38) (C. Hiruki, *unpublished data*). 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, *Phaseolus vulgaris* 'Red Kidney' over a period of 5 yr, and their respective antisera have been produced in different rabbits during the same period. The serological reactions

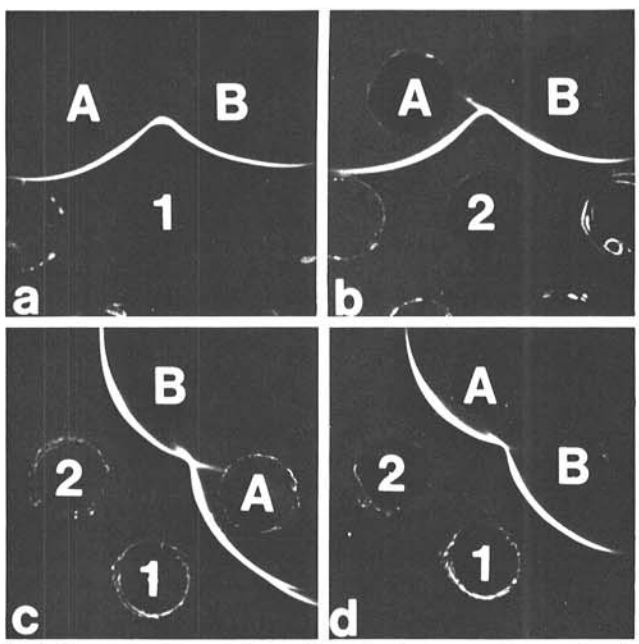

Fig. 3. Serological comparison of sweet clover necrotic mosaic virus (SCNMV) serotypes by double immunodiffusion in agar gel. A, SCNMV-38. B, SCNMV-59. 1, anti-SCNMV-38 serum. 2, anti-SCNMV-59 serum. Both antisera were used at 1/2 dilution. Letters a, b, c, and d are referred to in Results.

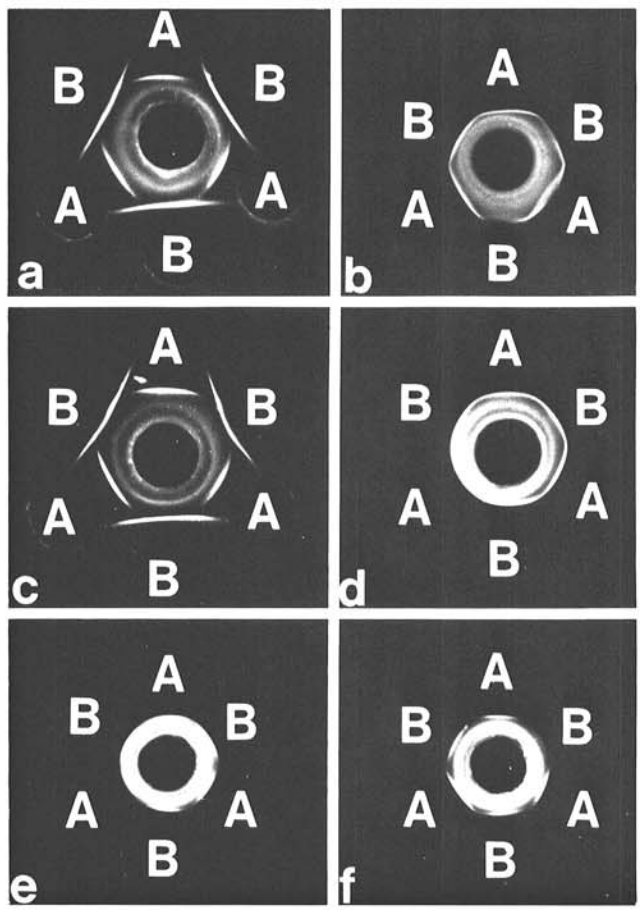

Fig. 4. Intra-gel cross-absorption tests of sweet clover necrotic mosaic virus (SCNMV) serotypes. A, SCNMV-38, B, SCNMV-59. a, Center well initially filled with SCNMV-38 antigen, 24 hr later with anti-SCNMV-38 serum. b, Center well initially filled with SCNMV-59 antigen, 24 hr later with anti-SCNMV-38 serum. c, Center well initially filled with SCNMV-59 antigen, 24 hr later with anti-SCNMV-59 serum. d, Center well initially filled with SCNMV-38 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.

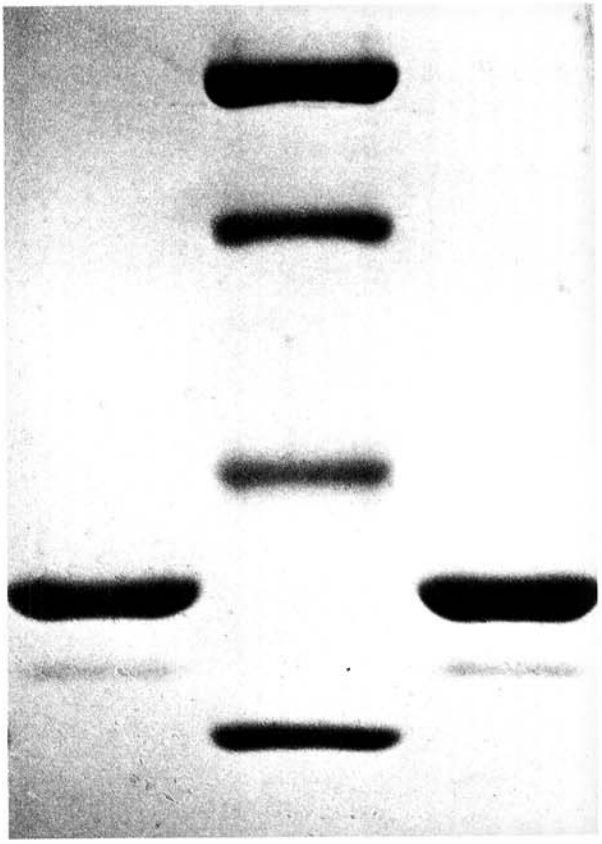

Fig. 5. SDS-10% polyacrylamide gel electrophoresis of dissociated coat proteins of sweet clover necrotic mosaic virus (SCNMV) serotypes. SCNMV-38 (left), SCNMV-59 (right). Molecular weight markers (from top) phosphorylase B (98 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase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

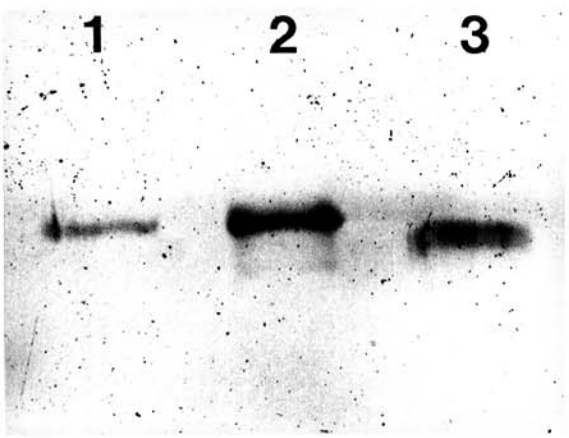

Fig. 6. Isoelectric focusing of sweet clover necrotic mosaic virus (SCNMV) serotypes. SCNMV-38 (lane 1), SCNMV-59 (lane 2), red clover necrotic mosaic virus (RCNMV)-TpM 34 (lane 3). pH gradient was generated by using Bio-Lyte ampholytes 4/7. Direction of migration is from positive (top) to negative (bottom).

determined by 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 (15) and by immunosorbent electron microscopy (3). The present results suggest that different sets of genes are available for

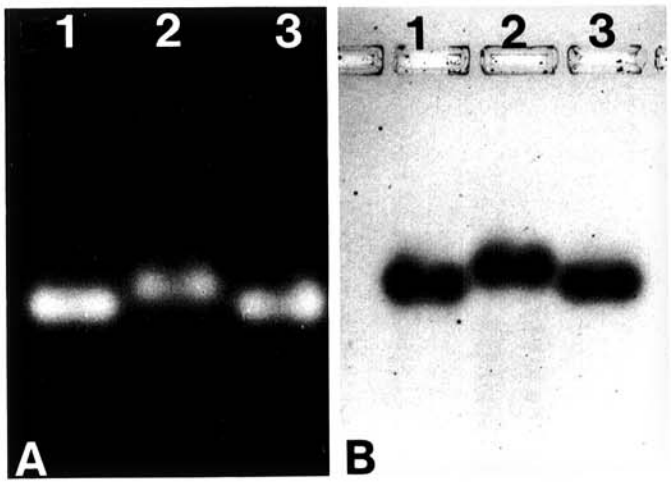

Fig. 7. Agarose gel electrophoresis of sweet clover necrotic mosaic virus (SCNMV) serotypes. **A,** Gel stained with ethidium bromide. SCNMV-38 (lane 1), SCNMV-59 (lane 2), and carnation ringspot virus (CRSV) (lane 3). **B,** Same gel stained with Coomassie Brilliant Blue R250. Direction of migration was from negative (top) to positive (bottom).

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 (19).

The isoelectric points of the SCNMV type strain and the new isolate are similar to those of RCNMV strains (5) 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 (18). 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 (20). Recently, this method has been used to characterize electrophorotypes of hibiscus chlorotic ring spot virus (11), panicum mosaic virus and its strains (21), and TMV (1). In this study, in addition to their demonstrated serological differences, SCNMV-38 and SCNMV-59 were also electrophoretically distinct.

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Here we report the first use of ELISA to detect TSWV in