Etiology

Purification, Serology, and Some Properties of a Mechanically Transmissible Virus Associated with Green Ring Mottle Disease in Peach and Cherry

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ABSTRACT

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Thin, flexuous, rod-shaped viruslike particles were purified from peach and cherry trees affected by green ring mottle disease. Purified particles ranged from 1,000 to 2,000 nm in length and were 5–6 nm in diameter. The presence of fibrillar inclusions in infected cells, together with virion properties such as buoyant density (1.24–1.25 g/cm^3) and nucleic acid and capsid protein molecular weights (2.5×10^6 and 2.5×10^4, respectively), are properties the virus shares with some closteroviruses. Direct and indirect enzyme-linked immunosorbent assay (ELISA) readily detected the virus in various *Prunus* spp. A disease resembling green ring mottle was reproduced in cultivar Montmorency sour cherry trees after slash inoculation with purified virus, and the thin viruslike particles were detected by direct ELISA and electron microscopy in these plants.

Additional keywords: *Prunus avium*, *Prunus cerasus*, *Prunus persica*, stone fruits.

Green ring mottle (GRM) is an important disease of *Prunus* species worldwide. The disease was first reported on cultivar Montmorency sour cherry (*Prunus cerasus* L.) in Michigan in 1937. A few years later it was observed in New York, Wisconsin, and Ontario (20). Since then the disease has been found to affect sweet cherry (*P. avium* L.), oriental flowering cherry (*P. serrulata* L.), peach (*P. persica* Batsch), and apricot (*P. armeniaca* L.) in fruit-growing regions throughout the United States, Europe, and Canada (18).

Symptoms in affected sour cherry trees include a yellow mottle with green islands or ringlike bands on leaves appearing several weeks after petal fall, along with constricting chlorosis along veins or midribs (18). Affected fruits are misshapen, bitter, and not marketable (15,19). Infected oriental cherry cultivars Shirofugen and Kwanzan exhibit foliar epinasty, midrib or veinal necrosis, shortened internodes, and roughened bark (18). Sweet cherry, peach, and apricot are symptomless hosts. The green ring mottle agent is usually detected in these hosts by indexing budwood onto Kwanzan flowering cherry.

Natural spread of GRM in sour cherry orchards is slow and in a pattern indicative of spread from a central source, suggesting spread via root grafts between adjacent trees (2,15). The GRM agent is readily transmitted through grafting but does not appear to be transmitted through seed (8). Attempts at mechanical transmission to woody and herbaceous species have been unsuccessful (14). No biological vectors have been identified.

Little is known about the properties of the GRM agent because it has not been isolated and has not been transmitted mechanically. The agent was resistant to conventional thermotherapy (14) but was inactivated by treatment of sour cherry buds at 38 C for 6 wk (17). Fridlund (7) found similarities in properties among several fruit tree viruses that are grouped together based on graft-

transmission rate. The GRM agent was transmitted at a rate similar to a group of viruses with elongate particles and known aphid or mite vectors. Several of the viruses in this group have been characterized as closteroviruses (1).

The effects of the GRM agent on sweet cherry and peach production are not completely known, although infected sweet cherry nursery trees were reported to be smaller than uninfected ones (13). The disease is important in sour cherry because fruits from infected trees often are unmarketable. Michigan is a major fruit-producing state, ranking first nationally in sour cherry production, second in sweet cherry production, and fifth in peach production. Little is known of the real and potential effects of GRM on fruit production in Michigan. Because the present method of diagnosis (indexing) takes 2–3 mo to complete, and because of the lack of information on the etiological agent, we now report studies on the possible viral etiology of GRM and development of a rapid detection method for a virus associated with this disease. A preliminary report of portions of this work has been presented (10).

MATERIALS AND METHODS

Transmission studies. For host range determination, leaves sampled in July from GRM-affected peach and oriental cherry trees, as determined by indexing done on Kwanzan cherry by the Michigan Department of Agriculture (MDA), were ground in 0.1 M sodium phosphate buffer (PB), pH 7.0, for use as inoculum. This tissue source from the MDA nursery was used for these experiments as well as for virus purification and characterization, antiserum production, and enzyme-linked immunosorbent assays (ELISAs). Three plants each of *Chenopodium quinoa* Willd., *C. amaranticolor* Coste & Reyn., *Nicotiana tabacum* L. 'Xanthi' and 'Turkish,' *Cucumis sativus* L. 'National Pickling,' *Vigna unguiculata* L. Walp. 'SR,' *Phaseolus hybrida* L., *Gomphrena globosa* L., and *Momordica balsamina* L. were inoculated and grown in a greenhouse at 23–25 C. Two weeks after inoculation, leaf-dip preparations from plants were examined for viruslike particles.

In an attempt to fulfill parts of Koch's postulates, we slash inoculated 2-yr-old Montmorency cherry trees (4) with purified virus (see below) in 20% (w/v) sucrose. Trees slash inoculated with 0.1 M Tris-Cl buffer, pH 8.2, in 20% (w/v) sucrose served as controls.

Electron microscopy. For cytopathological studies, both terminal (young) and older leaves from oriental cherry trees affected by GRM (as determined by indexing) from the MDA nursery as well as young and older leaves from symptomless, uninfected trees (also indexed) were examined. Leaf pieces were fixed in 5% glutaraldehyde in PB for 1 hr at room temperature. Tissues were dehydrated in a graded ethanol series, transferred to acetone, and embedded in Spurr's resin (21). Ultrathin sections were double stained with 5% (w/v) uranyl acetate and 0.4% (w/v) lead citrate and were examined at 30,000 × with a Philips 201 transmission electron microscope (N. V. Philips Gloeilampenfabrieken, Eindhoven, Netherlands).

For negative staining of crude extracts, leaf pieces were ground in liquid nitrogen with a mortar and pestle, and 0.1 M Tris-Cl buffer, pH 8.2, was added to the powdered tissue to obtain an aqueous suspension. Parlodion-backed, carbon-coated grids were floated on 20-μl drops of extract for 15–30 min at room temperature. Grids were rinsed in buffer and negatively stained with 1.5% (w/v) ammonium molybdate, pH 6.5.

For immunosorbent electron microscopy (ISEM), grids were floated on 20-μl drops of 1:100–1:800 (v/v) dilutions of antiserum for 1 hr at 23 or 37 C, rinsed in 0.06 M sodium phosphate buffer, pH 7.0, for 10 min, and floated on 20-μl drops of gradient-purified virus (see below) or extract prepared as above for 30 min to 1 hr at room temperature. Grids then were rinsed and stained as above.

Virus purification. Modifications of the methods described by Gugerli et al (9) and Zee et al (23) were used. Leaves freshly harvested from GRM-affected peach and oriental cherry trees, as determined by MDA indexing, or leaves that were stored for up to 2 wk at 6 C were ground in a Waring blender in 10 v (w/v) of 0.5 M Tris-Cl, pH 8.2, containing 4% (w/v) water-insoluble polyvinylpolypyrrolidone, 0.5% (w/v) bentonite, 0.2% (v/v) 2-mercaptoethanol, and 5% (v/v) Triton X-100. The homogenate was stirred for 1 hr at 6 C, squeezed through four layers of cheesecloth, and centrifuged for 30 min at 6,000 *g*. The supernatant was adjusted to 15% (v/v) $CHCl_3$, emulsified by stirring the solution at room temperature for 15 min, then centrifuged at 8,000 *g* for 15 min. Five percent (w/v) polyethylene glycol (PEG) MW 8,000 was added to the supernatant which was made 0.25 M with NaCl. The suspension was stirred for 1 hr, and precipitated virus was pelleted by centrifugation for 20 min at 12,000 *g*. Pellets were resuspended in one-fifth of the original sap volume with 0.1 M Tris-Cl, pH 8.2, (resuspension buffer) by stirring overnight at 6 C. The suspension was centrifuged at 7,000 *g* for 10 min, and the supernatant was subjected to a second cycle of PEG precipitation. After centrifugation at 7,000 *g* for 10 min, the virus preparation was layered on top of sucrose-cesium sulfate step gradients consisting of 1.0-ml layers of 0.0, 0.4, 0.8, and 1.2 M Cs_2SO_4 in 30% (w/v) sucrose in the resuspension buffer. Gradients were centrifuged in a Beckman SW41 rotor (Beckman Instruments, Inc., Fullerton, CA) for 2 hr at 38,000 rpm. Gradients were fractionated using an ISCO Model 185 density gradient fractionator (Instrumentation Specialties Co., Lincoln, NE). Fractions containing rod-shaped viruslike particles (as determined by electron microscopy) were combined, diluted fourfold with resuspension buffer, and pelleted at 26,000 rpm for 4 hr in a Beckman Type 30 rotor (Beckman Instruments).

Determination of virion properties. Virus pellets were resuspended in 1.0 ml of resuspension buffer overnight at 6 C and then centrifuged at 7,000 *g* for 10 min to remove host debris. Absorbances were measured in a Gilford Model 250 spectrophotometer (Gilford Laboratory Instruments Co., Oberlin, OH). The values obtained were used to determine yield and the $A_{260/280}$ ratio. An extinction coefficient of 2.0 was chosen for purified virus preparations, based on work with some viruses with similar properties (1).

Buoyant density of GRM virions was determined by equilibrium centrifugation in Cs_2SO_4. Purified virus (prepared as for absorbance measurements) was mixed with sufficient Cs_2SO_4 to yield a 25% (w/w) solution. Preparations were centrifuged at 35,000 rpm for 20 hr in a Beckman SW50.1 rotor (Beckman Instruments). Gradients were scanned at 254 nm, and 0.3-ml fractions were collected and examined for virus with the electron microscope. Refractive indices of fractions were read in a refractometer (Bausch & Lomb Inc., Rochester, NY) and were converted to densities (22).

For determination of capsid protein molecular weight, virus pellets and pellets from equivalent preparations purified from MDA-indexed uninfected peach or oriental cherry leaves were dissolved in 50–100 μl of dissociation buffer consisting of 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol, and 0.05% (w/v) bromophenol blue in 0.5 M Tris-Cl, pH 6.8. Samples were heated at 100 C for 3 min before electrophoresis. Proteins were separated in 12% SDS polyacrylamide resolving gels according to Laemmli (12) in a Protean Slab Cell apparatus (Bio-Rad Laboratories, Richmond, CA) at 130 V for 7 hr. Low-range molecular weight protein standards (Sigma Chemical Co., St. Louis, MO) were included as markers. Gels were silver stained according to Morrissey (16).

Double-stranded ribonucleic acid (dsRNA) was isolated and analyzed according to a modification of the method used by Jordan (11). Fresh leaves from infected and uninfected peach and oriental cherry trees, as determined by MDA indexing, were ground in liquid nitrogen in a mortar and pestle. The powder was transferred to a 250-ml centrifuge bottle, and 4-v extraction buffer consisting of 2× STE (single strength = 0.1 M NaCl, 0.05 M Tris-Cl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.0), 3% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol, and 0.5 mg/ml fractionated bentonite was added. After mixing briefly, 4-v STE-saturated phenol, pH 7.0, and 2-v chloroform were added to the bottles. After shaking on ice for 30 min, samples were centrifuged at 10,000 *g* for 15 min. The aqueous phase was adjusted to 15 to 18% (v/v) ethanol and then subjected to one cycle of CF-11

(Whatman Ltd., Clifton, NJ) cellulose chromatography on columns in 50-ml disposable plastic syringes. The dsRNA was precipitated overnight with ethanol and collected by centrifugation at 12,000 *g* for 30 min. Samples were treated with 5 μg/ml of DNase (Sigma Chemical Co.) in 30 mM $MgCl_2$ for 1 hr at 25 C, and the dsRNA was ethanol precipitated as previously described. The final pellet was dissolved in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.8) containing 20% glycerol (v/v). Nucleic acids were electrophoresed in 5% polyacrylamide gels for 16 hr at 90 V. Gels were either stained with ethidium bromide (0.5 μg/ml) in distilled water or silver stained as for protein gels. Molecular weight standards (kindly provided by R. Jordan, Agricultural Research Service, Beltsville, MD) were a pool of endogenous dsRNAs from *Phaseolus vulgaris* L. 'Black Turtle Soup' (8.0×10^6 M_r) and *C. sativus* 'National Pickling' (3.8 and $2.2-2.0 \times 10^6$ M_r).

Antiserum production. A female New Zealand white rabbit was initially injected intramuscularly with purified virus (obtained following sucrose-cesium sulfate gradient centrifugation and high-speed pelleting) mixed 1:1 (v/v) with Freund's complete adjuvant. The second and third injections (also intramuscular), administered 9 and 30 days, respectively, after the first injection, consisted of purified virus mixed 1:1 (v/v) with Freund's incomplete adjuvant. Antiserum was obtained from seven bleeds collected biweekly starting 5 wk after the first injection.

ELISAs. Immunoglobulins (Igs) were precipitated with ammonium sulfate, purified by DE 22 cellulose chromatography, and conjugated to alkaline phosphatase (AP) (Type VII-NT, Sigma Chemical Co.) (5).

Budsticks from various species of GRM-infected cherry were obtained from Paul Fridlund (Irrigated Agricultural Research and Extension Center, Washington State University, Prosser) and budded onto 1-yr-old Kwanzan cherry trees. Leaves from these trees were later tested in direct ELISA.

For direct ELISA, leaves from GRM-infected and uninfected cherry trees were ground in a 1:10 (w/v) ratio of phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBS-T). Immulon I microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 2.5 μg/ml of anti-GRM-associated virus IgG for 4 hr at 37 C. Because mixed infections in stone fruits are not uncommon, these tissues were tested for prune dwarf virus (PDV) and prunus necrotic ringspot virus (PNRSV) using 1.25 μg/ml of anti-virus IgG for both viruses. For determination of serological relatedness, anti-apple chlorotic leaf spot virus (ACLSV) and anti-apple stem grooving virus (ASGV) IgG at 2.5 μg/ml (courtesy of AgDia, Inc., Mishawaka, IN) and 1.25 μg/ml of anti-citrus tristeza virus (CTV) IgG (courtesy of D. Gonsalves, New York Agricultural Experiment Station, Geneva, NY) also were tested. After washing, test samples were added and plates were incubated overnight at 4 C. Alkaline phosphatase conjugate (diluted 1:400 [v/v] for GRM-associated virus and 1:800 [v/v] for CTV, PDV, and PNRSV) and horseradish peroxidase conjugate (diluted 1:200 [v/v] for both ACLSV and ASGV) were added to plates which were incubated for 4 hr at 37 C. Absorbances were measured at 405 nm for GRM-associated virus, CTV, PDV, and PNRSV, and at 490 nm for ACLSV and ASGV with a Bio-Tek EL 307 reader (Bio-Tek Instruments, Inc., Winooski, VT). Readings in which the mean of infected extract absorbances exceeded two times the mean absorbance of uninfected extracts were considered positive.

For indirect protein A-ELISA (PA-ELISA) (6), plates were coated with 1.0 μg/ml of protein A (Sigma Chemical Co.) for 2 hr at 23 C. A 1:1,000 (v/v) dilution of both coating and detecting whole antisera was added to plates which were incubated for 2 hr at 23 C. Heterologous sera used to test for relatedness included potato virus Y, tobacco etch, bean yellow mosaic, iris mild mosaic, and soybean mosaic (courtesy of J. Hammond, Agricultural Research Service, Beltsville, MD), clover yellow vein (courtesy of B. Reddick, University of Tennessee, Knoxville), and grapevine leafroll (courtesy of D. Gonsalves). Test antigens were prepared as for direct ELISA and were incubated for 2 hr at 4 C. Protein A-AP conjugate (Zymed Laboratories, Burlingame, CA) (1:1,000) was added and incubated for 3 hr at 23 C.

For indirect ELISA (goat anti-rabbit IgG [GAR]-ELISA) using alkaline phosphatase-labeled GAR (GAR-AP) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), plates were coated with test antigens (1:100, w/v) suspended in 0.05 M carbonate coating buffer, pH 9.6, containing 2% (w/v) polyvinylpyrrolidone for 2.5 hr at 23 C, then blocked with 1% (w/v) bovine serum albumin in PBS-T for 30 min at 23 C. Immunoglobulins (as used in PA-ELISA) were diluted 1:400–1:2,000 (v/v) and incubated for 2.5 hr at 23 C. After washing, GAR-AP (1:2,000 [v/v] dilution) was added and incubated overnight at 4 C. Substrate was added and absorbance was measured as for AP conjugate in direct ELISA.

RESULTS

Transmission studies. None of the mechanically inoculated herbaceous hosts developed virus symptoms, and no rod-shaped (or isometric) viruslike particles were seen in leaf-dip preparations from these plants.

Within 3 mo after inoculation, nine of the 11 Montmorency cherry trees slash inoculated with purified virus showed foliar epinasty and a yellow mottle, symptoms similar to those found on GRM-diseased nursery trees and graft-inoculated trees. Leaves from these nine trees tested postive in direct ELISA using GRM-associated virus IgG (Table 1), and viruslike particles resembling those in the inoculum were isolated from them. Two of the inoculated trees remained symptomless. Leaves from these trees, as well as leaves from trees slash inoculated with buffer alone, tested negative for particles and negative in ELISA.

Electron microscopy. Ultrathin sections of tissue from young and older infected Kwanzan cherry leaves revealed large aggregates of fibrillar material in crossed patterns and in parallel sheets in the cytoplasm of phloem parenchyma cells (Fig. 1A). Cytoplasmic contents of these cells were almost entirely replaced by fibrillar aggregates in older leaves. At higher magnifications, the fibrillar sheets appeared to be comprised of viruslike particles embedded in fibrous material (Fig. 1B). This fibrillar material was not detected in cells from uninfected tissues. Cylindrical or other types of discrete inclusion bodies were not found in these cells.

Viruslike particles were not detected in conventional leaf-dip preparations or in crude leaf extracts even after numerous attempts using various buffers and extraction methods (grinding in liquid nitrogen versus buffer). Similarly, particles were not detected in these extracts even when grids were coated with antiserum for ISEM.

Virus purification. Long, thin, threadlike particles were present in purified preparations following high-speed pelleting from GRM-infected oriental cherry and peach leaves but not in

TABLE 1. A_{405nm} values obtained in direct enzyme-linked immunosorbent assay with leaves from green ring mottle (GRM)-infected and uninfected cherry, using anti-GRM-associated virus IgG

Cherry cultivar	Range	Average[a]
Sweet cherry (bud inoculated)[b]		
Bing	>1.99	>1.99
Lambert	1.48–1.93	1.70
Black Republican	>1.99	>1.99
Black Tartarian	1.19–1.43	1.31
Kwanzan (uninoculated)	0.13–0.25	0.19
Sour cherry		
Montmorency (bud inoculated)	>1.99	>1.99
Montmorency (slash inoculated)[c]	0.43–1.99	1.23
Montmorency (uninoculated)	0.05–0.07	0.06
Oriental cherry		
Kwanzan (bud inoculated)	>1.99	>1.99
Kwanzan (uninoculated)	0.11–0.20	0.17

[a] Numbers are averages of four replicates per sample (bud inoculated).
[b] Uninfected oriental Kwanzan cherry trees were grafted with budwood from sweet, sour, and oriental cherry cultivars (obtained from P. Fridlund) suspected to be infected with the GRM agent. The Kwanzan tissue was tested for the presence of the GRM-associated virus.
[c] Trees were slash inoculated with gradient-purified virus preparations.

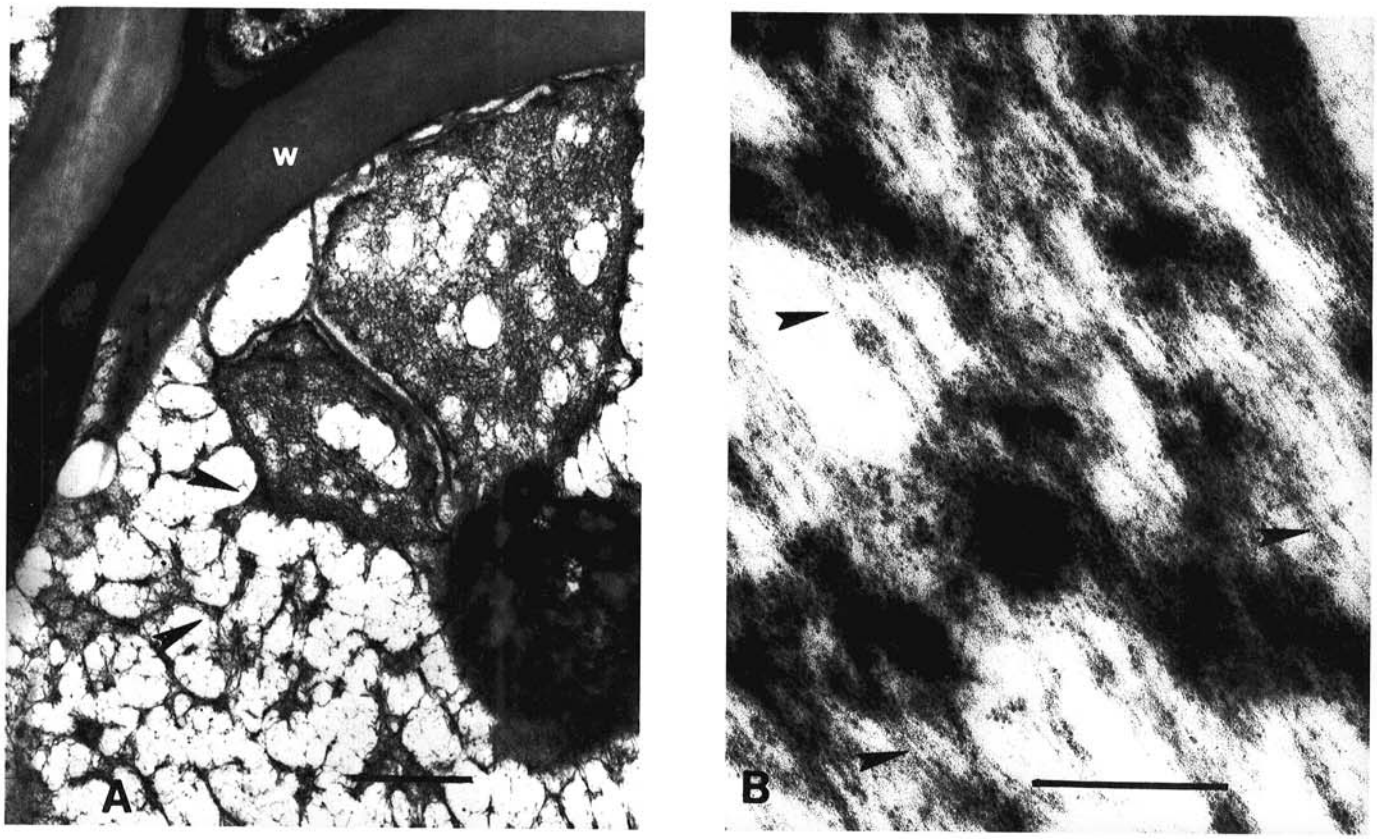

Fig. 1. Transmission electron micrographs of ultrathin sections of phloem parenchyma cells from green ring mottle virus-infected cultivar Kwanzan cherry leaves. **A,** Aggregates of fibrillar material (arrows) within the cytoplasm. W = cell wall. Bar = 1 μm. **B,** Higher magnification of infected phloem parenchyma cell containing viruslike strands of fibrillar material (arrows). Bar = 0.2 μm.

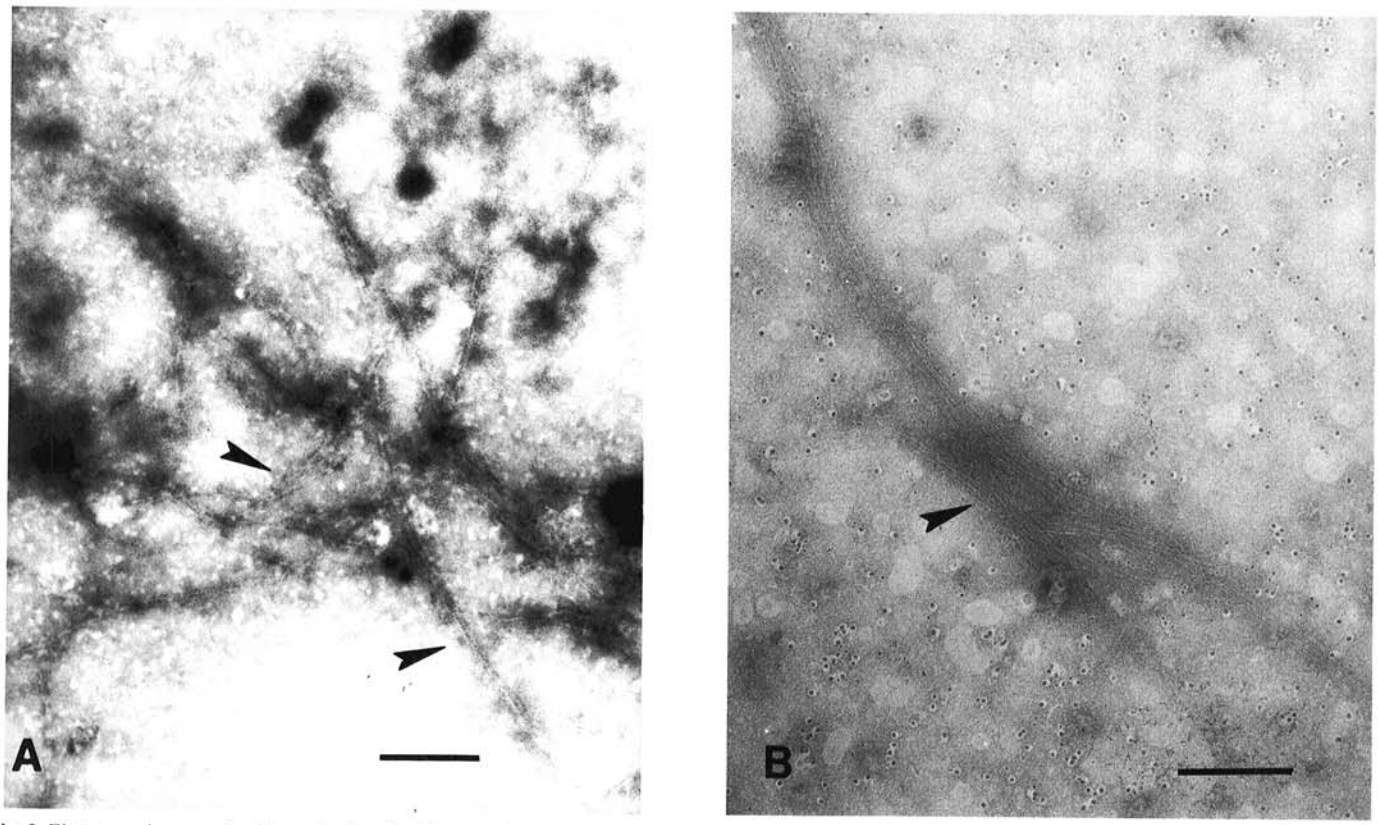

Fig. 2. Electron micrographs of negatively stained green ring mottle-associated virions purified from cultivar Harbrite peach. **A,** Purified particles (arrows) closely associated with host debris. Bar = 0.2 μm. **B,** Particles showing side-to-side aggregation. Bar = 0.2 μm.

preparations from uninfected trees. Particles were found only in preparations purified from leaf lamina tissues, not in preparations purified from only midribs and petioles. Highest concentrations of particles were found in fractions between 6.3 and 7.7 ml below the meniscus of the gradient. A small absorbance peak corresponding to these fractions was evident in the ultraviolet profile of the gradient. Virus yields were low; between < 1 and 100 particles per grid square were counted when a 20-μl sample was applied to the grid. When grids were coated with antiserum for ISEM, fivefold to tenfold more particles per grid square were found than when uncoated grids were used.

Purified particles were highly aggregated; individual particles were infrequently observed. Aggregates of particles in close association with host debris (Fig. 2A) and with other particles (Fig. 2B) were commonly found in most preparations. Attempts to reduce aggregation by varying the Triton X-100 concentrations were unsuccessful.

Particles were extremely thin, measuring 5–6 nm in diameter. The modal particle length could not be determined accurately because of excess aggregation. However, of 30 particles measured, all were between 1,000 and 2,000 nm in length, with most between 1,200 and 1,400 nm.

Only a few virus particles were recovered when purification was by the method of Zee et al (23). Virus was apparently lost after the high-speed centrifugation steps, even though virus pellets were thoroughly resuspended (overnight). Virus was readily recovered when PEG precipitation was used as the initial virus concentration step. This method of concentration may have induced aggregation, however. The addition of the organic solvent clarification step enhanced removal of green host material. However, PEG pellets contained a gelatinous material which was not precipitated by low-speed centrifugation. This material was separated from the virus-containing fractions and was found in a broad zone in the upper 1–2 ml of the centrifuged gradient.

Properties of virions. Virus yields were very low, usually 1–10 μg/g of fresh tissue. The highest yields were obtained from fresh peach tissue collected in mid-summer, whereas less virus was recovered from leaves collected in late fall and from cherry leaves. The $A_{260/280}$ ratio was somewhat high, usually 1.4–1.5. The buoyant density of purified particles in Cs_2SO_4 was 1.24–1.25 g/cm^3.

Two major proteins (2.5×10^4 M_r and 2.3×10^4 M_r) were consistently isolated from pooled purified virus preparations (Fig. 3). The larger protein was more abundant. Similar proteins were not present in preparations purified from uninfected tissues.

One major dsRNA band with a relative molecular mass of 4.9–5.0 $\times 10^6$ Da was isolated from infected Shirofugen and Kwanzan oriental cherry and peach (Fig. 4). These bands were readily detected in tissue extracts from 5.0 g of cherry leaves and 2.0 g of peach leaves. The dsRNA bands were unaffected by incubation with RNase A (5 μg/ml) in 0.3 M NaCl but were digested when incubated with RNase A in distilled water, thus confirming their double-stranded nature.

ELISAs. Leaves from GRM-infected sweet, sour, and flowering cherry gave high, positive absorbance values in direct ELISA (Table 1). Although the GRM-associated virus antiserum showed some anti-plant activity, absorbance values for uninfected samples generally were low. Leaves from these same trees tested negative for PDV and PNRSV, thus eliminating the possibility of a mixed infection with these viruses.

When anti-ACLSV and anti-ASGV Igs were tested against GRM-infected sweet cherry cultivar Black Republican in direct ELISA, absorbance values for infected tissues were only twofold greater than those for uninfected tissues, whereas the differences between uninfected and infected readings were fivefold to tenfold when anti-GRM-associated virus IgG was used. Similarly, clover yellow vein virus (CYVV), grapevine leafroll virus (GLRV), and CTV antisera tested positive in PA-ELISA with a twofold difference over uninfected sample readings, whereas reactions with homologous sera were much stronger. None of the other heterologous sera reacted in PA-ELISA. No reactions occurred with any homologous or heterologous antisera in GAR-ELISA. It is possible that the GRM particles were denatured by the high pH

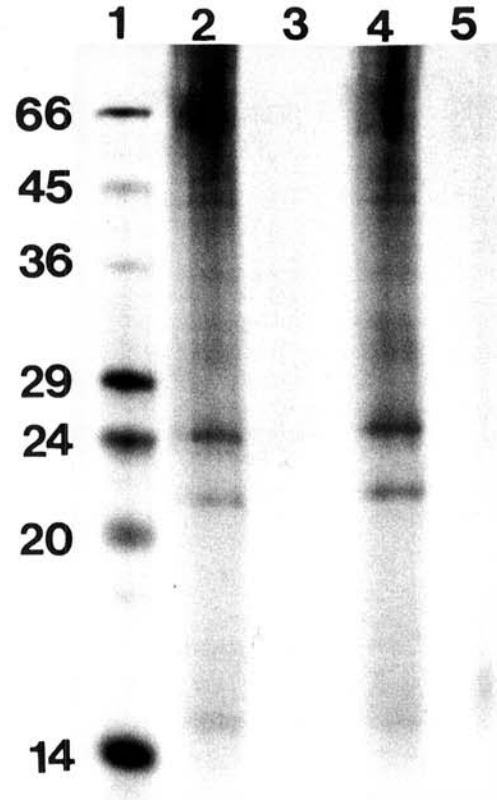

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel of proteins associated with purified green ring mottle (GRM) virions. Lane 1 = molecular weight standards (Sigma Chemical Co., St. Louis, MO): bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; and lactalbumin, 14.2 kDa. Lanes 2 and 4 = proteins from GRM virions purified from cultivar Harbrite peach and cultivar Kwanzan cherry, respectively. Lanes 3 and 5 = protein preparations purified from uninfected Harbrite peach and Kwanzan cherry, respectively.

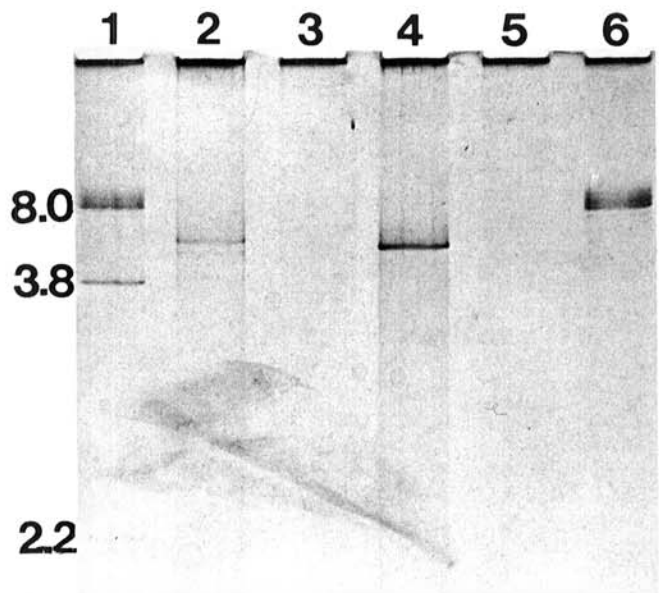

Fig. 4. Silver-stained 5% polyacrylamide gel of dsRNAs isolated from green ring mottle (GRM)-infected leaves. Lane 1 = molecular weight standards ($\times 10^6$ Da) from cultivar Black Turtle Soup bean and cultivar National Pickling cucumber (obtained from R. Jordan). Lanes 2 and 4 = dsRNAs from 5.0 g of GRM-infected cultivar Kwanzan cherry and cultivar Harbrite peach leaves, respectively. Lanes 3 and 5 = dsRNAs from 5.0 g of uninfected Kwanzan cherry and Harbrite peach leaves, respectively. Lane 6 = endogenous dsRNA (8.0×10^6 kDa) isolated from Black Turtle Soup bean by the first author.

of the coating buffer, but this has not been confirmed.

DISCUSSION

Thin, rod-shaped viruslike particles were isolated from peach and cherry trees affected by green ring mottle disease. A preparation containing threadlike particles purified from GRM-diseased leaves was inoculated into a suitable host which later developed symptoms typical of GRM-diseased nursery trees, yielded similar virus particles, and tested positive in ELISA with antiserum made to preparations purified from GRM-diseased nursery trees.

Based on cytopathology, particle morphology, and some virion properties, the virus most closely resembles some members of the closterovirus group (1). Particle lengths of 1,000–2,000 nm, the presence of nonspecific fibrillar inclusions in infected cells, a capsid protein of 2.5×10^4 M_r, a dsRNA species of about 5.0×10^6 M_r, and a particle buoyant density of about 1.25 g/cm^3 in Cs_2SO_4 are characteristics that coincide with some members of this group. The high $A_{260/280}$ ratio (1.4–1.5) also is characteristic for closteroviruses, although this value is of limited significance because of particle aggregation and the presence of some host material in purified preparations. Extrapolating from the size of the dsRNA, the genomic RNA of the GRM-associated virus is about 2.5×10^6 MW. Because of its probable genome size and the presence of ill-defined fibrillar material in infected cells, the GRM-associated virus most closely resembles the subgroup A closteroviruses (1), particularly heracleum latent virus (3). Particle length measurements most closely resemble those of subgroup B members. However, because particles were not detected in crude sap preparations, measurements were made from purified particles. Thus, these lengths may represent end-on-end aggregation (or possibly fragmentation) of particles induced by the purification processes and may not be indicative of actual particle length in vivo.

Antisera to ACLSV, GLRV, and CTV cross-reacted weakly in ELISA with the GRM-associated virus, which could be interpreted as additional evidence for inclusion of this virus in the closterovirus group. However, antisera to ASGV (unclassified) and CYVV (a potyvirus) also cross-reacted at the same level in ELISA with the GRM-associated virus. For these heterologous sera, the positive reactions were only twofold greater than those for healthy antigens, indicating very distant, if any, serological relationships between these viruses and the GRM-associated virus.

Although thin, closteroviruslike particles were consistently associated with GRM-diseased peach and cherry, their etiological significance remains uncertain. Source tissue used in this study indexed positive for GRM, tested negative in ELISA for PDV and PNRSV, and was apparently free from other known stone fruit viruses (e.g., tomato ringspot virus) based on lack of symptom development in herbaceous plants and absence of detectable rod-shaped or isometric virus particles in these plants. However, this does not discount the possibility that the disease is caused by another infectious agent, not detected as intact virions or occurring in a concentration too low for detection by the methods used, that co-purifies with the GRM-associated virus.

The etiological significance of the virus we have partially characterized may be somewhat difficult to determine because of its low concentration in tissues, lack of an herbaceous host, excess particle aggregation, and the presence of copious amounts of mucilaginous material in the *Prunus* leaf extracts. The use of large volumes of extraction buffer reduced the effects of the gelatinous material, enabling the determination of some physical and biochemical properties of pooled purified virus preparations. A purification method ensuring more effective release and separation of particles from host components and other interfering compounds may prove more useful in determining the exact relationship of these closteroviruslike particles with GRM. In addition, more rigorous testing of slash inoculations, that is, performing graft inoculations concurrently with slash inoculations followed by grafting suspect budwood from slash-inoculated trees onto healthy indicators, should confirm that the disease in slash-inoculated trees is identical to that in naturally affected and graft-inoculated trees.

Currently, GRM is diagnosed by indexing suspect budwood onto a suitable indicator and then watching for the development of characteristic symptoms in 2–3 mo. The production of an antiserum and subsequent development of ELISA methods as reported here should greatly improve detection of a virus that we have found to be closely associated with GRM and facilitate final proof of its involvement as the causal agent of the disease.

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