

Identification of Tobacco Ringspot Virus in *Clerodendrum thomsoniae*

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ABSTRACT

The causal agent of ringspot symptoms on *Clerodendrum thomsoniae* 'Glorybower' was mechanically transmitted to *Gomphrena globosa*. It was purified from *n*-butanol:chloroform-clarified sap of inoculated *Nicotiana tabacum* 'Xanthi-nc' by differential centrifugation and also by a polyethylene glycol (MW 6,000)-NaCl precipitation

procedure. The causal agent was identified as an isolate of tobacco ringspot virus because of its host range, physical properties, particle morphology, and serological relationships. This is the first identification of a virus disease of *Clerodendrum* spp.

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Additional key words: polyethylene glycol precipitation.

Because of the attractive flowers and perennial growth habit of *Clerodendrum thomsoniae* Balf. 'Glorybower', procedures are being developed for growing this plant in greenhouses as a potted flower for commercial producers (G. E. Beck, *personal communication*). In this program, a source plant originally obtained from the Mitchell Conservatory, Milwaukee, Wisconsin, was propagated by stem cuttings. Some of these asexually propagated plants showed prominent ringspot symptoms.

In 1962, Burnett and Youtsey (1) reported transmitting a pathogen from *C. thomsoniae* with zonate ringspot

symptoms to healthy plants by means of leaf grafting. They were unable to transmit the pathogen by sap inoculation techniques. No other investigations on virus diseases of any *Clerodendrum* spp. are known. This study was undertaken to isolate, characterize, and identify the causal agent of the ringspot symptoms in *C. thomsoniae*.

MATERIALS AND METHODS.—*Virus source.*—The *Clerodendrum* ringspot virus (CRSV) isolate was obtained from a local lesion on a *Gomphrena globosa* L. leaf that had been inoculated mechanically with the sap from a *Clerodendrum* leaf showing typical

ringspot symptoms. Inoculum was made by grinding the leaf in 30 mM potassium phosphate buffer, pH 7.0. This single-lesion isolate was propagated and maintained in *G. globosa* and *N. tabacum* L. 'Xanthi-nc' in the greenhouse.

Purification.—Clerodendrum ringspot virus was purified by slight modifications of the methods of Stace-Smith et al. (10) and Steere (11). Fresh or frozen infected Xanthi-nc or *N. tabacum* L. 'Cariollo' leaves, harvested 7-10 days after inoculation, were fragmented in a Waring Blendor with 0.5 M sodium borate buffer, pH 7.0 (1 ml/g leaf tissue) containing 10 mM sodium diethyldithiocarbamate (DIECA) and 20 mM 2-mercaptoethanol as a stabilizer. The fragmented tissue was squeezed through cheesecloth, clarified with an equal volume of *n*-butanol:chloroform mixture (1:2, v/v), and stored for 6-8 hours. The denatured protein was removed by low-speed centrifugation (10,000 *g* for 10 minutes) and the virus was then pelleted from the supernatant fluid by high-speed centrifugation [78,000 *g* (average) for 2 hours]. The resulting pellets were resuspended in 10 mM ethylenediaminetetraacetate (EDTA) buffer, pH 7.0, and clarified by low-speed centrifugation. After two additional cycles of high- and low-speed centrifugation, the partially purified virus preparation was dialyzed against three changes of 50 mM Tris [tris(hydroxymethyl)-amino-methane]-HCl buffer, pH 7.0. This virus preparation was used in antisera production.

The purified virus preparations of CRSV and tobacco ringspot virus (TRSV) used in serological studies were obtained with the polyethylene glycol precipitation procedure (3, 4, 5). Polyethylene glycol (PEG, MW 6,000; Union Carbide Corp., New York, N.Y.) and NaCl were added with stirring to the clarified extract to final concentrations of 10% and 0.3 M, respectively. The mixture was allowed to stand for 1 hour at 4 C, and then the precipitated virus was pelleted by a low-speed centrifugation (10,000 *g* for 10 minutes). The pellets were dissolved in one-fourth the initial volume of 30 mM potassium phosphate buffer, pH 7.0, containing 10 mM EDTA. After the insoluble material was removed by a second low-speed centrifugation, the virus was further purified and concentrated by differential centrifugation. The final preparation was suspended in 30 mM potassium phosphate buffer, pH 7.0.

Serology.—Antiserum against CRSV was produced by immunizing a rabbit through intramuscular injections of 1 ml of purified virus suspension (1 mg/ml, A_{260} of 10) emulsified with 1 ml of Freund's incomplete adjuvant at 3- to 4-day intervals for 4 weeks. Two more injections were given at weekly intervals; and, after the sixth week, serum was obtained and tested for antibody titer. Intramuscular booster injections were given 1 day after each bleeding. The sera collected from the bleedings were bulked and absorbed with protein prepared from healthy tobacco leaves. This tobacco protein was prepared by the same method as for CRSV with the exception that the pellets obtained from high speed (30,000 rpm in Spinco rotor 30 for 3 hours) were resuspended in 30 mM potassium phosphate buffer, pH 7.0.

An antibody fraction of this serum was precipitated by ammonium sulfate (24.3 g/100 ml of serum), pelleted by low-speed centrifugation, suspended in distilled water, and dialyzed for 36-48 hours against three changes of 50

mM Tris-HCl-saline (TS) buffer, pH 7.2, to remove sulfate ions. Sodium azide (0.1%) was added as a preservative.

Geranium isolates of tobacco ringspot virus (TRSV), type strain of TRSV, their antisera, and antisera against a gladiolus strain of TRSV were kindly supplied by Mostafa Abo El-Nil, R. W. Fulton, and G. A. de Zoeten (Department of Plant Pathology, University of Wisconsin, Madison), respectively. The type-strain TRSV antisera PV 45 was from ATCC.

Ouchterlony gel double diffusion tests were used for virus identification. These were carried out in plastic petri plates (9-cm diameter) containing about 15 ml of 0.9% Ionagar No. 2S (Wilson Diagnostic, Inc., Glenwood, Ill.) dissolved in TS. Sodium azide was added as a preservative. Six peripheral wells (antigen wells), 5 mm in diameter were punched in the solidified agar 5 mm from the central well (antiserum well). The antigen and antisera were placed in their wells and plates were observed for precipitin lines from 12 hours to 7 days. The antiserum titer was determined both by double diffusion and microprecipitin tests.

RESULTS.—*Host range and symptomatology.*—Naturally infected *C. thomsoniae* leaves showed characteristic ringspot symptoms consisting of definite alternating bands of chlorotic yellow and green rings (Fig. 1). Necrotic rings, puckering,

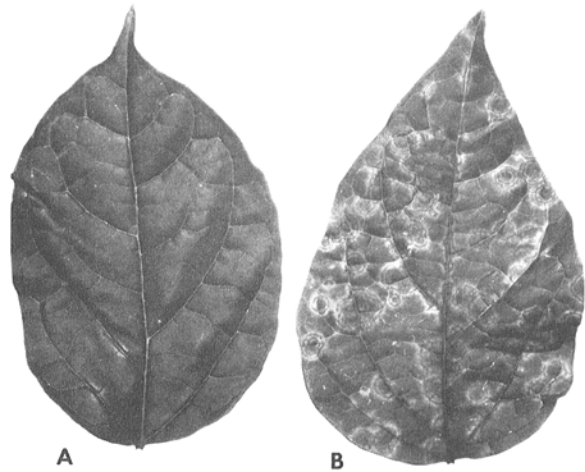


Fig. 1. *Clerodendrum thomsoniae* leaves **A**) from a healthy plant, and **B**) from a diseased plant showing ringspot symptoms.

chlorosis, and distortion of young leaves were also common. These symptoms were most pronounced during summer and fall.

Preliminary observations, particularly symptoms produced on tobacco, had indicated that CRSV might be a strain of TRSV. Therefore, differential hosts of TRSV, including 19 plant species and cultivars from 11 genera in six families, were tested for their reactions to CRSV. Five to 8 days after inoculation, necrotic rings appeared on inoculated leaves of *Nicotiana clevelandii* A. Gray, *N. rustica* L., *N. tabacum* L. 'Havana 38', Cariollo, and Xanthi-nc. Prominent systemic ringspot symptoms were observed by 2 weeks. *Nicotiana clevelandii* showed severe

chlorosis followed by necrosis and death of the entire shoot. Solid circular tan local lesions appeared within 1 week after inoculation on *Phaseolus limensis* L. 'Thorogreen' and 'Henderson's Bush', *Phaseolus vulgaris* L. 'Bountiful', *G. globosa* and *Vigna unguiculata* (L.) Walp. 'Blackeye'. *Phaseolus vulgaris* and *V. unguiculata* showed chlorotic spots followed by necrosis of veins and leaves. Systemic chlorotic spots and mottling, occasionally followed by necrosis, were produced on *Cucumis sativus* L. 'Straight 8', *Petunia hybrida* Vilm., and *G. globosa*. *Chenopodium amaranticolor* Coste & Reyn. and *Chenopodium album* L. developed only local lesions. *Antirrhinum majus* L. showed circular necrotic lesions followed by occasional systemic necrosis of the other leaves. *Datura stramonium* L. developed necrotic brown lesions on inoculated leaves, and these were followed by small necrotic spots on systemically infected leaves. After repeated tests, no symptom was observed on, and the virus was not reisolated from, *N. glutinosa* L., *Lycopersicon esculentum* Mill. 'Bonny Best' and *Pisum sativum* L. 'Darkskin Perfection' and 'Perfected Wales'.

CRSV isolate and the type strain of TRSV were compared in a parallel inoculation study which included these 19 plant species and cultivars. The symptoms produced by both (TRSV and CRSV) were similar on most of the plant species; however, TRSV produced severe necrosis on *D. stramonium* and *P. hybrida*, and a milder systemic symptom in *N. rustica* than CRSV.

When *C. thomsoniae* was mechanically inoculated with sap prepared from *G. globosa* or Xanthi-nc tobacco infected with either the CRSV or TRSV isolates, small necrotic spots were evident on inoculated leaves within 10 days, and systemic ringspot symptoms appeared after 3-4 weeks. Both virus isolates were recovered from inoculated and systemically infected leaves. Inoculum prepared from *C. thomsoniae* which showed prominent ringspot symptoms was not successfully used to produce symptoms on *C. thomsoniae*.

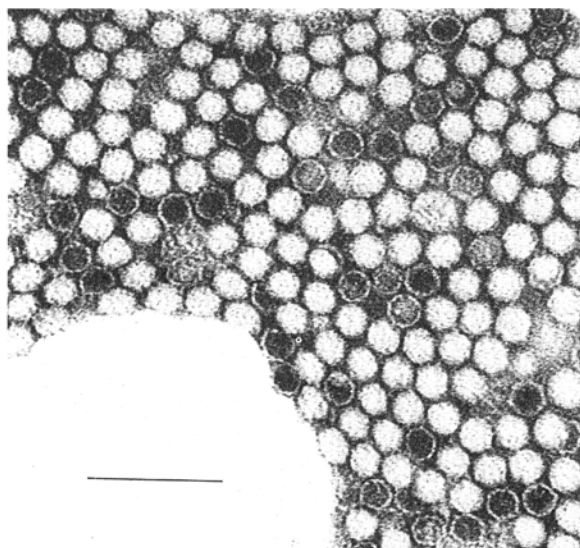


Fig. 2. Electron micrograph of a purified preparation of *Clero dendrum* ringspot virus particles stained with uranyl formate. Magnification bar equals 100 nm.

Purification of *Clero dendrum* ringspot virus.—CRSV preparations obtained by clarification of sap with the *n*-butanol-chloroform treatment followed by differential centrifugations were highly infective. Such preparations showed an ultraviolet spectrum of nucleoproteins typical of spherical plant viruses with a maximum absorbance at 260 nm, and an $A_{260/280}$ ratio of 1.65 (an average of six preparations). CRSV precipitation with PEG following *n*-butanol-chloroform clarification was an equally satisfactory means of purification, and was more convenient since it was simpler and larger volumes could be processed.

The efficiency of various PEG and NaCl concentrations in recovering CRSV was determined. Four concentrations of PEG (4, 6, 8, and 10%) and five concentrations of NaCl (0.1 M to 0.5 M) were used in various combinations to precipitate CRSV from 10 ml samples of clarified sap. Ten percent PEG with 0.3 M NaCl was optimum for maximum recovery of CRSV.

Morphology and physical properties of *Clero dendrum* ringspot virus.—Electron micrographs of purified CRSV preparations fixed with glutaraldehyde and stained with saturated solution of uranyl formate showed polyhedral particles with an average diameter of 28 nm (internal size standard was potato virus X, width = 13 nm) (Fig. 2).

The thermal inactivation point for CRSV was 65 C; however, more than 50% of the infectivity was lost at 55 C. Blackeye cowpeas developed local lesions when inoculated with infective sap from Xanthi-nc tobacco diluted to 1×10^{-2} , but not when diluted to 1×10^{-3} .

Serology.—The crude antiserum obtained from a rabbit immunized with the purified CRSV preparations had an antibody titer of 1:256 to the homologous antigen, CRSV, and a titer of 1:8 to healthy plant protein preparations. After this serum was absorbed with protein prepared from healthy tobacco leaves and then precipitated with ammonium sulfate, the final antiserum preparation had an antibody titer of 1:2,560 and did not react with the protein from healthy tobacco leaves.

Since the properties and host ranges of CRSV and TRSV were similar, their serological relationship was determined. In agar-gel double diffusion tests, CRSV reacted similarly with antisera against three different isolates of TRSV (TRSV from geranium, gladiolus, and ATCC PV45). Agar-gel double diffusion tests of CRSV, the TRSV from geranium, and PV45 against their antisera resulted in precipitin arcs without spurs between each virus isolate and their homologous or heterologous antiserum. Reciprocal cross-absorption between CRSV antiserum and TRSV antiserum with CRSV and TRSV was performed by intragel and liquid-phase methods (12). No precipitation lines formed when these cross-absorbed antisera were tested against CRSV or TRSV antigens.

CRSV did not react with antisera against several other spherical viruses, apple mosaic virus, prune dwarf virus, or tomato ringspot virus.

DISCUSSION.—The ringspot symptom on observed *C. thomsoniae* was similar to that of the zonate ringspot reported on *C. thomsoniae* from Florida (1). Burnett and Youtsey (1), in their preliminary studies, could not transmit the virus by sap inoculation from *C. thomsoniae* to *C. thomsoniae*. In our experiments, CRSV was mechanically transmitted from *C. thomsoniae* to cowpea or *G. globosa* and then back to *C. thomsoniae*. Purified

CRSV preparations or inoculum from infected cowpea, *G. globosa*, or tobacco incited the typical ringspot syndrome; however, CRSV was not transmitted from *C. thomsoniae* to *C. thomsoniae*. This failure in transmission may be partly attributed to the low concentration of CRSV in *C. thomsoniae*, indicated by serology and infectivity tests (6). The infectivity of inoculum from cowpea, *G. globosa* or tobacco was considerably higher than from *C. thomsoniae*.

The host range, general properties, and morphology of CRSV were similar to those reported for TRSV isolates (8, 9). Only minor differences in symptoms induced by CRSV and TRSV occurred on certain species, e.g., *P. hybrida* and *N. rustica*. These differences are not great enough to establish the CRSV isolate as a distinct strain of TRSV. CRSV is considered to be serologically identical to the type isolate of TRSV because of the lack of sput formation in agar-gel double diffusion tests and the complete cross-absorption of CRSV or TRSV antisera with their heterologous antigens. These findings indicated that CRSV is an isolate of TRSV.

From the results on transmission, properties, and serology, it is concluded that CRSV is the incitant of *Clerodendrum* ringspot in *C. thomsoniae*. Final proof of this assertion was obtained by reproducing the ringspot syndrome on *C. thomsoniae* with TRSV. *Clerodendrum thomsoniae* plants inoculated with sap from TRSV-infected *G. globosa* showed chlorotic ringspot similar to those incited by CRSV. TRSV has a wide natural host range and has been transmitted experimentally to many species in a large number of angiosperm families (2, 3, 7, 8). Its identification as the causal agent of *Clerodendrum* ringspot extends the natural host range of TRSV to the family Verbinaceae, and this is also the first report of a virus disease studied in detail from *C. thomsoniae*. Since little is known about the zonate ringspot described by

Burnett and Youtsey (1), it is not possible to conclude that this disease is also incited by TRSV even though the symptoms are identical.

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