Characterization of a Carlavirus Isolated from *Aster novae-angliae*

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ABSTRACT

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A previously unidentified carlavirus was isolated from *Aster novae-angliae* near Jordan Station, Ontario, and was found in one commercial greenhouse containing cultivars of *Aster* spp. Infected plants were stunted and showed systemic chlorosis and rosetting. Apical leaves were distorted, often showing necrotic flecking. The virus was readily transmitted mechanically and by several aphid species between *Aster* and *Chenopodium* spp. but was not seedborne. On the basis of host range studies, no similarity could be found between the virus and other viruses described in *Aster* spp. or within the carlavirus group. No serological relatedness was evident between the virus and several viruses described in the potexvirus, carlavirus, or potyvirus group. Physiochemical properties were similar to those of other carlaviruses. The virus was a single sedimenting nucleoprotein (590–640 nm long and 13 mm in diameter) with a buoyant density in sucrose of 1.29 g/cm^3, a sedimentation coefficient of 160 S, an ultraviolet extinction coefficient of 2.4, and an $A_{259/280}$ ratio of 1.38. Polyacrylamide gel electrophoresis revealed one protein species with an M_r of 35,400 daltons and RNA with an M_r of 2.78×10^6 daltons. The hyperchromic profile indicated a single-stranded RNA.

Aster novae-angliae L., or New England aster (4), is a prevalent weed throughout southern Ontario. This species grows abundantly in dry, sandy loam soils across the Niagara peninsula and is usually found in meadows, in abandoned fields, and along roadsides. In 1989, a carlavirus isolated from *A. novae-angliae* differed from any of the viruses previously described in *Aster* spp. and was tentatively named aster chlorotic stunt virus (AsCSV). The virus was transmitted mechanically and by several aphid species and may represent a potential threat to commercially grown *Aster* spp. This paper describes some of the biological properties of this virus.

MATERIALS AND METHODS

Virus purification. Virus was isolated from *A. novae-angliae* collected along fencerows near Jordan Station, Ontario. A single-lesion isolate of the virus was obtained from mechanically inoculated leaves of *Chenopodium quinoa* Willd. Small chlorotic lesions appeared on inoculated leaves after 3–4 days, followed by systemic foliar symptoms in 14–18 days. The virus was propagated and subsequently maintained in *C. amaranticolor* Coste & Reyn.

Virus was extracted from systemically infected *C. amaranticolor* leaves by differential centrifugation. Other procedures using organic solvents for clarification (8,13,26–28,30) or precipitants such as ammonium sulfate or polyethylene glycol (1,7) caused severe particle aggregation with the result that most of the virus was lost during the initial low-speed centrifugation. Tissue was homogenized in extraction buffer (0.5 M boric acid/NaOH buffer [pH 7.5] containing 0.01% sodium thioglycollate and 0.01 M sodium ethylenediaminetetraacetic acid). The homogenate was expressed through cheesecloth and the sap was clarified by centrifugation at 10,000 *g* for 10 min. The sap was adjusted to 1% Triton X-100 and stirred for 2 hr at room temperature. After low-speed centrifugation (10,000 *g*, 2 hr), pellets were resuspended in 6 ml of extraction buffer and reclarified (10,000 *g*, 5 min). Further purification of the virus was achieved by centrifugation at 104,000 *g* for 2 hr in 10–40% linear sucrose density gradients. The visible single broad band of virus was collected with an ISCO density-gradient fractionator (Instrumentation Specialities, Lincoln, NE), and virus was recovered by centrifugation at 24,000 *g* for 2 hr. Pellets were resuspended in 5 ml of extraction buffer, centrifuged (10,000 *g*, 5 min), and assayed for infection on *C. quinoa*. Virus was stored in extraction buffer at 4 C.

Pathogenicity tests. The virus isolate was examined on an extended host range. Systemically infected leaves of *C. amaranticolor* were triturated in 0.01 M neutral phosphate buffer (tissue:buffer, 1:9), and the extract was rubbed onto Carborundum-dusted leaves of three plants of each of the following: *Achillea filipendulina* Lam. 'Carnation Gold' and *A. ptarmica* L. 'Pearl'; *Ajuga reptans* L. 'Rubra'; *Althaea rosea* L. 'Rose'; *Aster* spp. 'Comet', *A. alpinus* L. 'Handy Blanc', *A. novae-angliae* L. 'Barrs Pink', 'Lilac', and 'Rose', and *A. novi-belgii* L. 'Cardinal', 'Crimson', 'Mini Lady', and 'Orlando'; *Aurinia saxatilis compactum* (L.) Desv. 'Wonderland'; *Begonia* × *tuberhybrida* Voss 'Midnight Beauty'; *Callistephus chinensis* (L.) Nees 'Bouquet Powderpuff'; *Chenopodium amaranticolor* and *C. quinoa; Chrysanthemum maximum* Ramond 'Silver Princess' and *C.* ×*morifolium* Ramat. 'Dunnettii'; *Coreopsis tinctoria* Nutt. 'Sunburst'; *Cucumis sativus* L. 'Improved Long Green'; *Dahlia pinnata* Cav. 'Figaro'; *Datura stramonium* L.; *Delphinium grandiflorum* L. 'Magic Fountain'; *Dianthus barbatus* L. 'Dwarf Single' and *D. caryophyllus* L. 'Noir'; *Gazania rigens* (L.) Gaertn. 'Mini Star'; *Gerbera jamesonii* H. Bolus ex J.D. Hook. 'Festival'; *Gomphrena globosa* L. 'Dwarf Buddy'; *Helenium autumnale* L.; *Helianthus maximilianii* Schrad. 'Zebulon'; *Impatiens wallerana* J.D. Hook. 'Elfin Blush'; *Iris pumila* L. 'Azurea'; *Liatris spicata* (L.) Willd. 'Kubold'; *Lycopersicon esculentum* Mill. 'Glamour'; *Myosotis scorpioides* L. 'Victoria Rose'; *Nicotiana clevelandii* A. Gray, *N. glutinosa* L., *N. rustica* L., and *N. tabacum* L. 'Harrow Velvet'; *Papaver nudicaule* L. 'Champagne Bubbles'; *Pelargonium* × *domesticum* L.H. Bailey 'Scarlet Orbit'; *Petunia* × *hybrida* Hort. Vilm.-Andr. 'Calypso'; *Phaseolus vulgaris* L. 'Frenchie'; *Portulaca grandiflora* Hook. 'Calypso'; *Primula* spp. 'Pacific Giant'; *Salvia splendens* Sellow ex Roem. & Schult. 'Scarlet Queen'; *Saxifraga bergenia cordifolia* L.; *Tagetes erecta* L. 'Inca Orange'; *Vigna unguiculata* (L.) Walp. 'Queen Anne Blackeye'; and *Zinnia elegans* Jacq. 'Peter Pan'.

Inoculated plants were maintained under greenhouse conditions (25 C), with supplementary sodium vapor and halide lamps providing a maximum light intensity of 350 $\mu E \cdot m^{-2} \cdot s^{-1}$. Plants were examined daily for virus symptoms. Five weeks after inoculation, plants were checked for the presence of virus by

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examining leaf triturates in the electron microscope and by back-inoculation onto *C. quinoa*.

Seed transmission. Seed was field-collected from 10 infected *A. novae-angliae* and *C. amaranticolor* plants and germinated in darkness. Seedlings were transplanted into 12-cm clay pots and maintained in the greenhouse for 8 wk. Apical leaves from each plant were tested for virus by electron microscopy and by bioassay on *C. quinoa* (23).

Aphid transmission tests. The green peach aphid (*Myzus persicae* (Sulz.)) and the melon aphid (*Aphis gossypii* Glover) were identified as the predominant aphid species collected from *Aster* spp. For transmission tests, nonviruliferous apterous aphids were reared on healthy *A. novae-angliae* plants. Aphids were allowed to feed for 12 hr on both systemically infected *A. novae-angliae* and *C. amaranticolor*. After feeding, 20 aphids were transferred to each of five healthy *A. novae-angliae* and *C. amaranticolor* plants and allowed to feed for an additional 12 hr. Plants were sprayed with a 0.026% aqueous solution of pirimicarb and maintained for 21 days in the greenhouse. Virus infection was confirmed as described previously.

Serology. Serological relationships with viruses in the potexvirus, carlavirus, and potyvirus groups were examined by the Ouchterlony double-diffusion technique (19) using 1% Difco Bacto agar containing 0.85% NaCl, 2% sodium dodecyl sulfate (SDS), and 0.1% NaN_3. Tissue from infected *A. novae-angliae* and *C. amaranticolor* was homogenized in distilled water, centrifuged (3,000 *g*, 10 min), and pipetted into the center well. Outer wells were loaded with antiserum to the various virus groups. Leaf extracts from *N. tabacum* infected with potato virus S or X or turnip mosaic virus and extracts from healthy *N. tabacum* and *C. amaranticolor* plants served as controls.

Antiserum to the following viruses were provided by R. Stace-Smith (Agriculture Canada, Research Station, Vancouver, British Columbia): the potexviruses clover yellow mosaic, cymbidium mosaic, potato aucuba mosaic, potato N, potato X, and white clover mosaic; the carlaviruses blueberry scorch, dandelion S, elderberry carlavirus, helenium S, poplar mosaic, potato S, and potato M; and the potyviruses bean yellow mosaic, henbane mosaic, pea seedborne mosaic, potato Y, and turnip mosaic.

In vitro tests. The dilution end point (DEP) was determined in sap extracted from virus-infected *C. amaranticolor* and diluted in a 10-fold series in 0.01 M potassium phosphate buffer (pH 7.5). Dilutions were rub-inoculated on *C. amaranticolor*.

Longevity in vitro (LIV) and the thermal inactivation point (TIP) were determined with 1-ml aliquots of infectious crude sap from *C. amaranticolor* in phosphate buffer. LIV was determined at 25 C stored in sealed ampules. A control sample was assayed immediately. At 1-day intervals, an aliquot was assayed by rub-inoculation onto two *C. amaranticolor* indicators. Tests were repeated twice.

To determine TIP, 5 g of systemically infected *C. amaranticolor* leaves was triturated in 10 ml of phosphate buffer and centrifuged for 5 min (5,000 *g*). Then, 1-ml aliquots were heated for 10 min at selected temperatures (40–90 C, 5-C increments) in a circulating water bath controlled to ±0.2 C. The heated solutions were cooled in an ice bath and assayed by rub-inoculation on two *C. quinoa* plants.

Electron microscopy. For observation of virus particles, carbon-stabilized collodion-covered grids (300 mesh) were floated on tissue macerates for 10 min. After being washed, the grids were stained with 2% phosphotungstic acid (pH 6.8) in phosphate buffer, then examined in a Philips 201 electron microscope. Particle measurements were made as described previously.

Buoyant density determination. Purified virus preparations (0.5 mg per tube) were layered onto 10–65% (w/w) sucrose gradients prepared in 0.5 M borate buffer (pH 7.5) and centrifuged at 24,000 *g* for 24 hr. The visible virus band was collected with a syringe and the refractive index was determined at 25 C in an Abbe refractometer (Carl Zeiss, Germany). Conversion of the refractive index to density was done from tables (22).

Sedimentation coefficient. Virus particles disintegrated when run in either $CsCl_2$ or $CsSO_4$ gradients. Sedimentation coefficients were therefore determined in a 10–40% linear sucrose gradient prepared in 0.5 M potassium phosphate buffer (pH 7.5) containing 0.01 M $MgCl_2$. Tomato blackring virus (TBRV) and tobacco mosaic virus (TMV) were used as markers. Marker viruses and the aster virus isolate were layered on gradients, run singly in sister tubes, or combined in the same tubes. Gradient tubes were centrifuged (24,000 *g*) over 6 hr, with the sedimentation depth of each virus being recorded at 1-hr intervals. Sedimentation coefficients were calculated as described previously (9).

Determination of ultraviolet extinction coefficients. The extinction coefficient of sucrose gradient-purified virus was determined as described previously (21).

Protein electrophoresis. Gels of 10% polyacrylamide (75 × 5 mm) were prepared as described by Allen and Dias (3). Virus dissociation and electrophoresis were done as described previously (19). Myoglobin (M_r 16,890 daltons), trypsin (M_r 35,000 daltons), and ovalbumin (M_r 43,000 daltons), 50 μg each, were used as markers for relative molecular mass determination.

RNA electrophoresis. RNA was separated from virus particles as described previously (24). Nucleic acid was electrophoresed in 0.1 M sodium phosphate buffer (pH 7) containing 0.1% SDS in 2.4% polyacrylamide gels (75 × 5 mm) for 2 hr. TMV-RNA (M_r 2.0 × 10^6 daltons), TBRV-RNA (M_r 2.4 × 10^6 daltons), and turnip mosaic virus RNA (M_r 3.1 × 10^6 daltons) were used as markers. Gels were scanned for absorbance at 265 nm and again after staining in 0.01% toluidine blue in 40% 2-methoxyethanol.

Thermal denaturation with extracted nucleic acid. Purified viral nucleic acid was adjusted to 1.42 absorbance units (265 nm) in 0.01 M phosphate buffer (pH 7.5) containing 0.1 M NaCl. The thermal denaturation profile of RNA was determined as described by Allen and Dias (3). Absorbance was corrected for light scatter by extrapolation from absorbance detected at 360–600 nm (11). The graphic determination of the midpoint of hyperchromic transition (T_m) was made as described (3).

Effect of RNase and DNase on viral nucleic acid. Virus, adjusted to 1.5 absorbance units in 0.01 M neutral phosphate buffer, was added to an equal volume of dissociation buffer and incubated at 38 C for 30 min. The sample was then divided into four equal aliquots, two of which received either 3 μg/ml of pancreatic ribonuclease (ICN Nutritional Biochemicals, Cleveland, OH) or 3 μg/ml of deoxyribonclease II (Sigma Chemical Co., St. Louis, MO). All aliquots were incubated for an additional 30 min at 38 C. Nuclease-treated and untreated samples were electrophoresed in polyacrylamide gels and stained as described.

RESULTS AND DISCUSSION

The carlavirus described here is dissimilar to other viruses reported in *Aster* spp. Bidens mottle virus (14,20), oat blue dwarf virus (5,6), tobacco rattle virus (12,17), and potato virus N (2), each previously reported in *Aster*, had distinctly different particle types and host ranges. The host range of the virus was limited to *Aster* and *Chenopodium* spp., which distinguished it from other viruses in the carlavirus group.

Symptoms on all *Aster* spp., both wild and domestic, were similar, with systemic chlorosis and stunting being the most distinguishing features (Fig. 1A). Apical leaves were often curled and elongated with necrotic flecking. Rosetting, associated with shortening of apical internodal length, was common in all infected plants. Irregularly shaped chlorotic lesions, often with necrotic centers, appeared on basal leaves. Commercially grown *A. novae-angliae* spp., including cvs. Barrs Pink, Lilac, and Rose, and all *A. novi-belgii* spp. were fully susceptible, producing severely stunted, unmarketable plants. Although low levels of virus infection were found in only one commercial greenhouse, the presence of virus in wild *A. novae-angliae* in fencerows may present a serious threat to commercial production of domestic *Aster* spp. In *C. amaranticolor* and *C. quinoa*, chlorotic lesions were formed on inoculated leaves. Within 10 days, plants showed prominent vein-netting and chlorotic spotting, most serious in *C. amaranticolor* (Fig. 1B).

Symptoms did not develop in seedlings grown from seed collected from infected *A. novae-angliae* plants, and virus was not detected in plant tissues by either electron microscopy or bioassay on *C. quinoa*. Virus was transmitted in a nonpersistent manner by both *M. persicae* and *A. gossypii*, which is consistent with other viruses in this group. Viruliferous aphids starved for 24 hr failed to transmit the virus into healthy aster plants.

Purification of the virus presented several difficulties. The use of both a boric acid buffer and EDTA was necessary to minimize particle aggregration. The modified procedure of Choi et al (10) failed to prevent virus aggregration and loss during centrifugation. Butanol or chloroform clarification destroyed virus integrity. Precipitation procedures using polyethylene glycol were unsuccessful, as virus was lost during low-speed centrifugation. Different trypsin procedures used for potyvirus purification (25; K. Raju, L. W. Stobbs, R. Kemble, and H. Bertrand, *unpublished*) similarly failed to yield significant amounts of intact virus, possibly because of degradation of virus coat protein (K. Raju, L. W. Stobbs, R. Kemble, and H. Bertrand, *unpublished*). Because of severe particle aggregation and disruption, $CsCl_2$ and $CsSO_4$ could not be used in gradient columns for M_r determinations. Differential centrifugation followed by sucrose rate-zonal centrifugation in sucrose yielded intact virus, with average yields of approximately 1.35 ng/ml, assuming an extinction coefficient of 2.4.

Sap extracted from infected *C. amaranticolor* retained infectivity when diluted to 10^{-6} but not to 10^{-7}. Thermal inactivation was exponential from 50 to 60 C, at which temperature inactivation was complete. Sap from virus-infected *C. amaranticolor* was noninfective after 5 days at 25 C. Infectivity, LIV, DEP, and TIP of the AsCSV isolate were typical of the carlavirus group (15).

Electron microscopy of negatively stained samples of both purified virus and crude plant sap from *C. amaranticolor* revealed numerous virus rods 590–640 nm long (modal length, 610–620 nm) and 13 nm in diameter (500 particles counted). In purified virus preparations, smaller fragments were evident, representing breakdown segments. End-to-end aggregration of rods was more prevalent in gradient-purified virus than in crude sap.

Rate-zonal centrifugation in sucrose gradients formed a single homogeneous band with a buoyant density (g/cm^3) of 1.29. The S_{20} of AsCSV, calculated relative to the standards, was 160, which is average for the carlavirus group. The purified virus in phosphate buffer had an absorption spectrum typical of a filamentous virus with a maximum absorption at 259 nm and a minimum at 244 nm. The $A_{259/280}$ and $A_{259/247}$ ratios were 1.38 ± 0.05 and 1.14 ± 0.03, respectively. The $A_{280/259}$ ratio of different preparations varied from 0.72 to 0.75 but indicated a nucleic acid content of the virus near 6%. On the basis of the optical density and the dry weight of purified virus, the molar extinction coefficient of the virus was $E_{260}{}^{0.1\%} = 2.4$. Average yields of virus, based on this value, were calculated to be 6.2 mg/100 g of fresh weight leaf tissue.

One protein component was resolved after virus dissociation with $M_r = 35{,}400$ daltons, as calculated by comparison with the protein standards. Electrophoresis of the viral RNA yielded a single RNA component with $M_r = 2.78 \times 10^6$ daltons, estimated in comparison with the marker RNAs. Viral nucleic acid was not dissociated with DNase but was with RNASE, as determined by PAGE, and was not infectious when treated with RNase and bioassayed on *C. quinoa*. Undissociated and dissociated virus untreated or treated with DNase systemically infected all inoculated *C. quinoa* indicators.

The hyperchromic profile of viral RNA in 0.01 M phosphate buffer (pH 7.5) showed a mean $T_m = 53$ C, with a mean percent hyperchromicity (absorbance 95.5/50 C) of 18%.

On the basis of particle size, aphid transmission, and biophysical properties, this virus has been tentatively classified as a carlavirus. The narrow host range was similar to that reported for helenium virus S (16,18) and carnation latent virus (29), although the virus failed to infect *Helenium*, *Impatiens*, and *Dianthus* species. Additionally, the virus did not react against antisera prepared against helenium virus S and carnation latent virus. The absence of any serological relatedness to viruses of the carlavirus group and the restricted host range suggest that the virus is unrelated to viruses currently described in the carlavirus group. On the basis of symptomatology, the virus has been tentatively named aster chlorotic stunt virus (AsCSV).

Fig. 1. (A) Rosetting, stunting, and distorted leaves of *Aster novi-belgii* 'Crimson' infected with aster chlorotic stunt virus (AsCSV). **(B)** Vein-netting and chlorosis on leaf of *Chenopodium amaranticolor* infected with AsCSV.

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