An Isolate of Tomato Ringspot Virus from *Trifolium ambiguum*

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ABSTRACT

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A *Trifolium ambiguum* plant growing in a field plot displayed a faint mottle typical of symptoms of virus infection in forage legumes. Sap inoculation to a series of herbaceous test plants indicated the presence of a virus. Preparations of crude sap examined in an electron microscope showed the presence of isometric particles. The virus was partially characterized and was identified as tomato ringspot virus (TomRSV) by gel double-diffusion serological tests. *T. ambiguum* has been proposed as a source of resistance to the common legume viruses. Although susceptibility of this species to TomRSV does not compromise the potential of some populations of *T. ambiguum* to be a source of resistance to other common legume viruses by means of interspecific crosses, the possibility of introducing susceptibility to TomRSV should be recognized.

Barnett and Gibson (4) reported sap inoculation test results showing that *Trifolium ambiguum* M. Bieb. possesses resistance to most of the viruses commonly found infecting white clover (*T. repens* L.) in the southeastern United States. A few plants of *T. ambiguum* were susceptible to clover yellow mosaic virus. The researchers concluded that the species *T. ambiguum* possessed resistance to all seven viruses tested, sometimes in a single plant, and that hybrids between it and *T. repens* offered a potential solution to virus disease problems in white clover. In addition, *T. ambiguum* is of interest to agronomists because it has a rhizomatous growth habit, is resistant to pests, and is winter-hardy and drought-tolerant, traits that may be useful in interspecific hybrids with other *Trifolium* spp. (6,22).

One of several plants of *T. ambiguum* transplanted into and growing in field plots at Clemson, SC, displayed a faint mottle typical of symptoms of virus infection in forage legumes. Sap-inoculated test plants developed symptoms unlike those caused by viruses commonly found in forage legumes. Before *T. ambiguum* could be used as a source of virus resistance in forage legumes, the infecting virus had to be identified. We partially characterized the virus and identified it as an isolate of tomato ringspot virus (TomRSV).

MATERIALS AND METHODS

Stock cultures of virus were maintained in *Nicotiana clevelandii* A. Gray

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after inoculation from *T. ambiguum*. The virus was also maintained in dried tissue of *N. clevelandii*, and cultures were periodically reestablished from the dried tissue.

Host range studies. We inoculated a range of plant species from 14 families with the virus. Sap prepared from leaves of *N. clevelandii* ground in 0.03 M sodium phosphate buffer, pH 8.0, containing 0.03 M 2-mercaptoethanol (1:5, w/v [tissue:buffer]) was rubbed onto leaves dusted with 600-mesh corundum. Symptoms on both inoculated and upper, uninoculated leaves were recorded, and tests for latent infection were completed by back-inoculation to either *Chenopodium amaranticolor* Coste & Reyn. or *C. quinoa* Willd. Once the virus had been identified as TomRSV, we compared the host range of our isolate from *T. ambiguum* (Bin 602) with those of two other isolates of TomRSV, an isolate from *Cornus florida* L. (DW 192) and the type isolate (ATCC 13) (9).

Persistence of infectivity in plant sap. Either inoculated leaves with local lesions or systemically infected tissue of *N. tabacum* L. 'X-73' was ground in 0.03 M sodium phosphate buffer, pH 8.0, and used to test the thermal inactivation point (TIP), longevity in vitro (LIV), and dilution end point (DEP) of the virus in crude sap. Sap was prepared using tissue diluted 1:5 (w/v) with buffer for the TIP and LIV tests and 1:10 (w/v) for the DEP test. Sap was heated in the TIP test for 10 min at the specified temperature and then rapidly cooled. LIV was tested in the greenhouse at 18–21 C.

Purification. The virus was purified from systemically infected tissue of *N. clevelandii*. All stages of the purification were completed at 4 C, with all solutions having been previously cooled to this temperature. Tissue was homogenized (1:4, w/v) in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.02 M 2-mercaptoethanol and bentonite, prepared as described by Dunn and Hitchborn (7), at the rate of 5 mg/100 g of tissue, in either a Virtis 45 high-speed homogenizer or a rotary leaf squeezer (13). The preparation was clarified by centrifugation (17,000 *g* for 20 min), and the resultant supernatant was retained and filtered through glass wool. Polyethylene glycol (PEG) (mol wt 8,000) and sodium chloride were added to concentrations of 10 and 1%, respectively, and the preparation was stirred for 1 hr. The precipitate that formed was recovered by low-speed centrifugation (10,000 *g* for 15 min) and resuspended in 0.03 M sodium phosphate buffer, pH 7.5, containing 0.005 M magnesium chloride (PM buffer) at the rate of 25 ml of buffer per 100 g of original tissue. After a further low-speed centrifugation (17,000 *g* for 20 min), the supernatant was put through another cycle of PEG precipitation. The precipitate was resuspended in a small volume of PM buffer.

The virus was further purified by rate zonal centrifugation in 10–30% isokinetic glycerol gradients (8) prepared using PM buffer. Samples containing approximately 2 mg of virus in 1.5 ml of solution were layered onto the gradients and then centrifuged at 160,000 *g* for 3.5 hr in a Beckman SW28 rotor. Gradients were scanned at 254 nm and fractionated. Fractions believed to contain virus were precipitated with PEG, and the precipitate was resuspended in PM buffer. Preparations were examined in an ultraviolet-visible spectrophotometer, and concentrations were determined using an extinction coefficient of 10 $(mg/ml)^{-1}$ cm^{-1} at 260 nm.

Serology. Gel double-diffusion tests were conducted using 0.75% agarose gels prepared with 0.03 M sodium phosphate buffer, pH 7.5, containing 0.85% sodium chloride and 0.02% sodium azide. Test samples were prepared in 0.03 M sodium phosphate buffer, pH 7.5. The virus was tested against antisera to cherry leafroll virus (provided by H. E. Waterworth); Arabis mosaic virus-type (AB-10), raspberry ringspot virus, and strawberry latent ringspot virus (Scottish Crop Research Institute); grape chlorotic mottle virus and artichoke Italian latent virus (G. P. Martelli); tobacco ringspot virus; and three isolates of TomRSV.

Properties of protein and nucleic acid. Capsid protein relative molecular mass (M_r) was determined by polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (10), with egg white lysozyme (M_r 14,300), β-lactoglobulin (18,400), trypsinogen (bovine pancreas) (24,000), pepsin (porcine stomach mucosa) (24,700), ovalbumin (45,000), and bovine serum albumin (66,000) as standards.

Nucleic acid was extracted from the virus with Pronase (14). Its M_r was determined by PAGE with the buffer described by Loening (11); gels were prepared and run as described by Bruening et al (5), and standards were RNA from tobacco mosaic virus (TMV)

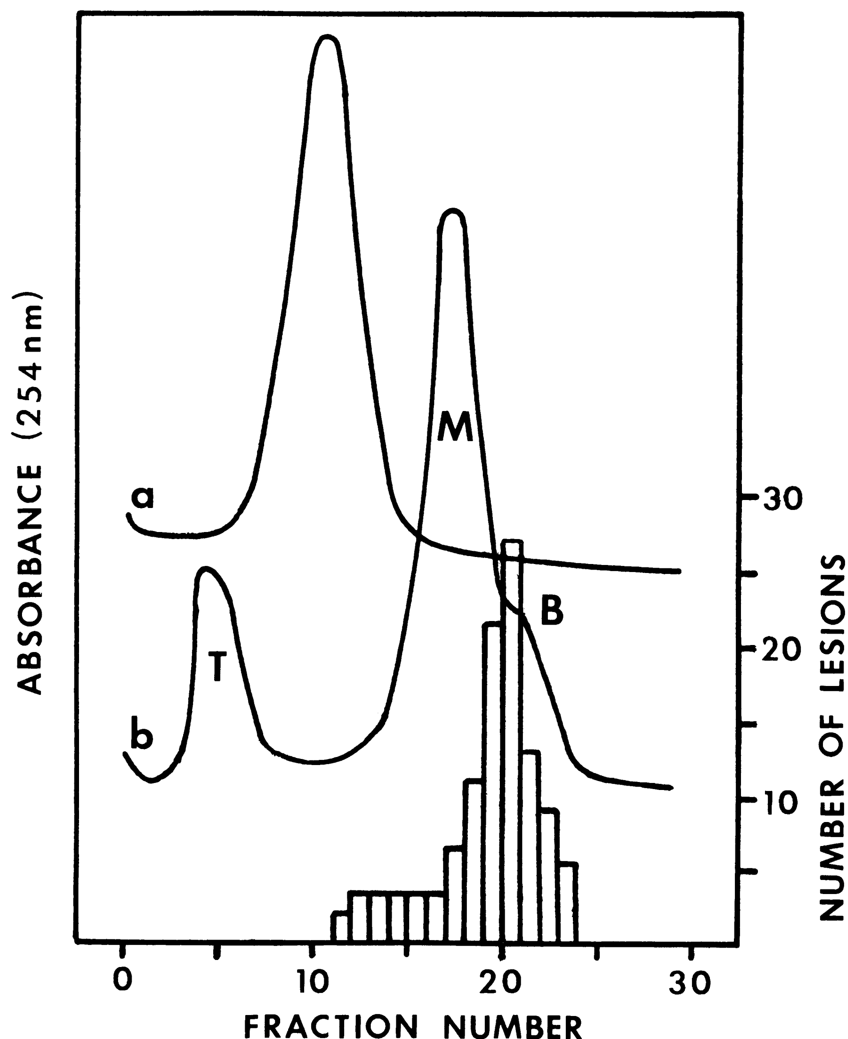

Fig. 1. Absorbance profiles in 10–30% glycerol gradients of preparations of Bin 602 purified using *n*-butanol for clarification (profile a) and using the procedure described in Materials and Methods (profile b). Columns show the infectivity of fractions from profile b. Centrifugation is from left to right. T = upper, M = middle, and B = lower band in rate zonal centrifugation.

(2.19×10^6) and ribosomal RNAs from *Escherichia coli* (1.009×10^6 and 0.534×10^6). For electrophoresis of RNA under denaturing conditions, freeze-dried samples were resuspended in a denaturing mixture of glyoxal and dimethyl sulfoxide for 1 hr at 50 C (15). Samples were layered immediately onto 0.9% agarose gels in 0.01 M sodium phosphate, pH 7, which had previously been subjected to electrophoresis for 15 min at 2 mA per gel. Electrophoresis was for 15 min at 0.05 mA per gel, then at 1–2 mA per gel until the bromophenol blue marker reached the bottom. Gels were scanned at 260 nm.

Double-stranded RNA (dsRNA) was extracted from *N. clevelandii* and subjected to electrophoresis as described by Abou-Elnasr et al (1). dsRNA from plants infected with cucumber mosaic virus (CMV) and the genomic dsRNA of cytoplasmic polyhedrosis virus (CPV) were used as markers with M_rs of $2.2–0.22 \times 10^6$ (3) and $2.29–0.55 \times 10^6$ (16), respectively. The dsRNA nature of the bands was confirmed by digestion with RNase A in 0.2 M NaCl.

RESULTS

Identification of Bin 602 proved difficult because the isolate failed to react with a number of antisera to isometric viruses when plant sap was used. In initial purification attempts using butanol as the clarifying agent, infectious, isometric virions were obtained that migrated as a single peak (Fig. 1, profile a) with a sedimentation coefficient of 90 S. Because virions from this peak did not react with antisera against cucumoviruses or tombusviruses (20), characterization of the virus was begun. The virus was identified only after other purification procedures were used.

Host range. The virus isolated from *T. ambiguum* infected species from the families Aizoaceae, Amaranthaceae, Apocynaceae, Chenopodiaceae, Asteraceae, Convolvulaceae, Cucurbitaceae, Fabaceae, Polemoniaceae, Scrophulariaceae, Solanaceae, and Apiaceae but did not infect the members of the Brassicaceae and Nyctaginaceae that were tested (namely, *Arabis alpina* L., *Brassica nigra* (L.) Koch, *B. juncea* (L.) Czernj. & Coss., and *Mirabilis jalapa* L. 'Petticoat Rose.'

Chlorotic or necrotic local lesions, together with symptoms of systemic infection, were observed on *Tetragonia expansa* Thunb. ex Murr., *Gomphrena globosa* L., *Beta vulgaris* L. 'Detroit Dark Red,' *C. album* L., *C. amaranticolor, C. foetidum* Schrad., *C. murale* L., *C. quinoa, Cucumis sativus* L. 'Chicago Pickling,' *Cucurbita pepo* L. 'Small Sugar,' *Mormordica balsamina* L., *Lablab purpureus* (L.) Sweet, *Glycine max* (L.) Merr. 'Bragg' and 'Davis,' *Phaseolus vulgaris* L. 'Black Turtle Soup' and 'The Prince,' *Pisum sativum* L. 'Bonneville,' 'Dark Skin Perfection,' and 'Perfected Wales,' *Vicia faba* L. 'Bell Bean,' *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye,' *Vigna unguiculata* subsp. *cylindrica* (L.) Verdc., *Phlox drummondii* Hook., *Antirrhinum majus* L., *Datura stramonium* L., *N. glutinosa* L., *N. longiflora* Cav., *N. occidentalis* Wheeler, *N. sylvestris* Speg. & Comes, and *N. tabacum* 'X-73,' 'Xanthi,' and 'Burley 21.'

Latent local infections, together with symptoms of systemic infection, were recorded on *Celosia argentea* var. *cristata* (L.) Kuntze, *Catharanthus roseus* (L.) G. Don, *Lactuca sativa* L. 'Buttercrunch,' *Lupinus albus* L., and *Medicago sativa* L.

Latent local and systemic infections were detected in *Atriplex nummularia* Lindl., *Zinnia elegans* Jacq. 'Wild Cherry,' *Lupinus hirsutus* L., *Lupinus luteus* L., *Torenia fournieri* Lind., *N. megalosiphon* van Heurck & Muell.-Arg., and *N. palmeri* Gray.

Symptoms of systemic infection without detectable local infections occurred in *Ipomoea quamoclit* L. 'Cardinal Climber,' *P. vulgaris* 'Bountiful,' *Pisum sativum* 'Little Marvel,' *Lycopersicon esculentum* Mill., and *Apium graveolens* L. var. *dulce* (Mill.) Pers.

Spinacia oleracea L. displayed local necrosis followed by systemic chlorosis and plant death. *Verbesina encelioides* (Cav.) Benth. & Hook. ex A. Gray developed necrotic local lesions, together with chlorosis and a latent systemic infection. *P. vulgaris* 'Pinto III' developed local necrosis and veinal necrosis on the systemically infected tissue. Locally infected leaves of *Pisum sativum* 'Ranger' became necrotic, and a mosaic developed on systemically infected leaves. Latent local infections without systemic infection were recorded in *Nicandra physalodes* (L.) Gaertn. and *Physalis alkekengi* L. No infections could be detected in *Beta patellaris* Moq., *Capsicum annuum* L. 'Carolina Hot Pepper,' *Citrullus vulgaris* Schrad. 'Sugar Baby,' *T. pratense* L., or *T. repens.*

The host range of Bin 602 differed only slightly from those of the other two isolates of TomRSV when the isolates were compared at the same time (Table 1). Symptoms induced by all isolates varied considerably depending on conditions at the time of year the tests were conducted, as shown by the differences between symptoms recorded during the comparative test and symptoms on the same host observed at other times (Table 1).

Persistence of infectivity in plant sap. The virus remained infective at a dilution of 10^{-2} but not 10^{-3} and at 60 C but not 65 C. It survived for 2 but not 5

Table 1. Host ranges of tomato ringspot virus isolates from *Trifolium ambiguum* (Bin 602) and *Cornus florida* (DW 192) and the type strain[a]

	Isolate		
Host species	Bin 602	DW 192	Type
Chenopodium quinoa	NLL:Mo,Chl,TN,Ma	NLL:Mo,NSp,TN	Chl:Chl,TN,Ma
C. amaranticolor	NLL,RB,Chl:Chl,TN(−)	ChlLL:Mo,Ma,Chl,NSp	NLL:Mo,Ma,Chl,TN
Nicotiana clevelandii	NRS:Mo,LP,RS	ChlLL(−+):Mo,N	NRS,NLL(−+):Mo,NSp
N. tabacum			
'Xanthi'	NRS,LP:−	NRS,RS:−	−+:−
'Burley 21'	NRS:ChlSp,RS(−)	NRS:−	NRS:ChlSp,RS(−)
Gomphrena globosa	−+:−+	−+:−+	−+:−
Lycopersicon esculentum 'Marion'	−+:ChlSp,VC,Mo	−+:ChlSp,VC,Mo	ChlLL:ChlSp,VC,Mo
Cucumis sativus 'Chicago Pickling'	ChlLL(−+):Chl(−+)	ChlLL,NLL:NSp,Mo,St	ChlLL(−+):VC(−+)
Phaseolus vulgaris			
'Black Turtle Soup'	−+:−+	−+:−	NRS:−+
'Pinto'	NLP,RS(−+):VC,Mo,Ma(−+)	−+:Mo,Ma(−)	NLL,RB:NSp,ChlSp
'Bountiful'	NRS:Mo,Chl,N	−+:−	NLP:NSp,TN,St
Pisum sativum 'Dwarf Gray Sugar'	−+:St	−+:St	−+:St
Petunia hybrida	−+:VC	−+:VC	−+:VC
Vigna unguiculata	ChlRS:Mo,TN	ChlRS:Mo	ChlLL:Mo

[a]Symptoms observed on inoculated leaves are listed before the colon; symptoms observed on systemically infected leaves are listed after the colon. − = no visible symptoms or virus detected by enzyme-linked immunosorbent assay; −+ = latent infection detected; Chl = chlorosis or chlorotic symptoms; N = necrosis or necrotic symptoms; LL = local lesions; RB = red border around lesion; RS = ringspots; TN = tip necrosis; VC = veinclearing; LP = line patterns; Mo = mosaic; Ma = malformed; St = stunting; Sp = spots. Symptoms in parentheses were observed after other inoculations at different times of the year.

days in sap at temperatures of about 20 C.

Serology. Serological reactions are shown in Figure 2. An antiserum produced against Bin 602 did not react well with the homologous virus or at all with TomRSV-type. Bin 602 virions produced good precipitin lines against antisera to TomRSV obtained from R. W. Fulton (type strain) and J. K. Uyemoto and against an antiserum to TomRSV-type produced in our laboratory. No spurs formed between the precipitin lines formed by Bin 602 virions and TomRSV virions. No reaction was observed with any of the other antisera.

Purification. The purification procedure yielded 2 mg of virus per 100 g of tissue. Preparations were faintly opalescent. Even after three cycles of centrifugation on glycerol gradients, slight quantities of green material were observed in the bottom of the tubes in which the gradients had been run. These preparations had an $A_{260/280nm}$ of 1.75–1.81, with an A_{min} at 240 nm.

Preparations sedimented as three components in rate zonal centrifugation (Fig. 1). The upper band (T) contained predominantly particles penetrated by stain ("empty" capsids), although a few particles that had not been penetrated by stain were seen (Fig. 3A). Particles not penetrated by stain predominated in the middle (M) and lower (B) bands, although a few that had been penetrated by stain were also present (Fig. 3B). Typically, the M and B components occurred in a 2:1 ratio (based on absorbance at 254 nm). Infectivity was associated with the region where peaks of M and B components overlapped. With a few preparations, the two peaks separated completely; in these instances infection was achieved only after the peaks were mixed.

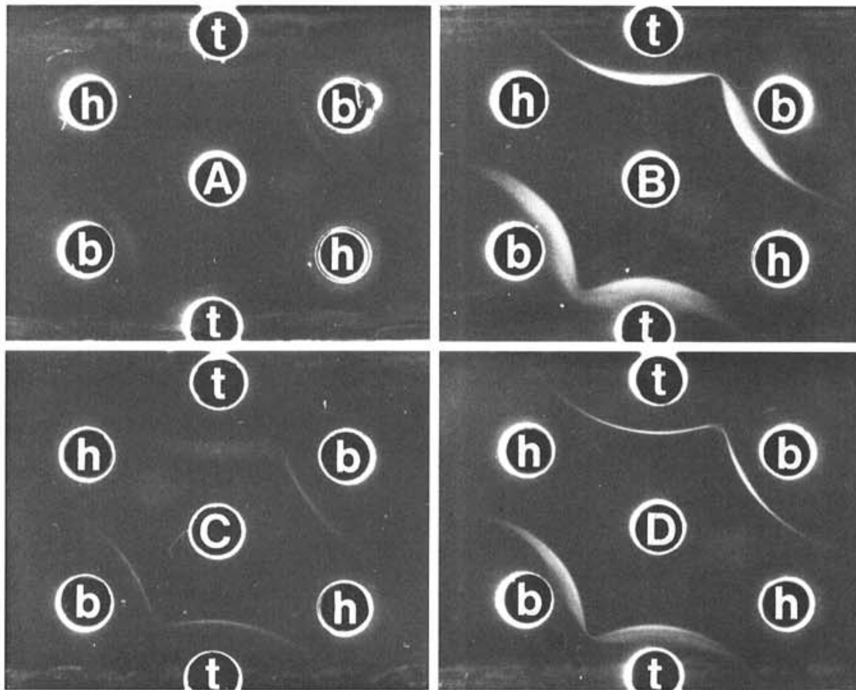

Fig. 2. Serological comparisons of the Bin 602 isolate with the type isolate of tomato ringspot virus (TomRSV). All viruses were used at a concentration of 0.762 mg/ml. The central wells contained antiserum against Bin 602 produced in our laboratory (A), against the type isolate of TomRSV obtained from R. W. Fulton (B), against TomRSV obtained from J. K. Uyemoto (C), and against the type isolate of TomRSV prepared in our laboratory (D). Samples of antigen were arranged in the outer wells: t = TomRSV, type isolate; b = Bin 602; and h = sap from healthy *Nicotiana clevelandii.*

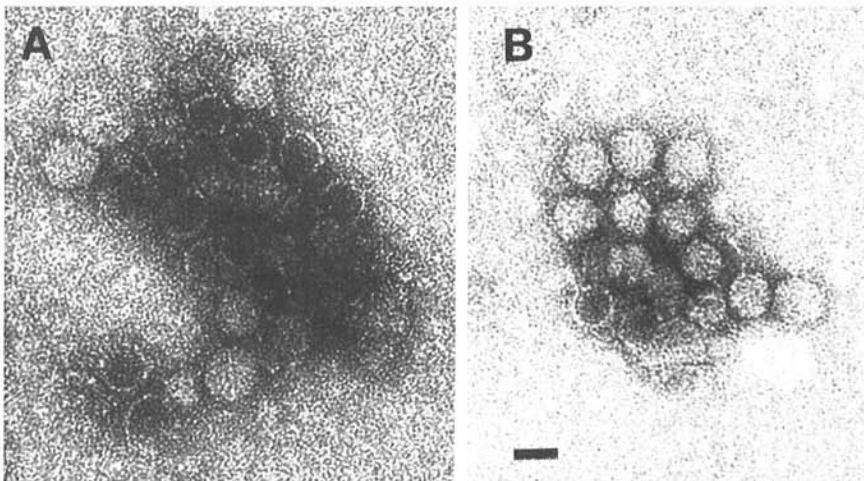

Fig. 3. Virus particles from **(A)** the upper band (T component) and **(B)** the middle and lower bands (M and B components) of preparations of Bin 602 centrifuged in 10–30% glycerol gradients (see Fig. 1). Particles were stained with 2% uranyl acetate. Scale bar = 30 nm.

Properties of protein and nucleic acid. Capsid protein M_r was determined as 54,000 ± 500. With some preparations faint bands also occurred at 13,000, 26,000, and 39,000.

Nucleic acids with estimated M_rs of 2.7×10^6 and 2.52×10^6 were detected in nondenaturing gels. M_rs were 3.35×10^6 and 3.10×10^6 in denaturing glyoxal gels. None of these values agree closely with those published for TomRSV (21).

Electrophoresis of extracts from samples of infected *N. clevelandii* (3–7 g) in 7% acrylamide gels (1) revealed two bands of dsRNA. The bands were diffuse and were no longer detected when electrophoresis was conducted in 5 or 6% acrylamide gels. Extractions and concentrations from greater quantities of tissue (up to 30 g) did little to improve the visibility of the bands at any concentration of acrylamide. The M_rs of the two bands exceeded the upper limits of both the CMV and CPV standards. An extract from 100 g of infected tissue was subjected to electrophoresis in 1% agarose gel using a *Hin*dIII digest of λ phage (BRL Life Technologies Inc., Gaithersburg, Maryland) as an additional standard and stained with ethidium bromide. Two indistinct bands were observed, with estimated M_rs (18) of 5.1×10^6 and 4.9×10^6.

DISCUSSION

When this virus isolate was first detected in 1977, no other viruses had been reported from *T. ambiguum.* Subsequently, Alconero (2) reported the presence of several viruses in this species. This outbreeding species exhibits phenotypic variation among and within populations (17). Thus, the unequivocal statement that this species is resistant to most viruses is no longer accurate and must be replaced by the modified assertion that certain populations of this species possess resistance to several viruses that infect forage legumes.

The fact that breeders are denied the opportunity to transfer an immune type of resistance from *T. ambiguum* into other clover species does not prohibit the use of *T. ambiguum* in interspecific

crosses designed to incorporate its many agronomic attributes into other suitable genetic backgrounds, with the added proviso that susceptibility to certain viruses may also be transferred. In addition, TomRSV and its vector, *Xiphinema americanum* Cobb, are neither native to, nor, if they have been introduced, prevalent in all parts of the world where hybrid perennial clovers might be used (for example, New Zealand).

Equally important, however, is the role that the incorporation of susceptibility to TomRSV into the genome of indigenous *Trifolium* spp. might play in the spread into and/or persistence of the virus in other crops where it poses a greater threat. Some populations of *T. repens* are a host of TomRSV (19), but some isolates of TomRSV, including Bin 602, do not infect white clover. Ectoparasitic nematodes, including *X. americanum,* are common in crops of forage legumes (12). The incorporation of susceptibility to TomRSV into *T. repens* or other perennial legumes might substantially increase the likelihood of infection of other hosts of agronomic and horticultural importance. For example, TomRSV causes stem pitting and tree death in peach (*Prunus persica* (L.) Batsch). In some areas (such as the Piedmont area of South Carolina), the preferred orchard floor cover is a mixture of fescue and perennial clovers, including *T. repens,* which, if infected by TomRSV, could lead to infection of peaches.

This isolate of TomRSV (Bin 602) differed slightly from two other isolates of the virus in host range. Symptoms produced by Bin 602 depended greatly on the conditions under which the test plants were grown. *N. clevelandii* developed necrotic ringspots on inoculated leaves and marked systemic veinal necrosis under subdued lighting at temperatures of about 20 C. The production of ringspots under these conditions was sufficiently reliable to be used as an infectivity assay. At higher temperatures and light intensities, however, no symptoms developed on inoculated leaves, and systemic infection was typified by a faint mosaic or mottle.

Bin 602 differed most noticeably from DW 192 and the type isolate of TomRSV in the difficulties encountered in purification and characterization. Before the virus had been definitely identified by serological tests, and while procedures for handling the virus were being decided upon empirically, several problems arose. Serological comparison was difficult because the concentration of Bin 602 in *N. clevelandii* frequently was low compared to those found with other isolates of TomRSV in the same host, even though test plants exhibited distinctive symptoms. The concentration of Bin 602 in *Cucumis sativus,* a host used by other researchers as a source of tissue for the purification of TomRSV (21), was even lower (comparisons were based on yields of virus obtained from identical quantities of tissue subjected to identical purification procedures and on the formation of precipitin reactions in double-diffusion serological tests). Serological identification became practical only when the virus was used in concentrated, partially purified preparations. Rate zonal centrifugation had to be done in glycerol, because although sedimentation profiles using sucrose were similar to those in Figure 1, no virus could be recovered by either dialysis or centrifugation. Extraction of nucleic acid was also not easy. Because the molecular weights of nucleic acids that we detected did not agree exactly with reported values (21), we speculated that the material was not pure and that some protein may still have been attached to the nucleic acid.

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