Etiology

Purification and Properties of Closteroviruslike Particles Associated with Grapevine Corky Bark Disease

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ABSTRACT

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Closteroviruslike particles, designated as grapevine corky bark-associated virus (GCBaV), were purified from mature leaves and stem phloem tissue of a corky bark-affected grapevine that had indexed negative for other grapevine viruses. Electron microscopy of purified preparations revealed the presence of flexuous rod-shaped viruslike particles that were about 13 nm in diameter and between 1,400 and 2,000 nm long, with a helical pitch of 3.4 nm. In purified preparations, the GCBaV particles degraded within a few weeks, unlike grapevine leafroll associated virus (GLRaV), which was stable for more than 1 mo under the same storage condition. The molecular weight of the coat protein of GCBaV was 24,000. A large dsRNA molecule (about 15.3 kbp), along with lower molecular weight species, was detected in tissues of corky bark-diseased grapevines, but not in healthy grapevines. Polyclonal antisera were produced in rabbits against purified or partially purified virus preparations. In direct enzyme-linked immunosorbent assay (ELISA), antisera to GCBaV did not react to the serologically distinct types (II and III) of the long closteroviruses associated with grapevine leafroll disease and grapevine virus A (GVA), and vice versa. This antiserum also reacted in ELISA with other corky bark-affected grapevines. Our data suggest that closteroviruslike particles, designated as GCBaV, may be the causal agent of corky bark disease. However, definitive proof is still lacking. The inclusion of GCBaV in the group of closteroviruses with citrus tristeza virus is proposed.

Corky bark, a graft-transmissible virus disease of grapevines, was first described as a viruslike disease under the name "rough bark" by Hewitt in 1954 (16,17). Since then, corky bark has been reported in a number of countries, including the United States (California), Mexico, Brazil, France, Spain, Switzerland, Italy, Yugoslavia, Bulgaria, South Africa, and Japan (1,5,12,13,17,22,23,30,31, 34,35,39). On most cultivars, the pathogen produces only a reduction of vigor. On Cabernet Franc and Gamay cultivars in California, it induces growth retardation at leaf burst or dieback of a few shoots (6,12). Some canes of diseased plants become soft and rubbery with longitudinal cracks at the base, resulting in a tendency to bend downward. Leaves often are smaller than normal, and on dark fruited cultivars, the leaves turn red and roll downward during the summer season. Red leaves of corky bark-affected vines differ from those with leafroll symptoms in that the entire surface of the leaf blade turns red, including the veins. On the diagnostic indicator LN-33 (Couderc 1613 × Thompson seedless), in addition to the aforementioned symptoms, an abnormal corky overgrowth of bark along with longitudinal cracks develops on canes, and the wood cylinder has deep pitting and grooving (6,12).

Although the graft-transmissible nature of corky bark has been demonstrated by many researchers (8,31,36,37), the etiology of the disease has not been determined. Several researchers have associated viruslike particles with grapevines that are affected with corky bark (4,10,38). Recently, Lee et al (25) detected closteroviruslike particles in tissue preparations of corky bark-affected St. George grapevines that were processed according to a purification procedure designed for citrus tristeza virus and other closteroviruses. In this report, we describe the purification and partial characterization of the closteroviruslike particles isolated from a corky bark-affected grapevine and the detection of these particles in crude tissue extracts using antisera produced to these particles. The comparative taxonomy of the rod-shaped grapevine viruses with this virus also is discussed.

MATERIALS AND METHODS

Corky bark isolate. An isolate from California, designated as CB100, was used extensively in our study. The CB100 isolate was diagnosed as corky bark approximately 35 years ago (16). It has been used as a corky bark positive control for many years in grapevine indexing programs because of its strong reaction on the indicator LN-33. The source of CB100 was a corky bark-affected grape cultivar Semillon from the virus collection at the University of California, Davis. It had been indexed on several woody indicators and was found to be free of other known grape

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virus diseases. Cuttings and leaf samples were collected from two selected vines maintained under field conditions at the University of California, Davis. A grape cultivar, Aledo, which indexed as free from corky bark, was used as a healthy tissue source. It originated from the California grape virus certification program. Greenhouse-grown CB100-affected Semillon also was maintained at Geneva, NY.

Virus purification. A modification of the method described by Zee et al (41) and Hu et al (18) was used. Sixty grams of stem phloem tissue or 80 g of petiole tissue, either harvested fresh, stored at 4 C or frozen at −80 C, was powdered in liquid nitrogen using a mortar and pestle. The pulverized material was added to 400 ml of extraction (E) buffer containing 0.5 M Tris-HCl, pH 9.0 at 4 C, 0.01 M $MgSO_4$, 4% water-insoluble polyvinyl pyrollidone (PVP), 0.5% bentonite, 0.2% 2-mercaptoethanol, and 5% Triton X-100, and agitated for 1 h at 4 C. The suspension then was squeezed through four layers of cheesecloth, and clarified by centrifugation for 20 min at 6,000 *g*. Pellets were resuspended in 40 ml of E buffer, clarified by low-speed centrifugation, and the resulting supernatant was combined with that from the first low-speed centrifugation. The extract was concentrated by high-speed centrifugation for 2 h at 80,000 *g* over 5-ml pads of TMS-20 buffer (20% sucrose [w/v] dissolved in 0.1 M Tris-HCl, pH 9.0, containing 0.01 M $MgSO_4$) in a Beckman type 30 rotor (Beckman Instruments, Palo Alto, CA). The resulting pellets were thoroughly dispersed in a small amount of TMS-10 buffer (10% sucrose [w/v] dissolved in 0.1 M Tris-HCl, pH 9.0, at 4 C, with 0.01 M $MgSO_4$), combined in a 40-ml centrifuge tube, and brought up to a total volume of 9 ml. The suspension was shaken at high speed, 4 C for 14 h, and left at 4 C for 1 h to allow host debris to settle. The clear upper phase was carefully removed with a pipet and the "settled" debris was centrifuged at 10,000 *g* for 15 min. The two supernatants were combined and loaded on a Cs_2SO_4-sucrose cushion-step gradient (10, 22.5, and 30% Cs_2SO_4 each in TMS-10 buffer), and centrifuged at 217,874 *g* (max) in a Beckman SW40Ti rotor for 2.5 h. Fractions containing viruslike particles were collected by a fractionator (Instrumentation Specialties Co., Lincoln, NE) and observed for viruslike particles by electron microscopy. This preparation is referred to as partially purified virus. A second Cs_2SO_4-sucrose cushion-step gradient was used to obtain a "purified" virus preparation.

Antiserum production. Polyclonal antisera were produced in two Flemish Giant × Chinchilla rabbits against purified or partially purified preparations of CB100. All preparations were injected subcutaneously along the back over a 3-wk period. The first injection consisted of 1 ml of purified preparation of the viruslike particles mixed with 1 ml of Freund's complete adjuvant. The two subsequent injections were with 1 ml of a purified preparation mixed with 1 ml of Freund's incomplete adjuvant. Sera were collected weekly starting 21 days after the first injection. Additionally, antisera to GLRaV II (unpublished data) and III (41) that were produced in our laboratory also were used in direct ELISA tests. The experimental conditions were those described above.

TABLE 1. Comparative effects of extraction buffers and reabsorption of enzyme-conjugated immunoglobulin for detecting grapevine corky bark-associated virus (GCBaV) by enzyme-linked immunosorbent assay (ELISA)

Extraction buffers[c]	ELISA[a] reading at 405 nm				
	No reabsorption		Reabsorption[b]		
	Infected[d]	Healthy[e]	Infected	Healthy	Buffer
Nonfat milk	0.650	0.030	>1.500	0.100	0.000
Nicotine	0.550	0.120	1.490	0.190	0.000
Regular	0.400	0.030	1.250	0.110	0.000
Purification buffer	0.450	0.100	1.070	0.220	0.080
$MgSO_4$	0.340	0.065	1.050	0.150	0.000
DIECA	0.360	0.070	0.980	0.180	0.000
Tris	0.330	0.080	0.830	0.170	0.000

[a]Each plate was coated with 2 μg/ml GCBaV immunoglobulin, loaded with each sample extracted with 10 × volume of each extraction buffer and 1:500 dilution of GCBaV enzyme-conjugate.

[b]Reabsorption of enzyme-conjugate was performed with extracts of healthy Aldeo petiole tissue as described by Gonsalves et al (14).

[c]Nonfat milk = regular extraction buffer containing 2% nonfat milk; nicotine = regular extraction buffer containing 0.1% nicotine; regular = PBS (pH 7.4) containing 0.8% NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 0.02% NaN_3; purification buffer = extraction buffer for purification of virus; $MgSO_4$ = regular extraction buffer containing 0.2% DIECA; Tris = Tris-HCL buffer (pH 8.2) containing 0.8% NaCl, 2% PVP, 1% PEG, and 0.02% NaN_3.

[d]GCBaV-infected Semillon petiole.

[e]Virus-free Aledo petiole.

Enzyme-linked immunosorbent assay (ELISA). The antiserum to CB100 was preabsorbed with antigens prepared from healthy LN-33 or Aledo vines as described by Zee et al (41). Immunoglobulin G (IgG) was purified from the antiserum with a protein A column (11) and conjugation of IgG to alkaline phosphatase (type VII, Sigma, St. Louis, MO) was done as described by Clark and Adams (9).

For indirect ELISA, plates were coated with test antigens (crude tissue extracts) suspended in ELISA coating buffer (9), blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, and then loaded with immunoglobulin. Before loading the plate, the immunoglobulin was preabsorbed a second time with extracts of healthy LN-33 or Pinot noir petiole tissue as described by Gonsalves et al (14). Direct double antibody sandwich ELISA (DAS ELISA) generally was done as described by Zee et al (41). Several antigen extraction buffers were tested (Table 1). Controls with buffer, healthy samples, and known infected samples that were used for virus purification were included in all ELISA tests. Absorption was measured at 405 nm with a Dynatech MR 580 reader (Dynatech Laboratories, Inc., Alexandria, VA). To be considered as a positive reaction in ELISA, the sample(s) had to have at least three times the absorbance of the healthy controls and be 0.10 or greater at 405 nm.

Coat protein characterization. To determine the molecular weight of the coat protein, a partially purified virus preparation (from 80 g of petiole tissue) was freeze-dried and then resuspended in 850 μl of distilled water for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (24). The gel was run using 5% stacking and 12% separating acrylamide gels at 100 V (constant voltage) for 2 h in a Mini-slab apparatus (Idea Scientific, Corvallis, OR) as described by Hu et al (18). Protein bands were visualized by Coomassie Brilliant Blue R-250.

Western blotting was done using a Trans-blot cell apparatus (Bio-Rad Laboratories, Richmond, CA) (18). After incubation of the membrane in a preparation of CB100-IgG, CB100-specific coat protein bands were incubated with a protein A gold conjugate solution (Bio-Rad), visualized by the enhancement method following the recommended procedure, and photographed.

DsRNA isolation. DsRNA from corky bark-diseased grapevines was extracted as previously described (18). Stem phloem tissue or mature leaves were used as a source of dsRNA. Purified dsRNA was electrophoresed on either a 5% polyacrylamide gel for 16 h at 20 mA or a 1% agarose gel for 2 h at 40 mA. The gel was stained with ethidium bromide (5 ng/ml) for 30 min, destained with distilled water for 5 min, and then photographed using Polaroid 667 film with a red filter. DsRNAs also were extracted from NY-1 leafroll-affected Pinot noir (41) and from rupestris stem pitting-affected Colobel (2).

Molecular weight standards for size estimation were dsRNA associated with infections by citrus tristeza virus (CTV) (19.6 kbp), tobacco mosaic virus (TMV) (6.3 kbp), cucumber mosaic virus (CMV) (3.7, 3.3, 2.4, 1.0 kbp), and genomic dsRNA of rice dwarf virus (RDV) (4.6, 3.7, 3.2, 2.6, 2.6, 1.5, 1.5, 1.1, 1.0, 1.0, 0.7, 0.7 kbp). CTV dsRNA was isolated from CTV-infected citrus bark tissue obtained from Dr. Garnsey, USDA; Orlando, FL. CMV dsRNA was isolated from CMV-WL-infected tobacco that was maintained in our laboratory. RDV genome dsRNA was obtained from the Laboratory of Plant Pathology, Tokyo Univer-

sity, Tokyo. The molecular weight of dsRNA was determined by the graphical method of Bozarth and Harley (7).

Electron microscopy. Leaf petiole or bark pieces were ground with a mortar and pestle in 0.1 M Tris-HCl buffer, pH 9.0, containing 2% water-insoluble PVP. Specimens were prepared by putting a drop of sample on a Formvar-coated grid for 30 sec, rinsing the grid with five drops of distilled water, and then staining with two drops of 2% uranyl acetate. A carbon grating replica of 5,000 lines per millimeter served as an enlargement standard. Particle length was measured using a micro Digi-Pad (GTCO Corp., Rockville, MD).

The immunosorbent electron microscopy (IEM) method of Milne and Lesemann (28) was used with antisera to CB100, CTV, grapevine virus A (GVA), and GLRaV types II (unpublished data) and III (41). Carbon-coated collodion-filmed 400-mesh grids were coated for 30 min with antiserum diluted 1:1,000 (1:100 for GVA) in PBS. Virions from purified preparations (CB100 and GLRaV type II) or leaf extracts (GVA) were trapped on the grids for 15 min, decorated with antiserum (diluted 1:500 in PBS) for 15 min, and then stained with 2% uranyl acetate. Grids were examined with a JEM-100SX transmission electron microscope (JEOL Ltd., Tokyo). All steps were performed at room temperature, and a 5-drop rinse of the grids with PB (= PBS without NaCl) followed by distilled water was done after each step, except after staining.

RESULTS

Virus purification. Closteroviruslike particles (Fig. 1) routinely were isolated from phloem tissue using slight modifications of the procedures described by Zee et al (41) and Hu et al (18) for GLRaV. Stem phloem tissue (bark scrapings from cane) and leaf petiole tissue could be stored at −80 C for more than a month. However, both the partially purified preparations (in TMS-10 buffer containing Cs_2SO_4) and the purified preparations (in 0.1 M Tris-HCl, pH 9.0, 4 C) of GCBaV particles were unstable and degraded within a few weeks at 4 C as shown by electron microscopy. They were stable, however, if 0.01 M $MgSO_4$ was present in the buffer. Yield comparison of GCBaV was done between petioles, leaves, and stem tissues by counting the number of particles trapped on one opening of a 400-mesh grid. Ten grid openings were checked in each fraction of the second Cs_2SO_4-sucrose cushion-step gradient after centrifugation. Yields from petioles were higher than from stem (Fig. 1) and leaf tissues (data not shown). For example, 100 g of leaf petioles gave a 0.6 ml peak fraction of partially purified preparation that had approximately 105 particles per grid opening, whereas stem tissue (260 g) had approximately 58 particles per grid opening, and leaf tissue (100 g) had approximately 24 particles per grid opening. These results were different from those of GLRaV, which yielded high amounts of virus particles from mature stem phloem and leaf laminae tissue (18,41).

Partial characterization of GCBaV. Electron microscopy of negatively stained preparations of GCBaV particles from partially purified preparations showed flexuous rod-shaped particles (Fig. 2) with a uniform diameter of 13 nm (Fig. 2A,B) and a helical pitch of about 3.4 nm (Fig. 2C). Of 609 particles that were mea-

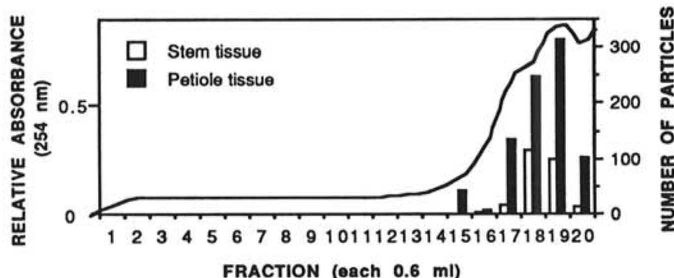

Fig. 1. UV-absorbance patterns and detection of grapevine corky bark-associated virus (GCBaV) (from petiole tissue) after a second Cs_2SO_4 sucrose cushion-step gradient centrifugation. Particle numbers are the average of GCBaV particles trapped from both petiole and stem tissues on one opening of a 400-mesh grid. Ten grid openings were checked for each fraction.

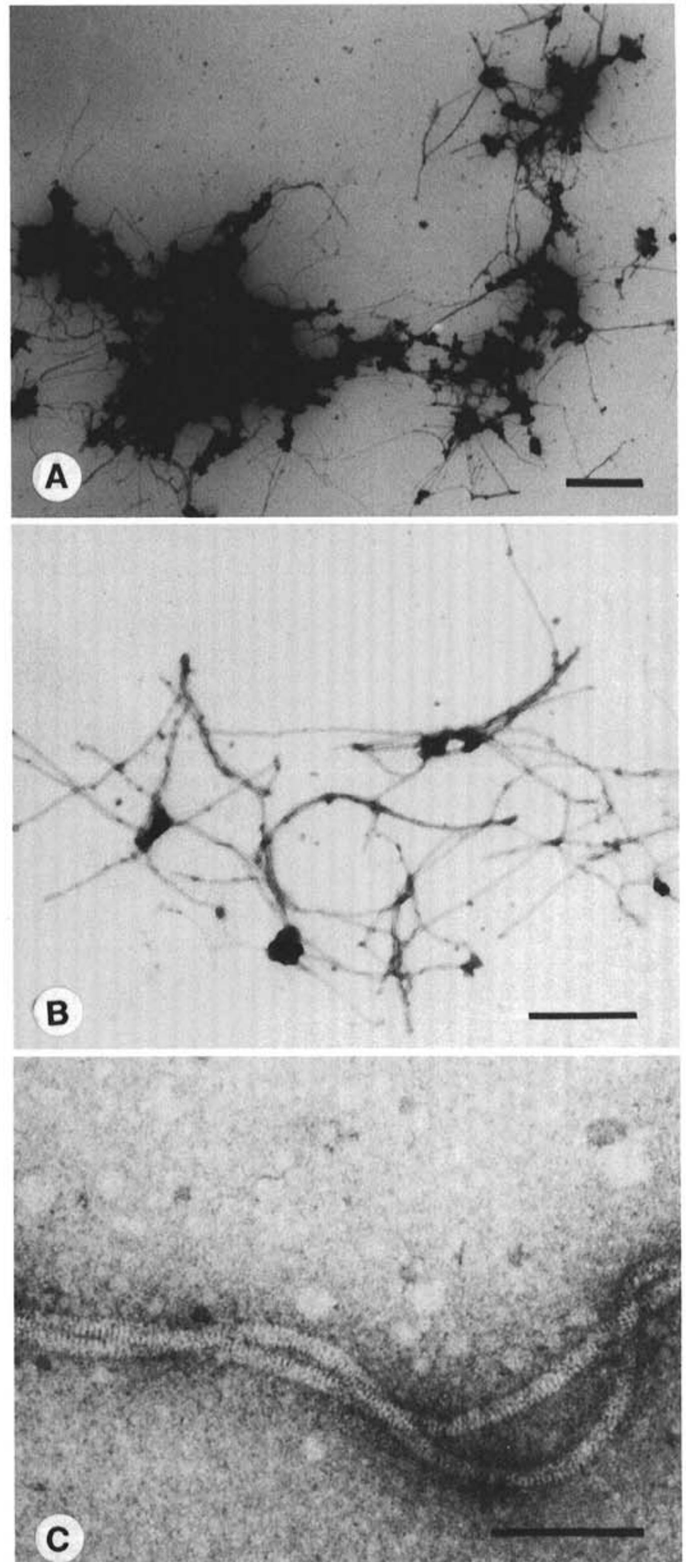

Fig. 2. Electron micrographs of closteroviruslike particles purified from grapevine petiole tissue affected with corky bark disease. **A,** Closteroviruslike particles from grapevine corky bark-associated virus (GCBaV) preparations after the first Cs_2SO_4-sucrose cushion-step gradient centrifugation; **B** and **C,** closteroviruslike particles from GCBaV preparations after the second Cs_2SO_4-sucrose cushion-step gradient centrifugation of virus-containing fraction from the first step gradient centrifugation. Note the largely aggregated particles after the first Cs_2SO_4 gradient centrifugation. Bars = 1,000, 500, and 100 nm for A, B, and C, respectively.

sured for length from partially purified preparations, the largest number (439) of particles measured between 1,200 and 2,000 nm with a modal length of 1,400–1,600 nm (Fig. 3).

Partially purified preparations had numerous protein bands when electrophoresed in SDS-PAGE and stained with Coomassie Brilliant Blue R-250. However, western blot analysis using GCBaV antiserum preabsorbed with healthy antigens revealed a coat protein MW of about 24,000 (Fig. 4).

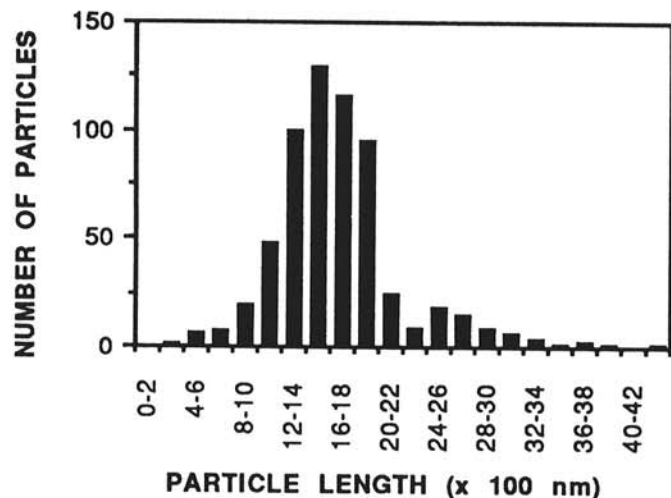

Fig. 3. Particle measurements of the grapevine corky bark-associated virus (GCBaV) isolate CB100 after the first Cs_2SO_4 gradient centrifugation. A carbon grating replica of 2,000 lines per millimeter served as a standard. Particle length was measured using a micro Digi-Pad (GTCO Corp., Rockville, MD).

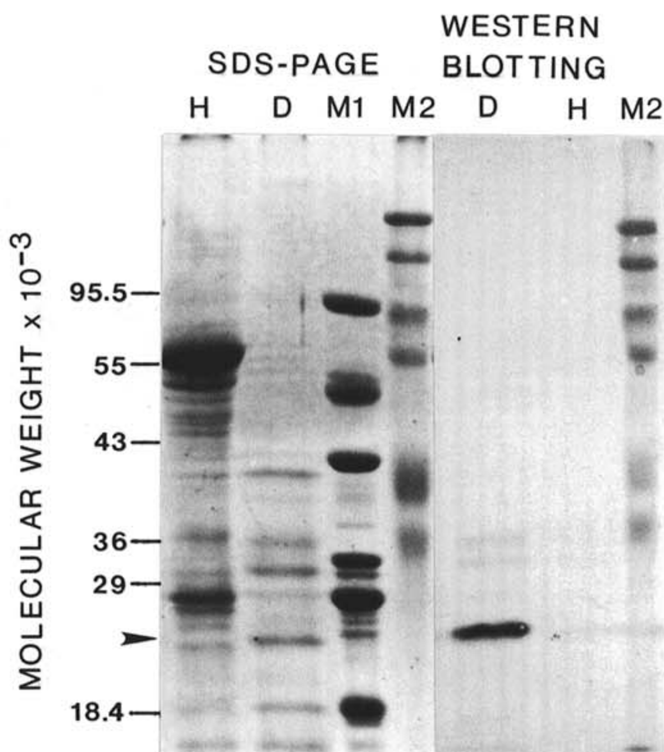

Fig. 4. Analysis of grapevine corky bark-associated virus (GCBaV) preparations by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. H = healthy grapevine preparation; D = GCBaV preparation; M1 and M2 = prestained protein molecular weight (MW) markers. Anti-GCBaV polyclonal antibody was used in the western blots. Western blot analysis shows a GCBaV specific coat protein band (arrow) of about 24,000 MW. Prestained MW markers were phosphorylase B (95,500), glutamate dehydrogenase (55,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), lactoglobulin (18,400), and cytochrome C (12,400).

A high molecular weight dsRNA (about 15.3 kbp), which was smaller than CTV, was identified in corky bark-diseased grapevine (Fig. 5). Several other dsRNAs ranging from 2.9 to 0.04 kbp also were observed. The sizes of the high molecular weight dsRNA of CB100 were similar to that of GLRaV NY-1, but the smaller dsRNAs varied in molecular weight. A high molecular weight dsRNA, identified in grapevine stem pitting-affected grapevine (2), was apparently smaller than those identified in both corky bark- and GLRaV-affected tissues. Stem phloem tissue was a reliable source of dsRNA.

Serology. Two polyclonal antisera were prepared to purified and partially purified GCBaV preparations. Both decorated the flexuous rod-shaped particles from partially purified preparations and from crude tissue extracts. However, they did not decorate closteroviruslike particles of GLRaV types II and III, GVA, and CTV. Reciprocal tests also showed that particles of GCBaV were not decorated by the heterologous antisera.

Initial work using standard ELISA procedures was not entirely satisfactory. This led to comparisons of several tissue extraction buffers using conjugated GCBaV immunoglobulin that had been reabsorbed with healthy antigens (Table 1). The combination of nonfat dry milk with regular ELISA extraction buffer (Table 1) and reabsorption of enzyme-conjugated IgG gave the best results. Interestingly, reabsorption raised the ELISA reading considerably with all buffers tried. A number of corky bark-affected cultivars were tested by ELISA using antisera to GCBaV and to serologically distinct types (II and III) of GLRaV (Fig. 6). Antisera to GCBaV reacted to six of seven other corky bark isolates. However, low ELISA reactions were obtained from corky bark isolates of Aledo and Fayoumi grapevine cultivars. Although a number of corky bark indexed positive vines also were infected with type II and/or type III particles of GLRaV, in other tests, antisera to GCBaV did not react with tissue extracts only infected with type II or III of GLRaV (data not shown).

DISCUSSION

Our data show that long closteroviruslike particles are associated with the corky bark disease. Although Lee et al (25) mentioned the isolation of closteroviruslike particles from corky bark-affected grapevines, this is the first report to fully describe the isolation and partial characterization of virus particles associated with corky bark disease. The purification procedure adopted for corky bark was very similar to that used for closteroviruslike particles associated with leafroll (18,41). However, it was observed that $MgSO_4$ was essential to stabilize the particles. Keeping the virions structurally stable is extremely important because the low yield of particles often necessitates the bulking of virus preparations that may involve combining purified materials over several weeks to months. We did not detect serological relationships between the closteroviruslike particles of GCBaV and of virus isolates of GLRaV by direct ELISA and by IEM. There exists the possibility that GCBaV particles simply represent a serologically distinct strain(s) of leafroll that does not cause typical symptoms on leafroll indicator plants while causing corky bark symptoms on LN-33. Since our data clearly indicate differences in the molecular weights of coat proteins of GCBaV (24,000) and GLRaV (36,000–43,000), we conclude that the closteroviruslike particles of GCBaV and GLRaV are different.

A number of the corky bark-affected grapevines also tested positive for GLRaV by ELISA (Fig. 6), which may suggest that GCBaV and GLRaV are needed to cause corky bark disease. Two factors argue against this suggestion. First, we have not detected GLRaV particles in the corky bark isolate (CB100) that is used as the standard for corky bark indexing. Second, it is not uncommon to have mixed infections of corky bark and leafroll. In fact, the isolates that were designated as corky bark affected (Fig. 6) also have indexed positive for leafroll (D. Golino, *unpublished data*).

Although our data suggest an association of closteroviruslike particles with vines affected with corky bark disease, the etiological significance of these particles remains to be determined. Several

Fig. 5. Electrophoretic analysis of dsRNA extracts from corky bark diseased grapevines in 2% agarose (lanes 1–3) and 5% polyacrylamide (lanes 4–9) gels. DsRNAs were isolated from tissue infected with: 1, citrus tristeza virus; 2, corky bark (cv. LN-33); 3, rice dwarf virus (note: this is dsRNA from virus); 4, cucumber mosaic virus; 5, grapevine stem pitting (cv. Colobel); 6, grapevine leafroll (the NY-1 isolate, cv. Pinot noir); 7, grapevine corky bark (cv. LN-33); 8, citrus tristeza virus; and 9, tobacco mosaic virus. Arrowhead indicates the high molecular weight dsRNA band (about 15.3 kbp) associated with corky bark-infected grapevines.

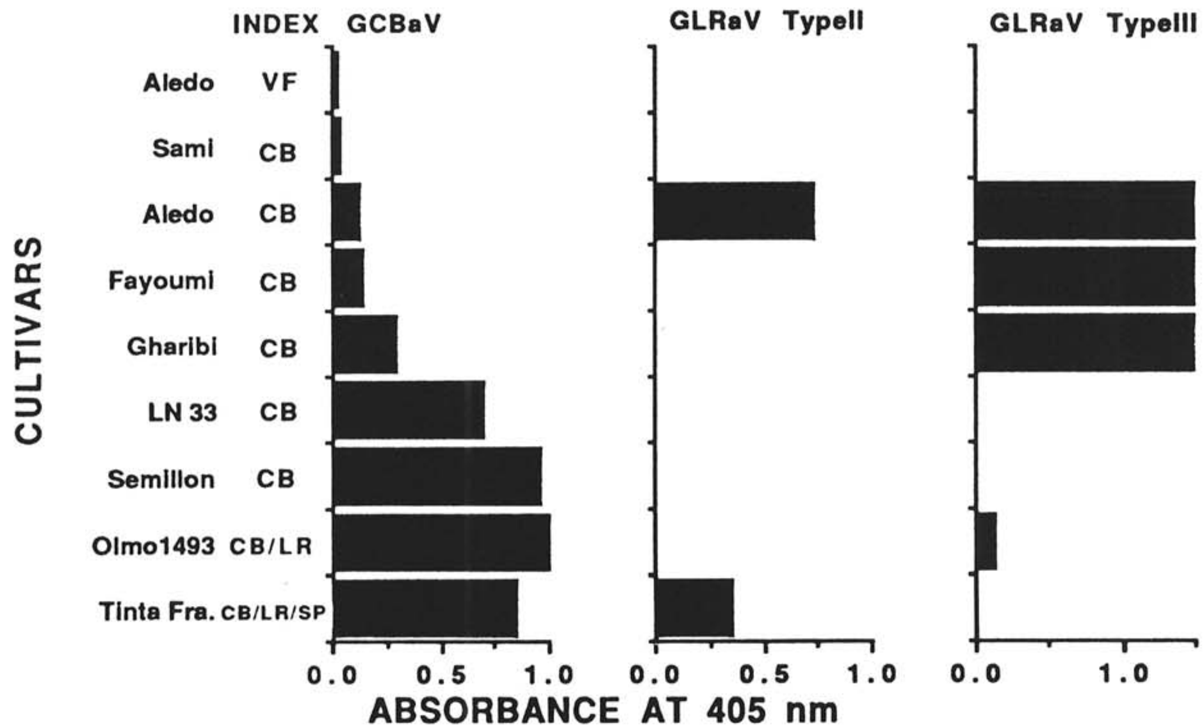

Fig. 6. Reactions of grapevine corky bark-associated virus (GCBaV) and grapevine leafroll-associated virus (GLRaV) types II and III antisera in enzyme-linked immunosorbent assay (ELISA) to different grape cultivars infected with corky bark (CB) disease. Designations above each bar graph indicate the antiserum that was used. Plates were coated with 1 μg/ml IgG for GLRaV types II and III and 2 μg/ml IgG for GCBaV. Homologous enzyme-conjugates were used in each ELISA test. ELISA reactions were recorded 45 min after loading the substrate. INDEX = virus status of the cultivars as determined by indexing on grapevine indicators. VF = virus free. CB/LR/SP = corky bark–leafroll–rupestris stem pitting affected. Note that all samples gave positive ELISA reactions to corky bark except virus-free Aledo and corky bark-affected Sami.

TABLE 2. Comparative properties of flexuous rod-shaped viruses of grapevines

Virus group	Modal length (nm)	CP MV[a] (kDa)	Literature citation
Closterovirus group	1,250–2,000	22.5–25	
GCBaV[b]	1,400–2,000	24	This report
Apple chlorotic leaf spot virus group	600–800	22–24	
GVA[c]	800	22	26
GLRaV group[d]	1,200–2,200	36–48	
GLRaV—type I	1,800–2,200	···	15,18
type II	1,800–2,200	36	15,18
type III	1,800–1,900	43	18
type IV	···	···	18
ASPaV[e]	800	48	21

major factors make it difficult to determine this conclusively. For example, as in the case of leafroll, the expression of corky bark symptoms in standard grapevine virus indicator plants takes about 2 yr to appear after inoculation. This makes it difficult to do follow-up experiments because of the long incubation period. Lack of local lesion and herbaceous hosts also adds to the difficulty in identifying and characterizing the causal agent(s) of corky bark.

Tanne et al (32) recently reported that corky bark can be transmitted by mealybugs. This information and the availability of antibodies to GCBaV now make it possible to determine the involvement of GCBaV in the etiology of corky bark.

Recently, several viruses were separated from the closterovirus group and transferred to a new group—the capillovirus group (26). The closterovirus group now contains two subgroups, the beet yellows virus (BYV) and the apple chlorotic leaf spot virus groups. However, this group also contains several possible members (e.g., GLRaV types II and III) that have larger coat protein molecular weights of 36,000–43,000 (Table 2). Recently, Koganezawa et al (21) reported a flexuous, filamentous virus associated with apple stem pitting disease that has a coat protein molecular weight of 48,000. Therefore, according to these virus characteristics, we suggest that the closterovirus group can be separated into three independent groups (Table 2; 3,15,18–20, 26,29,40,41). Interestingly enough, flexuous rod-shaped viruses from all three of these groups have been isolated from grapevines (Table 2). GVA falls into the group typified by apple chlorotic leaf spot virus while the GLRaV types (at least types II and III) fall into the new group typified by GLRaV type III. On the other hand, GCBaV falls into the group typified by beet yellows virus to which citrus tristeza virus also belongs. We did not detect any serological relationships between GCBaV and citrus tristeza virus. However, viruses in this BYV group do not show extensive serological relationships. Only carnation necrotic fleck is serologically related to BYV and more distantly related to wheat yellow leaf virus.

After this work was completed, we also isolated a closterovirus that was mechanically transmissible from the corky bark-affected Semillon to *Nicotiana occidentalis* (D. Boscia, S. Namba, and D. Gonsalves, *unpublished data*). Initial data suggest that the particles are about 13 × 800 nm and are serologically unrelated to GCBaV. Instead, its host range and particle length are similar to that reported for GVA (27). Others (e.g., 27,33) have observed GVA particles in leafroll, stem pitting, and even healthy-appearing vines that suggest that this mechanically transmissible virus is not the cause of corky bark. However, studies to determine if these particles have any association with the corky bark disease are under way.

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