Comparison of the RNAs and Some Physicochemical Properties of the Seed-Transmitted Tobacco Streak Virus Isolate Mel 40 and the Infrequently Seed-Transmitted Isolate Mel F

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Plant Pathology New Series 0151, College of Agriculture and Home Economics, Research and Extension Center, Washington State University, Pullman 99164.

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Accepted for publication 23 May 1995.

ABSTRACT

Walter, M. H., Wyatt, S. D., and Kaiser, W. J. 1995. Comparison of the RNAs and some physicochemical properties of the seed-transmitted tobacco streak virus isolate Mel 40 and the infrequently seed-transmitted isolate Mel F. Phytopathology 85:1394-1399.

The viral genetic basis of symptom severity and seed transmission was investigated with tobacco streak virus (TSV) pathotype I isolate Mel 40 and pathotype II isolate Mel F. These isolates differ in physicochemical properties and RNA secondary structure. Electrophoresis of RNA from the mild, infrequently seed-transmitted TSV isolate Mel F revealed many minor RNA species not detected in the severe, seed-transmitted isolate Mel 40. Inoculations with gel-purified Mel F genomic RNAs 1, 2, and 3 produced atypically severe symptoms on *Chenopodium quinoa* and bean (*Phaseolus vulgaris*). Severe symptoms were correlated with the absence of minor RNAs in the inoculum. Subisolate Mel FS arose as a rare seed transmission event of Mel F in bean. It was transmitted to approximately 30% of progeny seedlings but appeared otherwise indistinguishable from Mel F. Non-seed-transmitted Mel F encapsidated one minor RNA, designated RNA F5, which was not detected in seed-transmitted Mel FS viral RNA. Six heterologous RNA combinations from Mel F and Mel 40 were inoculated on *C. quinoa* by using gel-purified genomic RNAs. Only one pseudorecombinant isolate was sufficiently infectious for transfer and characterization, causing a slow-spreading infection that was not seed transmitted. Infectivity of TSV Mel 40 and Mel F appears to be dependent on homologous RNAs.

Although many seed-transmitted viruses have been studied (4), little is known about the physicochemical and genetic basis of seed transmission. Ilarviruses infect a wide range of woody and herbaceous hosts from which they are often seed and/or pollen transmitted. Several isolates of tobacco streak virus (TSV) have been characterized and differences in seed transmission rates in several hosts noted (8–10,17,18,31,33). TSV was used as a model to further study the physicochemical basis of seed transmission.

The TSV pathotype I isolate Mel 40 and the pathotype II isolate Mel F differ in symptoms, seed transmission, serology, and some physicochemical properties (9,17,24,30,31,33). Both Mel 40 and Mel F systemically infect *Chenopodium quinoa* Willd. and the bean (*Phaseolus vulgaris* L.) cultivar Black Turtle Soup (BTS) and replicate to approximately the same levels (17). Mel 40 seed transmission is approximately 15% in inoculated plants but about 33% in plants infected through seed transmission (17). Mel 40 appears to enter the bean flower ovule primarily though pollination (33). In contrast, Mel F is seed transmitted at approximately 0.5% from inoculated BTS and at a similarly low rate in other bean cultivars. Mel F is naturally seed transmitted in *Melilotus alba* Medik. (18).

The genetics of infectivity and seed transmission of multipartite viruses has been studied through pseudorecombination of both nucleoprotein and RNA components (11–13,15,16). Infectivity of pseudorecombinants depends on RNA compatibility, while symptoms and seed transmission may be determined by one or more RNAs. RNA 1 of tomato black ring virus strain G is necessary for infectivity of RNA pseudorecombinants between strains A and G (27). Heterologous combinations of RNAs 1 and 2 are incompatible in pseudorecombinants between cowpea chlorotic mottle (CCMV) and brome mosaic bromovirus (BMV) (2). Seed transmission, some host reactions, and replication of raspberry ringspot virus were assigned to RNA-1 of the bipartite nepovirus (13–16). Cucumber mosaic virus RNA-1 was linked to seed transmissibility in beans (12).

Our objective in this study was to assign low seed-transmission rate and disease severity to one or more of the viral genomic or minor RNAs by evaluating RNA pseudorecombinants of Mel 40 and Mel F and by studying the results of other inoculations with gel-purified RNAs.

MATERIALS AND METHODS

Virus source. Two previously described pathotypes of TSV (17,18,33) were used in our study. The TSV pathotype I isolate Mel 40 originated from a seedborne infection of *M. alba* at Central Ferry, Washington. The TSV pathotype II isolate Mel F was recovered from a symptomless infection of *M. alba,* also at Central Ferry (17). Both isolates were transferred to and main-

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tained on *C. quinoa* or transferred to BTS as previously described (33).

Virus detection and purification. Virus detection by indirect enzyme-linked immunosorbent assay (ELISA) and rub inoculation was carried out as previously described (33). Virus purification was based on a modified procedure by Ong and Mink (26). Chloroform-clarified homogenate from systemically infected *C. quinoa* plants was additionally clarified by low pH 5.3. Virus was purified by centrifugation for 2 h at 175,000 × *g* through a 20% sucrose pad.

Sucrose density gradient centrifugation. Samples (60 μg) of virions were loaded on 10 to 40% sucrose density gradients (0.02 M sodium acetate buffer, pH 6.4) for analytical rate zonal centrifugation. Virus was centrifuged for 2.5 h at 200,000 × *g* at 4°C.

RNA purification, electrophoresis, and recovery. Isolation and purification of TSV RNA was based on previously published methods (6). RNA was denatured according to McMaster and Carmichael (23) and purified by electrophoresis in 1.5% low melting point ultrapure agarose minigels (Bethesda Research Laboratories, Gaithersburg, MD). Gels were stained with ethidium bromide or 0.05% toluidine blue O (Fisher Scientific, Fair Lawn, NJ). RNA bands were cut from gels, phenol extracted, precipitated in ethanol, and reelectrophoresed for final separation and recovery of the single-species RNAs.

RNA inoculation. Local lesion assays of RNA infectivity were carried out on *C. quinoa* as described by Smit and Jaspars (29). RNA species composition was determined by inoculations with single or paired species of homologous RNAs onto young leaves of *C. quinoa*. All RNA inoculation mixtures contained 1 to 3 picomoles of RNA (1 mole of TSV RNA = approximately 1.8 × 10^6 g), 10 to 30 picomoles of alfalfa mosaic virus (AMV) coat protein (CP) (1 mole of AMV CP = approximately 24,500 g), 1 U of RNAsin, 0.01 M dithiothreitol, and 2% Celite in filter-sterilized 1× PEN buffer (0.01 M sodium phosphate, 0.001 M EDTA, and 0.01% sodium azide, pH 7.8) (20).

RESULTS AND DISCUSSION

Symptomatology. As in previous studies (17), rub inoculations with Mel 40 produced extensive tissue necrosis on inoculated leaves and apical necrosis on BTS and *C. quinoa*. Infected BTS plants often displayed severe systemic necrosis followed by production of new, slightly dwarfed, but otherwise symptomless shoots that also contained virus. Inoculations with Mel F resulted in mild stunting, leaf distortion, and yellow mosaic on trifoliolate leaves. Seedlings of BTS infected with Mel 40 by seed transmission displayed no obvious symptoms at the two-leaf stage, although virus was detectable by rub inoculation on *C. quinoa* and by ELISA.

Effects of pH on virus particle stability and sedimentation. Differences in particle stability have been observed in Mel F and Mel 40 and described in previous work (17). Mel F was also less stable during purification above pH 7.0. Decreased stability of Mel F could be linked to a possible difference in CP size (17) or to minor RNAs in Mel F virions, as discussed below. No experiments were carried out to test the effect of minor RNAs on particle stability.

In ultraviolet absorption profiles from sucrose gradient centrifugations of virions, Mel 40 typically displayed three peaks (Fig. 1), which agrees with previous work on the bean red node strain of TSV (5,17,22,24). Mel F displayed two peaks (Fig. 1). Differences in Mel F component ratios, as seen in a comparison of the profiles in Figure 1A and B, may have been linked to the lability of Mel F. Minor differences in age of infection, tissue condition, season, or tissue type or differences in extraction or resuspension buffer pH may have altered Mel F component ratios, as has been described for various TSV strains (21,24).

While the overall sedimentation rates of Mel 40 and Mel F were similar, our data suggest a differential sedimentation response to pH changes. In two experiments, virus preparations isolated at pH 6.4 were analyzed by centrifugation on sucrose gradients buffered at pH 5.5, 7.0, and 8.3. The decrease in sedimentation rate between pH 5.5 and 8.3 appeared greater for Mel F than for Mel 40 (Fig. 1B). The sedimentation rate of Mel F appeared

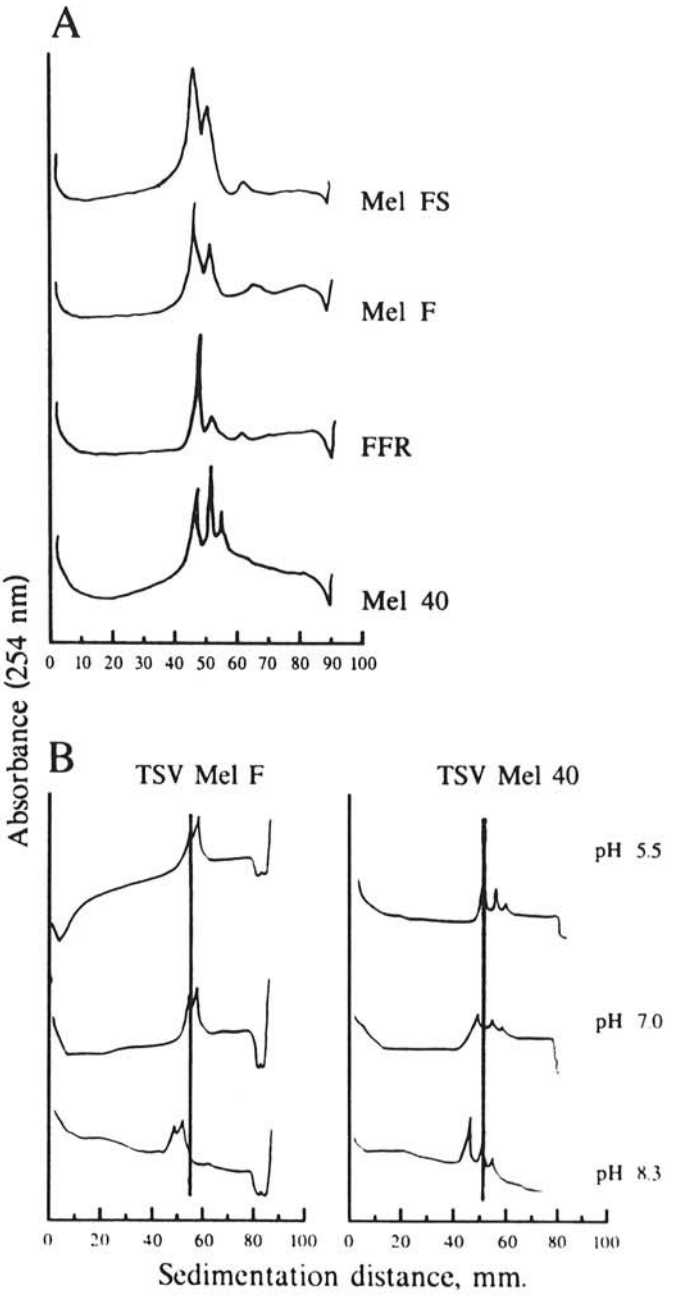

Fig. 1. Ultraviolet absorbency profiles of purified virions from isolates of tobacco streak virus (TSV) after rate-density gradient ultracentrifugation. **A,** Profiles of TSV pathotype I isolate Mel 40, TSV pathotype II isolates Mel F and Mel FS, and a pseudorecombinant isolate between Mel 40 and Mel F, FFR. The density gradient buffer was 0.02 M sodium acetate (pH 5.5). **B,** Effect of pH on sedimentation rates of TSV Mel 40 and Mel F. All virions were isolated from infected *Chenopodium quinoa* and purified at pH 6.4. Samples (60 μg) of purified virions were centrifuged for 2.5 h at 40,000 rpm on 10 to 40% sucrose gradients in a Beckman SW-40 rotor. The density gradient buffers were 0.02 M sodium acetate (pH 5.5), 0.03 M potassium phosphate (pH 7.0), and TAB (0.04 M Tris, 0.02 M sodium acetate, and 0.002 M EDTA, pH 8.3).

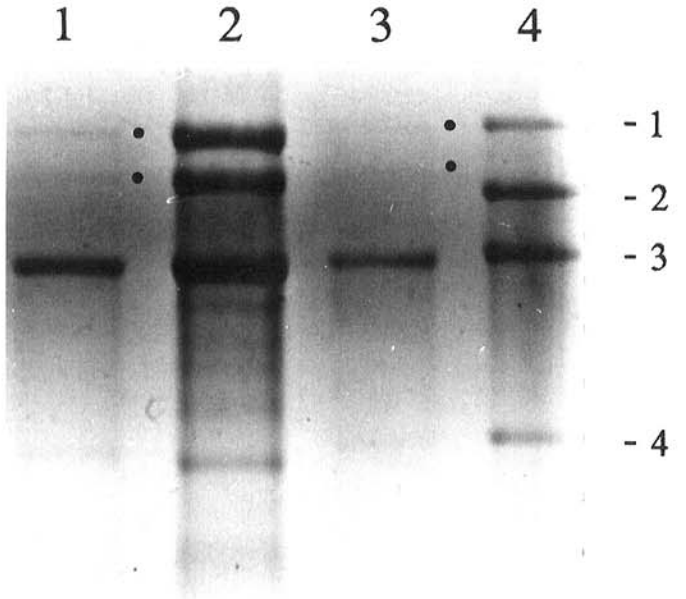

Fig. 2. Agarose gel electrophoresis of nondenatured viral RNA from infections established with tobacco streak virus pathotype I isolate Mel 40, pathotype II isolate Mel F, or pseudorecombinant isolate FFR. Lane 1, 12 µg of FFR RNA; lane 2, 18 µg of Mel F RNA; lane 3, 6 µg of FFR RNA; and lane 4, 15 µg of Mel 40 RNA. Purified RNAs were separated in a 1.5% low melt agarose gel. The gel was stained for 10 min in 0.05% toluidine blue O, destained, and photographed. Dots to the right of lanes 1 and 3 indicate the position of the faint bands representing RNAs 1 and 2 from FFR RNA. RNAs are numbered at the right of the gel.

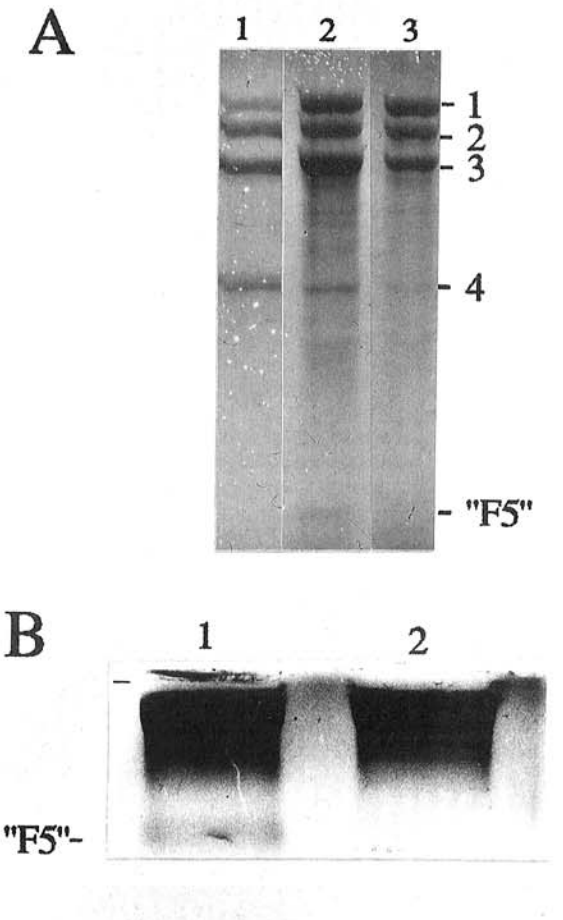

Fig. 3. Agarose gel electrophoresis of denatured viral RNA from tobacco streak virus pathotype I isolate Mel 40 and pathotype II isolates Mel F and Mel FS. **A,** RNA (6 µg) from Mel 40 (lane 1), 10 µg of RNA from Mel F (lane 2), and 6 µg of RNA from Mel FS (lane 3). Positions of genomic RNAs 1 through 4 and minor RNA F5 are indicated at right. **B,** RNA (10 µg) from Mel F (lane 1) and 10 µg of RNA from Mel FS (lane 2) electrophoresed for 45 min. RNAs purified from virions were denatured with glyoxal plus dimethyl sulfoxide and separated for 45 min or 1.5 h in an 11.5% low melt agarose gel. The position of F5 is indicated at left.

unaffected by the shift from pH 5.5 to 7.0 compared with a slight shift for Mel 40. This suggests that Mel 40 particles may change conformation (swell) gradually between pH 5.5 and 8.3 but that Mel F particles swell only when the pH is closer to 8.3. This observed difference in pH-induced conformational change parallels the observed differences in stability and infectivity of Mel F and Mel 40 under different pH conditions and is in agreement with previous work describing a general influence of pH on particle stability of TSV (17,21).

Agarose gel electrophoretic analysis. All four RNAs characteristic of TSV were clearly detectable when virions were purified at pH 6.4 (Fig. 2). Several features distinguished Mel 40 and Mel F RNAs under nondenaturing and denaturing conditions and were used to determine the origins of RNAs in the pseudorecombination study as described below. Electrophoretic migration of Mel F subgenomic RNA 4 appeared slightly greater than that of Mel 40 subgenomic RNA 4 under nondenaturing conditions (Fig. 2) but not under denaturing conditions (Fig. 3), suggesting the possibility of relatively greater secondary structure in the CP RNA of Mel F. There was no apparent difference in electrophoretic mobility between RNA 2 from Mel F and that from Mel 40 under denaturing conditions (Fig. 3). However, under nondenaturing conditions (Fig. 2), the electrophoretic mobility of Mel F RNA 2 was slower than that of Mel 40, suggesting relatively less secondary structure in RNA 2 of Mel F. These observations do not reflect any obvious trends in particle stability between Mel F and Mel 40, nor do we suggest that secondary structure of purified RNA reflects any capacity of that RNA to contribute to virion integrity. However, Ghabrial and Lister (10) speculated that TSV RNA may feature less secondary structure (hydrogen bonding) inside particles than RNA in other spherical viruses, either because of a lower overall proportion of RNA or because of the structural role of TSV RNA in virion integrity. If RNAs are differentially critical to virion stability, then secondary structure of the individual purified RNAs may reflect limitations in stability of particles encapsidating those RNAs.

There were no obvious size or secondary structure differences detected between the RNA 1 from Mel 40 and that from Mel F under denaturing or nondenaturing conditions. While RNA 1 from Mel F stained with approximately the same intensity as RNAs 2 and 3, the Mel 40 RNA 1 band appeared more faint compared with those of Mel 40 RNAs 2 and 3 (Figs. 2 and 3).

Electrophoretic profiles of RNA from Mel F featured minor bands under nondenaturing or denaturing conditions. No minor bands were observed in similar profiles of Mel 40 RNA (Figs. 2 and 3). We observed no increase in minor RNAs from Mel F in original stocks compared with virus that had been passaged three or more times on *C. quinoa.* Although specific nucleoprotein content was not experimentally determined in the present study, Mel F particles appear to encapsidate minor RNA species in addition to the major RNAs, as do some strains of AMV (3).

Effects of minor RNAs on symptomatology. Infections established with Mel F field isolates or with total RNA from Mel F displayed mild symptoms compared with the severe symptoms from infections established with gel-fractionated Mel F RNAs 1, 2, and 3 on *C. quinoa* or BTS. Such symptoms have not been previously described for Mel F and resemble Mel 40

TABLE 1. Symptoms, symptom timing, and serotypes of tobacco streak virus (TSV) infections from inoculation with genomic RNAs of isolates Mel 40 (RRR), Mel F (FFF), and pseudorecombinant isolate FFR on *Chenopodium quinoa* and *Phaseolus vulgaris*

	C. quinoa		*P. vulgaris*		
RNA	Symptoms on inoculated leaves	Symptoms on noninoculated leaves	Symptoms on inoculated leaves	Symptoms on noninoculated leaves	Serotype by ELISA[a]
FFF[b]	Chlorotic, necrotic lesions	Chlorosis; mosaic	Red, necrotic lesions; veinal necrosis	Chlorotic ring spot; mosaic	Mel F
RRR[c]	White, necrotic, coalescing lesions; stunting	Necrosis; stunting	Red, necrotic lesions; veinal necrosis; distortion	Necrosis; stunting	Mel 40
FFR[d]	Small, red lesions	No symptoms	Veinal necrosis	No symptoms	Mel 40

[a] Indirect enzyme-linked immunosorbent assay with polyclonal antiserum to TSV Mel F or Mel 40.
[b] Symptoms on inoculated leaves developed 2 to 3 days postinoculation (dpi) on *C. quinoa* and 3 to 4 dpi on *P. vulgaris*.
[c] Symptoms developed 1 to 3 dpi on *C. quinoa* and 2 to 4 dpi on *P. vulgaris*.
[d] Symptoms developed 4 to 6 dpi on *C. quinoa* and 5 to 7 dpi on *P. vulgaris*.

infections in severity (FFF inoculum, Table 1). In contrast, inoculations with gel-fractionated RNAs 1, 2, and 3 of Mel 40 caused symptoms similar to those of infections established by inoculation with total Mel 40 RNA or with purified virions (RRR inoculum, Table 1), ruling out RNA dose as an explanation for atypically severe Mel F symptoms. Minor RNAs were not detected in viral RNA preparations from infections established with gel-fractionated Mel F RNAs 1, 2, and 3, even after repeated passages.

One minor RNA species (RNA F5), approximately 300 bases in size, was best observed when preparations of denatured Mel F viral RNA were analyzed by agarose gel electrophoresis for only 45 min. RNA F5 was not detected in RNA preparations from Mel FS (Mel F-Seed), a seedborne subisolate of Mel F (Fig. 3A and B). Mel FS originated as a rare, seedborne isolate from BTS infected with the infrequently seedborne Mel F. We maintained Mel FS as an infected BTS seed line. Mel FS infected approximately 30% of progeny seedlings from BTS plants infected from seed, an infection rate similar to that of Mel 40, and was serologically and symptomatically indistinguishable from Mel F. Sucrose density gradient centrifugation profiles (Fig. 1) and electrophoretic patterns (Fig. 3) of Mel FS and Mel F RNAs were very similar. It has been proposed that Mel F usually fails to be seed transmitted in bean because it reaches only low levels in pollen-associated tissues (33). Although no floral-tissue infection studies were carried out with Mel FS, the high rate of seed transmission from Mel FS infections suggests that Mel F minor RNA F5 may be linked to inhibition of seed transmission.

Pseudorecombinant analysis. As with pseudorecombinants between BMV and CCMV, infectivity of pseudorecombinants between Mel F and Mel 40 was limited to combinations that suggested specific interactions between RNAs (1,19). Only one heterologous combination, consisting of Mel F RNAs 1 and 2 and Mel 40 RNA 3 (FFR inoculum, Table 1) gave rise to a stable hybrid isolate; the reciprocal combination (RRF) did not. The chlorotic areas on RRF-inoculated leaves had disappeared by 6 days postinoculation (dpi), and no virus was detected serologically or by rub inoculation. Lesion formation on FFR-inoculated leaves was slower by at least 1 day compared with that of parental RNA inoculations. FFR inoculations produced only small, noncoalescing, red lesions on inoculated leaves without subsequent necrosis, chlorosis, or stunting. Leaves inoculated with single and double RNA species as checks on RNA purity developed no lesions, with the exception of leaves inoculated with Mel F RNAs 1 and 2 (the source of FF in the FFR inoculum). Serologically mixed infections were not detected from any inoculations with mixed virions, nonfractionated RNAs, or fractionated RNAs. These results agree with previous findings (17). Mixed inoculations always resulted in the Mel 40 serotype (data not shown).

Single-lesion transfers from FFR-inoculated leaves were made at 5 dpi and produced small, red lesions on inoculated leaves of *C. quinoa* but no systemic symptoms. Systemic spread of the FFR infection was slower than that of infections from inoculation with the parental RNAs (Table 1). By 5 dpi, rub inoculation transfers suggested that systemic spread of the FFR infection into noninoculated leaves had not occurred (data not shown). Infections in FFR-inoculated plants could not be detected serologically in noninoculated leaves at 16 dpi (Fig. 4). Symptoms on noninoculated leaves appeared at 60 dpi. Virus was detected serologically in noninoculated leaves at 75 dpi but was probably present at 60 dpi (Fig. 4). Serial transfers were made from systemically infected leaves, and the same pattern of slow symptom development and spread was again observed, suggesting that reversion and/or mutation had not occurred.

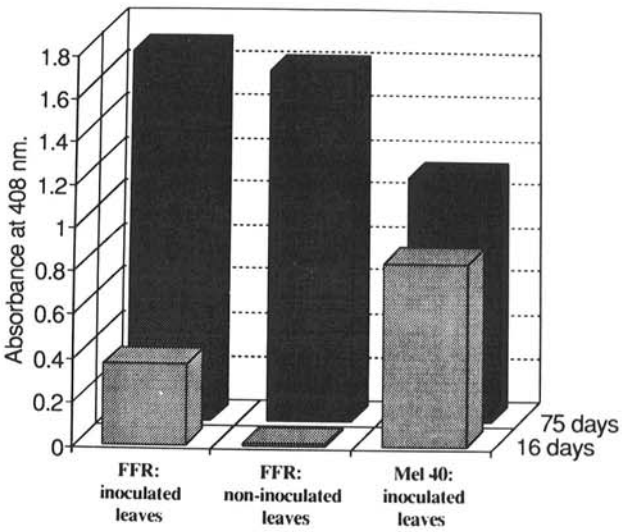

Fig. 4. Serological detection of systemic infections of tobacco streak virus (TSV) isolates at 16 and 75 days postinoculation. Results are from assays of leaf disks (0.5 cm in diameter) cut from noninoculated leaves of *Chenopodium quinoa* or leaves inoculated with FFR (RNAs 1 and 2 from TSV Mel F and RNA 3 from TSV Mel 40) or TSV Mel 40. Bar height represents average absorbance (408 nm) of two or four wells.

Although FFR infections were serologically of the Mel 40 type, sucrose gradient centrifugation and viral RNA electrophoresis profiles from FFR infections suggested that the nucleoprotein composition of FFR was unlike that of either parent isolate. Centrifugation profiles showed a very prominent top component but diminished bottom and middle components, unlike either parent isolate (Fig. 1A). Clark and Lister (5) suggested that RNAs 1 and 2 are predominantly encapsidated in the bottom and middle components of TSV, respectively. In agarose gel electrophoresis of both nondenatured (Fig. 2) and denatured (not shown) RNAs, RNAs 1 and 2 from FFR RNA preparations stained less intensely

TABLE 2. Symptoms and ELISA[a] absorbance values of *Chenopodium quinoa* tissue inoculated with various combinations of RNAs from tobacco streak virus strain Mel F and the pseudorecombinant FFR

RNA inoculum[b]	Symptoms on inoculated leaves[c]	ELISA antiserum	
		Mel F	Mel 40
1. F3 (F)	No symptoms	0.04 (0.01)[d]	0.00 (0.00)
2. Total RNA (F)	Coalescing necrotic lesions (100)	0.97 (0.04)	0.00 (0.00)
3. Total RNA (FFR)	1-mm necrotic lesions (9)	0.04 (0.00)	0.17 (0.00)
4. F1 and F2 (FFR) + F3 (F)	No symptoms	0.04 (0.00)	0.00 (0.00)
5. Total RNA (FFR) + F3 (F)	Coalescing, white necrotic lesions (12)	0.39 (0.04)	0.05 (0.01)
6. Total RNA (FFR) + total RNA (F)	Coalescing necrotic lesions (200); distortion	0.93 (0.07)	0.06 (0.01)
7. Noninoculated	No symptoms	0.02 (0.03)	0.00 (0.00)

[a] Indirect enzyme-linked immunosorbent assay.
[b] Inoculations were made on four to six leaves of four *C. quinoa* plants per treatment. Letters in parentheses indicate the type of infection from which the RNA was purified.
[c] Numbers in parentheses indicate approximate numbers of lesions.
[d] Mean and standard deviation (in parentheses) of ELISA absorbance values (A_{405nm}). Treatments were conducted in four to eight duplicate wells in standard microtiter plates.

than RNA 3, suggesting that RNAs 1 and 2 were either less abundant, less stable, or less efficiently encapsidated in FFR infections. Under nondenaturing conditions, RNA 2 of FFR appeared to have electrophoretic mobility similar to that of Mel F RNA 2 (Fig. 2) but less than that of Mel 40 RNA 2. Thus, RNA 2 from the FFR inoculation was probably Mel F RNA 2. Serological results suggested that RNA 3 from FFR was of Mel 40 origin. It was not possible to confirm the origins of RNA 1 by electrophoresis or phenotypic marker.

Using back inoculation to further investigate the identity of RNAs 1 and 2 from the FFR infections, we attempted to establish a Mel F infection with FFR RNAs 1 and 2 coinoculated with gel-purified Mel F RNA 3 or total Mel F RNA. Control inoculations demonstrated the purity of Mel F RNA 3 (inoculum 1, Table 2). Total FFR RNA was infectious, but no infection was produced by inoculation of gel-purified FFR RNAs 1 and 2 and Mel F RNA 3, possibly because of very low levels of RNAs 1 and 2 from FFR. Inoculation with total FFR RNA and Mel F RNA 3 produced a Mel F infection (inoculum 5, Table 2). Had either RNA 1 or 2 from FFR been of Mel 40 origin, we would have expected no infection or a weak infection on the basis of other experiments with heterologous combinations of RNAs. When taken together with results from RRF inoculations, this suggests that FFR RNAs 1 and 2 were of Mel F origin.

The FFR infection was also unlike either parental-type infection in BTS. Homologous (RRR and FFF) and heterologous (FFR) infections were transferred from *C. quinoa* to BTS, resulting in FFR infections that were slow to initiate (Table 1) and slow in systemic spread. We detected no seed transmission in 268 seedlings from 20 FFR-infected BTS plants.

The scarcity of stable pseudorecombinants from Mel 40 and Mel F suggests that these isolates are probably not closely related or that their genomic parts are incompatible. If isolates are closely related, then such stringent RNA compatibility within isolates might be unique to some ilarviruses among the tripartite viruses. This would contrast with the interstrain compatibility between multipartite viruses, such as CMV, in which all three RNAs can be exchanged without substantial loss of infectivity (12,28). Possible selection against interstrain RNA compatibility, particularly in connection with systemic spread and seed transmission, may also explain the lack of mixed infections observed between Mel 40 and Mel F.

Natural selection for compatibility between RNAs 1 and 2 may have limited the stability of pseudorecombinants heterologous for these RNAs. Contrary to our results, Fulton (8) determined that bottom and middle components (presumably containing RNAs 1 and 2) are infectious in homologous and some heterologous combinations between different TSV strains, although his work did not elucidate the exact RNA content of each nucleoprotein component. Replication of RNA 3 is apparently tied to RNAs 1 and 2 in bromoviruses (7) and AMV (25). However, unlike bromoviruses, ilarvirus RNAs 1 and 2 cannot replicate independently of RNA 3 or virus CP (1,19,32).

In summary, some TSV isolates may demonstrate a high degree of selection for homologous RNA compatibility. Minor RNAs appear to condition severity of TSV Mel F infections in *C. quinoa* and BTS. Data from experiments on the effects of minor RNAs on seed transmission are being evaluated.

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