Occurrence of a Vein-Clearing Tobamovirus in Turnip

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ABSTRACT

Lartey, R. T., Hartson, S. D., Pennington, R. E., Sherwood J. L., and Melcher, U. 1993. Occurrence of a vein-clearing tobamovirus in turnip. Plant Dis. 77:21-24.

A tobamovirus that infects turnips was identified as a contaminant of a preparation of cauliflower mosaic virus (CaMV). Because the virus caused vein clearing, it was designated turnip vein-clearing virus (TVCV). It was separated from CaMV in the preparation by alternate passage through a CaMV host and a nonhost or by treatment of the viral nucleic acids with DNAse. The purified TVCV contained a single species of single-stranded RNA similar in size to that of tobacco mosaic virus (TMV) and a single species of polypeptide similar in size to, but distinguishable from, the TMV coat polypeptide. Serological tests demonstrated TVCV was related, but not identical, to TMV *vulgare*. The reaction of several plant species to TVCV differed from the reaction of these plants to known crucifer-infecting tobamoviruses. On the basis of its physical, serological, and biological characteristics, TVCV appears to be a previously unreported tobamovirus.

We have routinely purified cauliflower mosaic virus (CaMV) from infected turnip (*Brassica rapa* L.) plants by the method of Hull et al (7). However, one such preparation was anomalous. The amount of hybridization of CaMV DNA to aliquots of the preparation was much lower than expected, based on the spectrophotometrically determined yield of CaMV. When the total nucleic acid from this preparation was analyzed by agarose gel electrophoresis, an unexpected nucleic acid species was observed. The preparation also was being used in a study of the ability of CaMV to adapt to and replicate in the nonhost tobacco (*Nicotiana tabacum* L. 'Samsun'). In that study, we passed the virus alternately through turnip cv. Just Right and tobacco. Tobacco plants inoculated with homogenates of turnips infected from the suspect preparation developed symptoms similar to those caused by tobacco mosaic virus (TMV) *vulgare*. Turnip plants inoculated with homogenates of these tobacco plants developed only vein-clearing symptoms. We report here the partial characterization of a previously unknown tobamovirus, turnip vein-clearing virus (TVCV), that was isolated and identified as the causal agent of the vein-clearing symptoms.

Oklahoma Agricultural Experiment Station journal article J-6183.

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Accepted for publication 24 August 1992.

MATERIALS AND METHODS

Virus propagation, purification, and extraction. Two methods were used to isolate TVCV from the contaminated CaMV preparation. In the first method, the nucleic acid was extracted from a viral preparation and treated for 15 min at 37 C with 100 μg/ml of RNAse-free DNAse I (BRL, Bethesda, MD), following the manufacturer's recommendations. The sample was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The resulting RNA, at 0.4 mg/ml, served as inoculum for turnip plants. In the second method, the contaminated CaMV preparation at 2 μg/ml was inoculated on turnips. Homogenates of infected turnip leaves (21) were used to inoculate tobacco.

Preparations for plant inoculation were diluted in 1% K_2HPO_4 with 3 mg/ml of Celite. When plants were at the five-leaf stage, the two oldest leaves were removed and each of the remaining leaves was inoculated by gently rubbing a 20-μl drop of inoculum over the leaf surface. The inoculated plants were maintained in a growth chamber under a mixture of incandescent and fluorescent lamps at a photosynthetic photon flux density of 270 $\mu E \cdot m^{-2} \cdot s^{-1}$ on a 12-hr photoperiod. The chamber temperature ranged from 23 to 20 C in the light and dark, respectively. The plants were watered daily.

From turnip or tobacco leaves infected with TVCV, separated from CaMV as described above, we extracted virions as described by Sherwood and Fulton (17), but without 2-mercaptoethanol. In some preparations, bentonite was added to the extraction medium to a concentration of 17 mg/ml. The purified virions were filter-sterilized by passage through a 0.2-μm syringe filter.

Alternate passage through turnip and tobacco. Turnip and tobacco were inoculated with TVCV virions. Virions were then extracted from diseased turnip and tobacco plants as described above. Each new preparation was used to inoculate additional plants of each of the two species. Virion extraction and inoculation were repeated twice. Control plants either were not inoculated or were inoculated with virion preparations from tobacco plants, which were infected with TMV *vulgare* by inoculation with in vitro transcripts of pTMV204 (3). All plants were assessed for symptoms over an 8-wk period.

Host range. Host specificity was examined by inoculating the following plant species with TVCV: *N. tabacum* 'Xanthinc', *N. clevelandii* A. Gray, *N. sylvestris* Speg. & Comes, *Phaseolus vulgaris* L. 'Top Crop', *Vigna unguiculata* (L.) Walp., *Petunia violacea* Lindl., *Chenopodium amaranticolor* Coste & Reyn., *Lycopersicon esculentum* Mill., and *Cucumis sativus* L. The plants and uninoculated controls were maintained in the growth chamber as described above for 4 wk and regularly observed for symptoms.

Nucleic acid and protein analysis. Nucleic acid was extracted from purified virions by a modification of the method for CaMV described by Gardner and Shepherd (4). Purified virions were digested with protease K (1 mg/ml) in 0.2 M Tris-HCl (pH 7.5), 10 mM EDTA, and 0.1% SDS. Nucleic acid was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The nucleic acid concentration was estimated by determining absorption at 260 nm.

Glyoxalated or undenatured nucleic acid was analyzed by 1% agarose gel electrophoresis in 10 mM sodium phosphate (pH 6.5) as described by Carmichael and McMaster (2). After electrophoresis, the gels were stained with 30 μg/ml of acridine orange in electrophoresis buffer (2) and destained overnight at 4 C with five to seven volumes of electrophoresis buffer. Nucleic acid bands were visualized with a UV light source. A kilobase ladder served as a size standard.

The molecular weight of the viral coat protein was determined by SDS-PAGE (9). A purified virion suspension was

mixed with sample buffer, boiled at 100 C for 5 min, and subjected to electrophoresis on a 0.75-mm-thick 12% polyacrylamide gel (1) along with samples of CaMV and TMV *vulgare* virions and polypeptide size standards (Bio-Rad Labs, Richmond, CA). The gel was stained with Coomassie blue. The molecular weight of the polypeptides from TVCV was determined by comparison with those of TMV *vulgare* polypeptides and with the size standards.

Serological tests. Antiserum against TMV *vulgare* was previously produced (18) and antiserum against TVCV was similarly produced. Briefly, a rabbit was injected intramuscularly twice weekly for 4 wk with 1 mg of purified virus in Freund's complete adjuvant. To determine the extent of serological relationship of TMV *vulgare* and TVCV, microprecipitin tests and reciprocal agar gel double-diffusion tests were conducted (18) with purified TMV *vulgare* and TVCV virions and antisera to each virus.

Fig. 1. Turnip leaf infected with turnip vein-clearing virus.

RESULTS

Virus purification. Both DNAse treatment of the nucleic acid of the contaminated CaMV preparation and alternate passage of the preparation in turnip and tobacco resulted in isolation of the contaminant from CaMV. Turnip plants inoculated with purified RNA or homogenates of infected tobacco plants developed limited vein clearing around major veins (Fig. 1). Serial passage of the contaminated CaMV preparation exclusively through turnips continued to result in turnips with symptoms typical of CaMV infection, i.e., chlorotic spots, chlorotic mottle, and fine vein clearing (12). Analysis of nucleic acid extracted from virions purified from plants infected with isolated TVCV failed to reveal any CaMV DNA.

Symptoms on turnips and tobacco. On turnips, symptoms first appeared usually about 4 wk after inoculation. The clearing of major veins appeared on emerging leaves. As the emerged leaves continued to grow, the symptoms spread along the veins. The symptoms did not spread to older mature leaves. No other symptoms were noted. In *N. tabacum* 'Samsun', TVCV infection caused a mosaic symptom (Fig. 2) similar to that caused by TMV *vulgare* (20). The symptoms first appeared about 5 days after inoculation. The TVCV symptoms on turnip and tobacco were reproduced when turnip and tobacco plants were reinoculated or cross-inoculated with virions extracted from infected plant tissues. Although TMV *vulgare* produced typical symptoms on tobacco plants, it did not produce any symptoms on turnip plants, and TMV could not be recovered from those plants (R. T. Lartey, *unpublished*).

Host range. The most severely affected species was *N. clevelandii* (Table 1); severe stunting was the initial reaction, followed by a typical mosaic 2 wk after inoculation. *N. tabacum* 'Xanthi-nc', *N. sylvestris*, and *C. amaranticolor* reacted with local lesions. In all cases, the lesions appeared in the first week after inoculation. None of the remaining test plants—*L. esculentum, P. vulgaris, V. unguiculata, C. sativus*, and *P. violacea*—showed any reaction to TVCV (Table 1). The possibility that asymptomatic species were tolerant hosts was not explored.

Nucleic acid and protein analysis. When undenatured TVCV nucleic acid was purified and analyzed by agarose gel electrophoresis and acridine-orange staining, a single orange band indicative of single-stranded nucleic acid was observed. The nucleic acid was sensitive to RNAse. After electrophoresis of glyoxalated TVCV nucleic acid, a single fluorescent band with a size of 6.4 kb was observed (Fig. 3). The migration of this RNA was indistinguishable from that of RNA isolated from TMV *vulgare*.

A single Coomassie blue-stained band was observed after SDS-PAGE of TVCV virions (Fig. 4). The TVCV polypeptide reproducibly migrated slightly faster than the coat protein of TMV *vulgare*. By reference to the migration of standard proteins, the apparent molecular weight of the TVCV polypeptide was 1.0 ± 0.5 kDa less than that of the TMV poly-

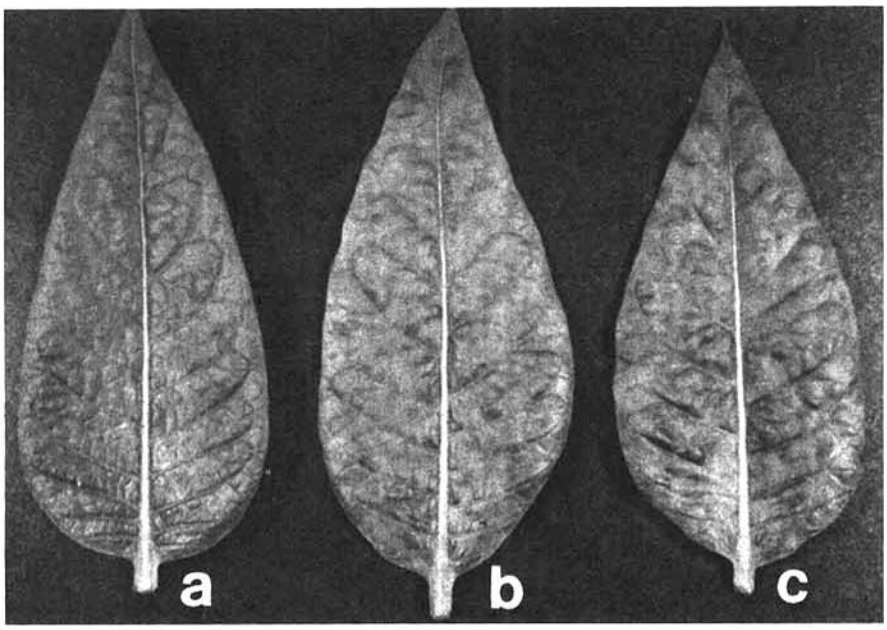

Fig. 2. Symptoms on tobacco (*Nicotiana tabacum* 'Samsun') leaves: **(A)** Uninoculated control, **(B)** infected with TMV *vulgare*, and **(C)** infected with TVCV.

Table 1. Reactions of indicator plants to inoculation with virions of turnip vein-clearing virus[a]

Host species	Symptoms
Brassica rapa	Late veinal chlorosis
Nicotiana tabacum 'Samsun'	Systemic; mosaic
N. tabacum 'Xanthi-nc'	Local lesions
N. clevelandii	Systemic; crinkling and severe stunting initially, severe mosaic later
N. sylvestris	Local lesions
Chenopodium amaranticolor	Local lesions
Phaseolus vulgaris	None
Vigna unguiculata	None
Petunia violacea	None
Lycopersicon esculentum	None
Cucumis sativus	None

[a] Plants were evaluated 4 wk after inoculation.

peptide, which had an apparent size of 18.5 kDa under the conditions used.

Serological tests. Homologous virus-antiserum combinations reacted more strongly than heterologous combinations in reciprocal microprecipitin and agar double-diffusion tests of antisera to TVCV and TMV *vulgare*. The dilution end point in the microprecipitin test was 1,024 for each homologous virus-antiserum combination. The dilution end points were 256 for anti-TVCV with TMV *vulgare* and 512 for anti-TMV *vulgare* with TVCV. Thus the serological differentiation index for TMV *vulgare* and TVCV (19) was 2 for the reaction to anti-TVCV and 1 for the reaction to anti-TMV *vulgare*. This difference in reaction also was evident in the agar double-diffusion assay. Although each virus reacted with its homologous antiserum, no band was evident in the assays with anti-TVCV and TMV *vulgare*, in which a number of serial dilutions of both antigen and antiserum were used. When anti-TMV *vulgare* was tested against TVCV and TMV *vulgare*, spur formation was observed on the precipitin band.

DISCUSSION

The virus isolated from the contaminated CaMV preparation shared several properties with TMV *vulgare*, the type member of the tobamoviruses. On *N. tabacum* 'Samsun', TVCV caused symptoms indistinguishable from those caused by TMV *vulgare*. The nucleic acids of the two viruses also were indistinguishable, both in number and type of components and in molecular size. The molecular sizes of the single viral polypeptides of the two viruses were similar. Anti-TMV cross-reacted with TVCV. We conclude that TVCV is a tobamovirus.

The specific origin of this tobamovirus is an enigma. The contamination was tentatively traced to a turnip mosaic virus (TuMV) culture obtained by the Melcher laboratory from the Sherwood laboratory (17). Plants inoculated with that culture were coresident in a growth chamber with plants that gave rise to the contaminated CaMV preparation. Plants inoculated with the suspect TuMV culture developed chlorosis around veins of late-emerging leaves and contained a virus indistinguishable from that described here as TVCV. Before and since the transfer of the culture to the Melcher laboratory, however, there has been no indication of contamination of the parent TuMV culture in use in the Sherwood laboratory.

The properties of TVCV did not correspond with those of any tobamoviruses reported replicating in crucifers. TMV *vulgare* replicates in *Arabidopsis thaliana* (L.) Heynh., a crucifer, but does not cause visible symptoms (16). That we and others (13) were unable to detect replication of TMV *vulgare* in turnip, coupled with the serological results, establishes that TVCV is not TMV *vulgare*.

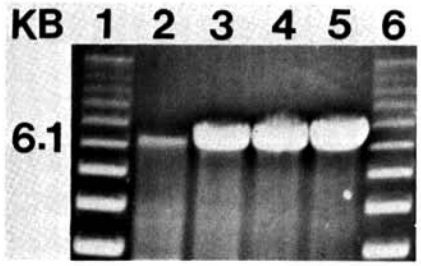

Fig. 3. Electrophoretic migration of TVCV and TMV RNAs. Glyoxalated nucleic acid preparations from the original contaminated CaMV preparation (lane 2), TVCV purified from turnips (lane 3) or *Nicotiana tabacum* 'Samsun' (lane 4), and TMV *vulgare* (lane 5) were separated in 1% agarose. A glyoxalated kilobase ladder (lanes 1 and 6) served as a size standard. The number at left indicates the size of the standard band closest to the sample bands.

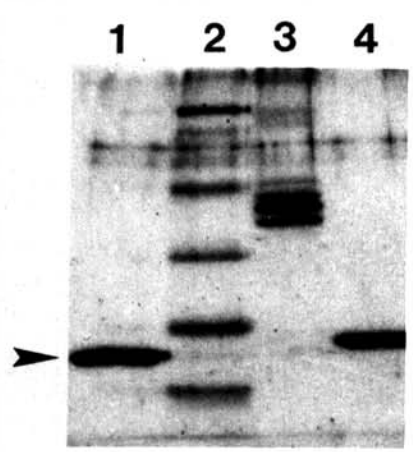

Fig. 4. SDS-PAGE of TVCV virions. Polypeptides of virions of TVCV (lane 1), CaMV (lane 3), and TMV *vulgare* (lane 4) were separated in 12% polyacrylamide. Polypeptide size standards are in lane 2. Arrow indicates position of TVCV coat polypeptide.

TVCV is not a known crucifer-infecting strain of TMV. TMV-C, isolated from the crucifer *Radicula sylvestris* Druce, causes vein clearing and a mosaic in *B. rapa* (13). TMV-C differs from TVCV in that no mosaic was associated with TVCV infection of *B. rapa* and that TMV-C symptoms on *N. tabacum* 'Samsun' are readily distinguished from TMV *vulgare* symptoms. TMV-Cg, isolated from garlic, is similar to TMV-C and infects turnips, causing both vein clearing and a mosaic (10). It infects *P. violacea* and *L. esculentum*, whereas TVCV did not. For species infected by TVCV and TMV-Cg, symptoms differ substantially. TMV-Cg induces necrotic spots within a mosaic in *N. tabacum* 'Samsun', whereas TVCV did not cause necrotic spots. A mosaic and distortion accompanying TMV-Cg necrotic spots on *C. amaranticolor* were not seen with TVCV infection of this species.

TVCV also is not ribgrass mosaic virus (RMV) or youcai mosaic virus (YMV). YMV is closely related to RMV (5,14) and may be a strain of it (15). RMV causes a light mosaic in *Arabis alpina* L. and intercostal light chlorosis in *Berteroa incana* (L.) DC. (8). Yet, RMV causes fine necrotic spots on *N. tabacum* 'Samsun', whereas TVCV caused typical TMV symptoms on this species. YMV infects crucifers and *N. tabacum* but, unlike TVCV, can also induce symptoms on *P. vulgaris* and *L. esculentum* (15). Thus, no tobamovirus previously reported to infect crucifers corresponds to TVCV.

The results of serological tests suggest that TVCV may be a virus distinct from TMV (6). The results were similar to those between other pairs of distinct tobamoviruses, such as YMV and TMV (15). The dissimilar reactions of indicator plants to TVCV and TMV support the hypothesis that TVCV is not a strain of TMV. Preliminary analysis of the nucleotide sequence of random cDNA clones of TVCV RNA (R. T. Lartey and U. Melcher, *unpublished*) also suggests that TVCV is no more closely related to TMV than it is to any other tobamovirus whose nucleotide sequence is available. The similarity in symptoms induced by TVCV and TMV in *N. tabacum* 'Samsun' underscores the hazards of relying exclusively on symptom classification in virus identification (6). TVCV may be extensively distributed, because the symptoms caused on *N. tabacum* could be easily mistaken for TMV symptoms and because symptoms on turnips appear only late during their growth.

We cannot rule out that TVCV is a strain of a tobamovirus other than TMV, such as YMV (or RMV), but we consider it unlikely. Occurrences of YMV in North America have not been reported. There is a considerable difference in the reactions of indicator plants to TVCV and to the other viruses. The RMV coat protein is identical in size to that of TMV (11), whereas that of TVCV is noticeably smaller (Fig. 4). The above considerations lead us to suggest that the designation of TVCV as a distinct tobamovirus is consistent with accepted criteria of virus identification (6).

Added in galley: Recent gel electrophoretic analysis by Leslie Lane (University of Nebraska) revealed that cyanogen bromide fragments of the coat protein of TVCV were indistinguishable from those of ribgrass mosaic virus.

ACKNOWLEDGMENTS

This work was supported in part by a Phillips Petroleum Foundation fellowship to the second author, the Oklahoma Health Research Program (HR8-048), and the Oklahoma Agricultural Experiment Station. We thank Ann Williams for technical assistance.

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