Effects of Low-Molecular-Weight RNA and Temperature on Tomato Bushy Stunt Virus Symptom Expression

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ABSTRACT

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Low-molecular-weight RNA associated with tomato bushy stunt virus (TBSV) infection altered symptom expression in several experimental hosts. The linear, single-stranded, low-molecular-weight RNA was encapsidated in TBSV-encoded protein. In addition, infected tissue contained a double-stranded (ds) form of this molecule. The most obvious symptom modification induced by the low-molecular-weight RNA was attenuation in *Nicotiana clevelandii*. At elevated temperatures (27 C), symptoms induced by TBSV alone and in combination with the low-molecular-weight RNA differed from those induced at a lower temperature (16 C). In *N. clevelandii*, high-temperature attenuation of symptoms was similar, but not identical to, that caused by the low-molecular-weight RNA. This symptom change was associated with changes in the dsRNA species accumulating in *N. clevelandii* as a result of virus infection. Among these changes was the suppression of low-molecular-weight RNA replication.

Tomato bushy stunt virus (TBSV) is the type member of the tombusvirus group which, in addition, contains six definitive and two possible members (22). The relationships among members remain somewhat confused. The type strain of TBSV, for example, is more distantly related serologically to TBSV-cherry strain than is petunia asteroid mosaic virus (PAMV) (16). Much of this confusion probably arises from early virus descriptions without serological comparison to known TBSV strains (20). All definitive tombusviruses except for cymbidium ringspot virus (CyRSV) show varying serological interrelationships.

Environmental conditions are known to affect tombusvirus symptom expression in different hosts. Symptoms often regress in the summer and early fall, as in artichoke mottled crinkle virus (AMCV)-infected artichoke (21). High temperature and extended photoperiod permit systemic invasion of *Gomphrena globosa* by TBSV, whereas the virus is normally confined to local necrotic lesions (26). Additionally, high temperature effectively controls pelargonium leaf curl virus (PLCV) (12). Infected plants can be freed of PLCV by holding them at 37 C for 4–6 wk. These environmental effects are major factors in tombusvirus etiology. We sought to determine if environment directly affected the pathogen.

The tombusvirus group has been well studied physicochemically, especially with respect to structure and morphology (8) and to virus-coded proteins (28). Tombusvirus nucleic acids have been somewhat less well studied. Virion RNA has been reported as a single species with molecular weight ranging from 1.5 to 1.8×10^6, depending on virus strain and molecular weight determination method (20). Henriques and Morris (9) reported double-stranded RNA (dsRNA) species of 3.2 and 1.5×10^6 daltons for the BS-3 strain of TBSV, but only a single 1.6×10^6-dalton virion RNA (9). The 1.5×10^6-dalton dsRNA species was suggested as a possible replicative form of a subgenomic 0.75×10^6-dalton single-stranded RNA (ssRNA), but no such species was identified. Here, we reevaluate this earlier work and correlate the effect of temperature on symptom expression with an alteration of dsRNA species within the infected plant. Portions of this work have been presented (10).

MATERIALS AND METHODS

Virus strains and purification. The BS-3 strain of TBSV (27) (originally obtained from C. A. Knight, University of California, Berkeley), and the Prunus strain of TBSV (1) (provided by R. Stace-Smith, Agriculture Canada, Vancouver, B. C.), were maintained in the greenhouse in *N. clevelandii* after three single-lesion passages of the original isolates. Virus was purified by extraction in 0.2 M sodium acetate, pH 5.0, and by using PEG precipitation and high-speed centrifugation as concentrating steps. In some experiments, virus was further purified on linear-log sucrose gradients (4) or on cesium chloride gradients centrifuged to equilibrium. Homogeneity of strains was monitored by double diffusion serology or by indirect ELISA as described by Lommel et al (19).

Single-stranded RNA analysis and plant inoculation. Single-stranded, virion RNA was isolated from purified virion preparations by cold phenol-SDS extraction as previously described (11). Sucrose gradient analysis was performed, by following the method of Brakke and Van Pelt (4), in a Beckman SW41 or SW50.1 rotor. Gradients were fractionated with an ISCO model 640 fractionator. Fractions were collected manually corresponding to 254-nm-absorbing peaks and were precipitated in ethanol at −20 C. RNA to be used as inoculum was centrifuged a second time. In the case of the low-molecular-weight RNA, further gel purification steps were employed as follows: RNA fractions were brought to 1% SDS, 4 M urea, and heated to 60 C for 5 min. This mixture was applied to a 2% agarose gel in 0.025 M sodium citrate, pH 6.8. Following electrophoresis for 3 hr at 50 V, gels were stained with ethidium bromide and examined at 302 nm. The major band containing low-molecular-weight RNA was excised and applied to an 8% polyacrylamide gel in 0.04 M tris, 0.02 M sodium acetate, pH 7.5, 0.001 M EDTA (TAE). Following electrophoresis for 5 hr at 80 V, the band of interest was stained and electroeluted into a buffer trough. The eluate was then extracted with water-saturated butanol, then ether, and RNA was precipitated in ethanol. RNA fractions were diluted in GKP buffer (4) for inoculation to assay plants.

Electrophoresis of purified ssRNA for analytical purposes was performed in denaturing gels by using methylmercury hydroxide (0.075 M) as the denaturing agent (3) in a 2% agarose gel in 0.025 M sodium citrate buffer, pH 6.8. Gels were electrophoresed 4 hr at 50 V in an Isolab electrophoresis apparatus. Following electrophoresis, gels were stained with ethidium bromide and photographed at 302 nm. Virion ssRNAs used as standards were tobacco mosaic virus (2.1×10^6 daltons) and cucumber mosaic virus with satellite RNA (CARNA 5) (1.1, 0.9, 0.7, 0.3, and 0.1×10^6 daltons).

Double-stranded RNA analysis. Double-stranded, replicative RNA was isolated from infected tissue essentially according to the procedure of Morris and Dodds (23). Electrophoresis was

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performed on a BioRad model 220 or an Isolab electrophoresis apparatus in 8% polyacrylamide gels with the TAE buffer system mentioned previously. Gels were stained in ethidium bromide and photographed as above. TMV and CMV dsRNAs were used as molecular weight markers. The sizes of the dsRNAs were estimated by doubling the ssRNA values.

Studies of temperature effects. Investigation of the effects of high and low temperatures on TBSV virulence and replication were conducted in constant 16, 21, 27, and 32 C greenhouse rooms without supplemental lighting. The diurnal temperature variability in these rooms was no greater than 4 C, as measured during a 2-wk period of the experiments. The work reported here was done with *N. clevelandii* used as a host.

RESULTS

Greenhouse studies. In uncontrolled greenhouse conditions, the BS-3 strain isolate induced large, necrotic lesions on inoculated leaves of *N. clevelandii* within 4–5 days. Chlorotic spots then developed at the bases of young, uninoculated leaves. Stunting and sporadic necrotic lesions were evident in systemically infected leaves of mature plants. These plants remained infected and continued to produce leaves with necrotic lesions for at least 4–6 mo. Virions isolated from systemically infected leaves sedimented as a major 135S species on sucrose gradients, while minor faster-sedimenting peaks of variable size were observed (Fig. 1B). The minor peaks were shown by serology, bioassay, and electron microscopy to be virion aggregates. Inoculation of virus from the major 135S peak at 50 μg/ml reproduced both local and systemic symptoms.

On extraction of ssRNA from virion preparations purified from these systemically infected leaves, three major species were observed on both gradients and denaturing gels (Fig. 2B, Fig. 3B). These RNA species had apparent molecular weights of 1.65, 0.75, and 0.15×10^6 daltons. The 1.65×10^6-dalton RNA (RNA A), the size expected for genomic RNA, was the predominant species, comprising about 80% of the total virion RNA by weight. The 0.75×10^6-dalton species (RNA B) accounted for about 10% and the 0.15×10^6-dalton species (RNA C) about 3% of the encapsidated RNA. The rest of the virion RNA was heterogeneous. The molar ratio of RNAs A, B, and C was estimated by integration of sucrose gradient peaks to be 3:1:1.

Extraction of dsRNA from the same tissue yielded four major bands with apparent molecular weights of 3.3, 1.5, 0.7, and 0.3×10^6 daltons in polyacrylamide gels (Fig. 4, lane B). Minor bands of 2.4 and 0.6×10^6 daltons were also consistently observed. A number of different BS-3 strain isolates from earlier studies had dsRNAs of $0.2 - 0.4 \times 10^6$ daltons, several having multiple species (Fig. 5).

RNA infectivity studies. The symptoms induced in *N. clevelandii* by inoculation of the three separated ssRNA species, alone and in the various combinations, are summarized in Table 1. RNA B was uninfectious alone and had no visible effect on symptom development in any combination. RNA A was the single component necessary for infection. When applied alone as inoculum, RNA A induced necrotic local lesions on inoculated leaves and a much more severe systemic reaction than the original virus inoculum. This severe syndrome was characterized by apical necrosis and collapse of the plant within 10–14 days postinoculation. RNA C was infectious only when applied with RNA A. Plants infected with this combination produced the typically milder systemic symptoms of the original virus isolate.

Virus preparations isolated from plants inoculated with the various RNA combinations produced identical sedimentation profiles on sucrose gradients (Fig. 1) and could not be distinguished serologically. The only apparent distinction was the presence of

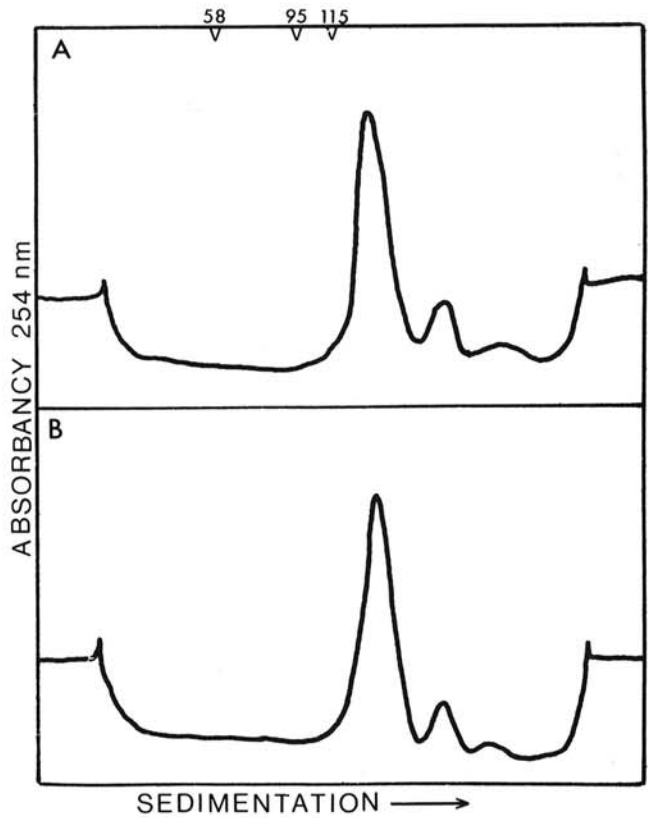

Fig. 1. Sedimentation profiles of tobacco bushy stunt virus virions following centrifugation in linear-log sucrose gradients. **A,** Virus preparation from dying plants of *Nicotiana clevelandii* that did not contain RNA C. **B,** Virus preparation from systemically infected plants of *N. clevelandii* that survived infection and contained RNA C. Gradients were centrifuged for 75 min at 45,000 rpm in a Beckman SW50.1 rotor. Arrows indicate the positions of cowpea mosiac virus components at 58, 95, and 115S.

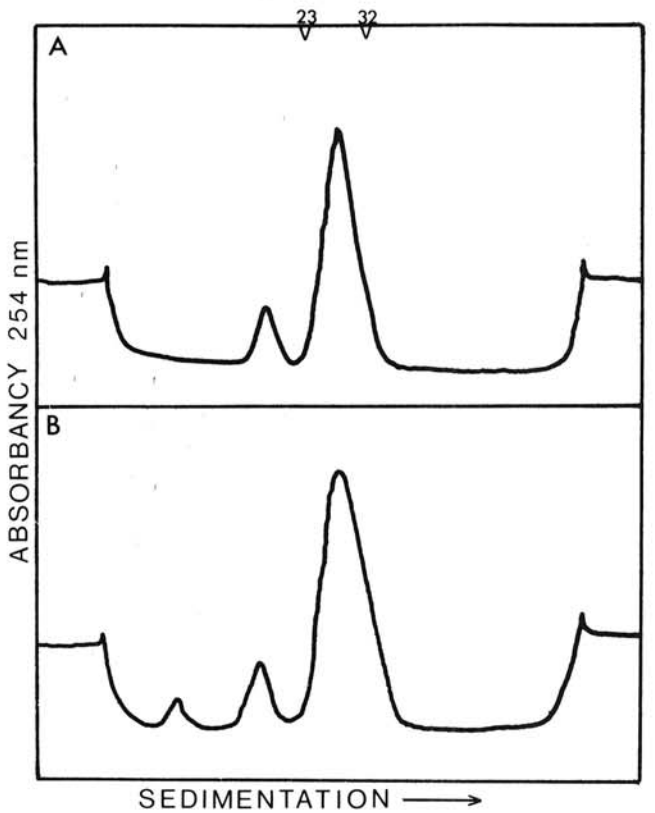

Fig. 2. Sedimentation profiles of tobacco bushy stunt virus virion single-stranded RNA (ssRNA) following centrifugation in linear-log sucrose gradients. **A,** Virion ssRNA from dying plants. **B,** Virion ssRNA from systemically infected plants that survived infections. Gradients were centrifuged for 4 hr at 45,000 rpm as described in Materials and Methods. Arrows indicate the positions of carnation mottle virus and tobacco mosaic virus genomic RNAs at 23 and 32S, respectively.

RNA C in virion preparations made from plants inoculated with RNA C-containing inoculum (Figs. 2 and 3). Virion preparations containing RNA C consistently gave the milder systemic syndrome. Infected plants of *N. clevelandii* containing RNA C also contained a 0.3×10^6-dalton dsRNA species (Fig. 4, lane B).

Heterogeneity of dsRNA C. When the heterogeneity of $0.2–0.4 \times 10^6$-dalton dsRNA species from different isolates of the BS-3 strain was noticed, it first appeared that the symptom modulating effects of each were identical. In each case, presence of a small RNA conferred protection against an otherwise lethal virus isolate. It later appeared that there may be differences in the protection conferred by different RNA C species. For convenience, we studied one particular RNA species. It was one that we were consistently able to select as a single electrophoretic species without repeated single-lesion passage. As we continued to work with this isolate, we noticed that infected plants of *N. clevelandii* did not survive as long as plants inoculated with some other isolates. For example, of over 30 plants inoculated with the isolate producing the dsRNA profile in Fig. 5, lane B, all survived a minimum of 2 mo after inoculation. Our experimental isolate (Fig. 5, lane A) was a single lesion isolate of that in lane B. Of the plants of *N. clevelandii* inoculated with the isolate in lane A, nearly 50% died within 2 mo. RNA C was extractable from inoculated leaves in all cases, indicating that there may be qualitative differences in the symptom-modulating effects of electrophoretically different RNA C species or that there may be a quantitative effect due to total RNA C accumulation.

Effect of elevated temperature on symptom expression. At 16 C, plants inoculated with the BS-3 strain reacted much as they had in uncontrolled greenhouse conditions. Plants inoculated with RNA A, either alone or with a virion isolate containing no RNA C, quickly became apically necrotic, wilted, and died, while plants inoculated with RNA A plus RNA C or with virions containing both RNAs survived for at least 6 wk postinoculation. The dsRNA isolated from plants grown at 16 C was similar to that from plants grown in uncontrolled greenhouse conditions (Fig. 4, lanes A and B).

N. clevelandii inoculated with the BS-3 strain isolates and held at 27 C behaved quite differently. Regardless of the presence or absence of RNA C in the inoculum, all plants survived infection without showing the apical necrosis symptoms, and all plants showed similar symptoms. The symptomatology of the plants held

TABLE 1. Symptoms induced at different temperatures by the tomato bushy stunt virus virion and RNA preparations

	Host grown at	
Inoculum[a]	16 C	27 C
RNA A	LL, AN, D[b]	LL, SSL, B
RNA B	–	–
RNA C	–	–
RNA A + B	LL, AN, D	LL, SSL, B
RNA A + C	LL, LSL	LL, SSL, B
RNA A + B + C	LL, LSL	LL, SSL, B
Virion, RNA C^+	LL, LSL	LL, SSL, B
Virion, RNA C^-	LL, AN, D	LL, SSL, B

[a]Virion preparations were purified through sucrose gradients and inoculated at concentrations of 50 μg/ml. RNA A was purified through sucrose gradient; RNAs B and C were purified by gel electrophoresis.
[b]Symptoms: – = no symptoms, LL = local lesions, AN = apical necrosis, LSL = large lesions on systemically infected leaves, SSL = small lesions on systemically infected leaves, B = bushy growth, D = death of plant within 2 wk postinoculation. Unless indicated with (D), plants continued to live at least 1 mo postinoculation.

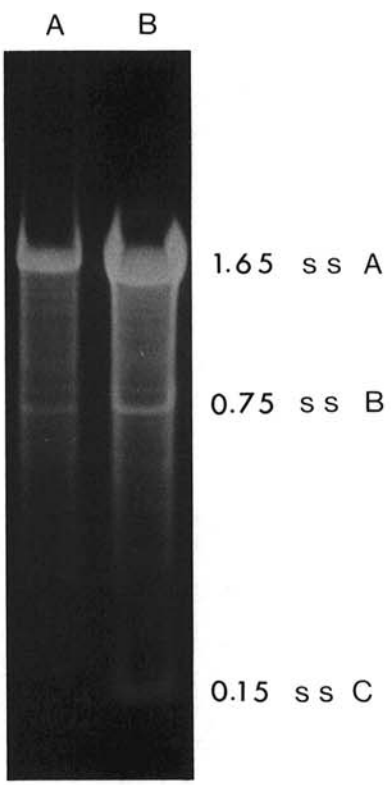

Fig. 3. Methyl mercury denaturing agarose gel electrophoresis profiles for tomato bushy stunt virus. Lane A, virion single-stranded RNA (ssRNA) from dying plants. Lane B, Virion ssRNA from systemically infected plants that survived infection. Gel electrophoresis was for 4 hr at 25 V in a 2% agarose gel as described. Molecular weights are daltons ÷ 10^6.

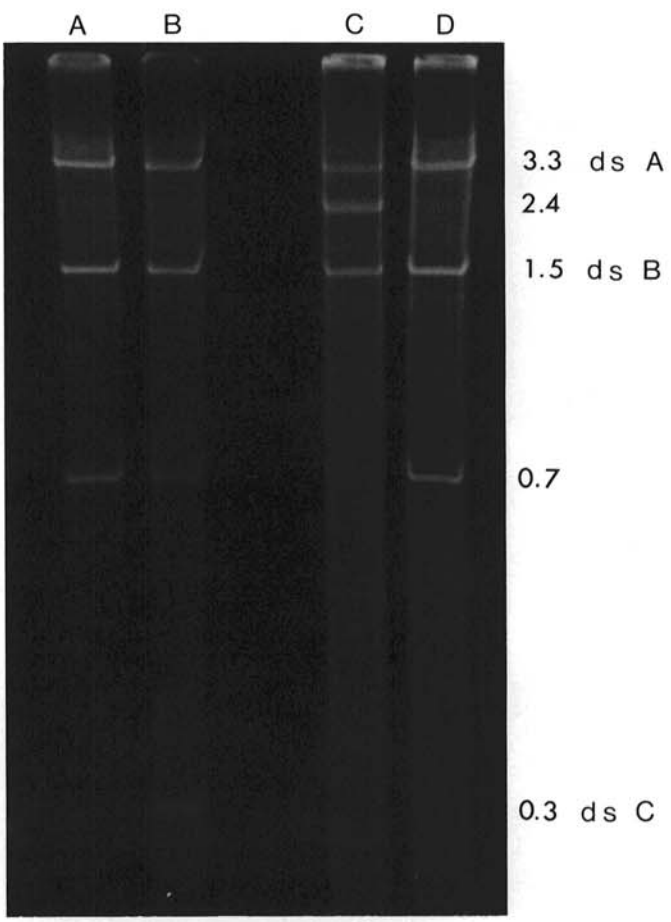

Fig. 4. Polyacrylamide gel electrophoresis profile of double-stranded RNA from tomato bushy stunt virus-infected plants of *Nicotiana clevelandii*: lane A, from dying greenhouse plants; lane B, from systemically infected greenhouse plants that survived infection; lane C, from systemically infected plants at 27 C; and lane D, from dying plants inoculated identically to those in lane C but held at 16 C. Molecular weights are daltons ÷ 10^6.

at 27 C differed somewhat from that of plants at 16 C which were systemically infected with an RNA C-containing isolate (Fig. 6). Plants held at the higher temperature showed greater proliferation of foliage, stunting, and mottling of systemically infected leaves. These plants had either very small necrotic lesions or none at all (Fig. 6C).

Nucleic acid analysis of plants grown at high or low temperatures revealed that there were differences in TBSV dsRNA accumulation at 27 C as opposed to 16 C. The dsRNA profile from plants held at 27 C included an additional major band corresponding to a species with a molecular weight of about 2.4×10^6 daltons (Fig. 4, lane C). Also, the quantity of the 0.7×10^6 species was greatly reduced at the higher temperature. Little or no dsRNA C could be detected at 27 C, even when RNA C was abundant in the inoculum. The only apparent difference in ssRNA isolated from virions from inoculated plants grown at 16 and 27 C was the reduction of RNA C in the plants grown at the higher temperature.

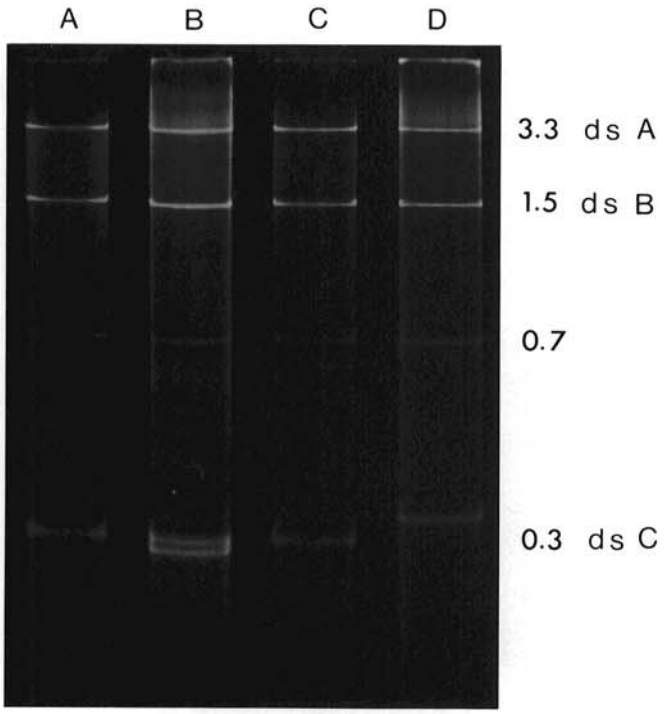

Fig. 5. Polyacrylamide gel electrophoresis profile of double-stranded RNA from tomato bushy stunt virus isolates and passages from the same original BS-3 strain. Lane A represents a local lesion isolate of lane B tissue, lane B represents an undiluted passage of lane C tissue. Lane D represents an isolate of different origin. Each isolate was serologically identical. Molecular weights are daltons ÷ 10^6.

DISCUSSION

Low-molecular-weight RNAs associated with isometric plant viruses are being identified in increasing numbers, but these molecules differ widely both in physical properties and in biological effects on the host plant. In the CMV-satellite system (7,14), a stable, linear, 0.1×10^6-dalton, encapsidated ssRNA that was not necessary for viral replication altered symptomatology in tomato. A number of nepoviruses have satellite RNAs that affect symptom expression (see reference 24 for a review). Turnip crinkle virus (TCV) also contains a linear satellite RNA (2). Several isometric plant viruses possess covalently closed, circular, satellite RNAs (eg, 25).

CMV satellite, like other plant virus satellites, accumulates to high levels relative to other viral RNA species (13). In the TBSV-RNA C system, presence of the low-molecular-weight RNA confers protection against an otherwise lethal virus strain. Though accumulation of RNA C in infected tissue is high, the relative quantity of encapsidated RNA C is very low. RNA C cannot be diluted out in inoculations from crude tissue extracts or virion RNA extracts; its infectivity is lost 2–3 logarithmic dilution units before RNA A infectivity in purified virus preparations, suggesting that the two RNA species may be encapsidated separately (Hillman et al, *unpublished*). No vector has been consistently associated with TBSV, and spread of the virus may well be by mechanical means and by uptake through the roots (15). These points raise the question of the epidemiological significance of RNA C. Continued spread of this RNA species would seem to require a high multiplicity of infection. With TBSV infection, the most obvious means for such spread in nonexperimental situations would be through vegetative propagation.

The following features suggest that RNA C should be considered a satellite RNA of TBSV: it is infectious and its replication requires TBSV genomic RNA, it is of low molecular weight and not required for TBSV replication, and it modulates symptoms in a manner similar to other satellite RNAs. We are, however, reluctant to assign satellite status to RNA C because of indications of homology between this molecule and TBSV genomic RNA (Hillman et al, *unpublished*). Should relatedness be confirmed, RNA C would seem more nearly analogous to the defective interfering RNAs that have been reported in many animal virus systems (eg, 17 for a review).

The effect of temperature on the symptoms of TBSV infection that develop in *N. clevelandii* is not in itself surprising. Such observations have been made in many plant virus systems and, as mentioned previously, the phenomenon is well documented for TBSV. Of considerable interest, however, is the marked effect of temperature on the dsRNA profile of the virus. It seems likely that the correlation of dsRNA profile at 27 C (eg, Fig. 4, lane C) and survival of TBSV infection is more than coincidental. Reduction of transcription of a subgenomic RNA coding for an integral product such as a protein required for cell-to-cell virus movement (18) is a mechanism worth consideration. In such a case, virus would spread slowly through the plant and be more easily limited by the plant.

Fig. 6. Tomato bushy stunt virus-infected plants of *Nicotiana clevelandii*. **A,** Necrotic whole plant inoculated with genomic RNA A and held at 16 C for 10 days. **B,** Large necrotic lesions on systemically infected leaf of plant inoculated with RNAs A and C and held at 16 C for 20 days. **C,** Small lesions on systemically infected leaf of plant inoculated with RNA A and held at 27 C for 20 days.

The dsRNA species of 3.2 and 1.5×10^6 daltons previously reported by Henriques and Morris (9) were consistently seen in our TBSV dsRNA preparations, though our molecular weight value for genomic dsRNA is slightly higher. Our finding additional 0.7×10^6-dalton dsRNA could be explained in several ways. For example, we used a more sensitive stain (ethidium bromide versus toluidine blue O) and growth conditions possibly more favorable for production of 0.7×10^6-dalton dsRNA. Growth conditions in the previous study were not mentioned, and they may have been suboptimal for generating that species. Indeed, a small peak can be observed in the region of our 0.7×10^6-dalton species in Fig. 4A of that paper. Also notable in that figure is the second arrowed species, interpreted by the authors as replicative intermediate (RI). This species had the same relative mobility as our 2.4×10^6-dalton species, which was induced at high temperature, and its increase would be expected to accompany a decrease in the 0.7×10^6-dalton species.

A subgenomic ssRNA of 0.75×10^6 daltons was postulated based on the 1.5×10^6-dalton dsRNA (9), but no evidence for a molecule of that size was presented in that communication, nor has it been noticed in other TBSV nucleic acid studies. In RNA preparations from strain BS-3 virions, a species of that size was consistently observed. Whether this represents an encapsidated subgenomic RNA or simply a breakdown product of genomic RNA generated in the extraction procedure is not known. Supporting the former explanation are the quantitative consistency with which this species has been observed and the lack of breakdown to a similar discrete species by gradient purified genomic RNA. Also supporting the idea of a subgenomic RNA is the presence of such a discrete species in six different tombusviruses, including the unrelated CyRSV, examined to date (Hillman, *unpublished*). Dougherty and Kaesberg (5) described translation of encapsidated subgenomic RNA in turnip crinkle virions, and it would not be surprising to find a subgenomic RNA associated with TBSV.

Symptomatology in various hosts is often a basis for separating tombusvirus strains (6,12). We have shown great diversity of symptom expression in *N. clevelandii* with a single serological strain of TBSV, depending on the presence or absence of RNA C and temperature. The fact that RNA C replicates poorly at elevated temperatures suggests that the two phenomena may in some way be related. The different symptoms in plants surviving TBSV infection due to RNA C and those surviving at elevated temperatures suggests that the plants are sustained by different mechanisms. It will be interesting to see how these observations relate to the documented warm weather reduction in severity of symptoms due to TBSV and related viruses (21).

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