# Mechanical Transmission, Identification, and Characterization of a Virus Associated with Mottle Leaf in Cherry

D. JAMES and S. MUKERJI, Agriculture Canada, Saanichton Plant Quarantine Station, 8801 East Saanich Road, Sidney, British Columbia, V8L 1H3

**ABSTRACT**

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A closteroviruslike particle was mechanically sap-transmitted from sweet cherry (*Prunus avium*) to the herbaceous host *Chenopodium quinoa*. Successful transmission was achieved by grinding fresh symptomatic leaves of cherry in a buffer containing magnesium, sodium thioglycolate, and nicotine. Stunting and chlorotic spots were observed on the *C. quinoa* plants 17 days postinoculation. The virus purified from *C. quinoa* had a modal length of 760 nm, a width of 10.2 nm, and striations 3.1 nm in pitch. The nucleic acid size was estimated at 8.2 kb. The thermal inactivation point of the virus was 50–55 C, and the virus-containing sap remained infectious for only 24 hr when stored at room temperature. Mottle leaf symptoms were observed on Bing cherry slash inoculated with purified preparations of the virus. The virus was subsequently transmitted from the inoculated Bing cherry to *C. quinoa*, and its identity was confirmed by indirect ELISA and Western blotting using a monoclonal antibody to the virus.

Cherry mottle leaf disease (CML) was first observed in Oregon in 1920 (26). The infectious nature of the disease was first established in 1935 (14,18). The pathogen occurs naturally in sweet cherry (*Prunus avium* (L.) L.), ornamental flowering cherry (*P. serrulata* Lindl.), peach (*P. persica* (L.) Batsch), and apricot (*P. armeniaca* L.) (4). Differences in symptom severity of this disease have been noticed, and CML is one of the most severe diseases of several cherry cultivars in some regions (4,16). *P. avium* (L.) 'Bing' is a woody indicator host for CML (16); the symptoms include irregular chlorotic mottling and distortion of the leaves, especially the terminal leaves. In some cultivars of sweet cherry, both the quality and the quantity of fruit are affected. The fruits of severely affected trees are small and tasteless with delayed ripening. Diseased trees appear rosetted because of a reduction of terminal growth and shortened internodes. The disease may be transmitted from woody plant to woody plant by budding or grafting; also, it appears that the disease may be transmitted by the scale mite *Eriophyes inaequalis* Wilson & Oldfield (4,16). The incubation period for the disease is short, with symptoms appearing 32 days after inoculation (19).

CML is a disease of both economic and quarantine importance in sweet cherry; however, the causal agent has yet to be identified. Detection of the agent is by indexing on woody indicators, a process that is time-consuming, and the results of which are greatly influenced by environmental conditions (20).

The objective of this study was to attempt to identify the pathogen associated with CML by mechanical transmission to a herbaceous host, purification of the agent, and subsequent fulfillment of Koch's postulates.

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## MATERIALS AND METHODS

**Virus source.** A sweet cherry tree that had been determined to be infected with green ring mottle, CML, apricot ring pox, and Prunus necrotic ring spot (PNRS) was identified as a good source of inoculum for attempts at mechanical sap-transmission to herbaceous indicators. The presence of PNRS infection of the cherry was determined by enzyme-linked immunosorbent assay (ELISA) (5). The presence of green ring mottle,

CML, and apricot ring pox infections was determined by budding onto the woody indicators *P. serrulata* 'Kwanzan,' Bing cherry, and Tilton apricot, respectively. The herbaceous indicator plants used in attempting transmission were seedlings of *Chenopodium quinoa* Willd., *C. amaranticolor* Coste & Reyn., *C. foetidum* Schrad., *Cucumis sativus* L., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Nicotiana benthamiana* Domin., *N. clevelandii* Gray, and *N. occidentalis* Wheeler '37B'.

**Transmission to herbaceous indicators.** Separate samples of fresh leaves, flowers, and bark were collected from the infected cherry tree and ground in buffer. The following buffers were tested: 1) TM buffer (0.05 M Tris, 0.01 M $MgSO_4$, final pH 8.5) containing 0.02 M diethyldithiocarbamic acid, sodium salt (DIECA), and 0.04 M sodium thioglycolate (ST); 2) TM buffer with 1 mg/ml poly-L-ornithine; 3) TM buffer with 0.04 M ST and 2.5% nicotine, final pH 9.5; 4) 0.01 M potassium phosphate buffer (pH 7.0) with 0.02 M DIECA and 0.04 M ST; 5) 0.01 M potassium phosphate buffer (pH 7.0) with 1 mg/ml poly-L-ornithine; and 6) 0.01 M potassium phosphate buffer with 2.5% nicotine (final pH 9.5). Approximately 0.5 g of tissue was ground in 5 ml of buffer. The inoculum was gently rubbed onto leaves dusted with corundum (grit 600). Three plants of each of the herbaceous indicators were used for each treatment. Control plants were buffer inoculated. All plants were kept in the dark for 24 hr prior to inoculation. Inoculated plants were kept in a greenhouse maintained at 20 C day and 18 C night, and illuminated for 16 hr each day. Supplementary lighting was provided by high-pressure sodium lamps.

**In vitro tests.** The thermal inactivation point and longevity in vitro were determined by procedures described by Hill (9). A sample from each heat-treatment and time-interval test was assayed by rub-inoculation onto three seedlings of *C. quinoa*. All tests were repeated three times.

**Virus purification.** The method of Takahashi et al (21) used for the purification of apple stem grooving virus (ASGV) was applied with some modifications. Infected leaves of *C. quinoa* were collected 14–21 days postinoculation. The leaves were homogenized in three volumes of TM buffer containing 0.02 M 2-mercaptoethanol and squeezed through four layers of cheesecloth; then activated charcoal was added (4.5 g/100 ml of homogenate). The mixture was stirred in an ice bath for 10 min and centrifuged at 7,000 *g* for 15 min in a GSA rotor; then a Mg-bentonite preparation (7) was added at 50–70 mg/100 ml of supernatant. This mixture was stirred in an ice bath for 10 min, stored in a refrigerator for 20 min, and then centrifuged at 7,000 *g* for 15 min. The supernatant was adjusted to 6% polyethylene glycol (w/v) and 0.02 M NaCl, stirred on ice for 1 hr, stored in a refrigerator for 1 hr, and then centrifuged at 14,500 *g* for 20 min in a GSA rotor. The pellet was resuspended in 20 ml of TM buffer, centrifuged at 7,000 *g* for 15 min in a Sorvall SS-34 rotor, and the supernatant centrifuged at 65,000 *g* for 4 hr in a Beckman Type-30 rotor. The pellet was resuspended in 10 ml of TM buffer and centrifuged at 7,000 *g* for 10 min. The supernatant was then layered onto sucrose gradients prepared the night before and stored at 4 C. The gradients consisted of 4 ml each of 10, 20, 30, and 40% sucrose (w/v) in TM buffer. The gradients were centrifuged at 65,000 *g* for 2.5 hr in a Beckman model L7 Ultracentrifuge using an SW25.1 rotor. The fraction containing virus, determined by electron microscopy, was diluted fourfold and centrifuged at 65,000 *g* for 4 hr in a Type-30 rotor. For the second sucrose-density-gradient centrifugation, the pellet was resuspended in 10 ml of TM buffer, centrifuged at 7,000 *g* for 15 min, and the supernatant layered onto sucrose gradients prepared the night before. These gradients consisted of 4 ml each of 20, 30, 40, and 50% sucrose (w/v) in TM buffer. The gradients were centrifuged at 65,000 *g* for 3.5 hr using an SW25.1 rotor. The fraction containing virus, determined by electron microscopy, was diluted fourfold and centrifuged at 65,000 *g* for 4 hr in a Type-30 rotor. The virus pellet was resuspended in TM buffer. The yield of virus was estimated by using an extinction coefficient of 2.02 $mg^{-1}$ $ml^{-1}$ $cm^{-1}$ at 260 nm, a value determined for apple chlorotic leaf spot virus (ACLV) (2), a similar virus.

**Electron microscopy.** Grids were examined in a JEOL JEM-100 electron microscope. Formvar-carbon backed copper grids were floated for 30 min at room temperature on purified virus preparations suspended in TM buffer. The grids were rinsed with 1 drop of filter-sterile double-distilled water and stained with 2% uranyl acetate. For particle-length measurements the instrument was calibrated using a JBS #401 grating replica (J.B. EM Services Inc., St. Laurent, Quebec). Measurements were made on 75 closteroviruslike particles (CVLPs). Normal length calculation was performed as described by Monette and James (15).

The serological relationship of the virus under study to some previously reported CVLPs was determined by immunosorbent electron microscopy similar to that described by Van Regenmortel (24). Grids were coated with the appropriate antiserum diluted 1:1,000 (v/v) with 0.06 M potassium phosphate buffer, pH 7.0 (6-PB). The virus particles were trapped and then decorated with the antiserum diluted 1:100 (v/v) with 6-PB. The grids were washed between steps with 6-PB, rinsed with filter-sterile double-distilled water, and then stained with 2% uranyl acetate. The grids were examined as described above. Antisera to the following CVLPs were tested: apple stem grooving virus (ASGV); apple stem pitting virus (ASPV); ACLV; Heracleum latent virus (HLV); potato virus T (PVT); and the grapevine viruses CA-4, GLRV-1, GLRV-3, GVA, and NY-1. These antisera were the ones available at the station with known reactivity to CVLPs.

**Nucleic acid analysis.** The viral nucleic acid was isolated by resuspending the purified virus in 0.5 ml of 0.2 M Tris-HCl (pH 7.5) containing 0.025 M ethylenediaminetetraacetic acid, 0.3 M NaCl, 2% sodium dodecyl sulfate (w/v), and 250 μg/ml proteinase K. Two extractions with phenol/chloroform (1:1, v/v) and one extraction with chloroform/isoamyl alcohol (24:1, v/v) were followed by precipitation with 0.10 volume of 3 M sodium acetate and 2.5 volumes of ethanol (13). The nucleic acid was pelleted by centrifugation at 16,000 *g* for 30 min, then washed twice with 70% ethanol. The pellet was dried and resuspended in sterile double-distilled water. An extinction coefficient of 25 mg $ml^{-1}$ $cm^{-1}$ at 260 nm was used to spectrophotometrically quantify the nucleic acid. Samples were treated with RNase A and DNase I and analyzed by electrophoresis in a 1% denaturing agarose gel containing 5 mM methylmercury hydroxide (13). The nucleic acid size was estimated by comparing its migration with an RNA ladder, with sizes from 0.24 to 9.5 kb (GIBCO, BRL).

**Transmission to woody indicators.** One-year-old, certified virus-free Kwanzan cherry, Bing cherry, and Tilton apricot trees were slash inoculated in the spring of 1991. Inoculations were repeated on the same trees in late summer. The inoculum consisted of purified preparations of the virus diluted to a concentration of 1 mg/ml in TM buffer, pH 8.5. Slash inoculation was performed by making a vertical incision with a sterile razor blade, carefully peeling back the bark, and pipetting 15 μl of the virus suspension (approximately 15 μg of virus) into the incision. Two incisions were made per tree for each inoculation. The wounds were protected by wrapping the stem with a water-resistant, self-sealing latex tape. The infectivity of the inoculum was checked by rub-inoculating three seedlings of *C. quinoa*. Plants of each *Prunus* cultivar were inoculated with TM buffer only and served as controls. The plants were maintained in insect-free screenhouses.

**Immunological assays.** All assays were completed using a monoclonal antibody prepared against virions purified from

*C. quinoa* (S. Mukerji and D. James, *unpublished*). Ascites fluid diluted 1:2,000 (v/v) was used as the detecting antibody in both ELISA and Western blotting.

The plate-trapped form of indirect ELISA as described by Torrance (22) was used. Leaf-tissue samples from healthy controls and infected plants were ground in carbonate coating buffer (1 g tissue/10 ml buffer) using a chilled mortar and pestle. The samples were centrifuged at 3,000 *g* for 10 min, and the supernatant was collected. Two-hundred-microliter aliquots of supernatant from each sample were placed in 16 wells of a microtiter plate and incubated overnight at 4 C. After washing with phosphate-buffered saline containing 0.05% Tween 20, pH 7.4 (PBST), a blocking solution consisting of 1% bovine serum albumin in PBST was added to each well and incubated at room temperature for 1 hr. Next, the monoclonal antibody, diluted 1:2,000 in PBST, was added and the plate was incubated at 37 C for 3 hr. This was followed by incubation with a goat anti-mouse IgG alkaline phosphatase conjugate (diluted 1:2,000) for 3 hr at 37 C. The substrate used was *p*-nitrophenyl phosphate (0.6 mg/ml). Absorbance readings at 405 nm were taken every 30 min for 2 hr using a Titertek Multiskan photometer (Flow Laboratories, Irvine, Scotland).

Leaf-tissue samples of healthy and infected plants were triturated by grinding 0.5 g of tissue in 5 ml of buffer, using a mortar and pestle. The buffers used were TM buffer for leaves of *C. quinoa* and 0.01 M potassium phosphate buffer containing 0.02 M DIECA, 0.04 M ST, and 0.5% nicotine for leaves of cherry. The sap was centrifuged at 3,000 *g* for 10 min and the supernatant was collected. The proteins were separated by polyacrylamide gel electrophoresis incorporating sodium dodecyl sulphate (SDS-PAGE) as described by Laemmli (11). Two identical gels were prepared. One gel was silver stained and used as a control. The other gel was electroblotted onto nitrocellulose (Schleicher & Schuell, Keene, NH, 0.45 $\mu$m pore size) using the procedure described by Towbin et al (23). Prestained markers (Bio-Rad) were used to estimate molecular weight and to assess transfer of the proteins from the gel to the membrane. Diluted ascitic fluid (1:2,000 in phosphate-buffered saline) was used as the primary antibody, and goat anti-mouse IgG alkaline phosphatase conjugate was used as the secondary antibody. The substrate consisted of *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad). These were used as recommended by the supplier.

## RESULTS

**Mechanical transmission.** Seventeen days postinoculation, systemic symptoms were observed on two of the three *C. quinoa* seedlings inoculated with infected cherry leaves ground in TM buffer containing 0.04 M ST and 2.5% nicotine (pH 9.5). The symptoms included chlorotic spots on the leaves (Fig. 1) and stunting of the plants. No symptoms were detected on any of the seedlings inoculated with infected bark or flower tissue. Subsequent mechanical transfer from *C. quinoa* to *C. quinoa* did not require the presence of ST or nicotine.

**In vitro tests.** The highest temperature to which virus-containing samples could be heated and still remain infectious was 50 C. Three of the nine seedlings inoculated with samples heated to 50 C produced symptoms. None of the plants inoculated with samples heated to 55 C or higher produced symptoms. Samples remained infectious after being stored for 24 hr at room temperature, but lost their infectivity when stored for longer intervals.

**Virus purification and electron microscopy.** Using the modified procedure of Takahashi et al (21) with a single sucrose-density-gradient centrifugation, preparations with numerous CVLPs were obtained, but with some host contamination. Attempts to improve the purity of the virus preparations by cesium sulphate-density-gradient centrifugation resulted in degradation of the virus and a loss of infectivity. However, the first preparation, after centrifugation through a second sucrose-density gradient, yielded relatively pure preparations of virus that remained infectious (Fig. 2). A virus yield of 2 mg/100 g of leaf tissue was estimated. Maximum absorption was observed at 260 nm with an $A_{260nm}$:$A_{280nm}$ ratio of 1.62. The purified virus particles had a modal length of 760 nm, and length distribution was normal. The particles were 10.2 nm in width ($n = 21$) with striations 3.1 nm in pitch ($n = 21$).

There appeared to be no serological relationship between the CVLP transmitted from cherry to *C. quinoa* and ASGV, ASPV, ACLV, HLV, PVT, or the grapevine viruses CA-4, GLRV-1, GLRV-3, GVA and NY-1. The immunosorbent electron microscopy tests were all negative.

**Nucleic acid analysis.** When analyzed spectrophotometrically, maximum absorbance of the viral nucleic acid was observed at 258 nm. The ratio of the absorption at 260–280 nm was 1.97. This indicated an acceptable level of purity of the nucleic acid (3). The nucleic acid

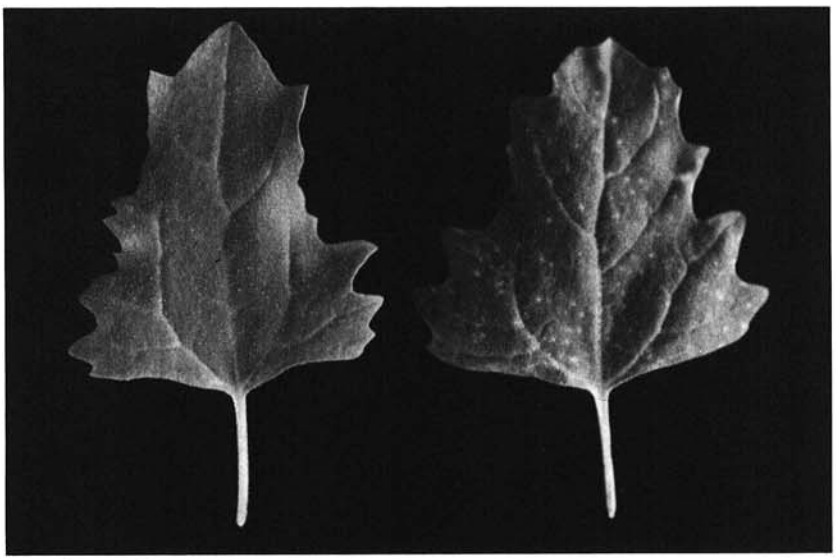

**Fig. 1.** *Chenopodium quinoa* leaves, buffer inoculated (left) and inoculated with extract from symptomatic leaves of cherry infected with cherry mottle leaf (right).

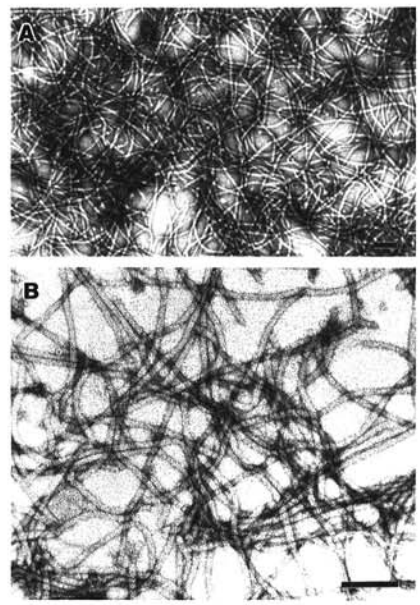


**Fig. 2.** Purified closteroviruslike particles transmitted from sweet cherry to *Chenopodium quinoa*, stained in 2% uranyl acetate. Bar = 100 nm. **(A)** Particles obtained using the purification procedure described. **(B)** Striations associated with closteroviruslike particles can be seen.

was unaffected by DNase but was digested by RNase. Electrophoresis of the nucleic acid in a 1% denaturing agarose gel revealed a band estimated at 8.2 kb in size, or approximately $2.79 \times 10^6$ (using a figure of $3.4 \times 10^5 = 1$ kb of RNA, sodium salt).

**Transmission to woody indicators.** In the spring of 1992, one year after the initial slash inoculations, symptoms were observed on a Bing cherry plant that was inoculated with the purified virus. The symptoms included irregular chlorotic mottling and distortion of the foliage, especially the terminal leaves (Fig. 3). No symptoms were observed on any of the inoculated Kwanzan cherry or Tilton apricot plants. Also, no symptoms were observed on any of the control plants. Symptomatic leaves of the infected Bing cherry were ground in TM buffer containing 0.04 M ST and 2.5% nicotine, pH 9.5. This was used to inoculate *C. quinoa* seedlings. Chlorotic spots were observed on four of six of the *C. quinoa* plants 9 days postinoculation. No symptoms were observed on any of the control plants. The identity of the virus was confirmed serologically (by ELISA and Western blotting) and by host-range studies.

**ELISA and Western blotting.** The virus was detected and identified by indirect ELISA in *C. quinoa* plants used for virus purification, the Bing plant inoculated with the purified virus, and *C. quinoa* plants to which the virus was retransmitted from the inoculated Bing plant (Table 1). The absorbance values given for ELISA are based on readings taken 1 hr after the substrate was added to the microtiter plate.

In Western blot analysis using a monoclonal antibody to the virus purified from *C. quinoa,* a distinct band with an approximate molecular mass of 20.5 kDa was detected in the propagation host, in the infected Bing, and in *C. quinoa* samples from plants to which the virus was retransmitted (Fig. 4B, lanes 4, 5, and 8, respectively). Samples taken from Bing plants that were inoculated but which did not show symptoms, were negative (Fig. 4B, lanes 6 and 7). All tests were repeated at least twice for each sample, and the results were consistent.

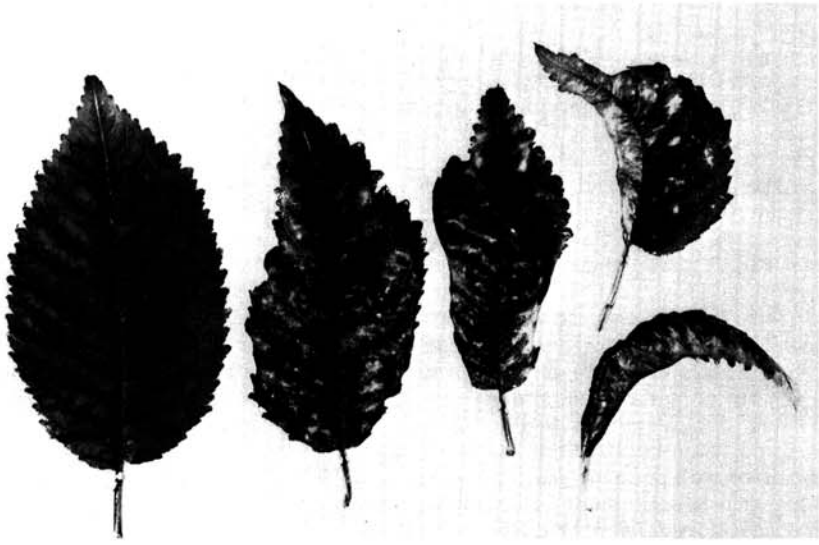

**Fig. 3.** Leaves of Bing cherry taken from a buffer-inoculated plant (extreme left), and from a plant inoculated with purified preparations of the closteroviruslike particle and showing symptoms. Leaves on the extreme right are terminal leaves showing mottling and severe distortion.

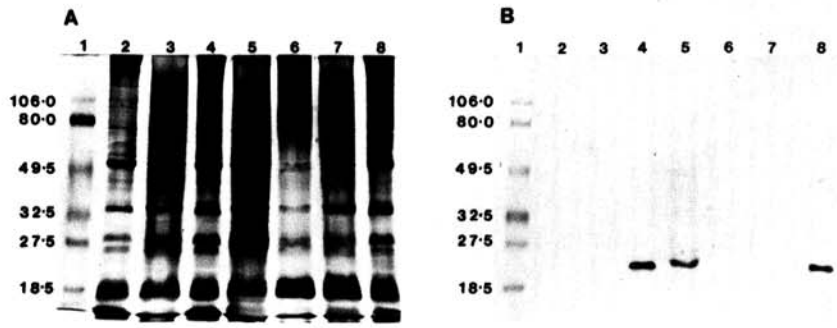


**Fig. 4.** Western blot analysis of crude leaf-sap samples. Patterns from 12% polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE). After **(A)** silver staining and **(B)** Western blotting and screening with a monoclonal antibody to the closteroviruslike particle purified from *Chenopodium quinoa.* Lane 1, prestained molecular mass standards (×1,000 Da); lane 2, *C. quinoa,* buffer-inoculated control; lane 3, Bing cherry, buffer-inoculated control; lane 4, *C. quinoa* with the originally transmitted virus; lane 5, Bing cherry inoculated with pure preparations of the virus and symptomatic; lanes 6 and 7, Bing cherry inoculated with pure preparations of the virus, but symptomless; lane 8, *C. quinoa* plant to which virus was retransmitted.

## DISCUSSION

The virus was isolated from cherry infected with PNRS, green ring mottle, apricot ring pox, and CML. The virus associated with PNRS is a well-characterized ilarvirus (8). CML symptoms were observed on a certified virus-free Bing cherry inoculated with the pure virus.

A CVLP was transmitted from an infected cherry to *C. quinoa* using a high-pH buffer containing magnesium, ST, and nicotine. Leaf extracts of *Prunus* contain large amounts of mucilage, a heteropolysaccharide (25). Phenolic substances such as tannins (17) and cell constituents consisting of proteins, polysaccharides, and enzymes (9) may be involved in the inhibition of virus infection. ST is a reducing agent that inhibits the action of phenols. Also, nicotine reduces the precipitation and inactivation of viruses by tannins (9). A high-pH buffer (6) and the divalent cation magnesium (12) promote the stability of some CVLPs. The presence of these elements in the inoculation buffer may have contributed to the successful transmission of the CVLP from cherry to *C.quinoa.* Neither nicotine nor ST were required for the subsequent transmission of this virus from *C. quinoa* to *C.quinoa.*

The virus was purified from leaves of infected *C. quinoa* plants using a modified version of the procedure of Takahashi et al (21). Addition of a second sucrose gradient improved the purity of the virus preparations. The virus either is unstable in cesium, or was degraded by the dialysis treatment after centrifugation. Lister and Hadidi (12) found that ACLV, a closterovirus, is unstable when subjected to dialysis. The purified virus, a striated flexuous particle, had

**Table 1.** Detection of a virus associated with mottle leaf disease in cherry by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody

| Sample | ELISA values[a] |
|---|---|
| *Chenopodium quinoa,* healthy | 0.093 ± 0.016 |
| *C. quinoa,* infected (propagation host) | 0.742 ± 0.045 |
| *C. quinoa,* infected (retransmitted virus) | 0.459 ± 0.043 |
| Bing cherry, negative control | 0.074 ± 0.018 |
| Bing, infected | 0.586 ± 0.078 |

[a] Mean absorbance values at 405 nm, ± standard deviation, n = 16.

a modal length of 760 nm, a width 10.2 nm, and a pitch of 3.1 nm. The coat protein subunit, detected by Western blotting, had a molecular mass estimated at 20.5 kDa, which was established in an earlier study (10). The viral nucleic acid appeared to be ssRNA, and the size was estimated at 8.2 kb. Given the above characteristics of this virus, it fits into the closterovirus group, subgroup II, based on the classification of Francki et al (8).

The characterized virus was detected by indirect ELISA and Western blotting using a monoclonal antibody, in the purification host, in the inoculated Bing cherry, and in *C. quinoa,* back to which the virus was transmitted. Also, the host range and symptomatology of the reisolated virus were identical to that of the originally transmitted virus (10). The monoclonal antibody was also used, subsequently, to detect the virus in the original *Prunus* host. Thus, this flexuous virus is associated with mottle leaf in cherry. Koch's postulates as described by Agrios (1) were fulfilled. To our knowledge this is the first report of the purification and association of a flexuous virus with mottle leaf in cherry.

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