

## Characterization and Detection of the Strawberry Necrotic Shock Isolate of Tobacco Streak Virus

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### ABSTRACT

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An isolate of tobacco streak virus (TSV) was recovered from *Fragaria vesca* displaying typical symptoms of necrotic shock (NS). Host reactions, serological relationships, and RNA sequence homologies were compared among TSV-NS and isolates from blackberry (R), bean (RN), tobacco (M), and white clover (WC). The five isolates could be distinguished from each other by the responses of three experimental host species. Serological and Northern hybridization tests established two distantly related subgroups comprised of isolates NS and R in one group and WC, M, and RN in the other. Recombinant plasmids containing complementary DNA sequences

derived from TSV-NS RNA 3 were used to ascertain the suitability of dot hybridization tests for detection of TSV-NS infection in *Fragaria* spp. Cloned DNA sequences were labeled with  $^{32}\text{P}$  by nick translation and hybridized to nucleic acid extracts spotted onto nitrocellulose. The dot hybridization assay readily detected TSV-NS in extracts representing both acute and chronic infections of *Fragaria* spp., and the sensitivity of the assay was equivalent to that of double antibody sandwich enzyme-linked immunosorbent assay.

*Additional key words:* cDNA probes, serology.

Strawberry is susceptible to numerous diseases caused by virus and viruslike agents that can result in pronounced losses of vigor, production quality, and yield, particularly when multiple infections occur in the same plant (1,10,27). Detection and diagnosis of most of the economically important strawberry viruses currently rely on bioassay via graft transmission to indicator varieties (11,12) and on study of vector relationships (31,33). The inherent limitations of these procedures have placed constraints on meaningful epidemiological research in major production areas such as California and have limited improvement and expansion of existing clean stock programs.

California currently recognizes 12 virus and viruslike diseases of strawberry as being sufficiently important to warrant assay for plant certification (2). The causal agents of only two of these diseases (tobacco streak and tomato ringspot viruses) have been adequately purified to allow production of antisera. Three additional viruses (veinbanding, crinkle, and mild yellow edge) have been sufficiently characterized by electron microscopy and vector relationships to permit tentative grouping (8). The other diseases (mottle, pallidosis, leafroll, witches'-broom, latent C, feather leaf, and pseudo mild yellow edge) are of unknown etiology. Viruses to which antisera are available have been propagated in hosts suitable for purification. Such hosts have not, however, been recognized for most of the other viruses, and such viruses have not been purified from viscous and rapidly oxidizing strawberry extracts. Thus, antisera of sufficient quality are not available for use in serological tests. These limitations have precluded definitive studies on the etiological agents of important strawberry viral diseases and prevented implementation of rapid, sensitive methods for routine detection and diagnosis.

Application of recombinant DNA methods to the production of virus-specific detection probes offers an alternative to serological detection methods. The use of nucleic acid probes in solid phase dot hybridization assays is a rapid, accurate, and sensitive alternative to serology for diagnosis of plant virus infections

(3,30,34). We evaluated recombinant, complementary (c) DNA probes for strawberry virus detection by dot hybridization using tobacco streak virus (TSV) as a model system.

TSV is associated with the necrotic shock (NS) disease of strawberry (6,10,13,16,36), and the symptoms have been produced in strawberry plants mechanically inoculated with purified virus (16). Although TSV infection is symptomless in most commercial cultivars, yield losses may still be significant (23). Antiserum prepared to a *Rubus* isolate of TSV has been used to detect the virus in strawberry (23,35), suggesting that strawberry necrotic shock disease might be caused by a strain of TSV closely related to the *Rubus* isolate. Serological properties and the viral RNA of TSV-NS have not been characterized. We report here the production of cloned cDNA probes to TSV-NS RNA, a direct comparison of serological and nucleic acid hybridization methods for detection of TSV in strawberry, and the relationship of the strawberry isolate to four other TSV isolates from different hosts and geographic locations.

### MATERIALS AND METHODS

**Virus isolates, maintenance, and purification.** The isolates of TSV used were originally recovered from strawberry, *Fragaria* × *ananassa* Duchesne (NS); blackberry, *Rubus ursinus* Cham. & Schlecht. (R); bean, *Phaseolus vulgaris* L. (RN); tobacco, *Nicotiana tabacum* L. (M); and white clover, *Trifolium repens* L. (WC). Isolate NS was obtained from R. Stace-Smith, Vancouver, BC; isolates WC and M from R. W. Fulton, Madison, WI; and isolates R and RN from R. H. Converse, Corvallis, OR. Isolate NS was initially transmitted mechanically from *Fragaria vesca* L. to *N. clevelandii* Gray in 0.02 M Tricine (Bio-Rad, Richmond, CA), pH 8.0, with 4% polyethylene glycol (PEG), and 0.1% celite. Thereafter, all TSV isolates were mechanically inoculated using 0.1 M potassium phosphate buffer, pH 7.0, with 0.1% 2-mercaptoethanol and 0.1% celite. All isolates were propagated under ambient greenhouse conditions, with the exception of isolate NS, which required controlled conditions (12-hr illumination, 24 C) during the summer. Virions of WC and M were purified from *N. clevelandii* as described by Van Vloten-Doting (39). The other isolates were purified from *N. clevelandii* (NS) or *Chenopodium*

*quinoa* Willd. (R and RN) using a procedure similar to the acid clarification protocol of Lister et al (25). Fresh or frozen tissue was homogenized in two volumes of 0.1 M potassium phosphate, pH 7.0, with 0.1% sodium diethyldithiocarbamate and 0.1% 2-mercaptoethanol. Homogenates were strained through cheesecloth, clarified by adjusting the pH to 5.1 with 1 N HCl, stirred for 1 hr on ice, and centrifuged at low speed (8,000 g, 20 min). Supernatants were strained through Miracloth (Cal Biochem, La Jolla, CA), adjusted to pH 6.0 with 1 N NaOH, and the virus was concentrated by PEG precipitation (8%, no salt). Precipitates were recovered by low-speed centrifugation, and pellets resuspended in 50 mM potassium phosphate, pH 7.0, 10 mM ethylenediaminetetraacetic acid (EDTA). Virions were further purified by three cycles of differential centrifugation (8,000 g, 20 min; 70,000 g, 3.5 hr), with virions pelleted through a 5-ml layer of 30% sucrose in the first high-speed centrifugation. Further purification of all isolates was accomplished by rate-zonal centrifugation in 5–20% sucrose gradients. Virus concentrations in purified preparations were estimated with an extinction coefficient of  $5.1 \text{ (mg/ml)}^{-1} \cdot \text{cm}^{-1}$  at 260 nm (14).

**Serology.** Antiserum directed against isolate WC was provided by R. W. Fulton, Madison, WI. Antiserum to isolate NS was prepared in a rabbit immunized by intramuscular injection of 60  $\mu\text{g}$  of virions (purified by two cycles of rate-zonal sedimentation) emulsified in 1 ml of Freund's incomplete adjuvant on days 1, 8, and 40. The serum used was from a bleeding on day 72. Both antisera were absorbed with protein from healthy *N. clevelandii* (26). The immunoglobulin (Ig) fraction was purified from the antisera using DEAE-Affi Gel Blue (Bio-Rad) (32). Enzyme-antibody conjugates were prepared by coupling alkaline phosphatase (Type VII, Sigma, St. Louis, MO) to purified immunoglobulin using the glutaraldehyde procedure (5). Direct, double antibody sandwich and indirect enzyme-linked immunosorbent assay (ELISA) conditions and buffers used were as described by Lommel et al (26). For sandwich ELISA, Ig was coated onto polystyrene ELISA plates at  $1 \mu\text{g} \cdot \text{ml}^{-1}$ . Plant extracts were prepared by grinding 1-g samples in 10 ml of sandwich ELISA buffer (SEB) and centrifuged 1 min. Antigen samples (purified virions or plant extract supernatants) were serially diluted in SEB and 200- $\mu\text{l}$  aliquots applied to Ig-coated plates. Conjugates were applied at dilutions (1/400 [NS] or 1/100 [WC]) previously determined to yield optimal reactivity against homologous antigen with minimal reactivity against healthy plant protein. Optical density ( $A_{405\text{nm}}$ ) was measured 1 to 2 hr after addition of substrate containing 0.5 mM  $\text{MgCl}_2$ . Indirect ELISA was performed as described for sandwich ELISA except antigen samples were coated onto plates after dilution in 0.05 M carbonate buffer, pH 9.6, Ig was applied to antigen coated plates at  $1 \mu\text{g} \cdot \text{ml}^{-1}$ , and goat-antirabbit alkaline phosphate conjugate (Bio-Rad) was diluted 1/4,000 for use.

**Virion RNA purification.** RNA of all TSV isolates was extracted from gradient purified virions by the hot phenol method (39). Following precipitation in 70% ethanol, 0.1 M sodium acetate (EtOH/NaOAc) at  $-20^\circ\text{C}$  and resuspension in sterile distilled  $\text{H}_2\text{O}$  ( $\text{dH}_2\text{O}$ ), genomic RNAs 1, 2, and 3 were separated from slower sedimenting material by rate-zonal centrifugation in linear-log sucrose gradients (in 10 mM Tris-Cl, pH 8.0, 100 mM NaCl, and 1 mM EDTA) using a Beckman SW 41 rotor at 37,000 rpm for 10 hr at  $4^\circ\text{C}$ . Gradient fractions containing genomic RNAs were pooled, concentrated by ethanol precipitation, resuspended in  $\text{dH}_2\text{O}$ , and stored at  $-20^\circ\text{C}$ .

**Random cDNA probes.** Random cDNA probes synthesized using gradient purified NS or WC genomic RNAs were prepared (4) in a 50- $\mu\text{l}$  volume containing 100 mM Tris-Cl, pH 8.3, 140 mM KCl, 10 mM  $\text{MgCl}_2$ , 1.0 mM each dGTP, dATP, and dTTP, and 40  $\mu\text{M}$  dCTP (Sigma), 2 mM dithiothreitol, 25 units of RNasin (Biotec, Inc., Madison, WI), 5  $\mu\text{g}$  of heat denatured virion RNA, 5  $\text{mg} \cdot \text{ml}^{-1}$  of calf thymus DNA random primers (37), 50  $\mu\text{Ci}$  [ $\alpha\text{-}^{32}\text{P}$ ] dCTP (ICN, Irvine, CA), and 50 units of avian myoblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). This mixture was incubated at  $42^\circ\text{C}$  for 1.5 hr, and the reaction terminated by addition of EDTA to 20 mM. RNA template was

digested by alkali treatment (final NaOH concentration of 0.25 M) at  $65^\circ\text{C}$  for 1.5 hr. The reaction mixture was then neutralized by the addition of Tris-Cl, pH 8.0 (final concentration 0.25 M), followed by addition of sufficient HCl to neutralize the NaOH added previously. Yeast tRNA (5  $\mu\text{g}$ ) was added as a carrier, and the  $^{32}\text{P}$ -labeled cDNA was separated from unincorporated nucleotides by two precipitations with 70% ethanol, 0.67 M ammonium acetate (EtOH/ $\text{NH}_4\text{OAc}$ ) at  $-20^\circ\text{C}$ . The percent incorporation of  $^{32}\text{P}$ -labeled dCTP into cDNA was determined by the DE 81 filter binding assay (29) with repeated washing in 0.5 M  $\text{Na}_2\text{HPO}_4$  to remove unincorporated nucleotides from the filters. cDNA prepared in this manner typically incorporated a total of  $1\text{--}5 \times 10^7$  cpm as measured by scintillation counting.

**Molecular cloning of TSV-NS RNA.** The first strand of cDNA was synthesized as described for random cDNA probes, but the calf thymus DNA random primer concentration was reduced to 0.5  $\text{mg} \cdot \text{ml}^{-1}$ , all four dNTPs were used at 0.5 mM, and the [ $\alpha\text{-}^{32}\text{P}$ ] dCTP was reduced to 20  $\mu\text{Ci}$ . After a 1-hr incubation at  $42^\circ\text{C}$ , the reaction was terminated and products were extracted with phenol-chloroform (1:1), precipitated twice with EtOH/ $\text{NH}_4\text{OAc}$ , vacuum dried, and resuspended in  $\text{dH}_2\text{O}$ .

When alkali treatment was omitted after reverse transcription, first-strand cDNA remained duplexed with template RNA, which permitted synthesis of the second DNA strand by the RNase H-DNA Pol I procedure (18). Second-strand cDNA synthesis was accomplished in a 50- $\mu\text{l}$  reaction volume containing the products of the first strand reaction in 20 mM Tris-Cl, pH 7.6, 5 mM  $\text{MgCl}_2$ , bovine serum albumin at 0.5  $\text{mg} \cdot \text{ml}^{-1}$ , 0.5 mM of each dNTP, 1 mM dithiothreitol, 100 mM KCl, 5 mM  $\text{MgSO}_4$ , 10 units of DNA Pol I (BRL, Gaithersburg, MD), 1 unit of RNase H (BRL), and 25  $\mu\text{Ci}$  of [ $^3\text{H}$ ] dCTP (ICN). Reaction mixtures were incubated for 1 hr at  $15^\circ\text{C}$  followed by 1 hr at  $22^\circ\text{C}$  before extraction with phenol-chloroform. Nucleic acids were precipitated twice with EtOH/ $\text{NH}_4\text{OAc}$  and once with EtOH/NaOAc. Final pellets containing double stranded (ds) cDNA were vacuum dried and resuspended in  $\text{dH}_2\text{O}$ .

Homopolymeric (oligo dC) tails were added to the 3' termini of both cDNA strands (4) in a 25- $\mu\text{l}$  reaction volume using 40 units of terminal deoxynucleotidyl transferase (BRL) in 100 mM potassium cacodylate, pH 7.2, 2 mM  $\text{CoCl}_2$ , 0.2 mM dithiothreitol, bovine serum albumin at 0.5  $\text{mg} \cdot \text{ml}^{-1}$ , and 2.5 mM dCTP. Reactions were incubated at  $37^\circ\text{C}$ , with half of the mixture terminated after 1 min with EDTA and half after 5 min. The mixtures were pooled and DNA precipitated with EtOH/ $\text{NH}_4\text{OAc}$  (with 1  $\mu\text{g}$  of yeast tRNA added as carrier). Oligo dG tailed Pst-I digested pUC8 (provided by J. C. Carrington) was prepared similarly.

Homopolymeric tailed ds cDNA and plasmid were annealed in 10 mM Tris-Cl, pH 7.8, 100 mM NaCl, and 1 mM EDTA (29). Mixtures were incubated for 5 min at  $65^\circ\text{C}$  in a water bath, cooled slowly to  $42^\circ\text{C}$ , and incubated for 1 hr at  $42^\circ\text{C}$ . Annealed cDNA-plasmid was used immediately for transformation (29) of strain JM 83 of *Escherichia coli* (40) made competent by the method of Mandel and Higa (28) as modified by Carrington and Morris (4).

**Screening of recombinant clones.** Cells of *E. coli* transformed with recombinant plasmids were plated onto Luria agar containing ampicillin at 50  $\mu\text{g} \cdot \text{ml}^{-1}$ , 40  $\mu\text{M}$  isopropyl  $\beta$ -D-thiogalactopyranoside, and X-gal (Sigma) at 20  $\mu\text{g} \cdot \text{ml}^{-1}$  (40). After a 20-hr incubation at  $37^\circ\text{C}$ , ampicillin-resistant, white-pigmented colonies (containing plasmid with no  $\beta$ -galactosidase activity) were screened by colony hybridization (17) using random cDNA probe synthesized with NS RNA template. Recombinant plasmids from clones that hybridized to NS random cDNA probe were extracted from ampicillin containing broth cultures and separated from chromosomal DNA by CsCl-ethidium bromide gradients (29). Selected clones were further analyzed using the restriction enzyme Pst-I (New England Bio Labs, Beverly, MA) and by electrophoresis in 1% agarose.

**Nick translation of cloned cDNA.** CsCl purified recombinant plasmids containing cloned NS sequences were  $^{32}\text{P}$ -labeled by nick translation (29) using 50  $\mu\text{Ci} \cdot \text{mg}^{-1}$ . Purification of labeled plasmids from unincorporated nucleotides and determination of

percent incorporation were performed as described for random cDNA probes. Specific activity of labeled plasmids usually ranged between  $1 \times 10^7$  and  $7 \times 10^7$  cpm  $\cdot$   $\mu$ g $^{-1}$ . Labeled plasmids were stored at  $-20$  C in 10 mM Tris-CL, 1 mM EDTA (TE), pH 7.6; aliquots were removed and alkali-denatured for use as a probe.

**Dot hybridization.** Dot hybridization tests employing random or cloned cDNA probes were done by a procedure similar to that for tobacco mosaic virus (TMV) (34). Total single-stranded (ss) RNA was extracted from plants by the method of Carrington and Morris (4). One-gram leaf samples were ground to a powder in liquid N<sub>2</sub> and resuspended in 4 ml of 50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.1% 2-mercaptoethanol. After two phenol-chloroform extractions, nucleic acids were precipitated in ETOH/NaOAc. Pellets were vacuum dried, resuspended in 0.5 ml of TE, pH 7.6, and adjusted to a final concentration of 2 M LiCl. RNA was precipitated during a 4-hr incubation at 0 C, pelleted by centrifugation, washed with 100% ethanol, and resuspended in dH<sub>2</sub>O. Plant ssRNA or virion RNA were denatured in 6% formaldehyde at 65 C for 15 min, diluted 10-fold with  $10 \times$  SSC ( $1 \times$  SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and applied to BA 85 nitrocellulose filters (Schleicher and Schuell, Keene, NH), that had been presoaked in  $2 \times$  SSC, using a BRL Hybri-Dot manifold with vacuum and Whatman 3MM paper as a backing. Samples on nitrocellulose filters were air dried for 1 to 2 hr, then baked for 2 hr at 80 C under vacuum on a Bio-Rad gel slab dryer (Model 224).

Prehybridization treatment (12–24 hr at 42 C) of filters was in sealed plastic bags containing 50% deionized formamide,  $5 \times$  SSPE ( $1 \times$  = 150 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.4),  $5 \times$  Denhardt's solution ( $1 \times$  = 0.02% each Ficoll, polyvinylpyrrolidone-10, and bovine serum albumin), and 200  $\mu$ g  $\cdot$  ml $^{-1}$  autoclaved salmon testes DNA. Conditions for hybridization of probes to bound samples were the same as for prehybridization, except that Denhardt's solution was used at a final concentration of  $2 \times$ . Hybridized filters were washed twice in  $2 \times$  SSC, 0.1% SDS for 30 min each at room temperature, and twice in  $0.1 \times$  SSC, 0.1% SDS for 30 min each at 65 C, air dried, and exposed to XAR (Kodak) film at  $-70$  C with an intensifier screen (DuPont-Cronex).

**Northern blots.** Virion RNA or plant ssRNA preparation and electrophoresis were as described by Gustafson et al (19). RNA

samples were treated in electrophoresis buffer (20 mM HEPES [Sigma], pH 7.8, 1 mM EDTA), 6% formaldehyde, and 50% formamide for 4 min at 65 C before electrophoresis for 8–12 hr at a constant 50 V through a 1.5% agarose gel containing electrophoresis buffer and 6% formaldehyde. The RNA was then transferred to nitrocellulose by blotting overnight in  $20 \times$  SSC. Baking, prehybridization, hybridization, washing, and autoradiography were as indicated for dot hybridization.

## RESULTS

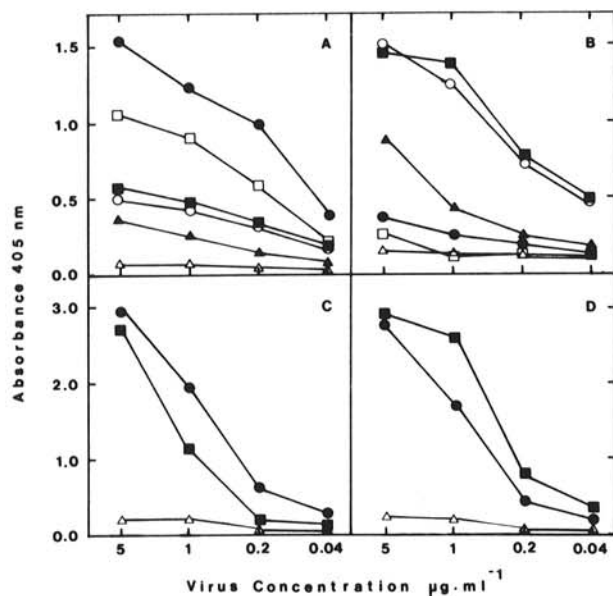
**Comparative analyses of TSV isolates. Host symptoms.** The host response of three experimental species to infection was sufficient to distinguish all five isolates. Only WC had the ability to induce toothed margins of new leaves of chronically infected (2 to 3 mo after inoculation). *N. tabacum* L. NS was distinct from other isolates producing symptoms in *Nicotiana* spp. (WC and M); NS caused mild symptoms on *N. tabacum* (generally only local necrotic lesions on inoculated leaves and sporadic, mild necrotic line patterns on systemic leaves) but caused more severe systemic necrosis on *N. clevelandii* than that caused by isolates WC and M. Infection by mechanical inoculation of NS was difficult to achieve during the summer months unless experimental species were grown under controlled conditions, although isolates WC and M readily produced symptoms year-round on plants under ambient greenhouse conditions. Isolates R and RN, which did not induce symptoms on *Nicotiana* spp., could be distinguished on *C. quinoa*. Isolate R readily produced systemic symptoms on *C. quinoa*, whereas RN produced only local lesions.

**Virus yields.** The five isolates varied in the amount of purified virions obtained per weight of tissue extracted. Isolates WC and M gave high yields, typically being 30–40 mg  $\cdot$  kg $^{-1}$ . Yields of isolates NS and RN were much lower, being 1.0–2.2 and 0.8–2.6 mg  $\cdot$  kg $^{-1}$ , respectively. Isolate R yields were intermediate, being 7–10 mg  $\cdot$  kg $^{-1}$ .

**Serology.** The two antisera differed in the ability to detect heterologous isolates in direct, double antibody sandwich ELISA (Fig. 1A and B). At equivalent virus concentrations, the R isolate reacted most strongly with the NS antiserum relative to the other heterologous isolates. NS antiserum reacted weakly to the other three isolates in sandwich ELISA. Conversely, WC antiserum reacted weakly in sandwich ELISA with the NS and R isolates, reacted to an intermediate degree with the RN isolate, and could not distinguish between isolate M and homologous WC isolate. Both antisera were more permissive in recognizing heterologous antigens by indirect ELISA as both NS and WC nucleoprotein were readily detected with the heterologous serum (Fig. 1C and D).

**Genomic RNAs.** Relative electrophoretic mobilities of genomic RNAs 1, 2, and 3 ( $M_r$  1.1, 1.0, and  $0.8 \times 10^6$ , respectively) were similar for all isolates under denaturing conditions as visualized by ethidium bromide staining (Fig. 2). RNA component ratios varied among isolates and generally reflected the ratios of nucleoprotein components in rate-zonal sucrose gradients. Isolate M contained a major RNA species (designated 3') that was not present in the other isolates. Levels of minor RNA components (including subgenomic RNA 4) varied in unfractionated RNA preparations of all isolates and were largely removed along with degraded RNA from genomic RNAs (and M RNA 3') by rate-zonal gradient centrifugation before electrophoresis.

**RNA hybridization.** Northern blot analysis was used to evaluate the relatedness of genomic RNAs of the five isolates (Fig. 3). Randomly primed cDNA probe synthesized using NS RNA template hybridized to all three genomic RNA species of the R isolate but less than to the homologous NS RNA species (Fig. 3A). No hybridization with RNAs of the other three isolates was detected under these relatively stringent hybridization conditions. With cDNA to the WC isolate (Fig. 3B), strong hybridization occurred with WC and M RNAs, and some hybridization occurred with RN genomic RNAs. To evaluate possible distant relationships, NS cDNA was reacted with the genomic RNAs of isolates WC, M, and RN, and TMV RNA using a probe with

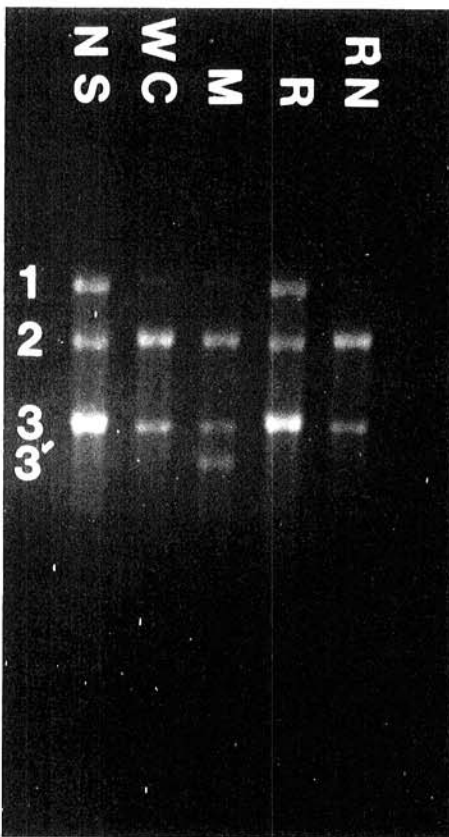


**Fig. 1.** Serological relatedness of tobacco streak virus isolates in enzyme-linked immunosorbent assays (ELISA). Direct sandwich ELISA (A and B) and indirect ELISA (C and D) using antisera to: A and C, the strawberry necrotic shock (NS) isolate; and B and D, the white clover (WC) isolate. All antigens were purified virions; ● = NS, ■ = WC, ○ = tobacco mild (M), □ = blackberry (R), ▲ = bean red node (RN), and Δ = virions of tobacco mosaic virus. Each point represents the mean of two replicates.

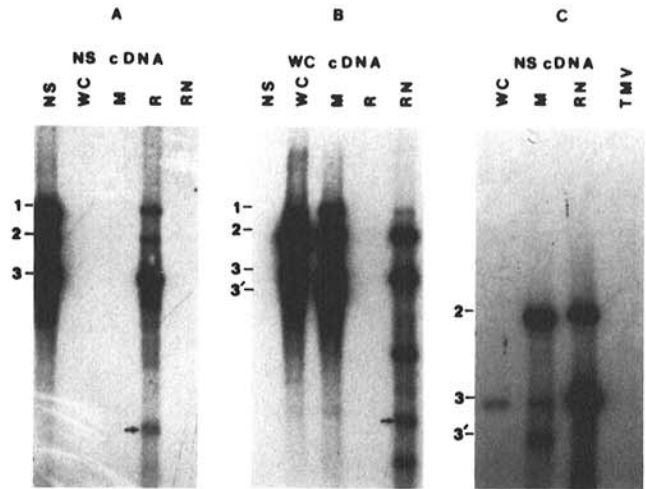
higher activity and prolonged autoradiographic exposure (Fig. 3C). Under these conditions, NS cDNA hybridized weakly to WC RNA 3 and M RNAs 3 and 3', and also hybridized to a greater degree to M RNA 2 and RN RNAs 2 and 3 as determined by the relative intensity of the bands on the autoradiogram. No hybridization of NS cDNA with TMV RNA was detected. Hybridization of random cDNAs to minor RNA components, particularly in R and RN preparations, indicated that fractionation of genomic RNAs from minor components and breakdown products was incomplete. However, this contamination allowed for detection of homology of R RNA 4 with NS cDNA and of RN RNA 4 with WC cDNA.

**Recombinant clones.** Two recombinant cDNA clones (designated pTSV-NS 2 and pTSV-NS 5) that hybridized strongly to NS random cDNA in colony hybridization tests were analyzed. Because homopolymeric tailing provides for regeneration of Pst-I sites flanking GC sequences on either side of the inserts, size estimates of cloned NS sequences were determined by electrophoresis in 1% agarose of Pst-I digested recombinant plasmids (Fig. 4). pTSV-NS 2 contained a 460-base pair (bp) insert, and pTSV-NS 5 contained a 320-bp insert (including GC borders of undetermined lengths). Inserts from these two plasmids were subcloned to form a composite plasmid (pTSV-NS 16) containing a single copy of each insert to increase the amount of <sup>32</sup>P-labeled residues incorporated into NS sequences by nick translation. Subcloning was accomplished by recovering the small Pst-I fragments from 1% agarose gels using Whatman DE 81 filter paper (7), ligation with T4 DNA ligase (BRL), and transformation of *E. coli* after ligation to Pst-I digested pUC8.

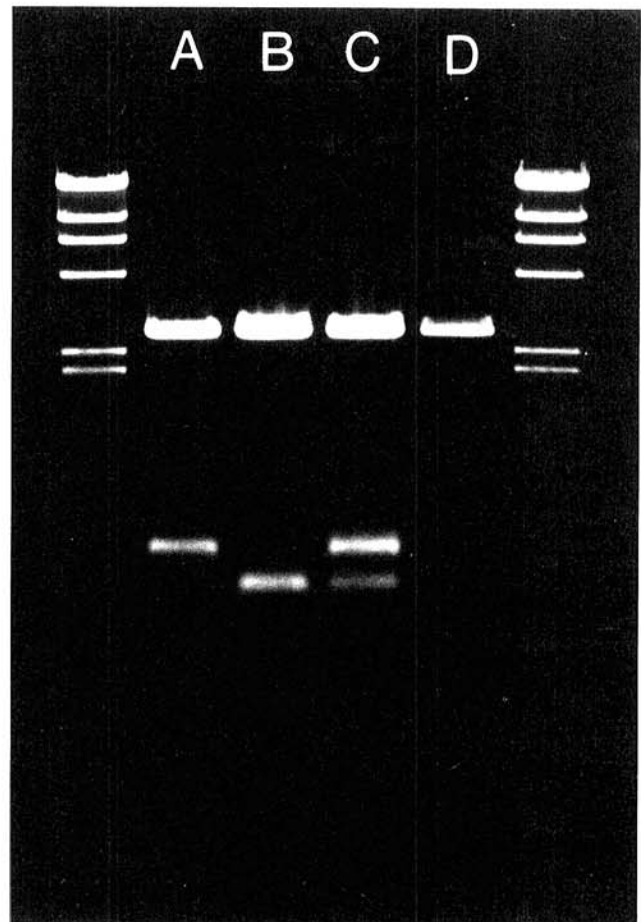
NS cDNA inserts of pTSV-NS 2 and pTSV-NS 5 (also pTSV-NS 16) hybridized with NS RNA 3 as shown by Northern blot analysis of genomic RNAs (Fig. 5). Southern blot analysis (29) indicated some cross-hybridization between the small Pst-I fragments of pTSV-NS 2 and pTSV-NS 5 (data not shown).



**Fig. 2.** Electrophoresis of genomic RNAs of tobacco streak virus isolates from strawberry (NS), white clover (WC), tobacco (M), blackberry (R), and bean (RN). RNAs (500 ng) were electrophoresed under denaturing conditions in 1.5% agarose, 6% formaldehyde for 8 hr at a constant 50 V and stained with ethidium bromide. Numbers refer to RNA species.



**Fig. 3.** Northern blot analysis of genomic RNAs of five tobacco streak virus (TSV) isolates. RNAs probed with  $1 \times 10^6$  cpm  $\cdot$  ml<sup>-1</sup> random cDNA to **A**, Strawberry necrotic shock (NS) isolate (2-hr autoradiogram) and **B**, White clover (WC) isolate (3-hr autoradiogram). **C**, TSV RNAs and TMV RNA probed with  $2 \times 10^6$  cpm  $\cdot$  ml<sup>-1</sup> NS random cDNA (72-hr autoradiogram). All samples were gradient fractionated genomic RNAs (50 ng). Electrophoresis was in 1.5% agarose, 6% formaldehyde for 8 hr (A and B) or 12 hr (C) at a constant 50 V. Numbers refer to major RNA species. Arrows indicate trace amounts of subgenomic RNA 4 remaining after fractionation of genomic RNAs.



**Fig. 4.** Electrophoresis in 1% agarose of Pst-I digested recombinant pUC8 plasmids bearing inserts complementary to RNA of the strawberry necrotic shock (NS) isolate of tobacco streak virus (TSV). Lane A, pTSV-NS 2; lane B, pTSV-NS 5; lane C, pTSV-NS 16; and lane D, pUC8. Outer lanes are Hind III digested lambda DNA size standards.

Whether the cross-hybridization between the two small Pst-I fragments reflects related NS sequences on both inserts or resulted from cross-hybridization of GC borders flanking the ends of NS sequences on both inserts was not determined. In dot hybridization tests, pTSV-NS 16 hybridized to RNA of the heterologous isolates R and RN (Fig. 6). All three clones hybridized strongly to R RNA 3 but not to R RNA 4 in Northern blots (data not shown).

**Detection of TSV-NS in plants. Analysis of NS RNAs in plant RNA extracts.** NS random cDNA and pTSV-NS 16 probes hybridized primarily with NS RNA 3 from infected plants in Northern blots (Fig. 7). RNAs 1 and 2 were detected in plant extracts with random cDNA but in lower concentrations than those of RNA 3. Minor components migrating faster than RNA 3 also hybridized with NS random cDNA and cloned probes. These bands appear to be virus specific, as no hybridization was detected with healthy control extracts. *N. clevelandii* ssRNA extracts appear to contain approximately eightfold more NS RNA

sequences than *F. vesca* ssRNA extracts, as similar hybridization signals were obtained from infected extracts when 2 µg of *F. vesca* ssRNA and 250 ng of *N. clevelandii* ssRNA were used in Northern blots. As the yield of ssRNA from *N. clevelandii* was consistently 5–10 times greater than *F. vesca*, infected *N. clevelandii* tissue appears to contain approximately 40–80-fold more NS RNA than an equal amount of *F. vesca* tissue.

**Random cDNA and cloned probes.** NS random cDNA and pTSV-NS 16 were compared in replicate dot hybridization tests for sensitivity of detection of NS sequences in plant extracts (Fig. 8). All three cloned probes readily detected NS RNA in plant extracts, but the random cDNA probe was more sensitive than cloned probes even when  $0.1 \times 10^6$  cpm · ml<sup>-1</sup> of random cDNA (specific activity  $2 \times 10^6$  cpm · µg<sup>-1</sup>) and  $1 \times 10^6$  cpm · ml<sup>-1</sup> of pTSV-NS 16 (specific activity  $2 \times 10^7$  cpm · µg<sup>-1</sup>) were used.

**Dot hybridization and ELISA.** The sensitivities of detection of NS and three field isolates from strawberry in graft-inoculated *F. vesca* by dot hybridization and by sandwich ELISA were approximately equal as determined by sample dilution end point (Figs. 9 and 10). A 200–800-µg tissue sample was required for detection in both tests. Although indirect ELISA was able to detect

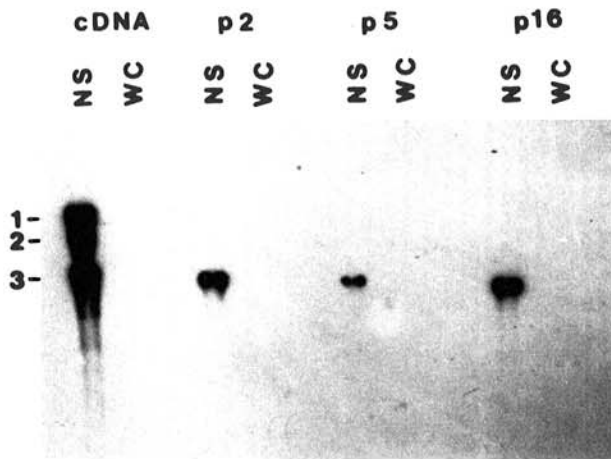


Fig. 5. Northern blot analysis of virion RNA (50 ng) from the strawberry necrotic shock (NS) and white clover (WC) isolates of tobacco streak virus (TSV) probed with  $1 \times 10^5$  cpm · ml<sup>-1</sup> NS random cDNA or  $5 \times 10^5$  cpm · ml<sup>-1</sup> pTSV-NS 2 (p2), pTSV-NS 5 (p5), or pTSV-NS 16 (p16). Numbers at left refer to genomic RNA species.

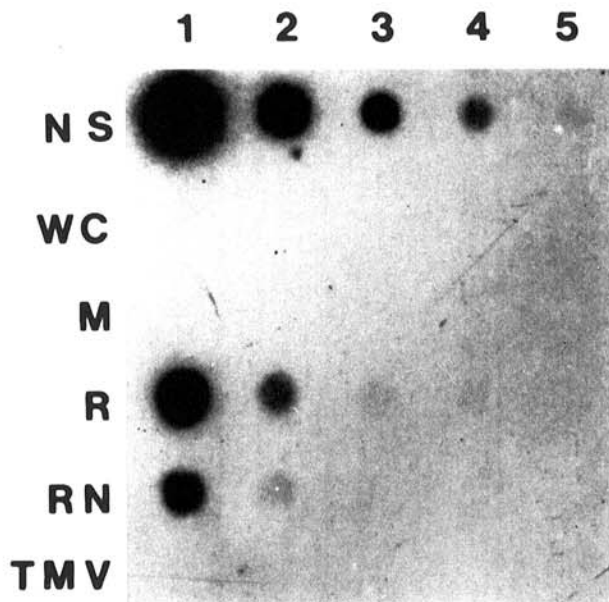


Fig. 6. Dot hybridization of RNAs of tobacco streak virus isolates from strawberry (NS), white clover (WC), tobacco (M), blackberry (R), bean (RN), and RNA from tobacco mosaic virus (TMV) probed with pTSV-NS 16 ( $1 \times 10^6$  cpm · ml<sup>-1</sup>). Numbers denote serial fivefold dilutions of virion RNA, with dilution 1 representing 200 ng.

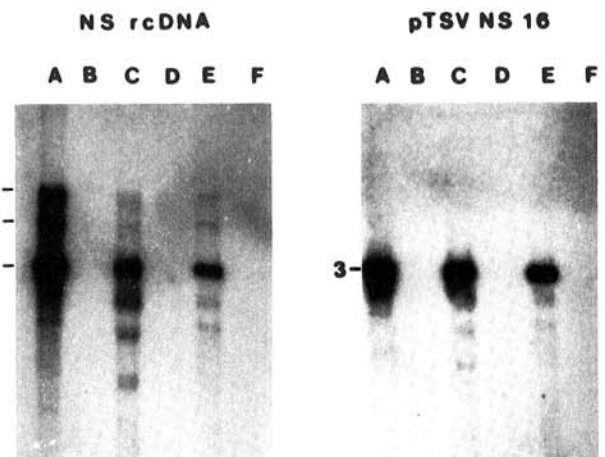


Fig. 7. Northern blot analysis of RNA extracts from plants probed with necrotic shock (NS) random cDNA ( $1 \times 10^5$  cpm · ml<sup>-1</sup>) or pTSV-NS 16 ( $5 \times 10^5$  cpm · ml<sup>-1</sup>). Virion RNAs (50 ng), from the strawberry NS (Lane A), and white clover (WC) (Lane B) isolates. RNA (250 ng) from NS infected *Nicotiana clevelandii* (Lane C), or uninfected *N. clevelandii* (Lane D). RNA (2 µg) from NS infected *Fragaria vesca* (Lane E), or uninfected *F. vesca* (Lane F). Numbers refer to genomic RNA species.

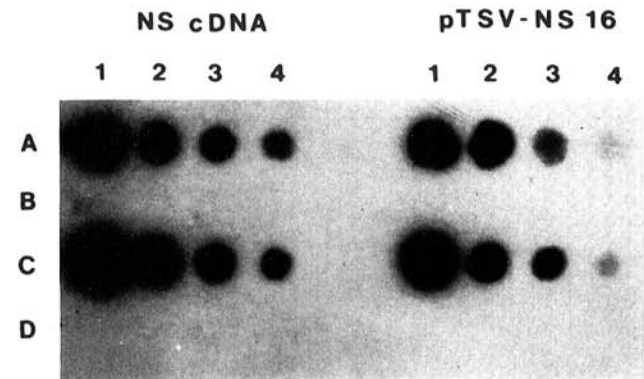


Fig. 8. Replicate dot hybridization assays of plant RNA extracts probed with NS random cDNA ( $0.1 \times 10^6$  cpm · ml<sup>-1</sup>) and pTSV-NS 16 ( $1 \times 10^6$  cpm · ml<sup>-1</sup>). Samples used were: A, *Fragaria vesca* infected with the strawberry necrotic shock (NS) isolate; B, uninfected *F. vesca*; C, *Nicotiana clevelandii* infected with the NS isolate; and D, uninfected *N. clevelandii*. Numbers denote serial fivefold dilutions of samples with dilution 1 representing 20 mg of tissue from *F. vesca* or 0.5 mg from *N. clevelandii* tissue.

NS infection in *Nicotiana* extracts, the assay was unsuitable for detection of virus infection in *Fragaria* because of high levels of nonspecific background associated with healthy control samples.

Attempts to mechanically inoculate TSV-NS to *F. vesca* were unsuccessful using crude inoculum or purified virions at concentrations up to  $1 \text{ mg} \cdot \text{ml}^{-1}$ . All mechanically inoculated *F. vesca* plants remained symptomless during a 6-mo period and tested negative by both sandwich ELISA and dot hybridization assays conducted 1, 4, and 6 mo postinoculation. In contrast, when inoculations were done by grafting, both tests readily detected NS infection in newly grafted symptomatic *F. vesca* and *F. virginiana* Duchesne and in symptomless commercial strawberry cultivars. Both assays were also capable of detecting NS infection in *Fragaria* spp. that had been graft inoculated up to 2 yr before testing. cDNA probes also detected NS infection of graft inoculated *F. vesca* that did not exhibit the acute necrotic shock phase of the disease (0–5%) upon which the bioassay by grafting to *Fragaria* hosts depends. In these cases, diagnosis by dot hybridization was confirmed by ELISA. Furthermore, both assays detected NS infection in *Fragaria* spp. during the summer months when bioassay by mechanical inoculation to *C. quinoa* (35) or *N. clelandii* is difficult to accomplish.

Detection of NS RNA sequences in strawberry extracts by dot hybridization was enhanced by an unknown mechanism. When NS genomic RNAs were added to ssRNA from healthy *F. vesca* before formaldehyde treatment, the signal was 25–100 times that of virion RNA alone (Fig. 11). This enhancement did not result from nonspecific hybridization to healthy RNA (no hybridization was detected with healthy ssRNA extracts), or from a carrier effect of healthy RNA (equal amounts of RNA from healthy *N. clelandii* or yeast tRNA had no effect on hybridization when added to NS virion RNA, data not shown).

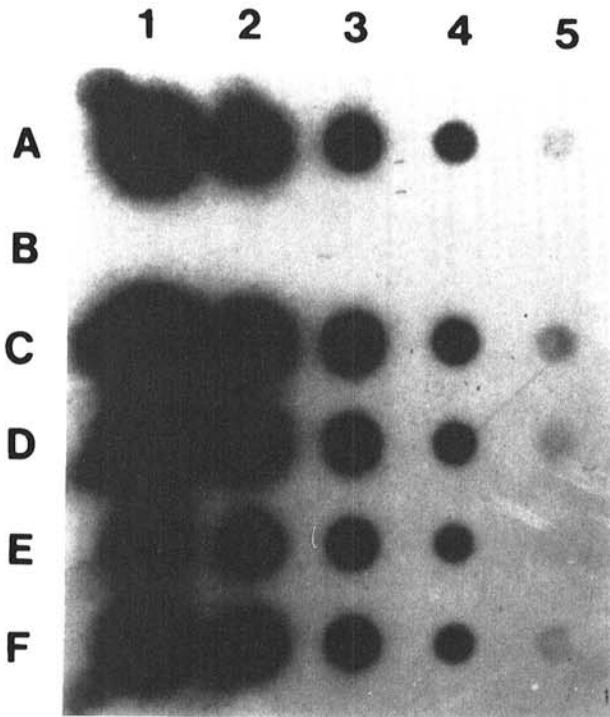


Fig. 9. Detection of three field isolates of tobacco streak virus (TSV) from strawberry by dot hybridization using pTSV-NS 16 probe ( $1 \times 10^6 \text{ cpm} \cdot \text{ml}^{-1}$ ). Samples A–F were applied to nitrocellulose as serial fivefold dilutions (1–5, with dilution 1 equivalent to 100 mg of tissue or 50 ng virion RNA). A, Virion RNA from the strawberry necrotic shock (NS) isolate. RNA samples extracted from B, uninfected *Fragaria vesca*, C, NS infected *F. vesca*, or D–F, *F. vesca* infected with three field isolates of TSV collected from strawberry in central California in 1985. All plant samples were extracted from half leaflets; the other halves were used in enzyme-linked immunosorbent assays (Fig. 10).

## DISCUSSION

**Strain relationships.** Isolates of TSV vary considerably in biological and biochemical properties. Isolate NS could be distinguished from the other four isolates by symptom expression in three experimental species. Our serological and RNA hybridization tests established two subgroups of closely related isolates. By serological comparisons, NS was distinct from isolates WC, M, and RN and most closely related to isolate R. Hybridization assays confirmed the close relationship between isolates NS and R and suggest that they compose a distinct subgroup with a distant genetic relationship to the second subgroup containing isolates WC, M, and RN. Using antiserum against another isolate from *Rubus*, field isolates of TSV from strawberry in the Pacific Northwest (23) and Israel (35) appear serologically closely related. Furthermore, as many TSV isolates

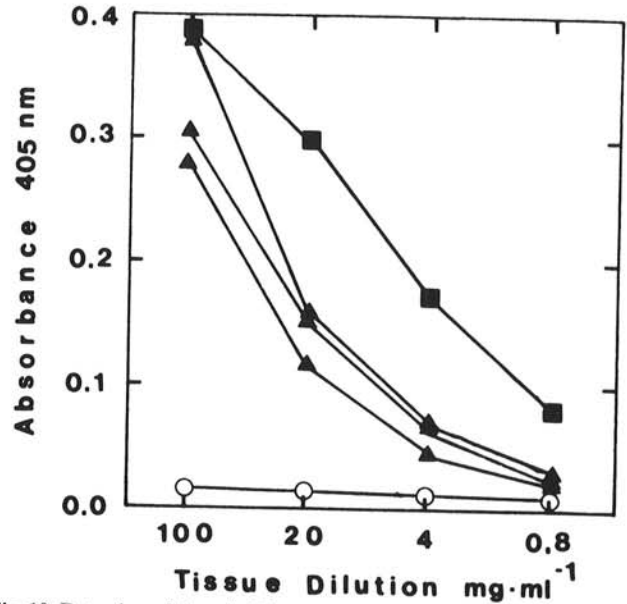


Fig. 10. Detection of three field isolates of tobacco streak virus (TSV) from strawberry by direct sandwich enzyme-linked immunosorbent assays using antiserum to the strawberry necrotic shock (NS) isolate. ■ = serial dilutions of extracts from *Fragaria vesca* infected with TSV-NS. ▲ = dilutions of extracts from *F. vesca* infected with field isolates of TSV from strawberry. ○ = dilutions of extracts from uninfected *F. vesca*. All plant samples were obtained from half leaflets; the other halves were used in dot hybridization tests (Fig. 9).

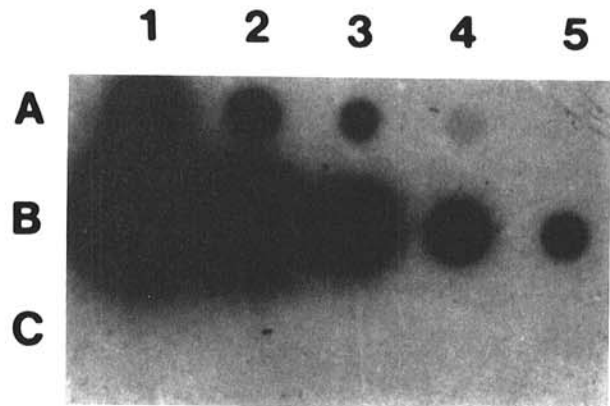


Fig. 11. Effect of RNA extracts from uninfected *Fragaria vesca* on detection of virion RNA of the strawberry necrotic shock (NS) isolate in dot hybridization tests. A, NS virion RNA alone; B, NS virion RNA added to RNA from uninfected *F. vesca*; and C, Uninfected *F. vesca* RNA alone. Numbers denote serial fivefold dilutions with dilution 1 representing 50 ng of virion RNA, and/or ssRNA extracted from 100 mg of tissue. Probe used was pTSV-NS 16 ( $1 \times 10^6 \text{ cpm} \cdot \text{ml}^{-1}$ ).

from *Rubus* spp. produce necrotic shock syndrome when graft-inoculated from *Rubus* to *Fragaria* (9), isolates from these roseaceous hosts may form a cluster of closely related isolates. However, the inability to introduce TSV isolates not already infecting graft-compatible species precluded us from determining if the ability to induce necrotic shock in *Fragaria* is a trait unique to roseaceous isolates. Isolates WC and M appear nearly identical by serological and hybridization tests and could only be distinguished by RNA 3' in M RNA preparations and by host responses of *N. tabacum*. M RNA 3' does not appear to contain novel genomic material, as WC random cDNA hybridized strongly to M RNA 3'. Production of RNA 3' by isolate M apparently depends on the species in which the isolate is propagated (25). Of the isolates closely related to WC, RN appears to have more in common with isolates in the NS subgroup, as determined by hybridization tests.

Apparent relatedness among isolates varied depending on the assay used. Host reactions were the most specific means of distinguishing among isolates closely related by serological and nucleic acid hybridization tests. Indirect ELISA was the most permissive in recognizing distantly related isolates, as shown for several other plant virus groups (24,38). Our results suggest that isolates of both subgroups should remain classified as strains of TSV, in keeping with the guidelines for the identification and characterization of plant viruses by Hamilton et al (21). Halk et al (20) used monoclonal antibodies against isolate WC to serotype five isolates of TSV, but isolates M, RN, R, and NS were not tested. Harrison (22) suggested that strain groupings into biological species for multipartite RNA plant viruses can be based on the ability to form pseudorecombinants. Although Fulton (15) described the production of pseudorecombinants between TSV isolates including WC and M, isolates NS, RN, and R were not included in the study.

**Etiology of the necrotic shock syndrome.** In contrast to a previous report (16), we were unable to infect *Fragaria* spp. with TSV-NS by mechanical inoculation. Although the acute symptoms of necrotic shock are typically associated with the presence of TSV in *Fragaria* indicator spp. (36), the inability to reintroduce purified virus to *Fragaria* prevented us from determining if TSV-NS is solely responsible for the necrotic shock syndrome. Despite this, the ability to detect TSV in commercial strawberry cultivars is important as yield loss has been associated with the presence of TSV in symptomless commercial cultivars (23). The ability to test breeding stock for TSV is crucial, as the virus is seedborne (23) and may be transmitted to the F<sub>1</sub> generation via an infected parent.

**Detection of NS in plant extracts.** Northern blot analysis of RNA extracts from plants revealed that the predominant species synthesized *in vivo* by NS was RNA 3, which was also the major species encapsidated. This was important in selecting cloned probes for use in diagnostic tests. Dot hybridization tests using cloned cDNA probes to NS RNA 3 readily detected NS infection in *F. vesca* and *F. virginiana* and in commercial strawberry cultivars. The probes we prepared from cloned viral sequences represent only a small portion of the viral genome and were predictably less sensitive than the random cDNA probes used for virus detection. Cloned probes, however, offer two important advantages over the use of random cDNA for routine virus detection: Preparation of nick translated probe from permanently available sequences is simpler, and cloned probes are more consistent in quality and less subject to background signal problems caused by host sequence contamination in some viral RNA preparations. Background hybridization was negligible with cloned probes and was seldom evident on autoradiograms after 24–72 hr of exposure.

Time required for sample preparation from *N. clevelandii* could be shortened by omitting the ethanol and LiCl precipitations. With *Fragaria* extracts, hybridization signals were weak and nonspecific using either the aqueous phase following phenol-chloroform extraction or total nucleic acid precipitated with ethanol; the same samples processed further with LiCl produced strong disease-specific hybridization signals. Other sample preparation protocols tested, including the method successfully used by Maule et al (30) for other RNA viruses were unsuitable for use with viscous

*Fragaria* extracts, which rapidly obstructed nitrocellulose filters. Northern blot analysis of ssRNA extracts indicated infected *N. clevelandii* contained 40–80-fold more detectable NS sequences than an equal weight of *F. vesca* tissue. However, since the enhancement effect of healthy *Fragaria* ssRNA extracts observed in dot hybridization was not examined for Northern blots, the actual difference in viral RNA titre may be even greater if the enhancement effect also occurs in Northern hybridizations. As only *Fragaria* ssRNA extracts, but not yeast tRNA or *N. clevelandii* ssRNA extracts, produced enhancement of signal it appears that enhancement in dot hybridization may be due to an unidentified contaminant(s) unique to *Fragaria* RNA extracts.

Sensitivities of dot hybridization and ELISA were roughly equivalent in detection of NS in *Fragaria*. Sample preparation of *Fragaria* tissue is more complicated for dot hybridization than ELISA, and for this reason dot hybridization may not become the method of choice for detection of TSV in strawberry. Several published reports (23,35) attest to the ability of antiserum made against a *Rubus* isolate to detect TSV in strawberry by ELISA. However, the demonstration that cloned cDNA probes can detect virus nucleic acids in strawberry extracts indicates that molecular hybridization tests should be applicable to the detection of other, more recalcitrant agents as cDNA clones to them become available. When diagnostic probes to the economically important disease agents of strawberry have been produced, molecular techniques will simplify the indexing of nursery, field, and breeding plants.

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